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Original paper

Antiproliferative Effect of Epilobium parviflorum Extracts on Colorectal Cancer Cell Line HT-29

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Abstract

The *Epilobium* species, rich in various active phytochemicals, have been widely used in folk medicine to treat several diseases including benign prostatic hyperplasia. Despite being demonstrated on some type of cancer cells such as prostate cancer, their potential anti-cancerous role on colorectal adenocarcinoma cells has not been studied yet. According to the World Health Organization (WHO), colon cancer is the third most common form of cancer, resulting over 800 000 deaths every year worldwide. The present study demonstrates the anti-cancerous activity of aqueous and ethanolic *Epilobium parviflorum* extracts in colon cancer cell line HT-29 cells *in vitro*. The both type of extracts reduced the cell viability of HT-29 cells in a dose dependent manner. Gene expression analysis of HT-29 cells demonstrated an increase at apoptotic genes, caspase 3 and caspase 8. Nuclear fragmentation of apoptotic cells was also demonstrated through TUNEL assay as well as immunostaining experiments. On the other hand, same lethal concentrations of *E. parviflorum* extracts were not profound on non-cancerous human fibroblast cell line BJ cells. Our results indicate that *E. parviflorum* may also be used as a therapeutic agent against colon cancers.

Keywords *Epilobium parviflorum*, colon cancer, HT-29 cells, apoptosis

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Introduction

The *Epilobium* genus from Onagraceae family contains more than 200 species distributed all around the world. Among them, *E. angustifolium* L., *E. parviflorum* Schreb. and *E. hirsutum* L. are the most well-known (Vitalone and Allkanjari, 2018), being widely used in traditional medicine to treat various infections, disorders and diseases. For instance, their extracts serve as a remedy in Chinese folk medicine to relieve menstrual disorders while in Native American traditional medicine they have been used to treat infected sores, swellings, and rectal hemorrhages (Granica *et al.*, 2014; Hevesi *et al.*, 2009; Stolarczyk *et al.*, 2013). *E. angustifolium*, *E. montanum* and *E. parviflorum* are also used in Austrian folk medicine to cure prostate, kidney and urinary tract diseases (Vogl *et al.*, 2013). Moreover, their uses in skin and mucosa infections were also reported (Granica *et al.*, 2014; Stolarczyk *et al.*, 2013). In parallel with its use in folk medicine, aerial parts of *Epilobium* species have been demonstrated to be rich in various active phytochemicals such as phytosterols, polyphenols, flavonoids and tannins. Granica *et al.* (2014), in a review of the literature, reported that *Epilobium* sp. contain 103 different compounds. The phytochemical characterization of *E. angustifolium*, *E. parviflorum* and *E. hirsutum* extracts based on UV and MS/MS analyses showed the presence of 38 compounds (Stolarczyk *et al.*, 2013).

In addition to the disorders/diseases mentioned above, *Epilobium* species are most commonly used as infusions to treat benign prostatic hyperplasia (BPH), urethral inflammation and micturition disorders. BPH is the most important urological disorder affecting the aging male population, due to excessive cellular proliferation of prostrate tissues (Vitalone *et al.*, 2001). Although its etiology is not fully known, BPH is regarded as an endocrine disorder caused by an age-related hormone imbalance (Ekman, 1989). Previous studies have demonstrated the inhibitory activity of *Epilobium* spp. extracts against 5 α -reductase (which converts testosterone to dihydrotestosterone) and aromatase (which converts testosterone to estrogens) enzymes, which are involved in etiology of BPH (Ducrey *et al.*, 1997). The main tannin component of *E. parviflorum*, oenothein B, is proven to inhibit the 5 α -reductase. Although BPH can be cured by medical operations, diuretic problems are often reported due to insufficient resection and persistent urinary infections. Therefore, infusions of *Epilobium* herbs still remain an attractive alternative for BPH (Akbulduk, 2002).

In addition to its anti-androgenic effect, an anti-proliferative effect of *Epilobium* extracts have also been demonstrated on various cell lines including androgen-independent prostate cancer cells (PC-3), androgen-sensitive human prostate adenocarcinoma cells (LNCaP), human mammary epithelial cells (HMEC), human astrocytoma cell line (1321N1) and human epithelial prostrate cells (PZ-HPV-7) (Kiss *et al.*, 2006; Stolarczyk *et al.*, 2013; Vitalone *et al.*, 2001; Vitalone *et al.*, 2003b).

