ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 1-9
doi: 10.25083/rbl/24.1/1.9

Received for publication: December 10, 2017
Accepted: April 14, 2018

Original paper

Content of Phenolics, in vitro Antioxidant Activity and Cytoprotective Effects against Induced Haemolysis of Red Cabbage Extracts

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Abstract

Consumption of plant foods has been associated with a reduced risk of several diseases including cardiovascular and cancer. This is supported by their content of phytochemicals which provide a variety of pharmacological properties. The aim of the present study was to evaluate the content of antioxidant compounds with polyphenolic structure from seven different red cabbage samples, as well as their antioxidant and antihaemolytic activity.

The mean values of total anthocyanins and flavonoids of the investigated ethanolic and acidified ethanolic extracts were 1228.81 mg 100g-1 DM and 2402.27 mg quercetin 100g-1 DM, respectively. The results indicate a positive correlation of anthocyanins with flavonoids. We report strong antioxidant activity of the crude extracts as measured by relevant assays such as Folin-Ciocalteu and ferric reducing antioxidant power (FRAP). Good positive correlations were found between FRAP and flavonoids, and between phenolics and anthocyanins, respectively. We also describe the cytoprotective effects of the ethanolic crude extracts against free-radical induced oxidative damage in erythrocytes.

The present study demonstrates the high content and activity of antioxidant compounds in red cabbages from different geographic regions. In addition, it provides the first scientific evidence for a protective role against cytotoxic effects of reactive oxygen species in erythrocytes linked to different sample sources.

Keywords : red cabbage, anthocyanins, phenolics, antioxidant, induced haemolysis

To cite this article: OANCEA S, MILA L, KETNEY O. Content of Phenolics, in vitro Antioxidant Activity and Cytoprotective Effects against Induced Haemolysis of Red Cabbage Extracts. Rom Biotechnol Lett. 2019; 24(1): 1-9. DOI: 10.25083/rbl/24.1/1.9

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Introduction

Oxidative stress, which is induced in human body by reactive oxygen species (ROS), plays a significant role in the development of several chronic diseases, e.g. cardiovascular, neurodegenerative, arthritis, autoimmune diseases, diabetes, and cancer. ROS, such as superoxide radical O2-, hydroxyl radical HO., hydrogen peroxide H2O2, and singlet oxygen 1O2 mainly produced in mitochondria, may lead to: lipid peroxidation in cell membranes, enzyme inactivation, activation of particular signalling pathways, DNA damages, generation of toxic compounds. Oxidative stress is produced as a result of an impaired balance between oxidative conditions and antioxidant mechanism. The rate of oxidative stress is related to the overall production of ROS, the efficiency of the endogenous antioxidant defence mechanism and the level of exogenous antioxidants.

An increase of antioxidants in diet provides one possible solution to excessive ROS production. Some in vitro and epidemiological studies indicate the association between a high intake of antioxidant compounds and a reduced risk of cardiovascular and cancer diseases (PRIOR [1], STANNER & al. [2], GOSZCZ & al. [3]). Fresh fruits and vegetables are excellent sources of phytochemicals with antioxidant properties. Health benefits of plant bioactives are often linked to the interactive and synergistic effects of the wide range of chemical entities highly correlated with each other.

Within the Brassicaceae family, red cabbage (Brasica oleracea var. capitata f. rubra) represents a dietary source rich in phytochemicals, in particular (poly)phenolics, flavonoids, anthocyanins, ascorbic acid, □-tocopherol, □-carotene, lutein, etc. (JAGDISH SINGH & al. [4]). Anthocyanins are water-soluble vacuolar pigments with proved health-promoting properties mainly based on their strong antioxidant activity (HE & GIUSTI [5]). The mechanism by which (poly)phenolics act as antioxidants is likely to involve: (i) a direct antioxidant activity through interaction with ROS and free radicals resulted from biomolecules; (ii) chelating metal ions (Fenton chemistry); (iii) the inactivation of enzymes responsible for superoxide radical generation (xanthine oxidase, protein kinase C); (iv) the activation of antioxidant enzymes (superoxide dismutase, glutathione S-transferase, glutathione peroxidase, catalase) [3]. Emerging theories from the current studies show that the (poly)phenolics molecular mechanism may be linked to the modulation of gene expression (RAY & al. [6]). These studies suggest that the modulation of genes such as those related to in vivo inflammation pathways or genes associated with the antioxidant activity of hydroxytyrosol, seems to be the molecular target for the antioxidant activity of polyphenolics, in particular from olives.

Epidemiological studies indicate that a high intake of Brassica vegetables may lead to a reduced risk of cardiovascular and cancer diseases (KRISTAL & LAMPE [7]). Other studies have reported hypocholesterolaemic (KOMATSU & al. [8]), hepatoprotective (SINGAB & al. [9]), neuroprotective (HEO & LEE [10]) and nephroprotective (KATAYA & HAMZA [11]) effects of red cabbage extracts in rats, in particular due to phenolics/anthocyanins content.

There are very few studies showing the antioxidant effects of red cabbage in erythrocytes against free-radical induced oxidative haemolysis. Thus, the aim of the present work was to evaluate and compare the content of antioxidant compounds with phenolic structure extracted from several red cabbage samples, and to determine their in vitro antioxidant and antihaemolytic activities.

Materials and Methods

1. Preparation of samples

Seven commercial samples of red cabbage (Brassica oleracea var. capitata f rubra) purchased in May 2015 from different local markets, three of which originated from Romania, two from Poland, one from Holland and one from Germany, were used for the present study. Cut slices of samples were freeze-dried using a lyophilizer (Alpha 1-4 LD plus, CHRIST) at -45oC. The freeze-dried cabbage was powdered using the knife mill (Grindomix GM 200, RETSCH).

Chemical reagents of analytical grade without further purification were used.

2. Extraction procedures

Extraction of bioactive antioxidant compounds (anthocyanins, phenolics, flavonoids) from lyophilized red cabbage was conducted using two solvent systems: (i) 70% (v/v) ethanol in water; (ii) acidified 70% (v/v) ethanol 0.05% HCl, for one hour at 4 oC. The obtained mixtures were centrifuged at 8000 rpm for 10 minutes

at 4 oC. The 320R refrigerated centrifuge (HETTICH) was used.

3. Physico-chemical characterization

Two quality parameters of the lyophilized red cabbage samples were measured, as follow: (i) water activity using the AW Meter LabMaster-aw (NOVASINA); (ii) moisture content determined at 105 oC using the moisture analyzer (ML-50, A&D Co. Ltd.). In addition, pH of the prepared extracts was measured using the Orion 2-star pH meter (THERMO SCIENTIFIC).

4. Total anthocyanins

The content of total anthocyanins was determined spectrophotometrically by the pH differential method (GIUSTI & WROLSTAD [12]). The Specord 200Plus UV-Vis spectrophotometer (ANALYTIK JENA) was used. The content was expressed as milligram cyanidin-3-O-glucoside (Cyn-3-O-G) equivalents per 100 g dry mass (mg 100g–1 DM).

5. Total flavonoids

The content of total flavonoids was determined using the aluminium chloride colorimetric method (KUMAR & al. [13]). The content was expressed as milligram quercetin equivalents per 100 g dry mass (mg 100g–1 DM).

6. Total phenolics

The content of total phenolics was determined according to the Folin-Ciocalteu spectrophotometric method (SINGLETON & ROSSI [14]). The content was expressed in milligram of gallic acid equivalents per 100 g dry mass (mg GAE 100g–1 DM).

7. Antioxidant activity by ferric reducing antioxidant power (FRAP)

The total antioxidant capacity was determined by the ferric reducing ability assay described by Benzie and Strain (BENZIE & STRAIN [15]). The results were expressed as milligram ascorbic acid per 100 g dry mass (mg 100g–1 DM).

8. Inhibitory effects on AAPH-induced hemolysis

The antihaemolytic activity of red cabbage extracts in 70% (v/v) ethanol was determined using the method described by Asgari et al (ASGARY & al. [16]). Sheep red blood cells (RBC) were separated by centrifugation and washed with PBS buffer pH 7.4 until the supernatant was colorless. A 7% suspension of RBC was prepared in PBS buffer and used for incubation at 37 ºC for 2 hours in the presence of 2,2'-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) (25 mM) and red cabbage extracts. Control sample without extracts was used. The inhibition of haemolysis was determined by measuring the absorption at 540 nm. It was calculated accordingly as percentage of inhibition (%).

9. Statistical analysis

All analyses were made in duplicate (n=2). Data are expressed as mean ± standard deviation from two parallel measurements. The processing of data was performed using mathematical and statistical methods with "IBM SPSS 21.0" software, following hypothesis testing and correlation between variables by calculating the Pearson's correlation coefficient, at a significance level of risk $\alpha \leq 5\%$ and probability $P \geq 95\%$.

Results and Discussions

1. Physical-chemical characterization of red cabbage powders/extracts

Seven commercial samples of red cabbage (Brassica oleracea var. capitata f rubra) of different geographical origin purchased from local markets were used in the present investigation, as follow: 1 (Romania), 2 (Romania), 3 (Romania), 4 (Poland), 5 (Poland), 6 (Holland) and 7 (Germany).

Extraction was performed by conventional extractive technology using 70% ethanol in water (v/v) as environmental friendly solvent. Because low amounts of acids facilitate extraction of polyphenolic-based compounds, acidified ethanol with 0.05% HCl was also investigated for the extraction of investigated antioxidant compounds. All extraction runs were conducted at 4 oC in order to minimize degradation of bioactives, in particular anthocyanins. We further used these two types of extracts to determine the content of anthocyanins, flavonoids, phenolics and antioxidant activity despite that other solvents such as methanol might have been better extracted particular compounds.

Some of the physical-chemical attributes of the lyophilized samples and extracts are given in Table 1. The moisture values of a sample indicates its total content of water, while the water activity values reveale the availability of water for biological processes, including microbial contamination. These two characteristics are often used to describe the food

safety and prediction of products' shelf life. By measuring the pH of the obtained hydroethanolic extracts, in accordance to Romanian Pharmacopoeia (FARMACOPEEA ROMANA [17]), we found that they are neutral, while the acidified hydroethanolic extracts are weak acids, as shown in Table 1.

Table 1. Physical-chemical characteristics of red cabbage samples of different origin.

Brassica **samples**	**Moisture of lyophilized sample (%)**	**Water activity (aw) of lyophilized sample at 25 ºC**	**pH of extract**	
			70% EtOH	*0.05% HCl in 70% EtOH*
1	5.915	0.282	6.489	5.457
2	9.709	0.454	6.623	5.660
3	4.460	0.208	6.585	5.820
4	7.260	0.382	6.624	5.591
5	7.563	0.358	6.600	5.720
6	4.000	0.150	6.378	5.630
7	7.335	0.365	6.505	5.513

2. Antioxidant compounds and activity of red cabbage crude extracts

Based on the significant contribution of red cabbage consumption to human health, hydroethanolic (70% EtOH) and acidified hydroethanolic (0.05% HCl in 70% EtOH) extracts were analysed in terms of anthocyanins and flavonoids contents, and of total antioxidant activity, as measured by Folin-Ciocalteu and FRAP assays. For comparison reasons, all values were calculated on percentage and dry mass (DM) basis.

No statistically significant differences ($P>0.05$) were found between the content of the antioxidant compounds and the extraction solvent used in this work.

The content of total anthocyanins and flavonoids in Brassica oleracea extracts is presented in Fig. 1.

The mean values of anthocyanins content in the investigated samples using two extraction solvent systems varied between 648.83 and 1471.54 mg 100g-1 DM, depending on the varieties originated from different regions. The statistical analysis showed that despite the fact that low amounts of acids added to the extraction solvent gave better extraction of anthocyanins, the differences were not significant. The highest concentration was obtained from sample 6 (red cabbage from Holland) regardless the applied extraction solvent systems. The anthocyanins level in samples grown in Romania varied between 648.83 and 1265.76 mg 100g-1 DM, most probably linked to different varieties and cultivation regions. Other reported values of anthocyanins concentration determined by the same pH differential spectrophotometric method, and on a dry mass basis, were similar and varied between 10-19 g kg-1 DM for the "Gradur" and "Roxy" hybrids from North-Central Italy (PICCAGLIA & al. [18]). The content of anthocyanins in red cabbage is influenced by several factors, such as genotypes and environmental conditions. The same authors have found that red cabbage variety seems to be a major factor compared to fertilization practises.

The results regarding the total flavonoids content indicate variations between 1727.36 and 3047.53 mg quercetin equivalents 100g-1 DM in the seven investigated samples, with the highest amount for sample 6 regardless the applied type of extraction solvent systems. The statistical analysis showed that despite the fact that low amounts of acids added to extraction solvent gave better extraction of flavonoids, the differences were not significant. Most previous studies on red cabbage reported the content of total flavonoids expressed on a fresh mass basis, and different equivalents (catechin, quercetin, rutin), so

that comparison with our data is not appropriate to perform, but a recent study on secondary metabolites from red cabbage (variety or region not specified) reported similar flavonoids content in methanolic extracts using the same analytical procedure (17.44 mg quercetin equivalents g-1 DM) (GAAFAR & al. [19]).

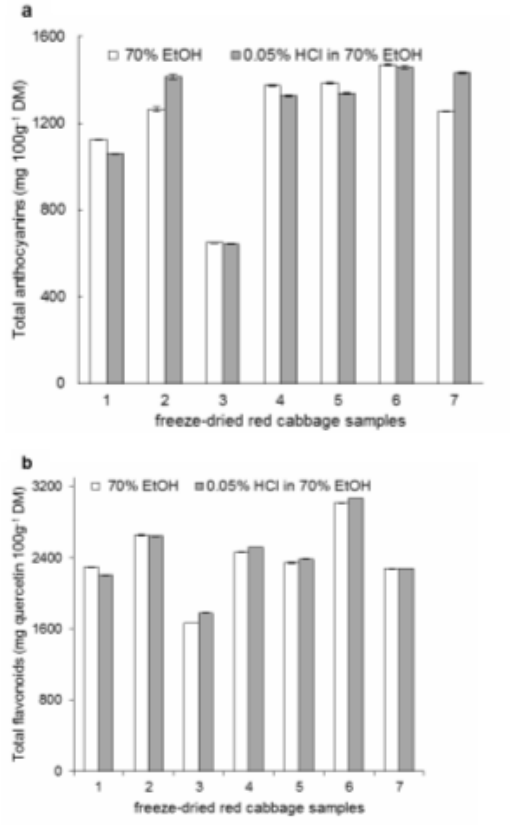

Figure 1. The content of total anthocyanins (a) and flavonoids (b) in two different extracts of red cabbage; vertical bars indicate SE of the means.

The mean value of total anthocyanins of investigated ethanolic and acidified ethanolic extracts was 1228.81 mg 100g-1 DM, while of total flavonoids was 2402.27 mg quercetin 100g-1 DM.

Polyphenolic compounds are antioxidant molecules which can interact with both radical and non-radical ROS, as well as with reactive species produced from cell molecules (QUIDEAU & al. [20]). The antioxidant properties of such compounds are considered to be responsible for their health benefits. Different investigative methodologies are used for this purpose, such as oxygen radical absorbance capacity assay (ORAC), total reactive antioxidant potential (TRAP), ferric reducing antioxidant power assay (FRAP), Trolox equivalent antioxidant capacity (TEAC), total phenolics assay by Folin Ciocalteu, thiobarbituric acid reactive substances assay (TBARS), DPPH radical scavenging activity or β carotene-linoleic acid assay. In our work, we used the FRAP and Folin-Ciocalteu assays to evaluate the total antioxidant activity of the hydrophilic red cabbage extracts. The results are presented in Fig. 2.

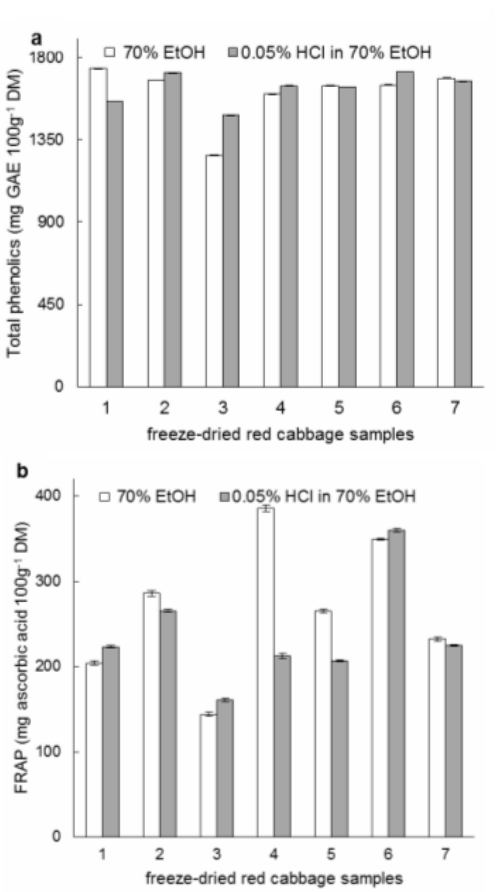

Figure 2. The content of total phenolics (a) and the antioxidant activity measured by FRAP assay (b) in two different extracts of red cabbage; vertical bars indicate SE of the means.

With the exception of sample 3, the others showed very similar total phenolics concentration (mean values between 1621.29 and 1693.84 mg GAE 100g-1 DM for both type of extracts). Our results showed that using 70% ethanol as extraction solvent proved better extractability of phenolics for sample 1, while acidified ethanol determined extraction of higher amounts of phenolics in sample 6. The statistical analysis showed that despite the fact that low amounts of acids added to ethanol improved the extraction of phenolics, the differences were not significant. Higher amounts of

phenolics in lyophilized red cabbages using the same Folin-Ciocalteu procedure have been reported by other authors, but on hydromethanolic extracts (GAAFAR & al. [19], DUCHNOWICZ & al. [21]), while others reported much lower phenolics content in the Romanian red cabbage cv. CB10089 (VICAS & al. [22]).

The mean values of FRAP of 70% hydroethanolic extracts varied between 141.45 mg ascorbic acid 100g-1 DM (sample 3) and 389.84 mg ascorbic acid 100g-1 DM (sample 4). The mean values of FRAP of 0.05% HCl acidified 70% hydroethanolic extracts varied between 159.28 mg ascorbic acid 100g-1 DM (sample 3) and 357.67 mg ascorbic acid 100g-1 DM (sample 6). As noticed in Fig. 2, sample 4 showed very high antioxidant activity compared to the other analysed samples.

Comparison of our results on the antioxidant activity of red cabbage hydroethanolic extracts with previous published ones is a difficult task, as different extraction procedures and methods of analysis of both water- and lipid-soluble antioxidants have been employed. However, using both phenolics and FRAP measurements, our results confirmed that the extract from sample 6 showed the highest value of total antioxidant activity, while the extract from sample 3 showed the lowest one. These results are in accordance to those regarding the determined content of anthocyanins and flavonoids.

The results of statistical correlation by regression analysis indicate a positive correlation of anthocyanins with flavonoids ($R2=0.843$, $P<0.01$). The total antioxidant activity positively correlates with anthocyanins and flavonoids, using both methods of measurements (Folin-Ciocalteu and FRAP). Good positive correlations were found between FRAP and flavonoids ($R2=0.817$, $P<0.01$) and between phenolics and anthocyanins ($R2=0.807$, $P<0.01$), respectively. Other authors have also found that flavonoids, anthocyanins and ascorbic acid are the best predictors for the antioxidant activity by FRAP of cabbage (KAULMANN & al. [23]).

This study affirmed that the level of polyphenolic-based antioxidant compounds of red cabbage varied extensively according to genetic and environmental conditions. Also, we do not exclude the influence of the period of commercial storage on the content of antioxidant compounds and activity.

3. Antihaemolytic activity of red cabbage hydroethanolic extracts

The oxidative damage induced by ROS can be investigated in vitro using erythrocytes as model of biomembranes as they may be disrupted in the presence of the reactive species causing haemolysis (CLEMENS & al. [24]).

Because erythrocytes are often used for in vitro studies regarding the nutritional impact on disorders induced by oxidative stress, in the present study we investigated the potential of red cabbage crude extracts to inhibit such oxidative damage. Erythrocytes are known for their high sensitivity to oxidative damage mainly because of the content of membrane proteins and polyunsaturated fatty acids (CHIU & al. [25]).

We used the water soluble azo compound AAPH as free peroxyl radical generator in erythrocytes, at 37 oC. Control samples consisting of erythrocytes and extracts incubated alone without AAPH were also prepared, suggesting the absence of abnormal oxidation. We recorded a significant inhibition of AAPH-induced oxidative haemolysis of erythrocytes in the presence of 70% hydroethanolic extracts of red cabbage after 2 hours of incubation, as presented in Table 2. Therefore, bioactive compounds (flavonoids, anthocyanins) from red cabbage may quench the chain propagating peroxyl radicals generated by AAPH in erythrocytes, thus decreasing the extent of peroxidation and diminishing haemolysis. As noticed in Table 2, the efficiency of the antihaemolytic activity varies in the order 4>1>6>2>3>5>7.

The most effective inhibition was produced by extract 4, which also demonstrated very high antioxidant activity by FRAP (Fig. 2) compared to the other samples. Interestingly, sample 6 which showed mean values of bioactive compounds higher than the other investigated samples indicated lower antihaemolytic activity. This may be related to the contribution of other important water soluble bioactive compounds existing in the red cabbage hydrophilic extracts, such as ascorbic acid, glucosinolates, minerals which may act synergistically and could explain the mechanism of interaction with erythrocytes membrane. In a previous study it was shown that chain-breaking ascorbic acid suppressed the haemolysis dose dependently (NIKI & al. [26]). We found that weak or

no statistical correlations exist between the antihaemolytic activity and the content of bioactive compounds in the investigated samples.

Table 2. The haemolytic inhibition of red cabbage hydroethanolic extracts of different origins; mean values ± standard deviation.

***Brassica* samples**	**Inhibition of haemolysis (%)**
1	74.91 ± 0.37
2	44.42 ± 1.06
3	43.72 ± 0.51
4	93.49 ± 0.09
5	30.78 ± 0.56
6	47.64 ± 0.61
7	5.35 ± 0.88

Considering all samples, we found a mean value of 48.61 % haemolytic inhibition caused by the different red cabbage extracts, indicating their protective effects against erythrocytes oxidative damage most probably through the inhibition of lipid peroxidation. As far as we know, no other similar haemolysis studies have been reported on red cabbage, but there are studies reporting: (i) the cholesterol decrease in blood samples from patients diagnosed with hypercholesterolemia caused by red cabbage extract added to collected erythrocytes without affecting membrane fluidity (DUCHNOWICZ & al. [21]); (ii) the inhibitory effect of red cabbage extract on peroxynitrite-induced oxidation in human erythrocytes, by measurement of nitrated tyrosine, protein carbonylation and lipid peroxidation (KOLODZIEJCZYK & al. [27]); (iii) the protective role of flavonoids, individual or in natural extracts, against damages in erythrocytes in a concentration and time dependent pattern (ASGARY & al. [16], SULAIMAN & al. [28]).

Antioxidant compounds, in particular flavonoids, have been shown to exert their biological/pharmacological properties through cell membrane interactions (ERLEJMAN & al. [29]). Using electron spin resonance technique (ESR) and erythrocytes model, a Polish research group showed that the flavonoid quercetin localizes at the polar-apolar interface of the lipid bilayer (PAWLIKOWSKA-PAWLÊGA & al. [30]). Such distribution may induce alteration of erythrocytes membrane permeability, thus protecting against peroxidation. Low concentrations of quercetin did not induce haemolysis, but higher levels lead to modifications of erythrocytes shape. It is considered that lipophilicity/chemical structure of the flavonoid molecules highly influences the flavonoid-lipid interactions.

The strong antioxidant activity and cytoprotective effects of red cabbage extracts support their health benefits and application in developing nutraceuticals, as well. Thus, some authors reported good results in developing probiotic products, using red cabbage juice as substrate for lactobacillli (BURULEANU & al. [31]).

Conclusions

The content of anthocyanins, flavonoids, phenolics, as well as in vitro antioxidant and antihaemolytic activity in seven samples of red cabbages were determined and compared.

The present study indicates that the level of polyphenolic-based antioxidant compounds varies according to genetic and environmental conditions of investigated samples. Statistically, we found that low acidification of ethanol solution used for extraction does not significantly influence the recovery of such bioactives. The mean value of total anthocyanins of ethanolic and acidified ethanolic extracts was 1228.81 mg 100g-1 DM while of total flavonoids content was 2402.27 mg quercetin 100g-1 DM. We found a positive correlation between anthocyanins and flavonoids. We report high total antioxidant activity of crude extracts as measured by Folin-Ciocalteu (1620.35 mg GAE 100g-1 DM) and FRAP (251.39 mg ascorbic acid 100g-1 DM) assays. The total antioxidant activity positively correlates with anthocyanins and flavonoids, using both methods of measurements.

In addition, the cytoprotective effects of the different ethanolic crude extracts from red cabbage against AAPH-induced oxidative damage in erythrocytes further indicates the beneficial role of red cabbage in preventing oxidative stress.

Thus, our studies demonstrate the potential health benefits of red cabbage extracts based on the high content and in vitro activity of polyphenolic antioxidant compounds, and provides the first scientific evidence for their protective role against cytotoxic effects of reactive oxygen species in erythrocytes.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PN-II-ID-PCE-2011-3-0474.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 10-19
doi: 10.25083/rbl/24.1/10.19

Received for publication: April 20, 2018
Accepted: December 2, 2018

Original paper

In vitro characterization of microbial biofilm on soft materials used in overdentures retained by mini implants

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Abstract

Study aim was to assess microbial biofilm (species composition, in vitro microbial development capacity, and virulence potential) developed on soft relining materials, used as soft matrices during osseointegration period, in overdentures retained by mini dental implants. Microbiota was dominated by Gram negative bacilli (Pseudomonas sp., Enterobacter sp.), followed by Gram positive facultative anaerobic cocci (Staphyloccoccus sp., Streptococcus sp.) and Gram positive anaerobic rods, yeasts belonging to Candida sp. being infrequently isolated. In implants with peri-implantitis clinical signs, with or without implant failure, the microbiota was dominated by Gram positive bacteria. The isolates showed the capacity of attachment on both inert and cellular substrata, with some differences according to bacteria type. The oral isolates produced different soluble enzymes, a higher production of gelatinase and lipase being recorded in Gram negative bacteria, and of haemolysins in Gram positive bacteria. In conclusion, knowledge of dental implants associated microbiota diversity and virulence characteristics is a key aspect for the understanding of the disease process, for selecting the best materials in terms of low colonization risk, and for establishing proper therapeutic measures to inactivate the microbial "artillery" and to prevent implant failure.

Keywords : dental prosthesis; peri-implantitis; matrices; soft relining; Gram-negative bacteria

To cite this article: PREOTEASA CT, PREOTEASA E, POPA M, PIRCALABIORU-GRADISTEANU G, GRIGORE R, MARUTESCU L. In vitro characterization of microbial biofilm on soft materials used in overdentures retained by mini implants. *Rom Biotechnol Lett*. 2019; 24(1): 10-19. DOI: 10.25083/rbl/24.1/10.19

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Introduction

Implant overdenture is well-recognized nowadays as an optimal treatment alternative for the aged edentulous patients, providing an increase of dentures' retention, promoting better mastication and phonation and higher physical and psychological comfort (PREOTEASA& al. [1]; VILLA& al. [2]).Similar to other dental implant prosthetic restorations, implant failure is one of the main complications which may occur due to various conditions, including peri-implantitis, a clinical condition similar to periodontitis, but with lower prevalence of common periodontopathogens, faster evolution and increased treatment resistance (TANDARA& al. [3]; KOYANAGI& al. [4]; FROUM& al. [5]; SUCIU& al. [6]). Implant survival and success are highly susceptible to bacterial infiltration, special consideration being given to certain periods, as the critical initial phase of osseointegration (PORTER& al. [7]; POLL& al. [8]; POLL& al. [9]). A relative large number of studies were conducted to gain knowledge on biofilms associated with dental implants used for fixed dental prosthesis, but fewer information is available in case of overdentures, especially for those retained by mini dental implants.

The purpose of this study was to assess features of microbial biofilm (species composition, microbial adherence assay, and virulence potential) developed on soft relining materials, used as soft matrices during osseointegration period, in overdentures retained by mini dental implants.

Materials and Methods

1. *Subjects and implant-prosthetic treatment*

This observational study was conducted on completely edentulous patients treated by overdentures retained by mini dental implants. Participants' inclusion was done in the Clinic of Dental Prosthodontics from "Carol Davila" University of Medicine and Pharmacy, Bucharest, in 2014 and 2015. There were included patients with previously made conventional complete denture, in which there were to be applied mini dental implants. Each patient was informed regarding study's main coordinates and a written informed consent was granted.

The overdentures were made like conventional complete dentures, with complete coverage of the support area, including the anatomical and functional borders, with a complete peripheral seal, with a lingualized denture occlusion. The mini dental implants used were one piece dental implants, with balls as attachments (mini1SKY, Bredent; MDI mini dental implants, 3M ESPE). A progressive implant loading protocol was used during osseointegration period. Soft acrylic or silicone-based materials (i.e. Mollosil, Detax; Tissue Conditioner, GC; Visco-gel, Dentsply; Elite H-D, Zhermack; Retention.Sil, Bredent) were used as matrices (were applied in the area of the overdenture base corresponding to the implant site).

For this study, the soft materials were applied for a period of 5 to maximum of 7 days, afterwards were sampled from around dental implants and transferred to a vial containing sterile thioglycolate broth and processed in the microbiology laboratory within 24 hours. The laboratory staff performing microbiological test was masked, by the usage of coding of the samples sent to them (Figure 1).

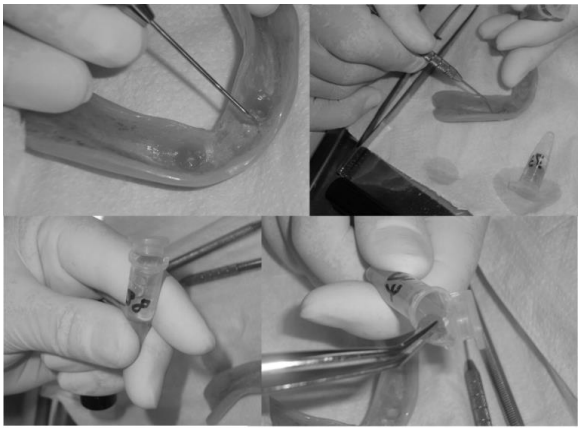

Figure 1. Sampling the soft matrices from overdenture base for microbiological assay

2. *Microbiological assays*

For microbiological analysis of the mixed-species biofilms, the inoculated thioglycolate media were plated on Columbia blood agar supplemented with 5% sheep blood plates (for incubation at 37 °C in aerobic and anaerobic atmosphere). The isolated colonies were identified based on culture, colony and biochemical characteristics.

For microbial adherence assay on inert and cellular substrata, expression of microbial surface attachment structures was investigated by microtiter plate method and the Cravioto's adapted method. The microtiter plate assay allowed the quantification of microbial adherence to an abiotic surface (polystyrene) (PANUS& al. [10]).Following incubation, planktonic cells were removed and the remaining biofilms were fixed with cold methanol, stained with 1% crystal violet dye, solubilized with 33% (v/v) acetic acid and estimated semi quantitatively by the color intensity of the obtained suspension, which is proportional with the number of microbial cells adhered to the inert substratum. Microbial adherence to the cellular substratum was investigated by Cravioto's adapted method. The HEp-2 line cell culture was inoculated with 10^8 CFU/ml suspensions obtained from overnight microbial cultures and incubated for 2 hrs. The cellular monolayers were rinsed with phosphate buffered saline (PBS), fixed with cold methanol, stained with 1% Giemsa solution and microscopically examined in order to establish the adherence patterns (localized, diffuse or aggregative) and the adherence index.

For soluble virulence factors expression, microbial virulence was investigated by growing the isolates on agar media containing specific substrate for different soluble enzymes: haemolysins (5% sheep blood agar), lecithinases (2.5% yolk agar) lipases (1% Tween 80 agar), caseinases (15% soluble casein agar), gelatinases (gelatin agar), DN-ases (DNA agar with toluidin blue), aesculin hydrolysis with the production of esculetol, a siderophore-like compound (10% esculin and iron sulphate agar) and amylase (10% starch agar). The production of the different enzymatic factors was evaluated after incubation by observation of the medium modification (i.e. clear, opaque, colored zones).

3. *Statistical analysis*

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software. For group comparison Mann-Whitney and Friedman nonparametric tests were used. The p-values < 0.05 were considered to be statistically significant.

Results and Discussions

1. *Samples*

Forty-seven samples of soft materials used as matrices during osseointegration period were analyzed. From these, 5 samples were categorized corresponding to implants with clinical signs of peri-implantitis that had a positive evolution afterword, and 3 samples to implants with clinical signs of peri-implantitis that failed (1 mandibular implant that failed in the second week after placement; and 2 maxillary implants that were placed in the same patient that failed after one and a half years). The rest of the samples were placed corresponding to healthy implants.

2. *Composition of microbiota sampled from soft relining materials, used as matrices*

All the soft materials sampled from around mini dental implants yielded viable microorganisms, between 1 and 5 microbial isolates per sample being found. From the total of 86 microbial isolates found, 78 were identified (Table 1). The microbiota around the implants in edentulous patients treated by overdenture was dominated by Gram negative bacilli (Enterobacter sp., Pseudomonas sp.), followed by Gram positive facultative anaerobic cocci (Staphyloccoccus sp., Streptococcus sp., Leuconostoc sp.,), and Gram positive anaerobic rods (Lactobacillus sp., Actinomyces naeslundii, Bifidobacterium sp.). Yeasts belonging to Candida sp. were infrequently isolated. Typical periodontal pathogens, like Aggregatibacteractinomycetemcomitans or Porphyro-monasgingivalis, were not detected. Microbiota identified in samples adjacent to implants with clinical signs of peri-implantitis and a favorable evolution was mainly Gram positive (Aerococcusviridans, Enterococcus faecium, Leuconostoc sp., Staphyloccocus epidermidis), being found only Pasteurella pneumotropicafrom Gram negative bacteria, and Candida tropicalis from yeasts. Microbiota identified in sample adjacent to implants with clinical signs of peri-implantitis that failed was only represented by Gram positive bacteria (Staphyloccoccus sp. and Streptoccocus sp.).

Table 1. Microbiological profile of the soft materials sampled from around mini dental implants.

Gram negative bacilli (n=38) *(Aeromonas caviae (n=2)* *(Bacteroides distasonis (n=1)* *(Burkholderiacepacia (n=2)* *(Chryseomonasluteola (n=3)* *(Citrobacter freundii (n=4)* *(Enterobacter sp. (n=8)* • *Enterobacter aerogenes (n=1)* • *Enterobacter cloacae (n=6)* • *Enterobacter sakazakii (n=1)* *Ewingellaamericana (n=3)* *(Klebsiella sp. (n=1)* *(Pasteurella pneumotropica (n=2)* *(Pseudomonas sp. (n=10)* • *Pseudomonas aeruginosa (n=9)* • *Pseudomonas putida (n=1)* *Salmonella sp. (n=1)* *(Serratia marcescens (n=1)*	**Gram positive cocci (n=24)** *(Aerococcusviridans (n=1)* *(Enterococcus(n=2)* • *Enterococcus durans (n=1)* • *Enterococcus faecium (n=1)* *Leuconostoc sp. (n=3)* *(Staphyloccoccus sp. (n=13)* • *Staphyloccoccus aureus (n=2)* • *Staphyloccocus epidermidis (n=2)* • *Staphylococcus cohniiurealiticum (n=1)* • *Staphylococcus lentus (n=4)* • *Staphylococcus warneri (n=1)* • *Staphylococcus xylosus (n=3)* *Streptoccocus sp. (n=5)* • *Streptoccocusbovis (n=2)* • *Streptococcus acidominimus (n=1)* • *Streptococcus intermedius (n=1)* *(Streptococcus mitis (n=1)*
Gram positive rods (n=11) *(Actinomyces naeslundii (n=3)* *(Bifidobacterium sp. (n=3)* *(Lactobacillus sp. (n=4)* *(Clostridium sp. (n=1)*	**Yeasts (n=5)** *(Candida sp. (n=3)* • *Candida albicans (n=1)* • *Candida magnolia (n=1)* • *Candida tropicalis (n=1)* (Other (n=2)

3. *Microbial adherence assay*

The majority of the oral isolates developed biofilms on inert substrata, this capacity being moderate and strong for most of them, and generally higher in Gram negative bacteria (Figure 2). A Mann-Whitney test showed there was a statistically significant higher slime production for Gram-negative bacteria than Gram-positive bacteria, U=173.5, z=-2.86, p=0.004.

Different adherence patterns to cellular substratum were recorded, i.e.: aggregative, diffuse and localized, with various values of the adherence index, generally higher for Grampositive and Gramnegative bacteria than for yeast (Figure 3). A Mann-Whitney test showed there wasn't a statistically significant difference of the adherence index between Gramnegative and Grampositive bacteria, U=154, z=-0.193, p=0.847.

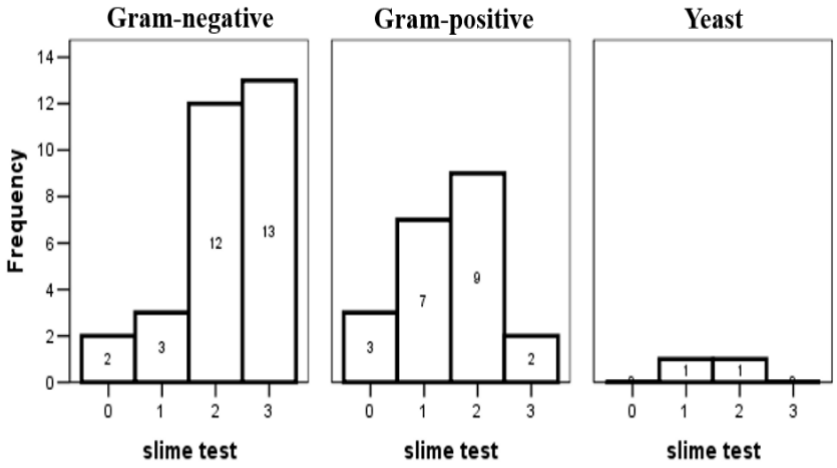

Figure 2. Slime production, according to bacterial type

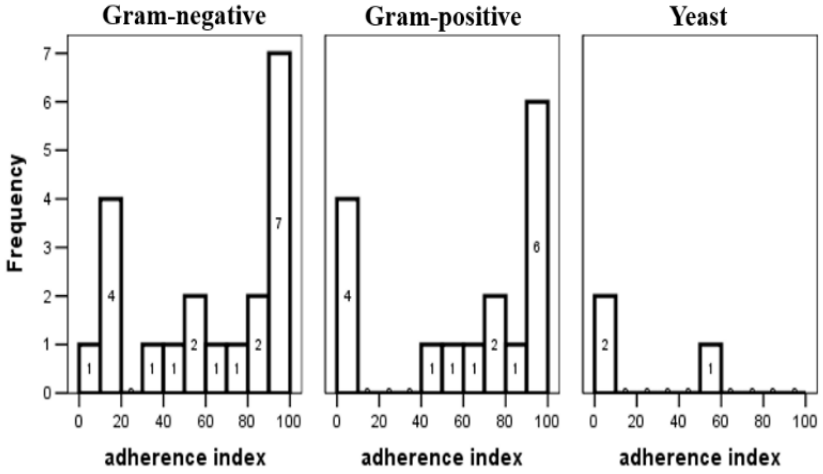

Figure 3. Adherence index, according to bacterial type

4. *Soluble virulence factors expression*

The oral isolates produced different soluble enzymes with variable rates of positivity and intensities (Friedman test, χ ;32.53=(6)2p<0.001). The microbial strains most frequently produced siderophore-like molecules, and least frequently lipase (Figure 4). Comparative analysis of Gram positive and Gram negative microbial strains showed a tendency of higher production of gelatinase and lipase in Gram negative bacteria, and a higher production of haemolysins in Gram positive bacteria, while yeasts expressed the narrowest spectrum of soluble virulence factors (Figure 5)

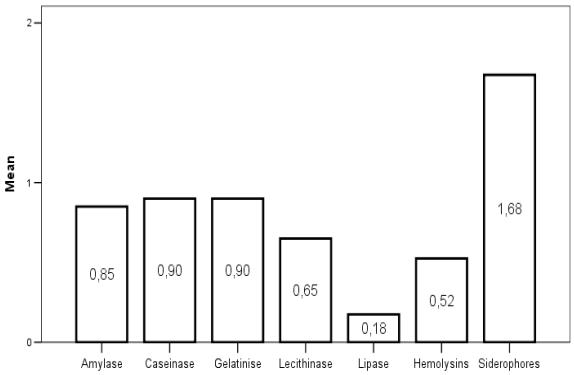

Figure 4. The expression level of different soluble virulence factors in the investigated strains

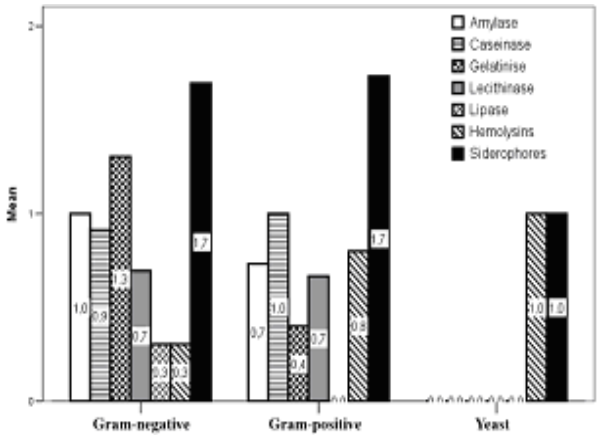

Figure 5. The expression level of different soluble virulence factors among different types of microbial strains.

Discussions

By this study's results, soft tissue materials used as matrices in mini dental implant overdenture treatment exhibit colonization with a diverse spectrum of microbial strains, dominated by Gram negative bacteria. In implants with clinical signs of peri-implantitis, with or without implant failure, the microbiota found was dominated by Gram positive-bacteria. The isolates showed the capacity of attachment on both inert and cellular substrata, with some differences according to bacteria type. The oral isolates produced different soluble enzymes, a larger spectrum being observed in bacteria (higher production of gelatinase and lipase in Gram negative bacteria, and of haemolysins in Gram positive bacteria), than in yeasts, which was expressed the narrowest spectrum of soluble virulence factors.

Adhesion-mediated infections developing of dental implants respond poorly to the antimicrobial treatment, thus suggesting the need to identify best materials in terms of low colonization risk, especially in critical phases, like the osseointegration period (PREOTEASA & al. [11]; CRISTEA & al. [12]; CINTEZA & al. [13]; KOSANIC & al. [14]; PERLEA& al. [15]). It is now recognized that microorganisms which colonize the inert biomaterials are the cause of infections associated with implant treatment (KOSTERTON & al. [16]). The peri-implant soft tissue resembles a scar fibrous tissue with poor vascularity and a reduced defense capability (HEYDENRIJK & al. [17]), being therefore easily colonized by oral bacteria. Peri-implant infectious process may initially affect only peri-implant soft tissues, but it could be also extended to bone tissue.

In the present study, the microbiota was dominated by aerobic Gram negative bacteria (enteric rods and pseudomonads), partially corroborating with previous studies. These state Gram negative bacteria are found in tissues around healthy dental implants, but usually state that in these cases microbiota is however dominated by Gram positive bacteria, and that typical periodontal pathogens are infrequently found(ATA-ALI & al. [18]; SANZ & al. [19]; SHIBLI & al. [20]; LEE & al. [21]; MIHALACHE (RADU) & al. [22]). Regarding the latter, in this study the soft material adjacent to healthy implants was colonized with several Enterobacteriaceae genera and opportunistic Pseudomonas aeruginosa, bacteria previously suspected to be associated with peri-implantitis (CANULLO & al. [23]; ALBERTINI & al. [24]). The present investigation detected also species of the genus Streptococcus sp. and Actinomyces naeslundii, confirming their role as primary colonizers (DIAZ & al. [25]; KOLENBRANDER [26]), in both peri-implantitis and periodontitis (PERSSON & al. [27]; RAKIC & al. [28]).

Candida albicans is the most common fungus found in the oral cavity, and its presence is strongly associated with oral candidiasis especially in patients wearing dentures. In the present study, the yeast recovery rates were of very low. Yeasts are only sporadically present in peri-implantitits lesions and preferentially in complete and partially edentulous cases (LEONHARDT & al. [29]).

P. gingivalis and A. actinomycetemcomitans were not found in the present study. It has been hypothesized that in completely edentulous patients, bacteria known as potential periodontal pathogens are eliminated together with the subgingival environment by extraction of all teeth (DANSER & al. [30]), although some studies have reported late (>5 years) occurring peri-implantitis in edentulous patients harboring a microbiota specific to dentate individuals(LEONHARDT & al. [29]).

This study indicated a high prevalence of biofilm-forming phenotypes among the oral isolates. The capacity to develop biofilms on inert substratum was higher in aerobic Gram negative bacteria, data that could explain the high recovery rates of this group in

our study. However, although poorer biofilm formers in vitro, Gram positive bacteria may still be important during polymicrobial infections where they can directly be incorporated into an established biofilm or interact synergically with other species.

The oral isolates produced different soluble enzymes. Microbial adhesins can also bind to epithelial cells receptors, subsequently bacteria destroying or invading them, initiating an inflammatory process. More than half of the isolates exhibited the ability to adhere to cellular substrata in vitro. Proteases (including collagenase with gelatinase activity) have been reported to be involved in periodontal and peri-implant tissue remodeling (INGMAN & al. [31]; BIRKEDAL-HANSEN [32]; EJEIL & al. [33]), by degrading components of the extracellular matrix of the connective tissue, activating host matrix metalloproteinases and subverting the host defense mechanisms by proteins inactivation (GUTHMILLER & al. [34]; TELCIAN & al. [35]). The gelatinase activity was higher in Gram negative strains, being the second most expressed virulence factor after siderophore-like compounds. Phospholipases are implicated in the early steps of host invasion and can be associated with dysfunction and/or physical disruption of host cell membrane. In our study most of the screened oral microbial isolates produced lecithinasein vitro. They could act synergically with haemolysins, triggering host cells lysis and subsequent inflammation. In addition to acting as virulence factors, soluble enzymatic factors such as amylase and caseinase can also provide bacteria a competitive advantage for growth and multiplication in environments rich in carbohydrates and proteins (SIQUEIRA & al. [36]), DN-ases reduce the viscosity of debris from dead host cells (like in abscesses) and may thus allow the spread of bacteria within an area where extensive damage to host tissue has occurred. Other virulence factors can act as pseudo-siderophore providing bacteria with iron required for metabolism and for successful colonization and proliferation (CHIFIRIUC & al. [37]).

This research may be used in future studies on more specific aspects on this topic – microbiota in implant overdenture, in different treatment related periods (e.g., during osseointegration, in osseo-integrated implants), by the use of different materials, in different implant overdenture alternatives, in healthy condition or related to different complications.

Conclusions

Based on this research, considering its limitations, it is suggested that the cultivable oral microbiota associated to soft relining materials used around mini dental implants is very diverse and dominated by aerobic Gram negative bacteria, demonstrating the necessity of further studies to elucidate their role in implants failure. The majority of isolates proved to be highly biofilm producers, particularly the aerobic Gram negative bacteria.

More than half of bacterial isolates exhibited the ability to adhere to the cellular substratum and to produce siderophore-like compounds and exo-enzymes (gelatinases, pore-forming toxins, DN-ases) that could be involved in the peri-implant tissue lesions. Knowledge of dental implants associated microbiota diversity and of virulence characteristics is a key aspect for the understanding of the disease process, for selecting the best materials in terms of low colonization risk, and for establishing proper therapeutic measures to inactivate the microbial "artillery" and to prevent implant failure.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

All authors have equally contributed to this work and are main authors. This work was also supported by the grant of Ministry of Research and Innovation, CNCS – UEFISCDI, Project Number PNIII-P4-ID-PCE-2016-0884, within PNCDI III.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 20-29
doi: 10.25083/rbl/24.1/20.29

Received for publication: June 30, 2018
Accepted: October 2, 2018

Original paper

Preliminary research on getting callus in Ginkgo biloba L.

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Abstract

The purpose of such preliminary research has been represented by the establishment of the influence of treatments by growth regulators on Ginkgo biloba L. explants (taken from plants in their second year of vegetation), so as to get callus.

Therefore, one has tested the influence of three such growth regulator treatments (6-benzylaminopurine, 6-benzylaminopurine and α-naphthaleneacetic acid, or simply α-naphthaleneacetic acid), on nine types of cauline explants for the following purposes: pre-initiation of callogenesis, inducing and generating the callus, callus development and maintaining such callus development.

The best influence in order to get callus in Ginkgo biloba L., has been achieved in the case of the experimental variant consisting of the mixture between the treatment by α-naphthaleneacetic acid and the explants of the type taken from position 4 from rosette (MBAV5).

Keywords : Ginkgo biloba L., 6-benzylaminopurine, α-naphthaleneacetic acid, callus

To cite this article: LIVADARIU O, RAICIU AD, BARBU LDN. Preliminary research on getting callus in Ginkgo biloba L. *Rom Biotechnol Lett*. 2019; 24(1): 20-29. DOI: 10.25083/rbl/24.1/20.29

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Introduction

In terms of Ginkgo biloba L. the current taxonomic classification is as follows: Plantae Kingdom, Gymnosperms Phylum, Ginkgoaceae Family, Ginkgo L type, Ginkgo biloba L. Species (http://www.theplantlist.org/tpl1.1/record/kew-334053 [1]).

The medical and economic relevance of Ginkgo biloba L. species is well renowned and is mainly based upon the use of leaves in various products (e. g., pills, tea, powders, juice, ointments, lotions and shampoo).

Murashige and Skoog (1962) basal nutritive medium - T. Murashige& F. Skoog [2] - supplemented by various growth regulators has proven to have a significant influence on the quality and proliferation of callus in plants.

In terms of Ginkgo biloba L., as early as of 1991, one has grown Ginkgobiloba L. cells on MS basal nutritive medium (1962), duly supplemented by 1.0 mg/l NAA and 0.1 mg/l KIN in 500 ml vases, or in bio-reactors with immobilization and the volume of 2 l or 6 l (D.J. Carrier & al. [3]). Yet, the quantity of ginkgolid B has been low in order to positively confirm the presence of this particular product. Also in terms of Ginkgo biloba L. one has tested the getting of callus from various types of initial inoculi (e. g., seeds, embryos, cotyledon tissue and embryos with removed cotyledons), grown on MS basal nutritive medium (1962), duly supplemented by 2.5 g/l NAA or by 2.5 g/l 6-BAP. However, one has got callus only from the inoculi of the embryo or cotyledon type. And the largest amount of callus has been got from the inoculum of the cotyledon type under the influence exercised by NAA 2.5g/l (https://link.springer.com/article/10.1023/B:BIOP.0000033460.75432.d1 [4]).

In 2009, Hao and the collaborators have brought proof to the fact that nitric oxide from an exogenous source triggers a growth in the latter's bio-synthesis activity if added to cultures of callus from Ginkgo biloba L., which growth has caused a better signaling in terms of: phenylalanine ammonium lyase activity coordination, flavonoids biosynthesis and stress tolerance (G. Hao & al. [5]).

Treating the cultures of Ginkgo biloba L. cells in suspension by endophytic fungi has triggered some beneficial effects, such as: the accumulation of flavonoids and the increase of abscisic acid production (G. Hao & al. [6]).

In 2014, from the callus induced from the embryos obtained from Ginkgo biloba L. seeds, that were in the cotyledon stage and which were grown on MS (1962) environment, duly supplemented by 2.0 mg/l NAA and 1 mg/l 6-BAP, one has extracted flavonoids and terpene lactones (S. CHENG & al. [7]).

Starting from foliar explants obtained by the due fragmentation of young leaves taken in august from mature speciments of Ginkgo biloba L., one has established some cultures of immobilized cells in Ginkgo biloba L. The latter have been achieved by growing 2 g of friable callus on 40 ml of MS (1962) basal nutritive medium, duly supplemented by 2.0 mg/l NAA and 0.1 mg/l KIN, at a temperature of 25ºC, under agitation at 100 rpm and incubation in the dark. In the presence of the jute fibres and under the synergetic effect of salicylic acid and of methyl jasmonate, such cultures of immobilized cells have generated conditions which seem to be optimum for the production of in vitro ginkgolides and bilobalides (A. SUKITO & S. TACHIBANA [8]).

Considering a large amount of information from the specialized literature (e. g.: getting callus in Ginkgo biloba L. especially from embryo structures; each cell of a zygotic type is unique; the biosynthesis of certain substances is dependant upon the age of the relevant source-plant, the stage of vegetation, the phytosanitary state; the tendency to use cell cultures for the biosynthetisation of certain substances of a pharmaceutical interest in view of replacing the acknowledged source, etc.), we found it necessary for someone to conduct some researches into the subject so as to enable both the valorization of the unicity of Ginkgo biloba L. plants obtained from zygotic type seeds and the accomplishment of biochemical or /and genetic tests which shall highlight the profile of such unicity.

As a consequence, considering the aforementioned, the purpose of such preliminary research has been represented by the establishment of the influence of the treatment by growth regulators on the Ginkgo biloba L. explants (taken from plants obtained by the germination of the previous year seeds and in a state of vegetation), in order to get callus. Basically, one has tested the influence of three treatments by growth

regulators (6-benzylaminopurine, 6-benzylaminopurine and α-naphthaleneacetic acid, or simply α-naphthaleneacetic acid), on nine types of cauline explants for the following purposes: pre-initiation of callagenesis, inducing and generating the callus, callus development and maintaining such callus development.

Materials and Methods

The biological material and the work methodology have complied with the criteria that are well acknowledged in the vegetal biotechnologies field, namely: the biological material shall be under good phytosanitary condition and the work methodology shall be characteristic to the in vitro work conditions.

The primary source of explants has consisted of Ginkgo biloba L. plants obtained out of the germination of seeds from the previous year and which were in the vegetation period (spring).

For the purpose of conducting the pre-aseptizationat the surface, the Ginkgo biloba L. explants have been washed by continuous coldwater jet for 30 minutes and then immersed into an 80% sodium hypochlorite solution (B. E. MOGHADDAM & al. [9]), for 15 seconds. Then, for the same purpose of surface aseptization, the explants have been immersed into 1.5% sodium hypochlorite solution (B. E. MOGHADDAM & al. [9]), for 10 minutes. After the aseptization, the explants required three successive washing operations (of 10 minutes each), in aseptized distilled water (E. M. BADEA & D. SĂNDULESCU [10]; D. CACHIȚĂ-COSMA& al. [11]).

The basal nutritive medium Murashige Skoog (1962) - T. Murashige & F. Skoog [2] -, has been duly supplemented by two types of growth regulators: 6-benzylaminopurine and α-naphthaleneacetic acid (D.-J. Carrier & al., [3]; https://link.springer.com/article/10.1023/B:BIOP.0000033460.75432.d1 [4]; S. CHENG & al. [7]; A. SUKITO & S. TACHIBANA [8]). Basically, one has prepared 3 types of nutritive mediums that were based on the basal nutritive medium Murashige and Skoog (1962), duly supplemented by:

- 6-benzylaminopurine (2 mg/l) = *MB treatment*,
- 6-benzylaminopurine (1 mg/l) and α-naphthaleneacetic acid (1 mg/l) = *MBA treatment* and
- α-naphthaleneacetic acid (2 mg/l) = *MA treatment*.

Prior to autoclavation, the pH has been adjusted at the 5.3 value. The nutritive mediums aseptization has been carried out by autoclavation, at a temperature of 121°C, for 15 minutes.

The *culturing conditions* have been achieved by incubation in a device of the Sanyo type (Versatile environmental test chamber), at a temperature of 21°C ± 2°C, for the photoperiod of 16 hours and the air relative humidity of 70%.

The *experimental plan* has included the mixture of the three treatments, as above described (MB treatment, MBA treatment and MA treatment), with nine experimental variants for the explants type under focus. The nine experimental variants have consisted of cauline explants of the following type:

- apical bud + one part of the stem = V_1;
- leaf prelevated from the position 1 in the rosette = V2;
- leaf prelevated from the position 2 in the rosette = V3;
- leaf prelevated from the position 3 in the rosette = V4;
- leaf prelevated from the position 4 in the rosette = V5;
- leaf with detached sides prelevated from the position 5 in the rosette = V6;
- leaf damaged by longitudinal sectioning prelevated from the position 6 in the rosette = V7;
- apical leaf section prelevated from the position 7 in the rosette = V8 and
- basal leaf section prelevated from the position 7 in the rosette = V_9.

Practically, the experimental plan consisted of twenty-seven experimental variants.

The methods of analysis of the biological material consisted of morphometric determinations (the diameter of hyper-hydrated explant and the diameter of callus).

Statistical procedures. Each experimental variant consisted of 3 repetitions and the experiments were repeated twice. Measurements were made for each individual inoculum. The data were statistically analysed and the standard deviation of mean was calculated.

Results and Discussions

A. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the pre-initiation of callogenesis

A.1. The preliminary testing of the influence exercised by the treatment by 6-benzylaminopurine on *Ginkgo biloba L.* explants for the pre-initiation of callogenesis has triggered the hyper-hydration of explants and implicitly the enlargement of the latter's volume (Figure 1).

Based upon the experimental results shown in Figure 1 can see that the experimental variant MBV2, which has included the treatment by 6-BAP and explants of the leaf taken from the position 1 in the rosette, has triggered the highest average value of the diameter of hyper-hydrated explants. Whereas, the experimental variant MBV9, which consisted of the treatment by 6-BAP and explants of the type of basal leaf section taken from the position 7 in the rosette, has triggered the lowest average value of the diameter of hyper-hydrated explants.

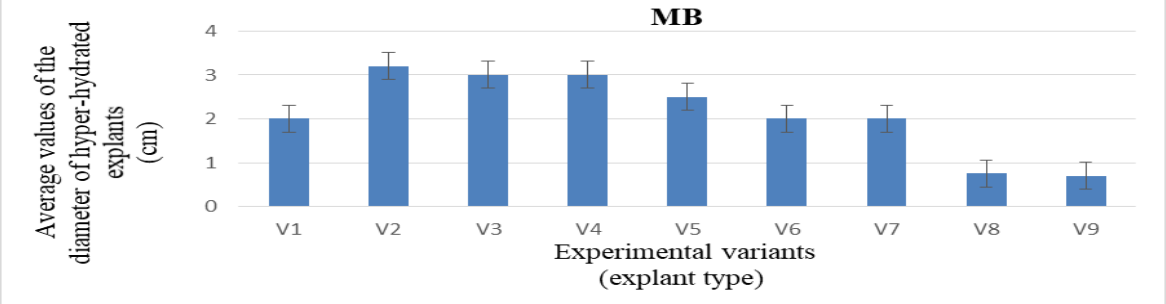

Figure1. Average values of the diameter of hyper-hydrated *Ginkgo biloba* L. explants obtained under the influence of treatment by 6-benzylaminopurine, subject to experimental variants

A.2. The preliminary testing of the influence exercised by the treatment by 6-benzylaminopurine and α-naphthalene acetic acid on Ginkgo biloba L. explants, for the pre-initiation of callogenesis has triggered both the hyper-hydration of explants and implicitly the enlargement of the latter's volume (Figure 2), which phenomenon has also been noticed in the case of the treatment by 6-BAP, as well as the intensification of the green color of explants.

Given the experimental results displayed in Figure 2 it follows that the experimental variant MBAV2, which consisted of the treatment by 6-BAP and NAA, and of explants of the leaf type taken from the position 1 in the rosette, has enabled the occurrence of the highest average value of the diameter of hyper-hydrated explants.

And the variant MBAV8, which included both the treatment by 6-BAP and NAA, and explants of the apical leaf section type taken from the position 7 in the rosette, has enabled the occurrence of the lowest average value of the diameter of hyper-hydrated explants.

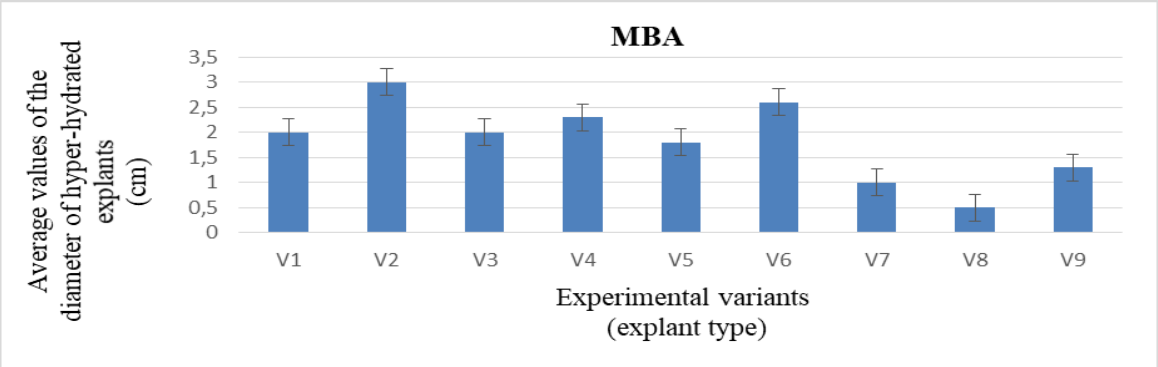

Figure 2. Average values of the diameter of hyper-hydrated *Ginkgo biloba* L. explants obtained under the influence of treatment by 6-benzylaminopurine and α-naphthaleneacetic acid, subject to experimental variants

A.3. The preliminary testing of the influence exercised by the treatment by α-naphthalene acetic acid on Ginkgo biloba L. explants, for the pre-initiation of callogenesis has triggered both the hyper-hydration of explants and implicitly the enlargement of the latter's volume (Figure 3), which phenomenon has also been noticed in the case of the other two treatments.
However, one has also noticed the intensification of the green color of explants, although such color has only be seen in the case of the treatment by 6-BAP and NAA.

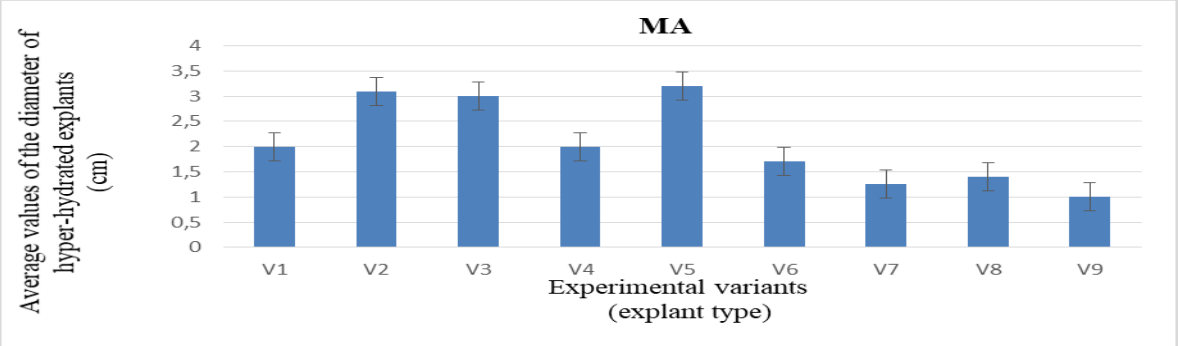

Figure 3. Average values of the diameter of hyper-hydrated *Ginkgo biloba* L. explants obtained under the influence of treatment by α-naphthalene acetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 3 can see that the experimental variant MAV5, which has included the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has triggered the highest average value of the diameter of hyper-hydrated explants. Whereas, the experimental variant MAV9, which consisted of the treatment by NAA and explants of the type of basal leaf section taken from the position 7 in the rosette, has triggered the lowest average value of the diameter of hyper-hydrated explants.

Reviewing the experimental results displayed in Figure 1, Figure 2 and Figure 3, one may notice that the preliminary testing of the influence of the treatment based on growth regulators on *Ginkgo biloba* L. explants, has enabled the modeling of explants metabolism so that one shall be able to carry out both the induction and the accomplishment of hyper-hydration. As one already knows from the renowned specialized literature, the aforementioned stand for the premises for the pre-initiation of callogenesis and, implicitly, the getting of callus.

B. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the induction and generation of callus

B.1. The preliminary testing of the influence of the treatment by 6-benzylaminopurine on *Ginkgo biloba* L. explants for the induction and generation of callus, has enabled the recording of experimental results (Figure 4), which show the inter-dependence between the treatment by 6-BAP and the type of explants, for callus materialization purposes. Inter-dependence which is also shown by the callus diameter.

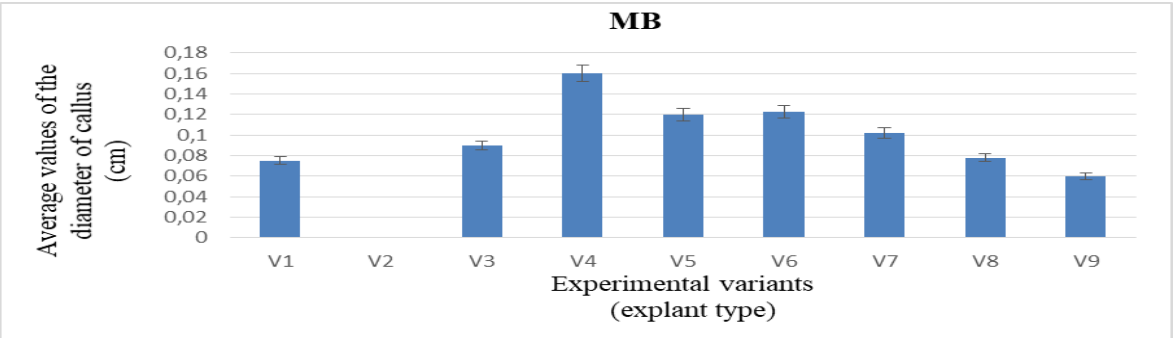

Figure 4. Average values of the diameter of callus from *Ginkgo biloba* L. obtained under the influence of treatment by 6-benzylaminopurine, subject to experimental variants

Given the experimental results displayed in Figure 4 it follows that the experimental variant MBV4, which consisted of the treatment by 6-BAP and of explants of the leaf type taken from the position 3 in the rosette, has enabled the occurrence of the highest average value of the diameter of callus. And the variant MBV9, which included both the treatment by 6-BAP and explants of the apical leaf section type taken from the position 7 in the rosette, has enabled the occurrence of the lowest average value of the diameter of callus. The recorded experimental results have also shown in case of the experimental variant MBV2, that there was a negative influence exercised by the treatment by 6-BAP on the explants of the leaf type taken from the position 1 in the rosette. Explants under the experimental variant MBV2 have shown senescence symptoms and they have been taken out of the experiment.

B.2. The preliminary testing of the influence of the treatment by 6-benzylaminopurine and α-naphthalene-acetic acid on *Ginkgo biloba* L. explants for the induction and generation of callus, has enabled the recording of experimental results (Figure 5), which show, just like in the previously described case, the inter-dependence between the treatment and the type of explants for the purpose of materializing the callus.

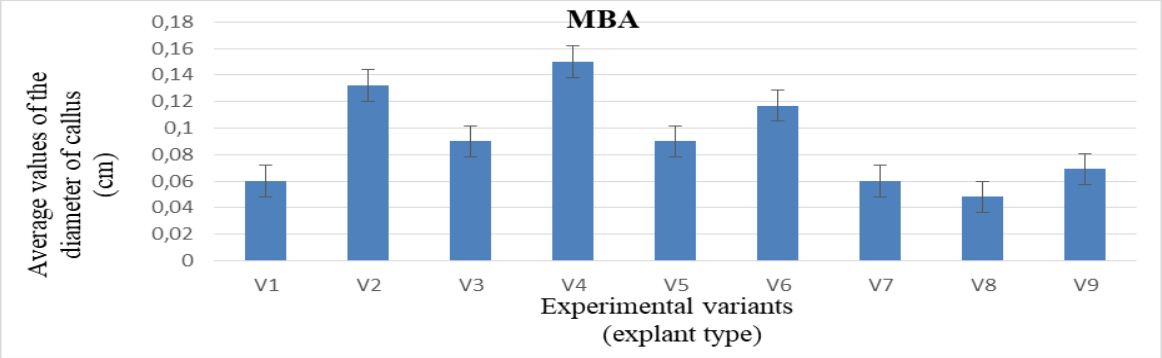

Figure 5. Average values of the diameter of callus from *Ginkgo biloba* L. obtained under the influence of treatment by 6-benzylaminopurine and *α-naphthaleneacetic acid*, subject to experimental variants

Based upon the experimental results shown in Figure 5 can see that the experimental variant $MBAV_4$, which has included the treatment by 6-BAP and NAA and explants of the leaf type taken from the position 3 in the rosette, has triggered the highest average value of the diameter of callus. Whereas, the experimental variant $MBAV_8$, which consisted of the treatment by 6-BAP and NAA and explants of the type of basal leaf section taken from the position 7 in the rosette, has triggered the lowest average value of the diameter of callus.

