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Veronica crista-galli Steven and *Veronica persica* Poir. as anticancer and antioxidant plants *in-vitro*

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ABSTRACT

The antioxidant activities and reducing power capabilities of *Veronica crista-galli* and *Veronica persica* were evaluated using DPPH radical scavenging assay, total antioxidant activity and MTT assay at incubation times of 24, 48 and 72 hours. A water fraction of methanolic extract of *V. crista-galli* with the highest amount of total flavonoid (16.19 ± 0.001 mg of QE/g of dried plant) demonstrated stronger antioxidant effect than *V. persica* extract with the highest content of phenolic compounds (60.75 ± 0.0013 mg GAE/g of dried plant). The IC_{50} values of *V. persica* and *V. crista-galli* extracts were found to be 153.7 and 79.64 µg/mL, respectively. *V. crista-galli* possessed considerable inhibitory effect at lower concentrations on MCF-7 and Hela cell lines by increasing incubation time. There was no cytotoxic effect observed in the normal HDF cell line. This study recommends that the water fraction of the methanolic extracts of *V. persica* and *V. crista-galli* with *in vitro* antioxidant and selective anti-proliferative activities can support the popular uses of *Veronica* species in traditional medicine as effective natural remedies.

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1. Introduction

Veronica L. (Plantaginaceae) with a Persian name 'Sizaab' is a genus comprising 450 species found in temperate regions of both hemispheres (Ghahreman et al., 2006). *V. persica* is an annual or perennial herb that mainly reproduces through its seeds. The leaves are paired on the lower stem and alternately arranged on the upper parts. The plant has weak stems that form a dense, prostrate groundcover. The flowers are sky-blue having dark stripes and white centres with one vertical plane of symmetry. *V. persica* can be distinguished from similar species by its heart-shaped fruit with two widely-separated lobes. In addition, *V. crista-galli* is an annual herb that is up to 40 cm tall. The cup is light blue. The time of flowering of this plant species is winter to summer and generally occurs as a weed in fields, margins of roads, gardens and ruined areas. Its geographical dispersion is in all parts of the world and in Iran this plant grows widely in the north, northwest, west and center regions of the country (Fischer, 1981). According to the plant names list (http://www.theplantlist.org), *Veronica crista-galli* Steven has a synonym of *Odicardis Cristal-galli* (Steven) Raf. The phytochemistry of the genus has been studied extensively and earlier investigations on *Veronica* species have resulted in isolation of mainly iridoid glucosides, benzoic and cinnamic acid esters of catalpol, some phenylethanoid and flavonoid glycosides. Isolated flavonoids were found as flavone glycosides, often with additional hydroxyl substitution at C-6 or C-8 of the A-ring. Acylation of the sugars is another characteristic feature of some of the glycosides (Harput et al., 2011). *Veronica* species are of paramount interest due to their wide use in folk medicine of many countries worldwide for the treatment of a wide range of disorders; in respiratory diseases against cough or as expectorant, antiscorbutic, diuretics, nervous system malfunctions, and for wound-healing properties (Živković et al., 2014). In spite of their widespread traditional uses, there are few physiological evidences to approve any claim of therapeutic values for *Veronica* species. A few studies have confirmed that certain *Veronica* species have remarkable bioactivities, such as antibacterial (Gusev et al., 2012; Stojković

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et al., 2013), antioxidant (Živković et al., 2012), anti-inflammatory (Grundemann et al., 2013) and cytotoxic effects (Saracoglu and Harput, 2012; Živković et al., 2014; Ignjatović et al., 2015). Referred to biological properties, numerous studies on the identification and exploitation of the various secondary metabolites of the genus have been reported so far (Albach et al., 2005; Taskova et al., 2006) and most of these reports seem to be beneficial for human health.

This study was planned to assess the *in vitro* antioxidant and anti-proliferative properties of water fraction of methanolic extracts of two *Veronica* species (*V. persica* and *V. crista-galli*) which are traditionally used for the treatment of cancer and other diseases related to the oxidative stress. The literature contains only a few reports of *in vitro* and *in vivo* antioxidant activity of *Veronica persica* (Harput et al., 2002; Harput et al., 2011). Nevertheless, to the best of our knowledge, there are no reports on biological activities of *V. crista-galli*, with the exception of one study on biodiversity of this plant (Vahdati et al., 2014). The other objective of this study was to profile the phenolic and flavonoid compounds of the aforementioned *Veronica* species, as well.

2. Experimental

2.1. Reagents and chemicals

1,1'-Diphenyl-2-picryl hydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma Chemical Co. (USA). Gallic acid, tert-butyl-4-hydroxy toluene (BHT) and Folin-Ciocalteu reagent were purchased from Merck Co. (Germany).

