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Platycladus orientalis Extracts with Antioxidant Activity from North of Iran

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Abstract

Recently, the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer. Plant secondary metabolites mainly phenolics and flavonoids are commonly found in various fruits, vegetables and herbs. The plant-derived antioxidants may use as therapeutics for before mentioned diseases. They play this role through acting as reducing agents by donating hydrogen, acting as chelators, quenching singlet oxygen and scavenging free radicals. In the present study, four extracts of *Platycladus orientalis* (L.) Franco leaves by maceration in water, methanol, ethanol and ethyl acetate, were prepared and the antioxidant activities of the extracts have been investigated by DPPH method, H_2O_2 scavenging activity and reducing power assays. The total phenol and flavonoid contents of these extracts were determined and compared with their antioxidant potential. The results showed that antioxidant effect of these extracts is increased in the following order: water < ethyl-acetate < ethanol< methanol extract. Among the extracts, the methanol extract showed as strong extract in both DPPH radical (IC_{50}: 11.45 µg/ml) and H_2O_2 (IC_{50}: 49.9 µg/ml) scavenging activity assays. The ethanol extract was shown a highest reductive potential in comparison with other extracts. There was positive and significant correlation between antioxidant properties and total phenol and flavonoid content of *P. orientalis.* It was concluded that the extracts of *P. orientalis* can use as a source of natural antioxidants in preventation of cellular oxidation.

Key words *Platycladus orientalis*; Extract; Antioxidant; Total phenol; Flavonoid

Introduction

Aerobic metabolism what occurs in living organisms is always under thousands of damages by reactive oxygen derivatives. These species are usually short-lived but possess a single unpaired electron, rendering them highly reactive against biologically important macromolecules (Nabavi *et al.,* 2012). Thousands of literature in clinical and experimental evidences accumulated in the past years suggested that free radical mediated peroxidation of biomolecules, membrane lipids and oxidative damage of DNA are associated with a variety of chronic health problems, such as cancer, atherosclerosis and ageing (Anderson *et al*., 2001; Cheng *et al.,* 2006). Cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidants, and on antioxidant enzymes. A high proportion of the antioxidant systems of the human body are dependent on dietary constituents. Therefore, inhibition of free radical–induced oxidative damage by supplementation of antioxidants has become an attractive therapeutic strategy for reducing the risk of these diseases (Wei *et al.,* 2006; Tsuda *et al.,* 2004).

Antioxidant phenolics and flavonoids as biologically active phytochemicals are commonly found in various fruits, vegetables, grains and herbs and they have been shown to provide an effective defence against oxidative damages from oxidizing agents and free radicals (Matkowski *et al*., 2006; Antolovich *et al.,* 2000). Today, many reports recommend the use of crude phenolic extracts from fruits, herbs, vegetables, cereals and other plant

materials in the food and supplement industry because they have been shown to retard oxidative degradation processes, which can damage biomolecules, such as lipids, proteins, DNA and carbohydrates (Liana & Shahidi 2007).

The evergreen shrub *T. orientalis* L. [=*Biota orientalis* (L.) Endl., *Platycladus orientalis* (L.) franco] (*Cupressaceae*) is a dense, compact large shrub tree that is extensively used as herbal medicine in some eastern Asian countries but not in Iran (Asili *et al.,* 2007). *Thuja orientalis* has its own Persian names: 'Sarve Khomrehi', 'Nush' and Sarve Tabari. Dried leaves of *T. orientalis* have been used as a hemostatic, expectorant and hypotensor in Korean folk medicine. In Chinese medicine this plant is an important herb as a hemostatic, expectorant, and cough remedy. Fresh leaves of the plant are used as an anti-inflammatory drug. Its seeds are used for bronchitis, insomnia and as antitussive. Recent laboratory studies showed that the extracts of the plant have a variety of actions, antimicrobial agent, a fungicide, an inhibitor of platelet-activating factor receptors and a free radical scavenger (Choi *et al.,* 2008; Han *et al.,* 1998; Xu *et al.,* 2009). The several flavonoid constituents of the leaves of *Thuja orientalis* such as rutin, quercetin, quercitrin, amentoflavone, aromadendrin, myricetin and hinokiflacone have been reported. The amount of non-volatile components such as flavonoids and tannins of the fruits and leaves were estimated by Emami (Emami *et al.,* 2007). GC-MS analysis of the essential oil of *P. orientalis* revealed that the percentage of monoterpene hydrocarbons was higher than other components and the major hydrocarbon was α-pinene in the leaf and fruit organs (Yoshimi & Safavi, 2012). It was shown that *P. orientalis* and its active component thujone (a ketone and a monoterpene that occurs naturally in two diastromeric forms: α-thujone and β-thujone) have the great potential against a various health problems. An aqueous extract of *T.* orientalis was shown significant anti-inflammatory activity and anticancer potential. The ethanol extract of *P. orientalis* leaf has been considered as natural nematicidal and molluscicidal extract, which thujone was identified as active molluscicidal component. Also, the leaf extracts in ether and acetone as solvents have shown insecticidal activity (Srivastava *et al.,* 2012).

