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Veronica persica Poir. extract – antibacterial, antifungal and scolicidal activities, and inhibitory potential on acetylcholinesterase, tyrosinase, lipoxygenase and xanthine oxidase

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Abstract: *Veronica persica* (Persian speedwell) is a flowering plant local to Eurasia. In this study, several analyses were done to discover the antimicrobial and scolicidal activities and acetyl cholinesterase (AChE), tyrosinase (TYR), lipoxygenase (LOX), and xanthine oxidase (XO) inhibitory activities of *V. persica* extract. The results presented that *B. subtilis* was the most susceptible to the extract (MIC = 40.3 μg/mL), while *P. aeruginosa* was the most resistant strain (MIC = 250.9 μg/mL) among all bacteria evaluated. The extracts demonstrated significant activity versus *E. granulosus* ($P < 0.5$) with dose-dependent inhibitions of the protoscolices. The analyzed plant extract exhibited a high AChE and TYR inhibitory activity 55.3% and 52.7% (at the highest utilized dose – 3 mg/mL), respectively. The extract also showed high anti-inflammatory activities in analyses tested. Our research proposed that extract of this plant could be promising to the human health, markedly in the infectious, neurodegenerative and inflammatory disorders.

Key words: Plantaginaceae; Antimicrobial activity; Neuroprotective ativity; Anti-inflammatory activity; Antioxidant activity.

Introduction

For thousands of years, medicinal plants represented a significant source of remedies, as well as the basis of traditional or indigenous healing systems still highly utilized by the population in the world (1-4). In recent years, medicinal plant therapy has been shown to be useful for treatment of various human and animal diseases (2-15).The unnecessary and frequent use of the same drugs used in modern medication has resulted in the evolution of antibiotic-resistant microbes (1, 2, 8-10, 16). In traditional medical science, a large number of therapeutic plants have presented antimicrobial effects and many of these have been used for the treatment of different infectious diseases (2, 8, 17-26).

Alzheimer's disease (AD) is an advancing neurodegenerative disease influencing 25 million people worldwide, and AChE is the most likely mark for the treatment of AD among cholinergic hypothesis (27). Several plants have been established as containing acetylcholinesterase inhibitory (AChEI) activity (28-31).

Tyrosinase (TYR), a multifunctional Cu-containing enzyme, catalyzes melanin synthesis in melanocytes (32). It has also been related to Parkinson's disease (33). Some TYR inhibitors from natural resources have been documented (34, 35).

Lipoxygenases (LOXs) are enzymes associated with inflammatory and allergic reactions because of the production of eicosanoid leukotrienes and lipoxins, and principal biological mediators of inflammatory processes (36). Alam et al. (37) indicated LOX herb inhibitor that can be a source of valuable alternative therapeutic agent.

Xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine into xanthine and uric acid during the metabolic processes of purines (38) accompanied by the production of reactive oxygen species (ROS) (39). XO is involved in the medical state known as gout, which is marked by hyperuricemia that causes uric acid deposition in the joints resulting in painful inflammation. Some research groups have done screening XO inhibitors from local medicinal plants that can perhaps be developed into clinical products (40-43).

The genus *Veronica* L. is the largest genus of the Plantaginaceae family, with about 500 species that showed a wide ecological variability. Recently, we reported an antioxidant capacity of *V. persica* phenolic-rich extracts (1); and according to the literature, there are no other data of its biological activity. In this study, antimicrobial, scolicidal activities and AChE, TYR, LOX, XO inhibitory activities of the extract prepared from *V. persica* were determined. The goal of this study was to discover a new source of effective medicine for treating infectious, neurodegenerative and inflammatory diseases.

Materials and Methods

Plant material and extraction conditions

The aerial parts (stems, leaves, and flowers) of *Veronica persica* Poir. were collected at flowering stage, in April 2016, from wild plants in the mountains of Meymand, Firuzabad County, Fars Province, Iran (Coordinates: 28°52′04″N 52°45′12″E). A botanist taxonomically identified the plant. All plant material collected dried in the shade, then pulverized into a fine powder using a grinder sieved through a No. 22 mesh sieve and stored in an air-tight container until required for the experiment. A volume of 200 mL of 70% methanol was added to 20 g of powder and kept on a mechanical shaker for 72 h. The content was filtered and concentrated under reduced pressure under controlled temperature to yield a dark gummy residue. The concentrated extract was stored dry in amber-colored flasks at 4 °C for upcoming experiments.