B.3. The preliminary testing of the influence of the treatment by α-naphthaleneacetic acid on Ginkgo biloba L. explants for the induction and generation of callus, has enabled the recording of experimental results (Figure 6), which shown, just like in the previously described two cases, the inter-dependence between the treatment and the type of explants for the purpose of materializing the callus. Yet, just like in the case of the treatment by 6-BAP, and unlike the treatment by 6-BAP and NAA, all the nine types of explants have induced and generated callus.

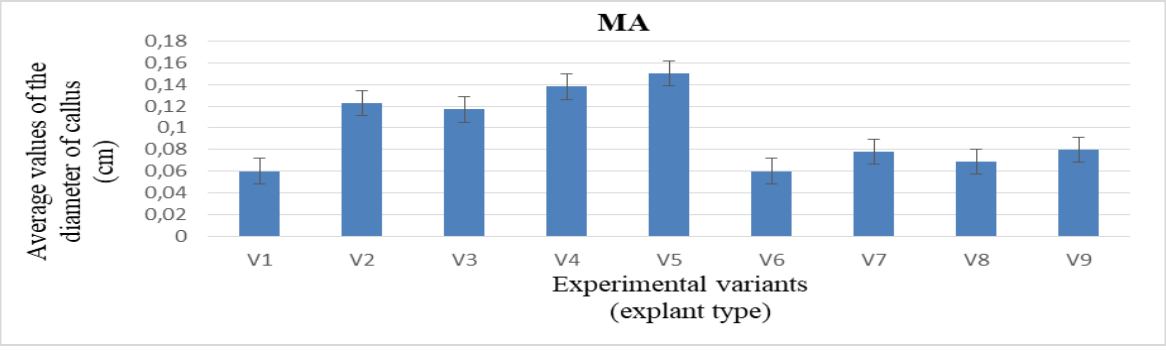

Figure 6. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 6 can see that the experimental variant MAV5, which has included the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has triggered the highest average value of the diameter of callus. Whereas both the experimental variant MAV1 (which consisted of the treatment by NAA and explants of the type of apical bud plus a part of the stem), and the experimental variantMAV6 (which consisted of the same treatment, yet applied to explants of the leaf type with detached margins taken from the position 5 in the rosette), have registered the lowest average value of the callus diameter.

C. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the callus development

C.1. The preliminary testing of the influence of the treatment by 6-benzylaminopurine on Ginkgo biloba L. explants for callus development, has enabled the recording of quantified experimental results by determination of the diameter of the callus, only in terms of the two experimental variants. Namely, the experimental variant MBV3 (which consisted of the treatment by 6-BAP and explants of the leaf type taken over from the position 2 in the rosette), which has had the highest average value of the callus diameter (0.078 ± 0.004 cm), and the experimental variant MBV1 (which consisted of the treatment by 6-BAP and explants of the type of apical bud plus a part of the stem) which has had the lowest average value of the callus diameter (0.100 ± 0.005 cm).

Explants cultivated under the other experimental variants (MBV2, MBV4, MBV5, MBV6, MBV7, MBV8 and MBV9), have shown senescence symptoms and have been taken out of the experiment.

C.2. The preliminary testing of the influence of the treatment by 6-benzylaminopurine and α-naphthaleneacetic acid on Ginkgo biloba L. explants for callus development, has enabled the recording of experimental results (Figure 7), which show the inter-dependence between the treatment and the type of explants for the purpose of materializing the callus.

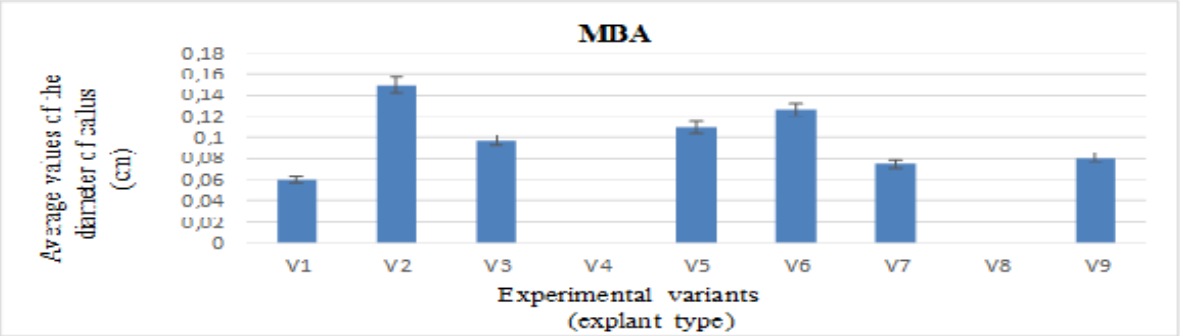

Figure 7. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by 6-benzylaminopurine and α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 7 can see that the experimental variant $MBAV_2$, which has included the treatment by 6-BAP and NAA, and explants of the leaf type taken from the position 1 in the rosette, has triggered the highest average value of the diameter of callus. Whereas, the experimental variant MBAV1, which included both the treatment by 6-BAP and NAA, and explants of the type of apical bud plus a part of the stem, has triggered the lowest average value of the diameter of callus. At the same time, the experimental variants MBAV4 and MBAV8 have not developed callus given the contamination with microorganisms of the fungus type. Both experimental variants that have been contaminated have been taken out of the experiment.

C.3. The preliminary testing of the influence of the treatment by α-naphthaleneacetic acid on *Ginkgo biloba* L. explants for callus development, has enabled the recording of experimental results which, just like in the case of the other two previously described treatments, support the idea of the existence a certain inter-dependence between the treatment and the relevant type of explants, for the purpose of materializing the callus (Figure 8).

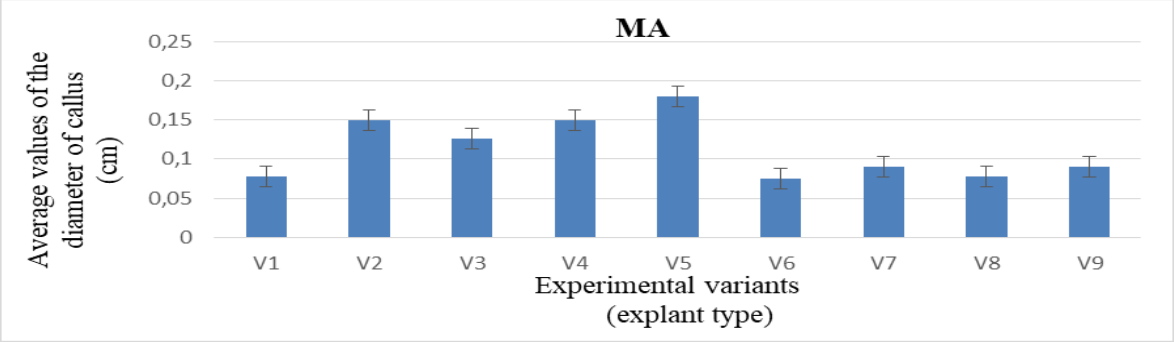

Figure 8. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 8 can see that the experimental variant MAV_5, which has included the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has triggered the highest average value of the diameter of callus.

Whereas, the experimental variant MAV6, which consisted of the treatment by NAA and explants of the type of leaf type taken from the position 5 in the rosette, has triggered the lowest average value of the diameter of callus.

D. The preliminary testing of the influence exercised by the treatments with growth regulators on Ginkgo biloba L. explants for the purpose of maintaining callus development

D.1. The preliminary testing of the influence of the treatment by 6-benzylaminopurine and α-naphthaleneacetic acid on Ginkgo biloba L. explants for the purpose of maintaining callus development, has enabled the recording of experimental results (Figure 9), which shape up the inter-dependence between the treatment and the type of explants, in view of maintaining such callus development.

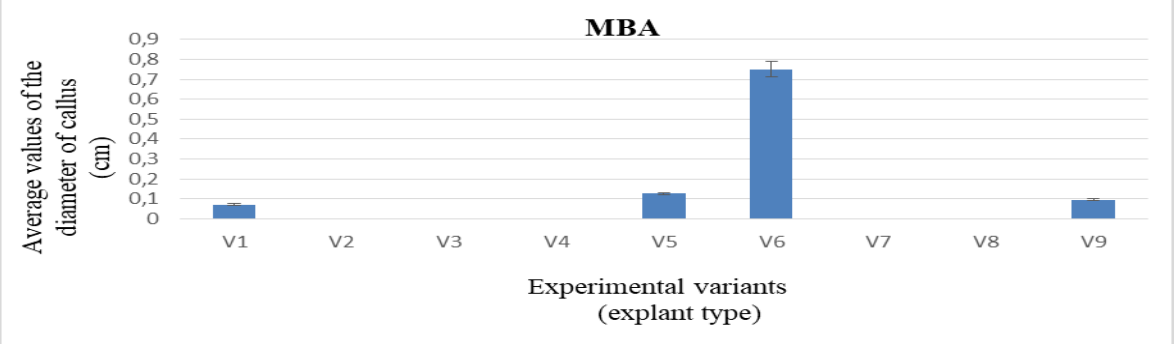

Figure 9. Average values of the diameter of callus from *Ginkgo biloba* L. obtained under the influence of treatment by 6-benzylaminopurine and α-naphthaleneacetic acid, subject to experimental variants

Based upon the experimental results shown in Figure 9 can see that the experimental variant MBAV6, which has included the treatment by 6-BAP and NAA, and explants of the type of leaf type taken from the position 5 in the rosette, has triggered the highest average value of the diameter of callus for the maintaining callus development.

And the variant MBAV1, which included both the treatment by 6-BAP and NAA, and explants of the type of apical bud plus a part of the stem, has enabled the occurrence of the lowest average value of the diameter of callus. Whereas the other experimental variants (MBAV2, MBAV3, MBAV4, MBAV7 and MBAV8), have been taken out of the experiment, given that they showed contaminations of the fungus type.

D.2. The preliminary testing of the influence of the treatment by α-naphthaleneacetic acid on *Ginkgo biloba* L. explants for the purpose of *maintaining callus development*, has enabled the recording of experimental results quantified by the determination of the callus diameter. The results in terms of callus development (Figure 10), show the inter-dependence between the treatment and the type of explants.

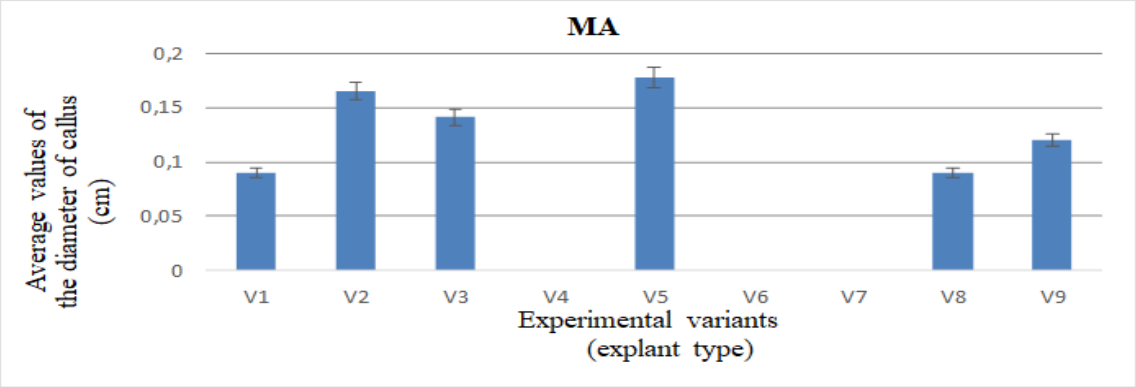

Figure 10. Average values of the diameter of callus from Ginkgo biloba L. obtained under the influence of treatment by α-naphthaleneacetic acid, subject to experimental variants

The experimental results shown in Figure 10 point out the fact that the experimental variant MAV5, namely the treatment by NAA and explants of the leaf type taken from the position 4 in the rosette, has recorded the highest average value of the callus diameter for the purpose of maintaining the callus development.

Whereas both the experimental variant MAV1 (which consisted of the treatment by NAA and explants of the type of apical bud and a part of the stem) and the experimental variant MAV8 (which consisted of the same treatment, however applied to explants of the apical leaf type taken from the position 7 in the rosette), have registered the lowest average value of the callus diameter. Since three of the experimental variants (MAV4, MAV6 and MAV7), showed some contamination of the fungus type, the same have been taken out of the experiment.

By reviewing the experimental results duly achieved in terms of the **pre-initiation of callogenesis, the induction and generation of callus, callus development and maintaining such callus development**, one may notice that the treatment by α-naphthaleneacetic acid has exercised the greatest influence on *Ginkgo biloba* L. explants in view of getting callus.

Conclusions

The treatment by 6-benzylaminopurine has had the best influence on the type of explants of the leaf type taken from the position 1 in the rosette (MBV2) as related to the pre-initiation of callagenesis in *Ginkgo biloba* L.

The treatment by 6-benzylaminopurine and α-naphthaleneacetic acid has exercised the best influence on the explants of the following type: leaf taken from the position 1 in the rosette (MBAV2) for the pre-initiation of callagenesis, leaf taken from the position 3 in the rosette (MBAV4) for the induction and generation of callus, leaf taken from the position 1 in the rosette (MBAV2) for callus development and leaf with detached margins taken from the position 5 in the rosette (MBAV6) for the purpose of maintaining callus development in *Ginkgo biloba* L.

The treatment by α-naphthaleneacetic acid has exercised the best influence on the explants of the leaf type taken from the position 4 in the rosette (MAV5) both for the pre-initiation of callagenesis, the induction and development of callus, callus development and for the maintenance of callus development in *Ginkgo biloba* L.

The best influence in terms of getting callus from *Ginkgo biloba* L. explants has been achieved in the case of the experimental variant that consisted of the combination of the treatment by α-naphthaleneacetic acid and the explants of the leaf type taken from position 4 in the rosette (MAV5).

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 30-40
doi: 10.25083/rbl/24.1/30.40

Received for publication: September 29, 2017
Accepted: January 28, 2018

Original paper

Optimization of microwave-assisted extraction of phenolics from blueberry

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Abstract

The influence of microwave-assisted extraction (MAE) on the recovery of phenolic compounds from blueberry was investigated. Response surface methodology (RSM) was applied to optimize the extraction conditions. Microwave power, extraction time and solvent to sample ratio were selected as extraction parameters. Ranges of independent variables were 100-300 W of microwave power, 2-16 min of extraction time and 5:1-50:1 ml/g of solvent to sample ratio. Responses of model were extraction yield and total phenolic content (TPC) and total anthocyanin content (TAC) for extraction of blueberry powder. Optimum conditions of MAE based on maximum levels of responses were 287 W of microwave power, 13 min of extraction time, 40:1 ml/g of solvent to sample ratio. Maximum levels of responses under optimum conditions obtained were an extraction yield of 78.35%, TPC of 30.75 mg GAE/ g blueberry powder and TAC of 8.92 mg Cyn-3-glu/ g blueberry powder.

Keywords : Microwave-assisted extraction, blueberry, phenolics, response surface methodology, optimization

To cite this article: AYSEL ELİK, DERYA KOÇAK YANIK, FAHRETTİN GÖĞÜŞ. Optimization of microwave-assisted extraction of phenolics from blueberry. *Rom Biotechnol Lett*. 2019; 24(1): 30-40. DOI: 10.25083/rbl/24.1/30.40

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Introduction

Blueberries (family Ericaceae; genus Vaccinium) are considered to be a rich sources of polyphenols, especially flavonoids such as anthocyanins (ELIK & al. [1]). Phenolics, especially anthocyanins, have been identified as having antioxidant properties which present beneficial effects in human health and prevent many degenerative diseases (BOBINAITĖ & al. [2]; NETO [3]). In addition to their antioxidant power, phenolics also have health benefits such as anti-inflammation action (JOSEPH & al. [4]), cancer enzyme inhibition (DUTHIE & al. [5]), antimutagenic activity (NILE & al. [6]), antimicrobial activity (DAGLIA [7]), neuroprotective property (DREISEITEL & al. [8]), nitric oxide production inhibition (DEL RIO & al. [9]) and chemoprotective enzyme inducement (YANG & al. [10]).

Extraction of natural phenolics using conventional methods is an expensive and time consuming process. Therefore, there have been numerous publications exploring that the use of modern extraction techniques such as ultrasound-assisted extraction (UAE), supercritical-fluid extraction (SFE), extraction by superheated water for food materials leads to time and solvent usage reduction, higher efficiency and increase of the extraction yields of valuable components (AZMIR & al. [11], GOGUS & al. [12]). One of the most promising techniques is microwave-assisted extraction (MAE) (CHAN & al. [13]). MAE utilizes microwave energy to heat solvents that are in contact with solid samples. In contrast with classical heating, the uniform heating by microwave energy allows for the sample to be heated. This allows for the solvent to heat rapidly, resulting in short extraction times. Furthermore, many studies have been reported that MAE can reduce solvent requirements, decrease extraction time and provide better extraction efficiency compared to conventional technique (JOKIĆ & al. [14]; NEMES & al. [15]; VENKATESH & al. [16]).

Studies about microwave assisted extraction from blueberry are limited in the literature (ZHENG & al. [17]). In the present study, phenolic extraction from blueberry using microwave technique has been investigated in detail. Therefore, the following points are considered in this study: (1) investigating the influences of MAE parameters (microwave power, extraction time and solvent to sample ratio) on the extraction yield, total phenolic content (TPC) and total anthocyanins content (TAC) (2) optimizing conditions of the MAE for the yield of extracts, TPC and TAC using response surface methodology.

Materials and Methods

Blueberry and reagents

Blueberries (Vaccinium Corymbosum, Bluecrop variety) were purchased from anorganic farm, Trabzon, Turkey. Blueberries were frozen at -40oC to perform freeze drying process. Whole blueberries were dried in a laboratory freeze-dryer (CHRIST Alpha 1-4 LDplus, Martin Christ, Germany) in 48 h. After drying, blueberries were ground and passed through to a 40-mesh sieve to produce blueberry powder. The moisture content of blueberry powder was under 1%. The freeze-dried blueberry powder was stored in airtight containers in refrigerated conditions until being used.

Folin–Ciocalteu's phenol reagent, ethanol, citric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trolox were purchased from Sigma-Aldrich. 2,4,6-tripyridyls-triazine (TPTZ) was purchased from Fluka. All other reagents and solvents used were of analytical or chromatographic grade.

MAE procedure

A quantity (quantity was varied as a function of solvent to sample ratio) of blueberry powder was weighed into the flask and mixed with ethanol–water mixture (60:40, v/v). Total volume of extraction solvent was kept constant at 72 ml. The sample solvent mixture was heated by application of microwave for a fixed amount of time and at a fixed power. MAE was carried out with a focused open-vessel microwave system (CEM Corporation, USA, 3100 Smith Farm Road, Matthews, NC 28105-5044). The microwave power level and the extraction time for which microwave was applied were selected according to the experimental design (Table 1). Upon completion of extraction process, extracts were transferred to centrifuge tubes and centrifuged at 6000 rpm at 25oC for 15 min and liquid portion has been collected.

Subsequently, solvent was evaporated using the rotary vacuum evaporator (Model VV 2000, Heidolph, Germany). Residual solvent in the extracts was evaporated using a freeze drier and then dry extracts were stored at -18oC until the analyses were performed.

Determination of moisture content

3-5 g of sample were weighed on aluminum tray and put in a drying oven at 105oC until achieving constant weight. The dried blueberries were weighed and the dried matter that remained was determined. All results were expressed on dry matter (DM) basis.

Table 1. Three-factor, three-levelface-centered central composite designand results for three variables studied

Standard Order	Microwave Power (W)	Extraction Time (min)	Solvent to sample Ratio (ml/g)	Extraction Yield[1] (%)	TPC (mg GAE/ g blueberry powder)	TAC (mg Cyn-3-glu/ g blueberry powder)
1	100	2	5:1	65.08	14.58	5.14
2	300	2	5:1	66.25	16.30	6.11
3	100	16	5:1	65.19	15.63	5.29
4	300	16	5:1	68.90	18.96	6.32
5	100	2	50:1	76.53	21.66	7.16
6	300	2	50:1	73.76	24.98	7.51
7	100	16	50:1	77.48	29.59	8.80
8	300	16	50:1	77.78	29.12	8.73
9	100	9	27.5:1	74.46	26.07	7.18
10	300	9	27.5:1	77.13	27.88	8.05
11	200	2	27.5:1	73.60	25.88	7.63
12	200	16	27.5:1	74.22	28.03	7.88
13	200	9	5:1	65.18	15.95	5.68
14	200	9	50:1	75.11	26.49	7.97
15	200	9	27.5:1	74.81	28.74	7.80
16	200	9	27.5:1	74.63	28.40	7.83
17	200	9	27.5:1	74.99	28.40	8.49
18	200	9	27.5:1	74.42	27.01	7.91
19	200	9	27.5:1	75.55	28.52	7.77
20	200	9	27.5:1	75.68	28.56	7.73

[1] (g dry extract/g blueberry powder)x100

1. *Total phenolic content (TPC) by Folin-Ciocalteu's assay*

The Folin-Ciocalteu method was used to determine total phenol levels in samples, which was adapted from SINGLETON &al. ([18]). TPC values were expressed as gallic acid equivalents (GAE) in mg per g of the powder. All measurements were done in triplicate.

2. *Total anthocyanin content (TAC) by pH-differential method*

The pH-differential method was used to determine anthocyanins content of extracts (LEE & al. [19]).

Total anthocyanins of samples were expressed as the amount of cyanidin-3-glucoside equivalents with unit of mg per g powder. All measurements were done in triplicate.

3. DPPH-scavenging activity assay

The DPPH radical scavenging activity of samples was measured according to the method of BRAND-WILLIAMS & al. ([20]). The results were expressed as a DPPH free radical scavenging activity EC50 value, which reflects 50% depletion of the free radical. DPPH tests were done in triplicate.

4. Ferric reducing antioxidant power (FRAP) assay

Antioxidant powers of samples were determined the FRAP assay, which is based on ferric to ferrous reduction in the presence of 2,4,6-tripyridyls-triazine (TPTZ) (BENZIE & al. [21]). Results were expressed as trolox equivalents (TE) in μmoles per g of the blueberry powder. All measurements were done in triplicate.

5. Experimental Design and Optimization by Response Surface Methodology

Response surface methodology (RSM) was chosen to determine the optimal conditions for MAE from blueberry. The RSM was performed using Design Expert (Stat-Ease, Design-Expert software, version 7). The effect of the independent variables; microwave power (100-300 W), extraction time (2-16 min) and solvent to sample ratio (5:1-50:1 ml/g) was investigated using a three-factor, three-level face-centered central composite design (FCCCD). The complete design consists of 20 runs, including six replications of the centre points for the three independent variables. Table 1 shows the effect of microwave power (100-300 W), the solvent to sample ratio (5:1-50:1 ml/g) and extraction time (2–16 min) on the extract yield, total phenolic content and total anthocyanin content of blueberry extracted by MAE.

The fitness of the model was determined by evaluating the Fisher test value (F-Value), and the coefficient of determination (R2) as obtained from an analysis of variance (ANOVA). The level of significance for all tests was set at 95% confidence level. FCCCD uses least-squares regression to fit the experimental data to a quadratic model. The quadratic model for the responses is as follows: (1) where Y are the dependent variable, β0, βi, βii and βij are the regression coefficients for the intercept, linear, quadratic and interaction terms of variables i and j, respectively, and Xi and Xj are the independent variables.

Results and Discussions

Preliminary studies

Extraction parameters chosen (microwave power, extraction time and solvent to sample ratio) in this study were based on preliminary experiments and previous studies (ZHENG & al. [17]; MANDAL & al. [22]). Microwave power was operated in range of 100-300 W.

Microwave power above 300 W was caused some troubles such as foaming and charring of blueberry. Studied extraction time ranged from 2 to 16 min. Longer extraction time did not cause considerable extraction of phenolics from blueberry. 5:1-50:1 ml/g of solvent to sample ratio was chosen. Higher ratio than 50:1 ml/g of solvent to sample ratio did not show significant recovery of phenolics and also caused waste of solvent.

In general, organic solvents such as methanol, ethanol, acetone and ethyl acetate are used for extraction of phenolic compounds. Organic solvents, however, such as methanol are potentially detrimental to human health. The use of ethanol has many advantages as it has high extraction efficiency and dielectric constant, low toxicity and cost (WU & al. [23]).

Therefore, ethanol was selected as extraction solvent for experiments. Aqueous solvent is considered more efficient than the pure solvent in phenolic extraction (BELWAL & al. [24]).

Extraction solvent with an ethanol-water ratio of 60:40 (v/v) was kept constant in all experiments due to obtaining highest yield of phenolics in preliminary experiment. Acidified solvents can increase extraction yield (BRIDGERS & al. [25]). Citric acid was used to obtain better yield in extraction.

Model fitting

Response surface models were used for experimental design in MAE. Regression analysis with backward elimination was used to obtain best fitting quadratic models. The backward elimination was employed to eliminate insignificant factors and interactions in the models. The regression models were significant ($p<0.001$) for MAE from blueberry powder with satisfactory coefficient of determination (R2) that were 0.9826 for the extraction yield, 0.9763 for TPC and 0.9653 for TAC.

Large F value and small value of probability of models also confirm that models are significant (Table 2). Relationship between independent variables and responses could be expressed by the following Eq. (2 - 4).

The extraction yield of blueberry as a function of the independent variables:

$$Y_1 = 74.72 + 0.51*Mw + 0.83*Ti + 5.01*Ra + 0.70*Mw*Ti - 0.92*Mw*Ra + 1.14*Mw^2 - 4.51*Ra^2 \quad (2)$$

TPC of blueberry as a function of the independent variables:

$$Y_2 = 27.75 + 0.97*Mw + 1.79*Ti + 5.04*Ra + 1.05*Ti*Ra - 6.42*Ra^2 \quad (3)$$

TAC of blueberry as a function of the independent variables:

$$Y_3 = 7.83 + 0.31*Mw + 0.35*Ti + 1.16*Ra - 0.21*Mw*Ra + 0.31*Ti*Ra - 0.96*Ra^2 \quad (4)$$

where Y_1, Y_2 and Y_3 are responses of models, Mw is microwave power, Ti is extraction time and Ra is solvent to sample ratio.

3D response surface and contour plots were used to show the effects of process variables on the responses. The response surface and contour plots indicated the effect of two variables on the dependent variable described by the quadratic polynomial equation while third variable was kept constant at middle level, 9 min for extraction time, 200 W for microwave power, 27.5:1 for solvent to sample ratio.

The effect of process variables on the extraction yield

Various factors showed significant effect on the extraction yield. Microwave power was evaluated in terms of the effect on the extraction yield. From Table 2, it was observed that microwave power has significantly ($p<0.05$) positive linear and quadratic effects on extraction yield. It could be explicated that increase in microwave power results in increased extraction yield. Increase in microwave power results in the rupture of the cell walls and enhance the extraction due to easier penetration of the solvent into the plant matrix (MENDES & al. [26]).

Microwave power had also an interaction effect with other process variables. The interactive effect between microwave power and extraction time was significant ($p<0.05$) and positive. It means that extraction yield obtained increases significantly as microwave power and extraction time increase simultaneously (Figure 1). In the study carried out by MILUTINOVIC & al. ([27]), a similar trend was found for interaction between microwave power and extraction time. Besides, it was shown that the interactions between microwave power and solvent to sample ratio affected extraction yield significantly (Table 2). As this interaction has negative constant coefficient, the effect of microwave power on extraction yield was negative or positive depending on solvent to sample ratio.

When the effect of extraction time on extraction yield is examined, it is able to understand that time is a more effective independent variable than microwave power due to its greater constant coefficient (Eq. 2). Extraction time displayed quite significant ($p<0.05$) positive effect on the extraction yield. It is concluded that longer extraction time had positive effects on the yield of extraction.

The linear effect of solvent to sample ratio was positive and significant ($p<0.05$) while its quadratic effect was significant and negative, which resulted in a curvilinear increase in extraction yield (Figure 1A and1B). The extraction yield went up rapidly up to

40:1 ml/g as solvent to sample ratio increases. After that point, however, the further increase of solvent to sample ratio affected the extraction yield negatively. When it was used large volume of solvent, it caused excessive swelling of the materials and extraction efficiency increased (MILUTINOVIĆ & al. [27]). However, in case of further increase in solvent volume, the most of microwave energy was absorbed by extraction solvent and very little microwave energy absorbed directly by the materials (YAN & al. [28]). Therefore, this phenomenon leads to the less efficient extraction.

Table 2. Analysis of variance (ANOVA) of responses for MAE experiments

Source	The extraction yield					TPC					TAC				
	SS[1]	DF	MS	F-Value	p-value Prob > F	SS[1]	DF	MS	F-Value	p-value Prob > F	SS[1]	DF	MS	F-Value	p-value Prob > F
Model	348.09	7	49.73	96.70	< 0.0001[2]	510.81	5	101.21	115.42	< 0.0001[2]	21.44	6	3.57	60.3	< 0.0001[2]
Intercept															
Linear															
Micro-wave Power (Mw)	2.58	1	2.58	5.02	0.0448[2]	9.43	1	10.02	10.63	0.0045[2]	0.99	1	0.99	16.74	0.0013[2]
Extraction Time (Ti)	6.97	1	6.97	13.56	0.0031[2]	32.15	1	31.08	36.37	< 0.0001[2]	1.2	1	1.2	20.32	0.0006[2]
Solvent to sample ratio (Ra)	250.6	1	250.6	487.32	< 0.0001[2]	254.22	1	251.2	287.18	< 0.0001[2]	13.53	1	13.53	228.23	< 0.0001[2]
Interac-tion															
Mw*Ti	3.93	1	3.93	7.65	0.0171[2]										
Mw*Ra	6.75	1	6.75	13.13	0.0035[2]						0.37	1	0.37	6.24	0.0267[2]
Ti*Ra						8.74	1	8.74	9.87	0.0072[2]	0.78	1	0.78	13.16	0.0030[2]
Quadra tic															
Mw^2	4.17	1	4.17	8.11	0.0147[2]										
Ti^2															
Ra^2	65.03	1	65.03	126.47	< 0.0001[2]	206.27	1	206.27	233.1	< 0.0001[2]	4.57	1	4.57	77.11	< 0.0001[2]
Residual	6.17	12	0.51			12.39	14	0.88			0.77	13	0.0059		
Lack of Fit	4.9	7	0.7	2.75	0.1418[3]	10.40	9	1.16	2.91	0.1264[3]	0.36	8	0.046	0.56	0.7775[3]
Pure Error	1.27	5	0.25			1.99	5	0.4			0.41	5	0.081		
Cor Total	354.26	19				523.19	19				22.21	19			
R^2	0.9826					0.9763					0.9653				
Adj R^2	0.9724					0.9679					0.9493				
Pred R^2	0.9462					0.9371					0.9294				

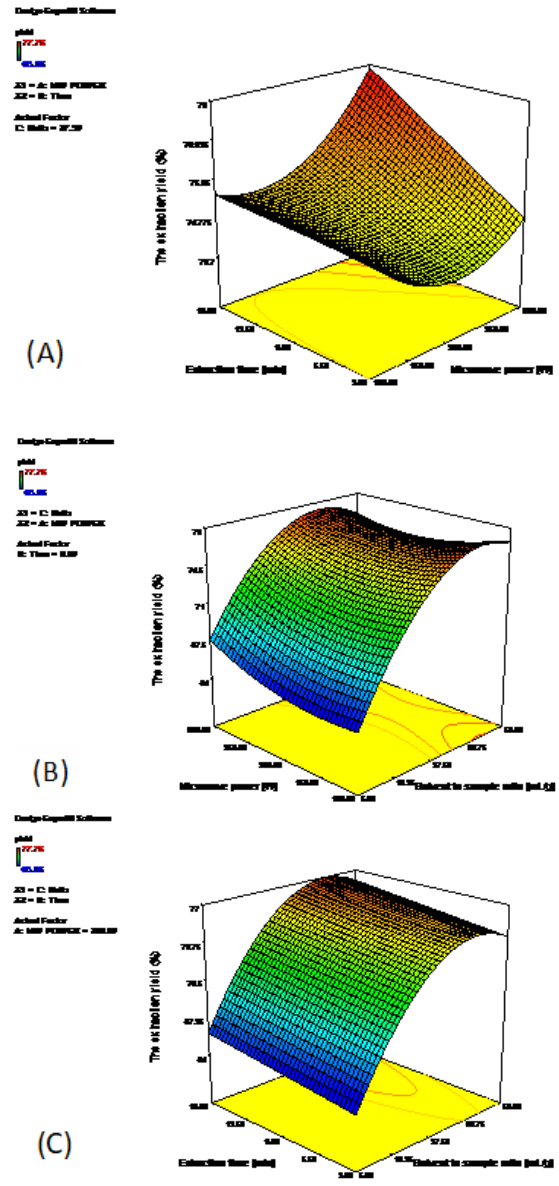

Figure1. Response surface plots of extraction yield as affected by extraction time, microwave power and solvent to sample ratio in MAE. (A) extraction time (X1) and microwave power (X2); (B) microwave power (X1) and solvent to sample ratio (X2); (C) extraction time (X1) and solvent to sample ratio (X2). The value of the missing independent variable in each plot was kept at the centre point

The effect of process variables on TPC

Microwave power showed a positive linear effect on TPC ($p < 0.05$). It indicates that increase in TPC is noted when microwave power increased from 100 to 300 W (Figure2A and 2B). However, the effect of microwave power was the least effective independent variable compared to other two variables, namely extraction time and solvent to sample ratio.

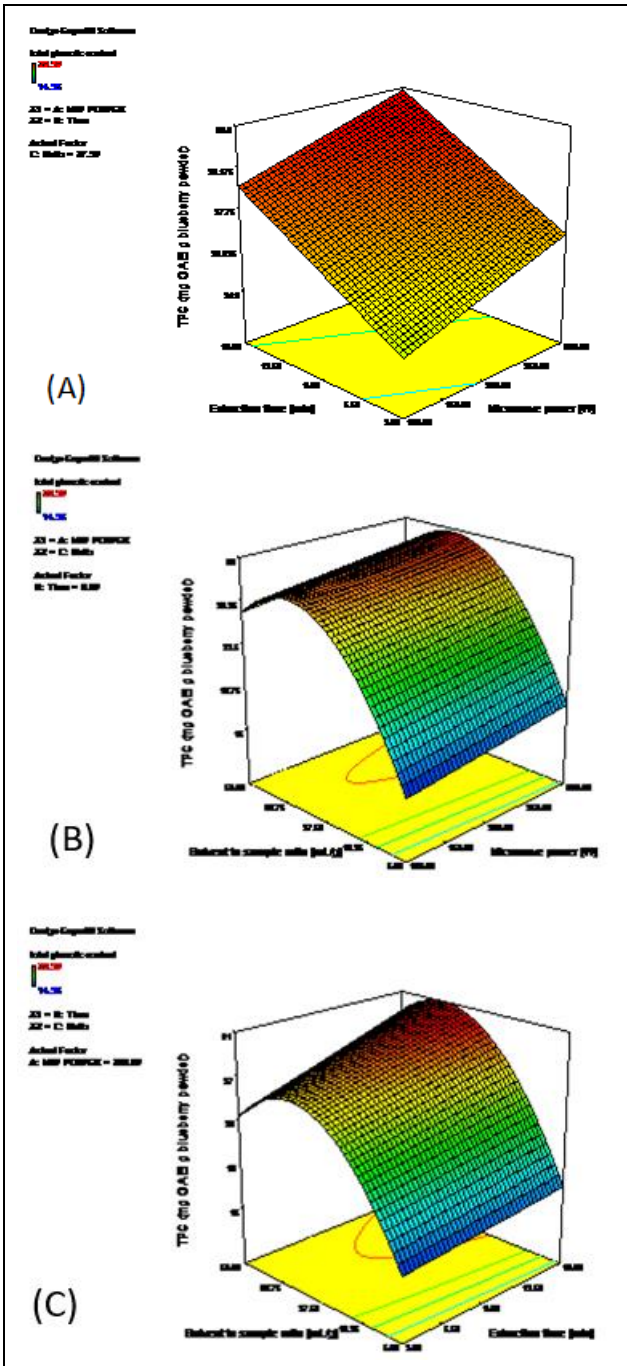

Figure 2. Response surface plots of TPC as affected by extraction time, microwave power and solvent to sample ratio in MAE. (A) extraction time (X1) and microwave power (X2); (B) solvent to sample ratio (X1) and microwave power (X2); (C) solvent to sample ratio (X1) and extraction time (X2). The value of the missing independent variable in each plot was kept at the centre point

ANOVA results showed linear effect of extraction time ($p<0.05$) on TPC. It can be interpreted that as extraction time increases, amount of phenolics extracted from blueberries also increase. Table 2 reveals that the interaction effect between extraction time and solvent to sample ratio is significant ($p<0.05$).

As seen in Figure 2B, more yield of phenolics extracted was resulted at higher solvent to sample ratio and microwave power. Solvent to sample ratio was the most important independent variable on TPC. As shown in Table 2, solvent to sample ratio had significant ($p<0.05$) positive linear and negative quadratic effect on TPC. This effect resulted in a curvilinear increase in TPC as in the extraction yield. LI & al. ([29]) reported that solvent to sample ratio was the most effective parameter in MAE of polyphenols from grape seed.

The effect of process variables on TAC

It was found that microwave power significantly influenced TAC as in TPC. It depicts that the increase in microwave power improves the yield of TAC. Negative interaction between microwave power and solvent to sample ratio was observed for TAC. (Figure 3B).

An ANOVA of extraction time indicated positive linear effect on TAC. Anthocyanins extracted increased linearly with extraction time. There were also significant ($p<0.05$) positive interaction effects of extraction time and solvent to sample ratio, indicating that TAC increases considerably with increase in solvent to sample ratio and extraction time.

Solvent to sample ratio was also observed as the most efficient factor for TAC as it has been observed for two other responses. Table 2 demonstrated positive linear and negative quadratic effects of solvent to sample ratio on TAC.

Solvent to sample ratio up to 40:1 ml/g caused increase in TAC however, further increase in solvent to sample ratio led to decline of quantity of extracted anthocyanins from blueberries. A similar observation was also reported in the study of ZHENG & al. ([17]).

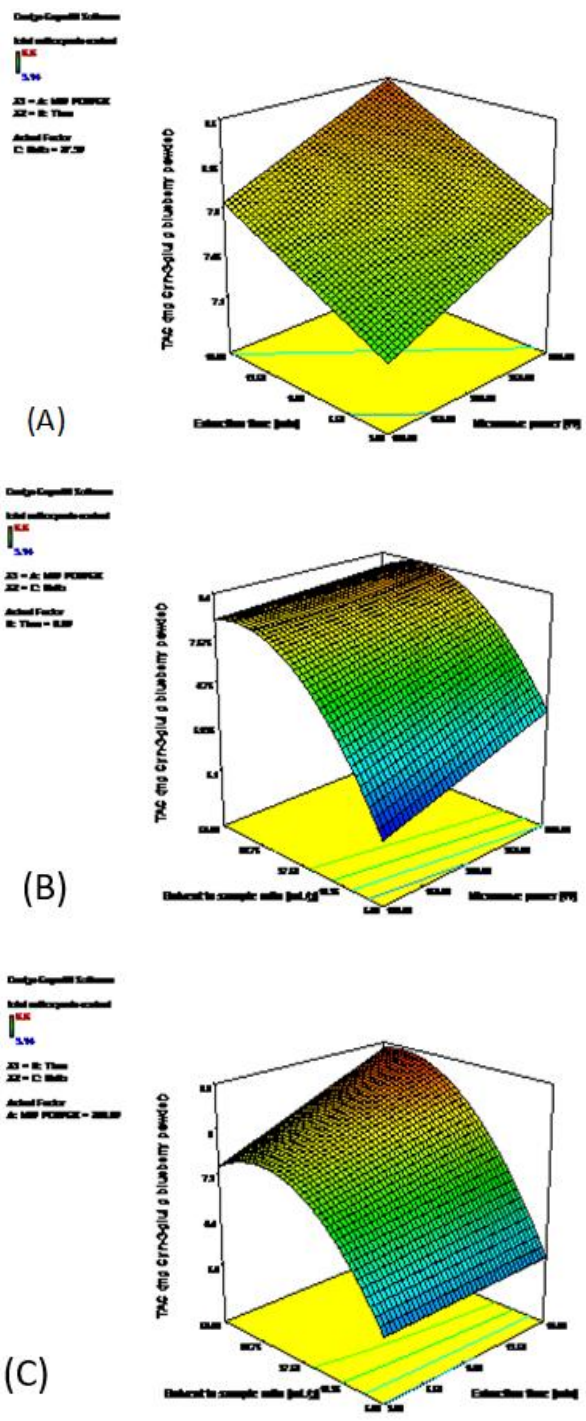

Figure 3. Response surface plots of TAC as affected by extraction time, microwave power and solvent to sample ratio in MAE. (A) extraction time (X1) and microwave power (X2); (B) solvent to sample ratio (X1) and microwave power (X2); (C) solvent to sample ratio (X1) and extraction time (X2). The value of the missing independent variable in each plot was kept at the centre point

Model optimization and verification

FCCCD was used for optimization of extraction parameters. Optimization of the MAE conditions was based on achieving the highest extraction yield, TPC and TAC. The optimized conditions for blueberry powder were 287 W of microwave power, 13 min of extraction time, 40:1 ml/g of solvent to sample ratio with the predicted extraction yield of 78.35%, TPC of 30.75 mg GAE/g blueberry powder and TAC of 8.92 mg Cyn-3-glu/ g blueberry powder.

Predicting the optimum response values is tested to determine the accuracy of the model using the selected optimum conditions. The experiments were carried out in triplicates and the average values were found as 78.47%, 30.38mg GAE/g blueberry powder and 8.78mg Cyn-3-glu/ g blueberry powder for extraction yield, TPC and TAC, respectively. The experimental results were quite close to the predicted ones. Consequently, it can be seen that indicating the RSM model was satisfactory and accurate.

Properties of berry extracts obtained at optimum conditions

Blueberry extracts were obtained at optimum conditions and total phenolic content (TPC), total anthocyanin content (TAC) and antioxidant activity (DPPH and FRAP) of extracts were determined. Table 3 shows TPC, TAC and antioxidant activity of blueberries before and after extraction. It can be understood from those results that 86.9%of phenolics of blueberry could be extracted by MAE. Anthocyanin content of blueberry powder constituted approximately 29% of total phenolics. Anthocyanins can be easily degraded by some factors such as temperature and long processing time (MANDAL & al. [22]; MARTYNENKO & al. [30]). Results from Table 3 have shown that most of anthocyanins content (87.1%) in blueberry could be extracted effectively by MAE without degrading. It might be explained by short processing time of MAE. Besides having rich phenolic content in extracts, their high antioxidant capacities were also very high. One of the most common method to evaluate antioxidant activity of extracts is the DPPH-scavenging activity assay which relies on the decrease of DPPH• absorbance at 517 nm induced by antioxidants. EC50 values (the concentration needed to decrease by 50% the initial DPPH concentration) of blueberry powder and extract were found 0.89 and 1.03 mg blueberry powder/ml, respectively. It indicates that blueberry extracts were very strong DPPH• scavengers. FRAP which is another common method to evaluate antioxidant activity was used to determine antioxidant activity of extracts. The FRAP values of blueberry powder and extracts were 277.57 and 242.28 μmoles TE/g blueberry powder, respectively. The FRAP values of blueberry powder and extracts are pretty close to each other. Both results of DPPH and FRAP demonstrated that blueberry extracts show high antioxidant activity. This is due to extracting high amount of phenolics, especially anthocyanins from blueberry powder.

Table 3. Comparison of TPC, TAC, DPPH and FRAP values before and after extraction

	TPC [1]	TAC [2]	DPPH·EC_{50} [3]	FRAP [4]
Before extraction (BE)	35.36	10.24	0.89	277.57
After extraction (AE)	30.75	8.92	1.03	242.28

[1]mg GAE/ g blueberry powder
[2]mg Cyn-3-glu/ g blueberry powder
[3]mg blueberry powder/ ml (EC_{50} =Blueberry concentration needed to decrease by 50% the initial DPPH concentration)
[4]μmoles TE/g blueberry powder

Conclusions

Optimization of MAE for TPC, TAC and the yield of extracts was successfully investigated using response surface methodology. The experimental values agreed with the predicted values, thus proving satisfactory and accurate of RSM model. All independent variables (microwave power, extraction time and solvent to sample ratio) demonstrated a significant effect on MAE from blueberry powder. The most effective variable on all responses was solvent to sample ratio, followed by extraction time and the least was microwave power. High amount of phenolics could be extracted from blueberry by using MAE.

Besides, it was found that extracts under optimum conditions had high antioxidant capacity. High-quality extracts was obtained in a short time using MAE. Our results showed that MAE was a fast and effective extraction technique and has a great potential to be used in phenolics extraction.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this articles; manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

Financial support for this project is provided by funding bodies within the FP7 ERA-Net CORE Organic Plus (618107), and with cofunds from the European Commission. We would also like to thank Bursa central research institute of food and feed control for financial support.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 41-49
doi: 10.25083/rbl/24.1/41.49

Received for publication: December 10, 2018
Accepted: January 4, 2019

Original paper

***Lactobacillus* spp. and *Enterococcus faecium* strains isolation, identification, preservation and quantitative determinations from turkey gut content**

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Abstract

From gut content (ileum and cecum) of eight turkeys, 73 days old, was isolated, phenotypic identified and preserved, nine strains from *Lactobacillus* genus (*L. acidophilus* IBNA 11, *L. acidophilus* biotype 3 IBNA 12, *L. fermentum* biotype 1, IBNA 13-18 and *L. salivarius* IBNA 19) and one strain of *Enterococcus faecium* (IBNA 10).

The parallel identification of strains was made by apiwebTM API50CHL V.5.1, BioMerieux (France) software, API20STREP, and ABIS online software. Quantitative level of *Lactobacillus* spp. strains (10^6 – 10^9 CFU/g intestinal content) and *Enterococcus faecium* strain (10^6CFU/g intestinal content) in ecological niche was determined.

The viability of *Lactobacillus* spp. strains preserved at 4^0C (from 45 to 90 days) and at room temperature (under 90 days), and *Enterococcus faecium* at 4^0C (more than 11 months) was tested.

Keywords : Lactobacillus spp., Enterococcus faecium, gut, turkey

To cite this article: SORESCU I, DUMITRU M, CIURESCU G. Lactobacillus spp. and Enterococcus faecium strains isolation, identification, preservation and quantitative determinations from turkey gut content. *Rom Biotechnol Lett*. 2019; 24(1): 41-49. DOI: 10.25083/rbl/24.1/41.49

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Introduction

Lactobacillus spp. and *Enterococcus* spp. strains are part of the normal avian intestinal microbiota (R.M. DUAR & al. [1], D.W. WAITE & M.W. TAILOR [2], S. WEI & al. [3], J. Lu & al., [4]) with beneficial effects for host health. The lactobacilli have symbiotic role in host fitness, their enzymes and metabolites aid in the digestive process and organic acids produced from fermentation prevent pathogens (R.M. DUAR & al. [1], M. DUMITRU & al., [14], M. DUMITRU & Ș. JURCOANE [15]). *Lactobacillus* account 7% of bacterial 16S rRNA gene sequences recovered from turkey gut (S. WEI & al. [3]). Initial poultry caecal microbiota, derived from shell contaminants, consists predominantly of *Enterococcus*, coliforms and clostridia (M.E. COATES & R. FULLER [5]). Starting with the fourth day afterhatching from the egg, *Lactobacillus* becomes a significant component of the intestinal microbiota (X.Y. ZHU & al. [6]).

The aim of our work was to isolate, identify and preserve the *Lactobacillus* and *Enterococcus* strains from turkeys gut content, for subsequent ”in vitro” and ”in vivo” testing of their probiotic characters and selection of strains as intestinal flora stabilizers in turkey nutrition.

Materials and Methods

Birds were treated in accordance with Romanian legislation (law no. 305/2006) for handling and protection of animals used for experimental purposes (G. CIURESCU & O. PANĂ, [8]).

1. *Isolation of bacterial strains and determination of CFU/g intestinal content*

The MOUNTZOURIS method (K.C. MOUNTZOURIS & al. [7]) was followed, which was completed with Gram staining of smears from colonies appearing on selective media for their morphological confirmation by microscopic examination. Sample preparation: 1 g of intestinal content (ileum and cecum, respectively) per capita from eight turkeys (73 days old) was homogenized with 7 ml Oxoid BHI (Brain Heart Infusion) broth and 2 ml glycerol, and immediately frozen at – 20ºC until testing (no more three months). After defrost, decimal dilutions in Oxoid PBS (Phosphate Buffered Saline) were performed, and 0.1 ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions, from every sample was inoculated on three Petri dishes with Oxoid MRS (Man, Rogosa, Sharpe) agar.

The MRS agar plates were cultural examined. The colonies of each cultural type were counted after 48 hours of anaerobic incubation in Oxoid jar with Anaerogen 2,5 L at 37ºC. Gram stained smears of each colony type were performed, for examining morphological characters and morphological confirmation of *Lactobacillus* or *Enterococcus* classification.

The catalase test was performed (negative for each genus) and CFU/g gut content was determined, as well. From every performed *Lactobacillus* colony type was inoculated one colony in one tube with Oxoid MRS broth for strains isolation and incubated 24-48 hours, in aerobic atmosphere, at 37ºC. From every presumptive Enterococcus colony type was inoculated one colony in one tube with Oxoid BHI broth for strains isolation and incubated 24 hours, aerobically, at 37ºC.

2. *Identification of bacterial strains*

Phenotypic identification of isolated bacterial strains was performed by morphological, cultural and biochemical characters examination, according to Bergey's Manual of Systematic Bacteriology (13), ABIS on line software (10), apiwebTM API50CHL software and API 20STREP BioMerieux (France). The results obtained by D.R. PELINESCU & al. [9] were also considered.

Morphological characters of isolated strains were analysed by Gram staining method. Cultures were grown in MRS or BHI broth, 24-48 h, at 37ºC, in aerobic conditions, and 48 h in anaerobic atmosphere, on MRS agar. Mainly, the shape of bacteria, tinctorial affinity, sporulation, grouping and the culture purity were followed by microscopic examination.

In broth medium we analysed the turbidity, deposit and the presence of surface formations, respectively the size, type, shape and colonies pigmentation on MRS agar plates.

The biochemical characters of *Lactobacillus* strains were analysed by the catalase and carbohydrate fermentation test using API50CHL, Biomerieux (France) strips, according to manufacturer's instructions. The incubation of strips was performed at 37°C, under aerobic conditions, for 48-72/120h. *Lactobacillus* sp. identification was done based on morphological characters, cultural and catalase assay. In the case of Enterococcus strain identification, following by catalase assay, biochemical characterization was performed with API 20 STREP. Test interpretation was done at 4 and 24h, at 37□C. The sensitivity to Vancomycin (30 μg) Oxoid was tested on Oxoid BHI agar. The *Enterococcus* sp. identification was based on morphological, cultural and catalase assays.

3. Preservation of bacterial strains

The medium-term preservation (months) was done by culture in broth medium (MRS for *Lactobacillus* spp. and BHI for *Enterococcus faecium*). The viability of bacterial strains was evaluated after 45 days, 3 and 4 months. Long-time preservation (years) was done at -80^{0}C, with addition of glycerol 20%. Bacteria viability will be assessed every 2 years.

Results and Discussions

The taxonomic classification of bacterial strains in *Lactobacillus* sp. was performed by morphologically (Gram positive, non-spore forming rods), culturally (anaerobic growth) and biochemically characters (negative catalase test). Identification of the *Lactobacillus* spp. was performed based of biochemical characters. Thus, 9 strains of the genus *Lactobacillus (L. acidophilus* IBNA 11, *L. acidophilus* biotype 3 IBNA 12, *L. fermentum* IBNA 13-18 and *L. salivarius* IBNA 19) and one strain of *Enterococcus faecium* (IBNA 10) were isolated, identified and preserved from the intestinal content (ileum and cecum), from 8 turkey chickens with the age of 73 days.

Figures 1-3 show smears from *L. fermentum* IBNA 15, *L. fermentum* IBNA 17 and *L. salivarius* IBNA 19 cultures on agar medium.

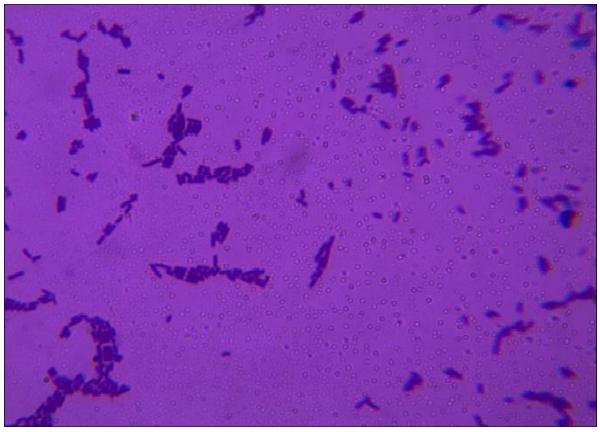

Figure 1. *L. fermentum* IBNA 15 anaerobe culture on MRS agar medium (Gram staining, x 1000)

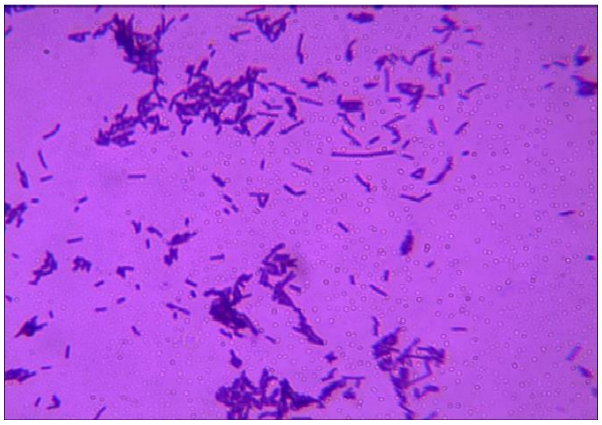

Figure 2. *L. fermentum* IBNA 17 anaerobe culture on MRS agar medium (Gram staining, x 1000)

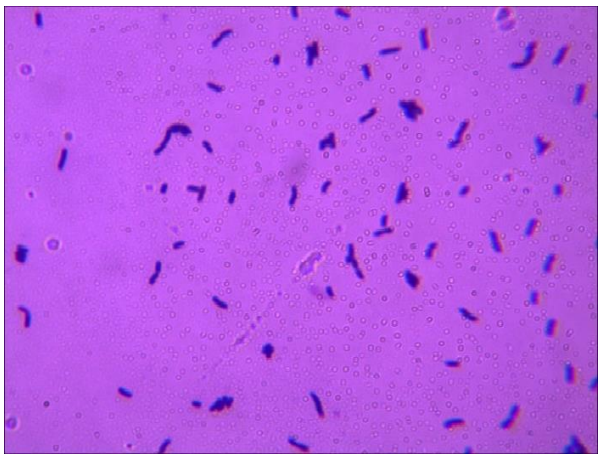

Figure 3. *L. salivarius* IBNA 19 anaerobe culture on MRS agar medium (Gram staining, x 1000)

The morphological, cultural and biochemical characteristics of these strains are presented in Table 1 and Table 2.

Table 1. Morphological, cultural and biochemical characteristics of the *Lactobacillus* strains isolated from intestinal contents of chicken turkey.

Tests	Strains								
	1	2	3	4	5	6	7	8	9
Morphological characters	a	a	b	a	a	a	a	a	b
Cultural characters	x	x	x	x	x	x	x	x	y
Catalase test	-	-	-	-	-	-	-	-	-
Fermentation (API50CHL, 48h)									
glycerol	-	-	-	-	-	-	-	-	-
erythritol	-	-	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-
D-ribose	-	-	-	-	-	-	-	-	-
D-xylose	-	-	-	-	-	-	-	-	-
L-xylose	-	-	-	-	-	-	-	-	-
D-adonitol	-	-	-	-	-	-	-	-	-
Methyl-βD-xylopyran.	-	-	-	-	-	-	-	-	-
D-galactose	+	+/5 days	+	+/3 days	+	+/5 days	+/5 days	+	+
D-glucose	+	+/5 days	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+
D-mannose	+	+/5 days	?/6 days	?	+/6 days	+/5 days	+/5 days	+/5 days	+
L-sorbose	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-
dulcitol	-	-	-	-	-	-	-	-	-
inositol	-	-	-	-	-	-	-	-	-
D-mannitol	+/3 d...	-	-	-	-	-	-	-	+
D-sorbitol	-	-	-	-	-	-	-	-	+
Methyl-αD-mannopyr.	-	-	-	-	-	-	-	-	-
Methyl-αD-glucopyr.	-	-	-	-	-	-	-	-	-
N-acetylglucosamine	+	-	-	-	-	-	-	-	+
amygdalin	-	-	-	-	-	-	-	-	-
arbutin	+	-	-	-	-	-	-	-	-
esculin	+	+/5 days	+	+	+	-	+	+	?
salicin	+	+	-	-	-	-	-	-	-
D-cellobiose	+	-	-	-	-	-	-	-	-
D-maltose	+	+	+	+	+	+	+	+	+
D-lactose	+	-	+/6 days	+/3 days	+	-	+/5 days	+/5 days	+
D-melibiose	-	+	+	+	+	+	+	+	+
D-saccharose	+	+	+	+	+	+	+	+	+
D-trehalose	-	-	-	-	-	-	-	-	+
inulin	-	-	-	-	-	-	-	-	-
D-melezitose	-	-	-	-	-	-	-	-	-
D-raffinose	+	+	+	+	+	+	+	+	+
amidon (starch)	-	-	-	-	-	-	-	-	-
glycogen	-	-	-	-	-	-	-	-	-
xylitol	-	-	-	-	-	-	-	-	-
gentibiose	-	-	-	-	-	-	-	-	-
D-turanose	-	-	-	-	-	-	-	-	-

D-lyxose	-	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-	-	-
potassium gluconate	-	-	-	-	-	-	-	-	-
potassium 2-ketogluc.	-	-	-	-	-	-	-	-	-
potassium 5-ketogluc.	-	-	-	-	-	-	-	-	-

1=*L. acidophilus* IBNA 11, 2= *L. acidophilus* biotype 3 IBNA 12, 3= *L. fermentum* biotype 1 IBNA 13, 4= *L. fermentum* biotype 1 IBNA 14, 5= *L. fermentum* biotype 1 IBNA 15, 6= *L. fermentum* biotype 1 IBNA 16, 7= *L. fermentum* biotype 1 IBNA 17, 8= *L. fermentum* biotype 1 IBNA 18 and 9= *L. salivarius* IBNA 19.
a= Gram positive, non - spore forming rods, grouped in short chains; b= Gram positive short rods, with rounded end, non-spore forming, arranged in irregular clumps / palisade
x= small colonies, 1.0-1.5 mm in diameter, S type, round, semi-transparent / transparent, whitish / unpigmented;
y = large colonies, 2.0-4.0 mm in diameter, S type, round, opaque, white
- = negative; += positive; ?= dubious, weekly positive.

Table 2. Morphological, cultural and biochemical characters of *Enterococcus faecium* IBNA 10 strain, isolated from the intestinal content of a turkey chicken

Morphological characters		a
Cultural characters		x
Catalase test		-
Production of acetoin		+
Hippurine hydrolysis		?
Esculin hydrolysis		+
Pyrrolidonyl arylamidase		+
α-galactosidase		+
β-glucuronidase		-
β -galactosidase		+
Alkaline phosphatese		-
Leucinaminopeptidase		?
Arginindihidrolase		+
Acidification		
	ribose	+
	arabinose	+
	mannitol	+
	sorbitol	-
	lactose	+
	trehalose	+
	inulin	-
	raffinose	-
	starch	+
	glycogen	-
Vancomycin (30 μg)		R

a = Gram positive coc, diplo, in short chains and in small staph groups (on solid media).
x = small colonies, 1,0-1,5 mm diameter, S type, opaque, whitish, round, with regular margins.
- = negative; + = positive; ? = doubtful, weakly positive; R = resistant

From the analysis of Table 1, is observed that, the differentiation of *Lactobacillus* isolated strains was performed, mainly on the basis of morphological characters (aspect of bacilli and grouping of them), some cultural characters (colony size and degree of transparency / opacity) and especially, biochemical characters (fermentation of D-mannitol, N-acetylglucosamine, arbutin, esculin, salicin, D-cellobiose, D-lactose, D-melibiose). Thus, the use of biochemical tests is essential for the differentiation of *Lactobacillus* species and for the identification of the *Enterococcus* faecium strain.

Table 3. The origin and the level of *Lactobacillus* spp. and *Enterococcus faecium* strains presence in the ecological niche (73 days old turkey intestinal content).

Strains	**Origin, sample number**	**CFU/g intestinal content (log10)**
Enterococcus faecium IBNA10	ileum content, 7	7,698 + E
L. acidophilus IBNA 11	ileum content, 7	8,525 + E
L. acidophilus biotype 3 IBNA12	cecum content, 9	8,342 + E
L. fermentum biotype 1, IBNA 13	ileum content, 10	6,176 + E
L. fermentum biotype 1, IBNA 14	ileum content, 11	6,903 + E
L. fermentum biotype 1, IBNA 15	cecum content, 11	9,681 + E
L. fermentum biotype 1, IBNA 16	ileum content, 12	6,352 + E
L. fermentum biotype 1, IBNA 17	cecum content, 13	9,556 + E
L. fermentum biotype 1, IBNA 18	ileum content, 14	6
L. salivarius IBNA19	ileum content, 14	6,176 + E

Table 3 presents the origin (ileon or cecum content) and the quantitative level of the isolates presence in ecological niche.

It should be noted that, at the respective dilutions, *Lactobacillus* and *Enterococcus* strains appeared as pure culture (one type of colony developed), with the exception of sample 14, where co-exist *L. fermentum* IBNA 18 and *L. salivarius* IBNA 19.

In Table 4 are presented the results of strains identification by apiwebTM soft, API20STREP, API50CHL V.5.1, BioMerieux (France) and ABIS online software.

Table 4. The results of parallel identification of strains by apiweb™ soft, API20STREP, API50CHL V.5.1, BioMerieux (France) and ABIS online software.

Strains	API, % ID	ABIS, % SIM
1. Enterococcus faecium IBNA 10	*E.faecium*, 99.2%	*E. mundtii*, 96% *E. gallinarum*, 92% *E. moraviensis*, 92%
2. *Lactobacillus acidophilus* IBNA 11	*L.crispatus*, 38.4% *L. acidophilus*, 37.2%	*L. acidophilus*,95%
3. *Lactobacillus acidophilus* 3 IBNA 12	*L. acidophillus 3*, 96.8%	*L. acidophilus*,92%
4. *Lactobacillus fermentum* 1, IBNA 13	*L. fermentum* 1, 90.6%	*L.acidophilus*,87.7% *L.fermentum*, 81.8%
5. *Lactobacillus fermentum* 1, IBNA 14	*L. fermentum* 1, 84.7%	*L.acidophilus*, 88% *L.fermentum*, 81.8%
6. Lactobacillus fermentum 1, IBNA 15	*L. fermentum*, 90.6%	*L.acidophilus*, 88% *L.fermentum*, 81.8%
7. Lactobacillus fermentum 1, IBNA 16	*L. fermentum 1*, 94.9%	*L. kefiranofaciens* subsp. *kefiranofaciens*, 94.8% *L. fermentum*, 81.9%
8. *Lactobacillus fermentum* 1, IBNA 17	*L. fermentum 1*, 88.3%	*L. fermentum*, 88%
9. *Lactobacillus fermentum* 1, IBNA 18	*L. fermentum 1*, 88.3%	*L. fermentum*, 88%
10. Lactobacillus salivarius IBNA 19	*L. salivarius*, 99.9%	*L. salivarius*, 97%

For apiweb identification is presented the % ID (percentage of identification), respectively % SIM for ABIS (percentage of similarity with respectively specie). % SIM for ABIS represents the percentage of similarity with taxa from the database, wich containing a matrix where probabilistic incidence values are allocated for every taxon and their corresponding morpho-biochemical characters. Apiweb™ % ID is a probabilistic calculation using bioMerieux own system procedure. API identification is based on the use of all 49 fermentation tests, while ABIS identification is based on 27 fermentation tests, generally those that differentiate between species. According to CATO & al. [11] *L. crispatus* are synonymous with *L. acidophilus* group A2 because the type strains of *L. crispatus* and *L. acidophilus* group A2 have 100% DNA homology.

In Table 5 are presented the results of viability test for *Lactobacillus* strains which are preserved at 4°C and at room temperature.

Table 5. Testing the viability of *Lactobacillus* and *Enterococcus faecium* strains preserved at 4°C and room temperature.

Strains	Viability at 4°C	Viability at room temperature
1. E. faecium IBNA 10	+/ 11 months	ND
2.*L. acidophillus* IBNA 11	-/4 months; +/45 days	-/4 months
3. *L. acidophillus* 3 IBNA 12	-/3 months; +/45 days	-/3 months
4. *L. fermentum* 1, IBNA 13	-/3 months; +/45 days	-/3 months
5. *L. fermentum* 1, IBNA 14	+/3 months	-/3 months
6. L. fermentum 1, IBNA 15	+/3 months	-/3 months
7. L. fermentum 1, IBNA 16	+/3 months	-/3 months
8. L. fermentum 1, IBNA 17	-/3 months; +/45 days	-/3 months
9. L. fermentum 1, IBNA 18	-/3 months; +/2 months	-/3 months
10. L. salivarius, IBNA 19	-/45 days	-/3 months

+ = positive, - = negative.

Note that, for E. faecium IBNA 10, 11 months is the minimum duration of resistance at 4ºC and their viability will be tested at 15 months. *L. acidophilus* IBNA 11, 12 resisted 45 days at 4ºC, a single passage. *L. fermentum* IBNA 14 and 15 strains survived 3 months at 4ºC, a single passage. *L. fermentum* IBNA 17 resisted 45 days at 4ºC, a single passage.

L. salivarius is included in the vertebrate adapted lifestyle lactobacilli group, with bile resistance and bacteriocins production as lifestyle-associated traits (R.M. DUAR & al. [1]). *L. salivarius* was isolated from human, pigs, chickens, hamsters and horses (R.M. DUAR & al. [1]). *L. acidophilus* belongs to the same group of lactobacilli and has recognized probiotic properties (R.M. DUAR & al. [1]). *L. fermentum* is included in the nomadic species group of lactobacilli (R.M. DUAR & al. [1]). *L. fermentum* was isolated from fermenting plant material, manure, sewage, milk products, mouth and faeces of humans, and intestines of pig, birds, cattle, mouse and rat (C. STOICA & I. SORESCU [10]).

These strains isolated from intestinal content of turkeys will be important for the development of a probiotic compound for same bird species, because the host-adapted strains of lactobacilli have a higher ecological fitness in their respective hosts and will be more competitive as probiotic when compared to strains that don't share an evolutionary history with the host (R.M. DUAR & al. [1]). The probiotic characters of these strains will be tested further. Higher fitness is relevant to outcompete pathogens, to have higher metabolic activity and to have an increased production of metabolic compounds. Moreover, tolerance interactions with the host immune system will be established. The count of lactobacilli/gram of intestinal content methodology of K.C. MOUNTZOURIS & al. [7] was completed/modified for the morphological confirmation of colonies which appear on selective medium by Gram staining of smears and microscopic examination. It was found that the microscopic confirmation is necessary because some colonies with common traits with *Lactobacillus* spp., by Gram staining, appear as Gram positive cocci or, more seldom, as Gram negative coccobacilli. Thus, the morphological confirmation of the presumptive positive colonies is necessary for fitting into the group of lactobacilli.

In the intestinal cecum content, the numbers of CFU lactobacilli/g are much higher (10^8-10^9) than in the ileum area (10^6-10^8). *L. fermentum* has a superior presence to other lactobacilli. This observation is very interesting, because the *L. fermentum* is considered to be nomadic specie, while the *L. acidophilus* and *L. salivarius* strain are adapted to vertebrate species. The higher numbers of CFU *Lactobacillus*/g intestinal content can suggest a better adaptation to the ecological niche, so a potential probiotic advantage.

As identification system, both software (apiwebTM and ABIS) are proved to be useful. In general was obtained the same taxonomic classification, sometimes even with identical percentage results, although the way of calculating them is totally different. The use of biochemical tests is essential for the differentiation of *Lactobacillus* species and for the identification of the *Enterococcus faecium* strain.