The aims of this work were (i) to evaluate content of total phenol and flavonoid in various extracts of *P. orientalis* (ii) to characterization *in vitro* antioxidant potential of the extracts in some solvents (water, methanol, ethanol and ethyl acetate) and (iii) to determine possible correlations between total phenolic content (TPC) and total flavonoid content (TFC) of the extracts with their antioxidant activity.

Materials and Methods

Chemicals

Sodium Carbonate, Folin–Ciocalteu reagent, Ferric Chloride, Potassium hexacyanoferrate (III), Butylated hydroxytoluene (BHT), Trichloracetic acid (TCA), Gallic acid, Quercetin, DPPH (2,2-Diphenyl- Picryl-Hydrazyl) radical, hydrogen peroxide 30% (Merck), Ascorbic acid (AA). Methanol, Ethanol and Ethyl acetate as solvents were purchased from Merck. All other Chemicals were of reagent grade and obtained from either Merck, Sigma-Aldrich or Fluka.

Plant material and preparation of the extracts

The plant material, *Platycladus orientalis* (L.) Franco was collected from Aliabad Katool area (Golestan province), during July 2011. The plant was identified in University of Mazandaran, where a voucher specimen (Nr. 1504) was deposited. The leaves were thoroughly washed with tap water in order to remove the dust particle and debris and then rinsed with distilled water. Leaves were dried in shade and then grinded to fine powder. The powder of *P. orientalis* leaves (1g) was soaked in 50 ml of each solvents: methanol, ethanol, water and ethyl-acetate (three times), for 72 h on a shaker in room temperature. These extracts were filtered through Whatman No.1 filter paper and collected after removing the solvent by rotary evaporator apparatus. The aqueous extract was dried in oven at 50 ºC. The dried extracts were stored at 4ºC in our refrigerator until use.

Determination of total phenolic content

The total phenolic content (TPC) was determined according to a modified version of

the procedure by Singleton and Rossi (Singleton *et al.* 1999). Briefly; 0.2 ml of different extract solution and 0.5 ml of Folin-Ciocalteu reagent were mixed thoroughly. 1 ml of 7.5% Na_2CO_3 was added to each tube. The contents in the tube were mixed by vortexing. Tubes were allowed to stand at ambient temperature for 30 min until the characteristic blue colour developed. Absorbance was measured at 760 nm. The content of total phenolic in each extract was calculated by employing standard curve prepared using gallic acid and expressed as micrograms of gallic acid equivalents (GAE) per gram dried plant.

All the experiments in this report were conducted in triplicate and the results are mean ± SD values. A blank was used for the evaluation without any extract added with same amount of solvents lonely.

Determination of total flavonoid content

Total flavonoid content (TFC) of the *P. orientalis* extracts were determined by using the aluminium chloride colorimetric method as described by Willet [Willet 2002], with some modifications. Ethanol, methanol, ethyl-acetate and aqueous extracts (2.8 ml), 10% aluminium chloride (0.1 ml), 1M potassium acetate (0.1 ml) and distilled water were mixed. After incubation at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm. Quercetin was used to make the calibration curve. The calibration curve was prepared by preparing quercetin solution in methanol. The calculation of total flavonoids in the extracts was carried out in triplicate and the results were averaged.