Antimicrobial activity assay

The antimicrobial activity of the extracts was assayed against two Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633), two Gram-negative bacteria (*Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9027) and two fungi (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 9142). All microorganisms were obtained from the Persian Type Culture Collection, Tehran, Iran.

The fungi and bacteria were cultured for 14-24 h at 37 °C and the densities were adjusted to 0.5 McFarland standards at 530 nm. The antibacterial experiments were performed by the disc diffusion method (44). Of the 100 µL microbial suspensions were spread on nutrient agar (Merck, Germany) plates (100 mm × 15 mm). Discs (6 mm diameter) were impregnated with 100 µL of different concentrations of extract (50, 100, 150, and 300 µg/mL) and placed on the inoculated agar. All the inoculated plates were incubated for 24 h at 37 °C. In this assay positive control discs used included ketoconazole, gentamicin and ampicillin (10 mg/disc) for fungi, Gram-negative and Gram-positive bacteria, respectively. Furthermore, we used 5% dimethyl sulfoxide (DMSO) as the negative control. Antimicrobial activity was appraised by measuring the zone of inhibition. Minimum inhibitory concentration (MIC) was determined using serial dilutions of the extracts (0-500 µg/mL) using microdilution experiment approved by Clinical and Laboratory Standards Institute (45). The bacteria and fungi were suspended in Luria-Bertani media and the densities were regulated to 0.5 McFarland standards at A530 nm (10^8 CFU/mL). The extract (100 µL) and the bacteria and fungi suspensions (100 µL) were added to microtiter plates and incubated at 37 °C for 24 h. The medium with bacteria and fungi but without extract was used as growth control and medium without bacteria and fungi was as sterility control. Growth in each well was compared with the growth in the control well. The MICs values were visually detected in comparison with the growth in the control well and delineated as the lowest concentration of the components with >95% growth inhibition.

Scolicidal activity

The scolicidal activity, *Echinococcus granulosus* protoscolices were gained from the infected livers of calves killed in an abattoir. Animals were treated humanely according to the Helsinki Convention. Hydatid fluid was collected together with protoscolices using the Smyth and Barrett (46) assay.

Briefly, the hydatid fluid was transferred to a glass cylinder. Protoscolices, which settled at the bottom of the cylinder after 40 min, were washed three times with normal saline and their viability was verified by motility under a light microscope (Nikon Eclipse E200, Japan). Protoscolices were transferred into a dark receptacle comprising normal saline and stored at 4 °C. Three concentrations of plant extract (5, 10, and 15 mg/mL) were assayed for 10, 20, 30 and 60 min. To prepare these concentrations 50, 150 and 100 µL of extracts, added to test tubes, were dissolved in 9.7 mL of normal saline supplemented with 0.5 mL of Tween-80 (Merck, Darmstadt, Germany) under continuous stirring. For each assay, one drop of protoscolex-rich solution was added to 3 mL of the extract solution, mixed slowly, and incubated at 37 °C. After each incubation period (10, 20, 30 and 60 min), the upper phase was carefully removed so as not to disturb the protoscolices, then 1 mL of 0.1% eosin stain was added to the remaining colonized protoscolices and mixed slowly. After incubating for 20 min at 25 °C, the supernatant was discarded. The remaining pellet of protoscolices (no centrifugation carried out) was then smeared on a manually scaled glass slide, covered with a cover glass, and evaluated under a light microscope. The percentage of dead protoscolices was determined after counting a minimum of 600 protoscolices. In the control, protoscolices were treated only with normal saline + Tween-80.

Acetylcholinesterase inhibition assay

In this study, the acetylcholinesterase inhibition activity was determined by the method illustrated by Ingkaninan et al. (47). In brief, 3 mL of 50 mM Tris–HCl buffer (pH 8.0), 100 µL of plant extract at various concentrations (0.5, 1.5, 3 mg/mL) and 20 µL AChE (6 U/mL) solution were mixed and incubated for 15 min at 30 ºC; a 50 µL volume of 3 mM 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to this mixture. The reaction was then started with by the addition of 50 µL of 15 mM acetylthiocholine iodide (AChl). The hydrolysis of this substrate was observed at 405 nm in a Hitachi U-2001 spectrophotometer (Tokyo, Japan). The formation of yellow 5-thio-2-nitrobenzoate anion was noticed as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide. The enzymatic activity was determined as a percentage of the velocities compared to that of the experiment by buffer instead of inhibitor (plant extract), as follows:

$EA= E\text{-}S/E \times 100$

In this formula, E is the activity of the enzyme wit-

Table1. Antibacterial activity of *V. persica* extract against Gram-positive and Gram-negative bacteria.