Colorectal cancer is one of the deadliest forms of cancer. If detected early, it can be cured by surgical

removal of the tumor however effective treatment options become increasingly limited as disease progresses. In addition to genetic predisposition, aging, smoking and lack of exercise, reports indicate that there is also a link between colon cancer and diet, and at least some colon cancers can be prevented by simply changing diet habits. Taking into consideration of the pathogenesis of prostate cells proliferation and application of *Epilobium* sp. extracts in folk medicine as well as its anti-proliferative activity on various cancer cell lines, in this work we investigated anti-cancerous activity of *E. parviflorum* extracts obtained through two different solvents, water and ethanol on human colorectal adenocarcinoma cell line HT-29.

Materials and Methods

Plant material and extract preparation

E. parviflorum seeds were purchased from The Strictly Medicinal Seeds Company (OR, USA). Seeds were individually sown into 20-cm square pots containing peat. Plants were grown under a regime of 16h-light/8h-dark period at 24°C and 60% relative humidity in a plant growth chamber. They were watered with half strength Hoagland's liquid medium every day. After reaching maturity (2-months old), aerial parts of the plants were harvested, and shade dried at room temperature. Thirty-five grams of the powdered plant material was macerated with 50% ethanol for 24 hours. Having filtered by filter paper, the extraction was repeated two times. The combined filtrate was concentrated under vacuum at 40 °C by rotary evaporator (Buchi R-100, Switzerland) to get the crude aqueous ethanol extract (13.61 g, Yield: 38.89%). Crude ethanolic extract was suspended in distilled water (500 mL), and consequently fractionated with n-hexane (10 × 500 mL), ethyl acetate (10 × 500 mL), n-butanol (10 × 500 mL) and distilled water (10 × 500 mL), using separating funnels. All the fractions were concentrated using rotary evaporator, and obtained 0.23 g hexane fr. (2.21%), 2.09 g ethyl acetate fr. (20.1%), 2.16 g n-butanol fr. (20.77%) and 5.095 g aqueous fr. (48.99%) (Ayaz *et al.*, 2014).

Cell Culture

Human colon cancer cell line HT-29 and noncancerous human fibroblast cell line BJ were purchased from ATCC (VA, USA). The cells were plated into T-75 tissue culture treated flasks (Corning, NY, USA) with DMEM (Gibco, MA, USA) medium supplemented with 10% fetal bovine serum (FBS) (Biological Industries, VT, USA), 1% antibiotic-antimycotic (Gibco), 1% L-glutamine (Gibco) and maintained at 37 °C in a 5% CO₂ humidified environment. The cells were passaged using trypsin (Gibco) once the plate became confluent. Before the plant extract application, the cells were seeded into a 96-well plate (Corning) as 10⁴ cells in each well and then incubated for 24 hours before the application. Powdered plant extracts of *E. parviflorum*, obtained using 2 different solvents (water and ethanol), were dissolved in distilled water. Ethanolic plant extract was diluted in the medium at a concentration of 500, 200, 100, 10 µg/ml. Aqueous

plant extract was diluted to 100, 50, 30, 10 µg/ml in the medium. Dilutions were then applied on HT-29 and BJ cells and incubated for 48 and 72 hours.

MTT (Cell Proliferation Assay)

HT-29 and BJ cells were plated into 96-well plates at 10^4 cells/well. The MTT (Millipore, MA, USA) solution was prepared at a concentration of 5 mg/ml. Following the application of plant extracts, 30 µl of stock MTT was added into each well on day 2 and day 3. Plates were incubated at 37 °C for 4 hours. After incubation, the medium was removed and 100 µl of DMSO was added into each well to dissolve the purple formazan. Using a spectrophotometer, formazan was read at 570 nm and the background was read at 690 nm. Based on the control group, the percentage of viable cells was calculated using the absorbance values found. The experiments were conducted with three biological and three technical replicates. The IC₅₀ values were calculated by GraphPad Prism 7 software.

RNA Isolation and cDNA Synthesis

Total RNA isolation was performed using GeneJET RNA Purification Kit (Thermo Scientific, MA, USA) according to the manufacturer's protocol. Any genomic DNA contamination was removed using RNase-Free DNase enzyme (Promega, WI, USA). RNA amounts were determined with BioDrop µLITE (Biodrop, UK). RNA samples were stored at -80 °C. 500 ng of total RNA from all samples were transcribed into cDNA using iScript cDNA Synthesis Kits (Bio-Rad, CA, USA) according to the manufacturer's protocol. The resulting cDNA samples were stored at -20 °C.

Quantitative PCR Assay

The level of gene expression was measured using LightCycler 96 system (Roche, Germany) and Maxima SYBR Green qPCR Master Mix (Thermo Scientific). Sequences of *Caspase-3*, *Caspase-8*, *p53*, *Bax*, and *Gapdh*, genes were obtained from NCBI database and the primers (Table 1) were designed using NCBI primer software. The specificity of the primers was confirmed by a single band image on the agarose gel. *Gapdh* gene was used as the reference. Gene expression levels were demonstrated using $\Delta\Delta CT$ method (Livak and Schmittgen, 2001).

