Romanian Biotechnological Letters *Vol. 23, No. 6, 2018*

ORIGINAL PAPER

Fungistatic potential of *Euphorbia hirta* L. against the cause of anthracnose

10.26327/RBL2017.105

Received for publication, August, 29, 2015
Accepted, July, 3, 2016

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Abstract

The present study was intended to evaluate the antifungal potential of Euphorbia hirta L. against the causal agent of anthracnose disease of mango i.e. Colletotrichum gloeosporioides Penz. Different concentrations of methanolic extract (1.5 - 3%) of E. hirta were tested in vitro against the test fungus and all these extract concentrations caused significant decrease in the colony diameter of C. gloeosporioides. However, 2.5% extract caused maximum (28%) reduction in radial growth of the test fungus. Phytochemical analysis of methanolic extract of E. hirta indicated the presence of glycosides, flavonoids, alkaloids, phlobatannins, tannins and coumarins. This methanolic extract was further partitioned between n-hexane, chloroform, ethyl acetate and n-butanol. In vitro bioassays of these isolated organic fractions were performed and chloroform fraction was found to be the most effective. This effective fraction was then analyzed by GC-MS and linoleic acid, oleic acid and stearic acid were found as the major constituents. The antifungal activity of chloroform fraction of E. hirta against C. gloeosporioides might be attributed to the presence of these compounds.

Keywords: Anthracnose, GC-MS, Mango, Methanolic extract, Phytochemicals

1. Introduction

Mango (*Mangifera indica* L.) is grown under a wide range of agro ecological conditions of the world with an annual production of 111.93 million tones. It is the 2nd largest fruit crop in Pakistan and annually 1658562 tons mangoes are produced in Pakistan (FAO, 2013 [1]). Mango is severely affected by anthracnose disease caused by *Colletotrichum gloeosporioides* Penz. This fungus belongs to class ascomycetes, produces sunken, prominent, dark brown to black rot spots on mango fruit and results in earlier fruit drop (KENGANAL & al. 2010 [2]). In areas where rain and humid weather is prevalent throughout flowering and fruit set, anthracnose can lead to huge loses by damaging the inflorescences and causing drop of immature fruits (PITKETHLEY & CONDE, 2007 [3]; NELSON, 2008 [4]).

Integrated management practices like induced resistance technique to decrease or eliminate the disease incidence in mango is the most practical approach for solving the problem (PLOETZ, 2003 [5]). Use of fungicides is the other effective method in controlling anthracnose but, the irregular treatment of various fungicides result in phytotoxicity, environmental pollution, health hazards and increased resistance to pathogens (ADHIKARY & al. 2013[6]). *Naturally occurring plants can become a substitute to synthetic chemicals as these have strong antifungal activity against mango anthracnose causal agent* (ONYEANI & OSUNLAJA, 2012[7]).

Earlier reports *had been available that test plant Euphorbia hirta* L. *exhibit antibacterial, antifungal, sedative, anti-inflammatory, antimalarial, antiviral and antihypertensive properties* (HORE & al. 2008 [8]; ABUBAKAR, 2009 [9]). *E. hirta* member of family Euphorbiaceae *contains coumarins, flavonoids, tannins, sugars, sterols and triterpenes including phytosterols, cardiac glycosides, diterpenes (phorbol esters), aromatic acids (shikimic and related acids),*

alkaloids and anthocyanins (ANONYMOUS, 2005 [10]). *Due to these characteristics of E. hirta,* this study was conducted to assess the antifungal activity of *E. hirta* specifically against *C. gloeosporioides* with a perspective of controlling anthracnose disease of mango.

2. Materials and Methods

E. hirta was collected from University of the Punjab, Lahore, Pakistan. The plant material was dried in sunlight and stored in polythene bags. Culture of *C. gloeosporioides* was isolated from the diseased mango plant on MEA (Malt Extract Agar) medium. MEA 2% was prepared by adding 2 g of ME and 2 g of agar in 100 mL distilled water. This culture was sub cultured and kept at 4 °C in refrigerator for further use (JAVAID & SAMAD, 2012 [11]).