Extract (µg/mL)	*Staphylococcus aureus*	*Bacillus subtilis*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
50	8.5 ± 0.2 e	9.6 ± 0.3 d	7.3 ± 0.1 e	5.5 ± 0.2 e
100	11.5 ± 0.1 d	11.2 ± 0.1 c	8.5 ± 0.4 d	7.7 ± 0.1 d
150	12.3 ± 0.1 c	13.8 ± 0.3 b	9.8 ± 0.1 c	8.3 ± 0.1 c
300	14.4 ± 0.3 b	17.9 ± 0.5 a	13.6 ± 0.3 b	9.5 ± 0.2 b
Ampicillin	15.5 ± 0.3 a	17.8 ± 0.5 a	-	-
Gentamicin	-	-	14.5 ± 0.2 a	11.9 ± 0.4 a
DMSO (negative control)	1± 0.0 f	1± 0.0 e	1±0.0 f	1 ± 0.0 f

Data are expressed as means ± SD of inhibition zone diameter (mm) for different concentrations of the plant extract and controls (µg/mL). The values with different letters within a column are significantly different ($P < 0.05$; HSD). DMSO: dimethyl sulfoxide; MIC: minimum inhibitory concentration.

hout the experiment sample and S is the activity of the enzyme with the experiment sample.

Tyrosinase inhibition assay

To assay, the tyrosinase inhibition used the method described by Liang et al. (48). The tyrosinase (EC1.14.1.8.1, Sigma) activity was spectrophotometrically measured on 3-(3,4-dihydroxyphenyl)-L-alanine ($_L$-DOPA) (Sigma-Aldrich) as substrate. Tyrosinase aqueous solution (100 µL, 0.5 mg/mL), plant extract (0.5, 1.5, 3 mg/L) and 1850 µL of 0.2 M phosphate buffer (pH 7.0) were mixed and incubated for 15 min at 30 ºC. Following, 10 mM L-DOPA solution (50 µL) was added and the absorbance at 475 nm was measured for 3 min against a blank in a Hitachi U-2001 spectrophotometer (Tokyo, Japan). The same reaction mixture having the plant extract replaced by the equivalent amount of phosphate buffer, as blank. The % inhibition of tyrosinase activity was measured based on the formula:

% Tyrosinase inhibition= $A_{control} – A_{sample\ /} A_{control} \times 100$

where $A_{control}$ is the change of absorbance at 475 nm without a test sample, and A_{sample} is the change of absorbance at 475 nm with a test sample.

Lipoxygenase inhibition assay

The lipoxygenase (LOX) inhibiting activity was measured using spectrophotometrically as illustrated by Lycklander and Malterud (49) with slight modifications. Briefly, 100 µL of the enzyme solution (at the final concentration of 200 U/mL) was prepared in boric acid buffer (0.2 M; pH= 9) and mixed with 25 µL of extract solution (1 mg/mL in boric acid buffer) and then incubated at room temperature for 3 min. Reaction was initiated by the addition of substrate solution (linoleic acid, 250 µM), and the velocity was recorded for 2 min at 234 nm against a blank in a Hitachi U-2001 spectrophotometer (Tokyo, Japan). Negative control was prepared with contained 1% methanol solution without fraction solution. Quercetin was used as positive control. The percentage of lipoxygenase inhibition was calculated according to the following equation:

% Inhibition = $[(V_o \text{ control- } V_o \text{ sample}) \times 100]/V_o$ control

Where V_o control is the activity of enzyme in absence of extract solution, and V_o sample is the activity of the enzyme in the presence of extract, quercetin or ibuprofen.