2.2. Plant samples and extraction

The aerial parts of two *Veronica* species were collected during their flowering period in June 2017 from Northern Province of Iran (Babel-kenar Forest, Mazandaran). Plant materials were taxonomically classified by Dr. A. Naqinezhad and the voucher specimens of the plant samples were deposited at the Herbarium collection of the University of Mazandaran, Babolsar. The voucher specimen numbers were VR 2408 and VR 2407 for *Veronica persica* and *Veronica crista-galli*, respectively. Each air-dried plant material was coarsed to a fine powder (1 g) and extracted with MeOH (10 mL) at 40 ºC in N-Biotek shaker incubator for three times. The combined extracts of each species were evaporated under vacuum to give crude methanol extracts. Then, the extracts were separately dissolved in water and partitioned with petroleum ether to remove chlorophylls. Water extracts were tested for further bioactivity and phytochemistry studies. The dried sample of each extract was weighed to determine the yield (w/w%) of soluble constituents and stored at 4 °C until use.

2.3. Total phenols determination

Total phenolic content of this study plants was determined using the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007). Accordingly, 1.6 mL of the extract at different concentrations (10-250 μg/mL) was made up with distilled water. In the next step, 100 μL of Folin-Ciocalteu reagent was added to each solution and the resulting mixture gently mixed. After 3 minutes, 300 μL of sodium carbonate (700 mM) was added. The contents were mixed and allowed to stand for 2 hours. The optical density of the blue coloured samples was measured at 765 nm using Spectrum sp-2100 UV instrument. As standard, gallic acid (1 mg/mL) was used.

2.4. Total flavonoid determination

Aluminium chloride colorimetric method was used for flavonoids determination (Mohadjerani, 2012). In our suggested procedure, each plant extract (in methanol) was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of potassium acetate (1 M) and 2.8 mL of distilled water. The obtained solutions remained at room temperature for 30 min; the absorbance of each mixture was measured at 415 nm using Spectrum sp-2100 UV instrument. The calibration curve was prepared by preparing methanol quercetin solutions over the linear range of 5-30 μg mL^{-1}.

2.5. DPPH$^{\cdot}$ scavenging activity assay

The DPPH$^{\cdot}$ test is based on the capability of antioxidants to quench the radical cation DPPH• (2,2'-diphenyl picrylhydrazyl). The purple coloured radical changes to yellow-coloured reduced DPPH• after the reaction with a radical scavenger. 1 mL of a 0.1 mM solution of DPPH radical in methanol was added to 3 mL of the extract at different concentrations. The mixture was shaken vigorously and the absorbance was measured at 517 nm using a spectrophotometer after 30 min incubation at room temperature in the dark (Salmanian et al., 2014). Inhibition of the DPPH free radical in percent (I%) was calculated by the following formula:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100 \qquad \text{(Eqn. 1)}$$

Where, A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. BHT and L-Ascorbic acid (AA) were used as positive controls.

2.6. Reducing power activity assay

The determination of reducing power of the prepared organic extracts was carried out as described

Table 1
Yield, phenolic, flavonoid content and DPPH· scavenging ability of the *Veronica* species.

Tested species	Yield of extraction (w/w%)	mg GAE / g of dried plant	mg QE / g of dried plant	IC_{50} values (µg/mL)
V. persica	14.20%	60.75±0.043	10.59±0.001	153.70
V. crista-galli	13.15%	53.58±0.034	16.19±0.002	79.64
BHT (standard)		-	-	16.26
AA (standard)		-	-	12.96

by Jayanthi and Lalitha (2011). Briefly, different amounts of extracts (12.5-800 µg) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and $K_3Fe(CN)_6$ (2.5 mL, 1%). The mixture was incubated at 50 °C for 30 min. A portion (2.5 mL) of trichloroacetic acid solution (10%) was added to the mixture. Then, 2.5 mL of this solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was then measured at 700 nm. The measurement was compared to the standard curve of prepared ascorbic acid solution.

2.7. Determination of total antioxidant capacity

The assay for the determination of the total antioxidant capacity of the plant extracts was based on the reduction of Mo (VI) to Mo (V) and subsequent formation of a green phosphate/Mo(V) complex in acidic medium (Chahmi et al., 2015). In this relation, a total volume of 1 mL extract was added to 1 mL of reagent solution which consisted of 0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate. The mixtures were incubated at 95 °C for 90 min. The absorbance was finally measured at 695 nm. The total antioxidant activity was expressed as the number of equivalence of ascorbic acid and BHT.

2.8. Cell culture

The human cervical carcinoma cell line (Hela, NCBI-115), estrogenic receptor positive human breast cancer cell line (MCF-7, NCBI C135) and human dermal fibroblast cells (HDF, NCBI C161) were purchased from the National Cell Bank, Pasteur Institute of Iran. The HDF normal cell line was grown in DMEM and the cancerous cell lines were cultured in RPMI 1640 medium. The cells were supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin.