General procedure

Antifungal activity of *E. hirta* against *C. gloeosporioides* was tested *in vitro.* Fifty grams of dried plant material was soaked in 250 mL methanol and left for three days at room temperature. Material was filtered through an autoclaved muslin cloth after three days. Finally 6 g methanol extract was obtained by evaporating at 35 °C in an electric oven and then diluted by adding 30 mL of distilled water to make 20 % of stock solution. The prepared extract was stored in refrigerator at 4 °C for further analysis.

After four days 60 mL of 2% MEA medium was made in 250 mL flask and autoclaved at 121 °C & 15 lb inch^2 for 30 minutes. Five concentrations of plant material viz. 1.0, 1.5, 2.0, 2.5 and 3.0% v/v concentrations of methanol extract were prepared. Control treatment was without any plant extract. In each concentration Chloromycetin (@ 50 mg 100 mL^{-1} of the medium) was added to avoid bacterial contamination. Twenty milliliter of each medium was poured in 9×9 cm sterilized Petri plate. *In vitro* antifungal bioassay was conducted with this methanolic extract. Five mm mycelial discs were prepared using sterilized cork borer from the tip of seven days old culture of *C. gloeosporioides* and were placed in all experimental Petri plates. Three replicates were made for each treatment. All these plates were incubated at 25 ℃ for one week. After one week incubation the fungal growth diameter was measured for each colony and percentage growth inhibition was measured by using the formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Phytochemical analysis

E. hirta methanolic plant extract was analyzed for different phytochemicals according to the methods of (EDEOGA, 2005 [12]; PAREKH & CHANDA, 2007 [13]).

Bioassay guided fractionation

Hundred grams dried plant material of *E. hirta* was extracted with methanol at room temperature. This extract was evaporated under vacuum on rotary evaporator at 40 °C; give up 11 g gummy mass. This gummy mass of *E. hirta* was further partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol at room temperature by using separating funnel (JABEEN & al. 2013 [14]). This extraction yielded as gummy mass of *n*- hexane (0.67 g), chloroform (0.29 g), ethyl acetate (0.17 g) and *n*-butanol (0.13 g). *In vitro* antifungal activity of these four isolated fractions was studied against *C. gloeosporioides* through agar serial dilution method (SHADOMY, 1991 [15]). Fungal culture was suspended in ME to prepare the inoculums. Two concentrations were made for each organic fraction i.e. 0.17 and 0.26%. Experiment was done by adding appropriate quantity of all crude organic fractions into 2% MEA medium making final volume upto 30 mL. Control medium was without any extract.

GC-MS analysis

Chloroform fraction was found highly effective in previous bioassay so this fraction was subjected to Gas chromatography mass spectroscopic (GC-MS) analysis. GC-MS-QP 2010

chromatograph was used to analyzed the sample separated on (30 m, 0.25 mm, 0.25 μm) DB-5MS capillary column. Applied program temperature were 40°C for 5 min, 40-70 °C at 2 °C/min, 70 °C for 2 min, 70-120 °C at 3°C min^{-1}, 120-150 °C at 5 °C min^{-1}, 150-220 °C at 10 °C min^{-1} and then 220 °C for 2 min, using helium as a carrier gas. The temperatures of detector and injector were 250 °C and 200 °C respectively. Mass detector conditions were: Ionization voltage 70 eV, mass scanning range m/z 29-540 and source temperature 230 ºC. The percentage composition of volatile compounds was computed from GC peak areas. Qualitative analysis was done on a comparison of indices, retention times and mass spectra with the corresponding data in the literature (NIST Library 2010 word software) (KUMAR & al. 2012 [16]).

