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*Original Research*

# Inhibitory activity on type 2 diabetes and hypertension key-enzymes, and antioxidant capacity of *Veronica persica* phenolic-rich extracts

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**Abstract:** *Veronica* genus (Plantaginaceae) is broadly distributed in different habitats. In this study, the inhibitory activity of free soluble and conjugated phenolic extracts of *Veronica persica* on key enzymes associated to type 2 diabetes (α-glucosidase and α-amylase) and hypertension (angiotensin I converting enzyme, ACE) was assessed, as well as their antioxidant power. Our results showed that both the extracts inhibited α-amylase, α-glucosidase and ACE in a dose-dependent manner. In particular, free phenolic extract significantly ($P < 0.05$) inhibited α-glucosidase ($IC_{50}$ 532.97 µg/mL), whereas conjugated phenolic extract significantly ($P < 0.05$) inhibited α-amylase ($IC_{50}$ 489.73 µg/mL) and ACE (290.06 µg/mL). The enzyme inhibitory activities of the extracts were not associated with their phenolic content. Anyway, the inhibition of α-amylase, α-glucosidase and ACE, along with the antioxidant capacity of the phenolic-rich extracts, could represent a putative mechanism through which *V. persica* exerts its antidiabetes and antihypertension effects.

***Key words:*** Plantaginaceae, α-amylase, α-glucosidase, angiotension I converting enzyme, traditional medicine, ethnopharmacology.

## Introduction

Type-2 diabetes mellitus accounts for over 90% of all instances of diabetes, both in developed and developing countries. About 382 million people are considered to be living with diabetes all over the world, with an impressive prediction of 471 million people with the disease by the year 2035 (1). Postprandial hyperglycemia has been implicated in the development of insulin resistance (2), being one of the first markers of glucose homeostasis deregulation (3). In addition, hypertension, cardiovascular disease and diabetic neuropathy are associate with this status (4, 5). A therapeutic approach in the prevention/control of hyperglycemia is through the delay of glucose absorption by inhibiting important enzymes involved in carbohydrate hydrolysis (α-amylase) and glucose release from disaccharide (α-glucosidase). However, some drugs widely used (acarbose and miglitol) to reduce blood glucose level are not exempt from a number of pharmacological side effects (6, 7). Therefore, there exists a continued necessity for alternative, natural α-amylase and α-glucosidase inhibitors with high specificity and less adverse effects.

As previously introduced, long term diabetes is generally associated with hypertension (8), which, in turn, represents risk factors for cardiovascular diseases and is chronic renal failure (8, 9). Angiotensin-I converting enzyme (ACE) is a zinc metallopeptidase that converts angiotensin-I to angiotensin-II, a strong vasoconstrictor, and breaks down bradykinin, a vasodilator (10). Inhibition of ACE is considered an effective therapeutic approach in the control of hypertension both in diabetic and nondiabetic patients (10). In this context, dietary phytochemicals, particularly phenolics, may play a protective role both in diabetes and hypertension (11, 12).

Nonetheless, chronic hyperglycemia in diabetes triggers oxidative stress at tissue and organ levels (6), which could be controlled by antioxidant agents. Interest on natural, dietary antioxidants is currently growing (13-16). Phenolic compounds represent a large group of bioactive phytochemicals which occur in almost all medicinal and food plants, because of their very high ecological relevance in plant organisms (17). Their in vitro antioxidant power, as well as other pharmacological activities, have been extensively demonstrated since years (18).

The genus *Veronica* is the most important genus belonging to the Plantaginaceae family, with about 500 species widely diffused in boreal hemisphere and present in many parts of the austral hemisphere. This is indicative of high ecological adaptability and genetic plasticity, with species growing in humid to dry environments, from the sea level to alpine biotopes (19). In traditional medicine, *Veronica* species are used as diuretic, wound healing, antirheumatic and anticancer agents (20-22). In addition, stems and leaves of some *Veronica* species are edible, either raw or cooked (23). Despite the use of *Veronica* species in traditional healing systems is extensively reported, there exists a paucity of

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information documenting their pharmacological activities and mechanisms involved in the observed health benefits. *Veronica persica* (common names: birdeye speedwell, common field-speedwell, Persian speedwell, large field speedwell, bird's-eye or winter speedwell) is a flowering plant native to Eurasia. To the best of our knowledge, no information is available on the possible mechanism(s) of action of *V. persica* extracts in decreasing blood glycemia and management of hypertension. Hence, the present study aims primarily at investigating the inhibitory activity of phenolic-rich extracts of *V. persica* on key enzymes involved in diabetes and hypertension, i.e. α-amylase, α-glucosidase and ACE.

