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Radical Scavenging Effects of Different *Veronica* Species

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Abstract: It is well known that the excessive production of reactive oxygen species is hazardous for living organisms and damages major cellular constituents such as DNA, lipid and protein. To find new products reducing free radical damage is very important researches in recent pharmaceutical investigations. Considering this information, fourteen *Veronica* species are decided to research in the view point of their antioxidant capacity and the chemical content. Water extracts of the plants were tested for their radical scavenging activity against 2,2-diphenyl-1-picryl hydrazyl (DPPH), superoxide (SO) and nitric oxide (NO) radicals spectroscopically. Dose dependent radical scavenging activity was observed and the results were found to be comparable to that of ascorbic acid, quercetin and BHA which are known antioxidative compounds. In addition, gallic acid equivalent total phenolic contents of the plants were also determined using Folin-Ciocalteau reagent. The most significant scavenging activity was found for *V. chamaedrys* against SO radical (IC50 113.40 µg/ml) and *V. officinalis* against DPPH and NO radicals (IC50 40.93 µg/ml, 570.33 µg/ml, respectively).

Keywords: *Veronica;* Plantaginaceae*;* radical scavenging effect; DPPH; Nitric oxide; Superoxide.

1. Introduction

The genus *Veronica* L. (Plantaginaceae) is a large genus of annual and perennial plants located in Central and Southern Europe and Turkey. It is represented by 79 species, 26 of which are endemic in Turkish Flora [1]. The phytochemistry of the genus has been studied extensively with many species surveyed for their iridoid [2, 3] and flavonoid constituents [4,5]. Earlier investigations on *Veronica* species were resulted in the isolation of mainly iridoid glucosides, especially benzoic and cinnamic acid esters of catalpol, some phenylethanoid and flavonoid glycosides [3, 6-10]. Isolated flavonoids were found 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 [4, 6]. A recent systematic study of *Veronica* based on sequence data from the internal transcribed spacer region of nuclear ribosomal DNA has renewed interest in the taxonomy of this genus [11] and it is transferred from the Schrophulariaceae to Plantaginaceae family [10,12].

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In addition to the chemotaxonomic and phytochemical importance of the genus, *Veronica* species were also become prominent from the view point of their traditional usage and biological activities. While *V. beccabunga* and *V. officinalis* were used as diuretic and for wound healing [13], *V. anagallis-aquatica* was used for the treatment of rheumatic pain as a poultice [14] in Anatolia. The usage of *V. hederifolia* in cough and influenza, and *V. polita* as expectorant and antiscorbutic were also found in traditional Turkish medicine [15]. In addition, anticancer usages of different *Veronica* species were determined in different countries [14, 16].

Previously, we have performed a lot of phytochemical and biological studies on the genus *Veronica* [7,8,17,18]. *V. persica*, *V. polita*, *V. hederifolia*, *V. anagallis-aquatica* , *V. cymbalaria* and *V. serpyllifolia* were studied phytochemically. We have isolated different iridoid, phenylethanoid and flavonoid glycosides from these species. However we have not studied their biological activity comparatively. Our previous researches and recent phylogenetic and pharmacological studies were turned us to focus on the researches on Plantaginaceae family from the view point of phytochemistry and biological activity. As a part of this ongoing research project, it is planned to test radical scavenging activity of *V. peduncularis, V. baranetzkii, V. officinalis, V. orientalis, V. persica, V. polita, V. cuneifolia* subsp. *cuneifolia, V. cymbalaria, V. hederifolia, V. anagallis-aquatica, V. fuhsii, V. chamaedrys,* Unknown *Veronica* and *V. serpyllifolia* against 2,2-diphenyl-1-picryl hydrazyl (DPPH), superoxide (SO) and nitric oxide (NO) radicals spectroscopically. Their total phenolic contents were also determined using Folin-Ciocalteau reagent.

Extensive studies with different model systems and biological materials have clearly shown that reactive free radicals are able to produce chemical modifications and damage to proteins, lipids, carbohydrates and nucleotides. Many diseases and degenerative processes can be associated with the overproduction of reactive oxygen species (ROS) including inflammation, brain ischemia, mutagenesis, dementia and physiological aging. In addition, it is well known the increased amount of free radicals in wounded and inflammated tissues, cancerous cells and organs [19, 20, 21]. For these reasons, several methods have been developed to measure antioxidant activity of the herbal *extracts in vitro* and here we have tested nitric oxide (NO), superoxide (SO) and 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity of the aqueous extract of selected species together with their gallic acid equivalent total phenolic content. There are no reported studies about these species relating to these properties to any biological activity.