Statistical analysis

All the data was statistically analyzed by using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test at 5% level of significance (DMRT) (STEEL & al. 1997 [17]).

3. Results and Discussion

Mango is a valuable fruit which plays a significant role in balanced human diet by giving about 64-86 calories of energy (SAUCO, 2002 [18]). Anthracnose is a severe fungal infection of inflorescence and premature mango fruits. This disease needs regular fungicide treatments on vulnerable cultivars of mango during favorable conditions (BALLY, 2006 [19]; NELSON, 2008 [5]). Though, the easiness of use and great efficacy of fungicide is a reason behind their use but excessive use of these results in environmental pollution and most importantly the presence of pesticide residues on food. Therefore, the use of natural product offer great benefits in comparison to chemical pesticides in their mode of action, in maintaining ecological balance and toxicity caused by them (CAWOY & al. 2011 [20]).

In present study antifungal potential of *E. hirta* was examined against phytopathogenic fungus *C. gloeosporioides.* All the applied concentrations (1- 3%) of plant material reduced the colony diameter of test fungus. However 2.5 & 3% concentrations of methanolic extract of *E. hirta* were more effective as they suppressed the test fungus growth upto 28 and 22% respectively. Other concentrations also inhibited the mycelium development of *C. gloeosporioides* (Fig. 1 & 2). Earlier (MOHAMED & al. 1996 [21]) studied the bioefficacy of ethanolic extract of fifty eight malysian plants including *E. hirta* against plant pathogens *C. capsici, Botryodiplodia theobromae, Fusarium pallidoroseum, Aspergillus niger* and *Phomopsis caricae-papayae* using the paper disc diffusion method. All the tested plants showed variable antifungal activity.

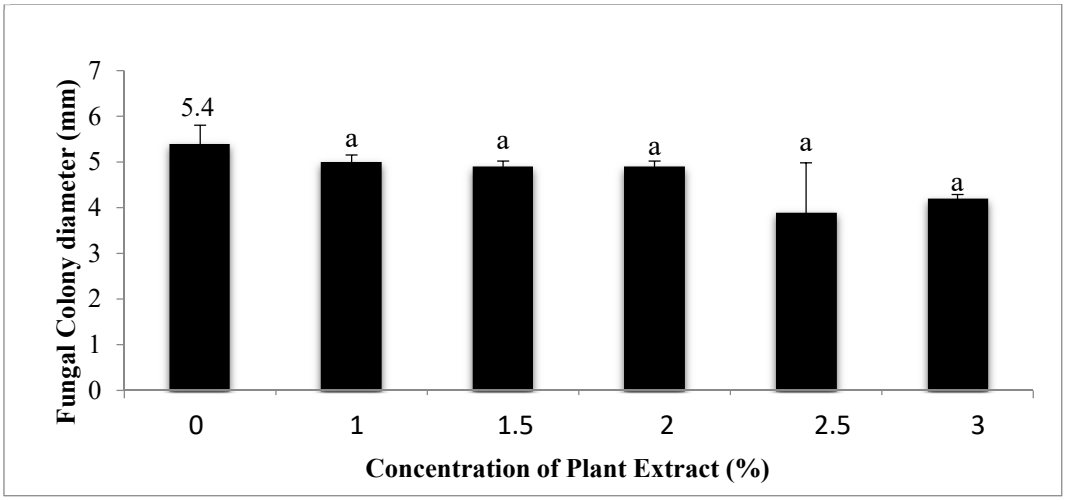

Figure 1: Effect of *E. hirta* plant extracts on *in vitro* growth of *C. gloeosporioides*. Vertical bars show standard error of means of three replicates. Alphabetical letters show insignificant differences as determined by DMR Test.