Table 1. Primer sequences used in qPCR

| Gene | Primer (5'-3') |
|------------------|-----------------------------------|
| <i>Caspase-3</i> | Forward: GCACTGGAATGACATCTCGGTC |
| | Reverse: ATGGCTCAGAAGCACACAAACA |
| <i>Caspase-8</i> | Forward: ATGAAAAGCAAACCTCGGGGATAC |
| | Reverse: GTGCCATAGATGATGCCCTTGTC |
| <i>p53</i> | Forward: CTGCTCAGATAGCGATGGTCTGG |
| | Reverse: AACCTCAGGCGGCTCATAGG |
| <i>Bax</i> | Forward: AGTGCTCAAGCGCATCGGG |
| | Reverse: TGTCCACGGCGCAATCA |
| <i>Gapdh</i> | Forward: GGGCTCTCCAGAACATCATCCC |
| | Reverse: GGTCACCACTGACACGTTG |

Tunel and LDH tests

The cells were plated into six-well tissue culture plates (Corning) at 10^6 cells/well and treated with IC₅₀

concentration of plant extracts. The next day apoptotic and necrotic cells were detected by flow cytometry (BD FACS Canto II) using APO-BrdU TUNEL Assay Kit (Invitrogen) and LDH cytotoxicity assay kit (Pierce) according to the manufacturer's protocol, respectively.

Statistical Analysis

The statistical significance of the MTT test results was determined by One-way ANOVA test (SPSS, IBM, USA) and Bonferroni test. qRT-PCR results were evaluated using the Holm-Sidak method in Sigma Stat3.5 software. The statistical significance of the LDH test results was determined by student's t-test. Values less than 0.05 (*) were considered to be statistically significant.

Results and discussion

Epilobium parviflorum extracts reduce cell viability of HT-29 cell line

The human colon cancer cell line HT-29 and human noncancerous fibroblast cell line BJ were treated with 2 different *E. parviflorum* extracts which were extracted with water and ethanol. Each extract were given to the cells at various concentrations; 10, 30, 50, 100 µg/ml for water and 10, 100, 200, 500 µg/ml for ethanol. Viability of HT-29 cells was significantly reduced in a dose dependent manner 48 hr and 72 hr after administration of each extract (Figure 1, 2). Water and ethanol extracts reduced HT-29 cell viability up to 50% when applied at a dose of 43,6 µg/ml and 191.0 µg/ml, respectively (Figure 1A, 1C; Figure 2A, 2C; Figure 3). On the other hand, notably these concentrations did not significantly reduce the viability of noncancerous BJ fibroblast cells (Figure 1B; Figure 2B).

The effect of Epilobium parviflorum extracts on gene expression profile

To further explore the effects of *Epilobium* extracts on HT-29 cells, the apoptotic gene expression profile of HT-29 and BJ cells were assessed 48 hr after administration of both extracts at their lethal concentration (LD₅₀). For this purpose, mRNA expression levels of three different apoptotic genes (*Caspase-3*, *Caspase-8*, *Bax*), as well as the *p53* gene, were demonstrated through qPCR (Figure 4). Results showed that both *E. parviflorum* extracts increased the expression level of apoptotic genes *Caspase-8* (1.72 ± 0.16-fold for ethanol, 1.82 ± 0.35-fold for water extracts) and *Bax* (1.74 ± 0.04-fold for ethanol, 2.45 ± 0.27-fold for water extracts) at different but significant degrees in HT-29 cells (Figure 4). However, both extracts significantly reduced the expression level of *p53* gene (0.57 ± 0.06-fold for ethanol, 0.41 ± 0.06-fold for water extracts) in HT-29 cells. However, the increase observed at apoptotic gene expression levels of HT-29 cells was not seen in BJ fibroblasts. Instead, their expression levels were reduced (0.34 ± 0.03, 0.52 ± 0.04, 0.37 ± 0.02-fold for *Caspase-8*, *Bax* and *Caspase-3*, respectively) (Figure 4). Aqueous extract, however, stimulated the expression of apoptotic genes (2.69 ± 0.34, 3.93 ± 0.17, 2.1 ± 0.14 for *Caspase-8*, *Bax* and *Caspase-3*, respectively) in BJ fibroblast cells which is consistent with 72 hr cell viability assays of aqueous extract on BJ fibroblast cells.

