

Assessment of Radical Scavenging Activities and Antiproliferative Properties of Two Cinquefoil (*Potentilla*) species with Their Phytochemical Contents

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ABSTRACT

Objective: *Potentilla* species have been used in traditional medicine for their antidiarrhoeal, antiviral, antidiabetic, anti-inflammatory, wound healer, coagulant and anticancer activities. We investigated the radical scavenging and cytotoxic activities of *P. recta* and *P. astracanica* which grown widely in Turkey to provide scientific contribution towards their traditional usage as well as to find a new candidate for natural antioxidant product.

Materials and Methods: Different extracts were prepared from the aerial parts of *P. recta* and *P. astracanica*. DPPH, $SO_2^{\cdot-}$ and NO radicals scavenging assays conducted to assess antioxidant activity. Cytotoxic activity of extracts in Hep-2 cell line were tested by MTT method. In order to evaluate phytochemical content of the species; total phenolic, total flavonoid and total flavonol contents of the extracts were estimated.

Results: All samples were tested at 50, 100 and 250 µg/ml concentrations for DPPH, $SO_2^{\cdot-}$ and NO radicals scavenging activities. All the extracts showed statistically significant radical scavenging activity dose-dependently. In general, *P. recta* was found to be more active than *P. astracanica*. According to the results, butanol extract of *P. recta* (2) exhibited very strong scavenging effect for $SO_2^{\cdot-}$ radical. Butanol extract of *P. astracanica* (5) scavenged 56% of the NO radicals at 50 µg/ml. The cytotoxic effects of the extracts were determined at 10, 25, 50, 100 µg/ml concentrations and ethyl acetate extracts of both plants showed cytotoxic effect at 100 µg/ml.

Conclusion: *Potentilla* species are good candidates to be promising possible sources for future novel antioxidants in food and pharmaceutical formulations. The strong scavenging activity partly explain the beneficial effects of *Potentilla* species for the treatment of degenerative diseases like cancer. Due to their active contents it seems that these plants are worth investigating for further studies.

Key words: *Potentilla*, antioxidant, cytotoxicity

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Introduction

Using antioxidants against oxidative stress in humans has attracted considerable interest for the last years. Free radical induced oxidative stress plays an important role in the etiology and pathogenesis of different pathologies such as diabetes, arteriosclerosis, cardiovascular illnesses, cancer and several neurodegenerative disorders. Antioxidant products are important to prevent several oxidative-stress related diseases [1,2]. Although synthetic antioxidants have often been used to protect against free radicals in medical practices, they may be unsafe because of their possible side effects and toxicity to the biological system. Thus, recently, health authorities draw

attention to the use of natural antioxidants against different pathologies [1].

The genus *Potentilla* L., (Rosaceae), which comprises more than 300 species, is mainly distributed in temperate regions of the Northern hemisphere [3]. These species are represented by 53 species in the flora of Turkey [4,5]. Some *Potentilla* species (cinquefoils) have been used for their antidiarrhoeal, antiviral, antidiabetic, anti-inflammatory, wound healing, coagulant and anticancer effects in traditional medicines [6-8]. Antiulcerogenic, antidiarrhoeal, antiinflammatory, antidiabetic, antioxidant, antiviral, antimicrobial activities of these species have

been shown both *in vitro* and *in vivo* studies [7,9]. Antiulcerogenic and rotavirus induced antidiarrhoeal activities of cinquefoils have been proven by different clinical studies [10-12]. Moreover, the European Medicine Agency Committee on Herbal Medicinal Products released an assessment report on *Potentilla erecta* (L.) Raeusch. Use of this plant for the symptomatic treatment of mild diarrhea as well as minor inflammations of the oral mucosa were assessed in this report [13]. Triterpenoids and flavonoids constitute the main compounds of *Potentilla* species [8,13-15]. Presence of phenolic compounds such as tannins, flavonoids in both *P. recta* and *P. astracanica*, neolignans and coumarins in *P. recta* have been shown in previous studies [14,16,17]. These traditional usages and the rich polyphenolic content of *Potentilla* species make these plants more remarkable from the viewpoint of biological and toxicological research. According to the literature the antioxidant activity of *P. astracanica* has not been examined. Although DPPH, $O_2^{\cdot-}$, H_2O_2 , radical scavenging activities of *P. recta* were investigated, NO and $SO_2^{\cdot-}$ radical scavenging activities of *P. recta* were evaluated for the first time in this study [18]. Subsequently, this is the first study to report total phenol, flavonoid, flavonol contents of *P. recta* and the biological activity of *P. astracanica*. We investigated the 2,2-diphenyl-1-picrylhydrazyl (DPPH), super oxide ($SO_2^{\cdot-}$) and nitric oxide (NO) radical scavenging and cytotoxic activities of different extracts of *P. recta* and *P. astracanica*. Moreover, total phenol, flavonoid and flavonol were determined to evaluate the correlation of quantity of these polyphenolic compounds and tested biological activities.