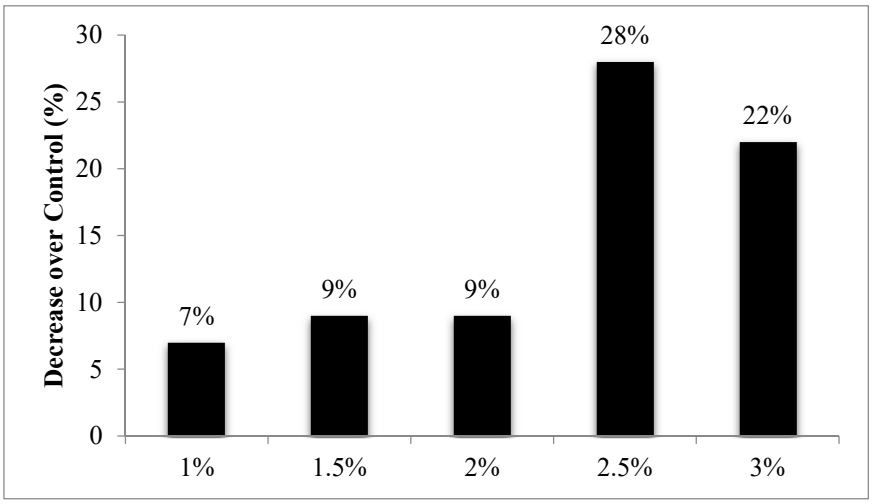

Figure 2: Percentage decrease in *C. gloeosporioides* due to different concentrations of methanolic plant extract of *E. hirta*

Phytochemical analysis of the methanolic plant extract of *E. hirta* indicated the presence of glycosides, flavonoids, alkaloids, phlobatannins, tannins and coumarins (Table. 1). The presence of these secondary metabolites in *E. hirta* might be responsible for its antimicrobial properties as supported by literature (UPADHYAY & al. 2010 [22]).

Table 1: Preliminary phytochemical screening of methanolic extract of *E. hirta* results.

Phytochemical	Expected result	*E. hirta*
Glycosides	Greenish-blue color appears	+
Alkaloids	Orange precipitates or creamish precipitates	+
Terpenoids	Blue-green ring	_
Saponins	Stable persistent froth	_
Flavonoids	Dark yellow color	+
Tannins	Blue-black or brownish-green	+
Phlobatannins	Red precipitates	+
Coumarins	Yellow fluorescence on filter paper detected under ultraviolet (UV) light	+

In vitro bioassays with different organic fractions showed that chloroform fraction was most effective against *C. gloeosporioides* as compared to other fractions. Maximum inhibition was showed by both applied concentrations of chloroform fraction (0.17 & 0.26%) upto 73 and 77% respectively (Fig. 3 & 4). BUSSAMAN & al. (2012 [23]) *in vitro* evaluated the fourteen crude plant extracts against *C. gloeosporioides* and found that ethanolic, chloroform and methanolic leaf extracts of *Piper sarmentosum* exhibited high antifungal potential. Treatment with crude chloroform extract of *Mentha cordifolia* leaves and crude methanol extract of *P. sarmentosum* also reduced the spore germination of *C. gloeosporioides*.