2. Materials and Methods

2.1. Plant Material

Tested *Veronica* species were collected from different places in Turkey. A voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey. Their locations and herbarium numbers were given in Table 1.

2.2 General

DPPH, nitro blue tetrazolium (NBT), sodium nitroprusside, Folin–Ciocalteu reagent, gallic acid, ascorbic acid were obtained from Sigma-Aldrich Chem Co (St. Louis, MO). 3-*t*-butyl-4-hydroxyanizole (BHA) was purchased from Nacalai Tesque Co. (Kyoto, Japan). Sulfanilamide and napthylethylenediamine dihydrochloride were obtained from Merck Co. (Darmstadt, Germany).

Table 1. Information about the plant species

Plant Material	Location	Herbarium No:
V. peduncularis Bieb.	Trabzon-Maçka	HUEF 09008
V. baranetzkii Bordz.	Trabzon-Maçka	HUEF 09007
V. orientalis Miller	Kars-Göle	HUEF 90026
V. polita Fries	Ankara	HUEF 99013
V. persica Poiret	Urfa	HUEF 99015
V. cymbalaria Bodard	Urfa	HUEF 99131
V. hederifolia L.	Urfa	HUEF 99016
V. cuneifolia subsp. *cuneifolia* D. Don	Antalya-Akseki	HUEF 06006
V. fuhsii Freyn et.Sint.	Bolu-Abant	HUEF 09327
V. chamedrys L.	Bolu-Abant	HUEF 09326
V. serpyllifolia L.	Bolu-Abant	HUEF 09328
Unknown *Veronica*	Bolu-Abant	-

2.3. Preparation of herbal extracts

The air-dried aerial parts of the plants were extracted with MeOH at 40^{o}C for three times. The combined MeOH extracts were evaporated under vacuum to give crude MeOH extracts. MeOH extract was dissolved in water and partitioned with petroleum ether to remove chlorophylls. Water extracts were tested for the bioactivity and phytochemistry studies.

2.4. DPPH radical scavenging effect

The DPPH radical scavenging effect of water extracts was assessed by the decoloration of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) spectroscopically; BHA, quercetin and ascorbic acid were used as standard compounds. MeOH solution (100 µl) of the extract at various concentrations was added to DPPH/MeOH (80 µg/ml) solution. The reaction mixture was shaken vigorously and the absorbance of remaining DPPH was measured at 520 nm after 30 min. The radical scavenging activity was determined by comparing the absorbance with that of blank (100%) containing only DPPH and solvent. All the analyses were done in 3 replicates [22, 7, 9, 23].

2.5. Superoxide radical scavenging activity by alkaline DMSO method

Superoxide radical scavenging activity of the extracts were determined by alkaline DMSO method according to the method of Elizabeth and Rao (1990) with slight modification In this method, the concentration of superoxide in alkaline DMSO system corresponds to the concentration of oxygen dissolved in DMSO. In tightly stoppered vessels, superoxide radical is stable more than 24 h; however in open vessels rapidly decreases its concentration. Briefly, superoxide radical was generated in non-enzymatic system. To the reaction mixture containing 10 µL of NBT (1 mg/mL solution in DMSO) and 30 µL of the extract or standard compounds were dissolved in DMSO, 100 µL of alkaline DMSO (1 mL DMSO containing, 5 mM NaOH in 0.1 mL water) was added to give a final volume of 140 µL and the absorbance was measured at 560 nm using microplate reader. The decrease in the absorbance at 560 nm with antioxidants indicated the consumption of generated superoxide [24-27].

2.6. NO scavenging activity

In order to determine NO radical scavenging activity of extracts, 60 µl of serial diluted sample were added into a 96-well flat-bottomed plate. Following this, 60 µl of 10 mM sodium nitroprusside, dissolved in phosphate buffered saline (PBS), were added to each well and the plate was incubated under light at room temperature for 150 min. Finally, an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, 2.5% H_3PO_4) was added into each well in order to measure the nitrite content. After chromofore was formed at room temperature in 10 min, absorbance at 577 nm was measured in a microplate reader [7, 28, 29].