## Materials and Methods

### Plant material

Aerial parts (stems, leaves and flowers) of *Veronica persica* were collected at flowering stage, in November 2014, from wild plants in the mountains of Meymand, Firuzabad County, Fars Province, Iran. The plant was taxonomically identified by a botanist and a specimen was deposited at the Herbarium of Pharmacognosy, Department of the Faculty of Pharmacy affiliated to Zabol University of Medical Sciences of Iran. The collected plant materials were dried in the shade, powdered and stored at 10 °C until required for analysis.

### Free soluble phenolic extraction

Free phenolic extraction was performed according to a slight modified method described by Chu *et al*. (24). Twenty grams of the powdered sample were extracted with 80% acetone (1:5, w/v) and filtered (filter paper Whatman No. 2) under vacuum. The filtrate was then evaporated by a rotary evaporator under vacuum at 40 °C until about 90% of the filtrate had been evaporated and then lyophilized to obtain a dry extract. The residues were kept for conjugated phenolic extraction, while extract was kept at -4 °C for next analyses.

### Conjugated phenolic extraction

The residue from free extraction was flushed with nitrogen and hydrolyzed with about 30 mL of 4 M NaOH solution at room temperature (24 ± 1°C) for 1 h with shaking. Afterward, the pH of the mixture was adjusted to pH 2 with concentrated HCl and the conjugated phytochemicals were extracted with ethylacetate. The ethylacetate fractions were then evaporated to dryness at 40°C.

### Total phenol content determination

The total phenol content was assayed according to the method described by Singleton *et al*. (25). Briefly, appropriate dilutions of the extracts were oxidized with 3 mL 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2 mL of 8% sodium carbonate. The reaction mixture was incubated for 45 min at 45°C and the absorbance was measured by using spectrophotometer-UV (Shimadzu A160) at 765 nm ($A_{765}$). The total phenol content was calculated as gallic acid equivalent.

### Reducing power determination

The reducing power of the extracts was determined by evaluating the ability of the extract to reduce $FeCl_3$ solution by method of Oyaizu (26). A 3 mL aliquot was mixed with 3 mL of 200 mM sodium phosphate buffer (pH 6.5) and 3 mL of 1% potassium ferricyanide. The mixture was incubated at 45 °C for 25 min and then was added 3 mL of 10% trichloroacetic acid. This mixture was centrifuged at 2600 rpm for 15 min. Five mL of the supernatant were mixed with an equal volume of distilled water and 1 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm ($A_{700}$) in the spectrophotometer-UV (Shimadzu A160) and ferric reducing power was subsequently calculated using ascorbic acid as standard.

### Total antioxidant capacity

The total antioxidant capacity was by 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate radical (ABTS) scavenging ability of the extracts according to the method described by Sharifi Rad *et al*. (27). The ABTS was generated by reacting ABTS aqueous solution (7 mM) with $K_2S_2O_8$ (2.45 mM, final concentration), in the dark, for 14 h and adjusting the absorbance at 734 nm to 0.700 with ethanol. Then, 0.2 mL of appropriate dilution of the extracts were added to 2.0 mL ABTS solution and the absorbance was measured at 734 nm in the spectrophotometer-UV (Shimadzu A160) after 15 min. The trolox equivalent antioxidant capacity (TEAC) was subsequently calculated using trolox (a synthetic antioxidant) as the standard.

### α-Amylase inhibitory activity

Phenolic extract dilutions (0-150 µL) and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing porcine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/mL) were incubated at 25 °C for 15 min. Then, 500 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) were added to each tube. The reaction mixture was incubated at 25 °C for 15 min and stopped with 1.0 mL of dinitrosalicylic acid colour reagent. Afterwards, the mixture was incubated in a boiling distilled water bath for 5 min, and cooled to room temperature (24±1°C). The reaction mixture was then diluted by adding 10 mL of distilled water, and absorbance measured at 540 nm ($A_{540}$) by using spectrophotometer-UV (Shimadzu A160). The α-amylase inhibitory activity was expressed as % inhibition (28).

### α-Glucosidase inhibitory activity

Phenolic extracts dilutions (0-150 µL) and 100 µL of α-glucosidase (EC 3.2.1.20) solution (1.0 U/mL) in 0.1 M phosphate buffer (pH 6.9) were incubated at 25°C for 15 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) were added. Before reading the absorbance at 405 nm ($A_{405}$) in the spectrophotometer-UV (Shimadzu A160), the mixtures were incubated at 25 °C for 5 min. The α-glucosidase inhibitory activity was expressed as % inhibition (29).

