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Research Article

ANALYSIS OF PHENOLIC ACIDS AND INVESTIGATION OF ANTIOXIDANT ACTIVITIES OF TWO *PYCNOCYCLA* SPECIES EXTRACTS

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ABSTRACT
The aim of this study was to determine the phenolic compounds and antioxidant activities of *Pycnocycla aucherana* and *Pycnocycla bashagardiana* extracts to find possible sources for future novel antioxidants in food and pharmaceutical formulations. The aerial parts of plants were extracted with 80% methanol, and hydro distillated for essential oil isolation. The antioxidant capacity of methanol extracts and essential oils were assessed using the 2, 2-diphenyl-2-picryl hydrazyl (DPPH) and β-carotene/linoleic acid bleaching (BCB) tests. Results showed that no phenolic and flavonoid compounds were identified in essential oils. The maximum values of the total phenolic (65.25 mg/g) and flavonoid (12.75 mg/g) were observed in *P. bashagardiana* and *P. aucherana*, respectively. The *P. bashagardiana* extract showed the maximum DPPH radical scavenging activity (79.94%) and inhibition (49.15%) of bleaching of b-carotene, and minimum IC_{50} (0.71 µg/ml). The obtained results revealed that the antioxidant activity of extracts was proportional with the phenolic compounds of extracts.

Keywords: *Antioxidant Activities, DPPH, β-Carotene, Pycnocycla Aucherana, Pycnocycla Bashagardiana*

***Abbreviations*:**
BCB - β-carotene/linoleic acid bleaching test,
TP - Total phenolic,
TF - Total flavonoid,
BHT - Butylated hydroxyl toluene,
DPPH - 2,2-diphenyl-1-picrylhydrazyl,
BHA - Butylated hydroxyl anisole,

INTRODUCTION
Free radicals can cause oxidative damage to lipoproteins which lead to aging and disease in human (Hussain *et al.*, 2008; Shahidi and Wanasundara, 1992). The activated oxygen incorporates reactive oxygen species (ROS) produce in our body on stress condition and damage cells (Sharififar *et al.*, 2003; Manda *et al.*, 2009).
The use of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-Butylhydroquinone due to their carcinogenicity and other toxic properties have been limited (Ahmad, 2004). Therefore, the use of natural antioxidants has considerably increased. The Natural antioxidants such as phenolic compounds (tocopherols, flavonoids, and phenolic acids) and carotenoids are able to prevent fatty acid oxidation. Several evidences have shown an inverse correlation between the intake of dietary antioxidants and the risk of chronic diseases such as coronary heart disease, cancer, and several other aging related health concerns (Hussain *et al.*, 2008; Hossain *et al.*, 2010).
Plants are a good source to produce a wide range of natural antioxidants. However, there are not enough knowledge and studies about the practical usefulness of plants. The secondary plant metabolites, antioxidant phenolics, and flavonoids are commonly found in various fruits, vegetables and medicinal plants. They have been shown to provide a successful defense against oxidative stress from oxidizing agents and free radicals (Matkowski, 2006; Sarikurkcu *et al.*, 2009; Antolovich *et al.*, 2000). Most of the

herbal infusions, commonly used as home medicines have antioxidative and pharmacological properties which can be attributed to the presence of phenolic compounds such as phenolic acids derivatives and flavonoids. Polyphenols such as phenolic derivatives and flavonoids are also able to prevent the fatty acids from oxidative decay (Fecka *et al.,* 2007).

The family *Umbelliferae* is one of the most numerous families within vegetable crops. This family is rich in secondary metabolites and has numerous genera with high economic and medicinal value (including essential oil and extract) (Margaris *et al.,* 1982). The *Pycnocycla* genus is belong to *Umbelliferae* family, and has eight endemic perennial species with spine leaves in Iran. Some pharmacological properties of *Pycnocycla* sp. such as anti-spasmolytic and anti-diarrhea effects, anti-microbial activity, relaxant and cardiovascular activities have been previously confirmed (Sadraei *et al.,* 2008; Sadraei *et al.,* 2006). The *P. aucherana* and *P. bashagardiana* plants are an endemic species of Iran. They are commonly distributed in South regions of Iran including Bandar Abbas and Hormozgan Province. However, there is no study about the antioxidant activity of these plants. In this work, the total phenols content and antioxidative properties of the essential oil and the extracts of the *P. aucherana* and *P. bashagardiana* were investigated by DPPH, b-carotene/linoleic acid.