DPPH radical-scavenging assay

The stable $DPPH^{\cdot}$ was used for determination of free radical scavenging activity of the extracts (Blois *et al.* 1958). Different concentrations of *P. orientalis* extracts were added, at an equal volume, to methanol solution of 100 mM, DPPH (2, 2-diphenyl-1-picrylhydrazyl) and vortexed thoroughly. After 15 min at room temperature, the absorbance of the remaining DPPH radicals was recorded at 517 nm. The scavenging of DPPH was calculated according to the following equation:

$$\%scavenging = \{(Abs_{control} - Abs_{sample})/Abs_{control}\} \times 100$$

Where $Abs_{control}$ = absorbance of DPPH radical + methanol; Abs_{sample} = absorbance of DPPH radical + *P. orientalis* extract/standard. The experiment was repeated three times. Vitamin C and BHT were used as standard controls. IC_{50} values denote the concentration of a sample, which is required to scavenge 50% of DPPH free radicals.

Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide (H_2O_2) was determined according to the method of Ruch (Ruch *et al.,* 1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH=7.4). Extract (0.1-1 mg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as following equation:

$$\% \text{ Scavenged } [H_2O_2] = [(A_o - A_1)/A_o] \times 100$$

Where, A_o was the absorbance of the control and A_1 the absorbance in the presence of the extract or standard.

Reducing power of *T. orientalis* extracts

The reducing power of *P. orientalis* extracts was determined according to Oyaizu (Oyaizu, 1986). According to this, 2.5 ml of extract (25-800 mg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixtures were incubated at 50°C for 30 min. Then a portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as positive control.

Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times from the same extract in order to determine their reproducibility. The IC_{50} values were calculated from linear regression analysis. Pearsons correlation analysis (SPSS 7.5 for windows SPSS Inc.) was used to test for the significance of relationship between the concentration of total phenols and flavonoids and IC_{50} values at a $p < 0.05$ significance level.

Results and Discussion

Total phenol and flavonoid content of the extracts

The yield of extraction from the leaves of *P. orientalis* (in ethanol, ethyl acetate, methanol and water) was determined as 4.6%, 2.3%, 3.03% and 2.6% respectively. Phenolic compounds are a class of antioxidant agents acting as free radical terminators (Shahidi & Wanasundara, 1992). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of the actions of flavonoids are through scavenging or chelating processes (Cook and Samman, 1996). The total phenol content was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent. The flavonoid content was determined by using the aluminium chloride colorimetric method and was expressed as quercetin equivalent in gram of dried plant (Fig. 1). The total phenolic and flavonoid content of the extracts have been reported using the corresponding standard curve equations. Table 1 shows the total phenol and flavonoid of the *T. orientalis* (T.o) extracts. Among all extracts T-MeOH appeared to have a higher concentration of total phenol and flavonoids, 19.71±0.008 mg GAE in gram of the dried plant and 14.308±0.005 mg QE in gram of the dried plant respectively, and the aqueous extract contain a lower content of total phenol and flavonoids 13.334±0.033 mg GAE / gram of the dried plant and 2.41±0.001 mg QE/ gram of the dried plant respectively. Therefore methanol was most effective solvent for extraction of these secondary metabolites from *T. orientalis* leaves.

Table 1. The results of DPPH and H_2O_2 scavenging activity of the extracts of *p. orientalis* and ascorbic acid and BHT as positive controls

Extracts of *P. orientalis* & standard	DPPH scavenging activity IC_{50} (µg/ml)	H_2O_2 scavenging activity IC_{50} (µg/ml)
Ascorbic acid	4.53 ±0.21	24.039±0.59
BHT	4.03±0.14	---
T-Et	14.47±0.52	98.6±0.48
T-Me	11.45±0.41	49.9±0.55
T-H_2O	25.56±0.50	149.3±0.82
T-EA	25.04±0.28	148±0.38

P: *Platycladus* ; Et: Ethanol; Me: Methanol; EtAc: Ethyl Acetate.

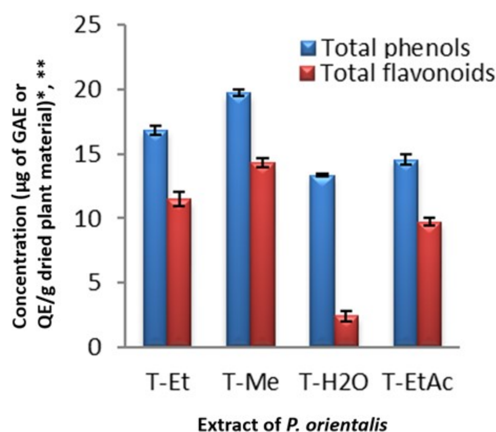

Fig. 1. Comparison of the amount of total phenolics and flavonoids in the extracts of *P. orientalis* L. Each value is expressed as mean value ± Standard Deviation (n=3). The vertical bars represent the standard deviation for each data point. * mg gallic acid equivalent / gram dried plant,** mg quercetin equivalent / gram dried plant (For abbreviation refer to Table 1).