Xanthine oxidase inhibition assay

The xanthine oxidase (XO) inhibition activity was measured on a spectrophotometer as illustrated by Owen and Timothy (50) with slight modifications. Briefly, the assay mixture consisted of 150 µL of phosphate buffer (0.066 M; pH 7.5), 50 µL of extract solution (1 mg/mL in phosphate buffer), and 50 µL of enzyme solution (0.28 U/mL). After pre-incubation at room temperature (25 °C) for 3 minutes, the reaction was initiated by addition of 250 µL of substrate solution (Xanthine, 0.15 M in the same buffer). A blank without enzyme solution was also prepared. The reaction was monitored for 3 min at 295 nm and velocity (V_o) was recorded. Phosphate buffer was used as negative control (activity of the enzyme without extract solution). Allopurinol was used as positive control. The percentage of xanthine oxidase inhibition was calculated using the following formula:

% Inhibition = [(Vo control- Vo sample) × 100]/V_o control

where V_o control is the activity of enzyme without macerate/fraction and V_o sample is the enzyme activity in presence of macerate/fraction or allopurinol.

Statistical analysis

All the experiments were carried out in triplicate. Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) post-hoc test at $P < 0.05$ using SPSS v. 11.5. Data are expressed as a mean ± standard deviation.

Results

The antibacterial activity of the extract is summa-

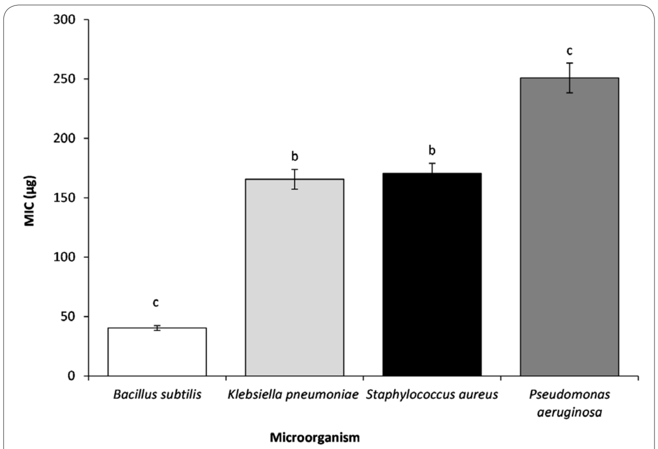

Figure 1. MIC values of *V. persica* extract against four tested bacteria. Values are mean of ± SD of three replicates. Means with different letters are significantly different ($P < 0.05$; HSD).

Table 2. Antifungal activity of *V. persica* extracts.

Extract (µg/mL)	*Candida albicans*	*Aspergillus niger*
50	3.9 ± 0.5 e	5.7 ± 0.5 d
100	4.7 ± 0.8 d	6.6 ± 0.3 c
150	7.9 ± 0.3 c	7.9 ± 0.2 b
300	9.9 ± 0.1 b	10.9 ± 0.5 a
Ketoconazole (µg/mL)	10.5 ± 0.1 a	10.5 ± 0.1 a
DMSO (negative control)	1.3 ± 0.1 f	1.1 ± 0.0 e
MIC	95.5 ± 0.1	66.7 ± 0.2

Data are expressed as means ± SD of inhibition zone diameter (mm) for different concentrations of the plant extract and controls (µg/mL). The values with different letters within a column are significantly different ($P < 0.05$; HSD). DMSO: dimethyl sulfoxide; MIC: minimum inhibitory concentration.

rized in Table 1. The results showed that the extract of *V. persica* demonstrated a dose-dependent antibacterial effect on the growth of all tested bacteria. The *V. persica* extract showed the maximum zones of inhibition at a concentration of 300 µg/mL of extract on the growth of all bacteria. Inhibition zones at a concentration of 300 µg/mL of the extract were 14.4 ± 0.3, 17.9 ± 0.5, 13.6 ± 0.3, and 9.5 ± 0.2 mm for *S. aureus*, *B. subtilis, K. pneumoniae, P. aeruginosa*, respectively. Among bacteria, *B. subtilis* (MIC = 40.3 µg/mL) revealed a high sensitivity to extract of *V. persica*. The results of antifungal assays are shown in Table 2. The *V. persica* extract inhibited the growth of *C. albicans* and *A. niger* in all the assayed concentrations ($P < 0.05$). The maximum inhibition zone was detected in concentration 300 µg/mL of extract. Indeed, the *V. persica* extract exhibited a strong activity against *C. albicans* and *A. niger* fungi with an inhibition zone of 9.9 ± 0.1 and 10.9 ± 0.5 mm at 300 µg/mL plant extract, respectively. MICs for *C. albicans* and *A. niger* were 95.5 ± 0.1 and 66.7 ± 0.2 µg/mL of *V. persica* extract, respectively. Mortality rates of *E. granulosus* protoscolices after treatment with various concentrations of *V. persica* extract is presented in Table 3. As exposure time and extracts concentration increased, % mortality was also increased. Therefore, exposure to the extracts for 60 min, at 5, 10 and 15 mg/mL led to 58.38%, 72.6%, 91.45% inhibition, respectively. The mortality in the control was 3.84%, after 60 min.