2.9. *In vitro* anti-proliferative effects of *Veronica* extract

To assess *in vitro* anti-proliferative effects of *Veronica* extract, a method described by Mosmann (1983) was used with some modification. The method is based on metabolization of MTT to blue formazan by mitochondrial dehydrogenases in living cells. Accordingly, cells were first preincubated in a 96-well plate at a density of 1×10^4 cells per well for 24 h in a humidified atmosphere of 5% CO_2 in air at 37 °C. After 24 h, the cells were treated with *Veronica persica* and *Veronica crista-galli* extracts over various concentration ranges of 38-614 µg mL^{-1} and 19-318 µg mL^{-1}, respectively. Cells were then reincubated for 24, 48 and 72 h. Thereafter, MTT reagent (5 mg/mL) was added to each well and plates were incubated for additional 4 h at 37 °C. The media were then removed, and the intracellular formazan product was dissolved in 150 µL of acidified β-isopropanol. The absorbance was then measured at 570 nm and the percentage of viability was calculated when compared to untreated control. Results were expressed as half-maximal inhibitory concentration of proliferation (IC_{50}) in µg/mL concentration.

2.10. Statistical analysis

Experimental results are presented as means ± SD, and all measurements and analyses were carried out in triplicate. Excel 2010 and SPSS V. 21 statistical package were used for the statistical and graphical evaluations in this study. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons and the Student's t-test. All p-values < 0.05 were considered significant.

3. Results and Discussion

3.1. Total phenolic and flavonoids contents

Phytochemical investigation of the *Veronica* genus revealed the importance of phenolic constituents for the scavenging activity (Harput et al., 2011). Total phenol compounds, as determined by Folin-Ciocalteu method, are reported as gallic acid equivalents (GAE) regarding the corresponding standard curve (y=0.0455x+0.0008, r^2=0.992). The total phenolic contents in water extract of *Veronica persica* were higher than that obtained from the extract of *Veronica crista-galli* (Table 1).

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are remarkable. As shown in Table 1, the total flavonoid contents (presented as QE) were higher in *Veronica crista-galli* (16.19 mgQE g^{-1}) with respect to *Veronica persica* (10.59 mgQE g^{-1}) considering the respective standard curve (y=0.0138x+0.0067, r^2=0.991).

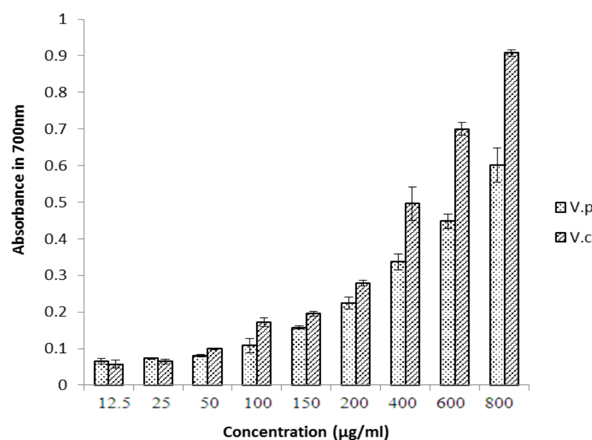

Fig. 1. Fe^{3+}-reducing power potential of water fractions of *Veronica persica* (*V.p*) and *Veronica crista-galli* (*V.c*).

3.2. DPPH. scavenging activity

In this study, water extracts of *Veronica* were investigated for their antioxidant activity with DPPH. scavenging assay. Although the investigated extracts of *Veronica* species exhibited a significant dose dependent inhibition of DPPH. activity, the activity was not stronger than that of the standard compounds (BHT and acid ascorbic, see Table 1). The concentrations required to scavenge 50% of DPPH. radicals were 153.70 and 79.64 µg/mL (Table 1), while the IC_{50} values of BHT and acid ascorbic were 16.26 and 12.96 µg/mL.

3.3. Reducing power

Different studies have indicated that the reducing capacity of bioactive compounds is directly associated with the antioxidant activity. The reducing power of a compound generally depends on the presence of molecules representing an antioxidant potential by donating a hydrogen atom to break the free radical chain. The obtained results showed an increase in reducing power of *Veronica* extracts in a concentration dependent manner. The reduction power varied over the range 0.057-0.907 and 0.065-0.601, both in terms of optical density units, for *Veronica crista-galli* extract and *Veronica persica* extract at the same concentration range, respectively (Fig. 1).