Material and Methods

Plant material

The aerial parts of *Potentilla recta* L. were collected on the 7th May, 2008, from Ankara province, *P. astracanica* were collected on the 3rd June, 2009, from Abant Lake, Bolu province, Turkey. The samples were authenticated by Prof. M.K. Sakar, and Prof. Dr. G. Akaydin Hacettepe University. Voucher specimen deposited in the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 08002, 09329).

Chemicals

DPPH, Nitroblue Tetrazolium (NBT), Diphenyltetrazolium Bromide (MTT), sodium nitroprusside, NaOH, $AlCl_3$, sodium acetate, gallic acid were

purchased from Sigma. Methanol (MeOH), butanol, (BuOH), dimethyl sulfoxide (DMSO), and Folin-Ciocalteu reagent were purchased from Merck. Ethyl acetate (EtOAc) was purchased from Riedel.

Preparation of extracts

Since the antioxidant compounds found in plants have different polarities, different solvents are used to extract them. Plant samples first extracted with methanol and then partitioned with petroleum ether, ethyl acetate and *n*-butanol, respectively. Radical scavenging and cytotoxic activities of six extracts from two *Potentilla* species were evaluated.

Air-dried and powdered plant materials (2 g) were extracted with 80% MeOH (4 × 200 ml) at room temperature, and combined methanol extracts were concentrated under reduced pressure. The resultant extract was then dissolved in H_2O and water-soluble portion was partitioned with petroleum ether (40–60°C) (4 × 100 ml), EtOAc (4 × 100 ml) and *n*-BuOH (4 × 100 ml), respectively to yield extracts: *P. recta*-EtOAc (1), *P. recta*-BuOH (2), *P. recta*- H_2O (3), *P. astracanica*-EtOAc (4), *P. astracanica*-BuOH (5), *P. astracanica*- H_2O (6).

DPPH radical scavenging effect

DPPH radical scavenging effect of the extracts of were assessed by the decolorization of the solution of DPPH; as previously described [19]. MeOH solution (100 µl) of the samples at various concentrations (50, 100 and 250 µg/ml) were added to DPPH/MeOH (1.5×10^{-5} M) solution. After incubating at 37°C for 30 minutes, the remaining DPPH was determined by spectrophotometry at 517 nm. The radical scavenging activity of each sample was expressed as % ±SD compare to blank. The experiment was conducted as triplicate.

$SO_2^{\cdot-}$ radical scavenging effect by alkaline DMSO method

The method of Elizabeth and Rao was used for the detection of $SO_2^{\cdot-}$ scavenging activity of the extract with slight modification [20,21]. The reaction mixture containing 10 µl of NBT (1 mg/ml solution in DMSO) and 30 µl of the extract (50, 100 and 250 µg/ml) were dissolved in DMSO. One hundred microliter 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 radical scavenging activities were given as % activity \pm SD compared to blank. The experiment was conducted as triplicate.

NO scavenging effect

NO radical scavenging activity of extracts was determined by the method of Tsai et al [22]. 60 μ l of diluted samples were added into a 96-well plate. Following this, 60 μ l of 10 mM sodium nitroprusside, dissolved in phosphate buffered saline (PBS), were added to each well and the plate was incubated at room temperature for 150 min. Finally, an equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₃PO₄) was added to each well in order to measure the nitrite content. After chromophore was formed at room temperature in 10 min, absorbance at 577 nm was measured in a microplate reader. This activity tested at 50, 100 and 250 μ g/ml concentrations and all tests were conducted in triplicate. The radical scavenging activity of each sample was expressed as % \pm SD compare to blank.

MTT assay for the cytotoxicity of the compounds

Cell viability was evaluated by the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) [23,24]. Briefly, Hep2 cells (10000 cells/well) were treated with different concentrations of the compounds (10 μ g/ml, 25 μ g/ml, 50 μ g/ml, 100 μ g/ml). After incubation with MTT, cells were lysed in DMSO and the MTT formazan was qualified by determining the absorbance at 570 nm. Cell viability was expressed as a percent of the control culture value.