The anti-neurodegenerative activity of *V. persica*

Table 3. Scolicidal activity of *V. persica* extract against *E. granulosus*.

Concentrations (mg/mL)	Exposure time (min)	Plant extract Protoscolices	Dead protoscolices	Mortality (%)
5	10	1829.00 ± 54.21	395.44 ± 18.22	21.59
	20	1453.94 ± 70.22	432.25 ± 51.11	29.73
	30	1222.53 ± 42. 45	588.33 ± 25.14	48.11
	60	1192.74 ± 32.17	696.55 ± 25.51	58.38
	Control	1455.00	56.00	3.84
10	10	945.92 ± 22.62	611.45 ± 77.22	64.65
	20	799.91 ± 32.81	527.22 ± 17.15	65.95
	30	993.74 ± 65.44	669.22 ± 25.33	67.37
	60	732.23 ± 33.17	532.00 ± 25.11	72.67
	Control	1455.00	56.00	3.84
15	10	934.91 ± 81.12	684.54 ± 17.82	73.23
	20	788.45 ± 13.17	675.39 ± 32.11	85.65
	30	888.29 ± 39.15	794.92 ± 45.82	89.41
	60	995.00 ± 32.00	910.32 ± 42.82	91.45
	Control	1455.00	56.00	3.84

Values are mean ± SD of three replicates. In the control, protoscolices were treated only with normal saline + Tween-80.

Table 4. Acetylcholinesterase inhibitory activity of *V. persica* extract.

Sample	AChE inhibition % 0.5 mg/mL	1.5 mg/mL	3 mg/mL
Veronica persica	35.5 ± 0.48 e	46.45 ± 0.32 d	55.32 ± 0.29 c
Galanthamine	99.86± 0.58 a	-	-
Rutin	55.35 ± 0.19 b	85.85 ± 0.54 a	90.42 ± 0.39 a
Caffeic acid	52.52 ± 0.32 c	75.99 ± 0.69 c	85.36 ± 0.19 b
Rosmarinic acid	50.45 ± 0.11 d	77.69 ± 0.51 b	85.55 ± 0.23 b

Data represent the mean ± SD of three independent replicates. The values with different letters within a column are significantly different ($P < 0.05$; HSD).

Table 5. Tyrosinase inhibitory activity of *V. persica* extract.

Sample	Tyrosinase inhibition %		
	0.5 mg/mL	1.5 mg/mL	3 mg/mL
Veronica persica	25.45 ± 0.31 e	35. 33 ± 0.12 e	52.7 ± 0.5 e
Ellagic acid	46.95 ± 0.47 b	66.5 ± 0.26 b	89.42 ± 0.29 b
Quercetin	35.65 ± 0.21 d	55.49 ± 0.11 c	79.84 ± 0.51 d
Rutin	39.59 ± 0.41 c	45.59 ± 0.33 d	80.45 ± 0.33 c
Kojic acid	75.55 ± 0.33 a	85.45 ± 0.39 a	94.44 ± 0.37 a

Data represent the mean ± SD of three independent replicates. The values with different letters within a column are significantly different ($P < 0.05$; HSD).

Table. 6. Lipoxygenase and xanthine oxidase inhibitory activities of *V. persica* extract.