The resistance at 4ºC is a relevant technical character of the strains. Thus, *E. faecium* IBNA 10 resisted, at 4ºC, for at least 11 months, while three of the six isolated strains of *L. fermentum* resisted for 3 months. *L. salivarius* strain IBNA 19 did not survive 45 days at 4ºC, whereas the *L. acidophilus* strains survived only 45 days a single passage. Obviously, a longer resistance is a plus, a positive character to the strains during their selection.

These results are very useful in screening the phenotypic characters of the candidate strains to prepare a probiotic product, involving resistance from the isolated strains, of at least 45 days at 4ºC, without significant loss of the biological value (viability, CFU/ml). From this point of view, *L. fermentum* and *E. faecium* strains will be selected for further testing of the probiotic characteristics. We did not find this type of information in the literature, perhaps because of the very specific benefices for industrial producers of probiotic products. However, it is accepted that the ability of probiotics to remain viable during culture handling, storage and gastric passage is an important

criterion during strain selection (A. UPADRASTA & al. [12]).

Conclusions

The gut content (ileum and cecum) of eight turkeys, 73 days old, was used to isolate, identify phenotypically and preserve nine strains of the *Lactobacillus* genus (two strains of *L. acidophilus*, six strains of *L. fermentum* and one strain of *L. salivarius*) and one strain of *E. faecium*.

The count of lactobacilli per gram of intestinal content methodology of K.C. MOUNTZOURIS & al. [7] was followed for the morphological confirmation of colonies which appear on selective media, by Gram staining of smears and microscopic examination. The quantitative level of *Lactobacillus* spp. strains (10^6 – 10^9 CFU/g intestinal content) and *E. faecium* strain (10^6 CFU/g intestinal content) in the ecological niche was determined.

It was found that the number of lactobacilli in cecum content is higher (10^8-10^9 CFU/g), than in the ileum (10^6-10^8 CFU/g) and *L. fermentum* is superior to other lactobacilli. The parallel identification of strains was made by apiwebTM API50CHL V.5.1, BioMerieux (France) software, API20STREP, and ABIS on line software.

Both softwares proved to be useful, generating the same taxonomic classifications. The viability of *Lactobacillus* spp. strains preserved at 4ºC (from 45 to 90 days) and at room temperature (under 90 days) and *Enterococcus faecium* at 4ºC (more than 11 months) was tested. From isolated *Lactobacillus* strains, those from *L. fermentum* are technically suitable for continual testing of probiotic qualities, as well as the strain of *E. faecium*.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

The study was funded by Romanian Ministry of Research and Innovation through Nucleus program and Program 1 – Development National Research-Development, Sub-program 1.2 – Institutional Performance - Projects funding excellence in R & D, Contract no.17 PFE/ 17.10.2018". The authors have no conflict of interest to report.

We thank Dr. Mihaela Habeanu for the logistic support and technician Dana Bulgaru for her excellent technical assistance.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 50-56
doi: 10.25083/rbl/24.1/50.56

Received for publication: June 23, 2017
Accepted: December 22, 2017

Original paper

Distillate composition of fermented media based on by-products of sugar beet processing

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Abstract

Composition of fermented media's distillate is very significant because it dictates choice of downstream processes to obtain appropriate ethanol quality. The aim of this research paper is the analysis of the distillates composition of fermented media based on raw, thin and thick juices and molassesin terms of ethanol, methanol, higher alcohols and aldehydes content. All applied substrates, which represent intermediate and by-products of sugar beet processing, are fermented by different commercial starter cultures of Saccharomyces cerevisiae - dried distiller′s yeast, dried wine yeast, dried wine yeast tolerant of high ethanol concentration, dried baker's yeast and fresh baker's yeast. If the fermentation is carried by commercial fresh baker's yeast there is statistically significant difference only in aldehydes content in distillate of fermented media based on molasses. However, if commercial starter cultures in dried forms are used as production strains, distillates of fermentation broths prepared from effluents of sugar industry are not statistically significant different regarding the content of the analyzed components.

Keywords : sugar beet, Saccharomyces cerevisiae, lower alcohols, higher alcohols, aldehydes

To cite this article: GRAHOVAC J, DODIĆ J, RONČEVIĆ Z, DODIĆ S, VUČUROVIĆ D. Distillate composition of fermented media based on by-products of sugar beet processing. *Rom Biotechnol Lett*. 2019; 24(1): 50-56. DOI: 10.25083/rbl/24.1/50.56

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Introduction

Ethanol is widely used for the production of strong alcoholic drinks and to less degree for medical or pharmaceutical purposes (BARAS& al. [1]). Nowadays it also represents a modern type of energy and an important substitute for liquid fossil fuels or, in mixtures with gasses, a substitute for natural gas. The basic concern over ethanol production expansion is depletion of natural resources and demands for environmentally acceptable fuels – biofuels from renewable feedstocks, emitting less carbon dioxide into the atmosphere (ERICSSON and NILSSON [2]; FARRELL & al. [3]).

Ethanol can be produced from a wide range of feedstock, which includes sugar-based (sugar beet or sugarcane juice and molasses), starch-based (corn and wheat) and cellulosic (bagasse and wood) resources. In particular, sugar-based feedstock contains readily available fermentable sugars and can be an ideal substrate for ethanol production, since starch and cellulosic substances require an additional pre-treatment step for conversion into fermentable sugars (HATANO& al. [4]). Less expensive production of sugar from sugarcane indicates that the application of sugar beet for bioethanol production has great potential. Also, increased yield and sugar production efficiency have led to a reduction in sugar beet requirements. For these reasons, the majority of existing sugar plants in Europe started simultaneous production of ethanol in refineries built as their extensions (POPOV& al. [5]). Introducing the concept of sugar and ethanol co-production is an attractive option for sugar factories, as it provides flexibility in terms of variation of produced quantities of sugar and ethanol, depending on the conditions prevailing on the market (GRAHOVAC & al. [6]). Molasses is commonly used feedstock for bioethanol production. In the sugar beet processing raw and thin juices are intermediate products with production costs considerably lower in comparison with molasses, obtained at the end of the process. The only disadvantage of these intermediate products is low storability and easy decomposition by the action of microorganisms. Thick juice is an intermediate product with significantly higher price mostly due to evaporation with storability comparable with molasses (HINKOVÁ and BUBNÍK [7]). Some domestic factories, that adopted co-production, use pure cultures of production microorganism for ethanol production. However, additional financial resources are needed for constructing part of the plant for inoculum preparation. To avoid these investments, most of existing factories apply commercial starter cultures.

Saccharomyces cerevisiae is the preferred fermenting microorganism for ethanol production because of its well documented industrial performances such as superior ethanol tolerance and high ethanol yield (PĂTRAȘCU & al.[8]). In addition to ethanol and CO_2, various by-products are also produced during ethanol fermentation, for example, glycerol, acetaldehyde, organic acids and higher alcohols (BAI & al. [9]). The presence of pectin in feedstock may also result in methanol generation in the fermented medium (ANLI & al.[10]). Ethanol obtained by fermentation is on the world market classified in ten quality groups. Potential use of commercial ethanol depends on characteristic of these groups (BARAS & al. [1]).

The aim of this research paper is the determination of distillates composition of fermented media based on raw, thin and thick juices and molasses as intermediate and by-products of sugar beet processing. Distillates of fermented media were analyzed in terms of ethanol, methanol, higher alcohols and aldehydes content. This research also includes comparison of distillates composition regarding the content of the analyzed components for media fermented by different strains of Saccharomyces cerevisiae, which are commercially available in the domestic market.

Materials and Methods

1. Microorganisms and inoculum preparation

Five different commercial types of Saccharomyces cerevisiae were used throughout this research: dried distiller′s yeast – DD (Lallemand Inc., Rexdale, Ontario, Canada), dried wine yeast – DW1 (Lallemand Inc., Rexdale, Ontario, Canada), dried wine yeast tolerant of high ethanol concentration – DW2 (Lallemand Inc., Rexdale, Ontario, Canada), dried baker's yeast – DB (Alltech, Senta, Serbia) and fresh baker's yeast – FB (Alltech, Senta, Serbia). In order to rehydrate dried yeasts and metabolically acclimatize the cells prior to fermentation, yeasts were suspended in a small quantity of culture medium under aerobic conditions for 2 h (temperature of 30°C, agitation rate of 200 rpm) and then introduced to the rest of the culture medium. On the other hand, fresh baker's yeast was also suspended in a small quantity of culture media and immediately used for inoculation.

2. Substrates

Raw, thin and thick juices and molasses obtained from a domestic sugar factory were used as fermentation media. Raw and thin juices were diluted with water to give total sugar mass fractions of 5 and 10% and also used without dilution with a total sugar mass fraction of approx. 13%, obtained from sugar beet processing technology. Thick juice and molasses were diluted with water to give a total sugar concentration of 5, 10, 13, 15, 20 and 25% (w w-1). The substrates were adjusted to pH 5.0 with 10% sulphuric acid (v v-1).

3. Fermentation

Fermentations were carried out in a 2.0 l laboratory bioreactor with the fermentation media of 1.5 l. The laboratory bioreactor with the substrate was sterilized by autoclaving at 121°C and pressure of 2.1 bars for 20 min. The sterile medium was inoculated to give the initial yeast cell concentration of 108 cells ml-1 (approx. 3 g of yeast dry solids per 1000 ml of media). The fermentations were carried out in batch mode under anaerobic conditions for 48 h at the temperature of 30°C and agitation rate of 150 rpm.

4. Analytical methods

Samples of the substrates were taken for analysis. The pH was measured directly in the media by the laboratory multiparameter analyzer Consort C863 (Consort, Turnhout, Belgium) with the glass electrode. Total dissolved salt (TDS) content and conductivity were determined using conductivity electrode. The amount of soluble ash was calculated based on the conductivity of the sample. Dry matter (dm) was determined by the standard drying method in an oven at 105°C to a constant mass (AOAC [11]).

The samples of the substrates were centrifuged at 4000 rpm for 15 min. Then sucrose and reducing sugar content (sum of glucose and fructose) of the supernatant were determined (Jasco, Inc, Easton, MD, USA, pump PU-980, detector RI-930, sampler AS-950, 20 ml injection loop, column sugar KS-801, eluent: water at flow rate of 0.6 ml min-1 and elution time 30 min).

Fermentable sugar content was expressed as the sum of sucrose and reducing sugars. Sucrose, glucose, and fructose standards were purchased from Supelco (Bellefonte, PA, USA). Free nitrogen content of the supernatant determined by Kjeldahl method (HERLICH [12]).

Ethanol content in distillate of fermented media was determined by gas chromatography, using a HP 5890 Series II GC (Agilent Technologies Inc, Santa Clara, CA, USA) equipped with a flame ionization detector, a Carbowax 20 M column at 85°C and the carrier gas was helium. Injector and detector temperature was maintained at 150°C.

The content of methanol, higher alcohols and aldehydes in the distillate of fermented media were determined by standard AOAC methods (AOAC [11]).

During this experiment three independent fermentations were carried out with raw, thin and thick juices and molasses from domestic sugar factories as fermentation media. The results were statistically tested by analysis of variance and the means were compared by Scheffe's test at a significance level of p=0.05, using the StatPro for Microsoft Excel 2003 software.

Results and Discussions

The compositions of substrates (raw, thin and thick juices and molasses) before dilution and fermentation were presented in Table 1. Results from Table 1 show that compositions of raw, thin and thick juices and molasses were characteristic and usual for sugar beet processing in domestic factories. With their compositions, the given by-product, as well as the intermediate products are to be considered as convenient raw materials for fermentation media preparation for bioethanol manufacturing process.

Table 1. Compositions of substrates (raw, thin and thick juices and molasses)

Parameter	Raw juice	Thin juice	Thick juice	Molasses
Dry matter, % ww^{-1}	14.56	14.37	60.31	81.32
Sucrose, % w w^{-1}	12.94	13.07	54.12	50.14
Coefficient of purity, % w w^{-1}	88.87	90.95	89.74	61.66
pH	6.41	9.09	8.03	7.05
Ash, % w w^{-1}	0.281	0.338	1.97	9.92
Reducing substance % ww^{-1}	0.0692	0.013	0.501	0.862
Total nitrogen, % w w^{-1}	0.129	0.128	0.158	1.841

Figure 1(A-D) shows effect of the initial concentration of fermentable sugars from raw, thin and thick juices and molasses on ethanol, methanol, higher alcohols and aldehydes content in distillates of fermented media. During these experiments, fresh baker's yeast (FB) was used as a production microorganism.

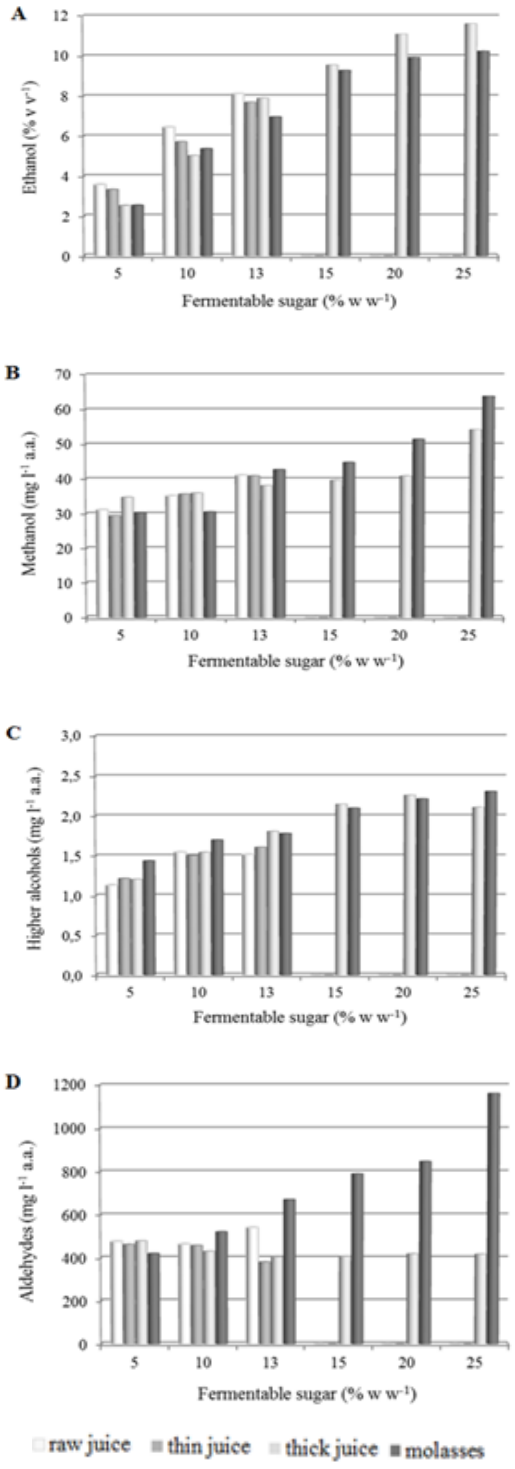

Figure 1. Effect of the initial concentrations of fermentable sugars from raw, thin and thick juices and molasses on ethanol (A), methanol (B), higher alcohols (C) and aldehydes (D) content in distillates of fermented media

The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or EMP pathway), through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced. Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO2. Theoretically, the yield is 0,511 for ethanol and 0,489 for CO2 on a mass basis of glucose metabolized (BAI & al. [9]). Results shown in Figure 1A indicate that with the increasing of fermentable sugars concentration from 5 to 20% (w w-1), ethanol content increased for all applied raw materials (raw, thin and thick juices and molasses). When initial sugar concentration of 20% (w w-1) was increased to 25% (w w-1), ethanol content in media based on thick juice and molasses were not significantly different. Low ethanol content at higher sugar concentrations were more expressed in fermentation medium prepared from molasses. This is a consequence of the higher dry matter content in molasses in comparison with thick juice. Higher dry matter content causes a higher osmotic pressure in the medium which adversely effects on yeast cell growth and ethanol production. In the industry, the ethanol yield that is calculated based on the total sugar feeding into the fermentation system without deduction of the residual sugar can be as high as 90–93% of its theoretical value of ethanol to glucose (BAI & al.[9]). The ethanol yields in applied experimental conditions were 0.61, 0.64, 0.61 and 0.54 (ml g-1 fermentable sugars) for media based on raw, thin and thick juices and molasses, which is 93.85, 98.46, 93.85 and 83.08% of theoretical yield, respectively. In terms of the ethanol yield, the obtained results suggest that all of the substrates are suitable for the ethanol production.

Methanol is formed by pectinolytic enzymes that split the methoxyl group from the pectin present in raw material (ANLI & al. [10]). Sugar beet contains 1-2 g pectic substances per 100 g of beet. Only relatively small amounts of pectin (0.1-0.3% w w-1) are dissolved in colloidal form in the beet cell juice. Under normal conditions, a very small fraction of pectic substances (4-6%) exceeds in raw juice during extraction in sugar beet processing (van der POEL& al. [13]). Throughout further processing in the sugar industry content of pectic substances are reduced. However, a small amount of pectin is sufficient for the formation of methanol during fermentation. Also, with less dilution of applied raw material, substrate contains a higher content of pectic substances. This means that the content of methanol increases with increasing fermentable sugars content in all substrates (see Figure 1B). For fermented media based on thin and thick juices and molasses with sugar concentration of 13% (w w-1) in comparison with raw juice with the same sugar concentration p-values were 0.9474, 0.1515 and

0.6647, respectively. The obtained results suggest that there is no statistically significant difference in the content of methanol in the distillates of fermented media based on raw, thin and thick juices and molasses.

Higher alcohols are produced by yeasts during alcoholic fermentation through the conversion of the branched chain amino acids present in the medium: valine, leucine, isoleucine, threonine and phenylalanine. They are also produced de novo from a sugar substrate (LAMBRECHTS and PRETORIUS [14]). Sugar beet contains 0.2-0.3 g amino acids per 100 g of beet. Amino acids are found in free form dissolved in the beet cell juice. Roughly 92-97% of free amino acids in sugar beet pass into the raw juice. The total amount of amino acids decreases during processing. The concentration of free amino acids is markedly higher in raw juice than in thin juice. The total amount of amino acids falls during juice purification to about 70% of its level in raw juice, mainly because of the formation of amides. However, this does not apply to certain amino acids such as valine, leucine, isoleucine and threonine which are precursors of higher alcohols. Content of these amino acids stays constant during sugar beet processing (van der POEL & al. [13]). This fact explains uniform content of higher alcohols in distillates of all fermented media with same concentration of fermentable sugars (see Figure 1C). For fermented media prepared from thin and thick juices and molasses with sugar concentration of 13% (w w-1) in comparison with raw juice with the same sugar concentration p-values were 0.6047, 0.1288 and 0.3104, respectively. These values suggest that there is no statistically significant difference in the content of higher alcohols in distillates of fermented media based on intermediate and by-product of sugar beet processing.

Aldehydes are usual products of alcoholic fermentation. The precursors of aldehydes, the 2-keto acids, are formed as intermediates in both the anabolic and catabolic formation of amino acids or higher alcohols. Conditions which favor higher alcohols production also favor formation of small quantities of aldehydes. These may be secreted but can be reabsorbed and reduced by yeast to the corresponding alcohol during the later stages of the fermentation. Acetaldehyde is the major component. It is an intermediary product formed from pyruvate. Oxidation of alcohols, degradation of amino acids and autooxidation of fatty acids may also produce aldehydes (LAMBRECHTS and PRETORIUS [14]). Results shown in Figure 1D indicate that fermentable sugars concentrations in fermented media based on raw, thin and thick juices no affected on change of aldehydes content in their distillates. Also, can be seen increase of aldehydes content with the increasing of fermentable sugars concentrations in fermented media prepared from molasses. This can be explained by the high concentration of non-sugar compounds in molasses from which arise aldehydes during distillation. There is no statistically significant difference in the content of aldehydes in distillates of fermented media based on thin and thick juices with sugars concentration of 13% (w w-1) compared to the raw juice with the same sugar concentration (p-values were 0.0703 and 0.0544, respectively). However, there is a statistically significant difference in aldehyde content in distillate if used medium based on molasses in comparison with medium based on raw juice (p=0.0269).

Fermentation media, prepared from raw, thin and thick juices and molasses with fermentable sugars concentration of 13% (w w-1), were also inoculated with different types of yeast in the dried forms in order to evaluate their fermentation productivity. Examined types of yeast are commercially available and extensively used as starter cultures in various branches of the fermentation industry. Dried distiller's yeast (DD) is intended for use in fuel ethanol and beverage alcohol production. Yeasts DW1 and DW2 are in use for wine production and are selected because of very short lag phase and fast fermentation. Strain DW2 is also tolerant on high ethanol concentrations. Strain of commercially produced fresh baker's yeast (FB) is also applied in form as dried yeast (DB).

Figure 2A-D shows the dependence of the ethanol, methanol, higher alcohols and aldehydes content on the applied media and yeast strains.

The effects of applied media and yeast strains on ethanol content are presented in Figure 2A. Average values of the ethanol content in distillates of fermented media based on raw, thin and thick juices and molasses were almost uniform. For ethanol content in media fermented by types of yeast in dried forms, DB, DD, DW1 and DW2, in comparison with fresh baker's yeast (FB), p-values were 0.9636, 0.3838, 0.8034 and 0.4912, respectively. The obtained results suggest that there is no statistically significant difference in ethanol production when commercial starter cultures are used as production strains.

Figure 2B illustrates the dependence of the methanol content on the applied media and yeast strains. For methanol content in the distillates of media fermented by strains DB, DD, DW1 and DW2, in comparison with fresh baker's yeast (FB), p-values were 0.3628, 0.7152, 0.0414 and 0.0417, respectively.

These results indicate that there is no statistically significant difference in methanol content in distillates of media fermented by the applied yeast strains.

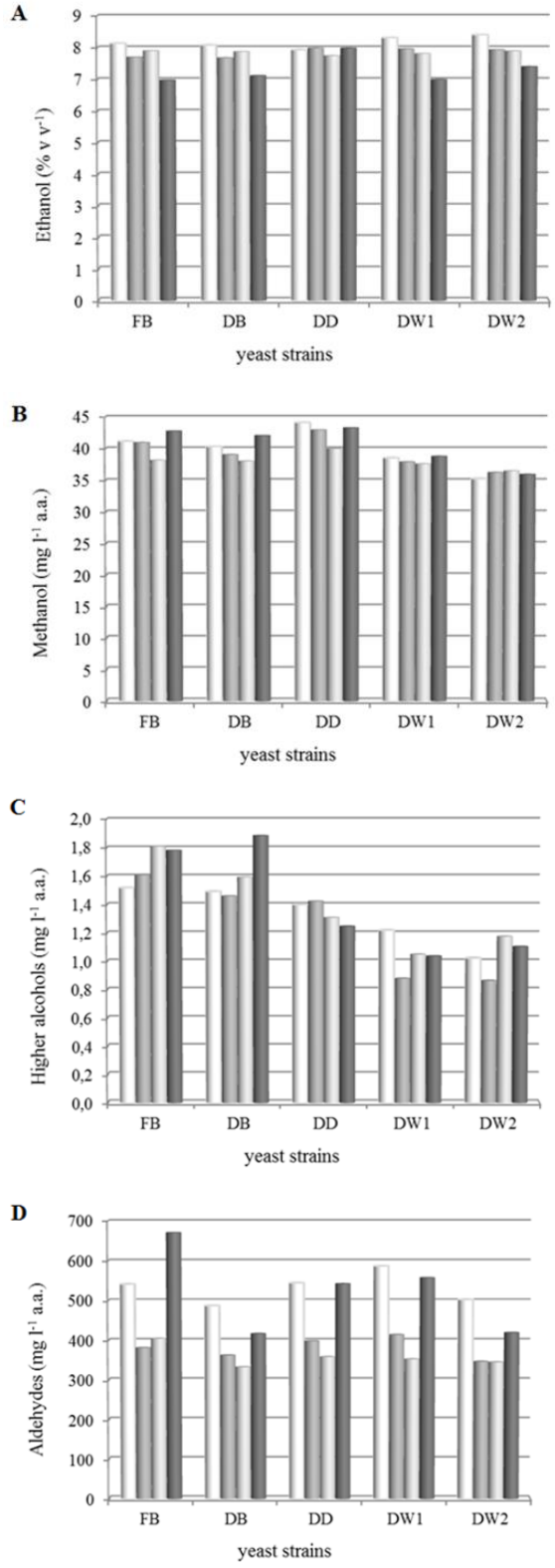

Figure 2. Dependence of the ethanol (A), methanol (B), higher alcohols (C) and aldehydes (D) content on the applied media and yeast strains

Experimental results indicate that yeast strains used for wine production give lower higher alcohols content in comparison with the other three yeast strains (see Figure 2C). For higher alcohols content obtained by fermentation with strains DB, DD, DW1 and DW2, in comparison with fresh baker's yeast (FB), p-values were 0.4278, 0.2228, 0.0979 and 0.0619, respectively. These results suggest that there is no statistically significant difference in higher alcohols content in distillates of media fermented by the applied yeast strains.

Dependence of the aldehydes content on the applied media and yeast strains are shown in Figure 2D. From this figure it is evident that aldehydes content in distillates of fermented media prepared from thin and thick juices is lower in comparison with raw juice and molasses for all applied yeast strains. For aldehydes content in the distillates of media fermented by strains DB, DD DW1 and DW2, in comparison with fresh baker's yeast (FB), p-values were 0.2531, 0.7021, 0.8607 and 0.2790, respectively. The obtained results suggest that there is no statistically significant difference in terms of aldehydes production during fermentation.

Conclusions

The obtained results suggest that there is no statistically significant difference in the content of ethanol, methanol and higher alcohols in distillates of fermented media based on raw, thin and thick juices and molasses if the fermentation is carried by commercial fresh baker's yeast. However, there is a statistically significant difference in terms of aldehyde content in distillate if used media based on molasses, in comparison with media based on raw, thin and thick juices. If commercial starter cultures are used as production strains - dried distiller′s yeast, dried wine yeast, dried wine yeast tolerant of high ethanol concentration, dried baker's yeast and fresh baker's yeast, distillates of fermentation broths are not statistically different in terms of ethanol, methanol, higher alcohols and aldehydes content, regardless effluent of sugar industry used as base of fermentation media.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

The authors gratefully acknowledge the support of the Ministry of Education, Science and Technological Development of the Republic of Serbia within the framework of TR-31002 project.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 57-65
doi: 10.25083/rbl/24.1/57.65

Received for publication: April 20, 2018
Accepted: July 10, 2018

Original paper

The influence of oenological tannins on the fermentation and color stability of a Romanian red wine

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Abstract

The study aimed to assess the influence of three different commercial oenological tannins characterized by the complexity of their composition, condensed, ellagic and gallic tannins on the Fetească Neagră red wine characteristics. In order to highlight the tannin effect, the color intensity was measured spectrophotometrically at various wavelengths 420 nm, 520 nm and 620 nm and displayed increased values for all the studied variants of wine compared to the control sample.

The total polyphenols content revealed an increase once the dose of oenological tannins was raised from 20 g/hL to 40 g/hL for the wines obtained from grapes with a corresponding phytosanitary status and for those obtained from grapes with Botrytis cinerea in a proportion of 10%. By using oenological tannins in different doses, many advantages were outlined such as color loss prevention by accentuating the violet tones and blocking the color evolution to orange-colored shades. Moreover, the intense extractions that allow the bitter and harsh tannins to pass through the solids into the liquid fraction were also limited.

Keywords : red wine, Fetească Neagră, oenological tannins, color stability, fermentation

To cite this article: BICHESCU C., STANCIU S. The influence of oenological tannins on the fermentation and color stability of a Romanian red wine. *Rom Biotechnol Lett*. 2019; 24(1): 57-65. DOI: 10.25083/rbl/24.1/57.65

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Introduction

The complexity of winemaking in terms of grape variety and wine composition gives each wine its uniqueness. To assess whether a wine has a high quality or not, the presence of polyphenols is imperative. These constituents give the wine a high antioxidant activity which accounts for the obvious diversity and for the ageing characteristics of red wines. Moreover, the quantity of total wine phenolics is a primary consideration. The phenolic compounds represent the secondary metabolites of plants that usually affect the organoleptic properties of wine, such as color intensity, aroma, bitterness and astringency (M. MONAGAS & al. [1], E. OBREQUE-SLIER & al. [2]), the flavonoids being of particular interest in winemaking as they are present in a wide variety. Besides the flavonoids, other types of compounds like non-flavonoid phenols are also present, hydroxycinnamic acids being the largest group found in grapes and wine. Regarding the red wine, the following phenolic compounds are present at a high concentration such as: tannins, which are responsible for the astringency and the color of red wines, anthocyanins responsible for the pigment of the grapes and also for the color of red wines, phenolic acids, flavonols and dihydroflavonols, stilbenes, which do not affect the sensory characteristics but are nevertheless important from the health perspective (J. MORENO & al. [3]).

During red wine production, the skin and seeds of the grape are in contact with the fermenting juice for a long period of time so that the pigments (primarily anthocyanins and tannins) and the polyphenols (predominantly catechins and proanthocyanidins) are extracted at a high rate. During the maturation and ageing processes, the biologically active compounds and other wine components react with each other to give the pigmented polymeric compounds that stabilizes the wine colour (Z. PENG & al. [4], V.I. PETROPULOS & al. [5]).

In winemaking, the use of oenological tannins is a common practice, their use being authorized by the International Organization of Vine and Wine (OIV). These types of compounds are primarily used for the clarification of musts and wines (O. PASCUAL & al. [6]). Furthermore, oenological tannins present many other useful properties such as the antioxidant activity (A. RICCI & al. [7]), the ability to scavenge for peroxyl radicals (L.M. MAGALHÃES & al. [8]), the direct consumption of dissolved oxygen (M. NAVARRO & al. [9]), the ability to chelate Fe2+, the oxidative damage prevention mediated by Fenton-based reactions (C.A. PEREZ & al. [10]), antibrowning activity (D. OBRADOVIC & al. [11]), improvement of sensory properties (N. VIVAS [12]), red wines color improvement and stabilization (V. CANUTI & al. [13]), copigmentation effect (A.C. NEVES & al. [14]), new pigments formation (A.VERSARI & al. [15]), reduction odors elimination (N. VIVAS [12]) and bacteriostatic effects (O. PASCUAL & al.[6]). Obviously, to all these benefits, the interactions between the oenological tannins and proteins in order to prevent protein haze must be also added, with effects on the wine's astringency and bitterness (O. PASCUAL & al. [6]).

Many commercial types of tannin of different plant origins and chemical compositions are available on the market (M. MALACARNE & al. [16], E. OBREQUE-SLÍER & al. [17]). The oenological tannins comprise different polymers of gallic and ellagic acids, whereas condensed tannins or proanthocyanidins are composed of flavan-3-ol subunits like catechin, epicatechin, epigallocatechinand gallocatechin (G. VAZALLO-VALLEUMBROCIO & al. [18]). The condensed tannins from the skins and seeds of grapes are extracted during the winemaking process, whereas the hydrolyzable tannins are transferred to the wine from oak wood barrels during aging. Different oenological products, such as chips, staves and commercial oenological tannins are widely used to add more phenolic compounds to a wine. These types of compounds are natural substances obtained from different botanical species that are rich inproanthocyanidins (from skins and seeds of grapes) and/or hydrolyzable tannins (from oak wood) (M. MALACARNE & al. [16]).

Also, the high antioxidant properties of red wines are also attributed to the high concentration of biologically active compounds. The oenological tannins act as fining agents that stabilize the wine color, improve the wine structure and contribute to a number of high beneficial biological effects (G. VAZALLO-VALLEUMBROCIO & al. [18]).

Romania, as a wine-consuming country, favors many varieties of wines, one of the most preferred types being the red wine. Among the red wines grape variety, Fetească neagră is one of the oldest Romanian varieties, whose origin and extraordinary quality are incontestable, being cultivated since the Dacian period. The wine obtained from Fetească neagră grape variety has a memorable scent of dry plums and dark fruits, a fragrant bouquet which is indisputable, being among the most popular types of wine.

The concept of modern wines, especially for the fruitful and expressive dry red wines intended for rapid commercialization, calls for the need to use complex oenological tannins in their biotechnology. In order to diversify the range of accepted oenological tannins by the current regulations, a very rigorous quality control is required, as well as numerous tests and experiments that are aimed to offer the desirability of their use in wine production and on the consumption safety of these products (lack of organic solvents, concentrations below critical limits in heavy metals, absence of various contaminants).

The most important properties of tannins are related to the ability to form complex compounds with proteins, the antiradical ability and the capacity of these compounds to consume the dissolved oxygen. In order to efficiently use exogenous oenological tannins, it is always necessary to perform laboratory experiments to determine the type of tannin required, the optimal dose and the expected sensory effects. Exogenous oenological tannins do not replace natural tannins in musts and wines, but they guarantee that their initial concentration and all the beneficial attributes resulting from the treatment will improve the chromatic, olfactory and gustatory characteristics, as well as provide the beneficial effect by protecting the human body against cardiovascular diseases.

Based on these characteristics, tannins exert interesting antioxidant properties both in the pharmacological, food and oenological fields as well as imprinting interesting taste properties reassembled under the astringency term with implications in the sensory quality of red wines. The main objective of this study was to emphasize the influence of the tannins addition in the fermentative phase on the color and phenolic composition of a red wine obtained from Fetească neagră grapes variety.

Materials and Methods

1. The grapes and the winemaking

The study highlighted the Fetească neagră grape harvest of 2016-2017, Murfatlar vineyard. The grapes were harvested at their phenolic maturity and were carefully transported to the cellar. The Fetească neagră red wine was obtained through a classical technology that started with the grapes destemming and crushing. The obtained mash was afterwards sulphited and moved to other recipients for maceration and fermentation. At the initial stage of the experiment, a quantity of 1 g/L tannins of different origin (condensed, ellagic and gallic) was added to a red wine obtained from the Feteasca neagră harvest.

2. Oenological tannins treatment

For the oenological tannins treatment, 3 different commercial products were purchased from Sodinal (Bucharest, Romania) such as: Fermotan characterized by the complexity of its composition, being a multi-component tannin product composed of condensed, ellagic and gallic tannins; Gallotan product containing gallic tannins and Protan Raisin composed only of condensed tannins. The initial phase was preceded by a repeated periodic aeration (at an interval of 15 days) using an oxygen intake of 5 mg/L at each aeration, for a period of three months after which the final stage of the experiment was completed. In order to highlight the tannin effect, the color intensity was measured spectrophotometrically at various wavelengths(λ = 420 nm, 520 nm and 620 nm) and also by determining the polymerized pigments index at the initial and final stage of the experiment for each variant in comparison to the control variant.

Further, the experiments followed the Fermotan product treatment at doses of 20 g/hL and 40 g/hL on the Fetească neagră wine. The grape samples used in the experiment were healthy grapes with a corresponding phytosanitary status and grapes partially contaminated with Botrytis cinerea mold in a proportion of 10%. The evaluation of the effect of Fermotan addition was achieved by monitoring the color intensity and total polyphenols indexes as well as the sensory properties of the wines.

In order to highlight the interaction between tannins and polysaccharides, the wine obtained from the Fetească neagră variety was treated with autolysed yeast derivatives (Batonnage Plus 150 KDa, 40 g/hL) and with a tannin complex consisting of condensed,

ellagic and gallic tannins (Fermotan, 20 g/hL) coupled with autolysed yeast derivatives (Batonnage Plus 150 KDa, 40 g/hL). The chromatic characteristics of the samples obtained from the three variants of wine were evaluated after 30 days.

3. *Determination of the polymerized pigments index*

By measuring the color intensity of the wine after adding SO2, compared to the control sample, allowed the determination of the polymerized pigments indexat the wine pH. To assess the polymerized pigments index (Equation 1), two assays were considered as follows: firstly, a volume of 45 mL of a synthetic solution with a pH of 3.2 and 0.2 mL of sodium metabisulphite 20% solution were added to 5 mL of wine, and after 5 minutes the first color intensity (Ci1) was determined; secondly, the same volume of synthetic solution and 0.2 mL of water were added to 5 mL of wine, and the second color intensity (Ci2) was determined. To calculate the Ci1 and Ci2, the absorbance of the two samples was read at 420, 520 and 620 nm, with water as blank.

$$I_{PP} = \frac{Ci_1}{Ci_2} \times 100 \qquad (1)$$

4. *Determination of anthocyanins ionization index*

The anthocyanins ionization index (Equation 2) represents the percentage of colored anthocyanins from wine (free and combined, decolorable by SO2). Thereby, two determinations were carried out that included the discoloration of the wine with an excess of SO2, at the pH of the wine and at pH 1.15. The index was given by the ratio of the two discolorations expressed as percentage.

$$I_i = \frac{\Delta DO_\alpha}{\Delta DO_\gamma} \times 100 \qquad (2)$$

The wine discoloration with SO2 at the pH of the wine consisted of two parts: the first optical density (DO1) was read at 520 nm for 10 mL of wine with 2 mL of distilled water; the second optical density was assessed after 15 minutes for 10 mL of wine with 2 mL 15% NaHSO3.Equation 3represents the contribution of colored anthocyanins at 520 nm.

$$\Delta DO_\alpha = (DO_1 - DO_2) \times \frac{12}{10} (3)$$

The wine discoloration with SO2 at the pH of 1.15 also included two parts: the first optical density (DO1') was read at 520 nm for 1 mLof wine with 7 mL 0.1 N HCl and 2 mL of water; the second optical density (DO2') was read after 15 minutes at 520 nm for 1 mLof wine with 7 mL 0.1 N HCl and 2 mL 15% NaHSO3. Equation 4 represents the contribution of all the colored compounds (anthocyanins and polymerized pigments) at 520 nm.

$$\Delta DO_\gamma = (DO_1' - DO_2') \times \frac{100}{95} (4)$$

5. *Determination of total polyphenols index or D280 index*

The total polyphenols index (TPI or D280 index) is a parameter that describes the content of total phenolic compounds (phenolic acids, tannins, anthocyanins, flavones etc.) from wines. It has values ranging from 3 to 15 for white wines and 20 to 100 for red wines. The principle of the method is based on the strong absorption of the ultraviolet light by the benzene nuclei, which is characteristic for the phenolic compounds and reaches a maximum absorption at the 275 - 280 nm wavelength interval. The D280 index is a spectral characteristic of all phenolic compounds (phenolic acids, tanning substances and coloring agents) present in the wine and was determined as follows: the wine was diluted 1% with distilled water, after which the absorbance was measuredat 280 nm in comparison to distilled water. The result thus obtained was multiplied by the dilution factor and the value of D280 was obtained.

Results and Discussions

1. *The effect of oenological tannins on the color intensity and on the polymerized pigments index*

The evolution of the main parameters that were analyzed (color intensity and percentage of condensed tannins in a polymerized form) in the initial and final phase of the experiment for each of the three studied variants compared to the control variant is presented in Figures 1 and 2.

The final stage of the experiment usually highlights higher values of the color intensity and higher proportions of condensed tannins in a polymerized

form, which was also the case for the three variants of wine compared to the control variant.

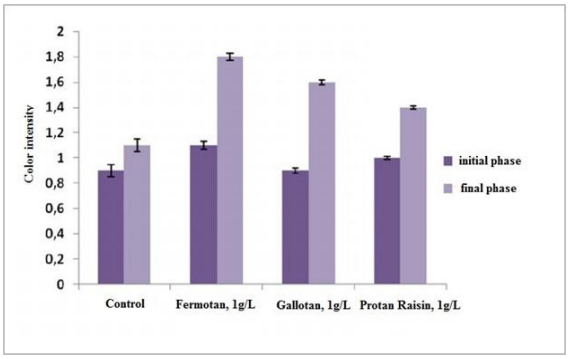

Figure 1. The color intensity of the wine samples in accordance to the type of tannin used during the alcoholic fermentation

The obtained results accentuated the synergistic action of tannin supplementation and micro-oxygenation on the color intensity increase and on the proportion of polymerized pigments. By using tannins in the fermentative step, a number of advantages were emphasized such as the ability to polymerize with anthocyanins and to form stable compounds thus avoiding the color losses as a result of the reactions between anthocyanins and other compounds. Moreover, another important property is the ability to react with oxygen hence avoiding the oxidation of polyphenols and attenuating the unwanted sensory character ("notes de réduction") by preserving varietal aromas. Also, they can prevent the early aging of wines by increasing the structural character of wines and reducing the doses of sulphurous anhydride in the pre-fermentative stage.

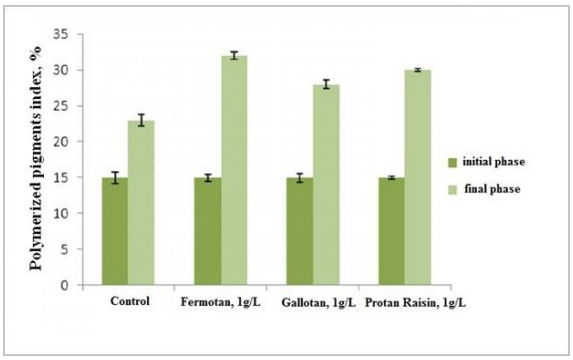

Figure 2. The polymerized pigments index of the wine samples in accordance to the type of tannin used during the alcoholic fermentation

C. GHANEM & al. [19] studied the chromatic properties and the antioxidant activity of a Cabernet Sauvignon red wine after the treatment with commercial tannins and mannoproteins. The addition of fining agents and oenological additives decreased the color intensity and increased the hue ofmost of the treated wines compared to the control. M.E. ALAÑÓN & al. [20] studied the behavior of different aging chestnut treatments that involve the exogenous tannins on the polymerized pigments index of Tempranillo red wines. Regardless of the type of wine, the values of the parameter were significantly higher, ranging from 30.14 to 41.78% when compared to the control sample.

The final step of the experiment emphasized higher values of the color intensity and higher concentrations of condensed tannins in a polymerized form for the three studied variants compared to the control variant. The obtained results alsodrew attention on the synergistic action of tannin supplementation and micro-oxygenation that produced the color intensity and the proportion of polymerized pigments increase. In the case of the wines obtained from crops with a suitable phytosanitary status and those obtained from crops partially contaminated with Botrytis cinerea in a proportion of 10%, an increase of the color intensity was also reported almost at the same level as the increase induced by the Fermotan doses used for both wines (Figure 3).

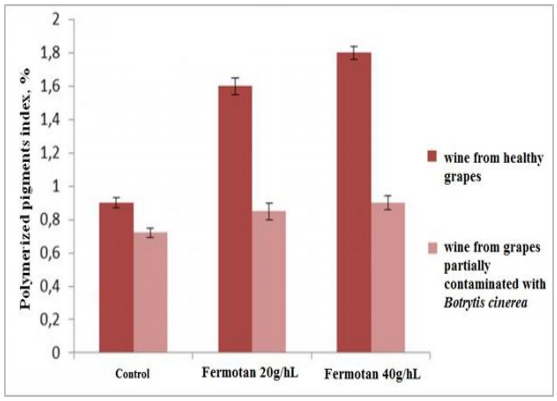

Figure 3. The color intensity evolution of the wine samples in accordance to the used Fermotan dose

The evolution of wine total polyphenols content revealed an increase once the dose of Fermotan, was raised from 20 g/hL to 40 g/hL for both wines obtained from grapes with a corresponding phytosanitary status and from those obtained from grapes with Botrytis cinerea in a proportion of 10% (Figure 4).The efficacy of Fermotanwas explained by the complexity of its composition, being a multi-component tannin

composed of condensed, ellagic and gallic tannins, designed to achieve a maximum efficacy in stabilizing the color of the red wines by precipitating the unstable protein.

G. VAZALLO-VALLEUMBROCIO & al. [18] displayed the characteristics of Carménère red wine that were assessed after the treatment with different commercial enological tannins.Regarding the color intensity and the hue values of the enriched wines, the third variant of wine registered the lowest color intensity values at the end of the study (90 days) in respect to the other sampling dates, while the rest of the wines presented similar values throughout the whole study. In contrast, all wines showed increased hue values at the third sampling date.

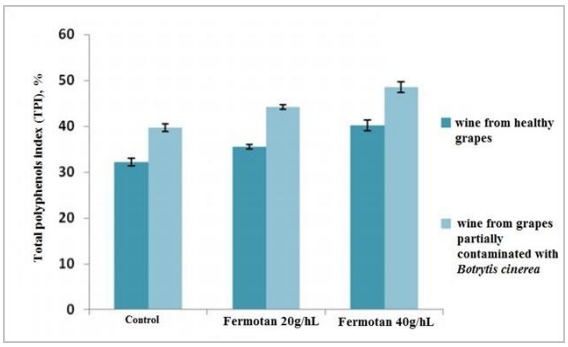

Figure 4. The total polyphenols index of the wine samples in accordance to the used Fermotan dose

M.E. ALAÑÓN & al. [20] presented the effect of 2 different oenological practices on the levels of different classes of phenolic compounds in the wines made from Vitisvinifera L. cv Tempranillo grapes.Several differences were observed between the phenolic composition of the wines made from the same grapes and with different oenological practices. The values of total polyphenols index and the color intensity for the treated variants of wine presented smaller values when compared to the control sample. G. VAZALLO-VALLEUMBROCIO & al. [18] evaluated the effect produced by the addition of different commercial enological tannins on the characteristics of Carménère red wine. All the wines enriched with tannins were analyzed after 5, 45 and 90 days, and they presented very high values of total phenols compared to the control wine (WT0) which exhibitedthe lowest concentration.

2. *The chromatic characteristics of the Feteasc*ă *neagr*ă *red wines*

Table 1 presents the chromatic characteristics of the wines 30 days after the completion of the alcoholic fermentation. Analyzing the data, it was observed that the yellow color component decreased in both cases compared to the control variant, while the red color values (as well as the intensity and brightness levels of the red color) underwent progressive increases compared to the blank sample.

The degree of polymerization and the degree of ionization also displayed an increase compared to the control variant. The evolution of the color intensity followed a progressive increase, while the wine tint (the ratio between the yellow and the red color, OD420nm/OD520nm) was stabilizedwhile the violet color decreased insignificantly.

The redness degree (derived from French "indice de rosissement" - the quantification of the wine oxidability with H2O2) diminished considerably in the variant treated only with Batonnage 150 KDa and showed a notable but less accentuated reduction in the variant treated with both Batonnage 150 KDa and Fermotan product.

Table 1. Chromatic characteristics of different variants of wine

Chromatic characteristics	**Control**	**Wine variant with Batonnage Plus 150 KDa, 40 g/hL addition**	**Wine variant with Batonnage Plus 150 KDa, 40 g/hL and Fermotan 20g/L addition**
Yellow color	30.0 ± 0.032	29.5 ± 1.060	29.0 ± 1.191
Red color	60.3 ± 2.054	61.1 ± 0.191	62.9 ± 0.097
dA, %	67.1± 0.140	68.5 ± 0.131	69.2 ± 0.543
Total polyphenols index (TPI)	54 ± 0.002	52 ± 0.028	53.5 ± 0.611
Color intensity	1.8 ± 0.531	2.3 ± 0.037	2.7 ± 0.468

Wine tint	0.5 ± 0.002	0.5 ± 0.106	0.5 ± 0.202
Polymerized pigments index	43 ± 1.412	44 ± 1.258	46 ± 1.907
Anthocyanins ionization index	74 ± 2.504	76 ± 1.384	81 ± 1.503
Redness degree (RS)	727 ± 5.201	532 ± 8.347	605 ± 9.011

Hence, it was demonstrated the ability of tannins to interact with polysaccharides so that the formed macromolecular aggregates decreased the astringency and improved the chemical and colloidal stability of the red wine in regards to aging abilities.

Other researchers evaluated and analyzed the effect of commercial tannins and mannoproteins on the pigment, color and tannins composition of a Cabernet Sauvignon red wine. The Total Polyphenols Index was largely affected by the used treatments. The decrease of TPI was explained by the removal ofsome polyphenolic compounds by the used treatments. The addition of tannins especially at a high concentration led to a significant increase of the TPI compared to the control wine. All the treated wines displayed a decrease in the content of total polyphenol except the wines in which the exogenous tanninswere added,although the increase was not significant except that for the maximum concentration. The obtained results were due to the effect of tannins on the anthocyanins contents of wines. The tannins addition increased the total polyphenols index by 9% at higher concentrations and the total tannins by 8% while it decreased the total anthocyanins content (C. GHANEM & al. [19]). W. TCHABO & al. [21] assessed the chromatic indicators in correlation to the phytochemical profile of a sulfur dioxide-free mulberry red wine.

The authors studied four different methods of color measurement of wine proposed by Boulton, Giusti, Glories and the Commission International de l'Eclairage (CIE). The change of the chromatic properties and phenolic composition of the non-thermal aged mulberry wine were examined by different statiscal tests. W. TCHABO & al. [21] studied many chromatic properties including the lightness, redness, yellowness, chroma, hue, angle, polymeric anthocyanin (PA) and saturation and also the total phenolic index (TPI), total flavonol index (TFI) and total anthocyanin index (TAI). The results revealed for all the parameters a positive effect of the non-thermal treatments on the phytochemical families and color stability of studied wines.

M.E. ALAÑÓN & al. [20] studied the behavior of different aging chestnut treatments that involved the exogenous tannins (chips, barrels, and aging time) on the phenolic profile and color characteristics of Tempranillo red wines. Practically, the formation of more polymerized pigments decreased the color saturation as a result of a more complex mixture between the pigments that provide the actual red color of the wine. The reduction of the anthocyanins during the aging process also caused a significant decrease of the copigmentation percentage that contributed to the total wine color and an increase of the increase of the lightness. In contrast, the yellow component of color and the hue angle did not significantly change after the aging period for the treated wines.

The wines red color presented observable purplish nuances similar to the original young wine. Furthermore, the authors identified significant differences between the control and the aged wines in terms of lightness and saturation. The color intensity of the aged wines was significantly lower than that of the control sample. This fact could be attributable to the decrease on the anthocyanin content, since they are involved not only in the reduction of copigmentation effect but also in the formation of insoluble polymeric pigments which represent the coloring matter of aged wines. On the other hand, the tint of the wine was evaluated based on the yellow color of wines. The highest values of tint observed were found in the wines aged for 6 months. The obtained values were also related to the high content of polymeric pigments that produced a color stabilization (M.E. ALAÑÓN & al. [20]).

Conclusions

The obtained results highlighted the synergistic action of the tannin supplementation and the microoxygenation on the color intensity increase and on the proportion of the polymerized pigments for the three studied variants compared to the control variant.

The synergy between the three categories of tannins developed a triple protective action on anthocyanins, namely the stabilizing action provided by the contribution of condensed tannins, the stimulation of acetaldehyde production which is indispensable for the formation of stable colored complexes and free radicals provided by the presence of ellagic tannins and the protective action against oxidation caused by the gallic tannin. Tannins possess the property to interact with polysaccharides in such a manner so that they can form macromolecular aggregates with a beneficial influence on the astringency, the chemical and colloidal stability and the ageing of the red wine.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 66-74
doi: 10.25083/rbl/24.1/66.74

Received for publication: January 22, 2018
Accepted: November 12, 2018

Original paper

Osteoclast recruitment and polymorphism during the healing process in dental implant surgery

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Abstract

Objectives: To demonstrate the role of plasma rich in growth factors (PRGF) in osteoclast recruitment and function during bone remodeling and healing of alveolar crest bone defect in dental implant surgery.

Method and materials: The histological examination was performed on bone carrots harvested after the implant surgery, in a patient with advanced alveolar bone atrophy in the posterior maxilla. Sinus floor elevation and bone augmentation were performed, associated with local application of PRGF. The samples were histologically examined using the Goldner's trichrome method.

Results: Microscopically, the particular aspects of new bone formation and remodeling were revealed: polymorphic bone trabeculae characterized by the association of woven, lamellar and necrotic bone. A prominent feature was the presence of numerous osteoclasts and osteoclast precursors on the surface of necrotic bone fragments, suggesting the intense bone resorption. Fewer macrophages were found on the bone trabeculae consisting of woven and lamellar bone, where remodeling was moderate.

Conclusions: Bone remodeling and healing at the site of implant surgery induced new bone formation and bone resorption by osteoclasts. Osteoclasts exhibited significant polymorphism and were associated with numerous osteoclast precursors. Local application of PRGF could influence bone remodeling by maintaining the balance between bone resorption and bone formation.

Keywords : alveolar crest bone defect, bone remodeling, osteoclasts, osteoclast precursors, plasma rich in growth factors PRGF.

To cite this article: RAȚIU C, BOȘCA AB, ILEA A, RUXANDA F, MICLĂUȘ V. Osteoclast recruitment and polymorphism during the healing process in dental implant surgery. *Rom Biotechnol Lett*. 2019; 24(1): 66-74. DOI: 10.25083/rbl/24.1/66.74

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Introduction

Nowadays, the use of dental implants is a common practice as tooth loss is a frequent problem occurring as a result of disease or trauma (POPA & al [1]). Dental implant surgery often requires grafting procedures in order to increase the bone quantity and quality at the recipient bed and to promote the optimal implant osseointegration and stability (F.S. SALMEN & al. [2]).

In dental implant surgery, free autologous bone grafts are the most widely used (N. VENKATARAMAN & al [3]). A frequent complication that occurs after autologous grafting is necrosis by insufficient blood supply (I.H. KALFAS [4]). In order to minimize graft necrosis and to improve the healing and integration process, several methods have been proposed, including the use of free vascularized or pedicle grafts (G.K.B. SÀNDOR & al. [5]). New biotechnologies, consisting in the association of bone grafts with various preparations such as platelet-rich plasma (PRP) and plasma rich in growth factors (PRGF) were proposed for stimulating and accelerating the healing process (E. ANITUA [6], F. MOLINA-MIÑANO & al [7]).

After the surgical trauma, a hemorrhage is formed around the graft; the mediators released from the recipient bed and from the blood act as chemo-attractants for the mesenchymal stem cells, inflammatory cells, and phagocytes. Depending on the local stimuli, the mesenchymal cells proliferate and differentiate into endothelial cells, fibroblasts or osteoblasts, resulting in the formation of blood vessels, connective tissue and bone; on the other hand, inflammatory cells and phagocytes play a role in graft incorporation, bone resorption, and the resulting remodeling process (I.H. KALFAS [4]).

Therefore, remodeling implicates not only the bone forming osteoblasts, but also the active osteoclasts that progressively eliminate the primary woven bone and allow the deposition of the bone lamellae leading to secondary lamellar bone formation during the healing process (VENKATARAMAN & al [3], I.H. KALFAS [4], S.N. KHAN & al. [8]).

The key cellular elements are the osteoclasts, multinucleated cells capable of bone resorption and essentially implicated in bone tissue homeostasis and remodeling (T. MARTIN & al. [9]).

Osteoclast formation and functions are regulated by systemic mediators such as: parathyroid hormone, Interleukin 1 (IL-1), tumor necrosis factor (TNF), transforming growth factor (TGF), monocyte colony stimulating factor (M-CSF), and 1,25-dihydroxyvitamin D3 (S.A. HIENZ & al. [10]); additionally, there are several cytokines released in the local microenvironment by the stromal cells in the bone marrow that control the osteoclastogenesis: interleukins, TNF-α, TGF-β, kinins and thrombin (R GRUBER [11]); the osteoclast is also capable to secrete cytokines that regulate its function (M.P. YAVROPOULOU & al. [12], T.J. MARTIN [13]).

From their formation site (bone marrow), mononucleated precursors circulate in the blood stream and upon arrival to the target site, they fuse to form the multinucleated osteoclast; alternatively, the mononuclear precursors may fuse with the local osteoclasts or even pre-existing multinucleated osteoclasts may fuse to form very large cells (T.J. MARTIN [13], G.R. MUNDY & G.D. ROODMAN [14], A.J. KAHN & D.J. SIMMONS [15], D.G. WALKER [16], G. GOTHLIN & J.L.E. ERICSSON [17], P.J. NIJWEIDE & al. [18]).

Numerous animal and clinical studies have investigated the effect of PRP and PRGF in combination with autologous bone and other types of graft materials, taking into consideration the following criteria: bone radiographic density, morphometric parameters of bone tissue, and the rapidity of healing. However, the results were controversial: Marx et al. reported increased bone density after using PRP with autologous bone for mandibular reconstructions, but Aghaloo et al. did not find significant radiographic or morphometric differences upon using PRP in bone lesions in rabbits (R.E. MARX & al. [19], T.L. AGHALOO & al. [20]). Molina et al. reported a higher amount of neo-formed bone in defects filled with autologous bone plus PRGF, but without significant differences compared with the controls. In vitro investigations confirmed platelet contribution in osteoclastogenesis and osseous resorption, but an exact mechanism is yet to be proposed (F. MOLINA-MIÑANO & al [7], C. FIORAVANTI & al. [21]).

Aim: Our study aimed at demonstrating the role of PRGF in bone remodeling by investigating the histological features during healing of alveolar crest bone defect.

Materials and Methods

The histological study was performed on bone carrots harvested from the alveolar crest 17 month after the initiation of the implant surgery, in a patient with advanced alveolar bone atrophy in the left posterior maxilla. The patient was clinically healthy and had no co-morbidities. Written and informed consent of the patient was obtained for tissue harvesting and histological examination.

Firstly, the sinus floor elevation and bone augmentation were performed, using autologous bone and bovine xenograft associated with PRGF; implants were inserted 6 months later. The outcome was not favorable due to an infectious complication involving left maxillary sinusitis and vestibular abscess after 3 months. Therefore, the implants and the necrotic infected bone were removed; the sinus was curetted and grafted with blood clot, fibrin clot and PRGF. The new implants were inserted 8 months later.

The harvested bone carrots were processed for histological examination; firstly, it was fixed in 10% buffered formalin for 7 days; then the sample was decalcified with 5% trichloroacetic acid, dehydrated with graded ethanol solutions (70°, 95°, absolute), clarified with n-buthanol and embedded in paraffin; finally, the tissue was sectioned at 5 µm and stained using the Goldner's trichrome technique. The slides were examined under an Olympus BX41 light microscope; images were taken with a digital camera and processed with Adobe Photoshop CS2 software.

Results and Discussions

The histological examination revealed characteristic features of new bone formation and remodeling; the new bone occupied the entire intervention area and displayed various morphological features, according to the location and the stage of development.

In the superficial area, woven bone had a dense structure, corresponding to the cortical bone on the surface of the alveolar crest. In the profound areas, the new bone had the structure similar to cancellous bone, with trabeculae of various shape and size delimiting areoles (Fig. 1a, 1b, 2a). In the areoles, next to the trabeculae, there was zonal chronic inflammatory infiltrate containing mononuclear cells, without tendency of acutization (Fig. 1a, 1b). The most significant feature was the polymorphism of the trabeculae due to the coexistence of woven bone, lamellar bone, as well as necrotic bone (Fig. 1a, 1b, 2a).

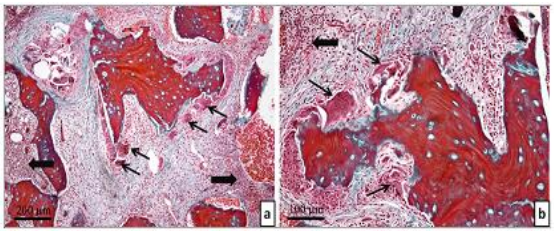

Figure 1. Bone trabeculae in various stages of remodeling. (a) and (b) trabeculae consisting of woven bone and lamellar bone, surrounded by osteoclasts (thin arrows); in the areoles, chronic inflammatory infiltrate associated with the trabeculae (thick arrows).

It could be easily estimated that the new bone had been initially primary woven bone, which still composed the main mass of the trabeculae, but by remodeling, it has been progressively replaced by secondary lamellar bone. However, in some areas necrotic woven bone could be identified; it was either associated with larger trabeculae (Fig. 2a), or formed isolated smaller fragments (Fig. 2b, 3b).

Larger bone trabeculae consisted of woven bone and lamellar bone in various proportions. Primary woven bone contained a large number of isodiametric osteocytes and lower mineral content in the matrix; lamellar bone contained irregularly arranged bone lamellae, and fewer flattened osteocytes embedded into the calcified matrix; small osteoblasts and the osteoid layer bordered the trabeculae (Fig. 2a). Bone resorption was moderate, and isolated osteoclasts or few osteoclast precursors were identified on the surface of the trabeculae (Fig. 1a, 1b).

Smaller bone fragments exhibited various degrees of necrosis and/or resorption; (Fig. 2b, 3b, 3c). Necrotic bone had fissures detaching smaller fragments from the main trabeculla; it contained dischromic bone matrix and degenerating osteocytes or empty osteocytic lacunae; osteoblasts were absent on the surface (Fig. 2b, 3b). Isolated or grouped giant multinucleated cells (osteoclasts) and numerous osteoclast precursors were present in the vicinity of these fragments (Fig. 2b, 3b, 3c).

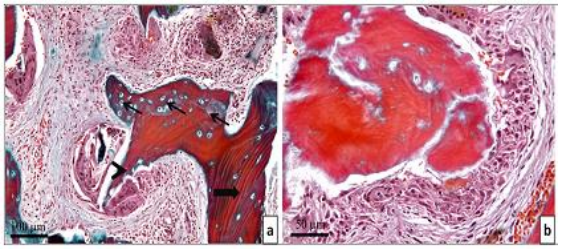

Figure 2. Bone trabecullae in various stages of necrosis and remodeling. (a) Bone trabeculla consisting of woven bone (thin arrows), lamellar bone (thick arrow) and necrotic bone covered by large osteoclasts (arrowhead) (b) Necrotic woven bone associated with the larger trabecula and surrounded by osteoclasts and osteoclast precursors.

Osteoclasts exhibited significant polymorphism regarding the shape, size, aspect of the cytoplasm and number of nuclei (Fig. 3). Osteoclasts' morphology seemed to be related to the formation stage and the functional state. Cells in early stages were smaller and contained three to six nuclei; these osteoclasts were either isolated in the areoles, or attached to the surface of small bone fragments (Fig. 3a). Mature active osteoclasts were larger and contained numerous nuclei, according to their size; their number depended on the type of bone tissue they were associated with. Active osteoclasts were fewer and rested in deep resorption lacunae on the surface of woven bone, and practically absent on the lamellar bone (Fig. 3b); by contrast, active osteoclasts were very numerous and completely surrounded smaller necrotic bone fragments, suggesting that the bone resorption was highly active (Fig. 2b, 3c). Resting and degenerating osteoclasts showed signs of apoptosis: condensed or vacuolated cytoplasm and shrunk irregular nuclei; these cells were in the proximity of intensely resorbed bone fragments or debris (fig. 3d).

Both early and active osteoclasts were associated with precursor cells that showed obvious tendency to fuse with one another (Fig. 2a, 3b). The osteoclast precursors were irregularly shaped, with many cytoplasmic processes, contained acidophilic cytoplasm and a single, round, euchromatic nucleus. The precursors located in the proximity and around bone fragments were recruited in variable number, according to the intensity of the bone resorption (Fig. 2a, 3b). Very numerous osteoclast precursors and active osteoclasts were associated with grouped small bone fragments, remnants of larger necrotic trabeculae that were intensely resorbed (Fig. 3c, 3d). In moderate numbers, mononuclear osteoclast precursors and osteoclasts were present inside the fibrous capsule organized around the small necrotic bone fragments (Fig. 4a, 4b).

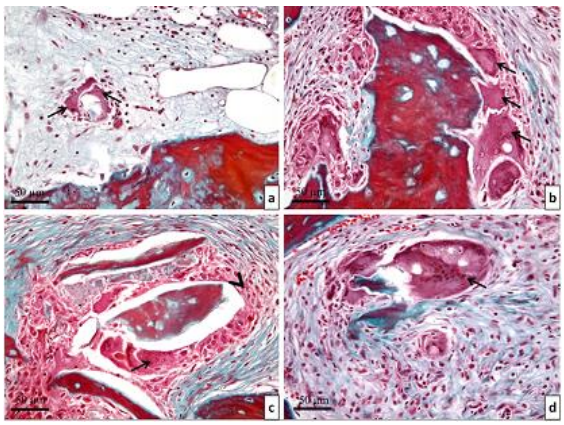

Figure 3. Polymorphic osteoclasts and osteoclast precursors. (a). Small osteoclasts isolated in the connective tissue within the areoles (arrows); (b). Active osteoclasts on the surface of necrotic bone with multiple euchromatic nuclei and abundant acidophilic cytoplasm (arrows); (c). Active osteoclasts (arrows) and numerous precursors with increased tendency to fuse (arrowhead). (d). Apoptotic osteoclasts with pyknotic nuclei and dark cytoplasm (arrow). (Goldner's trichrome)

Necrotic bone fragments were completely sequestered by osteoclasts and numerous precursor cells; at the periphery, a connective tissue capsule was formed, thus resulting in the organization of a structure similar to an encapsulated granuloma. (Fig. 4a, 4 b)

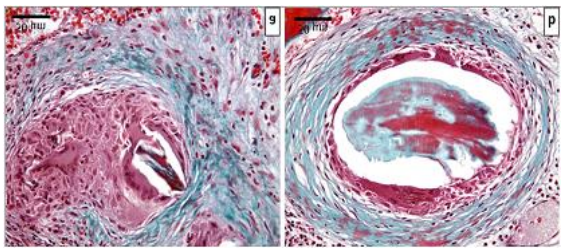

Figure 4. Encapsulated granulomas. (a) The connective tissue in the proximity of the necrotic bone showing tendency to condense and to enclose numerous osteoclasts and osteoclast precursors; (b) A thick capsule completely isolating the necrotic bone fragment and the osteoclasts on the bone surface (Goldner's trichrome)

Any surgical intervention in bone induces a complex tissue response due to the release of cytokines

such as: bone morphogenetic protein 2 (BMP-2), platelet derived growth factors (PDGF), TGF and vascular endothelial growth factor (VEGF), that activate osteogenesis and promote the regeneration of the traumatized bone (D. GERARD & al. [22], G. WEIBRICH & al. [23]). The healing process occurs in two steps: the initial woven bone is rapidly formed by osteoblasts to fill in the defect; but this immature bone has a low mineral content and a disorganized structure, thus its biomechanical properties are poor. In the next step, the woven bone is replaced by mature lamellar bone, due to coupled osteoclastic-osteoblastic activity – the remodeling process. The repair process will proceed until all the bone defect is filled with lamellar bone (N. VENKATARAMAN & al [3]).

Frequently, however, bone healing may be incomplete or delayed, depending on the influence of factors such as: nutrition (blood supply), mechanical forces (pressure or instability), and competition from the surrounding tissues (the ingrowth of the soft tissue in the bone defect) (F.S. SALMEN & al. [2], I.H. KALFAS [4], G.K.B. SÀNDOR & al. [5]).

Besides these factors, some other particular aspects are significant for healing of an autologous bone graft. When introducing a graft in a bone defect, the healing process may be influenced by the graft itself in two ways: passively, because the graft acts as a mechanical barrier, and actively, by the interaction between the graft surface and the molecules released from the graft and the biological environment. For example, an autologous graft may enhance the healing process due to the presence of the viable cells and bone inductive agents in the graft, while an allograft could have a negative effect, by eliciting an immunological reaction (F.S. SALMEN & al. [2], R.A. HOROWITZ & al. [24]). Moreover, even though the vascular supply at the recipient bed is a prerequisite, the survival of the osteocytes present within the bone graft seems to greatly influence the outcome, since these cells may be implicated in the repair process (N. VENKATARAMAN & al. [3]).

The repair process is different for the compact bone which consists of densely packed concentric bone lamellae, compared with the cancellous bone, which is porous and formed by trabeculae enclosing the marrow. Due to the medullary component, the bone graft in the spongy bone is in close relationship with numerous cells and blood vessels in the bone marrow, and thus the healing occurs more rapidly than in the compact bone (T. BAUER [25]).

However, in our case, the healing process was heterogeneous, and the evolution depended on the vascularization. In zones with deficient blood supply, the woven bone underwent necrosis and elicited intense osteoclastic activity. In bone fragments consisting of necrotic bone matrix and degenerating osteocytes, no osteoblasts were present on the surface and osteoid formation was not initiated. Instead, the necrotic bone had a chemotaxis effect and induced the massive recruitment of osteoclast precursors and their differentiation into active osteoclasts, leading to intense bone resorption. By contrast, in zones with appropriate nutrition, the remodeling of the woven bone into lamellar bone was favorable; trabeculae consisted of an association of woven and lamellar bone, and the bone tissue underwent remodeling by balanced apposition and resorption.

These particular aspects explain the presence of both necrotic bone fragments and trabeculae consisting of newly formed woven and lamellar bone.