### Angiotensin I converting enzyme inhibition

Angiotensin I converting enzyme (ACE) inhibitory activity was carried out using a slightly modified method of Cushman and Cheung (30). The phenolic extract dilutions (0-50 µL) and 50 µL ACE (EC 3.4.15.1)

solution (4 mU/mL) were incubated at 37°C for 15 min. The enzymatic reaction was initiated by adding 150 µL of 8.33 mM of the substrate Bz–Gly–His–Leu in 125 mM Tris-HCl buffer (pH 8.3) to the mixture. The reaction was arrested by adding 250 µL of 1 M HCl, after incubation for 30 min at 35°C. The Gly–His bond was then cleaved and the Bz–Gly produced by the reaction was extracted with 1.5 mL ethylacetate. Afterwards, the mixture was centrifuged to separate the ethylacetate layer; 1 mL of the ethylacetate layer was transferred to a clean test tube and evaporated. The residue was redissolved in distilled water and its absorbance was measured at 228 nm ($A_{228}$) by using spectrophotometer-UV (Shimadzu A160). The ACE inhibitory activities were expressed as % inhibition.

### $IC_{50}$ determination for α-amylase, α-glucosidase and ACE

$IC_{50}$ is defined as the concentration of phenolic-rich extracts (in our experiments) required inhibiting 50% of the enzyme activity. The $IC_{50}$ was calculated by using the formula described by Sharifi-Rad *et al.* (31): EXP (LN (conc > 50%) - ((signal > 50%-50)/(signal > 50%-signal < 50%)*LN (conc >50%/conc <50%))).

### Data analysis

Statistical significance was determined by Student's *t*-test and significance was accepted at $P < 0.05$. The results of triplicate tests were expressed as mean ± standard deviation (SD).

## Results and Discussion

Inhibition of enzymes involved in the metabolism of carbohydrates, such as α-amylase and α-glucosidase, and angiotensin I convertion (ACE) is a key therapeutic approach for decreasing postprandial hyperglycemia and hypertension (11, 32-35).

The α-amylase inhibitory activity of *V. persica* phenolic-rich extracts is reported in Figure 1. Both extracts dose-dependently inhibited the enzyme activity ($P < 0.05$), even if the bound (conjugated) phenolic extract was more effective ($IC_{50}$ 489.73 µg/mL) than the free phenolic extract ($IC_{50}$ 742.76 µg/mL) (Figure 1, Table 1). The inhibitory activity of the *V. persica* extracts on α-amylase activity was consistent with previous studies on phenolic extracts derived from other plants (36-41). The general trend of the extracts from *V. persica* to

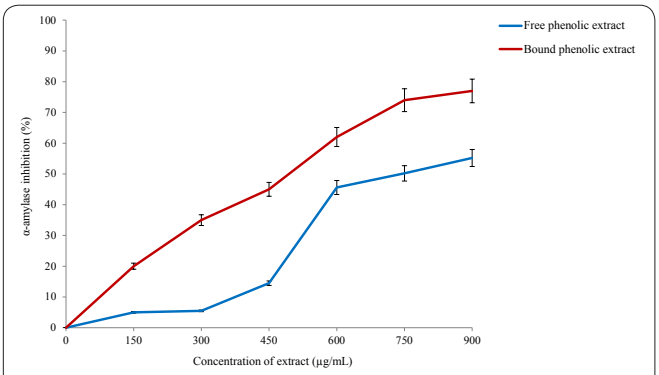


**Figure 1.** α-Amylase inhibitory activities of *Veronica persica* phenolic-rich extracts. Data represent mean ± standard deviation (n=3).

inhibit the α-amylase correlated with their total phenol content (Table 2). Therefore, inhibition of α-amylase activity, here *in vitro* demonstrated, could be one of the putative mechanisms supporting the efficacy of plant (including *V. persica*) extracts in the control of diabetes.

The inhibition of *V. persica* phenolic-rich extracts on α-glucosidase is shown in Figure 2. Both extracts dose-dependently inhibited α-glucosidase activity ($P < 0.05$), though with a different tend (Figure 2). Inhibition by free phenolic extract ($IC_{50}$ 532.97 µg/mL) was more active than the bound phenolic extract ($IC_{50}$ 825.78 µg/mL) (Table 1).