Experimental Procedures

Plant Materials, Extractions and Isolation of the Essential Oil

Aerial parts of *P. aucherana* and *P. bashagardiana* were obtained from Hormozgan, Bandar Abbas, Iran, and dried under air atmosphere at temperature of 25 °C for 5 days. The dried aerial parts of each plant was pulverized into the powdered form. The dried powder sample (100 g) was mixed with methanol solvent (80% methanol) under stirring at room temperature for 72 h. The extract was filtered through Whatman No. 41 filter paper to obtain particle free extract. The extracts were concentrated and dried under vacuum. For isolation of essential oil, 100 g of dried powder for each plant was subjected to hydro distillation for 3 h using a Clevenger type apparatus. Finally, the oil was dried with anhydrous sodium sulfate and was preserved in a sealed vial at 4 ºC.

Total Phenol (TP) Content Measurement

The level of TP in the crude extracts was determined by using Folin–Ciocalteu reagent and external calibration with gallic acid. Briefly; 0.5 mL of extract solution and 0.25 mL of Folin–Ciocalteu reagent were mixed thoroughly (Singelton *et al.,* 1999). After 4 min, 1 mL of 15% Na_2CO_3 was added, and then the mixture was further mixed for 2 h at room temperature. The absorbance was measured at 760 nm using a Shimadzu UV/VIS-120-01 spectrophotometer (Japan). The concentration of the total phenolic acids was calculated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve.

Determination of Total Flavonoid (TF) Content

The TF content of samples was assayed using the aluminum chloride colorimetric method as described previously by Chang *et al.,* (2002). The appropriate dilution of extracts (0.5 mL) were mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV/VIS-120-01 spectrophotometer (Japan). The TF content was calculated using a standard calibration curve.

Radical Scavenging Activity Using DPPH Method

Radical scavenging effects of the extracts were determined based on reducing the 2, 2-diphenyl-2-picryl hydrazyl (DPPH) solution. Different concentrations of the extract were added to 2 mL of a methanol solution of DPPH.

The absorbance of the solution was reported at 517 nm at room temperature after 30 min incubation. Inhibition of free radicals (I (%)) by DPPH was calculated as follows:

$$I(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \qquad (1)$$

Where A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. BHT (sigma) was used as a positive control. The IC_{50} was determined as the concentration of a compound that inhibits 50% of the DPPH solution (Sontag, 1980).

Antioxidant Assay Using B-Carotene-Linoleate Model System

The antioxidant activity was evaluated using b-carotene/bleaching (BCB) test as described by Jayaprakasha *et al.*, (2001) with some modification. Briefly, 0.1 mg of b-carotene were mixed in 0.2 mL of chloroform, 10 mg of linoleic acid and 100 mg of Tween-20 (polyoxyethylene sorbitan monopalmitate). The solvent was removed at 40 ºC under vacuum and the prepared mixture was diluted with 10 mL of water and was mixed well. Then, 20 mL of oxygenated water was added. Four milliliters aliquots mixture were pipetted into different test tubes containing 0.35 mL of extracts (100 and 200 ppm) and BHA (100 and 200 µg) in ethanol.

BHA was used for comparative purposes. A control containing 0.2 mL of ethanol and 4 mL of the above emulsion was prepared. The tubes were placed at 50 ºC in a water bath and the absorbance at 470 nm was taken at zero time (t= 0). The absorbance was continued until the color of b-carotene disappeared in the control tubes (t =60 min) at an interval of 15 min. the other mixture was prepared without b-carotene served as blank.

The antioxidant activity (AA) of each sample was evaluated in terms of bleaching of the b-carotene as follows:

$$AA(\%) = 100\left(1 - \frac{A_0 - A_t}{A_0^0 - A_t^0}\right) \quad (2)$$

where A_0 and A_0^0 are the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A_t^0 are the absorbance of test sample and control after incubation for 60 min. The results were expressed in % basis in preventing bleaching of b-carotene.