Estimation of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu reagent. In brief, a number of dilutions of gallic acid were obtained to prepare a calibration curve. 10 μ g/ml extracts and gallic acid dilutions were mixed with 150 μ l of diluted Folin-Ciocalteu's reagent (1:4 reagent:water) were added to 96 well-plates. The plate was incubated at 20°C for 3 min. Afterwards, 50 μ l sodium carbonate was added to mixture and left for another incubation for 2 hours. Absorption was measured at 725 nm using a Bio-Tek Instruments, M-Quant Biomolecular spectrophotometer. All tests were conducted in triplicate [25].

Determination of total flavonoids

Total flavonoids were determined using the method based on the formation of a flavonoid-aluminum complex [26]. A volume of 100 μ l of 2% AlCl₃ ethanol solution was mixed with 100 μ l of the sample solution (1 mg/ml). The resultant mixture was incubated for 1 h at room temperature for yellow color development which indicated the presence of flavonoids. The absorbance was measured at 420 nm using UV-VIS spectrophotometer. All tests were conducted in triplicate.

Determination of total flavonols

Total flavonol contents were determined using the method in the literature [27]. A volume of 40 μ l of the plant sample (1 mg/ml) was mixed with 40 μ l of 2% AlCl₃ prepared in ethanol and 60 μ l of (50 g/L) sodium acetate solution. The mixture was incubated at 20°C for 2.5 hours after which the absorption was measured at 440 nm.

Statistical analysis

The results are expressed as average values of three measurements \pm standard deviation. The results obtained in vitro were statistically processed using the SPSS program. Differences between the means of data were compared by the one way variance analysis (ANOVA) test, $p < 0.05$ was accepted for statistical significance.

Results

SO₂^{•-} Radical Scavenging Effect

The SO₂^{•-} scavenging effects of extracts are shown in Fig. 1A and Table 2. Similarly to DPPH assay, all extracts showed significant SO₂^{•-} radical scavenging activity in a dose dependent manner (50, 100 and 250 μ g/ml). Especially, **2** exhibited very strong scavenging effect for this radical ($p < 0,05$)

DPPH Radical Scavenging Effect

The DPPH assay is widely used for determining the antioxidant activity of natural compounds since it is cheap, sensitive and an easy technic to apply to many samples at the same time. Also it is useful to identify active ingredients at low concentrations. In our case, all extracts exhibited DPPH radical scavenging activity in a dose dependent manner. *P. recta* showed higher activity than *P. astracanica* as is shown in Fig. 1B and Table 1. The extracts were tested at 50, 100 and 250 μ g/ml concentrations. Maximum effect was observed at 250 μ g/ml concentration ($p < 0,05$).