Polyphenols have been shown to inhibit α-amylase and α-glucosidase, which may postpone rapid absorption of glucose (42). However, in our experimental conditions, free phenolic extract exhibited a higher in-

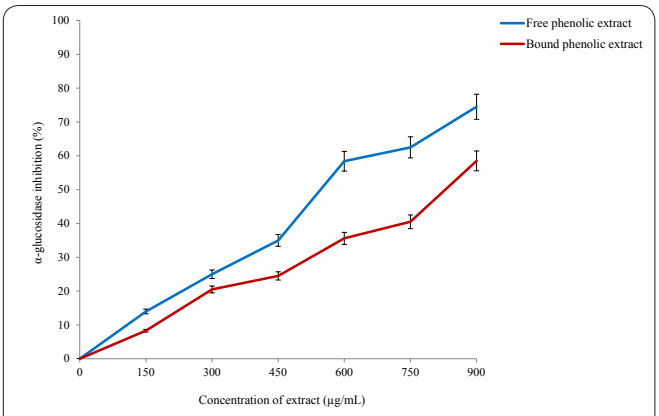


**Figure 2.** α-Glucosidase inhibitory activities of *Veronica persica* phenolic-rich extracts. Data represent mean ± standard deviation (n=3).

**Table 1.** $IC_{50}$ of the *Veronica persica* phenolic-rich extracts on α -amilase, α -glucosidase and angiotensin I converting enzyme (ACE).

| *V. persica* extracts | $IC_{50}$ (µg/mL) | | |
|---|---|---|---|
| | α-Amylase | α-Glucosidase | ACE |
| Free phenolics | 742.76 | 532.97 | 383.90 |
| Bound phenolics | 489.73 | 825.78 | 290.06 |

Data represent mean (n=3; $P < 0.05$).

**Table 2.** Total phenol content and antioxidant capacity of *Veronica persica* phenolic-rich extracts.

| *V. persica extracts* | TP (mg/100 g) | RP (mmol AAE/100 g) | TAC (mmol TEAC/100 g) |
|---|---|---|---|
| Free phenolics | 78.45 ± 1.3 | 8.86 ± 0.5 | 9.49 ± 1.2 |
| Bound phenolics | 54.77 ± 5.2 | 7.85 ± 1.39 | 6.99 ± 3.6 |

Data represent mean ± standard deviation (n=3; $P < 0.05$). TP: total phenols; RP: teducing power; TAC: total antioxidant capacity; AAE: ascorbic acid equivalents; TEAC: trolox-equivalent antioxidant capacity.

hibitory activity on α-glucosidase in comparison with α-amylase: interestingly, the use of *V. persica* phenolic-rich extracts could reveal an even better effect in diabete control over the commercial antidiabetic drugs currently in use, which have a relatively low α-glucosidase inhibitory activity.

Nevertheless, these findings are consistent with previous reports where phytochemicals showed higher α-glucosidase than α-amylase inhibition (34, 38, 39, 43-45). This aspect would avoid side effects associated with some of the anti-diabetes drugs currently in use (acarbose and voglibose), which possess an excess of α-amylase inhibition (46). Therefore, plant phenolics with mild α-amylase and high α-glucosidase inhibitory activities have been proposed as acceptable alternative for clinician to the corresponding synthetic inhibitors (34).

Moreover, the antihypertensive potential of phenolic extracts has also been investigated through the inhibition of angiotensin I converting enzyme (ACE). As shown in Figure 3, both extracts exhibited a high ACE inhibitory activity, in a dose-dependent manner. In particular, the bound phenolic extract showed significantly higher ($P < 0.05$) inhibitory activity on the enzyme than the free phenolic extract. The $IC_{50}$ values also confirmed these results, with bound phenolic extract significantly ($P < 0.05$) less active ($IC_{50}$ 290.06 µg/mL) than the free phenolic extract ($IC_{50}$ 383.90 µg/mL) (Table 1). Studies on the physical structure of human ACE have shown the presence of cysteine groups in the protein surface, which are available to form disulfide bridges (33). Therefore, ACE inhibitory activity of *V. persica* extracts may be due to the interactions between *V. persica* (free and bound) phenolics and disulfide bridges (oxidized cysteines) on the macromolecule surface, thus causing structural and functonal changes in turn associated with enzyme inhibition (33).