Statistical Analyses

Statistical analysis of the data was performed by Analysis of Variance (ANOVA) using SPSS (*version 18*) software. Each value is the mean ± SE of three replicates in each group. Differences between means were determined using Tukey multiple and comparisons least significant difference (LSD) at $P \leq 0.05$.

RESULTS AND DISCUSSION

Several techniques have been used to determine the antioxidant activity in vitro to provide the rapid screening of substances. Free radicals play a definite role in a wide variety of pathological manifestations. Antioxidants can combated the free radicals which protect the human from various diseases (Umamaheswari and Chatterjee, 2008). Phenolic compounds have strong antioxidant activities which protect the cells against the oxidative damage generated by free-radicals. The phenolic acids act as a radical scavengers, metal chelators, reducing agents, hydrogen donors, and singlet oxygen quenchers (Kahkonen *et al.*, 1999; Proestos *et al.*, 2006). The TP contents of the extracts and essential oils were determined by Folin–Ciocalteu methods which were reported as gallic acid equivalents (Singelton *et al.*, 1999). In the present study, the TP compounds content was found to be 65.25 and 50.1 mg GA/g d.s. for *P. bashagardiana* and *P. aucherana*, respectively.

Table 1: Total phenolics and flavonoid contents of *Pycnocycla aucherana* and *bashagardiana* extract and essential oils

Extract	TP (mg/g)	TF (mg/g)
P. aucherana extract	50.1 ± 2.47	12.75 ± 1.21
P. bashagardiana extract	65.25 ± 2.99	9.98 ± 1.68
P. aucherana essential oil	-	-
P. bashagardiana essential oil	-	-

The values are means ± SE of three replicates. Total phenolic content (TP) and flavonoid content (TF)

Also, no TP content was observed in the essential oil (Table 1). Mahboubi *et al.*, (Mahboubi *et al.*, 2014) showed the 57.8 and 33.9 mg/g phenolic contents in metanolic and aqueous extracts of *P. spinosa* plant, respectively. The results indicated that *P. bashagardiana* metanolic extracted with the higher TP amounts which showed this compound was a good source of natural antioxidant for scavenging of free radical.
Flavonoids have shown the positive effects on human health, and have a wide range of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activities (Montoro *et al.*, 2005). Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals (Montoro *et al.*, 2005; Bravo, 1998) implicated in several diseases. The TF content of *P. bashagardiana* and *P. aucherana* extracts were 9.98 and 12.75 mg rutin per 1g dry weight, respectively. Flavonoid content of *P. aucherana* was 27.75% higher than that of *P. aucherana* extract, and no TF content was observed in the essential oil of both plants (Table 1). Mahboubi *et al.*, (2014) showed the 24.4 and 3.8 mg/g flavonoid contents in metanolic and aqueous extracts of *P. spinosa* plant, respectively. Differences in TF contents could be due to genotypic and environmental variations (climate, location, temperature, fertility and diseases) within species (Shan *et al.*, 2005). Phenolic compounds of plants have several categories, and flavonoids and phenylpropanoids are the major contributor to the antioxidant properties of plants (Nunes *et al.*, 2012). According to the present study, flavonoid proportion of total TP content was low in *P. aucherana* and *bashagardiana* extract. Therefore, the other phenolic compounds could be responsible for antioxidant activity of *Pycnocycla* genus.
The DPPH is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant (Lu and Yeap, 2001). Thus, the lower IC_{50} value indicates a higher DPPH free radical-scavenging activity. In the present study, the IC_{50} of both essential oils was higher than the extracts and the lowest inhibitory activity against DPPH radical was observed in *P. bashagardiana* extracts (0.71 µg per ml) (Figure 1). It seems that the difference of IC_{50} between samples could be due to variation in polyphenolic composition in extracts and essential oil of both plants.