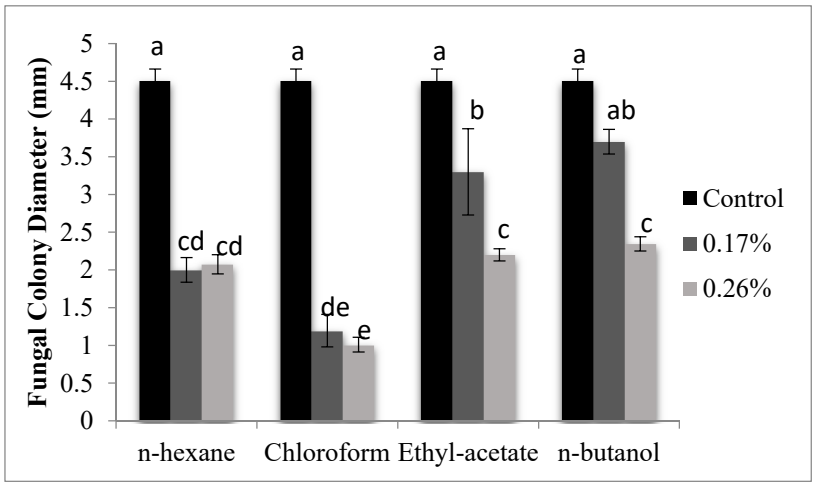

Figure 3: Effect of *E. hirta* extract of different concentrations on *in vitro* growth of *C. gloeosporioides*. Vertical bars show standard error of means of the replicates. Alphabetical letters show significant difference as determined by DMR Test.

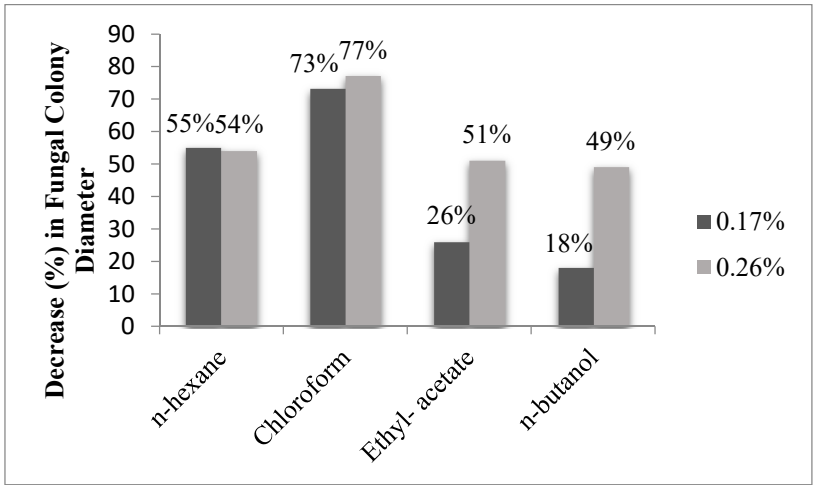

Figure 4: Percentage decrease in *C. gloeosporioides* due to different fractions of *E. hirta* plant extract.

The most effective fraction (chloroform) was then selected for GC-MS analysis and four bioactive phytochemical compounds were identified in this chloroform fraction of *E. hirta* (Table. 2). The identification of phytochemical compounds was based on the molecular weight, molecular formula and peak area. All identified compounds belong to fatty acids group. Among them, linoleic acid and 9-octadecenoic acid methyl ester were unsaturated fatty acids while stearic acid and octadecanoic acid were saturated fatty acids.

Table 2: GC-MS profiling of Chloroform fraction of *E. hirta*

Sr.#	R. Time	Compound name	Molecular Formula	M W	Peak Area%
1	46.383	Linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294	67.10
2	46.650	9-octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	296	55.05
3	48.983	Stearic acid	$C_{18}H_{36}O_2$	284	43.10
4	49.383	Octadecanoic acid	$C_{18}H_{36}O_2$	284	43.10

Octadecadienoic acid and linoleic acid possess antifungal properties as supported by literature (DILIKA & al. 2000 [24]; MCGRAW & al. 2002 [25]; CHANDRASEKARAN & al. 2011 [26]). WALTERS & al., (2004 [27]) also studied the effect of linoleic acid and oleic acid on the growth of plant pathogenic fungi *Pythium ultimum* and *Rhizoctonia solani*. Strong antifungal activity was observed against all the tested fungi. These compounds also possess anti-inflammatory, moisture retentive and antioxidant properties (MAILLARD & al., 2003 [28]).

4. Conclusion

It can be concluded that *E. hirta* has significant antifungal potential against *C. gloeosporioides* and this potential might be exploited in future to isolate potent antifungal compounds. The analogues of the compounds identified from GC-MS analysis can be synthesized to be used as nature friendly biopesticide against anthracnose disease of mango.

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