In our case, the healing process was favorable because in cancellous bone, that occupied most of the bone defect, regeneration is sustained by several local mechanisms. Due to the increased surface, more mesenchymal cells populate the bone surface and differentiate into osteoblasts (M. MOGA & al. [26]); the osteocytes in the bone matrix are closer to the periphery and their nutrition is better, and the vascular ingrowth was demonstrated to occur 30% faster than in compact bone. Osteoblasts lining the surface secrete osteoid which mineralizes to form the immature bone. In the final remodeling stage, the immature newly formed bone and the necrotic bone are resorbed by osteoclasts and replaced by mature lamellar bone; with time, the cancellous bone fills the entire bone defect, and the healing is complete (I.H. KALFAS [4], S.N. KHAN & al. [8], A.J. KAHN & D.J. SIMMONS [15], S.S. JENSEN & al. [27]).

Bone remodeling is essential for tissue homeostasis and regeneration of bone defects. Osteoclasts function and their differentiation from precursors play a key role in bone remodeling due to

their resorptive activity. Osteoclast precursors derive from the mononuclear hemopoietic cells: granulocyte/ macrophage progenitor cells that give rise to granulocyte and monocyte cell lineages (T.J. MARTIN [13], T. NAGASAWA & al. [28]). Monocytes are the common origin of several other types of multinucleated cells that have phagocytic activity, including Langhans giant cells, Touton giant cells and multinucleated giant cells in epulis (W.G. BRODBECK & al. [29], A.B. BOȘCA & al. [30]).

Osteoclast formation is a complex process encompassing several stages: proliferation and recruitment of progenitor cells; differentiation of progenitor cells; fusion of the mononuclear osteoclast precursors in order to form adult osteoclasts (T.J. MARTIN [31]).

Osteoclast differentiation and maturation is further regulated by the RANK-RANKL signaling mechanism. RANK (Receptor activator of nuclear factor kappa B) is a receptor molecule expressed on the surface of the osteoclasts that interacts with RANK ligand molecule (RANKL) produced and expressed on the stromal cells and osteoblasts' surface. Alternatively, immunocompetent cells, such as activated T lymphocytes can secrete RANKL molecules; thus, during inflammation, the osteoclast-mediated bone resorption is initiated (S.A. HIENZ & al. [10], T. NAGASAWA & al. [28], B.F. BOYCE & L. XING [31]).

In the bone tissue, the osteoclast binds to the bone surface by a mechanism that implicates the integrin αvβ3, the dominant osteoclast integrin and the marker of the osteoclast phenotype. This integrin is not present in macrophage precursors, but is induced in osteoclasts by RANKL. Integrin αvβ3 enables the osteoclast to recognize a tripeptide sequence (Arg-Gly-Asp) that is present in the macromolecules in the bone matrix: osteopontin, fibronectin, vitronectin and fibrinogen (W.G. BRODBECK & J.M. ANDERSON [29]).

In the present case, the local mediators were supplemented with growth factors present in the PRGF: TGF-β1, VEGF (Vascular endothelial growth factor) and IGF (Insulin-like growth factor); these proteins modulate cell proliferation, differentiation and migration (chemotaxis) (F. MOLINA-MIÑANO & al [7], R. FARINA & al. [32]). PRGF has the advantage of being an autologous product, it is a source of multiple growth factors and it is reabsorbed in several days after initiating local regeneration (E. ANITUA & al. [33]). Addition of growth factors such as TGF-β and IGF to the autologous graft stimulates the differentiation of cell lines implicated in bone regeneration and of the woven bone to lamellar bone (N. VENKATARAMAN & al [3], K.M. LACCI & A. DARDIK [34], J. ALSOUSOU & al. [35]).

Our results indicate that the recruitment of osteoclast precursors was induced by two mechanisms: the direct action of the chemotactic factors in PRGF and the indirect stimulation of osteoclasts, which secrete cytokines that control the migration and differentiation of the precursors. Moreover, the presence of numerous osteoclasts suggests an intense osteolytic activity necessary for removal of necrotic bone and the remodeling of woven bone in the intervention area.

The large number of mononucleated osteoclast precursors associated with multinucleated osteoclasts is explained by the life cycle of the osteoclasts. In vivo osteoclasts have an up to 2-week lifespan and a half time of 6 to 10 days (C. GRAY & al. [36]); in order to achieve an appropriate bone resorption, osteoclasts have to be renewed constantly.

Osteoclast precursors recruited in the proximity of the necrotic bone fragments formed groups close to active osteoclasts, suggesting that they were differentiating and fusing to form new osteoclasts, thus promoting the bone resorption and remodeling. A particular aspect was the presence of osteoclast precursors and active osteoclasts inside the granulomas sequestering necrotic bone fragments.

Conclusions

PRGF controls the resorption of the necrotic bone by osteoclasts, and new bone formation associated with bone trabeculae of whose viability was preserved.

The osteoclasts polymorphism is consistent with the formation stage and the functional state; moreover, their association with numerous osteoclast precursors is correlated with the intensity of bone remodeling.

Therefore, the association of osteoinductive agents, such as PRGF, sustains the remodeling process by maintaining the balance between bone resorption and bone formation.

The outcome of bone healing depends on the extent to which the circumstances in the environment

in the bone defect allow the new bone formation and remodeling in order to promote regeneration.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

All authors have equal contribution to this article.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 75-81
doi: 10.25083/rbl/24.1/75.81

Received for publication: May 2, 2018
Accepted: August 21, 2018

Original paper

The prevalence of hrHPV in a significant cohort of Romanian women

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Abstract

The aim of this study was to analyze the prevalence of hrHPV correlated to cytological abnormalities in a significant cohort of women from Romania. Methods: The study group included 625 Romanian female patients aged 16 to 68 years that were referred to Synevo laboratories for performing both a liquid-based Papanicolaou test and a high-risk HPV DNA test. Results: Cytological abnormalities were presented in 199 patients (31.84%) and HPV infections were detected positive in 215 patients (34.4%). When considering all 215 HPV positive samples, 13.02% of samples were associated with HSIL, 27.91% with LSIL, 31.63%, with ASC-US and 6.98% ASC-H. However, 20.47% from the samples presenting HPV DNA were included in NILM group. The prevalences of hrHPV were as follows: HPV16 27.82%; HPV18 7.26%, other hrHPV 64.92%. Analysis per cytological groups revealed that HPV 16 had a similar prevalence in LSIL and HSIL (34.29 vs. 34.48%) compared to HPV 18 that had the highest prevalence in ASC-H (27.78%). Other pooled hrHPV registered high prevalence in all abnormal cytologies (between 55.56% and 65.85%). Stratification by age showed a little variation of HPV16 and HPV18 prevalences in all age groups. Conclusion. Considering the high prevalence of hrHPV infections in Romanian women the support for vaccination programs and an increased availability of clinically validated HPV molecular tests are necessary.

Keywords : hrHPV, prevalence, Romanian women, cohort

To cite this article: STROE R, MAMBET C, CURICI A, IVAN F, ALEXA L, MORJAN C, LAZAR V, BLEOTU C. The prevalence of hrHPV in a significant cohort of Romanian women. *Rom Biotechnol Lett*. 2019; 24(1): 75-81. DOI: 10.25083/rbl/24.1/75.81

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Introduction

Cervical cancer is the fourth most frequent cancer in women all over the World and the second most common female cancer in the 16-44 years age group in Europe (BRUNI & al. [1]). According to statistics provided by GLOBOCAN 2012, 528000 new cases of cervical cancer and 266000 deaths are estimated annually. Developing countries account for more than 85% of new cervical cancer cases. The overall incidence of cervical cancer is growing, being expected an increase of 42% by the year 2020 (FERLAY & al. [2]). In Europe there is an unequal distribution of incidence and mortality rates per 100,000 population among different regions. Thus, in Eastern Europe the incidence and mortality rates are 16.3% and 6.2% compared to Western Europe countries where rates of 7.3% and respectively 1.8% are reported (BRUNI & al. [1]). Romania has the highest rate of incidence and mortality in Europe, a situation which has been constant since early 1980. Cervical cancer affects young, active women and is the leading cause of death for the Romanian female population belonging to the age category 20-44 years. (JSI RESEARCH & TRAINING INSTITUTE, INC./ ROMANIAN FAMILLY HEALTH INITIATIVE FOR THE USA AGENCY FOR INTERNATIONAL DEVELOPMENT [3]). Between 2000-2006 there were reported 22830 new cases of cervical cancer and 1273 deaths. In 2005 the incidence rate ranged between 17.8-31.2 and mortality between 12.3 - 21.5 (APOSTOL & al. [4]). In 2012 the age-standardized incidence rate of cervical cancer in Romania was estimated to 28.6% (FERLAY & al. [2]).

Persistent infection with high-risk (oncogenic) human papilloma virus (hrHPV) genotypes is the most important risk factor for the occurrence of premalignant lesions and cervical cancer, being a necessary, although not sufficient condition (ROSITCH & al. [5]). Other risk co-factors such as smoking, prolonged consumption (>6 years) of oral contraceptives, persistent immunosuppression, other sexually transmitted diseases and sexual behavior might play a role in cervical carcinogenesis (BURCELL & al. [6]). Based on their involvement in cervical cancer, HPV genotypes were classified into the following groups: HPV types with high oncogenic risk or carcinogenic (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59); HPV types probably carcinogenic (68); HPV types possibly carcinogenic (26, 53, 66, 67, 70, 73, 82); HPV types with low oncogenic risk (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108 (BOSCH & al. [7]; MARKOWITZ & al. [8]; SCHIFMANN & al. [9]). Virtually all cases of cervical cancer are caused by oncogenic HPV genotypes; among them, HPV16 and 18 are responsible for about 70% of all cases (SASLOW & al [10]). Although the progression to cancer is rare, the high prevalence of HPV infection among the population explains why the cancers related to HPV, including the cervical cancer and other anogenital cancers, are among the most frequent malignancies (DUNNE & MARKOWITZ [11]).

In order to prevent morbidity and mortality related to cervical cancer several strategies of screening have been attempted in the course of time, their aim being to detect cervical lesions that can progress to cancer. Adopting cervical cytology in Papanicolaou (Pap) smears as an effective mass screening method have significantly reduced cervical cancer incidence in the developed countries. However, important limitations of cytology have been identified, such as low sensitivity (50-60%) due to pre-analytical issues and subjective interpretation (COX & CUZICK [12]; SASLOW & al. [10]). Introduction of HPV testing in clinical settings aimed to improve the rate of cervical premalignant lesion detection (SASLOW & al. [10]).

There are only few Romanian studies concerning the prevalence of HPV infections and their association with cervical lesions, especially in ambulatory units. In this respect, the purpose of our study was to analyze the prevalence of hrHPV correlated to cytological abnormalities in a significant cohort of women that performed liquid-based cytology combined to a hrHPV test featured to detected clinically relevant infections.

Materials and Methods

Patients

The study group included 625 Romanian female patients aged 16 to 68 years that were referred to Synevo laboratories between October and December 2014 for performing both a liquid-based Papanicolaou test (BD SurePath™ liquid-based Pap test, Becton Dickinson) and a high-risk HPV DNA test (Cobas®

4800 Human Papillomavirus, Roche Molecular Diagnostics). The combined testing was recommended predominantly as a part of a routine control (70.77 % of the cases), or due to various medical conditions: cytological abnormalities detected during the last 3-12 months (16.93%), cervicitis (5.75%), recent surgery for cervical intraepithelial lesions (CIN, 5.11%), genital condylomatosis (0.80%), previous HPV infections without cytological abnormalities (0.64%).

After obtaining the informed consent from the patients, cervical samples were collected by specialized nurses using a soft cervical brush (Cervex-Brush®, Rovers Medical Devices B.V., Oss - The Netherlands) that allows simultaneously sampling of ectocervical, endocervical and transformation-zone cells for cervicovaginal cytology as well as for HPV test. Following sample collection, the detachable head of the brush was placed into the SurePath vial (Becton Dickinson) that provides efficient cell preservation and a proper transportation medium.

The liquid-based cytology

The liquid-based cytology was performed on Tripath platform (Becton Dickinson) including several sample processing steps: vial vortexing, centrifugal sedimentation through a density reagent (to partially remove non-diagnostic debris and excess inflammatory cells and to ensure sample enrichment with clinically relevant cells), automatic slide preparation and staining. The slides prepared in triplicate for each sample were examined under microscope by qualified cytopathologists using a digital image processing system (BD FocalPoint™ GS Imaging System). Results were reported according to the Bethesda 2001 guidelines.

HPV detection

HPV DNA test was performed on the automatic platform Cobas 4800 (Roche Molecular Diagnostics) using a qualitative Real-time PCR assay that detects 14 high-risk HPV clinically relevant genotypes. This test provides concurrently individual results for the highest risk genotypes, HPV 16 and 18, and pooled results for the other 12 high-risk HPV genotypes (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). Target DNA is a sequence of approximatively 200 bp located within the polymorphic L1 region of HPV genome. Human β-globin gene serves as an internal control, monitoring the whole test process.

Cervical samples were automated prepared to extract HPV as well as cellular DNA followed by amplification of target DNA and β-globin gene using specific primer pairs and real-time detection of the cleaved oligonucleotide probes that are labeled with four fluorescent dyes corresponding to the 12 high-risk HPV genotypes, HPV16, HPV 18, and β-globin gene. According to manufacturer instructions a set of negative and positive controls were used in each run in order to validate the results.

Statistical analysis

Frequencies and percentages were used to describe qualitative data while for quantitative data were used the range (minimum and maximum), mean, and standard deviation. The data were stratified by age (≤ 20 years, 21-25 years, 26-30 years, 31-35 years, 36-40 years, 41–45 years, 46–50 years and >50 years). For association between HPV infection in cervical lesions with cytology logistic regression analysis was performed. Data were analyzed by Student's t test and different categorical variables were compared by two ways ANOVA with Bonferroni posttests.

Results

Of 625 patients referred to Synevo laboratories, 199 patients (31.84%) presented cytological abnormalities with the following distribution according to the type of cervical lesions: 4.64% high-grade squamous intraepithelial lesions (HSIL), 11.2% low-grade squamous intraepithelial lesions (LSIL), 13.12% atypical squamous cells of undetermined significance (ASC-US), and 2.88% atypical squamous cells- HSIL cannot be excluded (ASC-H).

By employing Cobas HPV assay that is able to identify clinically relevant HPV infections we detected positive results in 215 patients (34.4%). When considering all HPV positive samples (215 patients), 13.02% of samples were associated with HSIL, 27.91% with LSIL, 31.63%, with ASC-US and 6.98% ASC-H. However, 20.47% from the samples presenting HPV DNA were included in NILM group. According to figure 1, the highest prevalence of HPV infection was observed in HSIL category (96.55%), while the lowest one was found in NILM patients (10.32%) ($p<0.002$). The other cytological abnormalities, ASC-US, ASC-H and LSIL were also associated with a high HPV prevalence: 82.93%,

83.33% and respectively, 85.71%. The overall prevalence of hrHPV in women with abnormal cytologies was 85.92%.

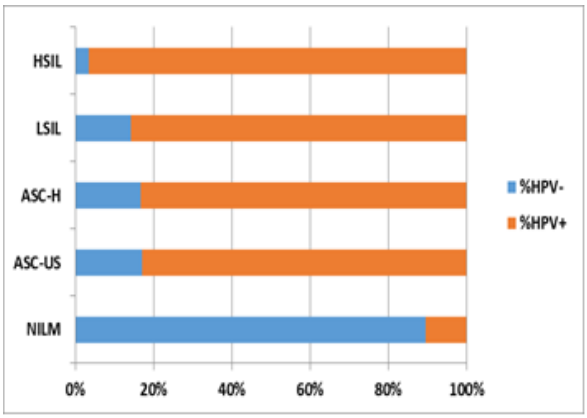

Figure 1. The percent of hrHPV+ in cytological groups.

Taking into account that age is a critical factor when assessing the relation between HPV infections and cervical cancer, the patients were furtherly stratified into 5 year age groups, starting with less than 20 years and ending with over 50 years. The prevalence of hrHPV infection progressively increased reaching maximum value in 26-30 years group and then registered a gradual decrease with age. Also, a similar pattern of abnormal cytologies in relation to age groups was observed (r=0.988) (Figure 2).

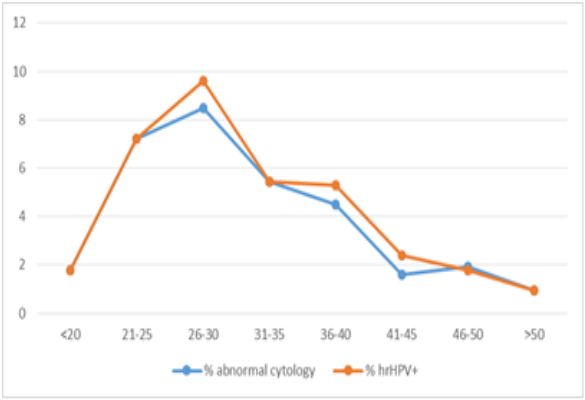

Figure 2. Prevalence of hrHPV infection and abnormal cytologies related to age groups (all 625 samples were considered).

When considering each age group separately we found that in women less than 25 years the prevalence of hrHPV was highest in LSIL and ASC-US cytology. In patients over 46 years, the presence of hrHPV was most frequently associated with HSIL cytology. The highest percent of HPV negative samples were associated with NILM cytology and older patients (Table 1).

Table 1. Prevalence of HPV in cervical lesions in each age group.

		NILM	ASC-US	ASC-H	LSIL	HSIL
<20	HPV(-)	43.48% (n=10)	4.35% (n=1)	(n=0)	4.35% (n=1)	(n=0)
	HPV+	8.70% (n=2)	13.04% (n=3)	(n=0)	26.09% (n=6)	(n=0)
21-25	HPV(-)	46.15% (n=42)	2.20% (n=2)	(n=0)	2.20% (n=2)	(n=0)
	HPV+	4.40% (n=4)	15.38% (n=14)	4.40% (n=4)	18.68% (n=17)	6.59% (n=6)
26-30	HPV(-)	54.55% (n=78)	2.10% (n=3)	(n=0)	1.40% (n=2)	(n=0)
	HPV+	8.39% (n=12)	16.08% (n=23)	3.50% (n=5)	9.09% (n=13)	4.90% (n=7)
31-35	HPV(-)	62.75% (n=64)	2.94% (n=3)	(n=0)	0.98% (n=1)	0.98% (n=1)
	HPV+	4.90% (n=5)	9.80% (n=10)	1.96% (n=2)	9.80% (n=10)	5.88% (n=6)
36-40	HPV(-)	61.76% (n=63)	2.94% (n=3)	1.96% (n=2)	0.98% (n=1)	(n=0)
	HPV+	10.78% (n=11)	6.86% (n=7)	1.96% (n=2)	9.80% (n=10)	2.94% (n=3)
41-45	HPV(-)	76.06% (n=54)	(n=0)	(n=0)	2.82% (n=2)	(n=0)
	HPV+	9.86% (n=7)	7.04% (n=5)	(n=1)	4.23% (n=3)	(n=0)
46-50	HPV(-)	72.92% (n=35)	(n=0)	(n=0)	2.08% (n=1)	(n=0)
	HPV+	(n=0)	10.42% (n=5)	4.17% (n=2)	2.08% (n=1)	8.33% (n=4)
> 50	HPV(-)	80.00% (n=36)	4.44% (n=2)	2.22% (n=1)	(n=0)	(n=0)
	HPV+	6.67% (n=3)	2.22% (n=1)	(n=0)	(n=0)	4.44% (n=2)

Analysis of all hrHPV positive samples per age group revealed a similar profile of increased association with LSIL in younger patients (Figure 3).

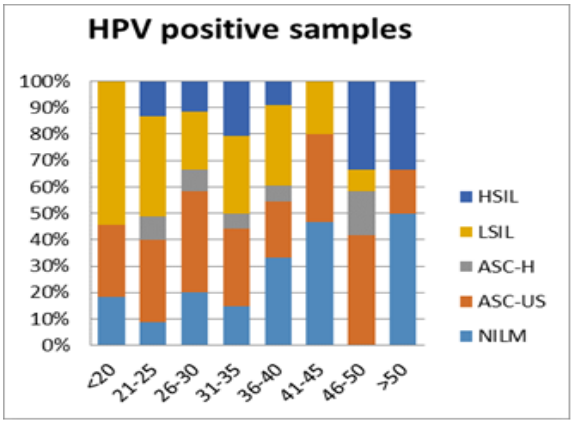

Figure 3. Distribution of cytological abnormalities per age groups in hrHPV positive samples.

As Cobas HPV assay provides individual genotyping information for HPV16 and HPV18 and reports all other 12 hrHPV as a pool we analysed in the next step the distribution of HPV genotypes in all HPV positive samples, within each cytological group and per age groups. Thus, considering all HPV positive samples, the prevalences were: HPV16 27.82%; HPV18 7.26%, other hrHPV 64.92%. Analysis per cytological groups revealed that HPV 16 had a similar prevalence in LSIL and HSIL (34.29 vs. 34.48%) compared to HPV 18 that had the highest prevalence in ASC-H (27.78%). Other pooled hrHPV registered high prevalence in all abnormal cytologies (between 55.56% and 65.85%) (Figure 4). Stratification by age showed a little variation of HPV16 and HPV18 prevalences in all age groups with a slight increase in women over 50 years (figure 5). Multiple HPV infections (confirmed through detections of at least tow fluorescent signals), were detected in 33 hrHPV DNA positive cases (15.3%), being more prevalent in younger women with ASC-US and LSIL cytology.

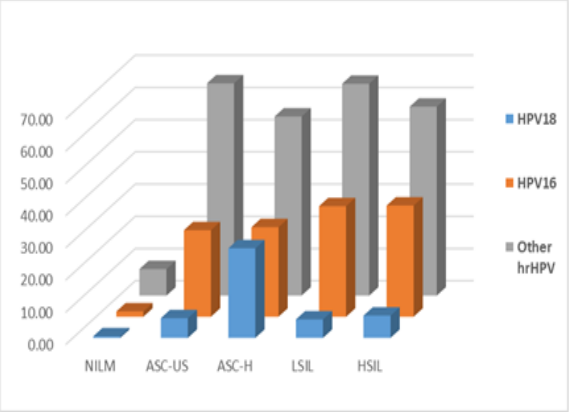

Figure 4. hrHPV genotypes distribution per cytological group

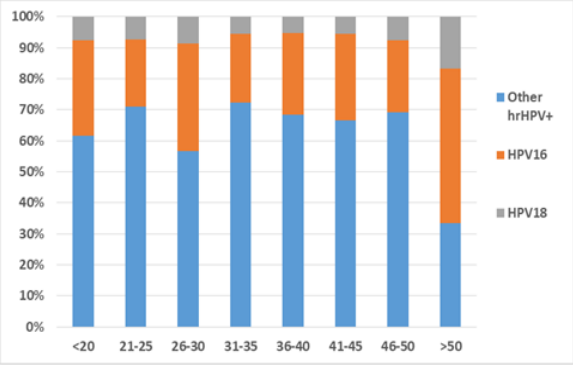

Figure 5. hrHPV genotypes distribution per age group

Discussions

The results of our study indicate a 34.4% prevalence of HPV infection with oncogenic genotypes that is closely correlated with the prevalence of cytological abnormalities (31.84%) among the population referred for co-testing to the Synevo laboratories from different geographical areas of Romania. The correlation has been observed also when we analyzed the prevalence by age-groups. These data support the good clinical performance of Cobas HPV assay that was previously validated by ATHENA (Addressing THE Need for Advanced HPV Diagnostics) study, the test being able to identify women at highest risk of developing premalignant lesions (CASTLE & al. [13]; WRIGHT & al. [14]).

The prevalence of hrHPV genotypes is slightly increased compared to the prevalence reported by the largest Romanian study that included 1000 women from Brasov county: 29%. Moreover, the prevalence of hrHPV in women with NILM (10.32%) is much lower and prevalence in cases with abnormal cytologies is much higher (85.92%) than in the above mentioned study: 24.2% and 48.24%, respectively (MOGA & al. [15]). These differences can be partly explained by the type of HPV assay employed - Cobas HPV versus LINEAR ARRAY HPV Genotyping Test.

When considering the prevalence of hrHPV genotpyes per age group, we found an obviously higher prevalence in women aged ≤ 25 years (49.1%) compared to women above 40 years (19.6%). While in younger women the hrHPV genotypes was mostly associated with ASC-US and LSIL cytology, in elder women the association of hrHPV was stronger with HSIL cytology. These data suggest a low progress of HPV infection acquired during early ages (MELON & al. [16]).

In our study, HPV 16 genotype was detected in 27.82% of all hrHPV DNA positive samples. Other Romanian studies reported the following HPV 16 genotype prevalence: 24.7% (Moga et al., 2014), 29.8% (URSU & al. [17]) and 34.7% (ANTON & al. [18]), indicating there might be some regional variations in hrHPV genotypes distribution.

In contrast with previously other Romanian studies, we found a higher prevalence of hrHPV genotypes and particularly HPV 16 genotype in LSIL cytology (85,71% and 34,28%, respectively). As the

risk of LSIL progression is dependent on the certain hrHPV genotype(s), being significantly higher in women carrying HPV 16 (MATSUMOTO & al. [19]), these data suggest an increased risk of cervical precursor lesion persistance and progression in LSIL women from our group. In order to avoid unnecessary invasive procedures (colposcopy and biopsy) and treatment in LSIL that is mostly prevalent in women under 30 years, immunocytochemical dual staining of p16/Ki-67 was proposed as an additional test for cervical cancer screening, being able to predict with high accuracy the high-grade cervical intraepithelial neoplasia in women ≤30 years diagnosed with LSIL (POSSATI-RESENDE & al. [20]).

Conclusions

As a limitation of our study, the results of the cervical biopsies performed on the female patients with cytological abnormalities were not available; these would have enabled a better correlation of hrHPV genotypes with cervical lesions.

Considering the high prevalence of oncogenic HPV infections in Romanian women with abnormal cytologies, both the support for vaccination programs and an increased availability of clinically validated HPV molecular tests are necessary.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

All authors have equal contribution to this article.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 82-90
doi: 10.25083/rbl/24.1/82.90

Received for publication: December 29, 2017
Accepted: January, 23, 2019

Original paper

Influence of age on sperm parameters in men with suspected infertility

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Abstract

Male fertility has become a very discussed topic among researchers in the last years. With the latest environmental changes, use of genetically modified foods, lack of physical exercise, work overload and frequent burnouts, the number of men prone to infertility is continuously increasing. In this study, we aimed to evaluate the influence of age on the sperm quality in presumably infertile males. In this purpose, we have investigated a significant number of semen samples, by using macroscopic and microscopic standard evaluation methods to determine the volume, viscosity, density, morphology, motility and vitality of spermatozoa. Our data suggest that spermatozoa viability and progressive motility, two of the most important indicators of sperm quality, decrease with age, suggesting the role of patient's age in male infertility, as well as the necessity for a better understanding of the intimate mechanisms and risks involved, as an increasing number of men are choosing to delay fatherhood.

Keywords : infertility, male population, age threshold, sperm parameters, motility, density, spermatozoa

To cite this article: SILEA C, CUCU IA, ZARNESCU O, STOIAN AP, MOTOFEI IG, BRATU OG, PIRCALABIORU GG, CHIFIRIUC MC. Influence of age on sperm parameters in men with suspected infertility. *Rom Biotechnol Lett*. 2019; 24(1): 82-90. DOI: 10.25083/rbl/24.1/82.90

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Introduction

Male infertility is defined as failure to produce spermatozoa of high quality, in terms of density, morphology or motility (VOGT 2004, [1]; W.H.O 2010, [2]). The term "male infertility" does not have a well-defined clinical concurrence, but rather constitutes a collection of different clinical conditions with a variety of possible etiologies and prognosis. (AITKEN et al. 1995, [3]; PASQUALOTTO et al. 2011, [4]). Factors such as urogenital infections, exposure to toxic substances and vascular diseases may impair seminal quality, resulting in embryonic defects, loss of pregnancy or children highly prone to develop genetic diseases and other pathologies (CAVALCANTE et al. 2008, [5]). The correct estimation of male fertility potential has become of great interest to researchers, but available tests are not always accurate for this specific pathology. On the other hand, when the results of these tests are under the normal values, they do not clearly state the diagnosis of sterility. Sperm analysis is one of the basic recommendations for the initial investigation in infertile couples. Since its first publication in 1980, the WHO manual used in andrology laboratories (with four updated editions, the last one published in 2010) recommends the standard methodology for human semen analysis (W.H.O. 2010, [2]). The first studies made to correlate the quality of seminal parameters with male fertility date back to 1930s (MACOMBER & SANDERS 1929, [6]). Although the analysis of seminal samples provides valuable information about male fertility, it is not always sufficient to determine the exact cause of infertility (WANG & SWERDLOFF 2014, [7]).

Materials and Methods

The current study was conducted over a period of 5 years (April 2013 - April 2017) on 500 semen samples from ambulatory care patients investigated for infertility. The samples were examined both macroscopically (to measure the volume, viscosity, appearance, odor and pH) and microscopically, to evaluate the presence of mucus filaments, aggregation or agglutination of spermatozoa (i) type A: "head-to-head"; ii) type B: "tail-to-tail"; iii) type C: through the tips of the tail; iv) type D: mixed - "head-to-head" and "tail-to-tail"; and v) type E agglutination: queue agglutination, but with non-free heads present in the agglutinate); to determine the presence of other cells: epithelial, immature germ cells, leukocytes, isolated sperm heads and ends; to assess sperm motility (i) "A"- fast, with beatings of the tail in the narrow beam, accompanied by lateral head movements and straight line progression; ii) "B" - slow, with head movements, but without tail beatings and with or without rectilinear motility; iii) "C" - low mobility, with disordered vibrations, frenzy, tail rotation and no movement; iv) "D" - immobile sperm, without tail movements, that can sometimes be confused with dead spermatozoa); to establish density, vitality and total number of spermatozoa (through eosin - nigrosin staining); to establish sperm morphology (through the inventory of head, intermediate piece and tail defects); to count white blood cells (C-Chip camera/Endtz Method).

According to the WHO criteria established in 2010, presented in Table 1, the seminal parameters values must fall within standard reference range, although some authors discuss in their papers about the drawbacks of using a single threshold value to distinguish the normal and abnormal values of seminal parameters (Patel et al. 2018, [8]).

Table 1. Semen parameters reference ranges defined by WHO, 2010 edittion.

Parameters	Reference range
Semen volume (mL)	1,5 (1,4-1,7)
Total number of spermatozoa (10^6/ejaculate)	39 (33-46)
Spermatozoa density (10^6/mL)	15 (12-16)
Total motility (PR + NP)	40 (38-42)
Progressive motility (PR)	32 (31-34)
Vitality (live spermatozoa, %)	58 (55-63)
Sperm morphology (normal, %)	4 (3-4)
Other threshold values	
pH	>7.2
Peroxidase positive leukocytes (10^6/mL)	<1

Legend: PR - Progressive motility; NP - Non-progressive motility; MAR - Mixed antiglobulin reaction

Results and Discussions

During the timeframe of the present study, it was possible to observe the increasing trend in the number of men who requested an investigation of their seminal parameters during. Thus, if 5% of the patients enrolled in this study were monitored in 2013, their number increased with time reaching 19% in 2014, 20-26% in 2016, growing to 29% in 2016 and ultimately reaching 21% in the first 8 months of 2017.

The patients enrolled in this study were distributed according to similar studies into 6 age groups, in order to be able to perform a comparative interpretation of the obtained results, i.e.: 18-25; 26-30; 31-35; 36-40; 41-48 years old; > 49 years. The age group of 31-35 years included the highest number of patients (165) (33%), followed by the group of 36-40 years (130) and ultimately by the 41-48 years old age group (118).

The age mean of the patients enrolled in this cohort study was 34.61 ± 5.85 years. Similar results were reported by CASTRO et al. 2012, [9] by analyzing a population sample with an average age of 32.47 ± 8.39 years. FARIA et al. 2012, [10] reported that the age of patients with infertility problems was ranging from 24 to 54-year-old.

From studying the quality of the sperm obtained from the 500 patients, we have recorded changes in all seminal parameters (Figure 1). It was noted that the most common abnormality was the sperm viability primarily found in 328 patients (66%), followed by sperm motility (with significant differences between progressive and total motility). In the case of progressive motility, 312 patients (63%) exhibited values inferior to the WHO normal range; whilst in the case of total motility, 214 patients (43%) presented lower values.

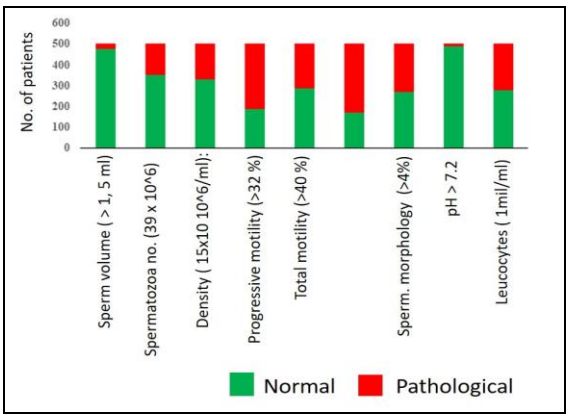

Figure 1. Distribution of normal and pathological values of different seminal parameters in the 500 investigated patients.

Similar results were obtained by Gao et al. 2008, [11] and Ramzan et al. 2015, [12] in a study performed on a population sample in China, uncovering abnornmal sperm viability in 73.8% of the analyzed samples. In the same group, it was found that 232 patients (50%) presented abnormal sperm morphology and leukocyte counts values.

In our study, the majority (90%) of the investigated patients had normal values of their semen volume. A volume below 1 mL of sperm indicates hypospermia, suggesting exposure to infections, retrograde ejaculation, congenital bilateral absence of the deferent vessels, poorly developed seminal vesicles, or an idiopathic origin (DOROFTEI et al. 2015, [13]).

This result is similar to the ones obtained from studies conducted in different regions of the world, suggesting that the volume mean of seminal material obtained from infertile and fertile subjects did not differ significantly (FISCH et al. 1996, [14]; HARRAWAY et al. 2000, [15]; JORGENSEN et al. 2001, [16]; AHMED et al. 2009, [17]; KHAN et al. 2013, [18]; RAMZAN et al. 2015, [12]).

The pH of seminal material was within normal range in most of the analyzed patients, except for a total of 11 males that presented a pH of less than 7.2. The changes in the pH value suggest anomalies of the auxiliary sexual glands or obstruction of the ejaculatory duct (DOROFTEI et al. 2015, [13]). The current study does not reveal significant differences in the pH values between subjects with normal/abnormal seminal parameters. Similar results were reported in studies performed on USA (Harraway et al. 2000, [15]), Norway (HAUGEN and GROTMOL 1998, [19]) and the Pakistani population (KHAN et al. 2011, [20]; RAMZAN et al. 2015, [12]).

Male infertility is multifactorial (AGARWAL et al. 2010, [21]) and any changes in the normal physiology of the reproductive organs can easily influence sperm functions and can lead to oligospermia (low sperm count), asthenozoospermia (reduced sperm motility), teratospermia (abnormal morphology of the spermatozoa), azoospermia (lack of sperm in the ejaculate) and, in some cases it can lead to oligoasthenoteratozoospermia (OAT), which is a major problem for successful fertilization (WHO 2010, [2]). In addition to many conventional causes, age plays an important role in the sperm physiology. Unlike women, men have the advantage of contributing to

conception at delayed ages (AMANN et al. 2008, [22]). However, starting with their 30's, degenerative changes of the seminal epithelium can be seen, and the decrease of the Leydig cell number and their function can lead to a reduced spermatogenesis through lower testosterone levels (WANG et al. 1993, [23]; JOHNSON 1986, [24]; HARMAN et al. 2001, [25]; AMARAL et al. 2009, [26]). Animal studies have shown that when the aging process starts, males produce sperm of lower quality (ESKENAZI et al. 2003, [27]). Age is correlated with a decrease in nitric oxide, which is associated with erectile dysfunction in older men. Moreover, with age, sperm quality decreases and a decline in the number of fertilized embryos is seen (DAIN et al. 2011, [28]; KIDD et al. 2001, [29]).

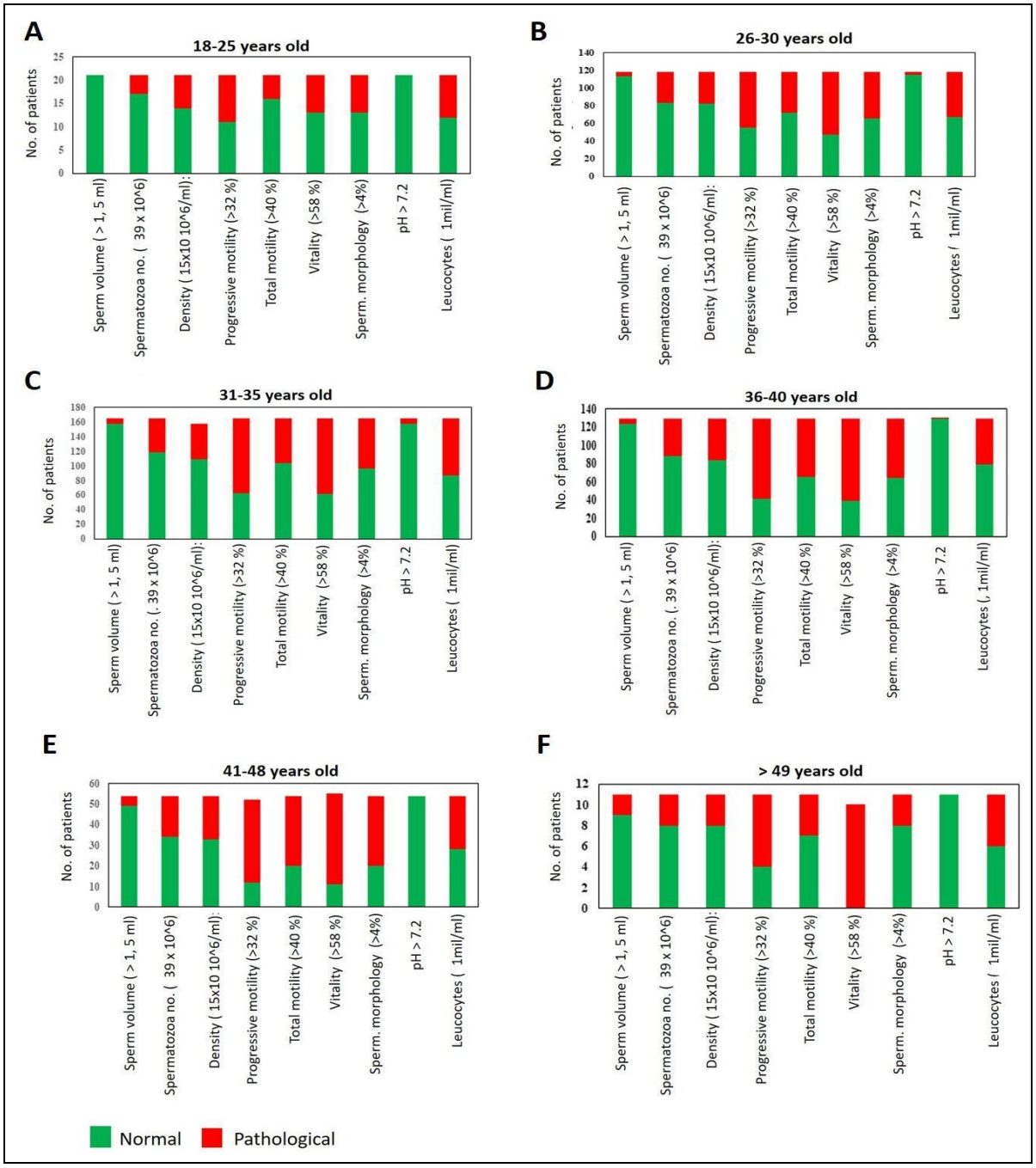

Figure 2. Distribution of patients belonging to different age groups, depending on the normal / pathological values of seminal parameters

Testicular volume and histopathological changes are strongly associated with age, resulting in hormonal changes. However, the age limit for sperm production is not defined (MAHOUD et al. 2003, [30]; SAMPSON et al. 2007, [31]). Moreover, delays of resorting to a better lifestyle can lead to the need for assisted reproduction (ZOFNAT et al. 2012, [32]).

As stated above, the males selected for this study were divided into six age groups. In the first group of 18-25 years old males, over 50% of patients had normal values for all seminal parameters (total sperm count/ejaculate - 17 patients, density -14 patients, progressive motility - 11 patients, total motility - 16 patients, sperm viability - 13 patients, sperm morphology - 13 patients and leukocyte count - 12 patients); as for two parameters (sperm volume and pH) - all 21 patients were within the normal range (Figure 2A).

Semen was collected from 118 patients within the age group of 26-30 years. After analysing the data, it was observed that over 96% of these patients had normal values of their sperm pH and over 55% of them also presented normal values for other seminal parameters (total sperm count/ejaculation - 83 patients, density - 82 patients, total motility - 72 patients, sperm morphology - 65 patients and leukocyte count - 67 patients). In the case of progressive motility, 55 patients (47%) presented normal values, while 47 patients (40%) had normal values of sperm viability (Figure 2B).

When compared with the first group of patients (ages from 18 to 25 years), it was observed that in the 26-30 years age range, over 50% of individuals present values below the WHO reference values for two seminal parameters (sperm viability - 55 cases and progressive motility of the spermatozoa - 47 patients).

A number of 165 males within the 31-35 years old age group had normal values of sperm volume and pH, meaning that they were within the normal range in over 96% of patients (Figure 2 C). The other seminal parameters were within normal range in over 50% of patients (72% - total sperm count, 66% sperm density, 63% total motility, 58% sperm morphology, 53% white blood cell count). When it comes to progressive motility and mobility, only 41% and 38% of cases had normal values.

Comparing these results with those obtained from the first two age categories, the decrease in progressive motility (50% in the 18-25 age group) and sperm viability (38% in the group of 31-35 years) is higher. All other seminal parameters showed similar values for the three age stages described in this study.

A number of 130 patients were included in the 36-40 years old age category. In Figure 2D, it can be noted that, as in the previous cases, sperm volume and pH values are within normal limits in over 95% of patients. The other seminal parameters turned out to have normal values in over 50% of patients (68% - total sperm count, 65% - sperm density, 51% total mobility, 50% sperm morphology, 60% leukocyte count). When it comes to progressive motility and viability, a decrease in their normal values was found in 25% and respectively 23% of men.

Semen samples were collected from 54 patients belonging to the age group of 41-48 years. Figure 2E shows normal values of their pH and volume in over 95% of cases. Also, three semen parameters were shown to have normal values in over 50% of patients, as follows: 63% - total sperm count; 61% - sperm density; 52% - the number of leukocytes; while four other seminal parameters presented abnormal values in over 50% of patients: 74% - progressive motility; 63% - total motility; 81% - sperm viability; 63% - normal sperm morphology. It can therefore be stated that with age, seminal parameters and sperm quality are significantly diminished. If the first two age categories (26-30 and 31-35 years old) showed only a slight decrease in viability and progressive motility, in the case of the 41-48 age group, there can be seen a much more pronounced decrease of the same seminal parameters (progressive motility and viability), plus a decrease in the total motility and sperm normal morphology.

In the age category of over 49 years, 11 patients presented for semen collection. Figure 2F shows that the values for pH and seminal volume are within the normal range in over 80% of the investigated men. Other seminal parameters presented normal values in over 50% of cases, as follows: 81% - total sperm count; 81% - semen density; 64% - total mobility; 81% - sperm morphology; 55% - the number of leukocytes. There are some exceptions, represented by progressive motility (although, 37% of patients are in the normal range) and viability, where all 11 patients present pathological values.

There are many studies that focus on the correlation between age and male / female infertility, all of them concluding that advanced paternal age is a strong risk factor of disorders that include production and sperm quality (CAVALCANTE et al. 2008, [5]).

Although there is no age limit for male fertility, studies reveal that fertility starts to decline at the age of 40 (TELÕKEN et al. 1999, [33]). Other studies indicate a decrease in semen volume in men after 40-50 years. In addition, some researchers demonstrate a reduction in progressive motility and normal sperm morphology with advanced age. On the other hand, information on the correlation between sperm density and aging is disputable (TELÕKEN et al. 1999, [33]; SILVA et al. 2012, [34]).

CASTRO et al. 2012, [9] compared the volume, progressive motility, normal morphology and sperm density in patients below/over 40 years of age. The authors found a statistically significant decrease in semen volume in patients over 40 years, while for the other parameters there were no significant differences present between these two age groups. Aging was also correlated with a decrease in sperm density and motility. On the other hand, a Brazilian retrospective study showed that sperm volume was the only parameter that diminishes with age, which corresponds to our findings. In our study the seminal volume was below the normal range in 11.2% of subjects, while other studies report abnormal values of this specific parameter in 23.4% of patients (CAVALCANTE et al. 2008, [5]).

Contradictory results that were reported in different studies may be due to country-specific cultural, environmental, economic and social factors, thus influencing the epidemiology of infertility (ELZANATY et al. 2005, [35]). It has been described that infertility patterns in developing countries differ from those in developed countries (BLACKWELL & ZANEVELD 1992, [36]).

Some studies have shown that with the growth of paternal age, sperm morphology deviates from normal (WALTER et al. 1998, [37]; ESKENAZI et al. 2003, [27]). ZHU et al. 2011, [38] performed sperm analysis in men, ranging between 20 and 60 years, showing that age has a negative effect on sperm morphology. Similar results have been reported by Kidd et al. 2001, [29] which demonstrated that normal sperm morphology decreases in 50-year-old patients compared to those that fit in the 30 years age category (WALTER et al. 1998, [37]).

Other studies have shown a decline in sperm viability and progressive motility starting with the group age of 31-35 years; lower values of these parameters being obtained as the subjects age (SUNANDA et al. 2014, [39]).

Similar results were obtained in a study performed on a population cohort in China by ZHU et al. 2011, [38] and Kuhnert et al. 2004, [40] who observed the downward trend in sperm viability, morphology and volume. Studies on population cohorts from Cordoba (Argentina) and the Arabian Gulf reported an age-related decrease in all seminal parameters (MOLINA et al. 2010, [41]; OMRAN et al. 2013, [42]). MUKHOPADHYAY et al. 2010, [21] have obtained similar results for motility, but not for sperm density. Since normal spermatozoa morphology is essential to ensure motility during sperm transit, it may be difficult to achieve a higher degree of motility, imperative for conception in older men. The decay of healthy germ cells in older men may be one of the reasons for motility loss (ESKENAZI et al. 2003, [27]). In our study, there was no significant change in semen volume, in contrast to another studies performed on elderly patients who switched to IVF and ICSI, and who had changes in their semen volume (DAIN et al. 2011, [28]).

Studies performed on laboratory animals have revealed histological changes of testes, seminiferous epithelium, germ cell defects, failure of preimplantation, and increased frequency of mutations in sperm DNA as men grow older. DNA damage was also more common in older men, leading to spontaneous abortion after IVF (KIDD et al. 2001, [25]).

ZHU et al. 2011, [38] presented a low reference value for motility (40.6%) and vitality in their paper (36.4%). But in the current study, the percentage of subjects with motility and viability parameters below the normal ranges established by WHO is very high in the case of total (50.8%) and progressive motility (43.4%). Good quality related semen was identified in patients from the ages of 20 until 34 years.

Since the entire spermatogenesis process is regulated by the Leydig-hypothalamus-pituitary gland and the androgen-dependent hormone secretions, continuous exposure to environmental toxic substances and today's lifestyle may be responsible for the alternations in the number and normal function of germ cells, Sertoli cells and Leydig cells, and this can explain the changes suffered by the reproductive system with age (CREASY 2001, [43]).

Conclusions

In conclusion, the results obtained from the evaluation of the 500 patients included in this study, distributed in different age groups, reveal that the sperm volume and pH are not influenced by age, these values being in the normal range in over 90% of patients, irrespective too their age group. In exchange, sperm viability and progressive motility decrease with age, targeting the group age of 31-35 years and over. The other seminal parameters (total sperm count, sperm density, total motility, sperm morphology, white blood cell count) fall below the normal range in approximately 50% of patients, being unrelated to any age group.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 91-99
doi: 10.25083/rbl/24.1/91.99

Received for publication: October 29, 2018
Accepted: January 3, 2019

Original paper

NGS data validated by Sanger sequencing reveal a puzzling small deletion of MYBPC3 gene associated with hypertrophic cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy (HCM) has a special place among genetic cardiomyopathies, being one of the main causes of sudden death in young patients, mainly in performance athletes. Herein we report a deletion in the myosin binding protein C (MYBPC3) gene identified in a female patient affected by HCM. The mutation was initially pinpointed in an NGS screening, then it was confirmed by Sanger sequencing with original primers. Bioinformatics analysis revealed a deletion previously reported as c.2441_2443delAGA, but the precise breakpoints mapping appears to be difficult to conclude. Since alternative three nucleotides deletions unambiguously result in a net Lysine missing from a specific poly-Lysine protein domain, the absolute mapping of the mutation is yet elusive, an aspect which should be considered when reporting the genomic coordinates of this deletion.

Keywords : Deletion mapping; MYBPC3 gene; Hypertrophic cardiomyopathy; NGS; Bioinformatics.

To cite this article: MICHEU MM, POPA-FOTEA NM, OPRESCU N, DOROBANTU M, RATIU AC, ECOVOIU A. AL., NGS data validated by Sanger sequencing reveal a puzzling small deletion of MYBPC3 gene associated with hypertrophic cardiomyopathy. *Rom Biotechnol Lett*. 2019; 24(1): 91-99. DOI: 10.25083/rbl/24.1/91.99

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Introduction

Hypertrophic cardiomyopathy (HCM) represents the leading structural cause of sudden cardiac death (SCD) at young ages, as well as in competitive athletes (MARON [1]), with an annual mortality among adults of 1-2% (PRIORI and BLOMSTROM-LUNDQVIST [2]). To date, over 1500 mutations in more than 11 causative genes have been identified (MARON & al. [3]). In more than half of cases the disease is caused by mutations in genes encoding for cardiac sarcomere proteins, the responsible mutation being inherited in an autosomal dominant pattern with variable expressivity and age-related penetrance (MARIAN [4], MARIAN and BRAUNWALD [5]). Mutations in the myosin binding protein C (MYBPC3) and beta-myosin heavy chain (MYH7) genes are the basis of up to 75% of the genotyped forms (ELLIOTT & al. [6], WALSH & al. [7]).

The genetic testing is currently acknowledged as a key step towards efficient management of patients with HCM. Importantly, the identification of the causative mutation in proband facilitates relatives' pre-symptomatic diagnosis and better prognosis through lifestyle modifications and timely targeted therapies (PRIORI and BLOMSTROM-LUNDQVIST [2]). On the other hand, the advent of next-generation sequencing (NGS) technologies allowed for a more comprehensive analysis of the links between phenotypes characteristic for various human genetic diseases and causative genetic mutations, represented especially by single nucleotide polymorphisms (SNPs) and small insertions and deletions. The Romanian population is poorly studied in terms of underlying genetic cause of inherited cardiac conditions, therefore our study stemmed from a real demand for genetic characterization of Romanian patients with HCM and their families.

In this paper, we describe NGS and Sanger sequencing results followed by a bioinformatics analysis, which revealed a deletion affecting the MYBPC3 gene. To our best knowledge, this mutation, symbolized as c.2441_2443delAGA, is reported for the very first time in a Romanian HCM patient.

Materials and Methods

Study subjects, disease criteria, and clinical evaluation

The study was approved by the Ethics Committee of the Clinical Emergency Hospital of Bucharest, and performed in compliance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

The proband, as well as first-degree relatives underwent clinical work-up, including medical history, physical examination, 12-lead electrocardiogram, and two-dimensional transthoracic echocardiography. The diagnosis of HCM was established according to the European Society of Cardiology (ESC) criteria (ELLIOTT & al. [6]). The ultrasound images were acquired using a Vivid E9 machine equipped with a 3.5-MHz probe (GE Healthcare), and subsequently analyzed off-line using dedicated software (GE EchoPAC). In index patient, a LV wall thickness of at least 15 mm detected by two-dimensional echocardiography in the absence of any other condition that could explain the hypertrophy was identified as HCM, while in first-degree relatives the cut-off value was 13 mm.

DNA isolation

Genomic DNA was isolated from the peripheral whole blood of one HCM patient and three first-degree relatives using MagCore Genomic DNA Whole Blood Kit (RBC Bioscience) following the manufacturer's protocol. The purity of resulted DNA was estimated with an Epoch spectrophotometer equipped with Gen5 software (BioTek). The DNA concentration was measured using the Qubit dsDNA HS assay kit (Life Technologies) the values ranging between 80-90 ng/µl. The extracted DNA was stored at -80°C (ULUF 490, Arctico Freezer) for later use in downstream applications.

Targeted Next-Generation Sequencing

Targeted sequencing was performed on an Illumina MiSeq platform using TruSight Cardio Sequencing Kit (Illumina) following the manufacturer’s instructions. The procedure targets 47 core and emerging genes (including ACTA1, BRAF, CRYAB, FHL1, KLF10, MYBPC3, MYH7, MYO6, MYOZ2, PLN, RAF1, TNNT2, VCL, etc.) associated

with HCM and allowed a depth of coverage of minimum 20x for 99% of the target regions. An initial amount of 50 ng of proband DNA was used for optimal enrichment.

Analysis of NGS variants

The sequencing run generates data files that are used by MiSeq Reporter software (Illumina) to generate FASTQ files and to perform the alignment of reads against the reference human genome (GRCh37) using BWA-MEM, which is the latest version of Burrows-Wheeler Alignment algorithm (LI [8]). For variant calling was employed GATK and Variant Call Format (VCF) files are produced as output. VCF files were processed with VariantStudio v3.0 software (Illumina).

The pathogenicity of a variant was determined based on allele frequency (AF) in the population and considering also in silico prediction. First, the variants of the 47 genes related to HCM were filtered according to the AF in general population. Common variants having an AF > 1% in the Exome Aggregation Consortium - ExAC (LEK & al. [9]) and the 1000 Genomes (http://www.internationalgenome.org) databases have been excluded. The mutations passing the frequency filter were analyzed individually; those included in the ClinVar repository as benign/likely benign were removed from the later analysis. The remaining variants were subjected to in silico analysis. To assess the potential functional impact of the identified mutation, we used two freely available online platforms: Protein variation effect analyzer - Provean (CHOI & al. [10]) and MutationTaster (http://www.mutationtaster.org/). A variant was considered to be functionally important if simultaneously marked as "deleterious" by Provean and "disease causing" by MutationTaster.

For variants classification in terms of clinical significance, we used the criteria issued in 2015 by American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) (RICHARDS & al. [11]) and also the interpretation provided by CardioClassifier, a specific on-line decision-support tool (www.cardioclassifier.org).

2.5. Variant databases and in silico tools

We accessed the following variant databases: 1000 Genomes Project (http://www.internationalgenome.org), the Exome Variant Server from the NHLBI Exome Sequencing Project (ESP) (https://esp.gs.washington.edu/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

In silico tools used in this study were as it follows: Provean (http://provean.jcvi.org/), MutationTaster (http://www.mutationtaster.org/), In-silico PCR (https://genome.ucsc.edu/), Cardio Classifier (https://www.cardioclassifier.org/).

Sanger sequencing

The mutation leading to a Lysine (Lys) deletion identified by NGS and supposed to have functional impact was further confirmed by Sanger sequencing. Also, a genetic testing for the presence of the mutation by Sanger sequencing was performed in selected first-degree relatives of index patient (two sons and one brother).

The original primers used to amplify the fragment of genomic DNA comprising the mutation were manually designed. Subsequently, in-silico PCR on-line tool was used to verify the primer binding specificity. The nucleotide sequences of the primers were as it follows: forward primer (PF) - 5'TGCACAGTACAGTGGGAGCC3', reverse primer (PR) - 5'ATGGCGTTGACCGCGTAGAC3'.

The PCR reactions were performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems). The amplification of the target sequence was carried out with 2 µl of DNA template, 0.5 µM of each primer, 1 X Buffer (Promega), 3 mM MgCl2 (Promega), 0.25 mM dNTPs (Promega), 2.5 U PQ Taq polymerase (BioTeke Corporation) in a final volume of 25 µl.

The PCR amplification program was run as it follows: 1 x (95°C - 10 minutes), 30 x (95°C - 30 sec, 59°C - 30 sec, 61°C - 30 sec), 1 x (72°C - 5 minutes), hold at 4°C. The expected amplicon lengths are illustrated in Table 1.

Table 1. Expected amplicons consecutive to PCR reactions

Amplicon	DNA	Amplicon lengths (bp)
II.2	Proband	301
Neg	Negative control	304
II.3	Brother	301/304
III.1	Son 1	301/304
III.2	Son 2	301/304

PCR amplicons were visualized by capillary electrophoresis with a QIAxcel system (Qiagen). The amplicons were purified using the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific) according to the protocol indicated by the manufacturer. For sequencing PCR, we used the Methods Development Kit (Beckman Coulter Life Sciences) procedure, where each reaction consisted in 0.6 µl of purified DNA amplicon, 0.3 µl of primer (PF and PR), and 4 µl of master mix in a final volume of 10 µl. The sequencing PCR reaction was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems) according to the following program: 30 x (96°C - 20 sec; 55°C - 20 sec; 60°C - 4 minutes), hold at 4°C.

The amplicons collections generated during sequencing PCR were purified using glycogen and ethanol mediated precipitation, resuspended in sample loading solution (Beckman Coulter Life Sciences), and then sequenced with CEQ 8800 Genetic Analysis System (Beckman Coulter Life Sciences).

Data collection and the primary analysis of the nucleotide sequences were performed using the CEQ 8800 dedicated software (Beckman Coulter Life Sciences).

Results

1. *Clinical features and pedigree description*

The proband (II.2) is a female patient of 49 years old, referred to our clinic for genetic testing. She has been diagnosed with HCM over 8 years ago, being under standard pharmacological therapy. Transthoracic echocardiography confirmed a LV asymmetrical hypertrophy with septum thickness of 20.5 mm in the absence of any other condition that could explain the hypertrophy. No clinical data were available for her parents, except that they died at the age of over 70 years old. She has a 53-year-old brother (II.3) who is asymptomatic and his echocardiogram showed no signs consistent with the disease. The two sons (24 and 16 years old respectively) of the proband are also asymptomatic with normal echocardiograms. No history of SCD has been recorded in the family.

2. *Mutation identification by targeted* NGS *sequencing and data analysis*

Following the processing of VCF files, 69 variants were detected within the 47 genes. Three out of these 69 mutations proved to have a lower than 1% frequency in general population (according to ExAC and 1000 Genomes databases). After discarding the variants classified as benign/likely benign in the ClinVar database, a heterozygous deletion in MYBPC3 gene was retained.

According to sequencing data analysis performed with MiSeq Reporter, the mutation in MYBPC3 consists in a deletion of 3 nucleotides (AGA) from exon 25 and spans the genomic interval 47359101-47359103 (GRCh37). This deletion leads to the synthesis of a structurally modified protein by the deletion of one Lys residue at position 814. The mutation nomenclature according to Human Genome Variation Society (HGVS) is c.2441_2443delAGA and p.Lys814del, respectively.

The corresponding protein, cMyBP-C, contains a short tandem repeat sequence consisting of 4 residues of Lys (Lys811, Lys812, Lys813, and Lys814). It worth mentioning that this is the only poly-Lys domain in the protein structure and the mutant protein is characterized by a Lys loss.

As revealed by MiSeq Reporter analysis, although the DNA deletion is not in-frame, at protein level it is equivalent to an in-frame mutation, because it coincidentally restores the serine codon (following the sequence of the 4 Lys) from the residues of the two adjacent codons (Figure 1).

Fig. 1. The figure depicts the partial nucleotide sequence of the sense strand of MYBPC3 coding for the poly-Lys domain (K811 to K814). Codons and corresponding amino acids are illustrated. The three deleted nucleotides according to MiSeq Reporter are underlined. With rectangles are marked the adjacent nucleotides that restore the serine codon, AGC. R – Arginine, K – Lysine, S – Serine, Y – Tyrosine.

The BWA-MEM alignment algorithm employed by MiSeq Reporter identified the coordinates of the mutation affecting the poly-Lys domain. A more detailed manual analysis reveals that actually, given the particular nucleotide structure of the gene region

containing the short Lys repeat, it is not possible to precisely determine which triplet is actually deleted. Moreover, a new question arose regarding the true nature of the missing triplet, which may be either AGA or AAG. In addition, when we processed the FASTQ file using alternative genomics tools, which are exploiting different alignment algorithms, we identified either c.2441_2443delAGA mutation (with Geneious Prime 2019.0.4), or c.2431-2433delAAG mutation corresponding to the deletion of Lys residue from the position 811 (with CLC Genomics Workbench 3.6.5). These data indicate that reporting the precise deletion coordinates by different alignment algorithms is rather a matter of convention than an absolute genomic reality. Single nucleotide sequences containing simulated AGA or AAG deletion within the poly-Lys domain were tested with BLAT (KENT [11]) and BLAST (ALTSCHUL & al. [12]) implemented by UCSC Genome Browser and NCBI. The results between these regularly used alignment tools are also conflicting, BLAT identifying c.2431-2433delAAG mutation, while BLAST is detecting the c.2441_2443delAGA mutation. Accordingly, a study reporting for the first time the identification in Finland of a Lys deletion within the poly-Lys domain, explicitly states that it is not possible to precisely know which AAG triplet is actually deleted (JÄÄSKELÄINEN & al. [13]), but the authors do not consider the alternative deletion of AGA instead. Nevertheless, in order to be consistent with the notations used in various databases, from now on we will use only the c.2441_2443delAGA notation for naming the mutation.

In ExAC database, the AF of c.2441_2443delAGA is reported with a frequency of 0.0323% in general population, and it was identified in 39 out of a total of 120,542 chromosomes tested. The presence of the respective mutant allele in homozygous condition was not reported. The deletion c.2441_2443delAGA is absent from the control cohorts according to the ESP and 1000 Genomes databases. The deletion was predicted to be "deleterious" by Provean and "disease causing" by MutationTaster.

3. *Sanger sequencing study of c.2441_2443delAGA*

The presence of a heterozygous deletion of AAG or AGA triplets within c.2431-c.2443 region of MYBPC3 was confirmed by Sanger sequencing (GenBank accession number MH595891) which revealed a mixture of apparently distinct DNA strands downstream the mutation. In Figure 2, the sequencing chromatograms obtained from proband (Figure 2a) and negative control (Figure 2b) are presented in a comparative manner. Sense genomic strand is shown, thus the deleted nucleotides are displayed as either TCT or CTT, instead of AGA or AAG.

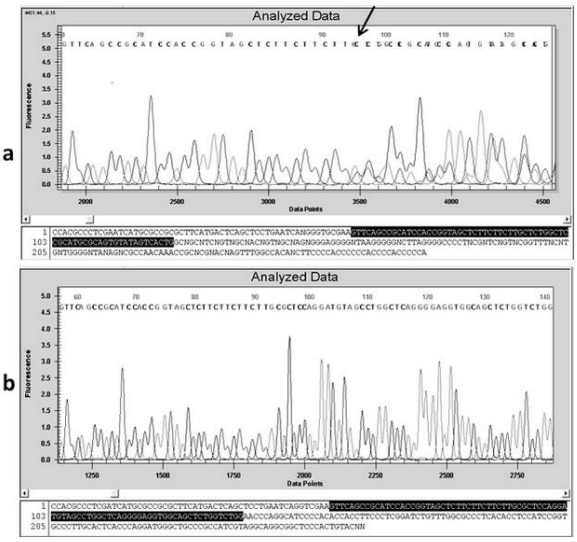

Fig. 2. Sequencing chromatograms showing the c.2441_2443delAGA heterozygous mutation (a) and a wild type, control sequence (b). Forward strand of chromosome 11 is displayed, thus the deleted nucleotides triplet could be either TCT or CTT. Downstream the poly-[CTT] sequence, a mixed G/C position follows, marking the start of the region (indicated by the arrow) where the reading is characteristic for a mix of amplicons that either harbor a deletion or not. When sequencing the amplicon from the control patient, a normal profile was obtained.

Sanger sequencing demonstrated the presence of heterozygous variant c.2441_2443delAGA in one of the two children (III.1). For the other two first-degree relatives (II.3, III.2) the variant is absent. Thus, the mutation was identified in two members of the family (II.2 and III.1), while the phenotype was displayed only by the proband. No clinical and genetic data were available for proband's parents.

Discussions

The "first modern description" of HCM was published in 1958 by the British pathologist Donald Teare (TEARE [14]). Since then, significant advancements have been made in understanding the

pathogenesis of this disorder, due to both the development of the field of cardiac imaging and the progress made with the analysis of human genome. Studies conducted so far have clearly demonstrated the implication of the genes coding for sarcomeric proteins in the disease aetiology, among them MYBPC3 having a central role (BONNE & al. [15], WATKINS & al. [16], VAN DRIEST & al. [17], VAN DRIEST & al. [18], COPPINI & al. [19], GESKE & al. [20]).

According to our data, this is the first study that identifies the c.2441_2443delAGA mutation in association with an HCM Romanian patient. A particular aspect of the DNA mutation is that, although it is not possible to know which nucleotide triplet is actually missing, it determines the net deletion of a Lys residue. Assuming that MiSeq Reporter analysis identifies the c.2441_2443delAGA deletion, the mutation may also restore the Serine codon from the residues of the two adjacent codons, as it is detailed in Figure 1.

Once a potential causal mutation is identified, the key point is to analyze its clinical significance. The differential diagnosis between a pathogenic variant and a rare variant with no clinical significance is not easy. The use of NGS led to an increased likelihood of detecting variants of uncertain significance (VUS) which are difficult to interpret. In an era where targeted gene sequencing and whole exome sequencing are routinely used, it is essential not only to centralize the resulting data, but also to analyze it in a standardized manner. Moreover, in the particular case of hereditary cardiac diseases, this aspect is of extended importance, since the result of genetic testing triggers specific interventions not only on proband, but also on first-degree relatives. Overdiagnosis based on a false-positive genetic test may result in inappropriate therapeutic measures and severe complications, as shown by a recent study conducted at a well-known clinic from the United States (GABA & al. [21]).

For variant classification in terms of clinical significance, we used the ACMG/AMP criteria (RICHARDS & al. [22]). Analyzing our data, we decided that c.2441_2443delAGA fulfils the following pathogenic criteria:

- one moderate evidence of pathogenicity - PM2 (absent from the control population),
- one supporting evidence of pathogenicity - PP3 (the harmful effect on the gene or its product is supported by multiple in silico tools).

These criteria alone were insufficient to classify the variant as likely pathogenic or pathogenic. The mutation c.2441_2443delAGA in MYBPC3 gene has been identified in several patients affected with HCM, but also in unaffected adult relatives (JÄÄSKELÄINEN & al. [13], ANDERSEN & al. [23], VAN DRIEST & al. [17], CARDIM & al. [24], SONG & al. [25], ZELLER & al. [26], BAHRUDIN & al. [27], EHLERMANN & al. [28]). Additional statistics has been provided by two private laboratories: Oxford Medical Genetics Laboratories (OMGL) in the United Kingdom and the Partners Healthcare Laboratory of Molecular Medicine (LMM) in the United States. According to data reported by the OMGL, the mutation was identified in eight HCM patients out of a total of 3,267 and was classified as likely pathogenic, while of the 2,912 HCM patients tested by LMM, it was identified in two cases, being classified as VUS (WALSH & al. [29]). In summary, the mutation c.2441_2443delAGA was identified in 10 patients with HCM in a 6,179 cohort, with a frequency of 0.16%.

One of the limitations of our study is the small number of family members and the lack of the allele segregation. Still, the existence of a normal phenotype does not exclude a subsequent onset of the disorder, especially in the case of the son detected positive for mutation, knowing that the initial diagnosis of the disease is on average in the fourth decade of life. In line with the recommendations issued by ESC, annual clinical evaluation for both children and the proband's brother will be performed.

Conclusions

Our study reports for the first time the identification of c.2441_2443delAGA variant in the MYBPC3 gene in a Romanian patient with HCM. Presently, there are insufficient arguments for classifying the mutation as disease causing one. Further family segregation analysis and functional studies yielding robust data are required to support reclassification of the identified variant. On the other hand, the precise mapping of the mutation is impossible, as revealed by the bioinformatics analysis with various specific tools.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

This work was supported by CREDO Project - ID: 49182, financed by the National Authority of Scientific Research and Innovation, on behalf of the Romanian Ministry of European Funds - through the Sectoral Operational Program "Increasing of Economic Competitiveness", Priority Axis 2, Operation 2.2.1 (SOP IEC -A2-0.2.2.1-2013-1) and co-financed by the European Regional Development Fund.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 100-107
doi: 10.25083/rbl/24.1/100.107

Received for publication: September 20, 2018
Accepted: December 18, 2018

Original paper

Genetic Risk Score for Prostate Cancer in the Romanian Population

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Abstract

In our study, we investigated the utility of genome-wide association study results in defining a risk score based on prostate cancer risk variants in the Romanian population. The study population consisted of 1301 unrelated histopathologically confirmed prostate cancer (PCa) cases and 1,073 male controls consisting of patients admitted for urological and surgical conditions, excluding cancer.