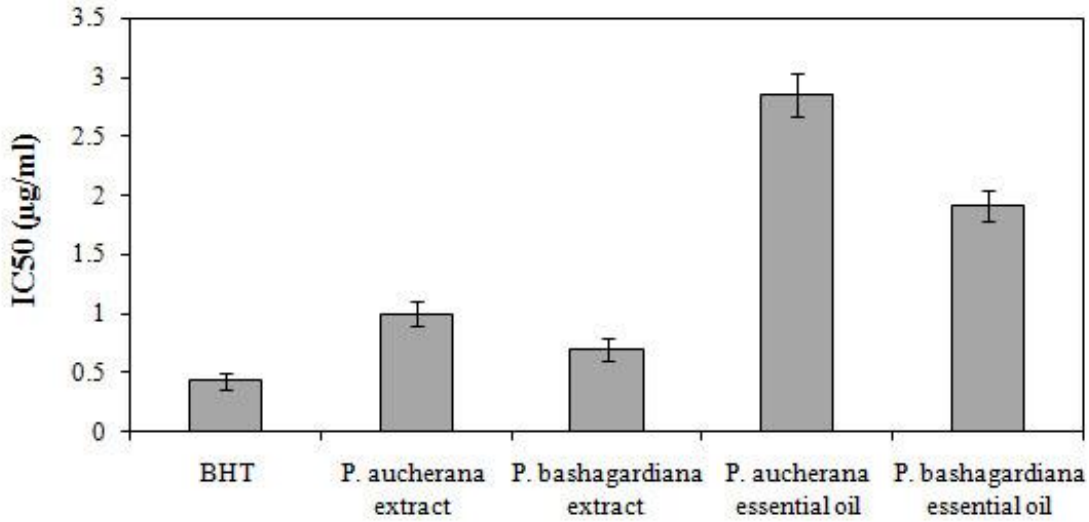

Figure 1: The IC50 (µg/ml) values of *Pycnocycla aucherana* and *bashagardiana* extracts and essential oils

The free radical scavenging activity of the aerial part extract and essential oil of *P. bashagardiana* and *P. aucherana* were tested through DPPH method (Figure 2). Free radicals play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals which protect human from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms (Krishnaiah *et al.*, 2011). The electron donation ability of natural products can be measured by DPPH purple-colored solution bleaching (Nunes *et al.*, 2012). The method

is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The degree of color change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates the remarkable free radical scavenging activity of the compound under test (Krishnaiah *et al.,* 2011). In the present study, the two extracts and essential oils of both plants were able to decolorize the DPPH with different degrees. Free radical scavenging potentials were in the order of BHT > *P. bashagardiana* extract > *P. aucherana* extract > *P. bashagardiana* essential oil > *P. aucherana* essential oil (Figure 2). Results suggest the *P. bashagardiana* extract contain special phytochemical constituents that are capable to donate the hydrogen to a free radical and scavenge the potential damage.

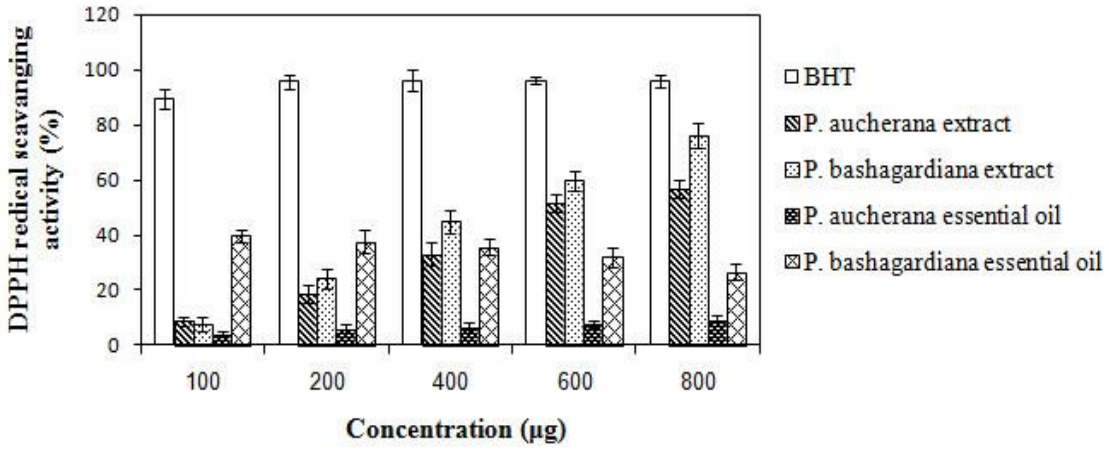

Figure 2: Radical DPPH scavenging activity (%) of *Pycnocycla aucherana* and *bashagardiana* extracts and essential oils