2.7. Estimation of total phenol content

Antioxidant compounds generally contain phenolic group(s) and the amount of phenolic compounds in the extract was estimated by using Folin–Ciocalteu reagent. Briefly, 10 µL sample or standard (50-500 mg/L gallic acid) plus 150 µL diluted Folin–Ciocalteu reagent (1:4 reagent:water) was placed in each well of a 96-well plate, and incubated at room temperature for 3 min. Following addition of 50 µL sodium carbonate (2:3 saturated sodium carbonate: water) and a further incubation of 2 h at room temperature, absorbance was read at 725 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram extract. All tests were conducted in triplicate [30, 31].

3. Results and Discussion

Involvement of free radical mediated cell damage in many different diseases and wide usage area of the *Veronica* species has led us to determine radical scavenging activity of fourteen *Veronica* species collected from different location in Turkey. Radical scavenging activity of the aqueous extracts was screened against DPPH, NO and SO radicals. All the tested extracts were found to show dose dependent DPPH radial scavenging ability from the concentration of 50 µg/mL. While they show strong radical scavenging activity against DPPH radical, their activity is not stronger than that of standard compounds BHA and ascorbic acid. The highest IC_{50} value was found for *V. officinalis* as 40.93 µg/mL. *V. cuneifolia* subsp. *cuneifolia*, *V. serpyllifolia* , *V. unknown*, *V. chamaedrys*, *V. baranetzkii* and *V. peduncularis* showed also high IC_{50} lower than 100 µg/mL (Table 2).

Nitric oxide (NO) is a small molecule that contains one unpaired electron, generated in biological system by nitric oxide synthase. Nitric oxide is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a variety of physiological process, such as blood pressure regulation, defence mechanism against pathogens, smooth muscle relaxation and immune regulation. However overproduction of NO may occur when the generation of nitrogen species exceeds the system's ability to neutralize and eliminate them. Increased NO concentration may lead to nitrosylation reactions that can alter the structure of protein and inhibit their normal function. When super oxide reacts with NO, it produces more oxidatively active molecules peroxy nitrite ion ($ONOO^-$), which causes DNA fragmentation and lipid peroxidation [26, 32]. NO scavenging effect of the aqueous extracts determined using Griess reagent. As seen on the Table 2, tested extracts were also showed dose dependent NO radical scavenging activity. While their IC_{50} values were very high comparing to DPPH radical scavenging activity, their effects were found comparable to that of known antioxidants BHA, quercetin and ascorbic acid (AA). *V. officinalis* showed highest NO scavenging

capacity (IC_{50} 570.33 µg/mL) and *V. chamaedrys* and *V. serpyllifolia* showed also high IC_{50} values comparing to other tested extracts (IC_{50}: 651.98 and 697.09 µg/mL, Table 2).

Table 2. DPPH, NO and SO radical scavenging activity and total phenolic contents of the *Veronica* species.

Tested species	IC_{50} (µg/ml)			Gallic acid equivalent total phenolic content (mg/g dry extract)
	DPPH	NO	SO	
V. orientalis	103.63	824.24	198.91	127.64
V. peduncularis	54.19	770.01	186.31	139.92
V. officinalis	40.93	570.33	223.44	200.20
V. baranetzkii	99.04	< 900	330.40	83.15
V.chamaedrys	57.31	651.98	113.39	79.50
V. unknown	63.03	727.84	< 900	98.50
V. fuhsii	117.99	< 900	697.05	46.52
V. serpyllifolia	53.96	697.09	475.23	78.11
V. cuneifolia	76.09	-	214.33	91.02
V. persica	154.49	811.43	142.81	82.04
V. polita	161.61	700.10	143.91	63.75
V. hederifolia	274.1	< 900	540.54	40.90
V. anagallis-aquatica	267.83	< 900	393.09	50.87
V.cymbalaria	390.09	< 900	588.89	-
BHA	> 10	306.64	502.47	
AA	> 10	< 900	13.36	
Quercetin	> 10	658.85	136.6	