Our results support the higher α-amylase and ACE inhibitory activities of the bound phenolic extract in comparison with the free phenolic extract. A main reason may be related to the higher hydrophilicity of bound phenolics, which are mainly in the form of glycosides, than the free phenolics which are mainly in the form of aglycones. Since the enzymes (α-amylase and ACE) are active in the aqueous phase, direct enzyme-inhibitor interaction is expected to be higher with bound phenolics compared with the free ones. A previous work with herbal extracts suggested the inhibitory activity of phenolics may involve the disulfide bridges situated on the external surface of α-amylase, thus modifying enzyme structure and function (47). Therefore, the inhibition of α-amylase and ACE by *V. persica* bound phenolic extract could be due to the direct interaction of the phenolics with the disulfide bridges of the enzymes.

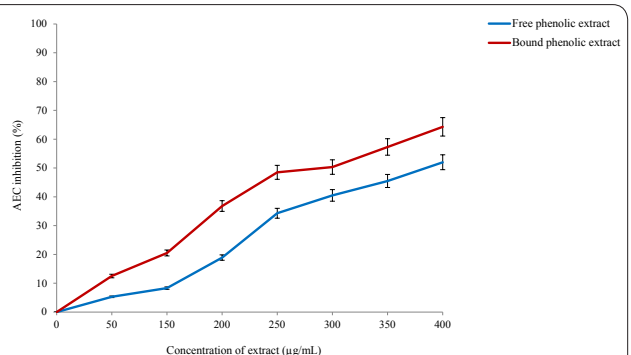


**Figure 3.** ACE (angiotensin I converting enzyme) inhibitory activities of *Veronica persica* phenolic-rich extracts. Data represent mean ± standard deviation (n=3).

Total phenol content of *V. persica* extracts is presented in Table 2. The results revealed that free phenolic content (78.45 mg/100 g) was significantly ($P < 0.05$) higher than the bound phenolic (54.77 mg/100 g) content. Our data on *V. persica* are in agreement with previously published articles on different plants (24, 39, 43-45, 48).

The total antioxidant capacity measured as trolox equivalent antioxidant capacity (TEAC) is reported in Table 2. Free phenolic extract exhibited significantly ($P < 0.05$) higher antioxidant capacity than the bound phenolic extract, showing the same trend observed for the total phenol content (Table 2) and suggesting that antioxidant properties of plant products are correlated with their phenol content (24, 49). Polyphenols are powerful plant antioxidants, which play a pivotal role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or breaking up peroxides (50). Phenolics are almost ubiquitous in plant organisms, where they can be found in different tissues as free aglycones and in conjugated (bound) forms either with sugars (primarily glucose) as glycosides or other moieties (51). Generally, conjugation occurs via the hydroxyl groups present in the phenolic backbone, consequently decreasing their antioxidant capacity, since the availability of free hydroxyl groups on the phenolic rings is essential for resonance stabilization of free radicals. Evidence about the antioxidant activity of *Veronica* species is scanty, although few studies support that certain *Veronica* species reveal antioxidant activity (52, 53). Persicoside, a phenylethanoid glycoside, was identified from the aerial parts of *V. persica* (54), while many phenylethanoid glycosides were found to have a broad range of biological activities, including antioxidant and anticancer properties (55-58). Nonetheless, the suppressive effect of *Veronica* species on NO production may arise from the presence of a high content of phenolic compounds such as flavonoids and phenylethanoids (59). Different phenolic compounds from *Veronica* species have been investigated (53, 60, 61), most of them beneficial for human health. The consumption of phenolic compounds is also useful for lowering or delaying the development of inflammation as result of their antioxidant activity (62). Along this direction, we also found the *V. persica* phenolic extracts showed reducing power, demonstrated as ascorbic acid equivalent (Table 2), revealing the free phenolic extract had higher reducing power than the bound phenolic extract. Reducing power is a strong antioxidant mechanism involving electron and hydrogen atom transfer (63, 64), and these results are consistent with the total phenol content of the extracts.

## Conclusions

*V. persica* phenolic-rich extracts showed the potential to inhibit *in vitro* α-amylase, α-glucosidase and ACE activities and, therefore, this plant could be a valuable dietary supplement for managing postprandial hyperglycemia and hypertension associated to type 2 dia-

betes, and as well as a promising antioxidant agent. Nevertheless, the enzyme inhibitory activities (except for α-glucosidase) were not associated with the phenolic content of the *V. persica* extracts, rather being possibly related with type and properties of the phenolic compounds. The potential pato-physiological relevance of these findings requires to be further investigated in order to reach a translational level.

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