None of the tested variants in the Romanian GWAS reached a genome-wide significance (p-value lower than 5x10-8), but 36 markers reached p-values of 1x10-7. 17 of the previously-reported SNPs replicated in the Romanian cohort. Evaluating the total risk observed in the general PCa GWAS compared with the GWAS performed in the Gleason Score <7 subcohorts, we found a 5.1 times difference in-between the two predicted risk scores in individuals carrying all 17 variants.

Keywords : prostate cancer, genetic epidemiology, GWAS, risk score, Romania

To cite this article: DANAU R, BADIU DC, IORDACHE P, URSU R, RADOI V, RASCU S, RADAVOI GD, SIMA CS, TOMA C, BRATICEVICI B, MANDU M, GRIGOREAN TV, JINGA V, Genetic Risk Score for Prostate Cancer in the Romanian Population. *Rom Biotechnol Lett*. 2019; 24(1): 100-107. DOI: 10.25083/rbl/24.1/100.107

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Introduction

Prostate cancer (PCa) is the most common cancer for the male population in Europe. The heritability of prostate cancer is contributed mostly to a large number of moderate to commonly occurring variants conferring lower risks and rarely to rare, occurring genetic variants with higher penetrance (BENAFIF & al. [1]) . The incidence rates of prostate cancer vary substantially worldwide, with a much higher incidence observed in the Western world than in Asian countries (MATSUDA & al. [2]).

The identification of genetic variation that increases susceptibility for PCa may help to inform screening strategies and clinical management of patients in the future. Despite the genetic variants individually only modestly influencing the risk, their cumulative impact is substantial (DADAEV & al. [3]). There are now more than 100 PCa risk-associated single nucleotide polymorphisms (SNPs) that have been described, and it has been estimated that these genetic loci increase the estimated proportion of the familial risk to 33% (Al OLAMA & al. [4]). These variants can be incorporated into a survival analysis to estimate their effects on the aggressiveness of prostate cancer and their impact can be estimated in the form of a genetic risk score.

The estimated age-standardized incidence of PCA in Romania is 37.9 per 100,000 men in 2012, and the estimated age-standardized mortality rate for PCA is 16.9 per 100,000 men (FERLAY & a [5]). From an epidemiological perspective, several studies were performed recently investigating the genetic profile of PCa in Romania. The results of these studies are indicating that many of the variants showing the most reliable statistical values in the Romanian population reside at loci that have been associated with several cancer types, so-called cancer hubs (IORDACHE PD & al. [6]). This clustering of the previously reported variants can provide a framework for possible risk score estimation in the Romanian population. In the present study, we investigated the utility of genome-wide association study results in defining a risk score based on prostate cancer risk variants in the Romanian population.

Materials and Methods

Study population

The subjects included in this study were male patients admitted between 2008 and 2016 in four clinics from Bucharest for various medical conditions. The study comprises 2374 hospital patients; 1301 unrelated histopathologically confirmed PCa cases, most of which had elevated PSA levels, and 1074 controls, consisting of patients admitted for urological and surgical conditions other than cancer. Blood samples and buccal swabs were collected for genotyping. PSA levels in plasma were measured for all subjects at hospital admission but were not used as exclusion criteria. All subjects gave written informed consent prior to enrolment and accepted the use of personal and clinical data and biological samples for genetic research (MAZILU & al. [7]).

Table 1. Clinical characteristics of the cohort

Decade	**% cases**
under 50	0.30%
50-60	1%
60-70	35.50%
70-80	44%
80-90	15%
Over 90	0.20%
T Staging	**% cases**
1A	2,32%
1B	1,11%
1C	15,65%
2A	1,71%
2B	1,91%
2C	4,04%
3A	43,80%
3B	6,06%
4	23,33%
Gleason score	**% cases**
2	0.20%
3	0.30%
4	1%
5	3.30%
6	13.20%
7	45.10%
8	20.30%
N Staging	**% cases**
N0	21.50%
N1	3.20%
Nx	75.30%
M Staging	**% cases**
M0	22%
M1	10%
Mx	68%

The Bioethical Committee of the Romanian College of Physicians approved the study, and the study protocols were approved by the National Ethical Board of the Romanian Medical Doctors Association in Romania. Trained interviewers performed face-to-face interviews, using standardized questionnaires, to collect personal data (ethnicity, marital status, education, height, and weight), lifestyle data (occupation, smoking, coffee, and tea consumption) and medical history (personal and familial). All subjects were of self-reported European descent. No significant differences were observed in other epidemiological features: BMI, smoking or alcohol consumptions (Table 1).

Genotyping and analysis of SNP data

DNA was extracted from whole blood and saliva at deCODE Genetics (Reykjavik, Iceland) and genotyped using Infinium OmniExpress-24 bead chips (Illumina). A total of 716,503 SNPs were genotyped for each included in the study. The genotype data were filtered using Plink! v1.07 (O'CONNEL & al. [8]) .Approximately 10% of the SNPs genotyped were removed using a Hardy–Weinberg equilibrium significance threshold of 5 × 10−6 and by excluding markers with a minor allele frequency lower than 1%. Prior to the imputation, each chromosome was phased in a single run using SHAPEIT (DELANEAU & al. [9]). Markers from Phase 3 October 2014 of the 1000 Genomes (GIBBS & al. [10]) were imputed into the 2024 chip-typed individuals using the IMPUTE2 software (HOWIE & al. [11]) with a posterior probability of 0.9 as a threshold to call genotypes. The set of genotypes were tested for population heterogeneity using principal component analysis in the ADMIXTURE software (ALEXANDER & al. [12]) and the results were consistent with a homogeneous population.

A total of 24,295,558 markers were generated by imputation for each in the study. Quality control for the imputation results was performed by removing markers with minor allele frequency less than 1%, call rate of 0.95 and info of 0.8. In total, 8,506,022 markers met the filtering criteria. An association test was performed between the 8.5 million imputed markers and a phenotype represented by a positive biopsy for prostate cancer. The association test was calculated using SNPTEST (MARCHINI & al. [13]), using a single binary variable as a response; all reported P-values are two-sided.

Selection of SNPs for replication of previous findings

A systematic literature review of variants associated with prostate cancer from previous GWAS' was completed on 01 September 2018 using the NHGRI catalogue of published genome-wide association studies (WELTER & al. [14)] as a starting point. A search query with 'prostate cancer' as a keyword was performed, and the inclusion criteria for selection were as follows: P-values <5 × 10−8 and a minor allele frequency above 5%. For each study, the following variables were collected: country and ethnicity of the participants, genotyping method, the source of controls and source of replication cohort, and several cases and controls in both discovery and replication study.

A total of 48 articles were initially obtained from the GWAS catalogue based on the keyword search. Twelve of the studies reported results only tangentially related to prostate cancer, while the remaining 25 studies reported associations with prostate cancer risk. We identified 123 unique markers previously associated with PCa.

Results

In an attempt to identify susceptibility loci for PCa, 1301 diagnosed PCa cases, and 1074 controls were tested. A total of 8.5 million imputed markers with a successful imputation score of >0.90% were included in the association test. Results across the genome are graphically illustrated in Figure 1, while the top findings are presented in Table 2.

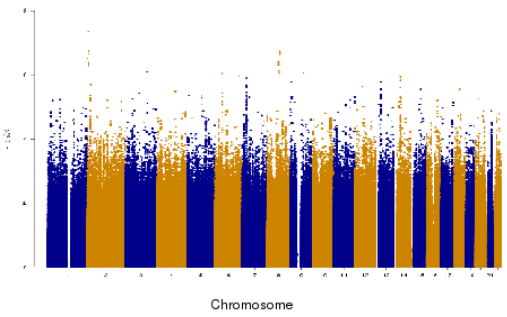

Figure 1. For the plot, the –log10 P-values (y-axis) of sequence variants are shown according to their chromosomal position(x-axis)

Table 2. Variants in the Romanian GWAS with lowest P-values for each locus MAF = Minor Allele Frequency

SNP	Chromosome	Position	Reference allele	Tested allele	MAF* in cases	MAF* in controls	OR	P-Value	Info
rs10490219	chr2	8400895	T	C	0.08	0.05	1.87	1.55E-07	1
rs3923300	chr8	126142476	C	G	0.11	0.07	1.65	2.33E-07	1
rs13253942	chr8	126154649	A	G	0.11	0.07	1.65	2.57E-07	1
rs34903473	chr8	126156276	C	G	0.11	0.07	1.65	2.57E-07	1
rs35215140	chr8	126200988	T	C	0.11	0.07	1.66	3.55E-07	1
rs35401436	chr8	126185276	A	T	0.11	0.07	1.66	3.70E-07	1
rs34246020	chr8	126246328	A	G	0.11	0.07	1.65	3.70E+47	1
rs12675943	chr8	126260092	A	G	0.11	0.07	1.69	4.47E-07	0.99
rs13273034	chr8	126134996	G	A	0.11	0.07	1.66	4.54E-07	0.99
rs35336507	chr8	126256028	G	A	0.11	0.07	1.7	4.82E-07	0.99
rs35955519	chr8	126256027	T	G	0.11	0.07	1.7	5.17E-07	0.99
rs72720569	chr8	126258407	T	G	0.11	0.07	1.68	6.11E-07	99
rs4601329	chr8	126254561	C	T	0.11	0.07	1.69	6.87E-07	0.99
rs72779189	chr2	8395447	G	A	0.08	0.05	1.88	7.99E-07	0.99
rs140930777	chr2	8395953	C	CTTGT	0.08	0.05	1.88	8.03E-07	0.99
rs72779193	chr2	8403566	A	G	0.08	0.05	1.85	8.13E-07	0.99
rs16866634	chr2	8375001	A	G	0.09	0.05	1.78	8.35E-07	0.98
rs6706154	chr2	8398487	C	T	0.08	0.05	1.84	1.07E-06	0.98
rs199704995	chr2	8402794	CA	C	0.08	0.05	1.84	1.17E-06	0.98
rs55937624	chr2	8388038	A	G	0.08	0.05	1.92	1.18E+46	0.97
rs72779188	chr2	8393977	T	C	0.08	0.04	1.95	1.20E-06	0.95
rs34313605	chr8	126271948	G	A	0.12	0.08	1.66	1.20E+46	0.94
rs72779172	chr2	8361400	C	G	0.08	0.05	1.85	1.30E-06	0.94
rs72779171	chr2	8359538	A	G	0.08	0.05	1.86	1.38E-06	0.94
rs16900462	chr8	126250796	A	G	0.14	0.1	1.61	1.43E+46	0.87
is112824993	chr8	126227755	T	G	0.11	0.07	1.65	1.44E-06	1
rs16900452	chr8	126243100	A	G	0.11	0.07	1.65	1.44E-06	1

None of the tested variants in the Romanian GWAS reached genome-wide significance (p-value < 5·10-8) but 36 markers had p-values < 1·10-7. In Table 1 there are presented the strongest association signals seen at 2p25.1 (LINCOO299 gene, intron 7/8) and 8q24.13 (NSMCE2 gene, intron 2/6). The data was obtained by filtering with the following conditions: imputation info >0.8, tested alleles frequency in controls >0.005.

Next, we tested the effect of 123 previously reported PCa variants in the Romanian population. 17 SNPs from 17 different loci were replicated in the Romanian cohort (P-value <0.05).

Also, for the 17 markers, we performed a second GWAS using a subcohort of PCa patients with the values of Gleason score above 7. The 17 markers replicated in the Romanian population are presented in Table 3.

Table 3. Previously reported PCa risk markers associated toPCa risk in the Romanian population with a P value < 0.05

RS-ID	Tested Allele	Ref Allele	OR	P-value	OR for the second test	The p-value for the second test
RS35148638	C	A	1,1905	0,000731	1,4213	0,000236
RS2242652	A	G	0,8721	0,002897	0,9412	0,002546
RS4713266	C	T	1,2541	0,0005421	1,8714	0,0002277
RS8102476	C	T	1,3323	0,003562	1,2224	0,0029581
RS10993994	A	G	0,9830	0,004372	0,9901	0,005376
RS2659124	A	T	1,1245	0,000939	1,3324	0,000672
RS4245739	A	G	0,4212	0,00321	0,7832	0,000913
RS11691517	G	A	0,9643	0,000574	0,7614	0,000729
RS6465657	T	C	1,0676	0,00473	1,1310	0,0061
RS9364554	T	C	0,8235	0,000671	0,9436	0,00064
RS35148638	C	A	0,9245	0,000731	0,9924	0,009722
RS12597458	G	C	0,8244	0,00095	0,9007	0,0012353
RS2005705	C	G	0,8543	0,003994	0,8130	0,003926
RS7127900	C	A	0,9676	0,000802	0,9887	0,000967
RS58262369	G	T	1,0557	0,0044	1,4412	0,00764
RS12791447	G	A	1,3215	0,00014	1,0324	0,00004
RS2238776	G	A	1,0400	0,000315	1,1900	0,000348

Evaluating the total risk observed in the general PCa GWAS compared with the GWAS performed in the Gleason Score <7 subcohorts, we found a 5.1 times difference in-between the two predicted risk scores in individuals carrying all 17 variants. The overall risk score for the 17 variants in the general PCa cohort is 0.63 compared to the risk score of 3.27 observed for the Gleason Score <7 subcohorts.

Discussions

A large amount of information on prostate cancer risk associated SNPs is available for multiple white case-control samples. However, less is known about whether these associations can be consistently replicated in Eastern European populations (JINGA & al.[15]; ARNOLD & al. [16]). Before trying to establish risk-based score on our results, we evaluated the expected p-values compared to the observed p-values. We observe no excess signal in the Q-Q plot when testing all marker (Figure 2-a); the observed p-values (blue line) show a comparable trend to the expected p-values (the red line).

However, we observe an excess of signals in the Q-Q plot when restricting to the set of previously reported variants (Figure 2-b); the observed p-values (blue line) show a comparable trend to the expected p-values (the red line). The Q-Q plots concurred with the plots from the study "Profile of common prostate cancer risk variants in an unscreened Romanian population," our study expanding the cohort of the above-mentioned paper (PANTEA S. & al [17]; NITIPIR C. & al [18]).

When we compare the odds ratios (OR) of the 17 variants in the general PCa GWAS to the GWAS performed in the Gleason Score <7 subcohorts, we observed a constant increase in risk for each replicated variant. The distribution of both OR sets is graphically illustrated in Figure 3.

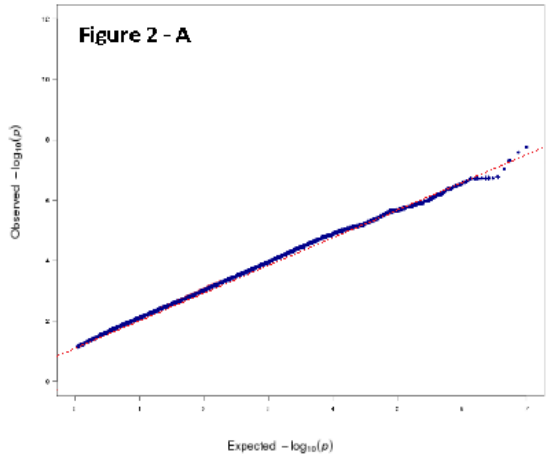

Figure 2(A) shows results from the genome-wide analysis;

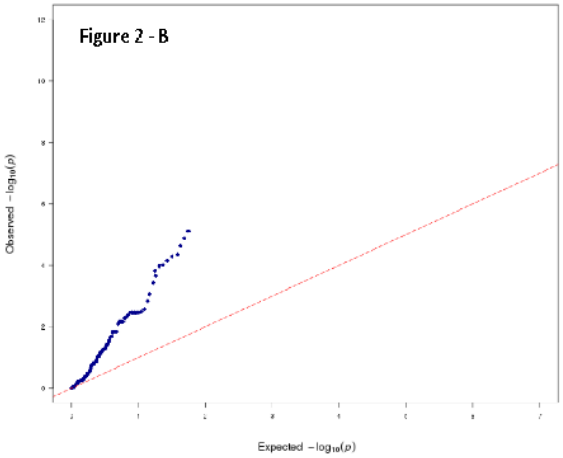

Figure 2 (B) shows results when restricted to GWAS catalog

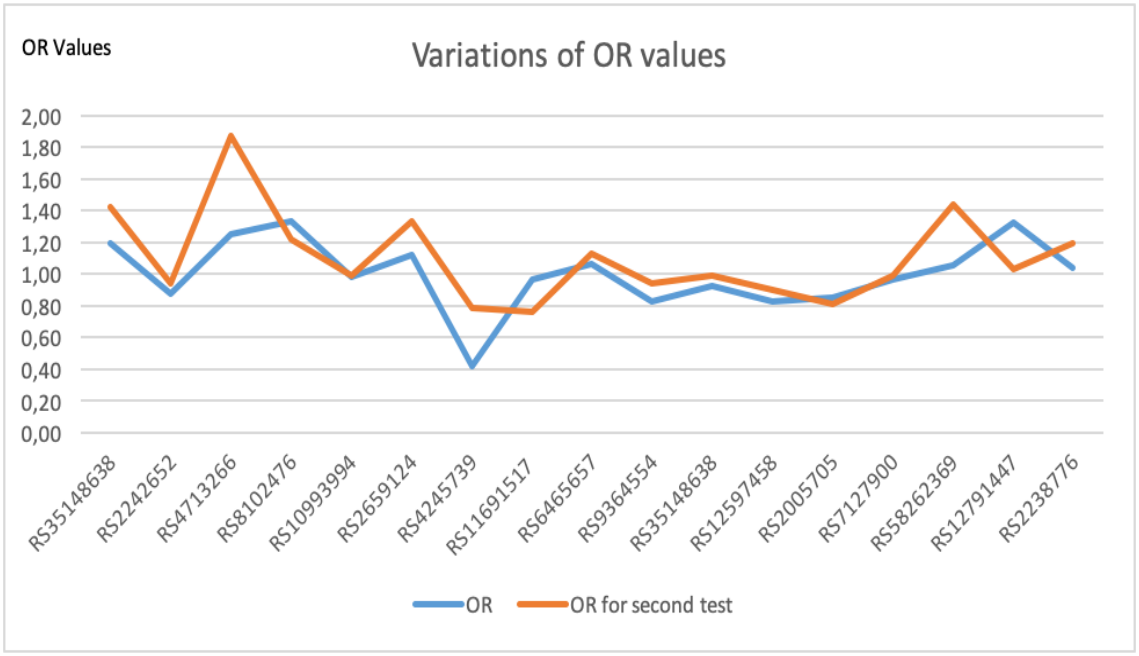

Figure 3: Distribution of OR's values for the 17 replicated markers

Conclusions

Including the 17 replicated Romanian SPNS can provide better risk assessment and can help guide relatives regarding the time of initiation and frequency of PCa screening. Although the genetic risk score can significantly improve screening practices in Romania, the complete clinical significance of this finding requires further evaluation. Furthermore, the inclusion of additional genetic variants from established prostate cancer susceptibility regions will improve disease prediction.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

This study was funded in part by the European Union FP7 Program (ProMark project 202059) and by the EEA grant (ROMCAN project RO14.0017; EEAJRP.RO.NO.20131.10191).

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 108-117
doi: 10.25083/rbl/24.1/108.117

Received for publication: June 2, 2018
Accepted: September 15, 2018

Original paper

Development and sequential analysis of a collagen-chitosan wound management biomaterial

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Abstract

An electrospinning method was used to fabricate a bilayer collagen sponge/chitosan (Col/CS) matrix to develop a new wound healing dressing. The aim of the study was to improve the mechanical properties and antimicrobial activity of a collagen sponge matrix used as medical device. Col/CS matrix was subjected to detailed analysis by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), Atomic force microscopy (AFM), comparative physico-chemical characteristics (swelling ratio and hydrolysis properties, enzymolytic properties, bilayer Col/CS morphology) between collagen sponge and Col/CS matrix, bioburden determination and antimicrobial activity. The physical characteristics, morphology and antimicrobial properties showed that this biomaterial has a high ratio of surface area and superior mechanical properties, than collagen sponge. After sterilization, the recovery efficiency of S. aureus and E. coli from Col/CS matrix was 36% and 52%, result confirmed through qualitative antimicrobial activity. These results suggest that Col/CS matrix could be a promising solution for chronic wounds management.

Keywords : Collagen, Chitosan, Electrospinning, AFM, SEM, FTIR, Antimicrobial activity

To cite this article: DOLETE G, TIHĂUAN BM, KAMERZAN CM, TUTUNARU O, MOCANU IC, BALAŞ C, ARDELEAN IL, MAIER SS, Development and sequential analysis of a collagen-chitosan wound management biomaterial. *Rom Biotechnol Lett*. 2019; 24(1): 108-117. DOI: 10.25083/rbl/24.1/108.117

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Introduction

Since interruption or failure of wound healing process may easily lead to non-healing chronic wounds, wound management is increasingly becoming a vital factor in the process of wound healing. The basic requirement for all wounds to heal is a clean and adequate environment. This may require removal of non-viable tissue, control of bacterial burden, maintenance of moisture balance, and an environment which can adequately stimulate epithelialisation (M. FLANAGAN [1], K. VOWDEN & P. VOWDEN [2]).

Despite the fact that characteristics of an ideal dressing were identified many years ago, the perfect product has not yet developed, even though, each year, more products are launched into an already crowded market. However, there is still a need for novel medical devices for wound management, because of the many interrelated factors that influence the selection of a proper wound dressing such as wear time, cost effectiveness, wound characteristics, as well as extrinsic factors related to patient, clinician, or the local health organization.

Wound care management has received much attention over the last two decades, leading to a substantial replacement of traditional wound dressing materials such as cottonwool, gauze and bandages, with new materials. These recently developed materials cover and protect the damaged tissue as a traditional wound dressing does, and beside that, they are designed specifically to offer improved healing rates by activating the cell proliferation and stimulating the healing process (L. O'NEILL & al. [3], C. VALENTA & al. [4]).

In this context, collagen-based dressings have been extensively studied over time (J.A.M. RAMSHAW & al. [5], S.H. LIU & al. [6], M. MIAN & E. BEGHE [7]). Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular proliferation and differentiation. Once dermis reconstruction is done, the covering of the wound surface with both *in vitro* expanded epidermis and autologous split-skin transplants is significantly easier, and it has an improved chance of success (Z. RUSZCZAK [8]).

A wound dressing may be a single product, or may combine two or more layers of dressing materials consisting of a primary wound contact layer, and a secondary retention or abosptive layer which is not in direct contact with the wound. Biopolymeric membranes can be successfully used for guided tissue regeneration, while antibacterial materials have a significant reduce in subsequent complications or infections.

Chitosan has a wide range of utility, being a natural, biocompatible, biodegradable polymer with a higly efficient wound healing effect. Chitosan has a structural characteristic similar to glycosaminoglycans, which are widely distributed among various tissues and seems to mimic their functional behaviour. There is a vast amount of literature on the extracellular matrix macromolecules including collagen, glycosaminoglycans and glycoproteins, playing a crucial role in the building up of new tissue. It is proposed that wound healing is accelerated by the oligomers of degraded chitosan by tissue enzymes, and this material was found to be effective in regenerating the skin tissue of wound area. Commercially products are already available to the market, like INTEGRA™ Bilayer Matrix Wound Dressing, which is an advanced wound care device comprised of a porous matrix of cross-linked bovine tendon collagen and glycosaminoglycan, and a semi-permeable polysiloxane (silicone layer). The semi-permeable silicone membrane controls water vapor loss, provides a flexible adherent covering for the wound surface and adds increased tear strength to the device.

Data in literature confirm that chitosan is a potential substitute for human novel wound dressings. A challenging area in the field of collagen-chitosan wound dressings is the use of electrospun nanofibrile membranes, which seems to potentially offer many advantages over conventional processes. On the account of the fact that electrospun nanofiber has a huge surface area and microporous structure, a nanofibrilar membrane could quickly start signaling pathway and attract fibroblast to the dermis layer, which can excrete important extracellular matrix components, such as collagen and several cytokines (e.g. growth factors and angiogenic factors),to repair damaged tissue (J. CHEN & al. [9], C.H. YAO & al.[10], H.P. FELGUEIRAS & M.T.P. AMORIM [11], S.B. QASIM & al. [12] X. GENG & al. [13]).

Another important aspect for new dressings consists in potential failures of the proper sterilization,

leading to significant costs associated with nosocomial infections of patients and mortality issues (G.C.C. MENDES [14]). Any dressing used in the clinic should be sterile in accordance with appropriate standards (E. MAZORA & M. ZILBERMAN [15]): EN ISO 11137-1:2015, EN ISO 11137-2:2015, EN ISO 11737-1:2006, EN ISO 11737-2:2009, EN ISO 13485:2016.

Sterile products can be obtained with methods using heat (dry heat or autoclaving) and the so-called "cold" processes, i.e. microbicidal gases (gas plasma of low-temperature hydrogen peroxide, low temperature peracetic acid gas, peroxide hydrogen vapors, ozone, chlorine dioxide) or high energy irradiation (M. GEIGER [16]).

In the case of collagen, sterilization with steam or dry heat is not applicable because can induce changes in protein chemistry and physical properties, affecting the absorption potential, mechanical strength or performance. An alternative to obtain sterile collagen products is ethylene oxide treatment, which is less common for drugs, but has a significant role in the sterilization of medical devices (R. PEACOCK [17]). Gamma irradiation is another established method of sterilization for collagen products (S.D. GORHAM [18]), and has been used for the preparation of bone grafts and human tendons (M. GEIGER [16]).

Since heat sterilization is not possible, sterilization with ethylene oxide is not applicable (not possible for liquid forms, and is difficult to validate for solids), and aseptic manufacturing is not an option given that the raw material is of animal origin (C. WIEGAND & al. [19]), according to E. MAZORA & M. ZILBERMAN [15], the irradiation is the most appropriate method of sterilization for wraps containing natural and sensitive polymers.

Although sterilization of collagen-based biomaterials is accomplished by low dose gamma irradiation (R. PARENTEAU-BAREIL & al. [20]), has been observed from previous studies that this method alters the molecular structure and decreases the mechanical and enzymatic resistance of the collagen scaffolds. Sterilization with ethylene oxide (ETO) or β-ray irradiation is less harmful than γ rays, but their applicability depends on the type of collagen products (E.M. NOAH [21]; W. FRIESS & M. SCHLAPP [22]). Immersion in a low peracetic acid concentration or formic acid are considered a potential collagen sterilizing agents (S.P. WILSHAW & al. [23], C.J. DOILLON & al. [24]). Immersion in ethanol with the combination of fungicides or/and antibiotics are techniques used in the laboratory for the sterilization of collagen scaffolds that have been physically crosslinked (L. MA & al [25]). However, according R. PARENTEAU-BAREIL & al. [20], no sterilization technique has proven optimal for sterilizing different forms of collagen. Investigating the effects of sterilization on the properties of collagen materials remains the best way to evaluate the performance of sterilized collagen.

Cross-linking is also required for biopolymers to control resistance to degradation and enhance mechanical integrity during sterilization (L.M. DELGADO & al. [26]). A bilayered collagen-chitosan wound dressing produced by using an electrospinning tehnique for the chitosan layer, is able to avoid molecular changes of collagen during sterilization, and the stability changes over time. More than that, chitosan layer has many advantages for wound healing, such as hemostasis, accelerating the tissue regeneration by enhancing the fibroblast collagen biosynthesis of (L. MA & al [25]), offering a much more promising effect of collagen dressings.

In this context, our aim was to improve the mechanical properties and antimicrobial activity of a collagen sponge matrices of medical device type, produced by a Romanian manufacturer, by the development and sequential analysis of a bilayer chitosan-collagen (Col/CS) product.

Materials and Methods

Sample Preparation

Chitosan solution was prepared at 2% (w/v) in 1 M acetic acid, at room temperature under magnetic stirring (100 rpm) for 3 days, to obtain homogeneous solution. The solution was centrifuged at 1500 RPM for 3 min prior to electrospinning, in order to remove air bubbles.

Collagen sponge production includes collagen extraction from animal tissues by solubilization with non-specific enzymes (5.2% w/w pepsin (250 units/mg) in HCl solution, at a pH value of 1.8), followed by (atello)collagen purification by

diafiltration against purified water. Briefly, the process had the subsequent steps: raw material (bovine tendons) mechanical processing; physico-chemical individualization of quasi-native collagen fibers by (i) peptization of collagenic tissue and (ii) solubilization of globular and denatured protein fractions; isolation of colloidal suspensions by successive filtration steps; non-stoichiometric neutralization of the mixture and advanced purification of the (atello)collagen dispersions by diafiltration (10 kDa MWCO membranes); freeze-drying of the (atello)collagen dispersions to obtain porous solid substrates. The porous substrate were subsequently laminated in order to obtain Col structures (colapsed porous structures as substrates for the chitosan electrospun solution).

Electrospinning

The prepared CS solution was placed in a 50 mL plastic syringe fitted with a 21-gauge stainless steel needle. The syringe pump delivered solutions at 7 mL/h flow rate, and electrospun with an applied voltage of about 20 kV between the electrodes, using a Tong Li Tech electrospinnig equipment. The CS fibrils were deposited on a rotating collector (140 RPM) of 10 cm diameter and 30 cm length, covered with a Col structure of 10x20x0.2 cm (lxLxh) kept at 15 cm away from at the tip of the needle. The experiments were carried out using a heat output of 0.500 W, with relative humidity ranging between 50% and 60%. The produced nanofibers matrices were left in ambient conditions to evaporate the excess of acetic acid and water prior to further analyses.

Material appearance and physical characteristics

Appearance: The visual inspection was carried out for material appearance assessment. Also, the overview of a bilayer product Col/CS as cross-section was observed by using a ZEISS Axio Imager 2 microscopy platform, and ZEN imaging software, used for length measurements, in order to establish the chitosan electrospun film thickness.

Determination of Swelling Ratio and hydrolytic loss: In order to induce swelling, Col/CS and Col matrices were used, sampled as 5 mm diameter discs. The pre-weighed samples (W0) of Col/CS and Col were immersed into deionized water at room temperature for 10 min. The samples were collected and the excess water was removed with filter paper, then were re-weighed (W1). Swelling ratio (ε) was calculated as follows:

$$\varepsilon(\%) = \frac{(W1 - W0)}{W0} x100$$

Hydrolytic loss of Col/CS and Col matrices was evaluated by immersing them in deionized water for 10 min, at 37 °C. Degradation was appreciated by visual inspection.

Enzymatic lysis: To determine the time required for complete digestion of the bilayer product, in the presence of pepsin, as a proteolytic enzyme, it was weighed as samples of 45 -50 mg, and then wetted with pure water. After removing the excess water, 100 mL of 1% pepsin in 0.1 N HCl, at 37° C, were added. The pepsin used has an enzymatic activity of 1.0 - 1.17 IU/mg. The mixture was kept in water bath at 37 °C, and checked every 2-3 minutes. The time required for pepsine digestion of the sample was determined when the product lost the integrity and structure, observing only light fibers in the solution. Col/CS and Col matrix as pozitive control were used, sampled as 5 mm diameter discs.

Col/CS bilayer morphology: The SEM analyses of the Col and Col/CS samples were performed using a FEI microscope. The samples were fixed with a double tape on a conductive support of aluminum. Afterwards, it was covered with a thin gold layer using sputtering for 60 sec. For the SEM measurements, the following parameters were used: high voltage 10 kV, working distance 10 mm, and an ETD detector for the detection of the secondary electrons.

The AFM analysis of the Col and Col/CS samples were performed using an AFM 5500 from Agilent Technology. The AFM measurements were performed in ambient atmosphere (23ºC, 35% relative humidity) in contact mode, using a silicon cantilever with elastic constant of 0.08 N/m, and a resonant frequency of 17 kHz. For the processing of the AFM images, the program Gwyddion was used to evaluate the morphological parameters (the average square roughness, RMS).

$$RMS = R_q = \left(\sum_{i=1}^{N}\left[\frac{(h_i - \bar{h})^2}{N}\right]\right)^{1/2}$$

ATR-FTIR analysis: Fourier Transform Infrared (FT-IR) spectroscopy is a valuable tool for characterizing and identifying compounds or functional groups. FTIR spectra of CS and Col/CS were recorded in triplicate, at room temperature, using the FTIR Cary 630 spectrometer from Agilent Technologies, in ATR mode, in the range of 4000 to 650 cm-1, with a spectral resolution of 2 cm-1 and 200 scans.

Bioburden determination

As stated by the standards for medical devices (ISO 11737 family), the appropriate method should be selected for bioburden determination, in order to ensure: removal of the viable microorganisms from the sample, culturing, and enumeration of the microorganisms. In this respect, we followed the pharmacopoeia rules for sterilization by using ionizing radiation sterilization, i.e. 25 kGy, in order to obtain a sterility assurance level (SAL) of 10-6. The sterile samples were used for establishing the factor for removal rate of the viable microorganisms from the samples. Two standardized strains were used for the tests, Staphylococcus aureus ATCC 6538 and Escherichia coli ATCC 8739. The product used for testing was defined as SIP (sample item portion) of 1 cm2 surface. Vortex mixing method was chosen for viable microorganisms removal from the SIP. Inocula adjusted according to McFarland Standard 0.5 were obtained from 18-24 hours cultures of S. aureus and E. coli developed on solid media. The sterile SIPs were immersed in 5 mL of suspension, maintained for 10 min for incubation at ambient temperature, and recovered on a sterile bottle. Excess inoculum was removed with a sterile filter paper, and SIP immersed in 5 mL of Eugon broth. Each tube was vortexed for 10 sec/min for 5 min at 100 RPM. Each sample was left for 10 minutes at rest, then 550 µL of supernatant were harvested and serial decimal diluted. 1 mL of the 10-6 and 10-7 dilutions were evenly plated. Positive control (decimal serial dilution inoculum) and negative (AFS, eugon broth and uncontaminated SIP) were used. The recovery factor (RF) was calculated as:

$$RF = \frac{UFC\ pos\ control}{UFC\ SIP}$$

Antibacterial activity assay

Qualitative screening for the antimicrobial activity of the Col/CS product (disks of 5 mm diameter) was made by using an adapted diffusimetric method. The antimicrobial qualitative screening was carried out against standardized microbial strains recommended by the European Pharmacopoeia (8th ed.) microbiological quality assessment for topical drug formulations: S. aureus ATCC 6538, E. coli ATCC 8739. The bacterial inoculum were made of 18-24 hours cultures grown on solid media, and adjusted to a density of 1.5 x 108 CFU/mL, using the 0.5 McFarland standard. The Col/CS samples were placed on the plates evenly spreaded with bacterial inoculum. Both Col and CS from the Col/CS product were placed in contact with the inoculated plate. The microbial sensitivity was expressed as the diameter of the growth inhibition zones.

Results and Discussions

The materials design and morphology must be controlled in accordance with the purpose of their use. The electrospun nanofiber has played a pivotal role in the area of biomaterials, especially in the biomedical field, by menas of biocompatible and biodegradable (natural or synthetic) polymers, in order to obtain products with high surface area and superior mechanical properties. The electrospun biomaterials structures are related to the electrospinning parameters, i.e. (i) the applied electric field, distance between the needle and collector, flow rate, needle diameter; (ii) the solution parameters, including the type of solvent, polymer concentration, viscosity and solution conductivity; (iii) relative humidity and temperature of the air inside the electrospinning cabinet.

A plethora of materials structures were developed by using collagen and chitosan (A. SIONKOWSKA & al. [29]; J.P. CHENA & al. [30]; B. KACZMAREK & al. [31]), as simple and or combined matrices, some of them being available in marketable form. Our purpose was to improve the mechanical and antimicrobial properties of a collagen sponge manufactured by a Romanian producer of medical devices. The description, embracing color and physical form is an important test for pharmaceutical materials, and could

be successfully adopted as a descriptor for the developed biomaterial. The term "white or almost white" was used for quality control purpose of the bilayer material, Col/CS. The electrospinning technique was chosen for obtaining a bilayer product, with electrospun chitosan and a laminated freeze-dried collagen support. A thin film of about 9.5 μm of the chitosan has been deposited (Fig. 1).

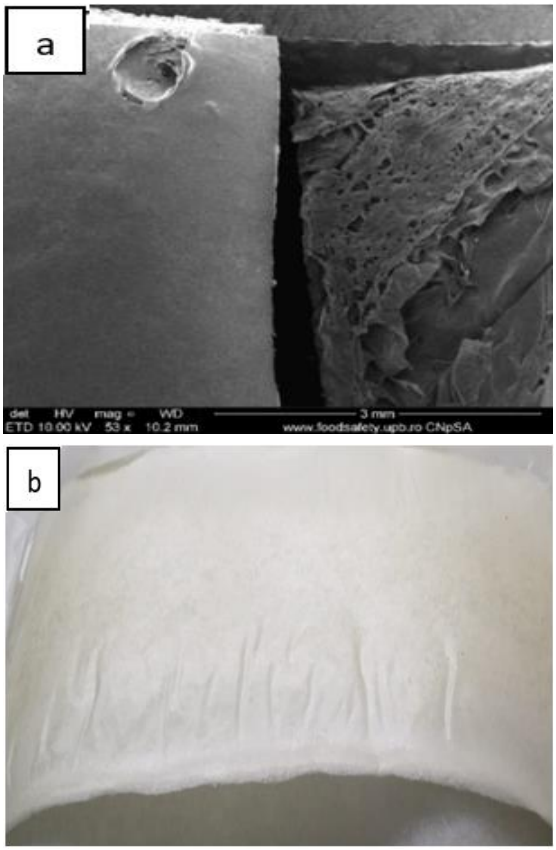

Figure 1. Overview of the bilayer product Col/Cs. a. SEM image, b. visual inspection aspect

Swelling ratio (ε) was calculated for the Col/CS product, and compared with the Col support matrix as positive control. Different swelling characteristics have been assessed for the support matrix, i.e. ε Col = 83.7%, and ε Col/CS = 43.5% for the Col/ CS product, presented as average of 3 separate determinations by using 5 discs of 5 mm diameter. A fractional hydration factor was calculated as:

$$FH = \frac{\varepsilon\ \mathrm{Col} - \varepsilon\ \mathrm{Col/CS}}{\varepsilon\ \mathrm{Col}} = 0.48$$

A certain humidity is essential for healing moist wounds, together with the abundance of wound exudate which raises severe problems in clinical practice. Having in view the exudates qualitative and quantitative influence on the healing phase of specific wounds, particular products could be developed for lightly to heavily exuding wounds (V. ANDREU & al. [32]). In this respect, the FH parameter could be used as quality control for the developed product, in preserving the collagen matrix properties, including the absorbance capacity.

The major drawbacks associated with the use of protein based-polymers are their low mechanical stiffness and rapid degradation rate in vivo (V. ANDREU & al. [32]). A plethora of methods were used for increasing the biological stability, i.e. crosslinking through physical or chemical methods. The biological stability of the Col/CS and Col support matrices was evaluated by exposing them to the selective endopeptidase pepsin, to assess degradation times and calculate a comparative rate of degradation. The average of 3 separate determinations by using 5 discs of 5 mm diameter, revealed degradation time of 12.5 min for the Col/CS bilayer product, and 4.3 min for the positive control Col. The comparative rate of degradation was 1.98, revealing a factor of about 2 in resistance increase for the bilayer product, Col/CS.

Fourier-transform infrared (FT-IR) spectroscopy is based upon the absorption of IR radiation by vibrational transition in covalent bonds of the biomolecules. The IR absorbance provides information about the sample contents depending on their structure, the molecular bounds and their environment (K. BELBACHIR & al. [27]).

FT-IR spectra of Col, CS and Col/CS are presented in Fig. 2. In the case of Col spectra, four amide bands can be observed at the wavelengths of 3301 cm-1, 3074 cm-1, 1630 cm-1 and 1542 cm-1. The Amide I band (1630 cm-1) originate from the ν (C = O) coupled to the δ (N – H) absorption. The Amide II band (1542 cm-1) is given by the δ (N – H) coupled with the ν (C – N) absorption. The other two Amides are from the δ (N – H) group, of medium to week intensity, appear at 3301 cm-1 and 3074 cm-1, respectively. In the spectra of CS, the ν (OH) group was observed at 3305 cm-1 and the ν (C – H) bond at

2911 cm-1. The absorbance peaks at 1650 cm-1, 1534 cm-1, 1400 cm-1 and 1308 cm-1 are attributed to the presence of ν (C = O), δ (N – H), ν (C – H) and ν (OH) characteristic to the chitosan structure. After the deposition, the peaks of Col/CS have modified values of absorbance. Since the intensity of –NH2 band in collagen molecule is stronger than that of –NH–, the changes of Amide II bands indicate that the free –NH2 group in collagen molecule were converted to –NH– groups (i.e. intermolecular links between chitosan and collagen or within collagen molecular formed) (X.H. WANG & al. [28]).

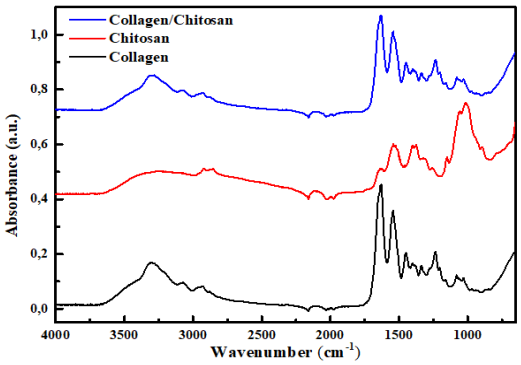

Figure 2. FT-IR spectra of Col (black line), CS (red line) and Col/CS (blue line).

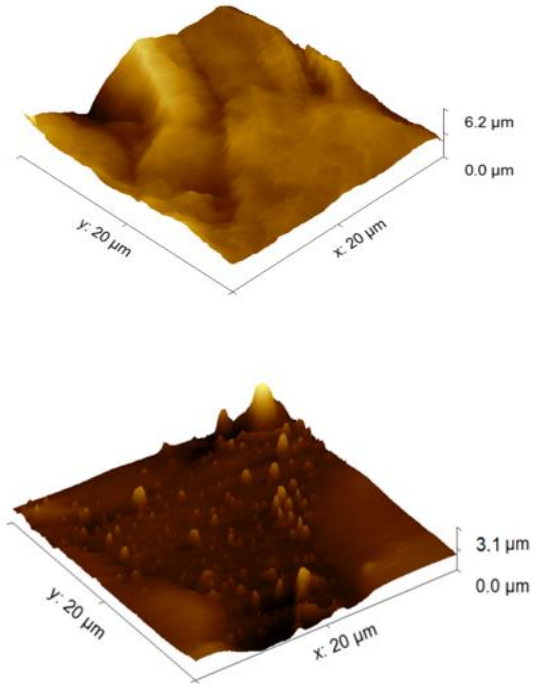

Figure 3. AFM images of Col (left) and Col/CS (right) samples

AFM images of the Col and Col/CS samples are presented in Fig. 3. From the processing of the AFM images we could evaluate the roughness morphology using a dedicated software. As expected the roughess of the Col sample (RMS of 620 nm) was much higher than the roughness of the Col/CS sample (RMS of 340 nm). The results showed a lot of beads disposed on the CS nanofibers surface. Further development would be necessary, in order to find the appropriate experimental conditions for obtaining the nanofibers layer without beads.

SEM images and diameter distribution of the Col and Col/CS samples are presented in Fig. 4 and 5. As could be seen in Figure 4, the results of the Col/ CS sample exhibits aligned nanofibres with the average diameter, dCol, of 102 ± 2 nm. In Fig. 5 we can observe that the Col support matrix exhibits evenly distributed and interconnected pores.

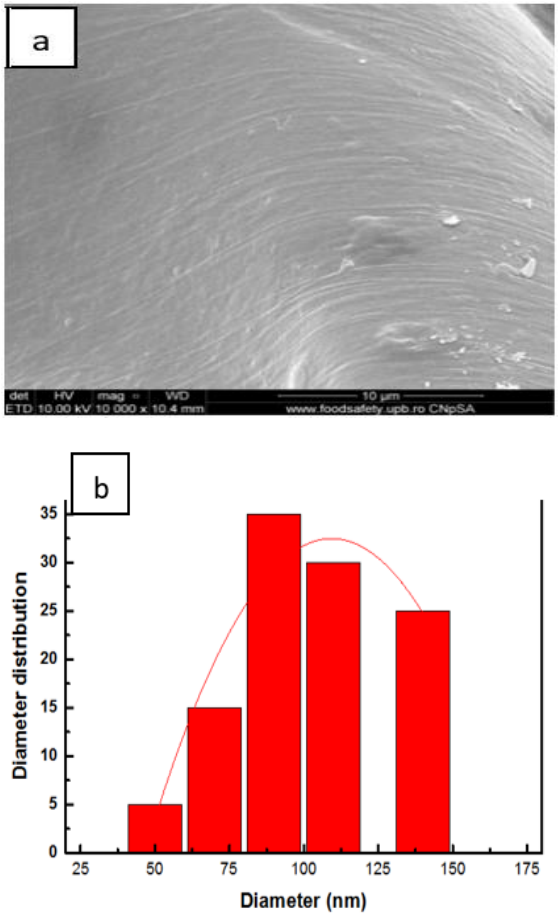

Figure 4. SEM images and diameter distribution (b) of the Col/ CS matrix (CS layer view –a)

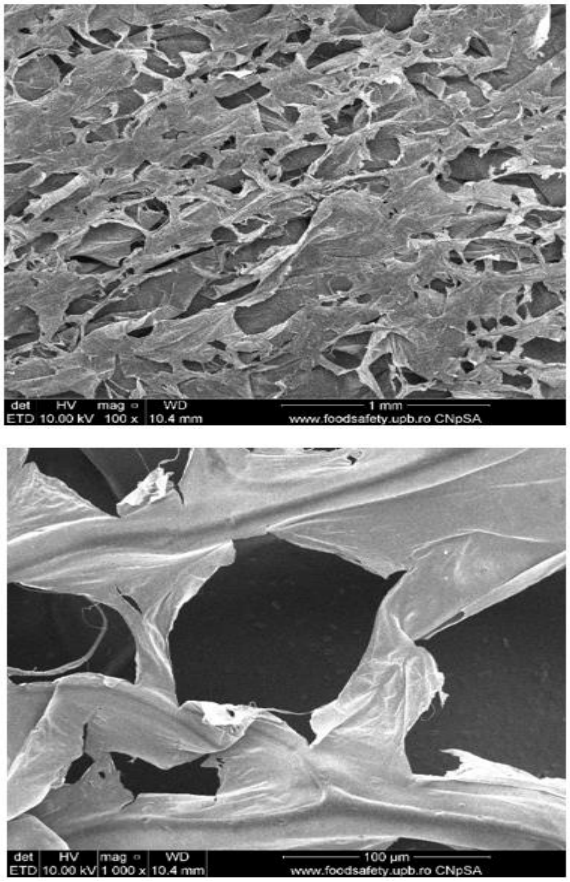

Figure 5. SEM images and pore distribution of the Col support matrix

The mandatory requirement for medical devices is the efficiency of microorganism recovery techniques in wound dressings. After sterilization of Col/CS matrix, the correction factor for recovery efficiency was determined, in accordance with European Pharmacopoeia, 8th ed. Five distinct stages are involved: sample selection, removal of microorganisms from sample, transfer of microorganisms to recovery conditions, enumeration of microorganisms with specific characteristics, and interpretation of data, involving application of appropriate correction factors determined during validation studies. In our study, the recovery efficiency of S. aureus and E. coli from Col/CS matrix was 36% and 52%, respectively. It was assumed that the results confirm the presence of the antimicrobial effect of the product. The recovery efficiency for E. coli was better than that for S. aureus, probably due to the difference in cell walls between Gram-positive and Gram negative bacteria. This result was also confirmed by the qualitative determination of antimicrobial activity (average diameter of the inhibition zone for the discs tested against standardized microbial strains was 9 mm for S. aureus, and 8 mm for E. coli).

Non-healing wounds has a worldwide negative impact, for example in the US, the chronic nonhealing wounds affected nearly 15% of Medicare beneficiaries (8.2 million). The annual cost was conservatively estimated at $28 billion when the wound was the primary diagnosis on the claim. When the analysis included wounds as a secondary diagnosis, the cost for wounds was conservatively estimated at $31.7 billion (S.R. NUSSBAUM & al. [33]). At the site the bacterial cells produce and secrete a variety of enzymes and toxins. A bacterial population of 105 colony forming units (CFU) per g or cm2 indicates an infected wound. Such a bacterial load can be reduced by using antimicrobial dressings (J. FONG & F. WOOD [34], BERTESTEANU &al. [35]). In this context, the proved antimicrobial activity and mechanical characteristics recommend the developed Col/ CS product as a practical solution for chronic wounds management.

Conclusions

In this study, certain steps were proposed for the development of a collagen–chitosan bilayer product, appropriate for biomedical use. The sequential analysis including product appearance and physical characteristics, morphology, and antimicrobial properties are the premises for laboratory validation of the new product. Also, important determinations for sterilization validation have been covered, i.e. sample selection, removal of microorganisms from sample, transfer of microorganisms to recovery conditions, enumeration of microorganisms with specific characteristics, allowing the calculation of the correction factor for the product contamination analysis. However, it is still necessary to implement further improvements of the electrospinning parameters of the chitosan layer.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNDI–UEFISCDI, project PN-III-P2-2.1-PTE-2016-0174 (Nano-ECol), Contract 51PTE/2016.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 118-128
doi: 10.25083/rbl/24.1/118.128

Received for publication: January 09, 2017
Accepted: March 5 , 2017

Original paper

Role of p38-mitogen-activated protein kinase in modulation of the response to therapy in FaDu Human pharyngeal carcinoma cell

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Abstract

Although cisplatin (CisPt) is used in treating head and neck squamous cell carcinomas (HNSCC), little is known about the cellular mechanisms triggered. We examined the morphological and functional changes in HNSCC cells when natural compounds such as resveratrol (RSV) are added to CisPt treatment of FaDu human pharyngeal carcinoma cell line. In addition, we analyzed the effects of CisPt and/or RSV treatments on cell proliferation and evaluated the ability to modulate the activation of p38 Mitogen-Activated Protein Kinase (p38MAPK). Moreover, we explored whether activation of p38MAPK is associated with p53 phosphorylation status in FaDu cells using SB203580 specific p38MAPK inhibitor. Proliferation vs. cytotoxicity induced by CisPt and/or RSV treatments in FaDu cells were studied using MTS colorimetric assay and evaluating cell viability. Whereas the activation of p38MAPK could be specific to HNSCC therapy, we investigated the phosphorylation status of p38 and p53 proteins by ELISA assays, using antibodies that recognize their dual forms (the active, phosphorylated and the total one). The obtained data showed that RSV enhanced the cytotoxic effect of CisPt in FaDu cells, having a synergic effect and the ability to promote p53 phosphorylation, suggesting a possible link between the p38MAPK and p53 activation pathways.

Keywords : HNSCC, cisplatin, resveratrol, p38MAPK, p53, SB203580

To cite this article: PETRICA-MATEI GG, ROMAN V, MIHAILA M. HOTNOG CM, BRASOVEANU LI, BOSTAN M. Role of p38-mitogen-activated protein kinase in modulation of the response to therapy in FaDu Human pharyngeal carcinoma cell. *Rom Biotechnol Lett*. 2019; 24(1): 118-128. DOI: 10.25083/rbl/24.1/118.128

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Introduction

Head and neck squamous cell carcinomas (HNSCC) include cancers of the nasal cavity, oral cavity, larynx, and pharynx (J.P. SHAH, Z. GIL [1]). The development of HNSCC is a multistep progressive process from precancerous lesions to malignant tumors, and despite the chemotherapy advances, the survival rate of HNSCC patients has not considerably changed over the last decades (R. P. TAKES & al. [2] and N. ZHANG & al. [3], BERTESTEANU & al. [4], POPESCU & AL. [5]). Treatment options are available, including chemotherapeutic agents such as cisplatin (CisPt) which is considered to be a standard treatment for patients with advanced HNSCC (R. BELCHER & al. [6]). CisPt is routinely used to treat the hypopharynx cancer, but almost 50% of the patients present tumor relapse or develop drug resistance in time. CisPT is a DNA damage-inducing agent and by its properties could block DNA replication and gene transcription (J.L. FISCHEL, G Milano [7]), but CisPt should not be related exclusively to the inhibition of DNA synthesis because it could also trigger other mechanisms, such as activation of p53 protein or Mitogen-Activated Protein Kinases (MAPKs), and thus could induce the apoptotic process (D. SANO & al. [8], A.C. JOERGER, A.R. FERSHT [9]).

In normal conditions, p53 - a nuclear transcription factor with pro-apoptotic function, is expressed at a low level, which is caused by proteasomal degradation largely mediated by E3 ubiquitin-protein ligaseMdm2 (Mouse double minute 2 homolog) a protein that in humans is encoded by the MDM2 gene. (R. KULIKOV & al. [10]). The cellular stresses like DNA damage might induce p53 accumulation in cell nucleus through post-translational modifications such as phosphorylation and acetylation. These chemical modifications convert p53 from a latent to an active form, which might be due to the dissociation of MDM2 from the former (N.D. MARCHENKO & al. [11]). When cells have serious DNA damages, p53 exerts its pro-apoptotic function to eliminate cells and thereby inhibit the transfer of damaged DNA to the daughter cells, thus, p53 has an important role to maintain genomic integrity (P. BRAGADO & al. [12]). Over 50% of human cancers have mutations in p53 gene and an oncogenic potential, and sometimes p53 mutated cells express a chemo-resistant phenotype. Phosphorylation of p53 in different serine (Ser)/ threonine (Thr) residues appears to be an essential key step, especially in the regulation of the response to some carcinogenic agents (J.J. MUKHERJEE & al. [13]). In addition, depending on the cellular context, CisPt could induce the cell death by mechanisms mediated by p53 activation but not clearly described. Several studies tried to demonstrate that activation of p38 MAPKs could lead to p53 activation, therefore influencing the apoptotic response of the cells. The MAPKs represent a highly conserved group of protein kinases that catalyze the phosphorylation of specific Ser and Thr residues in target substrates. The phosphorylation process is part of the cascade events that converts extracellular signals to intracellular pathways, and controls a wide range of cellular responses, such as proliferation, differentiation and apoptosis (I. DOLADO & al. [14]). MAPKs also play an essential role in signal transduction, and each group of MAPKs can be stimulated by a separate signal transduction pathway in response to different extracellular stimuli (E.F. WAGNER & al. [15]).

Since the cooperation between p53 and p38 MAPKs has not yet been established in the CisPt-treated HNSCC cell lines, our study focused on the evaluation of the crosstalk between p53 and p38 MAPKs in FaDu human pharyngeal carcinoma cell line in order to create an “in vitro” model. Moreover, we tested the modulation capacity of several compounds on p38 MAPK functional behavior, previously studied in inhibition assays of p38 MAPK and known for their therapeutic potential in inflammatory diseases (J. H. LOSA & al. [16] and J.L. LEFEBVRE & al. [17]). Like most chemotherapeutic agents, CisPt acts by forming DNA crosslinks within cells, leading to apoptosis and cellular senescence of tumor cells, but remission rates were often associated with severe side-effects, including nephrotoxicity, gastrointestinal or cardiovascular complications and bone marrow suppressive sequelae (E.E. COHEN & al. [18] and S. T. ELIAS & al. [19]). So, the main disadvantages of CisPt treatment are the associated side effects which often require a dose reduction or even discontinuity of CisPt-therapy (A.M. FLOREA & al. [20]). Efforts have been made to achieve a more effective management strategy to reduce this

phenomenon, and find alternative and less toxic cancer treatments like biotherapies, that use natural compounds (e.g. phytochemicals) which might be used in the treatment of various diseases, including head and neck cancers (T. KUNO& al. [21] and S.K. GOSWAMI & al. [22]). Despite the number of publications regarding HNSCC treatment, there are few data regarding the cellular mechanisms of CisPt action in association with natural compounds. One good example of such natural compounds is resveratrol (RSV) or 3,5,4'-trihydroxystilbene, a polyphenol found in red grapes, berries and peanuts, that has biological properties, such as antioxidant, anti-inflammatory, anticancer and anti-aging activities (L. MARZOCCHELLA & al [23]). In order to achieve our goal and further understand how cellular mechanisms might be influenced by CisPt and RSV combined treatment, the present study examined its cytotoxic effects on FaDu human pharyngeal tumor cell line, analyzed the modulation of p53 and p38-MAPK expression, and evaluated the capacity to alter the cell proliferation function. Since activation of p38 MAPK is associated with cancer progression, we further explored whether p38 MAPK activation is associated with p53 phoshorylation status in FaDu cells and might be influenced by CisPt and/or RSV treatments. Moreover, we studied the crosstalk between the two molecules and tried to evaluate the relevance of p53 and/or p38 MAPKs activation in order to improve the survival and quality of life of HNSCC patients, the main goal being the development of novel molecular targeted treatment strategies.

Materials and Methods

Reagents: Cisplatin (Cis-diammineplatinum (II) dichloride, DDP), Resveratrol (3,5,4'-trihydroxystilben), dimethyl sulfoxide (DMSO), etylenediaminetetraacetic acid (EDTA, PBS (Phosphate Buffered Saline), SB2035809 (4-[4-(4-fluorophenyl)-2-[4- methylsulfinyl) phenyl] - 1H-imidazol-5-yl]-pyridine) were purchased from Sigma (St. Louis, MO, USA).

Cell cultures: FaDu human pharynx squamous cell carcinoma cell line was purchased from the American Type Culture Collection (ATCC® HTB-43™). Cells were cultured either in 96-well plates or 25 and 75 cm2 culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-Glutamin and 100 U/ml penicillin/ 100 µg/ml streptomycin (Biochrom, Germany), and incubated at 37°C and in 5% CO2 humid atmosphere. When needed, the cells were grown for 24 hours to achieve around 60% confluence, and then treated with various concentrations of CisPt and/or RSV for different periods of time. Previously, CisPt was dissolved in 0.9% sodium chloride to a stock solution of 3.33mM, while RSV was dissolved in DMSO, and added with DMEM medium to obtain a 1mM stock solution. For inhibition studies of p38 MAPK function, cells were incubated for 2 h with 10 uM of SB203580 before CisPt and/or RSV treatments. After treatments, adherent cells were detached from flasks with a non-enzymatic solution of PBS/1mM EDTA, washed twice in PBS, and cell viability was evaluated using Trypan Blue exclusion test. Then FaDu cells were used for the evaluation of cell proliferation, cytotoxicity capacity or conserved as cell pellets at -80oC for preparation of cell lysates to further be used in ELISA assays. Non-treated cells were used as controls throughout all experiments.

Drug cytotoxicity assays: All assays were performed in triplicate using colorimetric CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA). The method is based on the reduction of yellow MTS tetrazolium salt by the viable cells and generation of colored formazan product that is soluble in the culture medium. Developed color can be spectrophotometrically quantified by measuring the absorbance at λ=490nm (J.A. BARLTROP & al. [24]). Briefly, 1 x 104/100µL/well of FaDu cells were seeded in 96-well microplates with flat bottom (Greiner, Frickenhausen, Germany), and incubated for 24 h at 37°C/5% CO2. After 24 h, supernatants were discarded, adherent cells treated with different concentrations of compounds under study in a final volume of 100 µL/well, and incubated for additional 12, 24, or 48 h. Then, 20 µl/well of MTS reagent containing (a) [3-(4,5 -dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl) -2-(4-sulfophenyl) -2H-tetrazolium] and (b) phenazine ethosulfate were added into each well, plates were further incubated for 0.5-4 h/ 37°C, and absorbance of color developed in each well was measured at λ=490 nm using a Dynex plate reader (DYNEX Technologies – MRS, USA). Results were expressed as mean values of three determinations

± standard deviation (SD). Untreated cells served as control and considered to have 100% viability.

Viability % = (T-B)/ (U-B) x 100, where T = O.D. of treated cells; U = O.D. of untreated cells, and B = O.D. of blank.

ELISA assay: Sandwich ELISA kits were used to measure both human P53 or p38 MAPK total proteins, and phospho-p53 or phospho-p38 α-MAPK proteins in cell lysates using a standard Streptavidin-HRP system. The kits consisted of: DuoSet_IC Human Total p53 ELISA [Cat. No DYC1043]; DuoSet_IC Human Phospho-p53 (S15) ELISA [Cat. No DYC1839]; DuoSet_IC Human/Mouse/Rat Total p38 ELISA [Cat. No DYC8691B] DuoSet_IC Human/ Mouse/ Rat Phospho-p38α (T180/Y182) ELISA [Cat. No DYC869B], and were purchased from R&D Systems Inc. (USA). In order to obtain the cell lysates, the untreated or CisPt and/or RSV treated FaDu cells were suspended in lysis buffer (1mM EDTA, 0,5% Triton X-100, 5mM NaF, 6M urea, 10ug/ml leupeptin, 10ug/ml pepstatin, 100uM PMSF, 3ug/ml aprotinin, 2,5mM sodium pyrophosphate, 1mM sodium orthovanadate in PBS, pH7.2-7.4), the Eppendorf tubes kept 30 min on ice with stirring every 5 min, then sequentially centrifuged 5 min/2000xg, and 15 min/14000xg (J.R. CROWTHER [25]). Protein concentration in lysates was evaluated using the Bradford assay. All experiments were performed in triplicate and sample O.D. was measured at λ=450 nm using Dynex plate reader.

Statistical Analysis. Data were analyzed using Student's t Test. One-way analysis of variation with p values of < 0.05 was considered statistically significant.

Results and Discussions

CisPt and/or RSV inhibition of human FaDu cell viability

CisPt or RSV concentration response curves in FaDu cells were generated by 6 h, 24 or 48 h incubation with 2-100μM CisPt and 0.5 - 200μM RSV treatments. Effects of CisPt or RSV monotherapy on FaDu cells were dose and time-dependent, and had statistical significance as compared with control untreated cells (Figure 1 A and B). The time-dependent effects of treatment with CisPt or RSV on FaDu cells viability indicated the 24h treatment as optimal.

The main objective of the present study was to investigate the potential synergistic effect of CisPt and RSV phytochemical combination on FaDu cells. The cell viability rate after 24 h treatment with 50μM RSV was 75% ($p<0.02$), while treatment with 10μM CisPt showed a cell viability rate of 64% ($p<0.003$) (Fig. 2A). After the simultaneous treatment of the FaDu cells with 50uM RSV and 10μM CisPt it was observed a reduction in cell viability till 52% after 24h treatment ($p<0.007$) as shown in Figure 2A. Moreover, combined CisPt-RSV treatments were compared to the single ones, and statistical significance were obtained: $p<0.005$ when compared to CisPt treatment, and $p<0.0045$ when compared to RSV, which demonstrated that combination could significantly reduce FaDu cell survival rate.

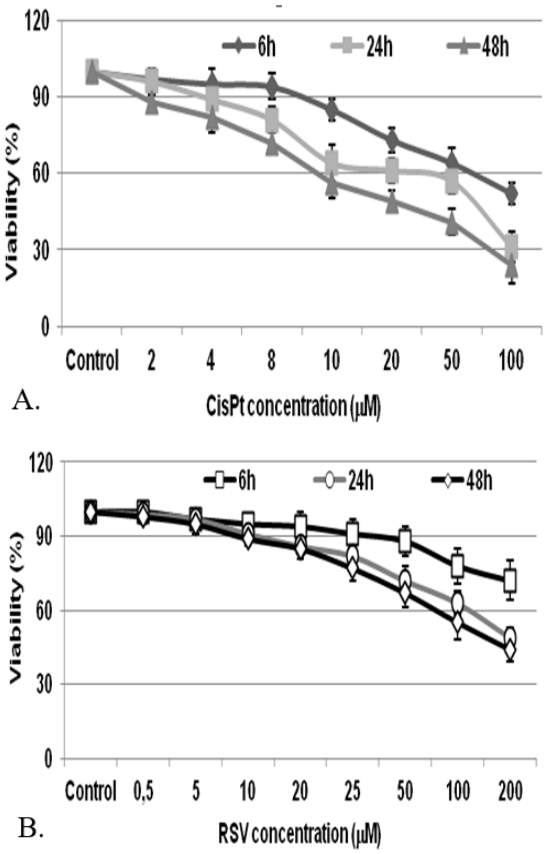

Figure 1. Sensitivity of the FaDu cells to CisPt and RSV treatments. Cell viability was measured by MTS assay after cell incubation with different doses of CisPt (A) or RSV (B) for distinct periods of time - 6, 24 or 48 hours. Data represent the mean ± SD values from three experiments.

In order to test whether p38 MAPK activation could control the cell proliferation in HNSCC, we blocked p38 signaling using SB203580 specific inhibitor. In this regard, we pretreated the FaDu cells

with SB203580 (10μM) for 2 h, followed by incubation with CisPt (10μM) and/or RSV (50μM) during 6, 24 or 48h. When the cells were pretreated with SB203580 for 2 hours, there were no significant differences in proliferation process of the cells treated with CisPt or RSV alone; however, the proliferation process of the cells simultaneously treated with CisPt and RSV was more suppressed in the presence of SB 203580 inhibitor, as shown in Figure 2 (CisPt+RSV+SB203580 versus CisPt+RSV-SB203580, $p<0.0095$), after 24h treatment.

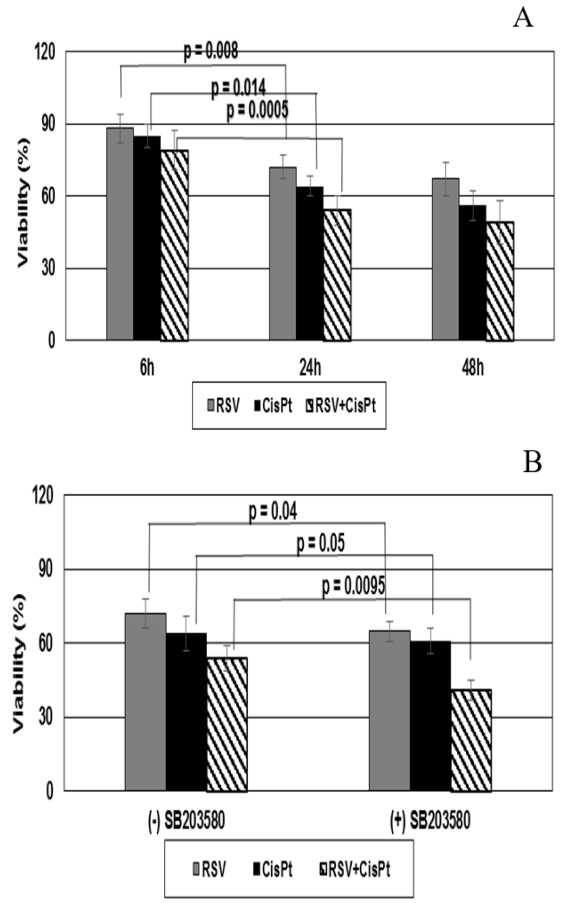

Figure 2. Effects of combined CisPt-RSV treatments on FaDu cells viability, in the presence or absence of SB203580 inhibitor of p38 MAPK.

(A) FaDu cells were incubated for 6, 24 or 48h with 10μM CisPt and/ or 50 μM RSV, then cell viability measured by MTS assay;

(B) Alternatively, cell viability was measured after preincubation of FaDu cell line with SB203580 inhibitor of p38 MAPK, and then with 10 μM CisPt and/or 50 μM RSV for 24h. Data represent the mean ± S.D. values of three experiments.

Effect of CisPt and/or RSV treatment on cell morphology

Cell morphology changes after 24h treatment with 10 μM CisPt and/or 50 μM RSV were recorded under light microscopy. The untreateated cells (CTR) showed a polygonal shape with a good appearance, typical for cell growth (Figure 3A). Treatment with 50 μM RSV induced morphological cell changes including shrinkage and a shift to a rounded shape, but they still adhered to the bottom (Figure 3B), while after 24 h treatment with 10 μM CisPt many cells rounded up, with some floating in the culture medium (Figure 3C). The combined treatment with CisPt and RSV induced a greater loss of cell attachment to ground matrix, membrane blebbing and more floating cells (Figure 3D), suggesting much stronger effects than single treatments.

Effects of CisPt and/or RSV on the activation of p38MAPK pathway in FaDu cell line.