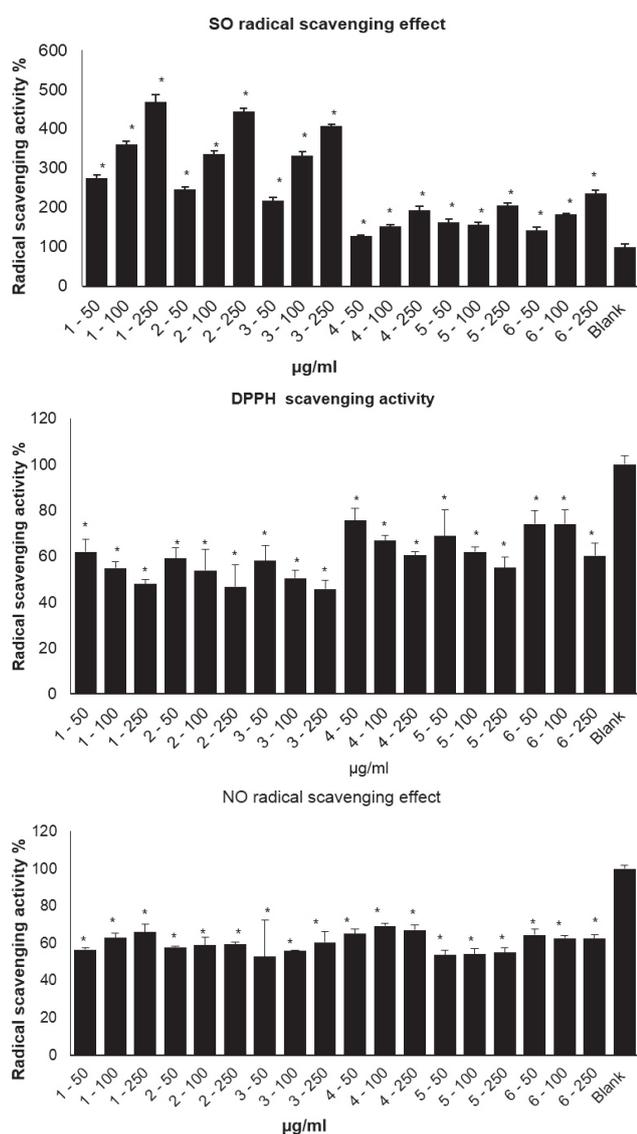


Figure 1. Radical scavenging effect of different extracts of *P. recta* and *P. astracanicum*. (A) SO_2^- radical scavenging effect of extracts. (B) DPPH radical scavenging effect of extracts. (C) NO radical scavenging effect of extracts. *P. recta*-EtOAc (1), *P. recta*-BuOH (2), *P. recta*-H₂O (3), *P. astracanicum*-EtOAc (4), *P. astracanicum*-BuOH (5), *P. astracanicum*-H₂O (6).

* $p < 0.05$, significantly different from blank.

NO Radical Scavenging Effect

All *Potentilla* extracts scavenged the NO radical very strongly (Fig. 1C and Table 3) at all the concentrations (50, 100 and 250 µg/ml). 5 scavenged 56% of the NO radicals at 50 µg/ml ($p < 0.05$).

Cytotoxic Activity by MTT Assay

All extracts were tested at 1, 10, 25, 50 and 100 µg/ml in Hep2 cell line. The cells proliferated up to 100 µg/ml concentration of the extracts (Fig. 2). Extracts 1, 2, 4 and 5 have cytotoxic effect at 100 µg/ml. Extract

Table 1. DPPH scavenging activity of the extracts at 50, 100, 250 µg/ml concentrations. *P. recta*-EtOAc (1), *P. recta*-BuOH (2), *P. recta*-H₂O (3), *P. astracanicum*-EtOAc (4), *P. astracanicum*-BuOH (5), *P. astracanicum*-H₂O (6).

	50 µg/ml	100 µg/ml	250 µg/ml
1	68,8±11,5	61,9±2,1	55,0±4,6*
2	62,0±5,5*	54,8±2,7*	47,9±2,0*
3	58,1±6,6*	50,5±3,4*	45,7±3,9*
4	59,0±4,8*	53,8±9,1*	46,7±9,4*
5	74,1±5,9*	74,1±6,2*	60,3±5,3*
6	75,6±5,2*	66,9±2,4*	60,4±1,7*

* $p < 0.05$, significantly different from blank
Activity % means of the compound versus blank (100%).
Results are given as activity % ±SD.

4 was the most cytotoxic extract.

Total Phenol, Flavonoid And Flavanol Contents

Total phenol content were calculated using the standard calibration curve of gallic acid ($y = 0.0037x + 0.0697$, $R^2 = 0.9999$) and expressed as gallic acid equivalents (GAE mg/g): 1 (277.05±0.31), 2 (157.42±0.23), 3 (118.70±0.23), 4 (134.29±0.83), 5 (98.88±0.43), 6 (88.60±0.42). 1 was the richest extract in terms of total phenol content and it was followed by 2.

Total flavonoid contents were determined according to the Default ($y = 0.0218x + 0.0993$, $R^2 = 0.9994$). Total flavonoid contents were expressed as quercetin equivalent (mg/g): 1 (32.2477±0.21), 2 (25.5541±0.43), 3 (9.2522±0.32), 4 (94.0688±0.58), 5 (64.9862±0.13), 6 (21.9128±0.22). *n*-BuOH extracts of both species (2 and 5) had the highest flavonoid content.

Total flavanol content were calculated in accordance with the Default ($y = 0.0187x + 0.2211$, $R^2 = 0.9993$). In this study, total flavanol content of the extracts of two *Potentilla* species were expressed as quercetin equivalent (mg/g): [1 (25.8946 ±0.21), 2 (32.8823±0.23), 3 (7.1604±0.12), 4 (23.877±0.82), 5 (21.3614±0.42), 6 (11.0085±0.84)].

Discussion

Phenolic compounds have strong antioxidant properties via their radical scavenging effects and protect the cellular molecules such as lipids and proteins against induced free radical DNA damage [28].