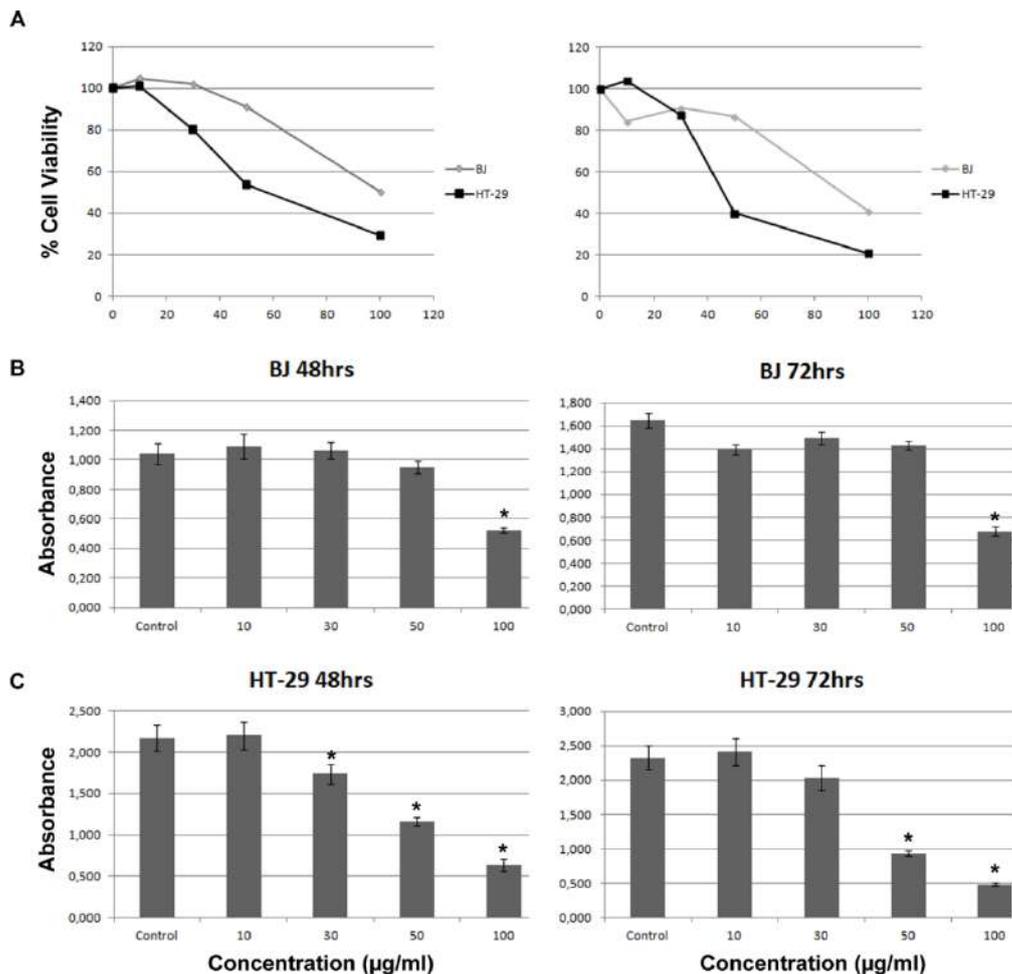


Figure 1. Cell viability percentage of HT-29 and BJ cells after 48 h (left panel) and 72 h (right panel) treatment with aqueous extract of *E. parviflorum* at different concentrations ($\mu\text{g/ml}$) (A). Absorbance values from MTT assay of BJ (B) and HT-29 (C) cells after 48 h (left panel) and 72 h (right panel) treatment with aqueous extract of *E. parviflorum* at different concentrations ($\mu\text{g/ml}$). Values are expressed as mean \pm S.D. ($n = 3$). Asterisk (*) indicates significant differences ($p < 0.05$).

Epilobium parviflorum extracts induce apoptotic cell death of HT-29 cells

In addition to profiling the apoptotic gene expression in HT-29 cells treated with *E. parviflorum* extracts, another morphological sign of apoptosis (nuclear fragmentation) in these cells was demonstrated by performing TUNEL assay. After labeling the 3'-OH edges of fragmented nuclear DNA through a TUNEL assay kit, flow cytometry analysis showed that 6.9% and 13.3% of HT-29 cells were fated to apoptosis when treated with water and ethanol extracts respectively (Figure 5A). Nuclear fragmentation in these cells was also demonstrated through immunostaining under a fluorescence microscope (Figure 5B). Moreover, LDH assay showed that both *E. parviflorum* extracts slightly but significantly stimulated the necrosis in HT-29 cells compared to untreated control cells (Figure 6).