The status of phosphorylation of p38MAPK in FaDu cells was investigated and data obtained showed a limited activity of p38 MAPK in FaDu untreated cells (control cells). These results indicate an association between a lower p38 MAPK activation with a high proliferative capacity of FaDu cells. In order to determine whether CisPt alone or in combination with RSV might activate a pathway leading to phosphorylation of p38MAPK, FaDu cells were treated with 10 μM CisPt and/or 50 μM RSV for 6, 24 or 48h. Then, cell lysates were prepared and analyzed by DUO IC ELISA method. CisPt and/or RSV induced a time-dependent phosphorylation of p38 MAPK of the incubation time (6, 24 h or 48h) (Fig. 4A). The results showed that p38 MAPK activity was significantly modified in treated cells when compared to control cells; thus the highest phosphorylation level of p38 MAPK was recorded after 24h treatments of FaDu cells with CisPt ($p=0.025$) or RSV ($p=0.005$). When cells were simultaneous treated with CisPt and RSV, it was noticed a significant enhancement of p38 MAPK phosphorylation (Figures 4B) as compared to control ($p=0.002$), CisPt ($p=0.003$) or RSV ($p<0.001$) treatments. After 48h, all single or combined treatments induced a significant decrease of phosphorylation level of p38 MAPK levels in FaDu cells as compared to 24 h treatments.

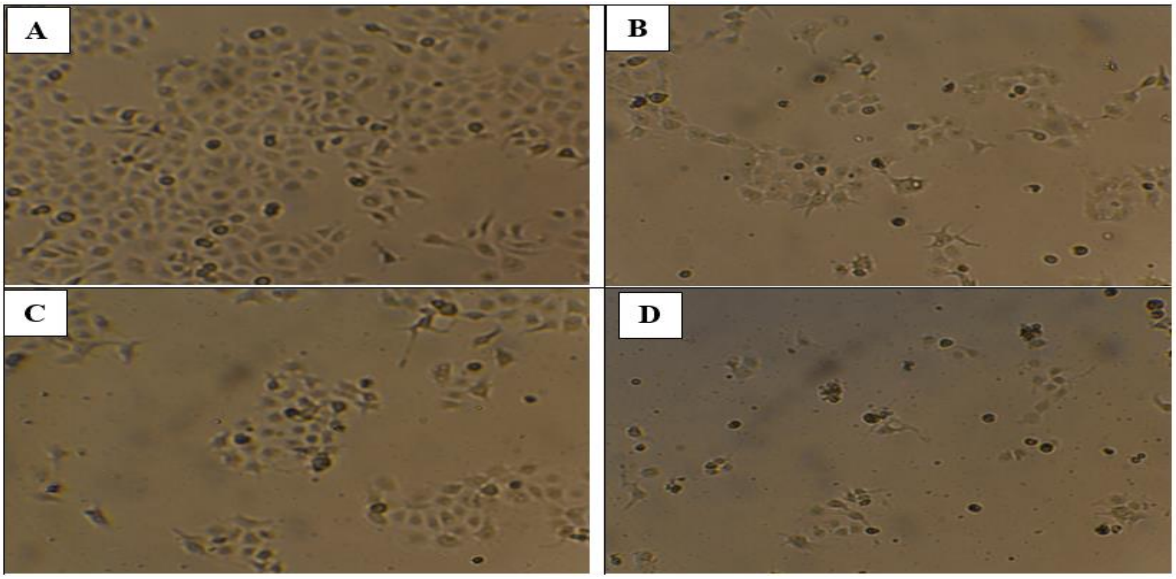

Figure 3. Effects of CisPt and/or RSV on morphology of FaDu cells.
FaDu cell line was cultured for 24h in complete DMEM medium (A), or added with 50μM RSV (B), 10μM CisPt (C), or 10μM CisPt and 50μM RSV (D), then morphological changes were examined under light microscopy and images taken with a digital camera (100x magnification).

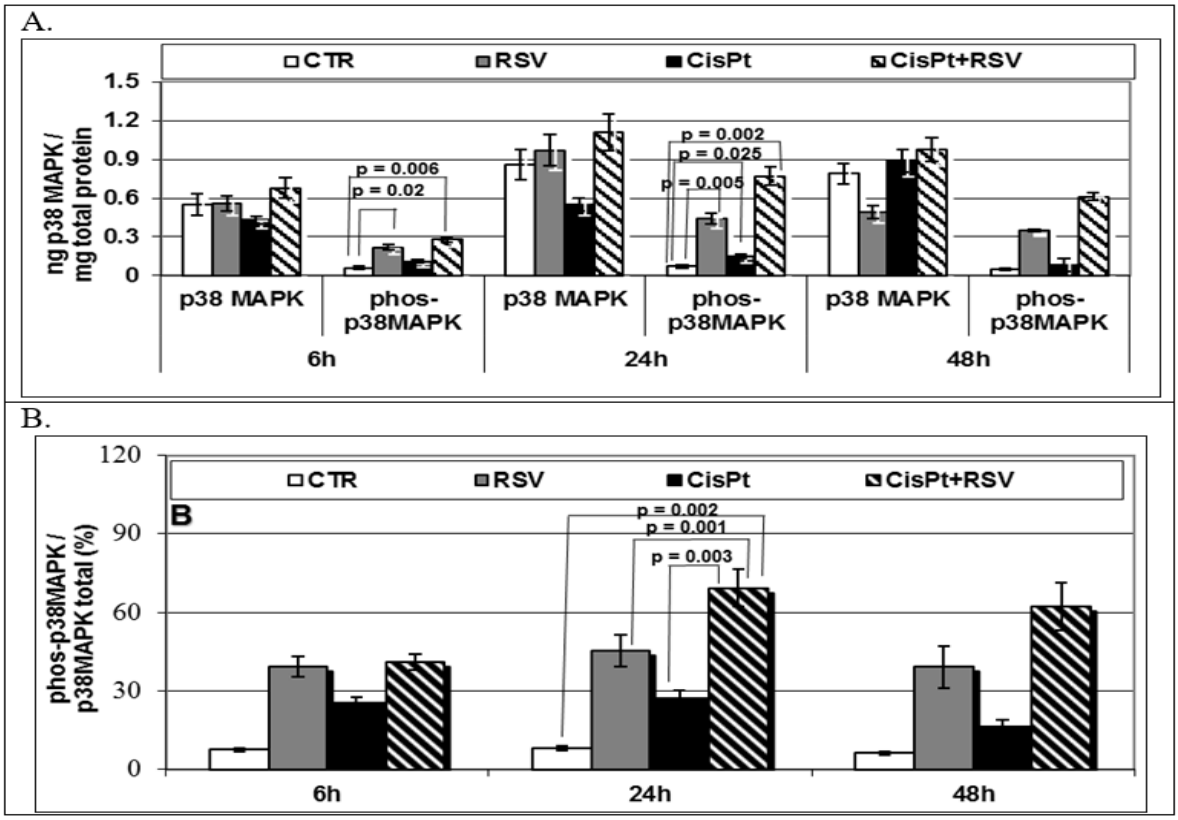

Figure 4. Effect of RSV and/or CisPt on p38MAPK activation in FaDu cell line.
Phosphorylated (phos-) and total p38MAPK levels were measured by ELISA assay in lysates of FaDu cells, untreated (CTR) or treated with RSV50μM and/or CisPt10μM for different periods of time (A). Raw quantitative data were used to calculate phosphorylated-p38MAPK / total p38MAPK ratios (B). Results are presented in graphs as mean ± S.D. values from three independent experiments.

Effects of CisPt and/or RSV on p53 phosphorylation in FaDu cell line

Phosphorylation is an important modification involved in the control of p53 activity. We hypothesized that phosphorylation of p53 could affect the sensitivity of cells to treatment. To test the effect of CisPt and/or RSV on the expression and phosphorylation of p53 in FaDu cells, we treated cells with 10μM CisPt and/or 50μM RSV for 6, 24 or 48h. We found that RSV treatment induced the phosphorylation of p53 in a time dependent manner, reaching a peak of maximum activation after 24 hours, while CisPt treatment slightly inhibited p53 phosphorylation (Figure 5). In addition, to analyze if phosphorylation of p53 depends on the activation of MAPKs, cells were pretreated with 10μM of SB203580 specific p38MAPK inhibitor for 2h before cells treatments with CisPt and/or RSV. We found that CisPt increased p38 MAPK phosphorylation, but not p53 levels in FaDu cells. In contrast, RSV induced p53 phosphorylation, as shown in Fig. 5 (Panel A and B), that was reduced when the cells were pretreated (2h) with SB203580 (p=0.002). Furthermore, the most significant changes were observed when the cells were simultaneously treated with CisPt and RSV (p=0.0006). Taken together, these evidences indicate that p38MAPK mediates phosphorylation of p53 protein in response to RSV treatment, the effect being even higher than in the cells simultaneously treated with CisPt and RSV.

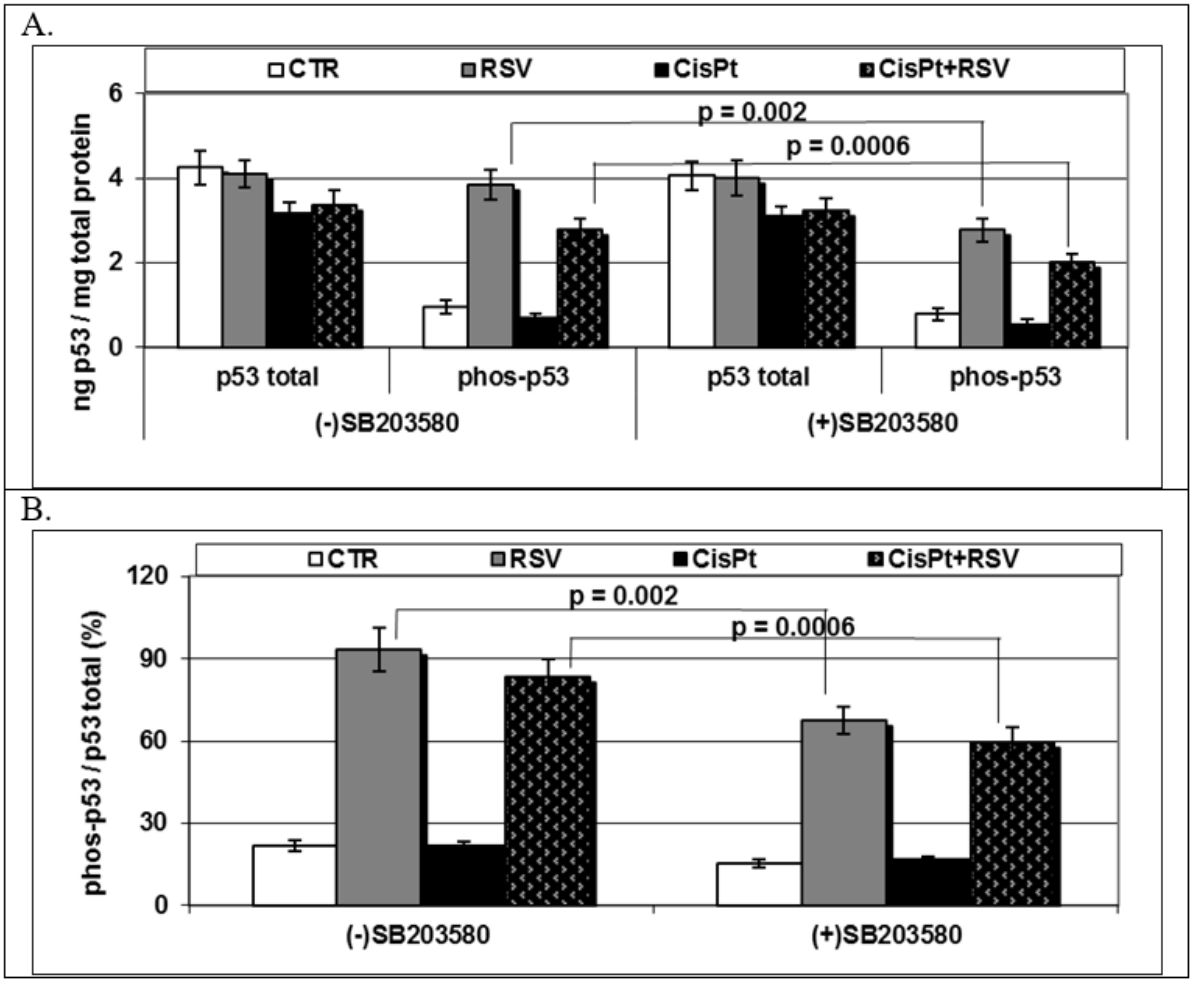

Figure 5. Effect of RSV and/or cisplatin on p53 phosphorylation in FaDu cell line.
Phosphorylated (phos-) and total p53 protein expression in FaDu cell line treated with RSV 50μM and/or CisPt 10μM for 24h in presence or absence of SB203580 inhibitor
(A). The results were analyzed and used to calculate the phospho-p53 /total p53 ratios
(B). Results are presented in graphics as mean ± standard errors from three independent experiments.

Discussions

Cisplatin is commonly used as a cytotoxic drug in chemotherapeutic approaches of several solid tumors, including neck and head cancers. Its mode of action has been linked to the ability to crosslink with the purine bases in DNA, interfering with DNA repair mechanisms and causing DNA damage. Therefore, DNA replication is blocked as well as gene transcriptions. The liaisons that CisPt forms with DNA are believed to be essential for the cytotoxic activity of the drug (T. C. HSIEH, & al. [26]). Following exposure to different agents implicated in DNA damage, cells show an increase in p53 expression levels as a consequence of its stabilization by post-transcriptional modifications. These modifications protect p53 from rapid degradation and lead to its activation as a fully active transcriptional factor able to regulate a set of p53 target genes (R. VIELBA & al. [27]). Phosphorylation at different Ser/Thr residues is a key step in the regulation of p53 function in response to various stimuli. Experimental data demonstrated elevated p38MAPK protein levels in the serum of HNSCC patients, which correlate with resistance to radiotherapy. The p38MAPKs were implicated in complex biological processes, such as cell proliferation, cell death, cell migration, and invasion. Abnormal p38MAPK levels in cancer patients were associated with advanced stages and short survival (K. GILL, & al. [28] and A. CUENDA, S. ROUSSEAU, [29]). The p38 MAPK plays a dual role as a regulator of apoptosis, mediating either cell survival or cell death, which depends not only on the stimulus type, but also of the cell type.

The study was focused on the activation status of p38MAPKs in FaDu cell line, and its relationship to other cell signaling events. To test whether p38MAPK signaling could control cell proliferation and p53 activation, we carried out experiments in the presence of a highly specific p38 MAPK inhibitor (SB203580) in order to block p38MAPK signaling in FaDu cells. SB203580 was effective to block p38MAPK functions when 10 μM concentration was used, similar to those reported by other research groups (N. YOSHIZUKA, & al. [30]). RSV, a potential cancer chemoprevention agent with no toxic or mutagenic effects, was previously reported that could be used to inhibit the growth of cancer cells because of its capacity to affect the activity of multiple targets involved in carcinogenesis (H. K. KOUL, & al. [31] and A. C. YUMUSAKHUYLU, & al. [32]). In addition, RSV seems to induce anti-tumor effects through activation of signal transduction pathways in various cancer types (M.R. ABEDINI, & al. [33] and S.R. CHANDANA, & al. [34]), and several reports mention the use of RSV in cancer treatment in combination with CisPt (E.M. VARONI, & al. [35] and L. MA, & al. [36].

All these data prompted us to analyze the role of RSV and/or CisPt in the inhibition of FaDu cell proliferation, and the possible cooperation between p38MAPK and p53 activation process. We found that exposure of cells to RSV induces activation of p38MAPK in association with an increase of p53 phosphorylation. The obtained results showed that FaDu cell line treatment with both RSV and CisPt has a higher effect than treatment with single drugs. We conclude that RSV treatment could amplify the antitumor activity of CisPt in FaDu cell line, reducing the proliferation rate after 24h of treatment, data being sustained also by the morphological changes observed in tumor cells treated with CisPt and/or RSV for 24 hours. In addition, RSV alone or in combination with CisPt treatment induced p53 phosphorylation which was abolished when the cells were pre-treated with SB203580 for 2 h.

Conclusions

Taken together, the obtained evidences suggest that p53 activation is linked to the activation of p38 MAPK pathway, and provide evidences that cell chemosensitivity could be associated with p53 activation. Therefore, the combined treatment of tumor cells with conventional chemotherapeutic drugs and RSV provides a promising direction for cancer chemotherapy. A better understanding of the role of p38MAPK in response to cancer therapy could lead to new therapeutic approaches in which modulating the activity of p38MAPK could be a cornerstone.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article, and there was no financial support that could have influenced the outcomes. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

We are grateful for the support from POS-CCE O2.2.1. 433/ 2012.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 129-139
doi: 10.25083/rbl/24.1/129.139

Received for publication: October 20, 2018
Accepted: November 30, 2018

Original paper

Bioprospecting Wild Biodiversity in Romania: Case Study - Gentiana lutea

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Abstract

The scope of this article is to discuss critical points in developing a bioprospecting protocol for Romania under the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity that entered into force in October 2014. By taking as a case study Gentiana lutea L., (yellow gentian) an endangered plant species of pharmaceutical importance, listed in the Annex D of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), our study reveals that bioprospecting is not officially implemented in our country. However, for ensuring the conservation of biodiversity as a whole it is needed to further strengthen and harmonize all legal provisions among international treaties such as CITES, Nagoya Protocol and Multilateral System adopted under the International Treaty on Plant Genetic Resources for Food and Agriculture or Plant Treaty.

Keywords : access to genetic resources, biodiversity conservation, bioprospecting, Romania, Gentiana lutea L.

To cite this article: Sava Sand C, Antofie Maria M. Bioprospecting Wild Biodiversity in Romania: Case Study - Gentiana lutea. *Rom Biotechnol Lett*. 2019; 24(1): 129-139. DOI: 10.25083/rbl/24.1/129.139

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Introduction

Plant species are today major sources for food and non-food industry. Two major technology areas from economic point of view are represented by pharmaceuticals and biotechnology followed by different other sectors such as: cosmetics, dyes and colorants, herbal health products, different intermediates, plant protection or essential oils (A. LUBBE & R. VERPOORTE [1]). Only for genetically engineered seeds was recorded a revenue of US$ 5.6 billion in 2013 (J. STRAUS [2]) and for biopharmaceuticals of US$ 150 billion in 2015 (J. NIOSI [3]). Today we assist to the increasing interest to synthetic biology, which arose with cephalexin synthesis (P.P. SAVIOTTI [4]). By funding research, development and innovation, pharmaceutical industry is continuously increasing worldwide even the costs for placing on the market of a single product is estimated to reach US$1 billion (B. DAVID & al. [5]; J.A. DIMASI & al. [6]). Over 25% of the nowadays pharmaceutical drugs are originating from plant natural products (A. LUBBE & R. VERPOORTE [1]) and 76% of the 1073 new chemical entities belonging to small molecules approved to be placed on the market up to 2010 are of natural origin (A.G. ATANASOV & al. [7]). These authors recognized among major challenges for pharmaceutical industry the accessibility of the starting material in their search for new bioactive compounds. Species important for their biological active compounds are widely spread all over the world. Some of them are common species and others are rare, endangered species that need conservation measures to be in place for their protection. It was recorded an increasing demand for plant species accession for research, development and innovation purposes (B. VANHEUSDEN & G. VAN DEN BERGHE [8]).

Romania is recognized as a very rich country in terms of biodiversity, mainly on native wild medicinal and aromatic plants. At least 300 wild plant species were recorded and described for their food potential (C. DRĂGULESCU [9]). A number of 24 plant species have been classified as important for essential oil production, 47 as aromatic, 48 for dyes and 32 for colorants, 186 are medicinal plant species, 79 poisonous species relevant for their alkaloids content and 223 are high pollen production species (A. BELDIE [10]). Today, it is agreed that the Romanian common wild flora is not seriously threatened by collecting from the wild (W. KATHE & al. [11]). The scope of this article is to identify and discuss some critical points regarding bioprospecting from the wild of Gentiana lutea in Romania for ensuring the full implementation of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits arising from their Utilization to the Convention on Biological Diversity (Nagoya Protocol), in harmony with already existing regulatory frameworks in order to further support the conservation of biodiversity as a whole. This wild species falls under the scope of Nagoya Protocol – prior accession (i.e. when discussing the access to genetic resources) and under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) for trade (i.e. when discussing the trade of species and specimens).

Materials and Methods

Description of the study site. The research was conducted in June, 2013 in Postăvaru Massive, Brașov county, Romania Lat. N: 45°34'60" Long E: 25°33'0" at an average altitude of 1,480 m, where the occurrence of Gentiana lutea L. was recorded before (E.C. MUICA & al., [12]).

Method. Critical identified points in developing a bioprospecting protocol as a step-by-step process is discussed following certain recommendations (T. GREIBER & al., [13]) and addressing as a case study on bioprospecting Gentiana lutea L. from the mountain range Postăvaru with the intention of collecting for scientific research and development purposes.

Biological material. Minimal standards guiding bioprospecting in the classical manner were applied for the characterization of selected genotypes (A.B. CUNNINGHAM [14]).

Results and Discussion

Bioprospecting under multilateral environmental agreements.

In 2010 in Nagoya (Japan) during the 10th Meeting of the Parties to the CBD the Nagoya Protocol was adopted and entered into force in the 12th of

October 2014. Romania signed the Protocol in the 20th of September 2011, as a European Union (EU) Member State approved the Protocol on the date of entering into force and nowadays must comply with the Protocol's provisions into the EU context (M. BUCK & al., [15]; G.S. NIJAR, [16]). Before the adoption of Nagoya Protocol, the Bonn Guidelines recognized by the EU provided recommendations on access for benefit sharing (ABS) upon the access of genetic resources, after its adoption at the 6th Conference of the Parties to the CBD (S. TULLY, [17]; U. SCHÜKLENK & al., [18]; S. BHATTI & al., [19]). The scope, of Nagoya Protocol, was established in the text of article 1: "the fair and equitable sharing of the benefits arising from the utilization of genetic resources", which in fact is the third objective of the CBD (S. BHATTI & al., [19]; M.W. TVEDT & al., [20]). In other words, granting access to genetic resources for an applicant from another country, for research and development aiming the commercialization of new products, must give back revenues to the country of origin of genetic resources (M. BUCK & al., [15]; J. DREXL, [21]). Thus, it is vital from economic point of view to integrate the objectives of biodiversity conservation into the national development strategies, to create synergies among different sectors in order to meet the mandates of the CBD (S.B. BRUSH, [22]). According to the provisions of Art. 22.5. f) of the Nagoya Protocol, entitled "Capacity": "each party have to develop measures including bioprospecting associated research and taxonomic studies". In this regard an official bioprospecting protocol should be adopted at the national level and in case of Romania it should be in line with the EU policy. This protocol should be the subject of a bilateral contractual agreement based on mutual agreed terms (MAT) between applicant and the Party (M. BUCK & al., [15]).

Some relevant terms need to be clarified for Nagoya Protocol for the current national regulatory framework such as following: applicant, country of origin and bioprospecting.

The term "applicant" was first defined into the text of Art. 2 (Definitions) of the Council Regulation (EC) 338/97 on the protection of species of wild fauna and flora by regulating trade therein. The same term was included in 2010 into the text of the Nagoya Protocol, namely into the provisions of Art. 13, regarding the national focal points and competent national authorities. In case of bioprospecting genetic resources in Romania the applicant will be defined according to the Council Regulation (EC) 338/97.

If "country of origin of genetic resources" for the CBD means the country which possesses those genetic resources into in situ conditions for Nagoya Protocol, it is noted a slight change: "Party or the Parties providing genetic resources that is the country or are the countries of origin of such resources or a Party or Parties that have acquired the genetic resources in accordance with the Convention". For EU countries based on the provisions of the Council Regulation (EC) 338/97, the term "country of origin" is defined as "the country in which a specimen was taken from the wild, captive-bred or artificially propagated" and fits with the terms agreed for the Nagoya Protocol. Thus, an applicant from another country will comply with the Council Regulation (EC) 338/97. In this case, in situ and ex situ origins of genetic resources may become "countries of origin" of the accessed genetic resources with the ratification of the Nagoya Protocol. Thus, ex situ collections of public interest may become the subject for bioprospecting as well.

The term "bioprospecting" is not mentioned in the text of the CBD but it is into the provisions of Art 22.5. (f) of the Nagoya Protocol. This term is also missing in the text of the Council Regulation (EC) 338/97 and therefore is missing too in the national regulatory framework for transposing this regulation. Bioprospecting is defined as the official granted search for plant and animal species from which commercially valuable compounds can be obtained. On contrary bioprospecting without permit is biopiracy (M.J.W. COCK & al., [23]). The term bioprospecting cannot be considered as an activity embedded or hidden under the term "scientific research" citing the Governmental Emergency Ordinance 57/2007 (GEO 57/2007) and transposing the Habitat Directive, Birds Directive and the Council Regulation (EC) 338/97. Based on the provisions of Art. 15 para. 2 let. d) of the GEO 57/2007, the term „scientific research" is related strictly to conservation measures of species and habitats that include scientific observations and monitoring activities. There is no connectivity to any potential sustainable use of genetic resources for

research and innovation or biotechnology. In case of protected species and habitats, scientific research is allowed based on derogations only for supporting the conservation, inside or outside protected areas, according to provisions of Art. 38 for setting down derogations according to the provisions of Art. 33 of the GEO 57/2007. Therefore, it can be concluded that in the national regulatory framework is missing an official procedure for bioprospecting as well as for accessing genetic resources according to Nagoya Protocol.

A bioprospecting protocol should be harmonized with already existing rules and procedures under other treaties and already existing domestic regulatory framework such as those of Multilateral System (i.e. this system is functioning under the International Treaty on Plant Genetic Resources for Food and Agriculture or Plant Treaty) and of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). We mention here that the Gene Bank of Suceava is the national focal point for the Plant Treaty, working under the Ministry of Agriculture (i.e. Romania adhered to the Treaty through the Low 42/2005). Ministry of Environment is the national competent authority for CITES implementation, closely working with the Commission for Nature Monuments of the Romanian Academy and The Forest Research and Development National Institute as scientific authorities according to provisions of Art 2. of the Law 69/1994 for the ratification of CITES Convention. Moreover, public ex situ collections may belong to public institutions functioning under the Ministry of Education or Ministry of Research.

Contracting with applicants under Nagoya Protocol

Mutual agreed terms (MAT) under a binding instrument such as a bilateral contractual agreement should be developed for bioprospecting implementation. In this case a guideline should be adopted to explain on case-by-case basis different situations that may emerge, by considering the specific of the country and targeted group of species. Some of deficiencies of the market and contractual approaches for governance all over the globe for these bilateral contractual agreements have been described (M. BUCK & al., [15]; T. DEDEURWAERDERE, [24]; L. ONOFRI, [25]; I.M. SOLIS, [26]). A bilateral contractual agreement for bioprospecting should include clear financial obligations for the applicant from another country in case of benefit arising from accessing genetic resources from the country of origin (S.P. SINGH & al., [27]). This regulatory act should be in line with the current regulatory framework for nature protection in the European countries (S.R. HARROP, [28]; L. MAGGIONI, & al., [29]). However, the current GEO 57/2007 works only for the conservation of biodiversity without considering the third objective of the CBD. Notifications under the scope of scientific research were received by our national environmental authorities from other countries such as: Serbia, Bulgaria, Hungary, Austria and Germany (personal communications) and therefore in case of accessing genetic resources it is necessary to comply to the objective of the Nagoya Protocol (E.C. KAMAU & al., [30]; E. GUNDU & al., [31]). In case of collecting from the wild yellow gentian for scientific research have been described different methods and some of them are highly destructive (D. PEĆANAC, [32]; M.R. POP & al., [33]). Thus, in case of endangered species only aerial parts of plants should be allowed to be collected, on a case by case basis, for ensuring first the maintenance of optimum population size of the species. The bilateral contractual agreement should also stipulate the beneficiary direct contribution to the national monitoring system that needs to meet the requirements of the provisions of Art. 17 of the Habitats Directive in the EU context.

Case study - Gentiana lutea

Gentiana lutea was described for its composition in different biological compounds and uses as a medicinal plant since 1821 (J.E. ATKINSON & al., [34]). Different compounds classes have been recorded (i.e. secoiridoid glicosides, flavonoid, xanthone, pyrone) and medicinal effects have been described. It contains unique compounds that are subject for cancer therapy or anti-inflammatory drugs development (S. POPOV & al., [35]; H. HARAGUCHI & al., [36]; O. PRAKASH & al., [37]). As an example, an amount of 10 mg of pure gentiopicroside, purified from yellow gentian, costs today 132 Euro (BOTANICERT & al., [38]). Yellow gentian is the subject of ABS for cloning

carotenogenic genes during the flowering for lutein production (C. ZHU & al., [39]), the isolation of new triterpenoids (Y. TORIUMI & al., [40]) or for biotechnology purposes (N.M. DROBYK & al., [41]).

In Romania, Gentiana lutea L. is a protected species since 1977 (i.e. the first group of protected species includes six species: Taxus baccata, Pinus cembra, Angelica archangelica, Gentiana lutea, Leontopodium alpinum and Cypripedium calceolus) all over the country territory (A. BELDIE [10]). The species, continues to be in a favourable status of conservation on the current territory of the country (G. DIHORU & al., [42]). Today, based on the current regulatory framework, the species is listed in the Annex no. 4A for protected species of community interest of the Governmental Emergency Ordinance 57/2007 (GEO 57/2007). Yellow gentian populates meadows and grasslands, and in Romania may be found in floristic associations with species belonging to the following genus: Nardus, Agrostis, Setaria (H. HELTMAN, [43]). Official data regarding the presence of the species in protected areas are supplied by the Order of Ministry of Environment 1964/2007, being recorded in several protected areas of community importance and listed under Chapter 3.3. of the Natura 2000 Standard Data Form (i.e. other species), in four sites of community importance according to already published management plans: ROSCI0013 (PARCUL NATURAL BUCEGI [44]), ROSCI0027 (PARCUL NAŢIONAL CHEILE BICAZULUI – HĂŞMAŞ [45]), ROSCI0122 (FĂGĂRAŞ NATURA 2000 [46]) and ROSCI0229 (REZERVAŢIE SIRIU [47]).

According to the European Commission data base EIONET Gentiana lutea is in a favorable status of conservation in the alpine regions for Austria, Germany, Bulgaria, Spain, France, Italy, Romania and Slovenia (EUROPEAN TOPIC CENTRE ON BIOLOGICAL DIVERSITY [48]).

At the global level as a non-CITES species Gentiana lutea is monitored for the trade purposes as it is listed in the Annex D of the CITES Convention (i.e. import, export and re-export as such or part of it). Romania do not officially trade yellow gentian as dried plants or roots for research and development based on CITES data base. However, according to official data provided by CITES, in 2012 14,301.00 kg roots of yellow gentian were officially imported in European countries from third Parties and collected from the wild for pharmaceutical purposes (CITES TRADE DATABASE [49]). The global market demands of the species, between 2010 and 2013, came only from the European Union's countries such as Germany (76,563.00 kg) and Italy (9,749.00 kg). Small quantities have been imported also by Spain (4.00 kg) and Slovenia (0.50 kg). A total amount of 86,316.50 kg (i.e. 28,129.50 kg of dried plants 58,187.00 kg roots) have been imported by the European Community's applicants from third parties also belonging to the European continent (e.g. Albania, Bosnia and Herzegovina, Croatia, Macedonia, Montenegro and Switzerland). The major contributor to yellow gentian export was Bosnia and Herzegovina with 72,310.00 kg followed by Albania (6,077.00 kg), Croatia (2,505.00 kg) Montenegro (1,958.50 kg), Makedonia (640.00 kg). Germany placed on the common market 2,714.00 kg of gentian originating from Albania and Switzerland exported a total amount of 112.00 kg gentian roots collected from the wild.

We underline that the unsustainable collecting from the wild may have negative impact on yellow gentian conservation. Thus, negative impact on the conservation of this species were already described for Bosnia and Herzegovina (S.U. PAULS & al., [50]), Albania and Macedonia, Croatia (Z. ŠATOVIĆ [51]; G. SAMY [52]) and Bulgaria (PETROVA & al., [53]). Moreover, in these countries of origin are not properly working financial mechanism for supporting the implementation of measurable conservation and/or ecological restoration indicators associated with gentian habitats (S. SULLIVAN [54]). Following the negative examples described above, the bilateral contractual agreement should include detailed risks assessment regarding the collection from the wild for each species.

Experiencing a potential bioprospecting Gentiana lutea in Postăvarul Massive

In Braşov area, in Bârsei Mountains, yellow gentian is best occurring in the grasslands of three mountains: Piatra Mare (ROSCI0195), Postăvarul (ROSCI0207) and Piatra Craiului (ROSCI0194) (E.C. MUICA & al., [12]). Protection measures are taken only in the Management Plan of Piatra Craiului, and by accident the species is not mentioned in the Ministerial

Order 1964/2007 (PIATRA CRAIULUI [55]; FUNDAȚIA CARPAȚI [56]; [57]). Granting the official access for bioprospecting the species in the wild and for collecting the minimum specimen numbers for analysis should include in situ description of the habitat and taxes for supporting biodiversity conservation (G.C. RAUSSER & al., [58]; N. PANASENKO [59]; G. KEPPEL & al., [60]).

In Romania, Gentiana lutea was the subject for scientific research and collecting from the wild, for biochemical analysis of pharmaceutical importance and for developing ex situ conservation protocols for in vitro micropropagation (I. HOLOBIUC & al., [61]; I. HOLOBIUC & al., [62]; L.S. MUNTEAN [63]). In case of an applicant from another country, there is a regulation gap regarding the access to yellow gentian that needs to be acknowledged and solved for the future. Moreover, the species was also considered for cultivation as a crop in Brașov area (C. SAVA [64]). It becomes obviously that there is a need for strengthening cooperation between the management units of protected areas or the custodians and the Ministry of Environment in developing clear provisions to be introduced into the bilateral contractual agreement regarding: specific period and areas for bioprospecting and collecting, imposed conditions, minimum training requirements for the involved personnel.

Species characterization based on filed observations

A large population of over 350 yellow gentian specimens for a single area of about 250 m2 on the South slope of the Postăvaru Massive according to the described GIS coordinates. The population is highly heterogeneous in terms of height but homogeneous for blooming stage. It is relevant to note that some morpho-anatomic descriptors related to species should be analyzed and provided to officials upon granting bioprospection (i.e. in case of yellow gentian it would be relevant to note the following descriptors: 3-4 rosette leaves, highly developed root, flowering stems of a height between 0.7 to over 1.3 m). It is relevant to note floristic associations with species belonging to the following genera Alchemilla, Carlina, Digitalis, Festuca. The bilateral contractual agreement should include on a case by case basis specific requirements regarding in situ measurements during bioprospection and collecting from the wild.

Conclusions

Following this study, it was highlight that Romania needs to develop and adopt an official bioprospecting protocol. This could help to contribute for fully addressing all commitments taken under the Convention on biological diversity with specific focus on the Nagoya Protocol. Such a protocol will protect the over-collecting from the wild, will ground financial revenues for the country of origin and at the same time will catalyze the optimization ratio between in situ and ex situ conservation strategies for ensuring the preservation of all genetic resources (M.R. BELLON & al., [65]).

Furthermore, the case study of collecting from the wild yellow gentian revealed already the need to include for a future bioprospecting protocol, specific requirements as taxes, minimal descriptors related to the place of origin, habitat and species. Such a protocol, based on a bilateral contractual agreement, may support officials for creating synergies with the monitoring system of endangered species and habitats (C.F. BLIDAR & al., [66]). Moreover, it will become compulsory that bioprospecting measures to be included into all current management plans for protected areas all over the country.

In terms of capacity building the future notification system for bioprospecting should empower the Ministry of Environment as a competent authority closely working in harmony with the Ministry of Agriculture in charge with Multilateral System as well as with the Ministry of Education, Ministry of Research and any other potential authority dealing with ex situ collections of genetic resources.

We also consider that the fully implementation of Nagoya Protocol will catalyze the development of research in genomics, metabolomics and proteomics in our country by creating awareness regarding the importance of patenting biodiversity by accessing genetic resources for creating new products and services of commercial use (M. NEGRUȚĂ & al., [67]; R.M. STOICA & al., [68]; C. P. CORNEA & al., [69]).

The appropriate implementation of Nagoya Protocol at the country level will further contribute for harmonizing policies, strategies including legal provisions that work among treaties such as Nagoya Protocol, Cartagena Protocol on Biosafety, CBD, CITES as well as the Plant Treaty highly important for Romania as well as for all countries in the region on this subject (N.M. ABUMHADI & al., [70]).

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 140-146
doi: 10.25083/rbl/24.1/140.146

Received for publication: January 19, 2018
Accepted: March 25, 2018

Original paper

Type II Diabetes Mellitus - Associated Risk Factor in the Onset and Evolution of Digestive Tract Carcinoma

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Abstract

Introduction: The number of digestive cancers cases has alarmingly increased within the past decades up to a current third place ranking in terms of global incidence. The association between type II diabetes mellitus and digestive neoplasms has been on an upward trend lately due to common risk factors as well as to mutually potentiating effects on the evolution of the two conditions.

Patients and methods: This clinical study evaluates a group of 34 gastro intestinal cancer patients suffering from type II diabetes mellitus who received treatment in 'Elias University Emergency Hospital', Medical Oncology division, between 2010 and 2016. A number of demographic features specific to the aforementioned group, as well as comorbidities linked to localization, the aggressiveness of primary digestive tumor and the presence of the biological inflammatory syndrome were evaluated.

Discussion: Digestive neoplasms associated with type II diabetes mellitus turn out to be more aggressive. The therapy is burdened by potential risks arising from all associated pathologies.

Conclusions: A set of clinical and biological data underpin the association between type II diabetes mellitus and digestive neoplasms as being detrimental. Therefore, screening measures for digestive neoplasms in type II diabetes mellitus patients should be carefully respected.

Keywords : diabetes, digestive cancers, risk factor, inflammatory syndrome

To cite this article: NITIPIR C, BARBU MA, ORLOV C, STANCIU AE, POPA AM, HAINAROSIE R, PITURU S, ARSENE AL, STOIAN AP. Type II Diabetes Mellitus - Associated Risk Factor in the Onset and Evolution of Digestive Tract Carcinoma. *Rom Biotechnol Lett.* 2019; 24(1): 140-146. DOI: 10.25083/rbl/24.1/140.146

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Introduction

The incidence of digestive cancers and diabetes has increased exponentially in the last decade. According to the International Diabetes Association [1], one out of 11 adults worldwide (415 million people) were diagnosed or were already suffering from diabetes. Of the many digestive tract cancer subtypes, colorectal cancer is the third most common cancer worldwide. Almost 1.4 million positive diagnosis were made in 2012, of which almost 95% being classified as adenocarcinomas [2]. Inquiries into any existing links between the two conditions have been made as early as 1959, starting with population-based studies. The majority of the later studies conducted have focused mainly on type 2 diabetes, since early data suggested that its presence increased the relative risk for digestive cancers to more than twofold (GIOVANNUCCI & al. [3]). Data shows that type 2 diabetes is strongly related to liver and pancreatic cancers (GIOVANNUCCI & al. [3]). Although the observed incidence numbers suggest this relation, it can also be stated that diabetes produces a number of hyperglycemia-related metabolic disorders, all of which directly impact the two organs. Moreover, given that diabetes is a worldwide underdiagnosed condition by a margin of at most 5%, this relation implies a higher cancer risk for the normal population (VIGNERI & al. [4]). Furthermore, some of the medications used for controlling diabetes have been shown to also have a negative link with certain types of cancer (NGUYEN& al. [5]). Although not entirely proven by the available data, this relationship has become more visible in recent years, given the existence of different types of drugs. It is important to note that the cancer risk for patients with uncontrolled diabetes is considerably higher than for patients with controlled disease (GIOVANNUCCI & al. [3]).

Type II diabetes is also linked both with a cytokine-induced inflammatory response and a long-term tissue necrosis response, which are considered to be relevant factors in determining either the relative risk of developing cancer or of the survival rate, for those already having a cancer-type condition (GIOVANNUCCI & al. [3]). As proven by other clinical studies, chronic inflammation facilitates tumor development and impairs the normal immune system response in an area, allowing for cancerous cells to proliferate [STANCIU & al. [6]). Thus, a chain of causality can be drawn starting from a diabetes condition that may lead to incidence or prognosis of cancers (LEROITH & al. [7]).

Materials and Methods

This work involved 34 patients with a history of type II diabetes mellitus, who were diagnosed with digestive tract neoplasms between 2010 and 2016 and who received treatment in “Elias University Emergency Hospital”, Medical Oncology division. The diagnosis of gastro-intestinal cancer was confirmed in all patients by histopathological results. All patients were initially evaluated by paraclinical (e.g. imagistic methods, such as computer tomography or magnetic resonance imaging - in selective patients), and by clinical findings. Individual data regarding sex, age at diagnosis, family history of diabetes and cancer, tobacco smoking, working in toxic environment and associated pathologies were collected. After receiving initial cancer treatment (surgery, chemotherapy or radiotherapy) the participants were re-evaluated. Tumor markers (CEA - carcinoembryonic antigen and CA 19-9 - carbohydrate antigen 19-9) and inflammatory markers (LDH - lactate dehydrogenase, ESR - erythrocyte sedimentation rate and CRP - C - reactive protein) were examined in correlation with tumor stage, evolution and response to oncological treatment.

Results and Discussions

Table 1 shows baseline characteristics of patients. Out of the 34 patients, more than 2/3 were men. Indexing the results by the age at cancer diagnosis, resulted that only one patient was diagnosed at an age lesser than 50 years old and 21 had over 65 years old at the time of the diagnosis. There was a family history of diabetes in 7 cases and a family history of cancer in 9 cases. From the latter group, only 3 patients had a family history of digestive tract cancers.

Tobacco smoking proved to be an inconclusive risk factor in developing gastro-intestinal cancers in patients with diabetes, given that the distribution of smokers and non-smokers was almost equal. On the same note, toxic working environments could not be looked upon as a risk factor in this study, given the small number of patients who have been exposed.

Table 1. Patient characteristics and diabetes treatment type

Characteristics	No patients
Sex distribution	
men	23
women	11
Age at cancer diagnosis	
< 50 years	1
50-64 years	12
>= 65 years	21
Family history of diabetes	
yes	7
no	27
Family history of cancer	
digestive cancers	3
other	6
none	25
Tobacco smoking	
current/ex-smokers	18
never smokers	16
Working in toxic environment	
yes	3
no	31
Current treatment for diabetes	
oral antidiabetics	22
insulin	8
diet only	4
Associated pathologies	
dyslipidemia	10
arterial hypertension	22
cardio-vascular diseases	20

Diabetes requires one or more drug types in order to control the disease. From the studied group, most participants (22) managed this condition by using oral antidiabetic medication, 8 were insulin dependent, while the rest (4) were only under dietary restrictions. Common comorbidities of diabetes mellitus were also present in the studied group. Most patients had a history of arterial hypertension (22) and/or heart disease (20). Dyslipidemia was observed in almost ¼ of cases.

Table 2. Primary tumor localization and tumor characteristics

	No patients	Percentage (%)
Types of cancer		
colorectal cancer	20	58.8
pancreatic cancer	9	26.4
gastric cancer	4	11.7
hepatocarcinoma	1	2.9
Tumor differentiation		
G1	8	23.5
G2	18	52.9
G3	6	17.6
undifferentiated	2	5.8
Tumor staging		
0	1	2.9
I	1	2.9
II	8	23.5
III	8	23.5
IV	16	47

Table 2 renders the distribution of digestive tract tumor primary sites, tumor staging and histological tumor differentiation. As previously mentioned, colorectal cancer has a wide incidence rate world-wide, that also reflects in the results of this study, being diagnosed in 58.8% of the patients, followed by pancreatic cancer at almost half the ratio (26.4%). Gastric and hepatic cancers were not as common as the previous two locations, all of them adding up to 14%. Tumor staging is an important factor that correlates with tumor prognosis and aggressiveness. Almost half of the patients presented metastases at diagnosis, while only 6% had early stage tumors. Tumor differentiation grade is another factor to take into consideration when

assessing prognosis. More than 50% of patients were diagnosed with moderately differentiated tumors.

As previously mentioned, 16 patients were diagnosed with metastases at the initial evaluation: 8 had colorectal cancer, 4 pancreatic cancer and 3 had gastric cancer. Table 3 concludes that hepatic metastases are the most prevalent, being present in 11 cases followed by lymphatic, pulmonary and peritoneal dissemination in almost equal distribution. Besides these, only 4 patients presented bone metastases.

Table 3. Location of metastases at diagnosis

Location of metastases at diagnosis
hepatic metastases
lymphatic metastases
pulmonary metastases
peritoneal metastases
bone metastases

Paraclinical investigations were conducted in order to determine a correlation between tumor staging, tumor markers and inflammatory markers. In early stage cancer patients (stages 0, I and II) these indicators had normal values at diagnosis. Similar results were also found in the majority of patients with stage III tumors. On the other hand, in metastatic cases, these values were elevated beyond normal ranges. In the case of tumor markers, from the total number of 16 patients who were diagnosed with metastases at the initial evaluation, 9 patients had (>1 time) elevated levels of CEA and 4 had (>2 times) elevated levels while in the case of CA19-9 marker, 14 patients had (>3 times) raised levels and 2 patients had (>2 times) raised levels. Inflammatory markers were also beyond normal ranges, 10 patients had elevated LDH levels (>2 times), 9 patients had raised ESR levels (>2 times) and 6 patients had elevated CRP levels (>1 time).

Diabetes diagnosis rates are increasing worldwide and, from a sex distribution standpoint, are fairly balanced. Statistics gathered in the United States show an increase for diagnosed males of 177% between 1980 and 2010, as opposed to an increase of 114% for females in the same time period, leading up to a ratio of 6.6% diagnosed men and 5.9% women, respectively, in 2014 [8]. Given that this study comprised a much higher ratio of men, more than two third of the studied group, it can be argued that cancer had a more significant impact in the sex distribution of the group. Digestive tract cancer types are proven to have a much higher prevalence in the male population than in the female one [9]. For example, in the United States, male colorectal cancer had an average annual incidence rate of almost 47 cases per 100.000 people, averaging 11 cases more than the female ratio. This evidence is supported also by the difference (between the sexes) of the incidence of liver, pancreas and stomach cancers, which is 7.8, 3.1 and 4.6 per 100.000 people, respectively [8]. Despite the small number of patients that this study had included, its results suggest the same global trends, with the number of male cancer diagnoses amounting to more than double that of the female cases (FERLAY & al. [10]).

Some pancreatic cancer statistics reveal that the average number of male cases per year is gradually higher than the female number until the age interval of 75 to 79 [11]. After this interval, the same studies show the exact opposite, with women having a higher incidence, peaking at a ratio of 2 over the age of 90. Overall, the cumulated average number of cases is highest starting with the age of 65. This study also showed the same ratios, with the number of patients diagnosed over the age of 65 being significantly higher than of other age groups.

Within the studied group, the majority of the patients did not have a family history of either digestive cancer or diabetes. However, those with a family history of any of the two had more aggressive types of cancer. Moreover, patients included in this category that were also diagnosed with metastatic cancer developed widely spread metastases, affecting multiple organs. Multiple global population studies argue that some diabetes drugs can be considered a risk factor for digestive tract cancers, but with inconclusive results (LI & al. [12]). This study also had inconclusive results in proving any relationship between the antidiabetic treatment and the aggressiveness of the diagnosed cancer. Diabetes did not deviate the distribution of cancer type in the studied group from the worldwide distribution of digestive cancers. As expected, the majority of patients were diagnosed with colorectal cancer, followed by pancreatic and gastric cancer.

Almost half of the patients comprised in this study were diagnosed with stage IV digestive tract cancers. In the case of colorectal cancers, which represented the majority of cancer cases, incidence by stage does not follow the patterns observed in international statistics [13]. It has been observed that these tumors were more aggressive, leading up to almost twofold the percentage of cases diagnosed with metastases at initial evaluation. On the other hand, the pancreatic cancer incidence indexed by stage was in line with international statistic results; 45% of the patients were diagnosed with stage IV tumors[14]. Gastric cases were mostly diagnosed in late stages. Furthermore, metastatic tumors were more frequently present in male patients than in female ones.

Correlations between cancer stadialization and inflammatory markers (LDH, ESR and CRP) had also been found. It was showed that advanced cases were corelated with elevated values of inflammatory markers while early stage cancers were not. From the studied group, in the case of the 16 patients who were diagnosed with metastases at the initial evaluation, 10 patients had elevated LDH levels (>2 times), 9 patients had raised ESR levels (>2 times) and 6 patients had raised CRP levels (>1 time).In the case of early stage cancer (stages 0,I and II) these indicators had normal values at diagnosis.

Endocan is a novel blood-and tissue-based biomarker. It is a product of endothelial cells, highly regulated by vascular endothelial growth factor and expressed during the switch between dormant to fast-growing angiogenic tumors studied in various types of cancer and inflammatory conditions. Multiple studies showed the bad prognosis signature of endocan biomarker in cancer (HUANG & al. [15]; BORCZUK & al. [16]; STANCIU & al. [17]).Sepsis and inflammation have associated endothelial dysfunction ranging from edema and vasodilation to ischemia and organ failure (DE FREITAS CAIRES& al. [18]; ARCAN & al. [19]; MIHAI & al. [20]). Since inflammatory mediators induce endocan expression, measured levels of this biomarker may closely reflect the severity of inflammation and of course the response to therapy (DE FREITAS CAIRES& al. [18]). It was shown that serum endocan levels are also increased in patients with inflammatory bowel disease (VOIOSU & al. [21]). Endocan may be a biomarker for both inflammatory disorders and tumor progression as well as a validated therapeutic target in cancer (SARRAZIN & al. [22]). More validation studies are however still required on larger cohorts on vascular endothelial growth factor-driven cancers. (MAZILU & al. [23]).

Conclusions

This study concludes that type II diabetes mellitus triggers a higher level of aggressivity in digestive tract cancers. The results have been confirmed by both paraclinical investigations and statistical results. Diabetes might also negatively impact the stage at diagnosis, as proven by the colorectal and gastric high incidence rates in advanced form. Thus, cancer screening is recommended for patients with type II diabetes and over 50 years old. In advanced stages, inflammatory markers were associated with tumor evolution and should be measured more frequently. The study also had inconclusive results in proving any relationship between the antidiabetic treatment and the aggressiveness of the diagnosed cancer.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

All authors have equal contribution to this article.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 147-153
doi: 10.25083/rbl/24.1/147.153

Received for publication: August 25, 2017
Accepted: February 11, 2018

Original paper

Biosequestration of heavy metals by microbially induced calcite precipitation of ureolytic bacteria

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Abstract

Microbial induced calcite precipitation (MICP) using urease producing bacteria has gained great importance of research in recent years. In this study, the efficiency of ureolytic, heavy metals resistant bacteria to biomineralize toxic heavy metals has been investigated. Twenty two bacterial strains were isolated from calcareous soil samples collected from Egypt and screened for urease and calcite production as well as the tolerance to heavy metals toxicity. Micrococcus sp. NCTC -1716 was selected for subsequent studies as it was the most potent strain. Resistance of Micrococcus to increasingly concentrations of zinc, cadmium, lead and iron varied according to the metal. The capacity of Micrococcus sp. to remove heavy metals ranged from 60.66 % for Cd2+ to 97.20 % for Pb2+after 48 h of incubation. Metal sequestration via calcite precipitation showed that heavy metals are removed according to the following sequence: Pb2+, Fe2+, Zn2+, and Cd2+. Scanning electron microscope was used to examine the morphology of calcite crystals and biomineralization products, while their structure was emphasized by X-ray diffraction (XRD) analysis. The study confirmed that MICP sequesters soluble heavy metals into biominerals and proved as a promising strategy for bioremediation process.

Keywords : Bioremediation, Heavy metals, SEM, Urease, XRD

To cite this article: Gomaa EZ. Biosequestration of heavy metals by microbially induced calcite precipitation of ureolytic bacteria. *Rom Biotechnol Lett*. 2019; 24(1): 147-153. DOI: 10.25083/rbl/24.1/147.153

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Introduction

In Nature, most heavy metals caused no toxicity as they persist at low concentrations. However, human activities such as combustion of fossil fuels, industrial progress, burning of wastes, use of agricultural fertilizers and pesticides give rise to release of non-biodegradable redundant amounts of heavy metals (H. GUO & al. [1]). Heavy metal contaminants pose a major threat to human health creating many diseases such as cancer, mental retardation and growth abnormalities. Therefore, it is necessary to search for an effective eco-friendly technique to dispose of such contaminants.

Traditional treatment mechanisms such as adsorption, filtration, evaporation, chemical precipitation, oxidation/reduction and electrolysis ion exchange used for remediation process are uneconomical, ineffective as well as the safe disposal issue of wastes (O. L. KANG & al. [2]). Many biological techniques have been used to get rid of such heavy metals from contaminated sites like bioaccumulation, bioleaching, biosorbents, biocoagulation and phytoremediation (S. CHOUDHARY& P. SAR [3]). Unfortunately, these methods are also not effective because they are expensive, take long time and result in the production of immobilized or adsorbed heavy metals back to the environment. Moreover, it cannot be applied in severe and dry climate of arid area (V. ACHAL & al. [4]).

Biomineralization based on microbially induced calcite precipitation (MICP) process provides a promising technique to remediate toxic metals from contaminated soils (C.H. KANG & al. [5]). Heavy metals are bound with calcite, which is responsible for heavy metals sequestration and ultimately resulted in significantly reduction of their levels in the environment. Provided such situation, heavy metals are removed by an effectively, economically and eco-friendly manner (C.H. KANG & al. [6]).

This study aimed at screening a number of bacterial strains isolated from calcareous soil samples collected from Egypt for urease production and calcite precipitation. The study was extended to investigate how toxicity of a range of heavy metals (zinc, cadmium, lead and iron) on growth of the most potent strain, then demonstrate the variations in heavy metals removal performance of these metals via bioprecipitation with calcite. Characterization of biomineralization products was done by scanning electron microscopy (SEM) coupled X-ray Diffraction (XRD) analysis.

Materials and Methods

Isolation of urease producing bacteria

Six calcareous soil samples collected from north of the western desert and Borg El-Arab of Egypt were used for isolation of urease producing bacteria. For enrichment technique, 5 g of soil sample were inoculated into 50 ml nutrient broth amended with 20 g/l filter-sterilized urea using 0.22-mm filter (Millipore, USA) and incubated at 35oC for 72 h under shaking conditions (V. ACHAL & al. [7]). Urease producing bacteria were isolated from enriched samples using serial dilution technique followed by culturing on Christensen's Urea Agar Base (UAB) (R.M. ATLAS [8]). Colonies that showed a pinkish color change around them were marked as urease producers and selected for subsequent experiments.

Measurement of urease activity and calcite production

Urease broth medium containing (g/l): urea, 20; NaHCO3, 2.12; NH4Cl, 10, nutrient broth, 3; CaCl2.2H2O , 25, pH 7 was inoculating with overnight grown seed culture and incubated under shaking condition at 37°C for 72 h. Ammonia released from urea hydrolysis was measured as an indicator for urease activity according to the phenol-hypochlorite assay method (K. R. NATARAJAN [9]). The culture filtrate (250 µl) obtaining after centrifugation of bacterial culture was added to a mixture of 1 ml of 2.5 ml of urea (0.1 M) and 0.1 M potassium phosphate buffer (pH 8.0) and incubated at 37°C for 5 min. Phenol Nitroprusside and alkaline hypochlorite, 1 ml each were added and incubated again at 37°C for 25 min. Optical density was measured at 760 nm. Ammonium chloride (100 µg/ml) was used as standard. The experiment was done in triplicate and the average absorbance was recorded. One unit of urease is defined as the amount of enzyme that catalyzed the hydrolysis of 1 µM urea per minute under the assay conditions.

The quantity of calcium carbonate (calcite) precipitated was measured using the same medium.

The precipitate collected after centrifugation was weighed after drying at 50°C to constant weight. Bacterial growth was determined by counting the colony forming units at different time intervals and the results were expressed as log CFU/ ml. A pH meter (pH Pen Jenco 610) was used for measuring the final pH of the culture. All experiments were conducted in triplicate and the mean values were considered.

Molecular strain identification

The most efficient bacterial strain was identified using 16S rRNA gene sequencing (V. ACHAL & X. PAN [10]). The gene sequencing was done by Macrogen (South Korea). Sequence analysis was performed at the National Center for Biotechnology Information (NCBI) database, USA.

Metal toxicity experiments

For metal toxicity experiments, nutrient broth media supplemented with different concentrations (0–10 mM) of heavy metal salts (zinc chloride, cadmium sulphate, lead nitrate, and iron sulphate) were inoculated with 2% overnight grown culture (6.5 Log CFU/ ml). Each metal concentration was tested in triplicate and incubated at 35oC for 48 h. Bacterial growth was measured to examine the viability of cells in the presence of these metals (C.E. RUGGIERO & al. [11]). The lowest concentration of each heavy metal that caused no visible growth was considered as the MIC (minimum inhibitory concentration) of that metal.

Metal bioprecipitation

Metal bioprecipitation tests were performed using urease broth media supplemented with ½ MIC of each heavy metal salt. The flasks were inoculated with 2% overnight grown seed culture and incubated at 35oC for 48 h under shaking conditions. An inductively coupled plasma atomic emission spectrometer (Perkin Elemer USA, Model 2400) was used to measure the initial and final concentrations of each heavy metal and the percentage of each heavy metal removal was calculated. Furthermore, the mass of precipitation was calculated after drying to constant weight. All experiments were performed in triplicate and treatments of urease broth media without any heavy metals added and those without bacterial inoculation were used as controls.

SEM and XRD analyses

The precipitates created during the bioremediation process for each heavy metals as well as calcite precipitated were further analyzed. After completely drying and grounded into powder form, samples were gold coated with a sputter coating Emitech K575. Morphological shape and size of the crystals were examined under scanning electron microscope (JEOL, JSM 5200).

X-Ray Diffraction (XRD) analysis was used to investigate the crystal structure of calcite and metal precipitates. The dried samples were spread uniformly onto glass plate and employed with X-ray diffractometer (INEL X-ray diffractometer). The samples were identified by comparing the X-ray profile of the samples with standards of International Center Diffraction Databases (ICDD) (C.H. KANG & al. [6]).

Statistical analysis

The results were presented as mean± standard deviation of triplicate experiments. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of $p<0.05$.

Results and Discussions

In the present study, 22 morphologically different bacterial strains were isolated from calcareous soil samples. These natural environments are regarded as potential candidate for isolation of ureolytic bacteria capable of yielding urease enzyme stable under harsh conditions such as high temperature and alkaline environments. As a primary test, the isolates were screened qualitatively for urease production using UAB medium. Based on the intensity of pink color observed on urea agar media, ten efficient bacterial strains were selected. Ammonia released from urea hydrolysis act to raise the pH of the culture to over 9.0 causing a color change of the indicator dye, phenol red, from yellow to pink (M.B. BURBANK & al. [12]). Further, the selected strains were quantatively evaluated for the rate of urease production and calcite precipitation.

Metal toxicity is an important factor to be considered for remediation process as it is directly related to the survival and activity of bacteria in contaminated sites (Y.H. LI & al. [13]). So, the strains were tested for their heavy metals resistance. Among the selected strains, WD-2, WD-5, WD-9, BA-3 and BA-7 were the highest urease producers and showed

significant heavy metals resistance efficiency as well. The results presented in Fig. (1) show that strain WD-9 exhibited the highest urease activity (6.50 U/ml), followed by WD-5 (4.14 U/ml), BA-3 (3.36 U/ml), BA-7 (2.20 U/ml) and WD-2 (1.98 U/ml). In addition, the highest calcite production was recorded for WD-9 (10.0 mg/ml), followed by BA-3 (9.32 mg/ml), WD-5 (8.22 mg/ml), BA-7 (7.40 mg/ml), and WD-2 (5.11 mg/ml).

Strain WD-9 was selected as the most effective isolate for urease activity and calcite precipitation. Furthermore, it showed the highest metal resistance against all metal tested (Zn2+, Cd2+, Pb2+ and Fe2+) compared to other strains. Strain WD-9 was identified using 16S rRNA gene sequence analysis. The sequence alignment for the comparison of 1,500 bp by BLASTN software showed a high homology of 98% to Micrococcus sp. NCTC -1716.

Type of bacteria, substrate concentration, pH, temperature and salinity are among the factors that control the ureolytic reaction rate (N. DHAMI & al. [14]). The urease production profile of Micrococcus sp. was recorded over 120 h. As shown in Fig. 2, maximum growth (9 log CFU/ ml) and urease activity (6.60 U/ml) were recorded at 72 h, afterwards a gentle reduction was observed, probably due to reduction of urea concentration in the medium. In the beginning of the incubation time, pH of the media was 7.0, and it raised to its maximum level (9.60) at 96 h. Shifting pH toward alkalinity correlated with increasing the density of calcite precipitate, reached its highest level (10.80 mg/ml) at 120 h. Under alkaline conditions, there is a considerable increase in carbonate production which binds calcium ions resulting in the deposition of calcite. This mechanism plays a vital role in biomineralization process (C.X. QIAN & al. [15]).

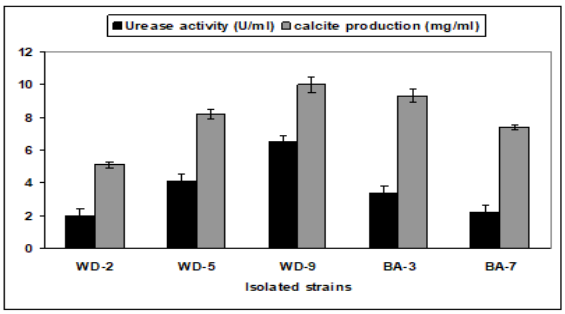

Figure 1. Urease activity and calcite production by isolated bacterial strains. Bars represent standard deviations.

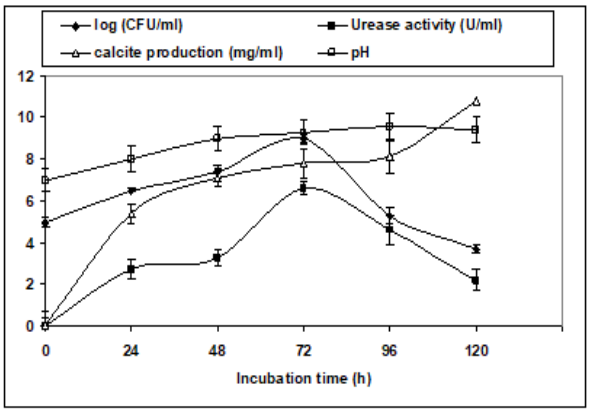

Figure 2. Time course of growth, urease activity, calcite production and final pH of Micrococcus sp. cultivated in urease broth medium. Values are averages from three independent experiments. Bars represent standard deviations.

Effect of heavy metals on bacterial growth

From the presented results, Micrococcus sp. possess high ureolytic and calcite deposition efficiency, be non-pathogenic and an alkalophilic strain that could grow under harsh conditions as well. This strain can be regarded as a possible candidate for bioremediation processes.

However, heavy metal toxicity affects the microbial growth and efficiency of MICP. Therefore, it is a prerequisite to select isolates exhibit ureolytic capability and heavy metal tolerance to upgrade the efficiency of bioremediation process (C.H. KANG & al. [16]).

Results illustrated in Fig. 3 showed that cadmium exhibited the highest toxicity on Micrococcus sp. with a minimum inhibitory concentration of 2 mM, whilst zinc and iron were similar, with an MIC of 3.5 mM. Resistance of the strain to lead was the highest, with growth unhindered below 5 mM. As a result, MIC values of Micrococcus sp. were superior to that reported for other strains. It is worth noting that survival of the strain at high metal concentrations cultivated in urea-amended medium suggested that the metal removal capacity offers a defense mechanism against metal toxicity.

Results showed that the least toxicity of lead to the cells. Lead has been recognized as one of the most hazardous heavy metals among environmental

pollutants. Electric battery manufacturing, mining activities as well as products including lead cause irregular supply of lead to the environment (D. HUANG & al. [17]).

Metal bioprecipitation

Biomineralization is defined as the extracellular production of minerals by a microorganism which has various potential applications in bioengineering field (L.C. VERONICA & al. [18]). Sequestration of inorganic pollutants, including heavy metals, is one of these applications that play an essential role in bioremediation process (S.H. RAUT & al. [19]). Microorganisms have to be enzymatically active to interact with the pollutants and convert them into harmless products. Calcite deposition capacity of urolytic bacteria results in mineralization of soluble heavy metal ions and their ultimate conversion to carbonates. Hence, production of calcium carbonate as a stable mineral phase displays permanent sequestration of contamination (A. LOPEZ-MORENO & al. [20]).

The ability of Micrococcus sp. to remove heavy metals via precipitation process catalyzed by urease hydrolysis is investigated. Upon cultivation of Micrococcus sp. in urease broth medium supplemented with ½ MIC of each heavy metal, it was found that Pb2+ recorded the highest removal rate (97.20%) while the lowest removal efficiency was recorded for Cd2+ (60.66 %) after 48 h (Fig.4). These results indicate that biosequestration process is superior to biosorption one which achieved only 36.07 % removal of lead by Micrococcus luteus DE2008 (Z.M. PUYEN & al. [21]).

Results presented in fig (4) showed that the highest precipitated mass was recorded for Pb 2+ (18.00 mg/ml), followed by Fe2+ (16.20 mg/ml), Zn2+ (14.98 mg/ml) and Cd2+ (11.73 mg/ml).

These metals can substitute for calcium as they are similar to it in atomic radius and form comparable metal carbonate structures (H.H. TENG & L. ZHAO [22]). However, on existing surfaces of calcite, rapid sorption of metal can form an extra outer layer, preventing further sorption and limiting dissolution of the mineral (Y. DU & al. [23]).

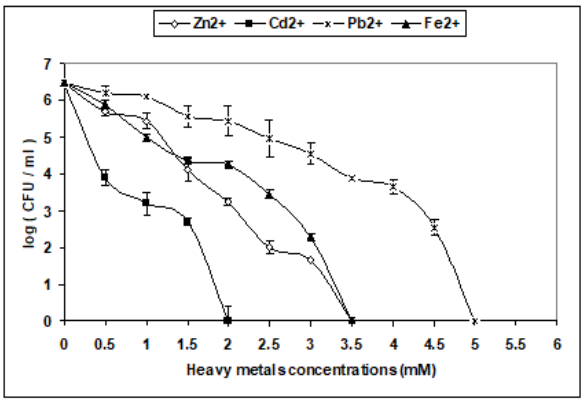

Figure 3. Metal toxicity experiments of zinc chloride, cadmium sulphate, lead nitrate, and iron sulphate on Micrococcus sp. cultivated in nutrient broth media supplemented with different heavy metal concentrations. Values are averages from three independent experiments. Bars represent standard deviations.

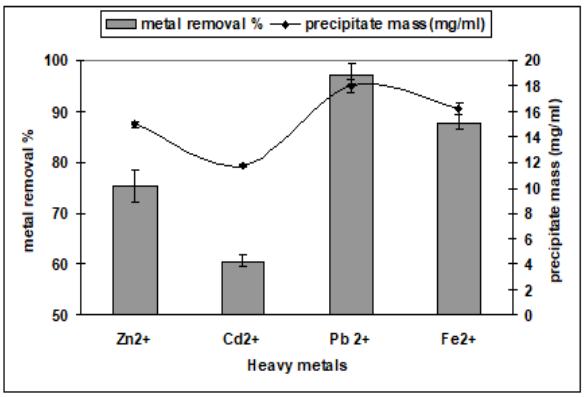

Figure 4. The removal rates and precipitated mass of individual heavy metals by Micrococcus sp. after 48 h of incubation. Values are averages from three independent experiments. Bars represent standard deviations.

SEM and XRD analyses

Many factors affecting the morphology and structure of metal carbonates precipitated during bacterial ureolysis such as bacterial strain, rate of urea hydrolysis, type of metal and activities of other enzymes such as carbonic anhydrase (N.K. DHAMI & al. [24]). In the present study, the biomineralization products as well as calcium carbonate crystals precipitated by Micrococcus sp. were visualized by SEM. Figure 5(A-E) showed that heavy metal crystals exhibited different morphological form, spherical, cuboid, rhombohedral, rod or irregular shape and their size is in the range of 10–50 µm.

To further confirm the role of MICP in heavy metals bioremediation process, mineral constituents of the precipitated samples were analyzed by X-ray diffraction (XRD) analysis. Fig 6A confirmed that the white precipitate produced by Micrococcus sp. is calcite. XRD peaks of Fig. 6 (B-E) showed that the metals are co-precipitated with CaCO3 in bioremediation experiment as ZnCO3, CdCO3, PbCO3 and FeCO3, respectively which showed evidence of transformations of metals into geochemically stable calcite species. Furthermore, this analysis indicated the presence of calcite crystals as principally mineral of all the carbonates precipitated.