The antioxidant activities through BCB test of the extracts and essential oils of *P. aucherana* and *P. bashagardiana* were compared in 100 and 200 µg/ml concentrations with butylated hydroxyanisole (Table 2). The addition of extracts of *P. aucherana* and *P. bashagardiana,* and butylated hydroxyanisole at 200µg/ml concentrations prevented the higher bleaching of β-carotene compared with 100 µg/ml concentrations. Inhibition (%) of bleaching of b-carotene was in the order of BHT > *P. bashagardiana* extract > *P. aucherana* extract > *P. bashagardiana* essential oil > *P. aucherana* essential oil. Extract of *P. bashagardiana* was 39.86% inhibition higher than that of *P. aucherana* extract (Table 2). In this method, β-Carotene undergoes the rapid discoloration in the absence of an antioxidant. This behavior could be attributed to the coupled oxidation of b-carotene and linoleic acid, which generated the free radicals. The linoleic acid formed free radical upon the abstraction of a hydrogen atom from one of its allylic methylene groups, which attacked the highly unsaturated b-carotene molecules. As a result, b-carotene was oxidized and broken down, subsequently the system lost its chromosphere and characteristic orange color, which could be monitored spectrophotometrically (Hossain *et al.,* 2010).

Table 2: Inhibitory effect of *Pycnocycla aucherana* and *bashagardiana* extract and essential oils on β-carotene bleaching

Extract/BHA	100 µg	200 µg
BHT	85.34 ± 2.35	93.23 ± 3.94
P. aucherana extract	18.33 ± 2.91	35.14 ± 4.38
P. bashagardiana extract	24.43 ± 2.83	49.15 ± 3.66
P. aucherana essential oil	14.29 ± 3.34	28.54 ± 5.91
P. bashagardiana essential oil	17.1 ± 2.48	30.32 ± 3.53

The values are mean s± SE of three replicates

The relationship between antioxidant activity of plant extracts and the phenolic composition is very difficult. Furthermore, plant extracts are complex mixtures of many different compounds with antioxidant properties, showing synergic actions in comparison to individual compounds (Kähkönen *et al.*, 2008). In this study, correlation analyses of values for radical scavenging activity (DPPH, BCB, IC_{50}) and total phenolic compounds (TP, TF) were listed in Table 3. A significant positive linear correlation was observed between TP, BCB ($R^2 = 0.945$; $P \leq 0.01$) and DPPH ($R^2 = 0.999$; $P \leq 0.001$) (Table 3). The TP content also showed a negative relation with TF and IC_{50}. Significant positive correlation between TP and antioxidant assays (DPPH, BCB) indicated TP compounds were the main phenolic constitutes with antioxidant properties in two medicinal plants. Antioxidant activity of phenolic compounds could be attributed to their redox properties, which were able them to act as reducing agents and radical scavengers (Kahkonen *et al.*, 1999; Mai *et al.*, 2009).

Table 3: Correlation analysis between radical scavenging activity (DPPH, BCB, IC_{50}) and total phenolic and flavonoid compounds of *Pycnocycla aucherana* and *bashagardiana* extracts

R^2	TP	TF	BCB	DPPH	IC_{50}
TP	-				
TF	-0.208	-			
BCB	0.945	-0.480	-		
DPPH	0.998	-0.235	0.963	-	
IC_{50}	-0.321	0.869*	-0.568	-0.345	-

Conclusion

The *P. bashagardiana* extract showed slightly more phenolic content compare with *P. aucherana*, while the *P. aucherana* extract showed the higher flavonoid content. The IC_{50} of extracts was lower than that of essential oils, and the lowest IC_{50} was obtained for *P. bashagardiana* extract which showed the highest antioxidant activity. The *P. bashagardiana* extract showed the highest DPPH radical scavenging activity and inhibition of bleaching of b-carotene. The results suggest that the extract of *P. bashagardiana* due to its partially antioxidant properties can be useful in defense against free radical damage possibly.

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