In consistent with being widely used in folk medicine, *Epilobium* spp. have been demonstrated to have various therapeutic effects such as analgesic (Pourmorad *et al.*, 2007; Tita *et al.*, 2001), antiandrogenic (Allkanjari and

Vitalone, 2015), anti-inflammatory (Hevesi *et al.*, 2009; Vogl *et al.*, 2013), antimicrobial (Battinelli *et al.*, 2001; Jones *et al.*, 2000), antioxidant (Stajner *et al.*, 2007; Steenkamp *et al.*, 2006), and antitumor activity (Vitalone *et al.*, 2003a; Voynova *et al.*, 1991) in various *in vitro* as well as *in vivo* animal studies. Anti-proliferative effect of *Epilobium* extracts on healthy (PZ-HPV-7) and cancerous prostate cells lines (LNCaP) was also reported by different groups and this effect is mainly associated with its ellagitannins; oenothien A and B which inhibit aromatase and 5- α -reductase enzymes involved in etiology of benign prostatic hyperplasia (Ducrey *et al.*, 1997; Lesuisse *et al.*, 1996; Piwowarski *et al.*, 2017; Yoshida *et al.*, 2018). However, demonstration of anti-tumorigenic and anti-proliferative effect of *Epilobium* extracts on leucosis (P388) (Voynova *et al.*, 1991) and astrocytoma (1321N1) (Vitalone *et al.*, 2003b) cell lines respectively indicates that this effect is not specific for prostate cells. Which biological compounds of *Epilobium* extracts are responsible for this anti-cancerous effect is not known yet,

even though there are studies demonstrating the anti-cancerous effect of ellagitannins on different cancer types (Ismail *et al.*, 2016; Wang *et al.*, 1999). Anti-cancerous effect of *Epilobium* extracts on various cancer cell lines

motivated us to investigate the effects of *E. parviflorum* on human colon cancer cell line HT-29 by leaving the question of which active molecule is responsible from this effect to further studies.