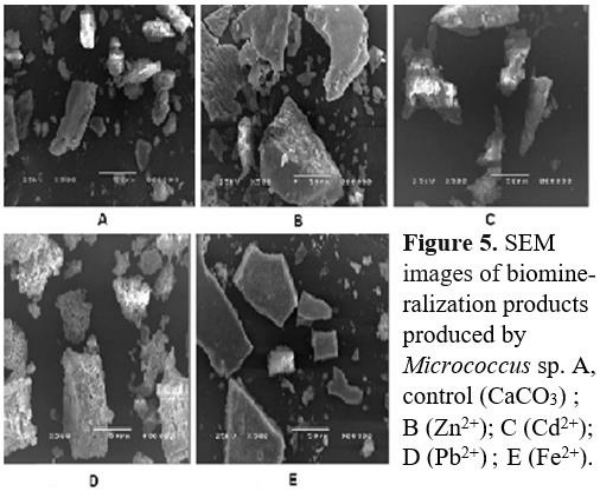

Figure 5. SEM images of biomineralization products produced by *Micrococcus* sp. A, control ($CaCO_3$) ; B (Zn^{2+}); C (Cd^{2+}); D (Pb^{2+}) ; E (Fe^{2+}).

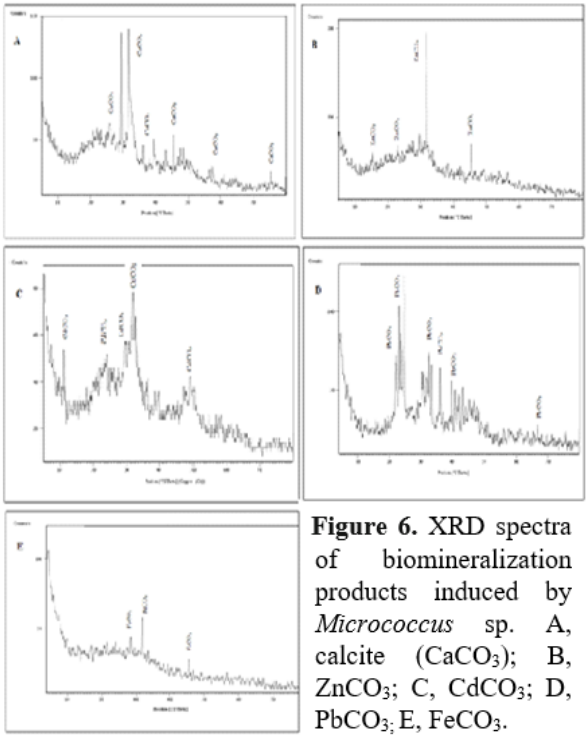

Figure 6. XRD spectra of biomineralization products induced by *Micrococcus* sp. A, calcite ($CaCO_3$); B, $ZnCO_3$; C, $CdCO_3$; D, $PbCO_3$; E, $FeCO_3$.

Conclusions

The results of the present study indicate that the biomineralization process based on ureolysis-driven calcium carbonate precipitation could be applied for soil bioremediation in a variety of environments.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 154-167
doi: 10.25083/rbl/24.1/154.167

Received for publication: April 24, 2017
Accepted: July 4, 2017

Original paper

In vitro Allelopathy at Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni

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Abstract

So far, the allelopathy is relatively little studied for in vitro cultures. The present study aims to highlight the allelopathic interactions of in vitro cultures between two plant species cultivated on one hand for their economic importance, and on the other for their beauty, respectively Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni; the research purpose was to determine the tolerability of one towards the other, in order to make in vitro floral arrangements, which are more and more popular and appreciated worldwide. Also, we plan to contribute in highlighting possible morphological and anatomical particularities of plants, which are in vitro co-culture, depending on the presence of growth regulators in the growing substrate. The analysis of this research revealed the existence of mutual allelopathy influence, synergistic from sequoia towards stevia, and antagonistic from stevia towards sequoia, the in vitro association being possible only until the age of 20 days of the in vitro cultures, up to which the allelopathy is mutually stimulating in the presence of 1 mg/l IBA. Keeping in vitro of both species in the same container of culture, over the age of 20 days, proved to be beneficial only for stevia plants, independently of the presence of growth stimulants.

Keywords : Sequoia sempervirens, Stevia rebaudiana, allelopathy, in vitro, plant biotechnology

To cite this article: BLIDAR CF, POP IF, FENESI B. In vitro Allelopathy at Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni. *Rom Biotechnol Lett*. 2019; 24(1): 154-167. DOI: 10.25083/rbl/24.1/154.167

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Introduction

The theory of influence exerted by some chemical compounds released into the environment by certain species on other species found in their vicinity was grounded since 1832 by the French botanist Augustin Pyramus De Condolle (J.L. HARPER [1]). After almost 100 years, the term "allelopathy" was launched in 1937 by the German botanist Hans Molisch (H. MOLISH [2]), who defines this process as including "any biochemical interaction, whether positive or negative, among plant of all levels of complexity" (Molish 1937 cited by J.V. LOVETT & A.H.C. HOULT [3]). This type of "socialization of superior plants" (R. KNAPP [4]) is achieved via biochemical compunds, such as phenolic acids, acetic acid, butyric acid, alkaloids, flovonoids, aliphatic compounds, hydroqiniones, terpenes, etc. (J.B. HARBONE, 1980 [5]; E. RICE [6]).

If millions of years ago, Sequoia trees genre were widespread, especially on southern continents, currently two species survived: Sequoia sempervirens (D. Don) Endl. and Sequoia giganteum (giant sequoia) (J.A. WOLFE [7]). Sequoia sempervirens (D. Don) Endl. (redwood) is a species belonging to gymnosperms from Pinophyceae class (Coniferophytes), order Pinales (Coniferales) widespread on the northern coast of California and Oregon coast (USA). It is the only hexaploid conifer (2n = 6x = 66) living today (W.L. LIBBY & al. [8]; M.R. AHUJA & D.B. NEALE [9]). Redwood is considered the highest tree in the world, reaching an average height of 60 meters and a trunk circumference of 3.6 - 4.8 meters. They are longevous trees, the oldest was reported as having 2,200 years; they reach maturity at 400-500 years old (H.A. FOWELLS [10]). Sequoia wood is used in timber industry, being mainly oriented to furnishings manufacture, construction elements (i.e. doors, windows, etc.), railway sleepers, structures of wooden houses (L. POP [11]).

This evergreen species, member of Taxadoiaceae family, unlike the others, has the ability to generate sprouts at the base of the stem that after cutting the trunk each sprout forms its own roots (L. POP [11]).

In vitro cultures from Sequoia sp. have been initiated since 1950, when explants taken from cambial tissue explants were in vitro cultivated, subsequently explants taken from various organs of plants were used (Y.P.S. BAJAJ [12]).

Boulay (M. BOULAY [13]) found that following repeated subcultivation, S. sempervirens inocula become more reactive, which is beneficial for micropropagation, by shortening the regeneration time of the plantlets. In order to obtain sprouts, positively reacting for in vitro cultures, it was recommended to handling parent trees that have been grown in vivo, by pruning, grafting, chemical treatment, etc. (A. FRANCLET [14]; A. FRANCLET [15]).

In vitro cultures initiated from stem fragments were widely studied by Boulay (M. BOULAY [13]) and Ball (E.A. BALL & al. [16]). By using in vitro multiplication and rooting, it was found that plants can be easier obtained starting from meristematic tissues than clones similar to those resulting from stem fragments. Small amounts of callus were also obtained at basal level of inocula, and the roots developed at the base of stem (M. BOULAY [13]).

Walker (N. WALKER & al. [17]) studied the initiation of in vitro culture originating from stem fragments, and they discovered that meristematic dome (showing attached the first two leaf primordia) develops well on the culture medium without growth regulators and supplemented with activated charcoal.

The animal-derived activated carbon added into the culture media in a concentration of 2% boosts the production of both hairy roots, multiple, and the generation of basal shoots at the level of in vitro plants of Sequoia sempervirens (D. Don) Endl. (L. POP [11]).

In their experiments on S. sempervirens, Pop and Cachiță (L. POP & C.D. CACHIȚĂ [18]) using culture media prepared with deuterium-depleted water (87.5 ppm. deuterium), proved to be more effective for increasing the stem of in vitro plantlets, reported to the usual double distilled water (150 ppm deuterium). On contrary, the complete replacement of the double distilled water in culture medium with deuterium depleted water (25 ppm deuterium), exerted an inhibitory effect on stem growth.

Among first studies on in vitro allelopathy tested S. sempervirens mini seedlings in relations with

protocorms of Cymbidium hybridum to determine the tolerability of a species towards the other was that published by Manci (A.M. MANCI [19]). The author found that the presence of cymbidium protocorms in the same jar stimulated the branching and stem growth in sequoia. Moreover they observed an increase in the number of leaflets compared to monoculture, a process that is more and more strong with age of in vitro culture. This partnership proved to be beneficial only up to 60 days age of the in vitro cultures, after which a process of slowing down of general development was observed.

Rogojan (P.A. ROGOJAN [20]) analyzed the reaction of mini cuttings of S. sempervirens in allelopathic conditions with another species: Drosera rotundifolia L. The authors reported that the in vitro association of these two plant species proved to be successful for long term cultivation.

Ward et al. (B.B.WARD & al. [21]) studied the effect of some monoterpenes produced by S. sempervirens on the in vitro development of Nitrosomonas europaea and identified adverse effects induced by four of them (i.e. limonene, sabinene, myrcene, and γ-terpinene compounds). Just one monoterpene (i.e. β-pinene) had a stimulatory effect on the in vitro development of N. europaea.

Stevia rebaudiana Bertoni is a herbaceous perennial plant and member of the Asteraceae family, Eupatorieae class, being known as Eupatorium rebaudianum. It can reach one meter height having many leaves of approx. 2-3 cm length. It is a short-day species, with an essential requirement of light for flowering, of about 13 hours. The aboriginal people in Brazil and Paraguay used stevia leaves for hundreds of years as a sweetener, but also as a remedy for various diseases (J. POL & al. [22]). Now, it is widely cultivated because of its sweet taste conferred by stevioside - a glycoside of the diterpene derivative steviol, especially contained in the leaves (A. DOSSIER [23]), a substance that is 300 times sweeter than sugar, which can successfully replace it (J.O. ATTEH & al. [24]). The plant is a good source of carbohydrates (61.93%), proteins (11.41% D.W.), crude fiber (15.52%) and minerals (i.e. K, Ca, Na, Mg, Cu, Mn, Fe, Zn); also essential amino acids were found in large quantities (A.E. ABOU-ARAB & al. [25]). This valuable natural sweetener is used worldwide as a substituting sugar, whose excessive consumption leads to multiple medical problems, such as obesity (J. POL & al. [22]; S. STRAUSS [26]).

Stevia seeds have a very low germination rate, and in vitro tissue culture is the fastest way for mass propagation of plants (M.B. AHMED & al. [27]).

Subsequently, Tawara (A.S. TAWARE & al. [28]) concluded that the MS62 culture medium containing 0.3 mg/l kinetin is most effective for in vitro cultivation of the stevia seedlings. The root induction was optimized on a MS62 culture medium supplemented with 0.2 mg/l IBA. The plantlets and the regenerated callus cultivated on a modified MS62 culture medium supplemented with 2,4-D and IBA proved to increase the production of stevioside. For these studies the authors used nodal explants that produced multiple shoots when cultivated on a modified MS62 culture medium supplemented with different combinations and concentrations of growth regulators, the supplemented variant with 3 mg / l BAP being the optimal (K.M. PANDA & al. [29]).

Badawi and collaborators (A.M. BADAWI & al. [30]) conducted a study that had as purpose the use of steviosides, the natural sweetener extracted from S. rebaudiana plants, as a substitute for sugar in dairy product for people suffering from diabetes.

Taware (A.S. TAWARE & al. [31]) studied the influence of various extracts of callus and seedlings of S. rebaudiana on seed germination of some agricultural crops, such as Triticum aestivum, Sorghum vulgare, Arachis hypogea, Glycine max, Cajanus cajan and Cicer arietinum. The experiments were carried out in Petri dishes and sterilized for five days at an average temperature of 28 °C. Callus proved to contain some metabolites showing some biological activities such as a significant inhibition of seed germination of C. cajan and C. arietinum. These inhibitory effects were proved by the browning of the roots' peak and a certain inhibition of their growth.

Tadhani (M.B. TADHANI & R. SUBHASH [32]) proved the antimicrobial effect of the in vitro stevia

leaves cultivation on two microbes: B. subtilis, S. aureus. Already in vivo, stevia proved to have an inhibitory effect on the development of some bacteria and other microorganisms, thereby successfully using the disinfection of wounds and gum disease (A.S. TAWARE & al. [27]).

Materials and Methods

Plant material. The used inocula consisted in mini seedlings of Sequoia sempervirens (D. Don) Endl. and Stevia rebaudiana Bertoni, with a size of approx. 1 cm length, taken from vitroplants, having attached small leaves at the level of the nodes, shortened by half, from the Plant Biotechnology Laboratory of the University of Oradea.

Growing and inoculation conditions. The mini seedlings were multiplied on a Murashige-Skoog (1962) (MS62) (T. MURASHIGE & F. SKOOG [33]) modified medium, supplemented with 7 g/l agar, 3-indoleacetic acid (IAA) and kinetin (K) and, the pH was adjusted to 5.7 before autoclaving. The medium was distributed in heat-resistant vials, with a height of 7 cm and 2.5 cm inner diameter. The medium sterilization was performed by autoclaving at 121 °C for 20 minutes.

Each vial was inoculated with one single redwood mini seedling, in case of the **VxSs**, or stevia to **VxSr** variants, and in the case of the **VxSsS**r variants the in vitro cultures contained both species in the same containers, one single inoculum of each plant.

To find the influence of the growth regulators on the associative vitro cultures, 3 variants of culture medium supplemented with growth regulators and one control, without regulators were organized as follows:

- **V0X** - basal medium (MB) - MS62 without growth regulators (control);
- **V1X** - MB-MS62 + 1 mg / l indole-3-butyric acid (IBA) (4.92 μM);
- **V2X** - MB-MS62+ 1 mg / l Kinetin (K) (4.64 μM);
- **V3X** - MB-MS62+ 1 mg / l IBA + 1 mg / l K;

where "**X**" represents the type of crop (monoculture sequoia - **Ss**, or stevia - Sr, or bi culture with both species - **SsSr**) (Table 1).

Table 1. Experimental variants used *in vitro* experiments of allelopathy between *S. sempervirens* and *S. rebaudiana*

The content of media in growth regulators	**Type *in vitro* culture**		
	V_x**Ss** (monoculture of *S. sempervirens*)	V_x**SsSr** (bi culture of *S. sempervirens* with *S. rebaudiana*)	V_x**Sr** (monoculture of *S. rebaudiana*)
V_0X (MB-MS without growth regulators)	**V_0Ss**	**V_0SsSr**	**V_0Sr**
V_1X (MB-MS + 1 mg/l IBA)	**V_1Ss**	**V_1SsSr**	**V_1Sr**
V_2X (MB-MS + 1 mg/l K)	**V_2Ss**	**V_2SsSr**	**V_2Sr**
V_3X (MB-MS + 1 mg/l IBA + 1 mg/l K)	**V_3Ss**	**V_3SsSr**	**V_3Sr**

The in vitro cultivation of inocula was conducted in growing room, under a lighting with fluorescent white day-light (6500K), with light intensity of 20 μM m-2s-1 PAR, under photoperiodical which corresponded to 16 h day light/ 24 hours, a temperature ranging from 22 °C to 24 °C.

Measurement of growth. After 20, 40 and 60 days of inoculation, observations following the monitoring of morphological parameters were: number of roots, root length, number of stems, length of stems and number of leaflets. Two gravimetric parameters were compared: fresh weight and dry weight (g).

We used two experimental variants as control, one for each plant species: VxSs – for the vitroplants of Sequoia sempervirens (D. Don) Endl., respectively VxSr variant - for the vitroplantlets of Stevia rebaudiana Bertoni from the sample variant (VxSsSr).

In total 12 experimental variants were developed and each was used in 60 vials.

Statistical analyses. For each of biometric parameter, at every experimental date, the values recorded in case of monocultures were considered as reference (100%) for the corresponding parameters in vitro plants belonging to the same species of co-culture. All statistical analyses were made using Microsoft Excel; the values are significantly different at $p<0.05$ according to t test. The experiment was repeated three times under the same conditions.

Results and Discussions

Biometric measurements and morphologic aspects at 20 days.

After the biometrizations performed at 20 days of in vitro cultures, we noticed that the seedlings of S. sempervirens exerted a stimulating allelopathic effect on S. rebaudiana. However, it was noticed an inhibitory activity exerted by the stevia on the sequoia seedlings development.

In case of culture medium without growth regulators, 93% of the S. sempervirens plantlets under co-culture had a weaker growth, showing obvious signs of senescence, consisting in a general mustard colour, with stem buds underrepresented or even absent, compared with V0Ss variant (Table 2, Figure 1).

Table 2. Statistical processing of the data measured in the *in vitro* seedlings of ***S. sempervirens*** cultivated in monoculture (V_xSs) and in co-culture with *S. rebaudiana* (V_xSsSr) at **20 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSs (control) (monoculture of *S. sempervirens*)		**V_xSsSr** (values for *S. sempervirens* found in co-culture with *S. rebaudiana*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.2 ± 0.71	0.50	1.0 ± 0.56	0.31	-0.2	-16.6	ns
	Strains length (mm)	8.3 ± 3.19	10.23	5.0 ± 2.13	4.55	-3.3	-39.7	***
	Leaf no.	6.6 ± 2.43	5.93	2.0 ± 0.99	0.99	-4.6	-69.6	***
	Fresh weight (mg)	23.5 ± n/a	n/a	10.6 ± n/a	n/a	-12,5	-54.8	n/a
	Dry weight (mg)	2.4 ± n/a	n/a	1.4 ± n/a	n/a	-1	-41.6	n/a
V_1	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.7 ± 0.91	0.84	2 ± 0.88	0.79	0.3	15.0	ns
	Strains length (mm)	6 ± 1.52	2.31	5.5 ± 1.73	3.00	-0.5	-8.3	ns
	Leaf no.	2.5 ± 1.02	1.05	2.5 ± 1.08	1.17	0.0	0.0	ns
	Fresh weight (mg)	13.6 ± n/a	n/a	30.4 ± n/a	n/a	16.8	123.5	n/a
	Dry weight (mg)	3.8 ± n/a	n/a	4.6 ± n/a	n/a	0.8	21.0	n/a
V_2	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.8 ± 0.86	0.74	1.6 ± 0.89	0.80	-0.2	-11.1	***
	Strains length (mm)	12.6 ± 2.63	6.96	7.6 ± 2.22	4.94	-5.0	-39.6	***
	Leaf no.	10.0 ± 2.24	5.03	5.2 ± 1.63	2.66	-4.8	-48.0	***
	Fresh weight (mg)	66.5 ± n/a	n/a	63.6 ± n/a	n/a	-2.9	-4.51	n/a
	Dry weight (mg)	16.5 ± n/a	n/a	13.5 ± n/a	n/a	-3.0	-18.1	n/a
V_3	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.0 ± 1.14	1.30	1.5 ± 0.98	0.96	-0.5	25.0	*
	Strains length (mm)	9.7 ± 2.29	5.25	7.7 ± 2.38	5.70	-2.0	-20.6	***
	Leaf no.	8.0 ± 1.72	2.96	5.2 ± 1.71	2.95	-2.8	-35.0	***
	Fresh weight (mg)	84.4 ± n/a	n/a	55.4 ± n/a	n/a	-29	-34.3	n/a
	Dry weight (mg)	7.2 ± n/a	n/a	5.2 ± n/a	n/a	-2	-27.7	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

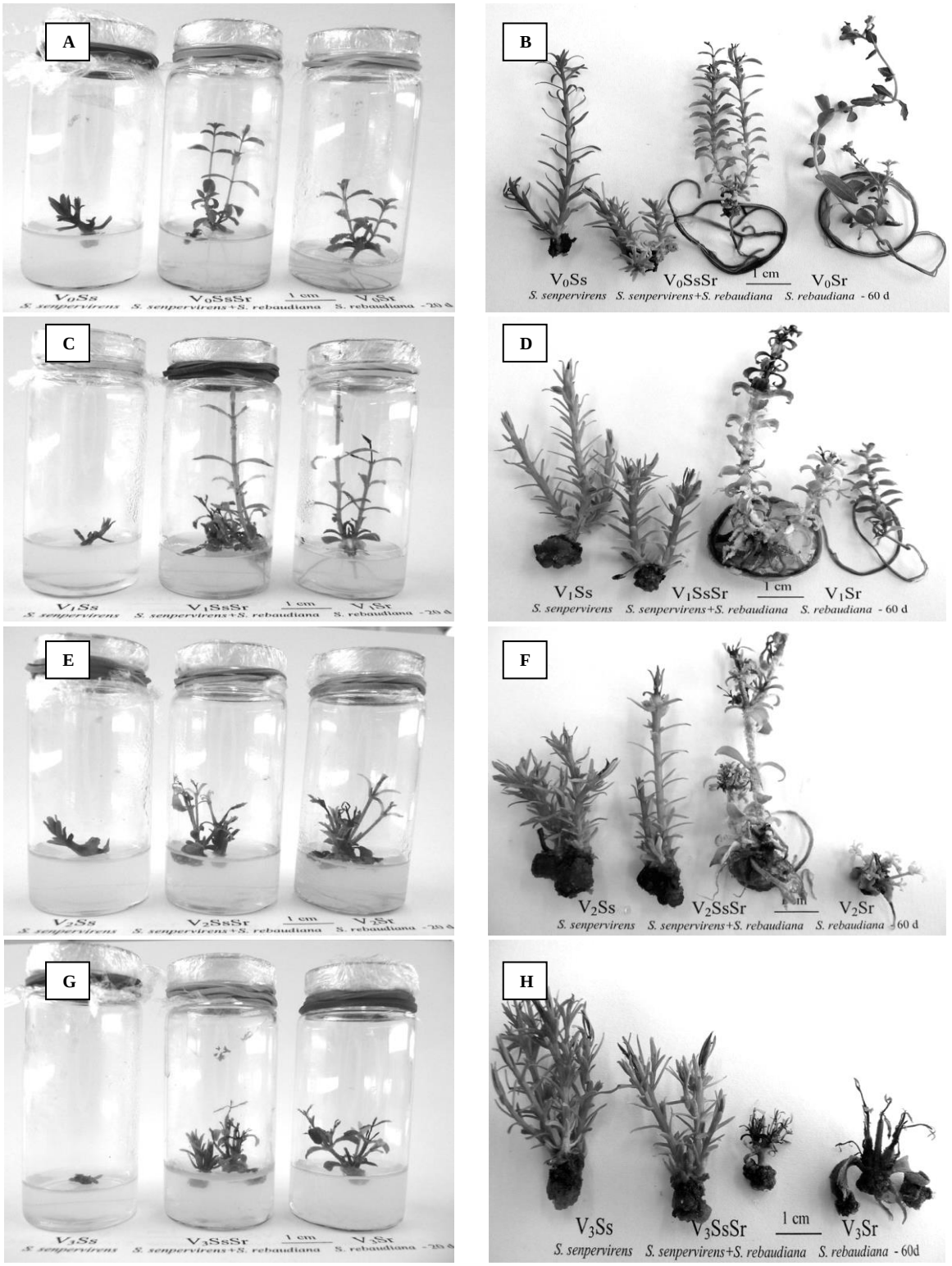

Fig. 1. Comparative morphological aspects of *in vitro* plantlets of *S. sempervirens* and *S. rebaudiana* under monoculture (VxSs respectively VxSr) and those of co-culture (VxSsSr) at 20 days - left column (**A, C, E, G**) and 60 days - right column (**B, D, F, H**) from the mounting experiments on culture media MS62 modified without added growth regulators (variants V_0X) with the addition of 1 mg / l IBA (var. V_1X) with the addition of 1 mg / l K (var. V_2X) or with the addition of 1 mg / l IBA and 1mg / l K (var. V_3X).

In case of co-cultivating plantlets of S. rebaudiana with those of S. sempervirens, a high positive reactivity was noticed compared to that recorded for the same plants separately as monoculture of stevia (V0Sr) (Table 3, Figure 1); the main morphological differences were a greater number of stems (8%) and an increased length (52,9%) and their degree of branching for the V0SsSr variant, the addition to control (V0Sr) (Table 3, Fig. 1A).

Table 3. Statistical processing of the data measured in the *in vitro* seedlings of ***S. rebaudiana*** cultivated in monoculture (V_xSr) and in co-culture with *S. sempervirens* (V_xSsSr) at **20 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSr (control) (monoculture of *S. rebaudiana*)		**V_xSsSr** (values for *S. rebaudiana* found in biculture with *S. sempervirens*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	1.6 ± 0.84	0.72	2.5 ± 1.29	1.68	0.9	56.2	***
	Roots length (mm)	24.6 ± 2.86	8.23	23.0 ± 3.23	10.49	-1.6	-6.5	*
	No. of strains	2.5 ± 0.87	0.75	2.7 ± 1.13	1.27	0.2	8	ns
	Strains length (mm)	15.5 ± 3.35	11.25	23.7 ± 2.74	7.54	8.2	52.9	***
	Leaf no.	10.2 ± 2.14	4.59	15.0 ± 2.53	6.42	4.8	47.0	***
	Fresh weight (mg)	59.1 ± n/a	n/a	47.9 ± n/a	n/a	-11.2	-18.9	n/a
	Dry weight (mg)	8.1 ± n/a	n/a	6.3 ± n/a	n/a	-1.8	-22.2	n/a
V_1	Roots no.	2.4 ± 0.87	0.77	3.0 ± 1.35	1.84	0.6	25.0	**
	Roots length (mm)	9.8 ± 2.01	4.05	8.2 ± 2.17	4.73	-1.6	-16.3	***
	No. of strains	2.6 ± 0.87	0.76	2.0 ± 1.01	1.03	-0.6	-23.0	**
	Strains length (mm)	24.4 ± 2.02	4.10	31.2 ± 3.37	11.35	6.8	27.8	***
	Leaf no.	19.4 ± 2.56	6.55	20.2 ± 2.48	6.17	0.8	4.1	ns
	Fresh weight (mg)	88.4 ± n/a	n/a	151.2 ± n/a	n/a	62.8	71.0	n/a
	Dry weight (mg)	9.8 ± n/a	n/a	20.8 ± n/a	n/a	11.0	112.2	n/a
V_2	Roots no.	1.0 ± 0.47	0.22	1.2 ± 0.46	0.21	0.2	20	**
	Roots length (mm)	6.6 ± 2.48	6.17	4.2 ± 1.08	1.17	-2.4	-36.3	***
	No. of strains	2.2 ± 0.76	0.58	1.8 ± 0.87	0.76	-0.4	-18.1	*
	Strains length (mm)	24.3 ± 1.84	3.41	20.8 ± 1.90	3.63	-3.5	-14.4	***
	Leaf no.	10.3 ± 2.39	5.72	9.2 ± 2.08	4.35	-1.1	-10.6	*
	Fresh weight (mg)	149.3 ± n/a	n/a	128.3 ± n/a	n/a	-21.0	-14.0	n/a
	Dry weight (mg)	16.9 ± n/a	n/a	29.3 ± n/a	n/a	12.4	73.3	n/a
V_3	Roots no.	0.0 ± n/a	n/a	0.0 ± n/a	n/a	0.0	0.0	n/a
	Roots length (mm)	0.0 ± n/a	n/a	0.0 ± n/a	n/a	0.0	0.0	n/a
	No. of strains	3.0 ± 0.91	0.84	2.5 ± 1.18	1.41	-0.5	-16.6	ns
	Strains length (mm)	16.0 ± 2.03	4.14	17.5 ± 2.20	4.87	1.5	9.3	**
	Leaf no.	10.8 ± 1.94	3.78	6.0 ± 1.67	2.81	-4.8	-44.4	***
	Fresh weight (mg)	189.0 ± n/a	n/a	200.6 ± n/a	n/a	11.6	6.13	n/a
	Dry weight (mg)	22.6 ± n/a	n/a	25.9 ± n/a	n/a	3	14.6	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

The number of leaflets of the plantlets reached the highest values of the V0SsSr variant compared to control (V0Sr), the percentage differences were of 47% and 4.8 leaves/vitroplant (Table 3, Fig. 1A). Also, the number of roots registered values of 56.2% higher for stevia cultivated on culture medium without growth regulators (V0SsSr) compared to V0Sr (control).

In case of V2SsSr variant 55% of the stevia plantlets presented signs of senescence, at the level of the leaves in a proportion of 20-30% (Table 2, Figure 1). In these cultures, the plantlets of S. rebaudiana had a higher rate of growth compared to those which were viable, without senescence (Table 3, Fig. 1).

Regarding the viability of in vitro plantlets of sequoia, positive results were obtained on the culture medium MB-MS supplemented with 1 mg/l IBA + 1 mg/l K (V3SsSr). The plantlets had an overall color of deep green (Fig. 1G). The largest accumulation of fresh weight and dry matter for the plants of sequoia were recorded in case of the V1SsSr experimental variant, the percentage differences compared to control being of 123.5% for fresh weight and 21% for dry weight (Table 2). For in vitro plantlets of stevia on these two parameters, higher values than the control variants, were registered in the experimental V1SsSr variant, the differences being of 71% and 112.2% (Table 3, Fig. 1C).

Biometric measurements and morphologic aspects at 40 days. In the second stage of experimental observations, an increased inhibitory allelopathic influence exerted by the stevia plantlets on sequoia plantlets was observed, both in terms of morphogenesis of plants and biomass accumulation. We could not find any parameter where the values recorded at the sequoia co-cultivated with stevia to be higer compared to those of monoculture as a control, whatever the composition of the used culture medium (Table 4).

Table 4. Statistical processing of the data measured in the *in vitro* seedlings of ***S. sempervirens*** cultivated in monoculture (V_xSs) and in co-culture with *S. rebaudiana* (V_xSsSr) at **40 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (**V_2**), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSs (control) (monoculture of *S. sempervirens*)		**V_xSsSr** (values for *S. sempervirens* found in biculture with *S. rebaudiana*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	1.5 ± 0.75	0.75	1.1 ± 0.56	0.32	-0.4	-26.6	*
	Strains length (mm)	17.2 ± 2.65	7.02	5.3 ± 1.89	3.58	-11.9	-69.1	***
	Leaf no.	23.5 ± 3.17	10.10	2.0 ± 0.99	0.99	-21.5	-91.4	***
	Fresh weight (mg)	123.4 ± n/a	n/a	18.8 ± n/a	n/a	-104.6	-84.7	n/a
	Dry weight (mg)	15.9 ± n/a	n/a	2.3 ± n/a	n/a	-13.6	-85.5	n/a
V_1	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.3 ± 1.19	1.42	2.1 ± 0.80	0.64	-0.2	-8.6	ns
	Strains length (mm)	17.6 ± 3.81	14.5	14.4 ± 3.25	10.5	-3.2	-18.8	***
	Leaf no.	29.5 ± 4.0	16.18	7.0 ± 1.84	3.41	-22.5	-76.27	***
	Fresh weight (mg)	98 ± n/a	n/a	78.4 ± n/a	n/a	-19.6	-20.0	n/a
	Dry weight (mg)	12.1 ± n/a	n/a	9.6 ± n/a	n/a	-2.5	-20.6	n/a
V_2	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.9 ± 1.15	1.32	1.7 ± 0.77	0.59	-1.2	-41.3	***
	Strains length (mm)	26.6 ± 3.14	9.87	18.4 ± 3.75	14.11	-8.2	-30.8	***
	Leaf no.	20.9 ± 1.94	3.77	9.7 ± 2.21	4.90	-11.2	-53.5	***
	Fresh weight (mg)	240.7 ± n/a	n/a	57.4 ± n/a	n/a	-183.3	-76.1	n/a
	Dry weight (mg)	23.2 ± n/a	n/a	17.1 ± n/a	n/a	-6,1	-26.2	n/a
V_3	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.3 ± 1.17	1.38	1.8 ± 0.85	0.73	-0.5	-21.7	ns
	Strains length (mm)	33.5 ± 2.19	4.80	12.8 ± 2.53	6.40	-20.7	-61.7	***
	Leaf no.	25.9 ± 2.58	6.68	15.5 ± 2.21	4.89	-10.7	-40.1	***
	Fresh weight (mg)	217.5 ± n/a	n/a	161.5 ± n/a	n/a	-56.0	-25.7	n/a
	Dry weight (mg)	21.3 ± n/a	n/a	13.1 ± n/a	n/a	-8.2	-38,4	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

Table 5. Statistical processing of the data measured in the *in vitro* seedlings of ***S. rebaudiana*** cultivated in monoculture (**V_xSr**) and in co-culture with *S. sempervirens* (V_xSsSr) at **40 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSr (control) (monoculture of *S. rebaudiana*)		**V_xSsSr** (values for *S. rebaudiana* found in biculture with *S. sempervirens*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	2.6 ± 0.89	0.80	5.2 ± 1.36	1.84	2.6	100	***
	Roots length (mm)	65.3 ± 2.47	6.10	56.8 ± 2.76	7.64	-8.5	-13.0	***
	No. of strains	3.5 ± 1.02	1.04	3.2 ± 1.16	1.34	-0.3	-8.5	ns
	Strains length (mm)	25.3 ± 1.53	2.36	34.3 ± 2.34	5.51	9.0	35.5	***
	Leaf no.	15.6 ± 2.49	6.24	24.4 ± 2.63	6.96	8.8	56,.4	***
	Fresh weight (mg)	82 ± n/a	n/a	198.9 ± n/a	n/a	116.9	142.5	n/a
	Dry weight (mg)	8.6 ± n/a	n/a	19.6 ± n/a	n/a	11.0	127.9	n/a
V_1	Roots no.	4.1 ± 1.62	2.64	3.2 ± 1.16	1.35	-0.9	-21.9	**
	Roots length (mm)	18.3 ± 2.4	5.7	15.5 ± 2.63	6.92	-2.8	-15.3	***
	No. of strains	2.7 ± 0.88	0.78	2.4 ± 1.03	1.06	-0.3	-11.1	ns
	Strains length (mm)	72.4 ± 2.75	7.58	52.2 ± 2.38	5.67	-20.2	-27.9	***
	Leaf no.	22.7 ± 1.95	3.80	21.1 ± 1.95	3.80	-1.6	-7.04	***
	Fresh weight (mg)	298.9 ± n/a	n/a	542.0 ± n/a	n/a	243.1	86.9	n/a
	Dry weight (mg)	35.7 ± n/a	n/a	47.6 ± n/a	n/a	11.9	33.3	n/a
V_2	Roots no.	4.4 ± 1.45	2.10	4.5 ± 1.50	2.25	0.1	2.2	ns
	Roots length (mm)	25.3 ± 2.36	5.57	20.9 ± 1.99	3.96	-4.4	-17.3	***
	No. of strains	2.3 ± 0.81	0.67	2.8 ± 0.98	0.96	0.5	21.7	**
	Strains length (mm)	34.2 ± 2.06	4.24	35.5 ± 2.72	7.44	1.3	3.8	*
	Leaf no.	11.1 ± 2.33	5.46	13.7 ± 1.77	3.15	2.6	23.4	***
	Fresh weight (mg)	532.5 ± n/a	n/a	594.6 ± n/a	n/a	62.1	11.6	n/a
	Dry weight (mg)	52.6 ± n/a	n/a	62.3 ± n/a	n/a	9.7	18.4	n/a
V_3	Roots no.	0.1 ± 0.44	0.20	0.9 ± 1.82	3.32	0.8	800	**
	Roots length (mm)	0.3 ± 1.19	1.43	0.2 ± 0.46	0.21	-0.1	-33.3	ns
	No. of strains	3.2 ± 1.28	1.66	3.4 ± 1.34	1.79	0.2	6.25	ns
	Strains length (mm)	26.6 ± 2.32	5.39	26.2 ± 2.1	4.41	-0.4	-1.5	ns
	Leaf no.	8.5 ± 1.5	2.25	6.4 ± 1.47	2.17	-2.1	-24.7	***
	Fresh weight (mg)	377.1 ± n/a	n/a	267.4 ± n/a	n/a	-109.7	-29.0	n/a
	Dry weight (mg)	46.7 ± n/a	n/a	34.9 ± n/a	n/a	-11.8	-25.2	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

In the case of the co-culture of in vitro plants of stevia, the most increased rootedness was recorded when cultivated on V3SsSr culture medium that was with 800% higher compared to control (V3Sr). The values of number and length of stems, was higher observed in stevia seedlings obtained in the case of V2SsSr, compared to control (Table 5). Regarding the number of leaflets, the highest value was found at the experimental V0SsSr variant, due to the stimulating effect exerted by the sequoia plants (Table 5).

Regarding gravimetric parameters, for sequoia cultivated with stevia, the recorded values were lower than those registered for control as monocultures, which might be due to the allelopathic inhibitors influence exerted by the biochemical compounds released by stevia plants on that of sequoia (Table 4).

In the case of the S. rebaudiana plants, the fresh and dry weights reached the highest differences values was recorded on the culture medium without growth regulators (V0SsSr): being of 142.5% of the fresh weight and 127.9% for dry weight (Table 5).

As a result, we can conclude that at 40 days of co-cultivating stevia and sequoia, the most effective variant was found to be the control without growth regulators, but only for S. rebaudiana.

In the case of the Sequoia plants, the allelopathic inhibitor influences induced by stevia led to recording some negative effect of the development compared to control, no matter the used experimental variant.

Biometric measurements and morphologic aspects at 60 days.

Throughout the experimental period (60 days), the presence of the S. rebaudiana in co-culture with S. sempervirens inhibited their growth and development, a process increasingly stronger with the aging of the in vitro cultures, being able to record during the measurement of the parameter value leaflets number value -63.8% on variant V0SsSr, and -59% for V3SsSr variant, in plants co-culture compared to controls (Table 6, Fig. 1B).

Table 6. Statistical processing of the data measured in the *in vitro* seedlings of ***S. sempervirens*** cultivated in monoculture (V_xSs) and in co-culture with *S. rebaudiana* (V_xSsSr) at **60 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSs (control) (monoculture of *S. sempervirens*)		**V_xSsSr** (values for *S. sempervirens* found in biculture with *S. rebaudiana*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	2.0 ± 0.78	0.61	2.0 ± 0.59	0.35	0.0	0.0	ns
	Strains length (mm)	30.3 ± 2.32	5.38	25.1 ± 1.60	2.58	-5.2	-17.1	***
	Leaf no.	38.7 ± 2.34	5.50	14.0 ± 1.85	3.43	-24.7	-63.8	***
	Fresh weight (mg)	187.5 ± n/a	n/a	116.0 ± n/a	n/a	-71.5	-38.1	n/a
	Dry weight (mg)	21.8 ± n/a	n/a	11.5 ± n/a	n/a	-10.3	-42.2	n/a
V_1	Roots no.	0.0 ± n/a	0.00	0 ± n/a	0.00	0	0	ns
	No. of strains	3.0 ± 1.41	2	2.8 ± 0.81	0.66	-0.2	-6.66	ns
	Strains length (mm)	35.5 ± 2.13	4.57	18.2 ± 2.61	6.83	-17.3	-48.7	***
	Leaf no.	38.1 ± 2.04	4.18	17.8 ± 2.05	4.22	-20.3	-53.2	***
	Fresh weight (mg)	293.7 ± n/a	n/a	167.4 ± n/a	n/a	-126.3	-43.0	n/a
	Dry weight (mg)	16.5 ± n/a	n/a	21.9 ± n/a	n/a	5.4	32.7	n/a
V_2	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	3.5 ± 1.26	1.59	2.2 ± 0.97	0.95	-1.3	-37.1	***
	Strains length (mm)	32.6 ± 2.00	4.02	21.9 ± 1.66	2.78	-10.5	-32.8	***
	Leaf no.	47.8 ± 1.76	3.11	23.2 ± 2.28	5.20	-24.6	-51.4	***
	Fresh weight (mg)	448.2 ± n/a	n/a	193.8 ± n/a	n/a	-254.4	-56.7	n/a
	Dry weight (mg)	48.7 ± n/a	n/a	20.7 ± n/a	n/a	-28	-57.4	n/a
V_3	Roots no.	0.0 ± n/a	0.00	0.0 ± n/a	0.00	0	0	ns
	No. of strains	4.3 ± 1.70	2.9	2.6 ± 1.00	1.01	-1.7	-39.5	***
	Strains length (mm)	35.8 ± 2.28	5.20	23.2 ± 2.67	7.16	-12.6	-35.1	***
	Leaf no.	68.3 ± 2.83	8.01	28.0 ± 2.67	7.14	-40.3	-59.0	***
	Fresh weight (mg)	440.9 ± n/a	n/a	339.7 ± n/a	n/a	-101.2	-22.9	n/a
	Dry weight (mg)	44.8 ± n/a	n/a	44.3 ± n/a	n/a	-0.5	-1.1	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

At 60 days of starting the experiments, the plantlets of stevia cultivated on V2SsSr variant were characterized by strong vitality marked by a broad caulogenesis, showing a large number of stems and high lengths.

The most intense caulogenesis in case of the stevia plant in coculture was recorded in the variant V2SsSr (medium MB-MS supplemented with 1 mg/l K). Regarding the number of roots, the highest value was found in the experimental variant V0SsSr variant (Table 7, Fig. 1F).

Regarding the parameters of fresh and dry weight, in case of the stevia plantlets, reaching the highest values at the in vitro plants cultivated on the culture

medium supplemented with 1 mg/l K (V2SsSr), increases recorded compared to group control being seven times higher for fresh biomass and three times higher in case of dry biomass (Table7, Fig. 1F).

Table 7. Statistical processing of the data measured in the *in vitro* seedlings of ***S. rebaudiana*** cultivated in monoculture (V_xSr) and in co-culture with *S. sempervirens* (V_xSsSr) at **60 days**, on modified MS62 basic medium without growth regulators (V_0 culture media), with 1 mg/l IBA (V_1), with 1 mg/l K (V_2), or with 1 mg/l IBA and 1 mg/l K (V_3)

		V_xSr (control) (monoculture of *S. rebaudiana*)		**V_xSsSr** (values for *S. rebaudiana* found in biculture with *S. sempervirens*)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signi-ficance (*p*)**
V_0	Roots no.	3.1 ± 1.15	1.33	6.8 ± 1.10	1.21	3.7	119.3	***
	Roots length (mm)	71.7 ± 2.38	5.68	66.6 ± 2.30	5.31	-5.1	-7.11	***
	No. of strains	3.9 ± 0.99	0.99	3.5 ± 1.02	1.04	-0.4	-10.2	ns
	Strains length (mm)	34.4 ± 2.86	8.18	42.2 ± 3.06	9.42	7.8	22.6	***
	Leaf no.	21.4 ± 2.16	4.68	35.2 ± 2.03	4.13	13.8	64.4	***
	Fresh weight (mg)	158.2 ± n/a	n/a	311.0 ± n/a	n/a	152.8	96.5	n/a
	Dry weight (mg)	17.1 ± n/a	n/a	24.0 ± n/a	n/a	6.9	40.3	n/a
V_1	Roots no.	22.4 ± 2.21	4.92	5.2 ± 1.44	2.10	-17.2	-76.7	***
	Roots length (mm)	54.5 ± 2.30	5.29	47.8 ± 2.41	5.85	-6.7	-12.2	***
	No. of strains	3.6 ± 1.06	1.13	3.1 ± 0.98	0.96	-0.5	-13.8	ns
	Strains length (mm)	73.3 ± 3.20	10.30	88.6 ± 2.94	8.67	15.3	20.8	***
	Leaf no.	29.3 ± 2.12	4.55	32.4 ± 2.45	6.03	3.1	10.5	***
	Fresh weight (mg)	314.1 ± n/a	n/a	692.4 ± n/a	n/a	378.3	120.4	n/a
	Dry weight (mg)	39.5 ± n/a	n/a	55.3 ± n/a	n/a	15.8	40.0	n/a
V_2	Roots no.	5.5 ± 1.39	1.95	5.8 ± 1.18	1.41	0.3	5.45	ns
	Roots length (mm)	27.6 ± 2.31	5.35	30.3 ± 2.49	6.23	2.7	9.78	***
	No. of strains	2.5 ± 0.88	0.77	3.8 ± 0.81	0.67	1.3	52.0	***
	Strains length (mm)	37.2 ± 2.17	4.71	46.8 ± 2.01	4.05	9.6	25.8	***
	Leaf no.	11.5 ± 2.42	5.88	14.6 ± 1.54	2.39	3.1	26.9	***
	Fresh weight (mg)	43.4 ± n/a	n/a	357.2 ± n/a	n/a	313.8	723.0	n/a
	Dry weight (mg)	9.6 ± n/a	n/a	39.7 ± n/a	n/a	30.1	313.5	n/a
V_3	Roots no.	-	-	-	-	-	-	-
	Roots length (mm)	-	-	-	-	-	-	-
	No. of strains	-	-	-	-	-	-	-
	Strains length (mm)	-	-	-	-	-	-	-
	Leaf no.	-	-	-	-	-	-	-
	Fresh weight (mg)	-	-	-	-	-	-	-
	Dry weight (mg)	-	-	-	-	-	-	-

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

In conclusion, at 60 days from the experiments, in the case of S. sempervirens and S. rebaudiana, was recorded a mutual allelopathy influence, synergistic in part sequoia on stevia, and antagonistic in part stevia on sequoia.

Keeping into in vitro culture under allelopathy conditions both plant species, over 20 days, it is effective only in the case of stevia, which is positively influenced by redwoods plantlets, while stevia plants have had a negative allelopathic effect on the redwoods, wherever the used culture medium.

In similar studies, the allelopathic effects exerted by sequoia plants on those of cultivated Drosera rotundifolia L. were reported, which were grown in the same container on the MS62 medium without growth regulators. The negative effects of Sequoia plants exerted in the first 60 days of the study generated the inhibition, but not by stopping the increase of the number of roots, leaflets and rosettes belonging to Drosera rotundifolia L. species.

Subsequently, between 60 to 90 days to have a reversal effects exerted by the Sequoia seedlings,

respectively to evolve from antagonistic to synergistic (P.A. ROGOJAN [20]). A reversible allelopathic effect was recorded by Blidar (C.F. BLIDAR & al. [34]) at in vitro cocultures of Cymbidium hybridum protocorm like bodies (PLB) and Drosera rotundifolia L., where up to 30 days the orchid exerted a negative effect on the drosera plantlets, and then an synergistic one.

It was also reported negative in vitro allelopathic effects of stevia plantlets on the seed germination of C. cajan and C. arietinum (A.S. TAWARE & al. [31]), as well as on some bacteria development (A.S. TAWARE & al. [28]).

Manci (A.M. MANCI [19]) analyzed the reactivity of the S. sempervirens mini seedlings under allelopathic conditions with protocorms of C. hybridum. In this case, the redwood plants stimulated the growth and development of protocorms, the combination of both species, being beneficial up to the age of 60 days of in vitro cultures for orchids.

The results obtained in our experiments confirmed the in vitro allelopathic properties of the two species, S. sempervirens respectively S. rebaudiana.

After 60 days, choosing a modified MS62 culture medium supplemented with kinetin (K) at a concentration of 1 mg/l (V2SsSr) was the first choice, but only for the plantlets of S. rebaudiana. Taware (A.S. TAWARE & al. [28]) also showed the effectiveness of the use of 0.3 mg/l kinetin in the culture medium in which the explants of stevia are transferred.

Conclusions

The present study showed that the identified allelopathic synergistic influences, allow the in vitro association of S. sempervirens with S. rebaudiana, but only until the age of 20 days of in vitro culture, the positive growth and the development being recorded, but it was relatively insufficient for possible methods of sub-cultivation or efficient acclimation.

However, the use of a modified MS62 culture medium supplemented with 1 mg/l IBA, was found to be most favourable for the proliferation and growth of the plantlets for both species.

Economically speaking, namely marketing the in vitro floral arrangements containing both Sequoia sempervirens and Stevia rebaudiana species is not recommend due to the inhibitory allelopathic influences exerted by the stevia plantlets over the sequoia. However, the phenomenon of inhibition induced by the stevia plantlets, may represent a possibility of in vitro conservation of this, thus adding the conservation variants in the literature (A. PĂUNESCU, [35]). This requires studies to revive the culture maintained for a long term under inhibition (A. MANOLE & C. BANCIU [36]) and genetic, too (A. KARP & al. [37]).

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 168-175
doi: 10.25083/rbl/24.1/168.175

Received for publication: January 15, 2017
Accepted: September 20, 2018

Original paper

Anticancer effects of curcumin in luminal B and HER2 breast cancer cell line models

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Abstract

In this study we aimed to test the activity of curcumin, a polyphenolic compound with known anti-cancer properties, in two breast cancer cell lines with different phenotypes: MCF-7 cell line as luminal B model and SK-BR-3 as HER2-amplified cancer cell model. As assayed by flow cytometric measurements 72 h curcumin treatment blocked the cell cycle progression of MCF-7 cells in G2/M phase, while for SK-BR-3 cells the cell cycle evolution was blocked less efficiently in the S phase. Curcumin reduced the clonogenic survival of both cell lines with EC50 values in the low micromolar range for the 48 h treatment period, SK-BR-3 being slightly more affected. In MCF-7 cells, curcumin reduced the expression level of ERK and its phosphorylation (Y204). Short term (0 to 120 min) treatment with curcumin increased the ROS production in both cell lines, but the effect was more pronounced in MCF-7 cells. In both cell lines, 48 h of 25 μM curcumin treatment induced mitochondrial membrane depolarization, with similar EC50 values. In conclusion, curcumin had anticancer effects in both cancer cell lines phenotypes, indicating its potential therapeutic usefulness against different types of breast cancer.

Keywords : curcumin, breast cancer cells, HER2, luminal B, clonogenic potential, mitochondrial membrane potential, reactive oxygen species

To cite this article: FILIPPI A, MARU N, CHIFIRIUC MC, GRIGORE R, GANEA C, MOCANU MM. Anticancer effects of curcumin in luminal B and HER2 breast cancer cell line models. *Rom Biotechnol Lett*. 2019; 24(1): 168-175. DOI: 10.25083/rbl/24.1/168.175

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Introduction

Breast cancer is the most frequent malignancy in women world-wide, accounting for 23% of the total new cancer cases and is responsible of 14% of cancer deaths in this subpopulation (Jemal & al. [1]). Breast cancers as a whole have a relative low mortality rate (Ferlay & al. [2]) but they are also a highly heterogeneous group of diseases, with particular treatments and different prognosis. According to their extracellular receptors they can be divided in luminal A (ER+ and/or PR+, HER2- and Ki-67 low), luminal B (ER+ and/or PR+, HER2+/- and Ki-67 high), HER2-amplified and triple-negative (Yersal & al. [3]). The most frequent breast cancer subtype, luminal A, accounting for 50-60% of all breast cancers, has a good prognosis and its treatment is mainly hormonal, the triple-negative phenotype accounts for about 17% of cases and has an intermediary prognosis, the worst prognosis being that of luminal B, and HER2-positive phenotypes, each accounting for about 15-20% of breast cancer cases (Cheang & al. [4, Yersal & Barutca & al. [3]).

The adjuvant therapy of luminal B cancer includes tamoxifen and endocrine treatment (Coates & al. [5]) while the treatment of HER2-positive breast cancers is centered on HER2 targeting, either by humanized monoclonal antibodies such as trastuzumab and pertuzumab, or by inhibiting the tyrosine kinase activity of the receptor by lapatinib, in combination with chemotherapy with paclitaxel or docetaxel. However, resistance to HER2 therapy often occurs and the treatment regimens using more therapeutic agents to overcome this resistance have severe side effects (Kumler & al. [6]).

Curcumin is a polyphenol from Curcuma longa studied for a plethora of effects including anti-infective (Mazumder & al. [7]), anti-inflammatory (Zhang & al. [8]), hepato- and cardiovascular protector (Fujise & al. [9, Kapakos & al. [10]), anti-trombotic (Kim & al. [11]) and anticancer. Among anticancer effects of curcumin, apoptosis inducement through the increased expression of death receptors 4 and 5 (Jung & al. [12, Yang & al. [13]), and decreased expression of a large number of kinases, enzymes and anti-apoptotic proteins such as Bcl-2 were shown (Shanmugam & al. [14]). Also, curcumin was shown to decrease HER2 levels and phosphorylation in cancer cells overexpressing this protein (Hong & al. [15]) through ubiquitination and degradation by carboxyl terminus of Hsc70-interacting protein (Jung & al. [16]).

As the many anticancer effects of curcumin were shown before in different cancer models, in this study we compared the effect of curcumin on models for the more aggressive forms of breast cancer, luminal B and HER2 overexpressing, using as models (Prat & al. [17]) the MCF-7 cell line, and HER2-amplified SK-BR-3, respectively.

Materials and Methods

Cell cultures and treatments

Experiments were performed using mammary adenocarcinoma MCF-7 and SK-BR-3 cells from American Type Culture Collection. The culture medium was DMEM (Sigma) supplemented with 10% heat inactivated FBS (Sigma), 1% Penicillin/Streptomycin (Gibco), 2mM L-glutamine (Gibco) and, in the case of MCF-7, 10 µg/ml insulin. The cells were grown in a humidified incubator at 37°C, 5% CO2. Adherent cells were detached by trypsinization and cells were washed in PBS before reseeding or use in experiments. Curcumin (Sigma) was used from fresh 50 mM stock solutions prepared in DMSO (Sigma) in the day of the experiment. In all control samples, the same volume of DMSO as in the treated samples was added.

Clonogenic assay

MCF-7 and SK-BR-3 cells (1000 and 2000 cells/well, respectively) were seeded in 6 well plates and let to adhere. The cells were treated for 48 h with 0 (control), 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µM curcumin in complete medium and incubated at 37 °C for 7 days, followed by 5 min fixation in 3.7% formaldehyde and 15 min staining with 0.5% crystal violet. The excess dye was washed with tap water. After drying, macroscopic pictures were taken. Colonies were counted automatically using the ImageJ v.1.48 software, EC50 values were calculated from inhibition curves fitted in GraphPad Prism v.5 using the following formula 1:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogEC50 - X) \times HillSlope}} \quad (1)$$

where X is logarithm with base 10 of the concentration of EGCG, LogEC50 is the logarithm with base 10 of the half-maximal effective concentration, Y is number of colonies normalized to control, “top” and “bottom” are plateau values.

Cell cycle evaluation

A number of 2 x 105 MCF-7 and SK-BR-3 cells were seeded in 6 well plates and let to adhere for 24 h. After that, the cells were synchronized in G0/G1 phase of the cell cycle by 24 h incubation in serum free media, then treated for 72 h with 0 (control), 5, 10, 25, 50, 100 μM curcumin, stained with propidium iodide in RNase staining buffer (BD Pharmingen) and measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emission: 620 ± 30 nm).

Intracellular staining of signaling molecules

Cells were plated in T75 flasks and let to adhere for 24 h and then treated with 0 (control) or 25 μM curcumin for 48 h. Trypsinized cells were aliquoted to 106 cells/sample, fixed in 3.7% paraformaldehyde (Sigma), washed in PBS, permeabilized with 0.2% Triton X-100 (PerkinElmer), washed and stained with 20 μl anti-ERK PE (Santa Cruz), 20 μg/ml monoclonal anti-pERK (E-4) FITC (Santa Cruz), antibodies for 1 h at 4 °C. The excess of the antibodies was removed during the final wash in PBS. The samples were measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emission: 525 ± 40 nm or excitation: 488 nm, emission: 575 ± 30 nm for FITC and PE, respectively).

Reactive oxygen species production assay

Cells were plated in 6 well plates at a concentration of 3-5x105 cells/well and let to adhere for 24 h and then treated with 25 μM curcumin for 0 (control), 10, 30, 60 and 120 min. Trypsinized cells were stained for 30 min with 5-(and-6)-carboxy-2′,7′-dichloro- dihydro-fluorescein diacetate (carboxy-H2DCFDA, Molecular Probes) and washed in PBS; the samples were measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emission: 525 ± 40 nm).

Mitochondrial membrane potential assay

The MCF-7 and SK-BR-3 cells were grown in 6 well plates, incubated for 72 h with 0 (control), 5, 10, 25, 50 or 100 μM curcumin, stained 15 minutes with 5 μg/ml JC-1 (Thermo Fisher) at 37°C, trypsinized, washed in PBS and measured with Gallios Beckman Coulter flow cytometer (excitation: 488 nm, emissiongreen: 525 ± 40 nm, emissionorange: 575 ± 30 nm). Curcumin fluorescence was subtracted from treated, non-stained samples. EC50 values were calculated from inhibition curves fitted in GraphPad Prism v.5.

Data analysis

Data are represented as mean ± standard error of the mean (sem). Significance was assayed using unpaired t test or one way ANOVA with Bonferroni post-test for multiple column analysis. A value of $p < 0.05$ was set as level of significance. Raw data were analyzed using ImageJ v.1.48 (NIH, USA) and Flowing Software 2.5.1 (University of Turku, Finland). For statistical analysis, Microsoft Excel 2010 (Microsoft Inc., USA) and Prism 5 (GraphPad Software Inc., USA) were used.

Results and Discussions

Curcumin inhibits the colony formation

In both MCF-7 and SK-BR-3 cell lines, 48 h curcumin treatment reduced the number of colonies in a dose dependent manner (Figure 1). For the more sensible to treatment cell line, SK-BR-3, curcumin concentrations of 25 μM and higher completely inhibited clonogenic growth, while in the MCF-7 cell line clonogenic growth was completely inhibited by concentrations higher than 50 μM. Thus, SK-BR-3 showed an EC50 value of 2.2 μM (best fit, 95% confidence intervals from 1.08 to 4.49 μM), and MCF-7 showed an EC50 value of 12.3 μM (best fit, 95% confidence intervals from 5.06 to 29.74 μM). The overall lower concentrations needed for colony growth inhibition than those needed for other anticancer activities of curcumin shown in this study are in agreement with previous results showing lower curcumin concentrations needed for the inhibition of clonogenic formation than for the inhibition of proliferation as assayed in an MTT test (Hong & al. [15]).

This might be explained by the fact that for a colony to reach macroscopic measurable sizes not only individual cells must survive but also they must proliferate. It was also shown before that in breast cancer cells oxidative stress hinders proliferation as NADPH is funneled from lipogenesis to ROS detoxification (Jerby & al. [18]). As we further show, curcumin blocks the evolution of the cell cycle and induces oxidative stress in both cell lines tested.

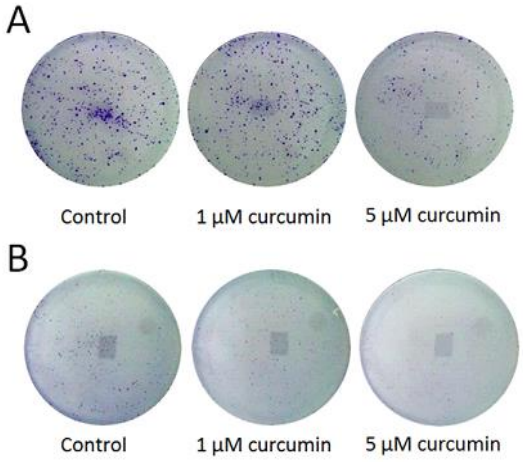

Figure 1. Curcumin reduces clonogenic potential of MCF-7- and SK-BR-3 cell lines. Representative images of curcumin treated MCF-7 (A) and SK-BR-3 (B) wells.

Curcumin affects cell cycle progression dependent on cell line

Curcumin blocked the cell cycle progression in different phases of the cell cycle depending of the cell line tested. Thus, in the MCF-7 cells, 25 µM curcumin treatment led to a reduction in G0/G1phase cells (from 70.1 ± 7 % to 48.3 ± 0.3 % of the viable cells), associated with an almost doubling in the number of cells in the G2/M phase (from 19.9 ± 4.9 % to 36.6 ± 3.7 %), as shown in Figures 2A and 2B. There is contradicting data on curcumin ability to block the cell cycle in luminal B cancer cells as some studies suggest that curcumin blocks MCF-7 cells in G2/M phase (Berrak & al. [19]) while others in G0 (Choudhuri & al. [20]), our data coming in support of the former theory.

In SK-BR-3 cell line, the reduction in G0/G1 cell numbers was observed starting with the 10 µM curcumin treated sample (55.3 ± 1.1 % compared to 66.4 ± 0.3 %, unpaired t-test, $p<0.05$) associated with an increase in the S phase number of cells (22.1 ± 2.8 from 15 ± 0.03). The reduction in G0/G1 phase was maintained in the 25 µM curcumin treated sample (62.4 ± 0.4 % compared to 66.4 ± 0.3 %, unpaired t-test, $p<0.05$), in correlation with the increase of the number of cells in S phase (19.7 ± 1 from 15 ± 0.03, unpaired t-test, $p<0.05$), as shown in Figures 2C and 2D.

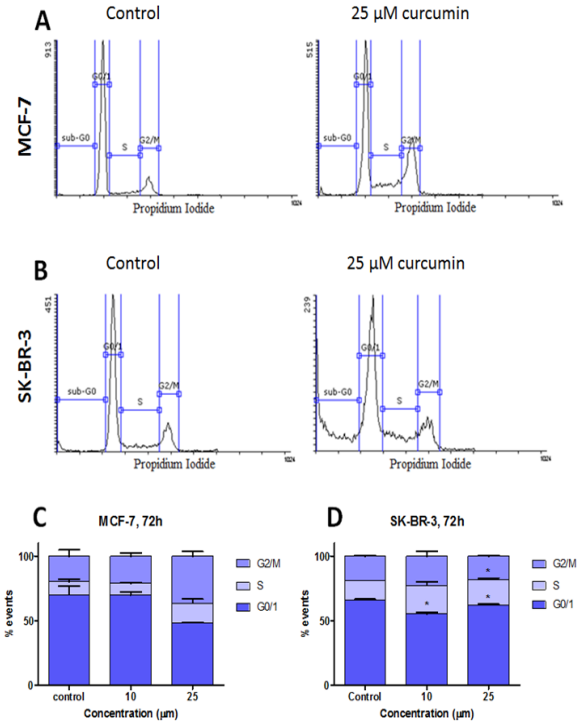

Figure 2. Curcumin blocks cell cycle evolution. Representative histograms for control and 25 µM curcumin treated MCF-7 (A) and SK-BR-3 (B) cells. (C) and (D) show barghraphs of percent cells in each cell cycle phase for the curcumin concentrations tested. *$p<0.05$.

Curcumin inhibits ERK expression and its phosphorylation at Y204 in cell line dependent manner.

Extracellular signal regulated kinase (ERK) activation occurs downstream to insulin receptor as well as HER2 signaling and controls transcription factors, leading to increased proliferation and survival. Thus, we have been interested to see if the curcumin induced growth inhibition might be associated with a reduction in ERK expression or phosphorylation. Our experiments showed that in MCF-7 cells 48 h treatment with 25 µM curcumin greatly affects ERK expression (a threefold reduction in mean fluorescent intensity ratio, MFIR, from 53.3 ± 3.5 to 17.8 ± 0.1, unpaired t-test, $p<0.01$, Figure 3A) and, consequently, a drop in the phosphorylated form of ERK (pERK) was also observed (a MFIR reduction from 25 ± 2.3 to 13.2 ± 1.1, unpaired t-test, $p<0.05$). In contrast to this marked effect, in the SK-BR-3 cells the curcumin treatment had no effect in reducing ERK expression (MFIR for ERK was 24.8 ± 2.5 in control and 25.3 ±

7.3 in the 25 μM curcumin treatment condition) or phosphorylation (MFIR for pERK was 20.66 ± 0.7 in control and 20.2 ± 6 in the treatment condition, Figure 3B). As ERK is over activated in many cancers and plays a role in cell survival and proliferation (Boulton & al. [21]) they are a promising therapeutic target with specific inhibitors being developed and patented (Samatar & al. [22]). A reduction in ERK expression in luminal B cancer cells by curcumin is likely to lead to a less aggressive form of disease.

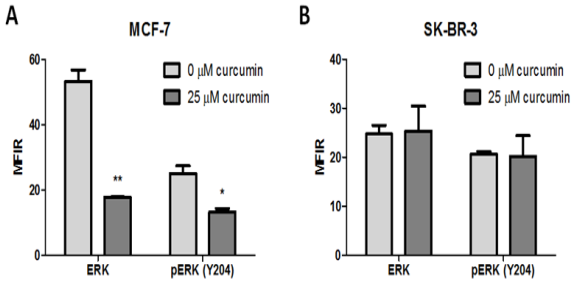

Figure 3. Curcumin can affect the expression of signaling molecules. Bargraphs showing ERK expression and tyrosine 204 phosphorylation reduction effect of curcumin in MCF-7 cell line (A) but no effect in the case of SK-BR-3 cell line (B) *p<0.05;**p<0.01.

Curcumin induces reactive oxygen species production

Next, we evaluated the ability of 25 μM curcumin to induce oxidative stress depending on the incubation time. Curcumin induced reactive oxygen species production in both cell lines in time dependent manner, MCF-7 cell line being more sensitive to this action (Figure 4).

Thus, in this cell line curcumin induced a maximum 2.46 ± 0.18 fold increase in ROS production at 2 h (one-way ANOVA, p<0.001). In the SK-BR-3 cell line, ROS production reached a plateau after 1 h incubation at about 1.5 fold ROS production (1.55 ± 0.06 (one-way ANOVA, p<0.001) for the 1 h incubation and 1.57 ± 0.6 (one-way ANOVA, p<0.001) for the 2 h incubation). Rapid ROS production is perhaps the most important anti-cancer action of curcumin, being the initial signal in different pathways of apoptosis, both caspase-dependent and caspase-independent (Thayyullathil & al. [23]).

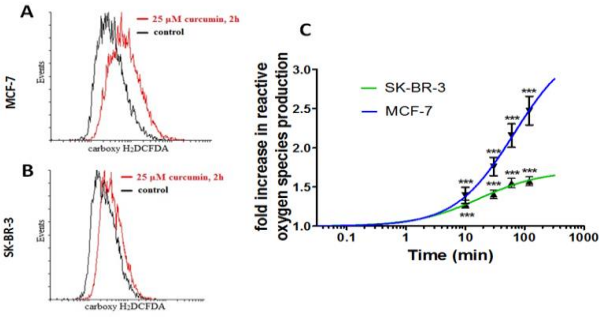

Figure 4. Curcumin induces reactive species formation dependent of the cell line. (A) and (B) show overlay histograms of 120 min vs. 0 min (control) 25 μM curcumin treated MCF-7 and SK-BR-3 cells. (C) The graph is showing treatment time dependence in the ROS production for the two cell lines.

Curcumin reduces the mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential collapse is observed in all dead cells no matter the mechanism of this death: necrosis or apoptosis. However, in the case of apoptosis ΔΨm is affected early and thus, ΔΨm collapse before morphological changes is considered a hallmark of apoptosis (Lugli & al. [24]). To assay the death inducing actions of curcumin we tested the ΔΨm after short (0-120 minutes) and long (48 h) treatment incubation periods. In the short term incubation, even if curcumin seemed to increase the percent of cells with depolarized mitochondrial membranes, the effect wasn't strong enough to reach statistical significance (Figure 5).

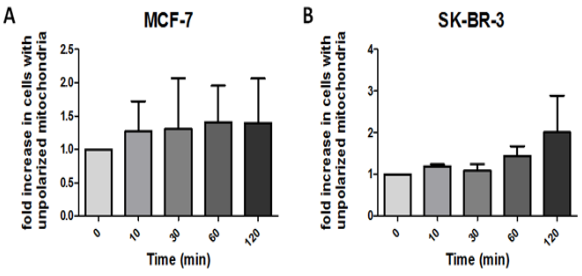

Figure 5. Up to 120 minutes incubation with 25 μM curcumin did not significantly induce ΔΨm colapse. Shown are bargraph of fold increase in MCF-7 (A) or SK-BR-3 (B) cells with depolarized mitochondrial membranes at different time points after 25 μM curcumin treatment.

When ΔΨm was assayed after 48 h treatment, curcumin induced mitochondrial membrane potential collapse in dose dependent manner in both cell lines (Figure 6), with similar efficiencies. In the MCF-7 cell line the EC50 value was 44.1 µM (best fit, 95% confidence interval from 42.40 to 45.94 µM) and in the SK-BR-3 the EC50 value was 34.7 µM (best fit, 95% confidence interval from 25.3 to 47.7 µM).

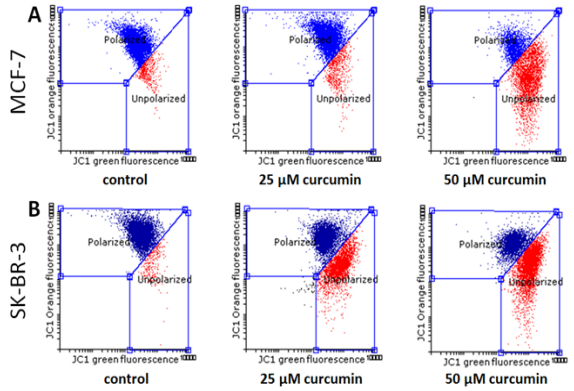

Figure 6. Curcumin treatment for 48h reduces the mitochondrial membrane potential. shows dot-plots of JC1 green vs. orange fluorescence in (A) MCF-7, (B) SK-BR-3 cells treated with 0, 25 or 50 µM curcumin. The cells with more JC1 aggregates, emitting in orange, have normal polarized mitochondrial membranes, while those with JC1 mainly in monomeric form, emitting in green, have depolarized mitochondrial membranes.