3.4. Total antioxidant capacity

Total antioxidant capacity (TAC) of the water extracts was determined using phosphomolybdate assay. The assay is based on the fact that molybdenum (VI) is reduced to molybdenum (V) in the presence of a reducing agent which is typically an antioxidant. Many natural products, including phenols and flavonoids, can cause this reduction. As shown in Fig. 2, the two extracts exhibited different levels of activity in a concentration-dependent manner. The water extract of *V. crista-galli* showed a much higher activity than the *V. persica* extracts in same concentration.

3.5. *In vitro* antiproliferative effects of *Veronica* extracts

Although *Veronica* species have been widely used in folk medicine as anticancer agents (Harput et al., 2002), their pharmacological effects have not been fully investigated. In this study, cell lines were treated with different concentrations of *V. persica* (38-614 µg/mL) and *V. crista-galli* (19-318 µg/mL) extracts for 24, 48 and 72 h. The water fractions of both *Veronica* species inhibited the proliferation of Hela and MCF-7 cells in a dose and time-dependent manner. *V. crista-galli* at lower concentrations possessed considerable inhibitory effect on two mentioned cancerous cell lines by increasing incubation time. Among all the cell lines studied in three times of incubation, the highest IC_{50} value for fibroblast cell line (HDF) and the lowest rate one for cervical cancer cell line (Hela) were observed, respectively (Table 2). Taking into account the previous reports on stringent endpoint criteria with IC_{50}-values

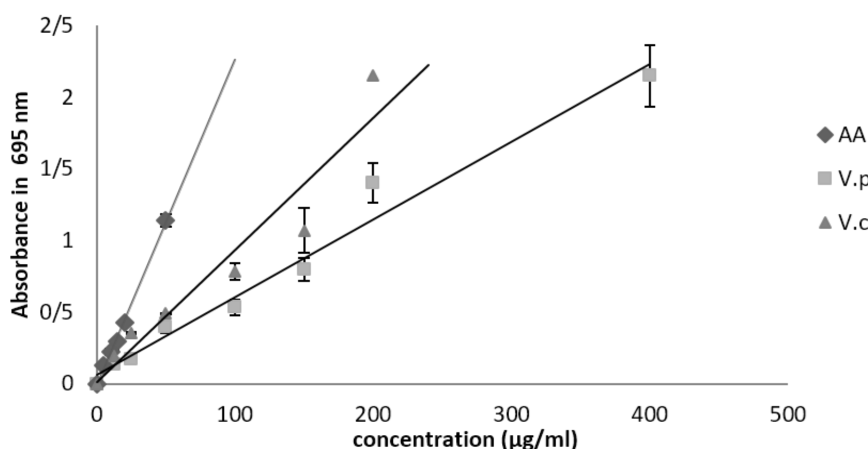

Fig. 2. Total antioxidant capacity of two extracts of *Veronica persica* (*V.p*) and *Veronica crista-galli* (*V.c*), according to phosphomolybdate assay, expressed as µg/mL of ascorbic acid equivalents (AAE), (n=3).

Table 2
In vitro, cytotoxic effects of water fractions of methanolic extracts of *V. persica* and *V. crista-galli* on MCF-7, Hela and HDF cell lines.

Extract		***V. persica***	***V. crista-galli***
Cell line	**hr**		
MCF-7	24	145.82	118.28
	48	112.94	77.40
	72	56.92	55.64
Hela	24	132.81	94.85
	48	98.03	71.35
	72	97.19	52.48
HDF	24	>614	340.82
	48	>614	145.02
	72	>614	274.01

Results were expressed as half-maximal inhibitory concentration of proliferation (IC_{50}) in µg/mL concentration of two *Veronica* fractions.

generally lower than 100 µg/mL and 25 µM respectively for extracts and pure compounds (Cos et al., 2006), our study on two *Veronica* extracts showed antiproliferative activities against tumourigenic Hela and MCF-7 cells, without appreciable toxicity against normal fibroblast cells. These data could be of prime interest as they suggest that the *Veronica* extracts exhibited higher toxicity for cancer cells compared to normal cells.

4. Concluding remarks

Previously, many phytochemical and biological studies have been conducted on the genus *Veronica*. It was reported that water fraction from *Veronica* species contain different iridoid, phenylethanoid and flavonoid glycosides. Moreover, there was a direct correlation between the high phenolic content of the plant derived extract and its radical-scavenging activity, reducing power and etc. The results obtained in this study showed that both water fractions of *V. persica* and *V. crista-galli*, which contain high amounts of flavonoid and phenolic compounds, exhibited *in vitro* antioxidant and antiproliferative activities. Antioxidant and anticancer potentials of phenolic contents of these plants might provide a new source for some areas of food industries, health benefits, medicine and pharmacology. However, our findings in the present study give us new insight into an explanation of the usage of *Veronica* species in traditional medicines. In fact, selective anti-proliferative and possible chemotherapeutic properties of *Veronica crista-galli* deserve deeper research attention.

Conflict of interest

The authors declare that there is no conflict of interest.

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