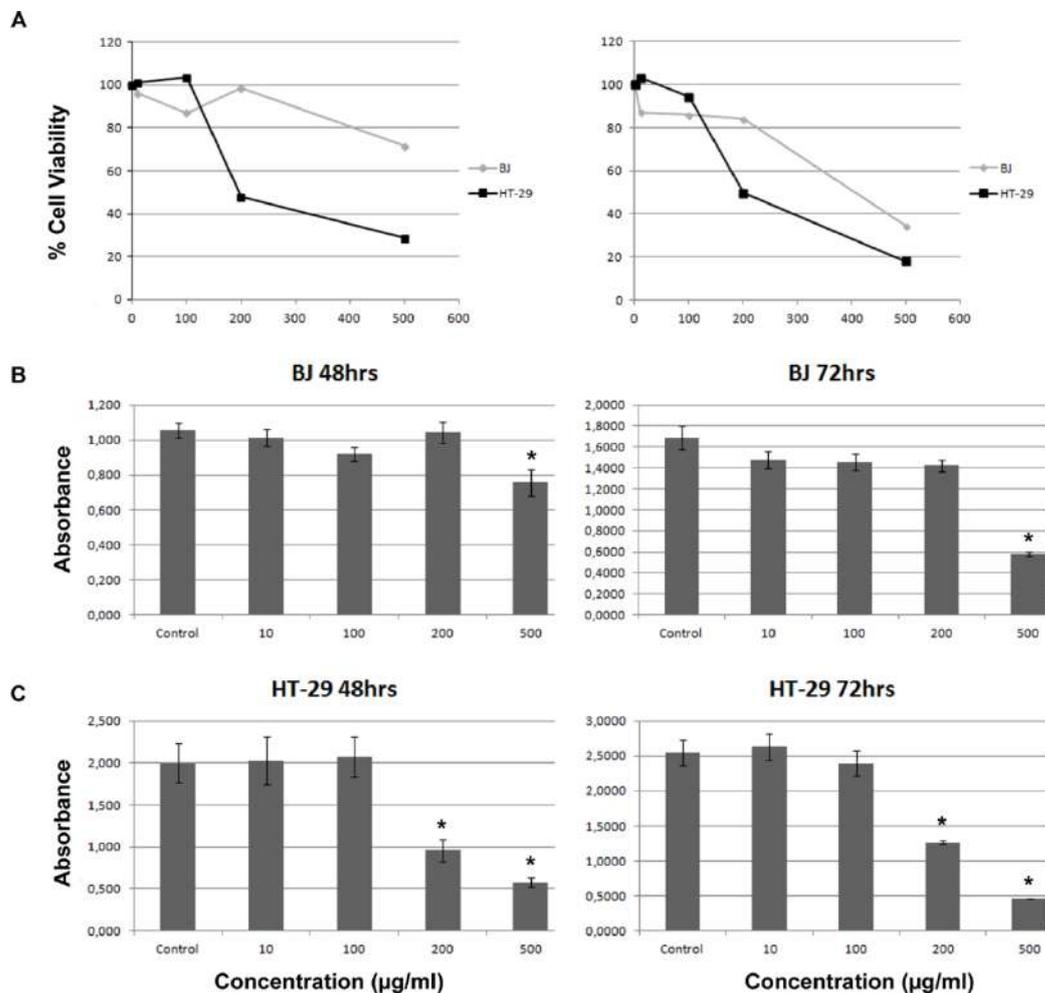


Figure 2. Cell viability percentage of HT-29 and BJ cells after 48 h (left panel) and 72 h (right panel) treatment with ethanolic extract of *E. parviflorum* at different concentrations (µg/ml) (A). Absorbance values from MTT assay of BJ (B) and HT-29 (C) cells after 48 h (left panel) and 72 h (right panel) treatment with aqueous extract of *E. parviflorum* at different concentrations (µg/ml). Values are expressed as mean ± S.D. (n = 3). Asterisk (*) indicates significant differences (p < 0.05)

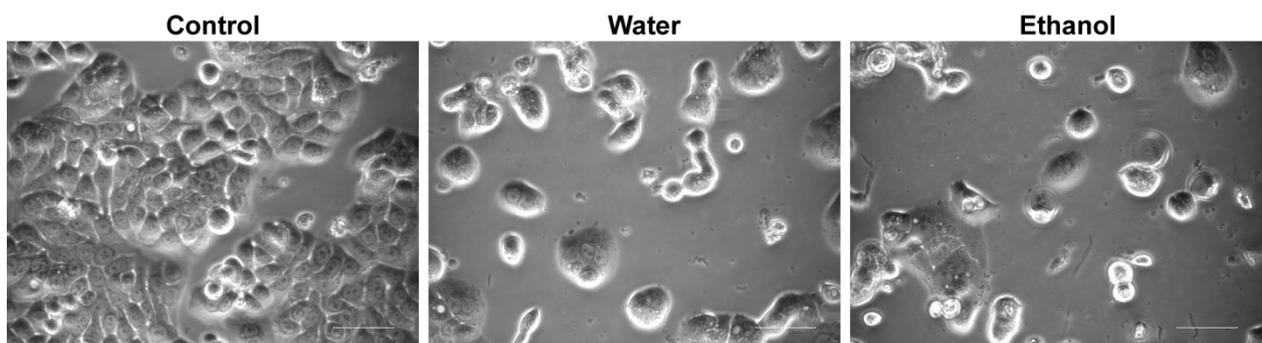


Figure 3. Reduced viability of HT-29 cells treated with aqueous and ethanolic extracts of *E. parviflorum*. Scale bars, 50µm.

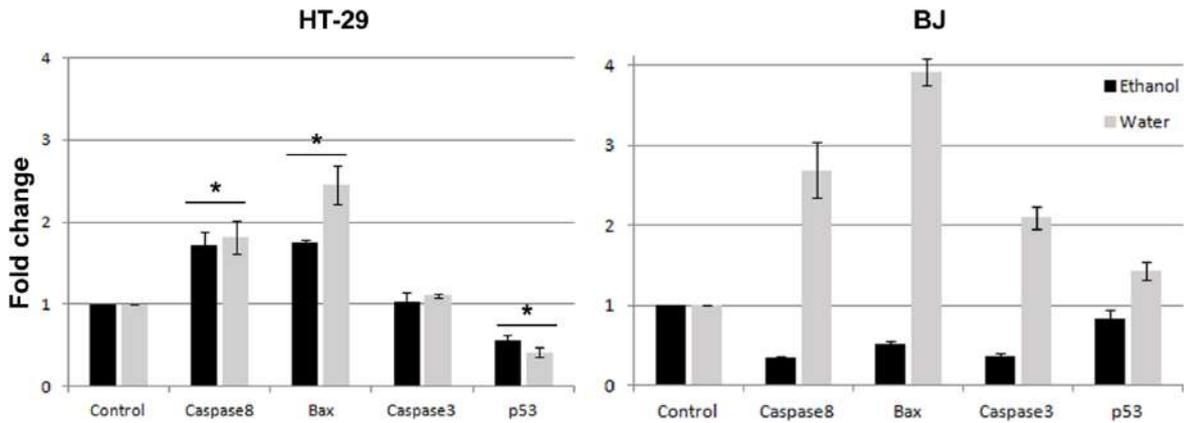


Figure 4. Apoptotic gene expression profile of HT-29 (left panel) and BJ (right panel) cells treated with ethanolic (black bars) and aqueous (grey bars) extracts of *E. parviflorum* for 48 hours. Data are represented as fold change relative to untreated control cells. Each gene expression was normalized according to *Gapdh* expression. Values are expressed as mean \pm S.D. (n = 3). Asterisk (*) indicates significant differences ($p < 0.05$).