Sample	Lipoxygenase inhibition (%)	Xanthine oxidase inhibition (%)
Veronica persica	55.35 ± 0.45 b	84.99 ± 0.22 b
Quercetin	55.69 ± 0.22 b	85.44 ± 0.32 b
Allopurinol	ND	92.54 ± 0.43 a
Ibuprofen	80.38 ± 0.25 a	ND

Data represent the mean ± SD of three independent replicates. The values with different letters within a column are significantly different ($P < 0.05$; HSD).

extract was examined at concentrations of 0.5, 1.5, and 3 mg/mL using AChE and TYR assays (Tables 4 and 5). Inhibition of both enzymes by the extract varied between 35.5 to 55.32% for AChE, and from 25.45 to 52.7% for TYR. A statistically marked difference was observed among the different concentrations of the extract. The extract exhibited statistically weaker activity than the standards galanthamine, rutin, caffeic acid and rosmarinic acid for AChE assay, and ellagic acid, quercetin, rutin and kojic acid for TYR experiment. The extract of *V. persica* presented stronger inhibitory effect at the highest concentration (3 mg/mL) in both assays.

V. persica extract demonstrated high anti-inflammatory properties, both in the LOX and XO experiments. In the XO experiment, the results showed a high inhibition of enzyme activity. Finally, when assayed using the LOX assay, the extract presented moderate inhibition (Table 6).

Discussion

It is well known that active phytochemicals are produced for protection of plants against microbial pathogens and those plants can be hopeful sources of new compounds with biological activities such as antioxidants and antimicrobials (7, 51, 52). This study demonstrate that the extract of *V. persica* possesses extensive antibiotic activity against Gram-positive and Gram-negative bacteria, fungi and *E. granulosus*. The genus *Veronica* exhibited strong antibacterial activity probably due to the high content of flavonoids, phenol carboxylic acids and tannin (53). Dunki et al. (54) showed that *V. spicata* is beneficial as phytotherapeutical, antioxidative and antimicrobial agent. Also, Mocan et al. (2015) indicated that the use of three *Veronica* species *V. officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz as antimicrobial agents (55). Probably, the antimicrobial potential of *V. persica* we observed can be ascribed to the presence of polyphenolic compounds.

AD influences memory and other features of human mind and is characterized by the loss of activities referring to the acetylcholine in the cerebral cortex. Noteworthy, most of the AChE inhibitors such as galanthamine were originally isolated from plants (56). Furthermore, it has been demonstrated that TYR might be related to the damaged neurons typical for another progressive neurological disorder, Parkinson's disease (57). A number of polyphenols isolated from plants were established as AChE and TYR inhibitors, such as quercetin, kaempferol and caffeic acid (58). Our study documented the activity of *V. persica* extract against enzymes involved in neurodegenerative disorders. Although inhibitory activities of the extract were lower than the standard ones for those enzymes, the results of our study propose that *V. persica* could be of interest for the development of food supplements that could prevent neurodegenerative diseases. In a previous study, a moderate neuroprotective activity of *V. jacquinii* and *V. teucrium* extracts was reported on human neuroblastoma SH-SY5Y cell line (59). In addition, Živković el al. (2017) showed that *V. teucrium* and *V. jacquinii* methanol extracts inhibit AChE and TYR enzymes (60).

Although *Veronica* species have been widely utilized in the oriental medicine for inflammatory disorders, the pharmacological effects of these species have not been fully examined. In the present study, *V. persica* was assessed for its inhibitory effect on LOX and XO. XO is an enzyme that generates ROS (reactive oxygen species) from the chemical reaction it catalyzes. ROS react with cellular lipids, resulting in the formation of lipid peroxides, which are metabolized to malondialdehyde, a major product of lipid peroxidation (61). In our paper, the high inhibition of XO by *V. persica* extract may contribute to its antioxidant effect. *V. persica* extract also demonstrated a moderate inhibition of LOX enzyme. LOX are involved in the metabolism of leukotrienes (62). The moderate LOX inhibition by *V. persica* extract could partially contribute to the anti-inflammatory activity of the plant extract. The anti-inflammatory effects of *V. offcinalis* extract on human lung epithelial cell line A549 were previously (63). Similarly, the extracts of three *Veronica* species, *V. jacquinii* Baumg., *V. teucrium* L. and *V. urticifolia* Jacq exhibited LOX inhibitory activity (64). In conclusion, our results indicate that *V. persica* extract could be a promising food supplement for human health, particularly in the prevevtion of infectious, neurodegenerative and inflammatory disorders.

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Conflict of interest

The authors declare no financial or other conflicts of interest.

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