On the other hand, NBT assay was carried out to test whether the extracts scavenge superoxide anions or not. Alkaline DMSO, used as a superoxide generating system, reacts with NBT to give colored diformazan. SO radical scavenging activity of the extracts was observed from the concentration of 25 µg/mL and *V. chamaedrys* showed the highest scavenging activity with IC_{50} 113.39 µg/mL. When the results compared with the activity of standard compounds, only AA showed higher activity, quercetin (IC_{50} 136.6 µg/mL) and BHA (IC_{50} 502.47 µg/mL) showed lower scavenging activity then *V. chamedrys* (Table 2). Since superoxide anion is the most common free radicals *in vivo,* a precursor for other ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen that have the potential reactivity with biological molecules and inducing tissue damage, the concentration of superoxide anions is very important under conditions of oxidative stress.

Table 3. Isolated iridoid glycosides from tested species (1. *V. cuneifolia,* 2. *V. cymbalaria*, 3. *V. persica*, 4. *V. hederifolia*, 5. *V. polita*, 6. *V. anagallis-aquatica* 7. *V. fuhsii,8. V. serpyllifolia)*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Aucubin [1]	+	+	+	+	+			
Catalpol [2]	+	+					+	
Veronicoside [3]	+	+	+	+	+	+		+
Catalposide [4]	+	+	+	+	+	+		+
Verproside [5]	+	+	+			+	+	+
Amphicoside [6]	+	+	+	+	+			
6-O-veratroyl catalposide [7]		+	+				+	
6-O-izovanilloil katalpol [8]		+						
Verminoside [9]	+	+	+	+	+	+	+	
8-epiloganin [10]	+	+			+			
6′-*O*-benzoyl-8-epiloganic acid [11]						+		
6′-*O*-*p*-hydroxybenzoyl-8-epiloganic acid [12]						+		
6′-*O*-benzoyl-gardoside [13]						+		
Urphoside A [14]				+				
Pikuroside [15]				+				

Concerning DPPH, NO and SO radical scavenging activity of the tested extracts, DPPH radicals found to be the most scavenged radicals. Radical scavenging activity of the extracts indicated the presence of high phenolic contents of the extract. It is obvious that the total phenolic content measured by the Folin–Ciocalteu procedure does not give a full picture of the quality or quantity of the phenolic constituents in the extracts. However, correlation between the radical scavenging activity and the total phenolic contents of the different extracts was shown in a lot of different study [29]. Therefore, total polyphenols in the extracts were determined by Folin-Ciocalteu reagent. This assay has been extensively used to measure the total phenolics in plant materials for many years and based on electron transfer reaction and actually measures a sample's reducing capacity. Therefore, it is accepted as a routine assay for rough estimation of the antioxidant capacity of herbal samples. Here, total phenolic content of the aqueous extracts was expressed as gallic acid equivalent in mg/g dry extract. As shown in Table 2, highest phenolic content was determined for *V. officinalis,* 202.20 mg/g dry extract. Since *V. officinalis* has been found to show strongest DPPH and NO radical scavenging activity, direct correlation between the high phenolic content of the plant and its radical scavenging activity indicated that radical scavenging activity of *V. officinalis* was arising from phenolic content of the plant. On the other hand, radical scavenging activity of tested species was found close to each other against NO and SO radicals. Well known high ester-iridoid and phenylethanoid contents for *Veronica* species, and above results show us to the importance of phenolic constituents for the

scavenging activity [7,8,18]. In addition, since highest phenolic content was found for *V. officinalis* together with its strongest DPPH and NO scavenging capacity, bioactivity guided isolation studies will be performed on this species to find the responsible compounds. In our previous studies on genus *Veronica*, *V. cuneifolia* subsp. *cuneifolia*, *V. cymbalaria*, *V. persica*, *V. hederifolia*, *V. polita*, *V. anagallis-aquatica* and *V. fuhsii* were studied phytochemically. As seen on Table 3, their iridoid contents are very close to each other with minor differences. We have mostly focused on iridoid contents of the plants up to now because of their chemotaxonomic importance. In future studies, it should be investigated for phenolic contents of the *Veronica* species together with their iridoid contents. Our phytochemical and biological researches on the member species of Plantaginaceae family are still continuing and further researches are needs to clarify chemotaxonomic and biologic significance of these species

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