Table 2. SO₂^{•-} scavenging activity of the extracts at 50, 100, 250 µg/ml concentrations. *P. recta*-EtOAc (**1**), *P. recta*-BuOH (**2**), *P. recta*-H₂O (**3**), *P. astracanica*-EtOAc (**4**), *P. astracanica*-BuOH (**5**), *P. astracanica*-H₂O (**6**).

	50 µg/ml	100 µg/ml	250 µg/ml
1	163,0±7,2*	156,4±6,2*	206,2±5,9*
2	274,2±9,2*	360,1±9,6*	469,2±17,4*
3	217,9±8,4*	331,9±10,3*	406,6±5,5*
4	246,2±6,1*	335,5±8,0*	443,4±8,6*
5	142,4±8,0*	181,9±3,6*	236,6±8,2*
6	127,7±2,6*	151,8±5,0*	193,0±9,7*

*p < 0.05, significantly different from blank.

Activity % means of the compound versus blank (100%).

Results are given as activity % ±SD.

In our study, we investigated the radical scavenging and cytotoxic activities of the extracts obtained from *P. recta* and *P. astracanica*. The free radical DPPH has been used in many applications due to its high stability and intense purple color that changes whenever it reacts. Moreover, the DPPH assay is the most extensively used method for screening the antioxidant activity of natural products since it can be applied to many samples in a rational time and identify active ingredients at low concentrations [29]. All tested extracts showed DPPH radical scavenging effect and maximum effect was observed for 250 µg/ml concentration for each extract. *P. recta* found to be a better DPPH scavenger when compared to *P. astracanica* (Fig. 1A). Extracts of *P. recta* scavenged almost half of DPPH radical at 250 µg/ml: **1** (47.09±2.03%), **2** (46.70±3.92), **3** (45.74±3.92).

SO₂^{•-} is one of the most powerful reactive oxygen species among the free radicals that could be generated and all tested extracts scavenged this radical in dose dependent manner (Fig. 1B). *P. recta* were found to be better radical scavengers when compared to *P. astracanica* against SO₂^{•-} radical, too. **1** (EtOAc extract of *P. recta*) was the most powerful SO₂^{•-} radical scavenger and was followed by **2** (n-BuOH extract of *P. recta*). After treating sample with **1**, SO₂^{•-} radical diminished more than 4-fold comparing the blank at 250 µg/ml.

NO is a very unstable radical species under the aerobic conditions. It reacts with O₂ to produce its stable products nitrate and nitrite. All tested *Potentilla* extracts showed a NO radical scavenging

Table 3. NO scavenging activity of the extracts at 50, 100, 250 µg/ml concentrations. *P. recta*-EtOAc (**1**), *P. recta*-BuOH (**2**), *P. recta*-H₂O (**3**), *P. astracanica*-EtOAc (**4**), *P. astracanica*-BuOH (**5**), *P. astracanica*-H₂O (**6**).

	50 µg/ml	100 µg/ml	250 µg/ml
1	53,7±2,3*	54±2,9*	55±2,2*
2	56,5±0,9*	62,8±2,5*	65,9±4,5*
3	52,8±19,4*	55,8±0,3*	60,5±5,6*
4	57,8±0,5*	58,9±4,1*	59,3±1,1*
5	64,4±3,4*	62,7±1,4*	62,4±2*
6	65,1±2,3*	69,3±1,4*	67±2,9*

*p < 0.05, significantly different from blank.

Activity % means of the compound versus blank (100%).

Results are given as activity % ±SD.

effect (Fig. 1C). **5** (n-BuOH extract of *P. astracanica*) was the strongest NO scavenger among the tested extracts, and it scavenged almost half of the present NO radical in the tested conditions. However, scavenging effect of **5** at tested concentrations did not significantly differ from each other.

Our evaluation of the literature showed that there are no biological activity studies on *P. astracanica* until now. NO and SO₂^{•-} radical scavenging activities of *P. recta* had not been investigated, either. However, DPPH, O₂^{•-}, H₂O₂, radical scavenging activities of *P. recta* have been investigated, and it has been shown that it has concentration dependent radical scavenging activity. While ethyl acetate extract was found to be the most powerful DPPH scavenger, diethyl ether extract was the most powerful O₂^{•-} radical scavenger. Researchers showed that a hydrolysable tannin, which is also a polyphenolic compound, was responsible for the antioxidant activity [29]. In our present study, we found that DPPH radical scavenging activities of ethyl acetate and n-butanol extract were not statistically different at the tested concentrations except at 250 µg/ml.