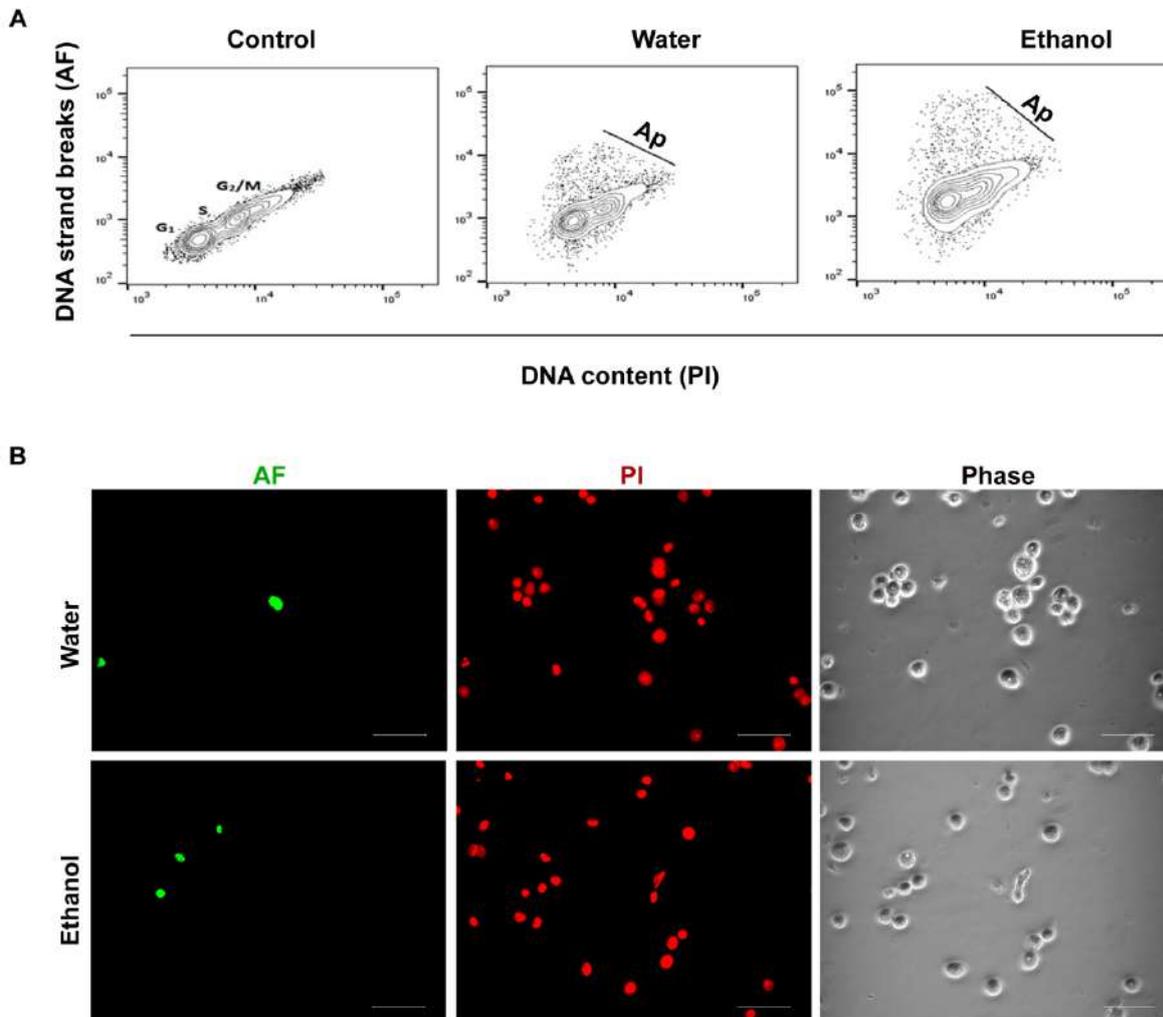


Figure 5. Demonstration of apoptotic HT-29 cells treated with *E. parviflorum* extracts through FACS (A) and fluorescence microscope (B). Cells were analyzed after 24 h extract treatment. DNA strand breaks in apoptotic cells were labeled with Alexa Fluor 488 (AF; green). Cell nuclei of all cells were labeled with propidium iodide (PI; red). Scale bars, 50 μ m.