Conclusions

As shown here, curcumin treatment leads to similar outcomes in the luminal B and HER2 amplified cell lines tested, with similar curcumin concentrations required for mitochondrial membrane depolarization, with oxidative stress induction and inhibition of clonogenic formation in both models but with slightly different sensitivities. Curcumin also presented cell line specific effects, blocking the cell cycle evolution in different stages – G2/M phase for luminal B MCF-7 and S phase for HER2-amplified SK-BR-3 – and reducing the expression and Y204 phosphorylation of ERK only in the luminal B model. The results presented here provide insights in the anti-cancer effects of curcumin in different breast cancer models and could offer some use in developing new treatment strategies.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

This work was supported by the Romanian Ministry of Education. The financial support of the UEFISCDI project PN-III-P2-2.1-BG-2016-036 is gratefully acknowledged.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 176-183
doi: 10.25083/rbl/24.1/176.183

Received for publication: August 3, 2018
Accepted: October 20, 2018

Original paper

In vitro test of inhibition effect of extracts from three seaweed species distributed at Black sea on different pathogens potentially dangerous for aquaponics

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Abstract

Aquaponics is innovative recirculation system where hydrobionts are cultivated together with the plants. The possibilities for control of pathogens in these systems are highly restricted. One possible strategy for inhibition of pathogenic microorganisms in aquaponics is the usage of seaweeds extracts. The study connected with the investigation of inhibition effect of seaweeds from Bulgarian Black sea cost on different pathogens is rare. The aim of current study was to test In vitro the inhibition effect of three seaweed species (Ulva rigida, Cladophora vagabunda, and Ceramium rubrum) distributed at Black sea in front of Bulgarian cost on different pathogens (bacteria and fungi) which are potentially harmful to hydrobionts, plants and consumer of aquaponics products. The ethanol and methanol extracts from investigated seaweed species were prepared. They were tested with agar well diffusion method against the following fish, food borne and plant pathogens: Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Salmonella typhimurium, Candida albicans, Penicillium verrucosum var. verrucosum, Fusarium graminearum, Fusarium moniliforme and Aspergillus ochraceus. The current study showed that the following extracts from seaweeds distributed in front of Bulgarian Black sea cost possess high inhibition effect (size of inhibition zone higher than 10 mm) against potentially pathogenic microorganisms in aquaponics: ethanol extract of C. vagabunda against B. cereus and A. ochraceus, methanol extract of C. vagabunda against C. albicans, ethanol extract of C. rubrum against E.coli, B. cereus and C. albicans.

Keywords : Aquaponics, in vitro tests, inhibition effect, seaweeds, pathogens

To cite this article: SIRAKOV I, VELICHKOVA K, RUSENOVA N, DINEV T. In vitro test of inhibition effect of extracts from three seaweed species distributed at Black sea on different pathogens potentially dangerous for aquaponics. *Rom Biotechnol Lett*. 2019; 24(1): 176-183. DOI: 10.25083/rbl/24.1/176.183

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Introduction

Earth's population will continue to grow up to 9 billion by 2050 (UN, [1]). In order to meet the needs of the continuously growing population, it is necessary to develop technologies that allow a significant proportion of food products to be produced directly in the cities. Thus will reducing the pollution of the environment and lowering transportation costs. One such technology, which allows the cultivation of hydrobionts and plants to be integrated into the urban environment, is aquaponics (GRABER & al. [2]).

Along with the many advantages that this technology possess, one of its disadvantages is that the pathways for reducing the pathogenic microorganisms in it are restricted as the use of pesticides can lead to the toxic effects in the cultivated hydrobionts from one side and on the other hand, the use of the therapeutics on the hydrobionts can lead to their accumulation in plants (RAKOCY & al. [3]).

In aquaculture a large number of microorganisms are referred as a fish pathogens, or potentially dangerous for the humans i.e. S. aureus (SHAH & al. [4]), E. coli (NEWELL & al. [5]; SHIN & al.[6]), B. cereus (KOLANJINATHAN & al. [7]), S. typhimurium SHIN & al.[6]), P. aeruginosa (CHOUDHURY & [8]), C. albicans (PEREIRA & [9]). Therefore, their control in aquaponic systems is of the great importance for the aquaponics hobbyist as well as for the commercial aquaponics farms.

From the other side, the higher humidity in aquaponics greenhouses contributes to the development of pathogenic fungi (from genus Aspergillus, Fusarium, and Penicillium) which are potentially harmful to the plants and could decrease their productivity or deteriorate its commercial realization.

One method proposed by SIRAKOV & al. [10] to solve this disadvantage is the isolation of beneficial bacteria and their use in the control of pathogenic microorganisms associated with hydrobionts and plants. Another possibility is the use of synbiotics in the aquaponic systems, which can improve the immune system of the fish and the metabolism in the cultivated plants (SIRAKOV & al. [11]).

Different approach to diseases control in aquaponic systems is the use of extracts from different plants and it still remains not investigated. Numerous studies have shown the possibility of using extracts of different plants to control pathogens in hydrobionts, plants or for consumers of agriculture production. Studies investigating the possible double effect of the application of extracts from different plants on hydrobionts and plants pathogens as well as on potentially harmful for the consumer of aquaponic products microorganisms are rare.

One of the possible groups of plants that could be used in this battlefield against pathogenic microorganisms is algae. The effect of their use is strongly dependent of intraspecific variability in the production of second metabolites related to seasonal variation and location of their yield (LIMA-FILHO & al. [12]; TÜNEY & al. [13]; PÉREZ et al. [14]). Studies investigating the antimicrobial potential of seaweed from the Black Sea, in front of the Bulgarian coast on various pathogens, are very limited (KAMENARSKA & al. [15]). The antibacterial effect of the following seaweeds species collected from Bulgarian Black sea cost was tested: Bangia fuscopurpurea, Haliptilon virgatum, Corallina elongata, Gelidium spinosum, Callithamnion granulatum, Ceramium diaphanum var. elegans, Chondrophycus papillosus, Laurencia coronopus, Polysiphonia denudate, Polysiphonia denudate f. fragilis, Stilophora tenella, Punctaria latifolia, Punctaria plantaginea, Colpomenia peregrina, Scytosiphon lomentaria, Zanardinia prototypus and Cystoseira crinite (KAMENARSKA & al.[15]) .

The aim of current study was to test in vitro the inhibition effect of three seaweed species (Ulva rigidaC. Agardh, Cladophora vagabunda (L.) C. Hoek,and Ceramium rubrum (Huds.) C. Agardh) distributed at Black sea in front of Bulgarian cost on different pathogens (bacteria and fungi) which are potentially harmful to hydrobionts, plants and consumers of aquaponics products.

Materials and Methods

1. Preparation of Seaweed for extraction

Three seaweeds species (Ulva rigida C. Agardh, Cladophora vagabunda (L.) C. Hoek, and Ceramium rubrum (Huds. C. Agardh) were collected from coastal areas of Black sea, south from Burgas (Tzarevo municipality, Lozenets village, Bulgaria)

(42°12'32.1"N 27°48'40.0"E). The seaweeds were determined by DIMITROVA-KONAKLIEVA [16]. They were prepared for extraction according to SUJATHA & al. [17] with slight modification. The collected seaweeds were transported to laboratory prior washing them with sea water. The remaining animal castings, sand particles attached debris were removed by soaking them in tap water, followed by rinsing with sterile water. Afterward, the excess water removed by blotting them on filter papers and dried on shade in an air-conditioned room for one week at 20°C and cut into small pieces. They were dried in an oven at 37ºC and powder of them was prepared with the help of electric grinder.

2. Extraction procedure

The process of extraction was made by soaking algae's powder in 100% ethanol solvent (1:10 w/v) for 24 hours. The received materials were concentrated in a rotary evaporator to reduce the volume. The received extracts were filtered through a syringe filter with 0.2 µm pore size and collected in plastic containers. The containers were placed at refrigerator until antimicrobial tests to be started. The same procedure was used for the preparation of methanol extracts of seaweeds. The concentration of the methanol used for the extraction procedure was 100%.

3. Tested pathogens

In the current study the antibacterial activity of seaweed's extracts against the following potentially harmful for fish and humans pathogens were tested: referent bacterial strains-Staphylococcus aureus ATCC25923, Escherichia coli ATCC25922, Pseudomonas aeruginosa ATCC 27853 and clinical isolates - Bacillus cereus, Salmonella typhimurium, and Candida albicans. The strains were kept at refrigerator at -20°C. Prior to their use in inhibition tests, they were recovered at tryptic soy blood agar (Himedia, India).

The plant's mould strains examined were Penicillium verrucosum var. verrucosum 2003 NRRL F-143, Fusarium graminearum 2294 IMI 155426, Fusarium moniliforme 394 Strain FN-9 and Aspergillus ochraceus 2002 IM-BAS.

4. Test of antimicrobial activity of seaweed's extracts against pathogens

Antimicrobial tests with fish and foodborne pathogens

The extracts (methanol and ethanol) were tested for antimicrobial activity by the diffusion method in wells (agar well method) (GHOSH & al. [18]) with some modifications consistent with the type of strains tested: instead of brain heart infusion agar was used Mueller Hinton agar (Himedia, India). The plates were incubated for 24 hours under aerobic conditions instead of microaerophilic. Briefly, from 24 h bacterial and yeast colonies of trypticase soy blood agar, inoculums were prepared in physiological saline corresponding to 0.5 of the McFarland standard ($1.5x10^8$cfu.mL^{-1}). The wells were formed with a sterile 6 mm diameter well probe at the distance of 2 cm from the center after pre-application of the inoculum with a sterile cotton swab. The wells were filled with 100 µl of the extracts. Positive control with gentamicin at a concentration of 12.5 µg.mL^{-1} and negative with the respective solvents was performed. The wells were incubated at 37°C for 24 hours under aerobic conditions. Antimicrobial activity was assessed in the presence of a growth suppression zone of ≥ 8.0 mm (MOHAMMADI-SICHANI & al. [19]).Tests were performed three times to determine the reproducibility of the results.

5. Antimicrobial tests with plant pathogens

The methanolic and ethanolic plant extracts were screened for antifungal activity by agar well diffusion method (PEREZ & al. [20]). The 72 hours old fungal cultures were grown on potato glucose agar (glucose 20.0 g, potatoes 200.0 g, yeast extract 2.0 g, agar 20.0 g, pH 5.6). 20 mL of potato glucose agar was poured in every Petri dish. After solidification, 0.1 mL inoculum of the fungal strains (1-2 x 10^4 CFU.mL^{-1}) was introduced on the surface of the agar plate and the wells were made by using sterile cork borer of size 6.0mm. 0.1ml of methanolic and ethanolic plant extracts were introduced in the wells. An incubation period of 3-7 days at 23^0 C was maintained for observation of antifungal activity of plant extracts. The antifungal activity was evaluated by measuring zones of inhibition of fungal growth surrounding the plant extracts in the wells. The zones of inhibition were

measured in mm and the experiment was carried out in triplicates. Antifungal activity was assumed in the presence of aninhibition zone ≥ 8.0 mm. The complete antifungal analysis was carried out under strict aseptic conditions. Methanol and ethanol were used as a negative solvent control.

6. Data analysis

The antimicrobial tests were conducted in a completely randomized design with three repetitions, for each treatment. The received data were analyzed by ANOVA single factor at a significance level of $P<0.05$. The analyses were made by using the SPSS program.

Results and Discussions

1. Antimicrobial tests with fish and foodborne pathogens

The current In vitro test showed advantageously low and moderate inhibition effect of tested seaweeds extracts (U. rigida, Cl. vagabunda, and C. rubrum) against fish and foodborne pathogens (Table 1). The ethanol and methanol extracts of U. rigida showed lower than 10 mm inhibition zone in antagonistic tests against tested pathogens (Table 1). They showed the highest inhibition effect against fish pathogen S. aureus and it was higher with 60% than the average size of inhibition zone found out for the control variant. The ethanol and methanol extracts of Cl. vagabunda showed the highest inhibition effect against B. cereus and C. albicans and the inhibition zones were higher respectively with 71.6% and 103% compared with the sizes of inhibition zone found for the control variants. The sizes of inhibition zone of ethanol extract of C. rubrumin In vitro tests with pathogens E.coli, B. cereus and C. albicans was higher respectively with 68.1%, 66.6%, and 84.3% compared with the size of inhibition zone in control variant where only ethanol's inhibition effect was tested.

The positive control showed the higher inhibition effect of Gentamicin against tested fish and foodborne pathogens compared with the tested seaweeds extracts except for S. typhimurium where the ethanol extract of Cl. vagabunda showed the highest inhibition effect (8.6±0.4) (Table1).

Table 1. The diameter of inhibition zone of antimicrobial screening of algae extracts determined by theagar diffusion method

Test algae extract	*S. aureus*	*E. coli*	*B. cereus*	*S.typhimurium*	*P. aeruginosa*	*C.albicans*
***Ulva rigida* EtOH**	9.6±1.15*	9.3±0.5*	8.3±0.5*	8±0.5*	8±0.5*	9±0.5*
***Ulva rigida* MeOH**	9.6±1.15*	8±0.2*	8±0.5*	7±0.7^{ns}	8.06±0.15*	9.1±0.1*
***Cladophora vagabunda*EtOH**	9.6±0.5*	9.3±0.5*	10.3±0.5*	8.6±0.4*	8.3±0.5*	9.3±1.15*
***Cladophora vagabunda* MeOH**	9.5±0.5*	8.1±0.1*	7.9±0.45*	7.4±0.5*	7.1±0.1*	12.2±0.8*
***Ceramium rubrum* EtOH**	9.3±0.5*	10.1±0.4*	10±0.3*	7.9±0.2*	8.2±0.2*	11.06±0.2*
***Ceramium rubrum* MeOH**	7.9±0.4*	7.7±0.3	6.2±0.2^{ns}	6.1±0.1^{ns}	8.1±0.1*	9.03±0.3*
EtOH	-	-	-	-	-	-
MeOH	-	-	-	-	-	-
Gentamicin	21.1±0,4*	17.3±0,2*	23.0±0,4*	-	15±0,2*	

Diameter of inhibition zone (mm) against plant's mould (Staphylococcus aureus ATCC25923, Escherichia coli ATCC25922, Bacillus cereus (clinical isolate), Salmonella Typhimurium (clinical isolate), Pseudomonas aeruginosa ATCC 27853) and fungal strain Candida albicans (clinical isolate), EtOH=Ethanol, MeOH=Methanol; diameter of inhibition zone equal to 6 mm =absence of inhibition effect (-), diameter of inhibition zone in range from 6 mm to 8.5 mm=low inhibition effect, diameter of inhibition effect in range from 8.6 mm to 10mm=moderate inhibition effect; diameter of inhibition zone equal or higher than 10 mm =higher inhibition effect;
Asterisk (*) denotes a significant different at P<0.05, ns Difference not statistically significant;

Antimicrobial tests with plant pathogens

The inhibition effect of tested seaweeds extracts against plant's pathogenic fungi was lower than this found out for fish and foodborne pathogens (Table 2). The highest inhibition effect was found out for ethanol extract of Cladophora vagabunda against Aspergillus ochraceus and the inhibition zone was higher with 68.3% compared with the size of inhibition zone in the control variant. The moderate inhibition effect was also observed in tests conducted with the ethanol extracts of Cladophora vagabunda and C.rubrum respectively against Aspergillus ochraceus and Fusarium moniliforme (Table 2).

Table 2. Inhibition of mycelial growth of different plant pathogens affected by different herbal extracts

Test algae extract	***Aspergillus ochraceus***	***Fusarium moniliforme***	***Fusarium graminearum***	***Penicillium verrucosum***
***Ulva rigida* EtOH**	-	-	-	7,1±0.1*
***Ulva rigida* MeOH**	-	-	7±0.3^{ns}	-
***Cladophora vagabunda* EtOH**	10.1±0.1*	7.5±0.5^{ns}	-	-
***Cladophora vagabunda*MeOH**	8.5±0.3*	9±0.4*	8±0.2*	7±0.1*
***Ceramium rubrum* EtOH**	9±0.1*	-	-	-
***Ceramium rubrum* MeOH**	8±0.2*	-	-	-
EtOH	-	-	-	-
MeOH	-	-	-	-

Diameter of inhibition zone (mm) against plant's fungi strains (Penicillium verrucosum var. verrucosum 2003 NRRL F-143, Fusarium graminearum 2294 IMI 155426, Fusarium moniliforme 394 Strain FN-9 and Aspergillus ochraceus 2002 IM-BAS.), EtOH=Ethanol, MeOH=Methanol; diameter of inhibition zone equal to 6 mm =absence of inhibition effect (-), diameter of inhibition zone in range from 6 mm to 8.5 mm=low inhibition effect, diameter of inhibition effect in range from 8.6 mm to 10mm=moderate inhibition effect; diameter of inhibition zone equal or higher than 10 mm =higher inhibition effect; Asterisk (*) denotes a significant different at P<0.05, ns Difference not statistically signifcant;

Our study showed the low antagonistic activity of methanol and ethanol extracts of Ulva rigida against tested pathogens and received results were similar to the results received from TÜNEY & al. [13] where the same seaweed species from Aegean sea were tested for the antimicrobial activity. The current tests were conducted with a dry matter of seaweeds which is distinguished with lower antagonistic effect against pathogens compared to the higher antimicrobial activity found out in a fresh matter of seaweeds possible due to the lower quantity of volatile compounds or fatty acids in the samples according to TÜNEY & al. [13]. We did not find differences in antimicrobial activity in an extract of Ulva rigida when different solvents were used (methanol and ethanol). TÜNEY & al. [13] found strong inhibition effect

against different pathogens when diethyl ether was used as a solvent resulted probably in different content or quantity of active substances which were extracted during the extraction process.

The current test with Cladophora vagabunda showed high antagonistic effect against bacterial pathogen B. cereus and pathogenic fungus C. albicans and A. ochraceusand moderate antagonistic effect against pathogenic micro-organisms S. aureus, E. coli, S. typhimurium and Fusarium moniliforme. Our study is in confirmation of research made by HORINCAR & al. [21] which determined the antagonistic effect of C. vagabunda collected from the Romanian Black sea against S. enteritidis, B. subtilis, and E.coli. The same authors explored the volatile composition and fatty acids content and found that the main volatile components presented in C. vagabunda are hexanal (11.2%), octane (9.8%), nonanal (6.7%), octanal (6.7%), 2,5,5-trimethyl-2-hexene (4.7%), 3-hexen-2-one(4%), and o-cymene (3.6%) and fatty acid composition was composed generally of palmitic and arachidonic acids. HORINCAR & al. [21] found out that MUFAs and PUFAs content in C. vagabunda was 42%.

Ethanol extract of Ceramium rubrum showed a high antagonistic effect against E.coli, B. cereus and C. albicans during conducted from us In vitro tests. The study made by CORTÉS & al. [22] showed that the antimicrobial ability of C. rubrum primarily results from the availability of the lipophilic extract. Other study showed that the type of solvent has a primary meaning for antibacterial activity of C. rubrum because a different type of solvent has different antimicrobial capabilities and it was demonstrated that that methanol extract from C. rubrum seaweed had a higher inhibitory effect than nonpolar n-hexane extract (DUBBER & al. [23]). In our study, the ethanol's extract showed a higher antagonistic effect in C. rubrum.

Another factor influenced antimicrobial activity in seaweeds is the geographical location (HELLIO & al. [24]). The microbiological investigation of antagonistic activity of seaweeds in front of Bulgarian cost are limited (KAMENARSKA & al. [15]) and present commercial interest.

The antagonistic effect of seaweeds against pathogenic microorganisms is promoted mainly from the content of two group compounds- volatile compounds and fatty acids. Inhibition effect of volatile compounds is connected with the significant changes in membrane permeability in cells of pathogens (TROMBETTA & al. [25]). It is already known that polyunsaturated fatty acids could kill pathogenic microorganisms by disrupting their cell membrane, leading to cell lysis (LE et al. [26]). These characteristics made from seaweeds possible object for biological treatment of pathogens in such multifunctional system as the aquaponics where the fish and plants are presenting.

Conclusions

The current study showed that the following extracts from seaweeds distributed in front of Bulgarian Black sea cost possess high inhibition effect against potentially pathogenic microorganisms in aquaponics: ethanol extract of C. vagabunda against B. cereus and A. ochraceus, the methanol extract of C. vagabunda against C. albicans, the ethanol extract of C. rubrum against E.coli, B. cereus, and C. albicans. The future study connected with different doses and the ways of application of seaweeds extracts possess antimicrobial activity should be tested in vivo in the aquaponics.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

This study was supported by the project 1AF/17 and 8AF/18.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 184-192
doi: 10.25083/rbl/24.1/184.192

Received for publication: December 28, 2016
Accepted: October 10, 2017

Original paper

Effects of production system on the content of organic acids in Bio rhubarb (Rheum rhabarbarum L.)

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Abstract

Rhubarb is a perennial vegetable crop well adapted to the temperate climate but it is not well known and cultivated as a traditional crop. The aim of the present study is to highlight the influence of the rhubarb cultivar and crop density on the content of some organic acids (malic, tartaric, oxalic, citric, ascorbic) in an organic crop. The highest content of organic acids was determined in a local population from Moldova at a density of 9090 plants·ha-1; the following quantities of acids for 100 g fresh weight (FW) were determined: tartaric – 486 mg, oxalic – 343 mg, citric – 41 mg, malic – 686 mg and ascorbic –437 mg.

In this present paper, we have demonstrated that content of organic acids from edible rhubarb parts varies according to crop density and cultivars, except for malic acid.

Keywords : Rheum rhabarbarum, cultivar, density, tartaric, oxalic, citric, malic, ascorbic compounds

To cite this article: STOLERU V, MUNTEANU N, STAN T, IPĂTIOAIE C, COJOCARU A, BUTNARIU M. Effects of production system on the content of organic acids in Bio rhubarb (Rheum rhabarbarum L). *Rom Biotechnol Lett*. 2019; 24(1): 184-192. DOI: 10.25083/rbl/24.1/184.192

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Introduction

The rhubarb (Rheum rhabarbarum L.) is a perennial species which grows best in a cold and temperate climate [1], but it is not well known and cultivated as a crop in Romania [2]. Rhubarb plants are cultivated as vegetable for their leaves and stems. Fresh rhubarb is generally available across from April to October, in open fields, harvested from March to December. Rhubarb is one of those iconic vegetables that transport us from one season to the next, from the dull days of winter to the rebirth of spring [3, 4]. Interest from the European consumer in recent times for this vegetable is a good opportunity to study the influence of some technological factors on the quantity and quality of the yields [5].

The main quality characteristics are the taste and aroma, and these depends on the chemical composition. The chemical composition in organic acids, minerals, carbohydrates, proteins and vitamins highly depends on the cultivation and harvesting period [6]. A special interest has been shown by scientists regarding the quantitative and qualitative content of the organic acid.

Gherghi, 1985 identified 32 organic acids in vegetable rhubarb products. Although malic, citric and oxalic acids have been known since the time of Scheele. The tartaric acid, at least in the form of its salts, the exact position and length of these widely distributed substances in the general scheme of metabolism in plants is still a matter of speculation and debate [8, 9, 10].

Bio–synthesis of organic acids in plants is depending on biotic and abiotic factors that in organic cultivated crops are very important elements which determine the organoleptic qualities of the edible part, since synthetic fertilizers are prohibited [11]. A balance content in organic acids provide or increase immunity in the plants to the harmful organisms, which is an important fact when it comes to organic crops because of the limited application of the chemical synthesis pesticide [12]. Dynamics of acidity is an important technological indicator for harvesting of the yield, knowing that a balance between acids reduces the changes during ripening which could decrease organoleptic quality [11, 13]. Such biochemical analysis opens a new horizon to establish the optimal time to harvest the edible parts.

The organic acids have a well-established role in the plant's growth, such as the cell wall structure, storage of phosphates and building blocks of lignin. Sometimes these present functions of antibacterial, antifungal, anti-parasitic; they contribute to the appearance, color and smell of food they contain [14, 15].

Malic acid is a predominant one, and citric acid and oxalic acid are present in a smaller quantity in the rhubarb plants [16]. Because the malic acid is degraded more quickly than other acids, it is also clear that the rhubarb's edible parts lose their acidity and therefore storage lasts a short period [17].

The citric and tartaric acids in fruits and vegetables are the hardest to degrade, and a higher content in these acids favors and maintains a higher percentage of the ascorbic acid content [9, 11, 18].

It is known that oxalic acid is found in large quantities in plants of the Polygonaceae and Chenopodiaceae families, where the content can be up to 40–50 mg/100 g [19, 20]. In these plants, a high content of oxalic acid may cause in-solubilisation of the other organic acids in the human body's cells and deposition of crystallized calcium oxalate. Oxalic acid is an anti-nutritive substance, the in–solubilizing part of calcium and magnesium, thus causing toxic effects [21].

Ascorbic acid has a strong reducing effect, passing to dehydro-ascorbic acid. The role of ascorbic acids is very complex, since in plants they cause the formation of unsaturated fatty acids, degradation of amino acids or carbohydrates, affecting the iron metabolism etc. [7, 11, 21]. In the last 20 years, increasing experimental evidence has associated the metabolism of organic acids with plant tolerance to environmental stress.

This new perspective of organic acid metabolism and its potential manipulation may represent a way to understand the fundamental aspects of plant physiology and lead to develop new technologies [22, 23]. Content of vitamins, minerals, organic acids of rhubarb petioles is directly influenced by these technological benefactors, including: cultivar, planting distance and the interaction of these vital role factors [25].

The aim of the research was to evaluate the organic acid content from rhubarb plants cultivated in an organic system in relation with cultivar, crop density and the combination of cultivar x density.

Materials and Methods

1. Experiment trial

This research was carried out in the experimental field of the Agricultural University from Iasi, managed in the organic system on a rhubarb crop established in 2013. To achieve the goal and objectives of this research, a bi-factorial trial was established: Factor A–cultivar (cv.) with two graduations, "Glaskin's perpetual" (GP) and "Local population" (LP) from Moldova, and factor B–plant density, with two graduation, 9,090 plants•ha-1 and 12,120 plants•ha-1. The trial was organized in a split plot design, with three replicates. A plot has a surface of 16.50 m2 (2.2 m x 7.5 m).

2. Cultivation practical

The cultivation practices were done according to recommendations of adequate literature [5, 24, 25], using only organic and biological measures for fertilizing, disease and pest control according to our previous study [12]. Petioles harvesting was carried out once every five days.

3. Identification of organic acids

The analyses were carried out at the Chemistry & Biochemistry Laboratory of Banat's University, using methods recommended by [26]. Juice extract samples were obtained from each petiole variant. The samples were extracted in a Soxhlet-type apparatus in which the solvent is provided by boiling the extract. This method is based on the difference between the boiling points of the solvent and the extracted analyses. Based on this property, the extract is brought to the boiling point of the solvent, which will condense the refrigerant and returned to the cartridge containing the sample to be extracted. Through achievement of several cycles of extraction, the process efficiency can be controlled so that the extraction efficiency is maximized. The obtained extract was centrifuged at 3000 rpm for 10 minutes, and the supernatant is diluted in a 1:50 ratio for the determination of the citric acid in a ratio of 1:5 acids. The dilutions were filtered before determination of the concentration using colorimetric analysis & Beer's Law. The two samples are analyzed in duplicate. A mixed standard solution was prepared containing 1000 mg·L-1 citric acid, 2000 mg·L-1 of malic acid, 300 mg·L-1 oxalic acid and ascorbic acid, 700 mg·L-1 of tartaric acid and 400 mg·L-1 lactic acid. The standard solution and appropriate dilutions were prepared with distilled water and kept in a dark place at low temperature (+40C). The organic acids in the samples were separated and determined using absorbance, then quantified with the calibration graphs, on wavelengths of λ = 254 nm for ascorbic acid, and λ = 214 nm for other organic acids [27].

The method recommended by Ionel in 2013 was used for the identification of organic acids. The sample is titrated with a sodium hydroxide solution, from a burette, with the order of 0.1 cm^3, 0.5 cm^3 portions and toward the end portion than 0.1 cm3. A preliminary probe is determined when passing portions of 0.1 cm^3. After each additional portion of NaOH, mixed with a glass rod, a drop of titration is passed on a porcelain mesh plate, in which one drop of phenolphthalein is added. The titration is terminated, when at drop wise addition of the solution changes to pink colour [29]. The results were expressed as mg·100 g^{-1} fresh product of rhubarb.

4. Data processing and statistical analyses

The experimental data processing was carried out using specific mathematical and statistical methods [30]. All analyses were carried out in the three replications. Standard deviation (±SD) was calculated for each data series as an indicator of dataset scatter (n=3). The significance of acid content and yield, due to cultivar x density combinations was established by ANOVA [31, 32], based on the Fisher test. The differences among the average values for each experimental variant were compared by using the Student test and the least significant difference (LSD) test at $p<0.05$ probability level, computed by the SPSS version 20. To determine the degree of correlation between the combinations of the studied factors and content of organic acids the regression coefficient (r^2) was determined.

Results and Discussions

1. Influence of the cultivar and planting distances on the total content of organic acids

The cultivar and planting density are two technological factors which directly influences the quantity and quality of the yield. Four experimental combinations of the two factors appear in the interaction study (V_1, V_2, V_3, V_4, with three replication) (Table 1)

Table 1. The organic acid content of rhubarb

Variants	Average content of organic acids (mg·100 g^{-1} FW)					
	tartaric	oxalic	citric	malic	ascorbic	Total acid
V_1 (GP x d_1)	230^b	256^b	**534^a**	670	339^b	2029
V_2 (GP x d_2)	324	340	500^a	676	359^b	2199
V_3 (LP x d_1)	388	**377^a**	465^b	683	386	2299
V_4 (LP x d_2)	**520^a**	343	441^b	**687**	**438^a**	2429
$\overline{x}$	**365.5**	**329**	**485**	**679**	**380.5**	2239
p<0.05%	88.33	39.58	10.78	14.91	25.06	-

GP- Glaskin's perpetual; LP - Local population; d_1 - 12,120 plants•ha^{-1}; d_2- 9,090 plants•ha^{-1}; a–positive significant difference at p<0.05; b–negative significant difference at p<0.05

In general, depending on the combination of the two factors, except malic acid, the total content of organic acids varies widely, from 2029 mg•100 g^{-1} FW on V_1 till to 2429 mg•100 g^{-1} FW on V_4. Tartaric acid varied between 230 and 520 mg•100 g^{-1}, oxalic acid varied between 256 and 377 mg•100 g^{-1}, citric acid varied between 441 and 534 mg•100 g^{-1} and ascorbic acid between 339 and 438 mg•100 g^{-1}.

2. Tartaric acid content in rhubarb

The trend is upward from V_1 to V_4, with R2 = 0.9811, which indicates that LP planted at a density of 9,090 plants•ha-1 accumulates a larger amount of tartaric acid (Fig.1). In the terms of the tartaric acid content, it can be seen that it ranged between 230 mg•100 g^{-1} FW in V_1 and 520 mg•100 g^{-1} FW in V_4. Concerning the tartaric content, the variation depends on the combination of two factors analyzed.

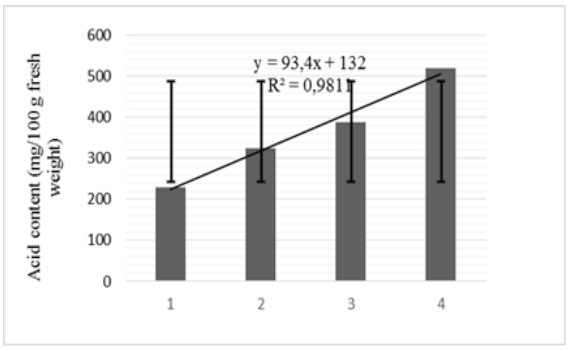

Fig. 1. The tartaric acid content according to cultivar and plant density

3. Oxalic acid content in rhubarb

The oxalic acid content is an indicator that depends on the quality of the edible part of rhubarb. In the experiment the oxalic acid content ranged from 257 mg•100 g^{-1}FW in V_1 up to 377 mg•100 g^{-1} FW in V_3, with an average from 329 mg•100 g^{-1} FW. Referring to the oxalic acid content compared with tartaric acid, the variation content is lower with R^2= 0.5585 (Fig. 2).

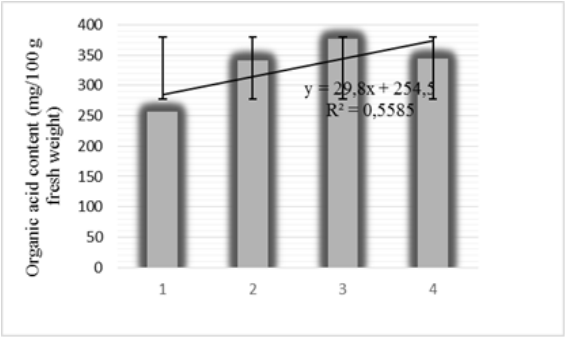

Fig. 2. The oxalic acid content according to cultivar and plant density

4. Citric acid content in rhubarb

The content of citric acid in rhubarb ranged between 441 mg•100 g^{-1} FW in V_4 and 534 mg•100 g^{-1} FW in V_1, with an average content of 485 mg•100 g^{-1} fresh weight. The higher content in oxalic acid was obtained on GP cv., but we can say that the variation in the content of citric acid in the edible parts differ, depending on the distances from planting, so the highest content was observed in variants with higher density, respectively 12.120 pl•ha^{-1}. Compared with the other four analyzed acids from rhubarb, citric acid

is one in which the regression line descends, from GP x 12,120 plants•ha^{-1} by the LP x 9,090 plants•ha^{-1}, with a probability of 99.35% (Fig. 3).

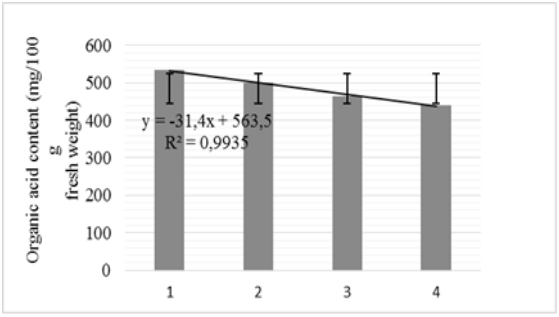

Fig. 3. The citric acid content according to cultivar and plant density

5. *Malic acid content in rhubarb*

Among the organic acids, the highest content was found in the case of malic acid, with average values from 679±2.88 mg•100 g^{-1} fresh weight, with very small differences between the experimental variants; this shows that the four variants of this content are not influenced by cultivar and planting density, that fact being confirmed also by other studies [33]. However, the highest content in malic acid was found in the LP planted at a density of 9,090 plants•ha^{-1}.

6. *Ascorbic acid content in rhubarb*

In the case of ascorbic acid, we can say that the values of this acid in rhubarb are close to the values of oxalic acid. The ascorbic acid content ranged from 339 mg•100 g^{-1} FW in GP cv. with a density of 12,120 plants•ha^{-1} to 438 mg•100 g^{-1} FW in the case of LP cv.at density of 9,090 plants•ha^{-1}.

Perspective to accumulation the ascorbic acid is upward from a high density to a lower crop density, especially in the LP, which supports the assertion with a probability of 95.07% (Fig. 4). So, the higher content of ascorbic acid of 438 mg•100 g^{-1} obtained by LP cv.at density of 9,090 plants•ha^{-1}.

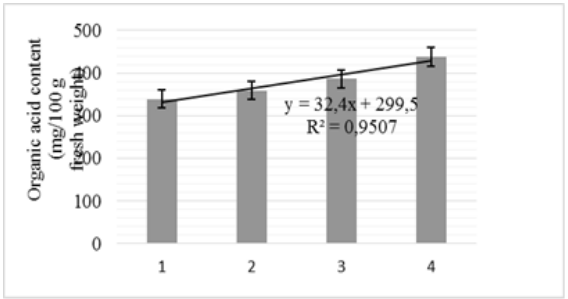

Fig. 4. The ascorbic acid content according to cultivar and plant density

7. *Effect of cultivar and planting distance on rhubarb yield*

The cultivar and planting distance, as technological measures influenced the yield of rhubarb, cultivated under organic growing conditions, in the second year after established. The production dates are presented in Table 2.

Table 2. Effect of cultivar and planting distance on rhubarb yield (n=3)

Experimental version	Yield (t/ha)	Differences *vs.* mean (t/ha)	Significances of the differences *vs.* mean
V_1 (GP x d_1)	39.08±5.21	7,37	**
V_2 (GP x d_2)	33.85±1.51	2,14	ns
V_3 (LP x d_1)	27.52±2.96	-4,19	ns
V_4 (LP x d_2)	26.39±3.76	-5,32	o
Average $\bar{x}$	31.71±0.00	-	

LSD 5%=4.45 t/ha; LSD 1%=6.75 t/ha; LSD 0.1%=10.84 t/ha; GP- Glaskin's perpetual; LP - Local population; d_1 - 12,120 plants•ha^{-1}; d_2- 9,090 plants•ha^{-1}; ns-no significant differences; ** distinct significant positive differences at $p<0.1$; o-significant negative differences at $p<0.05$.

The distinct significant positive differences was obtained in the experimental version were GP cv. planted at a density of 12,120 plants•ha^{-1}. Local population planted at distance of 12,120 plants•ha^{-1} (26.39 t/ha) and 9,090 plants•ha^{-1} (27.52 t/ha) obtained productions less than average experiment (31.71 t/ha).

Discussions

The technological measures were applied on a rhubarb crop, to ensure the optimum plant growth and development as well as the good quality of the vegetable product under natural circumstances, according to the requirements of plants [12, 34]. The role of the optimization of technological factors is to achieve sufficient production in quantitative terms but mostly qualitative.

Analysis of the variance (ANOVA) of the experimental data revealed the importance of the studied factors: cultivar, crop density and cultivar x crop density on the organic acid content. The Fisher

test for $p<0.05$ level of significance highlighted that the cultivar is a significant factor for the following organic acids: tartaric, oxalic, citric, malic and ascorbic.

The crop density influence, according to the same test, is positive significant influenced for the following content of organic acids: tartaric, citric, and ascorbic.

Except for the analyzed citric acid, all four acids have a smaller quantity in GP, compared to LP, underscoring the influence is determined by cultivar. This result might be explained by a genetic adaptation of the LP to certain abiotic or biotic stress factors, during the long period of cultivation, which favors a higher acidity [34].

Thus, we can say that tartaric, oxalic and ascorbic contents are lower in GP than the mean value of experiment, regardless of plant density per hectare. Higher values are an indication of this acid, which shows that the LP from Moldova are ready faster than GP this is a very important clue in storage balance among the acids in petioles from rhubarb.

Regarding the variation of organic acids in varieties, based on calculated statistical values, we can say that the value of this coefficient is higher in GP cv. ($r^2 = 0.236$) compared to LP where $r^2 = 0.0937$, which means that the differences between acid contents are lower in LP cv. This is of practical interest because, LP is more balanced in terms of acidity.

The distance between plants is a technological factor that directly influences the crop density, nutrition space, light regime and, as a consequence of organic acid content.

The content total un-azotic organic acids depend on the distance of planting. Thus, in terms of larger nutritional space content of organic acids, it is 2,239 mg•100 g^{-1} FW compared to a smaller space where the nutritional content of organic acids is lower at 2164 mg•100 g^{-1} FW. From the data, it follows that to obtain products with higher acidity, less crop densities or greater distances between plants should be used.

At a density of 12,120 plants•ha^{-1}, the organic acid content was higher compared to the situation when rhubarb is planted at lower densities. On the basis of the statistical factor r^2 regarding how plant density plays an important technological factor when it comes to creating optimal conditions for balanced accumulation of acids, a last resort is influenced by light and photosynthesis.

This could be explain by the fact that the rhubarb petioles overall did not reach maturity from consumption and thus there is an imbalance between acid [20, 35].

The total content of non-azotic acids varies according to the interaction between cultivar and planting distances. The highest content of organic acids was obtained in the experimental variant V_4 (LP x d2).

Concerning to tartaric acid content, variation depends on the combination of two factors analyzed. The trend with $r^2 = 0.9811$ indicate that LP cv. planted at a density of 9,090 plants•ha^{-1} accumulates a larger amount of tartaric acid.

The oxalic acid content is an indicator that depends on the quality of the edible part of rhubarb. The higher content of oxalic acid is observed in the LP from Moldova, but we can say that the variation in the oxalic acid content did not differ significantly according with planting distances. Even though the literature notes that in some cases oxalic acid can be 50% of the total acidity in rhubarb [20]. The results of oxalic acid are in accordance with Son, (2000) which showed in rhubarb a content of 336 mg•100 g^{-1} FW. Through technology applied and varieties, this content is reduced which means a lower presence oxalate in the body and therefore a lower risk to human health. Oxalic acid content in rhubarb higher than our results was obtained by [37]. Other studies and surveys reported oxalic acid contents higher in other plants, as Amaranthus sp., Tea Chinese, Atriplex hortense, Tetragonia tetragonoides, Spinacia oleracea [1] or less in tomato, parsley, apple, cabbage or lettuce [37, 38].

The higher content in oxalic acid was obtained on GP cv., but we can say that the variation in the content of citric acid in the edible parts differ, depending on the distances from planting, so the highest content was observed in variants with higher density, respectively 12, 120 pl•ha^{-1}.

However, the highest content in malic acid was found in the LPs from Moldova, planted at a density of 9,090 plants•ha-1. In the case of ascorbic acid, we can say that the values of this acid in rhubarb are close to the values of oxalic acid. Generally, in major type of vegetable the contents of malic acid are less in rhubarb [39, 40].The ascorbic acid content ranged from 339±14.00 mg•100 g^{-1} FW in GP cv. with a density of

12,120 plants•ha^{-1} to 438±35.23 mg•100 g^{-1} FW in the case of LP cv.at density of 9,090 plants•ha^{-1}. Lower contents of ascorbic acid can be found in other leaf (spinach, cabbage) and root vegetables [40].

In general, the pleasant sweet and sour taste from the fruit is given by high total free acidity, like in rhubarb [20]. Concerning the total yield of rhubarb there is not a positive correlation between the cultivars production and total content of organic acids. LP get lower production than GP cv., but organic acid content is higher at lower plant density. Similar results of production, on Victoria variety to the mulching conditions were obtained [41]. Rumpunen (1999) based on a research study to 71 commercial cultivars mentions productions from 3 to 7 t/ha at density of 8870 plants•ha^{-1}. The higher yield in GP cv. is clearly influenced by density, but the total production does not positively correlate with a higher content in organic acids.

Conclusions

This study demonstrated the clear impact of the cultivars and planting density as well on the organic acid content and yield.

The better suited variety to environmental conditions planted at less distances favored getting a higher total acidity rate of rhubarb stalks and thus in a balance between acids.

In terms of quality exception to the rule, citric acid content is higher in improved cultivars and small planting distances. Malic acid, regardless of the cultivar and planting distance was found in the same quantity. The average ratio of tartaric, oxalic and ascorbic acids is approximately 1.

The tartaric and oxalic acids are lower in improved cultivar planted a higher density, it should be considered the establishment for future rhubarb crops, as a possible design solutions.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

We thank Dean Hufstetler for revised English.

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ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(1): 193-199
doi: 10.25083/rbl/24.1/193.199

Received for publication: August 20, 2018
Accepted: October 2, 2018

Original paper

Evaluation of the effect of ^{15}N-labeled fertilizers on maize plant

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Abstract

The effects of the experimental factors studied concerning isotopic indicators ($^{15}N/^{14}N$ isotopic ratio, degree of $\delta^{15}N$ isotopic accumulation, export of ^{15}N isotope, ^{15}N isotope concentration, degree of ^{15}N isotope uptake) were evaluated in maize plant, Cortes hybrid. Experimental foliar fertilizers were obtained: Nutrifert (complex NPK matrix, secondary nutrients and trace elements); Nutrifert vegetal (complex NPK matrix, secondary nutrients, trace elements and vegetal natural organic compounds); Nutrifert Plus (complex NPK matrix, secondary nutrients, trace elements and animal proteins). The natural organic compounds (compounds resulted by neutral hydrolysis of some proteic substances which have biostimulating and chelating action) showed the positive effect of foliar fertilization on nutrients use efficiency by biostimulating nutrients uptake by plant. The experiment was bifactorial with two factors: A, chemical nature of the ^{15}N-labeled nitrogen from the fertilizers applied radicular and B, chemical nature of the foliar fertilizers (with or without vegetal and animal proteic hydrolisates). By combining these two factors, 12 variants were established. The effects induced on isotopic indicators were evaluated. Analytical and isotopic determinations were performed from dried plant material samples. The experimental data were statistically processed by variance analysis using the Duncan multiple comparison test, multiple comparison threshold α=0.05. For the graphic and statistical processing, the Excel, XLSTAT and SPSS 14.0 programs were used.

Experimentally, it has been found that ^{15}N isotope can be used in agrochemical research to improve: the nutrition studies, the research concerning the absorption from soil and mobility in plant of nutrients and of different forms of nitrogen (amide N-NH_2, nitric N-NO_3, ammonical N-NH_4) from nitrogen fertilizers, the experiments for demonstration of the utility of different fertilization methods.

Keywords : plant, ^{15}N isotope, foliar fertilizer, radicular fertilizer, agrochemical testing

To cite this article: MIHALACHE D, VRÎNCEANU N, TEODORESCU RI, MIHALACHE M, BACAU C. Evaluation of the effect of 15N-labeled fertilizers on maize plant. *Rom Biotechnol Lett*. 2019; 24(1): 193-199. DOI: 10.25083/rbl/24.1/193.199

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Introduction

Agrochemistry deals with plant nutrition issues in a close connection with the application of fertilizers, amendments, pesticides and bioregulators. Based on soil-plant-fertilizers relationships, it sets out rules and practical methods for plant nutrition regulation, fertilization and soil fertility evaluation.

In the field of agriculture, it is recommended to use extraradicular fertilizers for: the treatment of certain plant nutrition diseases, preventing the plant diseases, increasing crop yield, product quality and reducing the negative impact of classical fertilizers on the environment. Also, plants treated with fertilizers containing chelating natural organic compounds are more resistant to frost, to drought, to biotic and abiotic stress(D. MIHALACHE et al., 2015 [1]).

The analysis of the existing literature data on classical extraradicular fertilizers or those with growth-enhancing compounds in their formulas has indicated that only the use of biostimulators in crops treatment, often does not result in some significant effects. In these cases, the „explosive“ vegetative development of the plant is not sustained by an additional, rapid supply of macro and micronutrients (CIOROIANU Tr.M., C. SÎRBU, 2010 [2]).

The widespread use of foliar fertilizers is a current European Community policy which has the aim of reducing the fertilization with classical fertilizers and, mitigating the soil and groundwater pollution, their management following the European environmental protection standards and that's of promoting a sustainable agriculture. In the last years, there have been concerns about the production of new types of foliar complex fertilizers containing natural compounds with biostimulating action. In the same time, it has been highlighted that biostimulators represent a special category of agro-compounds obtained synthetically or naturally, which affect the regulation of the physiological processes in the plant. Generally, bioregulatory products are organic compounds. When they are applied in low concentrations, have positive effects on the physiological processes of plant growth and development and on the quality and quantity of crop yield. (CIOROIANU Tr. M., C. SÎRBU, 2011 [3]).

The nuclear techniques with 15N isotope is useful for some agrochemical aspects related to: the efficient use of the different nitrogen forms (amide, nitric, ammoniacal) from fertilizers containing complex NPK matrix and organic substances with biostimulating and chelating properties (applied foliar and by incorporating in soil) for plants; biostimulating action of nutrients absorbtion from the natural soil resources (D. MIHALACHE et al., 2017 [4]).

For a rigurous evaluation, the processes of nutrients absorbtion and metabolization by plants, determined by complex microscopic mechanisms, were studied by using stable radioactive isotopes (^{15}N, ^{2}H, ^{35}S, ^{13}C, ^{14}C, ^{32}P etc.). (BLAIR G., TILL R., 2000 [5]). ^{15}N is very widely used in the agricultural research. The use of stable ^{15}N in agrochemical testing is very helpful in understanding the nutritional processes of plants, which may lead to productivity and sustainability increases of the agricultural systems.

The use of the stable ^{15}N isotope in agrochemical studies related to evaluation of nitrogen cycle and its transformation from the nitrogen fertilizers is well established, but in case of long term testing (perennial crops), there are some deficiency because of the plants size, sampling procedures, detection levels and of interferences in analytical and isotopic determinations (A. QUIÑONES et al., 2003 [6]; BUSTAMANTE C. et al., 1997 [7]; LIMA F., MALAVOLTA E., 2003 [8]; RAPISARDA P. et al., 2005 [9]).

The objective of the study was to estimate the combined effects of chemical nature of the ^{15}N-labeled nitrogen from the fertilizers applied radicular and of the chemical nature of the foliar fertilizers on the isotopic indicators determined on the maize plant.

Materials and Methods

The research was carried out within at the National Research and Development Institute for Soil Science, Agro-chemistry and Environment – ICPA Bucharest, within the Physical-Chemical Analysis Laboratory and the Laboratory of Fertilizer Quality Control and Testing, and the isotopic analyses (determination of $\delta^{15}N$ parameter and $^{15}N/^{14}N$ percentage) were determined at Cornell Isotope

Laboratory (COIL), Cornell University, Ithaca, United States of America. In Table 1 the experimental variants and types of fertilization are presented.

The following experimental foliar fertilizers were obtained: Nutrifert (complex NPK matrix, secondary nutrients and trace elements); Nutrifert vegetal (complex NPK matrix, secondary nutrients, trace elements and vegetal natural organic compounds); Nutrifert Plus (complex NPK matrix, secondary nutrients, trace elements and animal protein). In Figure 1 is presented the technological flow of production of NPK foliar fertilizers used in agrochemical testing.

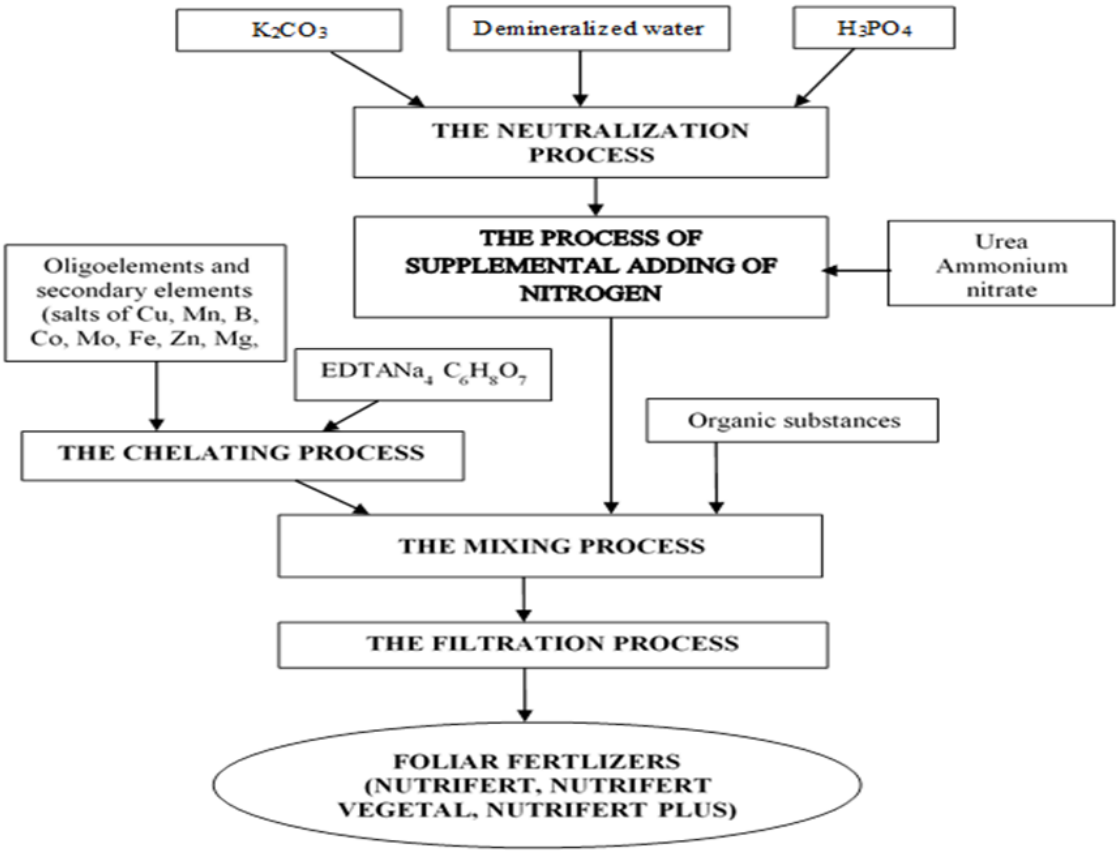

Figure 1. Technological flow of production of NPK liquid fertilizers with secondary nutrients and trace, and organic substances

Within these agrochemical tests, for radicular fertilization, ^{15}N-labeled fertilizers (urea and ammonium nitrate) were used by incorporating in the soil: $(^{15}NH_2)_2CO$, urea with 10% ^{15}N-labeled ($-NH_2$); $^{15}NH_4$-NO_3, ammonium nitrate with 10% ammonia nitrogen ^{15}N-labeled ($-NH_4$); NH_4-$^{15}NO_3$, ammonium nitrate with 10% ammonia nitrogen ^{15}N-labeled ($-NO_3$)

Working conditions:

- Mitscherlich pots with 10 kg of dry soil, type Chernozem.
- Test plant: maize, CORTES hybrid.
- Nutrients dose for basic fertilization was $N_{45}P_{45}K_{45}$ using NPK 15:15:15 (0.8 g/pot), equivalent of 300 kg fertilizer/ha.
- Basic fertilization was supplemented with ^{15}N-labelled fertilizer, $(^{15}NH_2)_2CO$, $^{15}NH_4$-NO_3 and NH_4-$^{15}NO_3$, incorporated in soil.
- The quantity of ^{15}N isotope applied was 30 mg/pot.
- Foliar fertilizer treatments: 30 mL/pot (15 mL/plant) as 1% concentration solution.
- Irrigation 50% from the water pot capacity.
- Plants maintenance: daily irrigation up to 2/3 of the water capacity of the pot.
- 12 experimental variants; 3 replicates, 2 plants/pot, for each of the experimental factors combination (experimental variants).
- The control was basic and foliar fertilized.
- Harvesting maize biomass was done 60 days after seeding.

Table 1. Experimental diagram and types of fertilization

TYPES OF FERTILIZATION		
RADICULAR		FOLIAR
BASIC	^{15}N-labeled fertilizer	
NPK 15-15-15 (nutrients dose $N_{45}P_{45}K_{45}$)	—	NUTRIFERT PLUS
	UREA (^{15}N-NH_2)	
	AMMONIUM NITRATE (^{15}N-NO_3)	
	AMMONIUM NITRATE (^{15}N-NH_4)	
	—	NUTRIFERT VEGETAL
	UREA (^{15}N-NH_2)	
	AMMONIUM NITRATE (^{15}N-NO_3)	
	AMMONIUM NITRATE (^{15}N-NH_4)	
	—	NUTRIFERT
	UREA (^{15}N-NH_2)	
	AMMONIUM NITRATE (^{15}N-NO_3)	
	AMMONIUM NITRATE (^{15}N-NH_4)	

The experimental data were statistically processed by variance analysis using the Duncan multiple comparison test, multiple comparison threshold α=0.05. For the graphic and statistical processing, the Excel, XLSTAT and SPSS 14.0 programs were used.

Results and Discussions

The effects induced by combining the experimental factors on isotopic indicators (15N/14N isotopic ratio, degree isotopic accumulation δ15N in the maize plant, export of 15N isotope with maize plant, 15N isotope concentration in the maize plant, absorption degree of 15N isotope from soil in maize plant) were evaluated in maize plant.

The experiment was bifactorial with two factors:

- A - chemical nature of the ^{15}N-labeled nitrogen from the fertilizers applied radicular;

Graduations: a_1 Control; a_2 ^{15}N-NH_2; a_3 ^{15}N-NO_3; a_4 ^{15}N-NH_4

- B - chemical nature of the foliar fertilizers.
- Graduations: b_1 Nutrifert; b_2 Nutrifert Vegetal; b_3 Nutrifert Plus. By combining the graduations of these two factors, AB, 12 variants were established.

a_1b_1 Control + Nutrifert	a_1b_2 Control + Nutrifert Vegetal	a_1b_3 Control + Nutrifert Plus
a_2b_1 ^{15}N-NH_2 + Nutrifert	a_2b_2 ^{15}N-NH_2 + Nutrifert Vegetal	a_2b_3 ^{15}N-NH_2 + Nutrifert Plus
a_3b_1 - ^{15}N-NO_3 + Nutrifert	a_3b_2 ^{15}N-NO_3 + Nutrifert Vegetal	a_3b_3 ^{15}N-NO_3 + Nutrifert Plus
a_4b_1 ^{15}N-NH_4 + Nutrifert	a_4b_2 ^{15}N-NH_4 + Nutrifert Vegetal	a_4b_3 ^{15}N-NH_4 + Nutrifert Plus

Figure 2 shows the obtained values that were ordered in descending order resulting in a hierarchy of the effects of the application of the 12 experimental treatments. The highest values were:

- ammonium nitrate^{15}N-NH_4+Nutrifert Vegetal (3,08%)
- ammonium nitrate^{15}N-NH_4+Nutrifert Plus (3,07%)
- ammonium nitrate^{15}N-NH_4+Nutrifert (3,04%)

Values ranked in the 1-3 hierarchy do not differ statistically from each other.

Ammonium nitrate ^{15}N-NH_4 (radicular applied) generated the highest values for ^{15}N/^{14}N isotopic ratio in the maize plant.

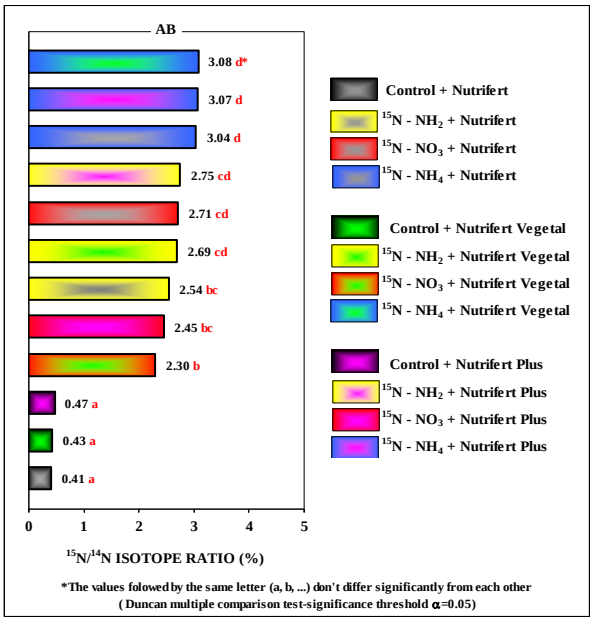

Figure 2. Effects induced by combining the chemical nature of radicular nitrogen source (A) with foliar fertilizers (B) on 15N/14N isotopic ratio in the maize plant

Figure 3 shows the obtained values that were ordered in descending order resulting in a hierarchy of the effects of the application of the 9 experimental treatments. The highest values were:

- ammonium nitrate ^{15}N-NH_4+Nutrifert Vegetal (7728‰)
- ammonium nitrate ^{15}N-NH_4+Nutrifert Plus (7699‰)
- ammonium nitrate ^{15}N-NH_4+Nutrifert (7616‰)

Values ranked in the 1-3 hierarchy do not differ statistically from each other.

Ammonium nitrate ^{15}N-NH_4 (radicular applied) generated the highest values degree for isotopic accumulation $\delta^{15}N$ in the maize plant.

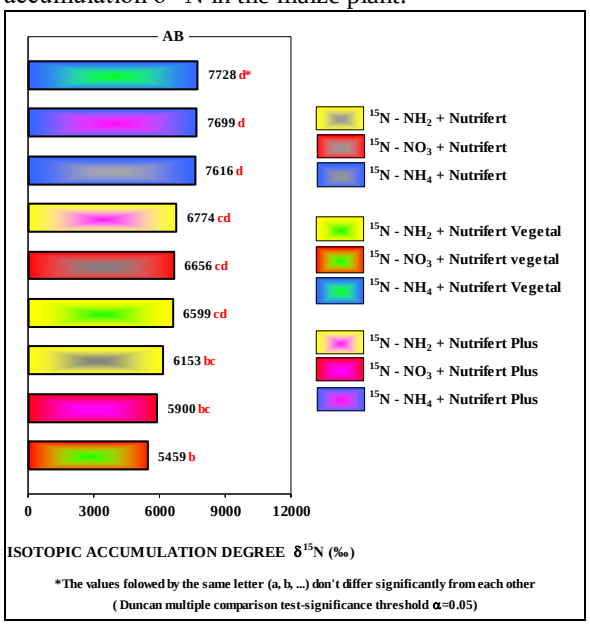

Figure 3. Effects induced by combining the chemical nature of radicular nitrogen source (A) with foliar fertilizers (B) on degree isotopic accumulation δ15N in the maize plant

Figure 4 shows the obtained values that were ordered in descending order resulting in a hierarchy of the effects of the application of the 12 experimental treatments. The highest values were:

- urea ^{15}N-NH_2+ Nutrifert Plus (0,0036%)
- ammonium nitrate ^{15}N-NO_3+ Nutrifert Plus (0,0036%)
- urea ^{15}N-NH_2+ Nutrifert Vegetal (0,0035%)

Values ranked in the 1-3 hierarchy do not differ statistically from each other.

Urea ^{15}N-NH_2 and ammonium nitrate ^{15}N-NO_3 (radicular applied) generated the highest values for concentration ^{15}N isotope in the maize plant.

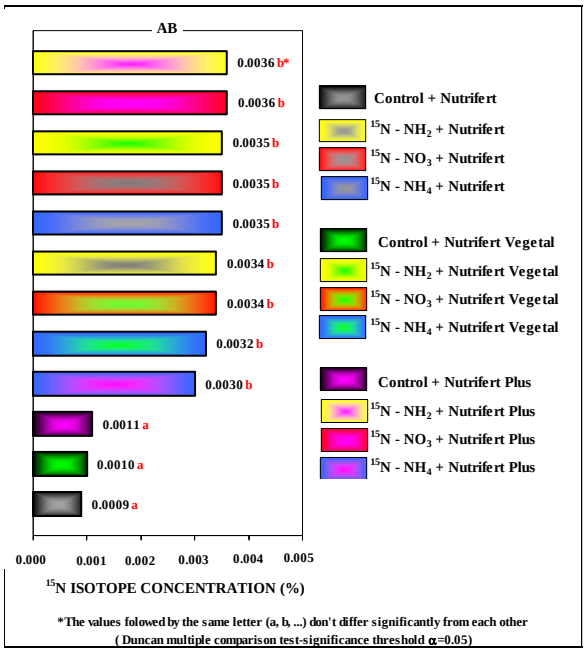

Figure 4. Effects induced by combining the chemical nature of radicular nitrogen source (A) with foliar fertilizers (B) on concentration the 15N isotope in the maize plant

Figure 5 shows the obtained values that were ordered in descending order resulting in a hierarchy of the effects of the application of the 12 experimental treatments. The highest values were:

- urea ^{15}N-NH_2 + Nutrifert Plus (9,34 mg)
- urea ^{15}N-NH_2 + Nutrifert Vegetal (8,94 mg)
- ammonium nitrate ^{15}N-NO_3 + Nutrifert Plus (8,87mg)

Values ranked in the 1-3 hierarchy do not differ statistically from each other.

Urea ^{15}N-NH_2 (radicular applied) generated the highest values for ^{15}N isotope export with the maize plant.

Figure 6 shows the obtained values that were ordered in descending order resulting in a hierarchy of the effects of the application of the 9 experimental treatments. The highest value was: urea ^{15}N-NH_2 + Nutrifert Plus (26,0%)

Values obtained in the hierarchy of this 9 treatments do not differ statistically from each other. Urea ^{15}N-NH_2 (radicular applied) generated the highest values for absorption degree of ^{15}N isotope from soil in maize plant.

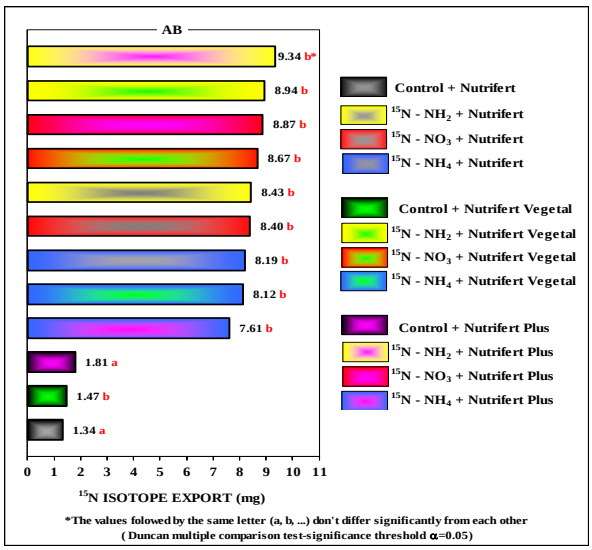

Figure 5. Effects induced by combining the chemical nature of radicular nitrogen source (A) with foliar fertilizers (B) on 15N isotope export with the maize plant

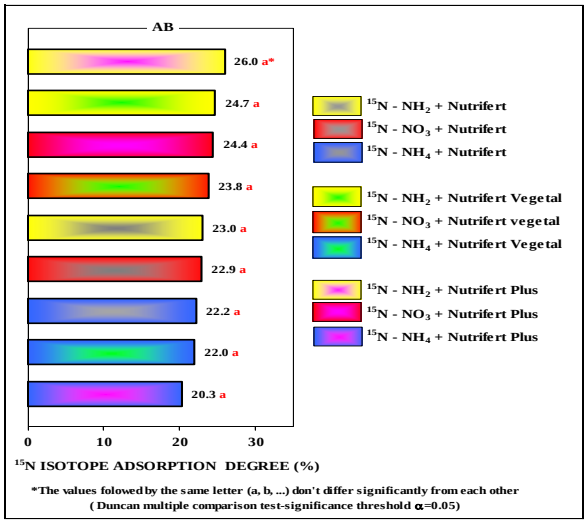

Figure 6. Effects induced by combining the chemical nature of radicular nitrogen source (A) with foliar fertilizers (B) on absorption degree of 15N isotope from soil in maize plant

Conclusions

1. Four experimental foliar fertilizers (NUTRIFERT NUTRIFERT, NUTRIFERT PLUS, NUTRIFERT ECO) with complex matrix type N and NPK, with secondary nutrients, trace elements and natural organic substances with a chelating and biostimulating role were obtained.

2. The values of $^{15}N/^{14}N$ isotopic ratio, values of concentration ^{15}N isotope in maize plant and values of ^{15}N isotope export with the maize plant, that were obtained as a result of treatments including radicular fertilization with ^{15}N-labeled fertilizers and foliar fertilization, differ statistically significantly when compared to the values obtained for Control.

3. The degree of isotopic accumulation $\delta^{15}N$ values in the maize plant, obtained as a result of treatments including radicular fertilization with ^{15}N-labeled fertilizers and foliar fertilization, differs statistically significantly between them.

4. The values of absorption degree of ^{15}N isotope from soil in maize plant do not differ statistically significantly between them.

5. The highest values for $^{15}N/^{14}N$ isotopic ratio and degree of isotopic accumulation $\delta^{15}N$ in the maize plant were generated by the combined effects of radicular application of ammonium nitrate ^{15}N-NH_4 with foliar fertilizers.

6. The highest values for the ^{15}N isotope concentration in maize plant were generated by the combined effects of radicular application of ammonium nitrate ^{15}N-NO_3 with Nutrifert as foliar fertilizer and urea ^{15}N-NH^2 with Nutrifert Vegetal, respectively and Nutrifert Plus as foliar fertilizers.

7. The highest values for ^{15}N isotope export with the maize plant were generated by the combined effects of radicular application of urea ^{15}N-NH_2 with Nutrifert Vegetal, respectively and Nutrifert Plus as foliar fertilizers.

8. The chemical nature of the three foliar fertilizers Nutrifert, Nutrifert Vegetal, Nutrifert Plus did not affect the values obtained for ^{15}N isotopic indicators in maize plants.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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