Natural polyphenolic compounds can exhibit prooxidant properties under certain conditions as well as antioxidant effects. Possible prooxidant action of the compounds could contribute to their anticancer properties at higher doses [30,31]. In our study, cytotoxic activity of extracts against to Hep-2 cells were evaluated, and generally extracts enhanced the cell proliferation (except **2**) up to 100 µg/ml and started to show cytotoxic effect at

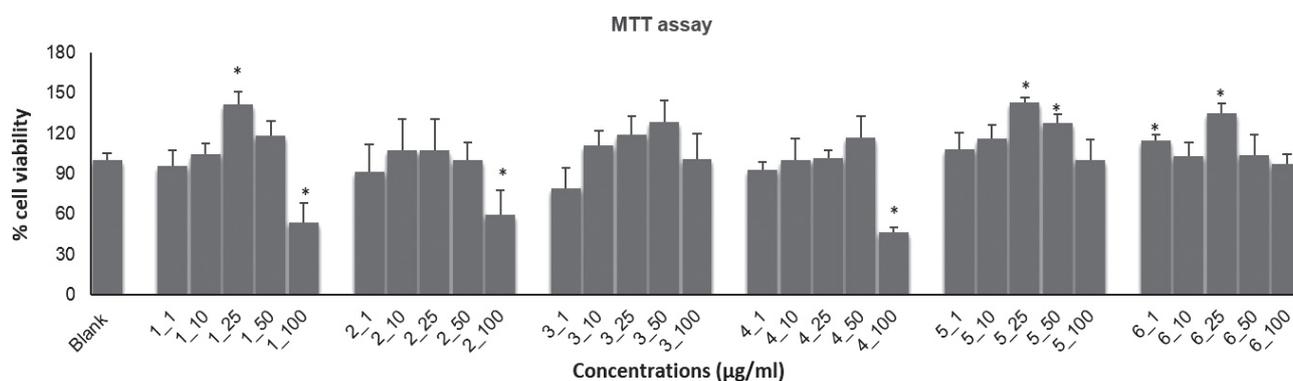


Figure 2. Cytotoxic activity of extracts. *P. recta*-EtOAc (1), *P. recta*-BuOH (2), *P. recta*- H₂O (3), *P. astracanica*- EtOAc (4), *P. astracanica*- BuOH (5), *P. astracanica*- H₂O (6).

*p < 0.05, significantly different from blank

this concentration. 5 and 6 did not show any cytotoxicity at the tested concentrations. Extracts 1 and 4 were diminished cell viability to 44.33% and 46.39%.

Plant phenolic constitutes one of the major groups of compounds acting as primary antioxidants [32]. Of all the phytochemicals tested, total phenol contents were the highest, 277.05 mg/g, and 157.42 mg/g (catechin equivalent) for ethyl acetate (1) and n-butanol (2) extracts of *P. recta*, respectively. n-butanol extract of *P. astracanica* was the richest extract (5) 98.88±0.43 mg/g (quercetin equivalent); in terms of total flavonoid content. Ethyl acetate extract of *P. recta* (1) also had the highest total flavonols 25.8946 mg/g (quercetin equivalent). According to the literature, polyphenolic compounds have been shown to possess significant antioxidant activity. The antioxidant properties of phenolic compounds originate from their properties of proton loss, chelate formation, and dismutation of the radicals. The high level of phenol content of plant extracts might account for the strong scavenging activity against DPPH, SO₂⁻ and NO radicals. This scavenging activity could be due to the presence of hydroxyl groups attached to the aromatic ring structures. They give hydrogen

atoms from their hydroxyl groups to radicals and form stable phenoxyl radicals; hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of phenolic compounds is very important in order to determine the antioxidant capacity of plant extracts [33,34]. Thus, the high phenolic content in this plant may be responsible for the strong antioxidant activity observed in this study. However, we cannot conclude this assumption for the total flavonoid content.

Our results obtained from radical scavenging assays provide important evidence that both *P. recta* and *P. astracanica* are potent source of protective agent against oxidative stress. On the other hand, it has been reported that plant polyphenols are naturally occurring antioxidants, and they also exhibit pro-oxidant properties under certain conditions. For instance, the prooxidant action of the plant polyphenols could contribute to their anticancer properties [31]. However, our results suggest that further investigation is necessary to determine the medicinal properties of the plant *in vivo* and *in vitro*.

Declaration of interest

The authors report no conflicts of interest.

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