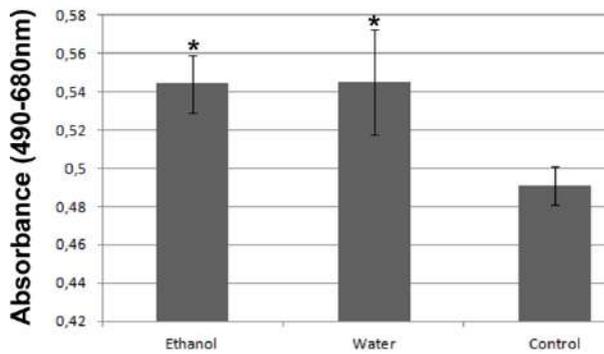


Figure 6. Necrosis of HT-29 cells treated with *E. parviflorum* extracts. Cells were analyzed after 24 h extract treatment. Values are expressed as mean \pm S.D. (n = 3). Asterisk (*) indicates significant differences ($p < 0.05$).

Our results from this study demonstrated that different *E. parviflorum* extracts, which are prepared by using water and ethanol solvents, reduced the viability of HT-29 cells significantly (Figure 1; Figure 2; Figure 3). The potency of plant extracts depends on the extraction method. The most potent extraction solvent also depends on the plant species to be studied as well as the active molecule to be isolated. In the present study the aqueous extract (43,6 $\mu\text{g/ml}$) of *E. parviflorum* is more potent than the ethanolic extract (191.0 $\mu\text{g/ml}$) on HT-29 cells (Figure 1; Figure 2). This may come from the different degree of polarity of these solvents. Ethanol is less polar than water and more hydrophilic components are known to be retained in aqueous extracts compared to ethanolic extracts (Abarca-Vargas *et al.*, 2016; Rauf *et al.*, 2018). On the other hand, it should also be noted that ethanol is a better solvent for organic compounds than the water is. Another reason why water extract was more potent may be due the fact that we re-suspended both extracts in water which may not dissolve the non-polar components extracted with ethanol. It is also notable that these concentrations reducing the viability of HT-29 cells up to 50% did not have much effect on healthy fibroblast cells. qPCR results showed that both extracts increased the expression of apoptotic genes *Caspase-8* and *Bax* (Figure 4). The tunel assay, in which 3'-OH ends of fragmented DNAs are fluorescently labeled, also verified cell death through apoptosis (Figure 5). Apoptosis can be initiated by intrinsic and extrinsic pathways. Extrinsic pathways activate Caspase-8 whereas intrinsic pathways activate Caspase-9; both of which are called initiator factors, and can separately induce executioner factors to initiate apoptosis (McIlwain *et al.*, 2013). Bax is also a regulator of intrinsic pathways of apoptosis and induces mitochondrial dysfunction by mitochondrial membrane permeabilization (Liu *et al.*, 2016). Permeabilization of mitochondrial membrane causes the release of cytochrome c which in turn stimulates apoptosis through Caspase-9 (Liu *et al.*, 2016). Our qPCR results indicate that *E. parviflorum* extracts may induce apoptosis both through intrinsic and extrinsic pathways. Consistent with our

results, Stolarczyk *et al.* (2013) also demonstrated the apoptotic effect of different *Epilobium* extracts on human hormone dependent prostate cancer cells through intrinsic pathways (Stolarczyk *et al.*, 2013). In addition to stimulation of apoptosis, we also found that HT-29 cells treated with *E. parviflorum* extracts also underwent necrosis (Figure 6). The observed necrosis could be secondary necrosis due to the toxic content of apoptotic cells which are not cleaned out by macrophages in *in vitro* culture (Silva, 2010). On the other hand, there are also reports showing that the same compounds can even cause apoptosis and necrosis simultaneously (Arakawa *et al.*, 2015). We also showed that expression of *p53* gene is downregulated in HT-29 cells treated with *E. parviflorum* extracts. Wild type *p53* is a tumor suppressor protein and *p53* gene is mutated in many cancer cell types including HT-29 cells (Freed-Pastor and Prives, 2012; Levine and Oren, 2009). In HT-29 cells, *p53* gene has a R273H mutation (Rodrigues *et al.*, 1990). There are many reports demonstrating that R273H substitution in *p53* is a gain of function mutation and providing oncogenic functions to the cells to drive invasion and metastasis (Lang *et al.*, 2004; Muller *et al.*, 2013; Olive *et al.*, 2004; Sigal and Rotter, 2000). Based on the literature, reduction of mutant *p53* expression, as we saw in our study, may be useful to reduce the oncogenic properties of HT-29 cells as well.

Even though no clinical study verifying the anti-cancerous effects of *Epilobium* extracts has been conducted yet, and more *in vivo* studies are still required, the results of our study and others suggest that *Epilobium* extracts can be used to eliminate cancerous cells including colon carcinoma cells at least *in vitro* conditions.

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Author contribution statement

MAA and EA conceived the study; TS, EA, NE and MAA conducted the experiments. EA and MAA wrote the manuscript, and all authors read, edited and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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