DPPH radical-scavenging activity

DPPH radical is usually used as a substrate to evaluate antioxidative activity of antioxidants. The method is based on the reduction of DPPH solution in the presence of a hydrogen donating antioxidant, due to formation of the non-radical form DPPH-H by the reaction. All extracts were able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. Blois found that cystein, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines reduce and decolorize DPPH radical by their hydrogen donating ability (Oyaizu 1986). Our work appears that the *P. orientalis* extracts possesse hydrogen donating

capabilities and act as antioxidant. Fig. 2 shows the effect of different concentrations of *P. orientalis* L. extracts in comparison with BHT and vitamin C on the inhibition of DPPH radical.

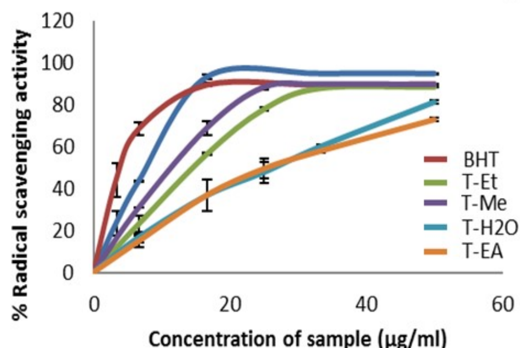

Fig. 2. Free radical scavenging activity of the ethanol, methanol, water and ethyl acetate extracts of *P. orientalis* in comparison to ascorbic acid and BHT using the DPPH˙ inhibition method. For abbreviation refer to table 1.

The radical-scavenging activities of T-extracts increased with increasing concentration. According to this chart, ethanol and methanol showed the most similar treatment compared to standard. The IC_{50} values (concentration inhibiting the 50% of DPPH radicals) for ascorbic acid and BHT were 4.53 and 4.03 mg ml^{-1}, respectively. Based on these experiments, the highest antioxidant activity of the extract is related to methanol extract with IC_{50} equal to 11.54 µg/ml. However, scavenging activity of ascorbic acid and BHT as known antioxidants, used as positive controls, were relatively more pronounced than that of T-extracts.

Reducing power of the extracts

Figure 3 shows the reductive abilities of *P. orientalis* extracts in comparison to ascorbic acid. In order to evaluate the reductive ability, we monitored the Fe^{3+}-Fe^{2+} transformation in the presence of *T. orientalis* extracts using the method of Oyaizu (Oyaizu, 1986). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Mier *et al*., 1995). The antioxidant activity of putative antioxidants have been attributed to various mechanisms, including the prevention of chain initiation, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997). The reducing power of the extracts increased with their concentration and exhibited moderate reducing power that were comparable with that of vitamin C ($p < 0.05$). All amounts of four extracts showed higher activities than that of the control. The reducing power of water, methanol, ethanol and ethyl acetate extract of *P. orientalis* and ascorbic acid is rated in the following order: AA>T-Ethanol>T-Methanol>T-Ethylacetate>T-H_2O.

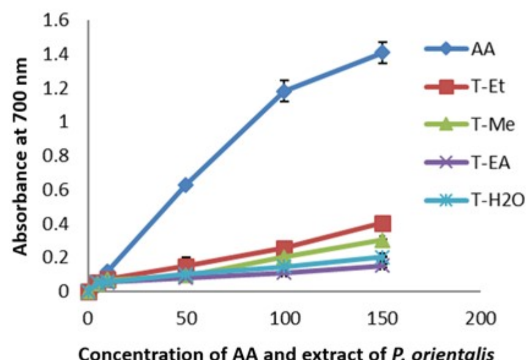

Fig. 3. Reducing power of the ethanol, methanol, ethyl acetate and water extracts of *P. orientalis* as monitored by means of spectrophotometric detection of the Fe^{3+}-Fe^{2+}. AA: ascorbic acid, was used as positive control. For abbreviation refer to table 1.

Scavenging of hydrogen peroxide radicals

The ability of the *P. orientalis* extracts to scavenge hydrogen peroxide was determined using the method of Ruch (Ruch *et al.,* 1989). Fig. 4 shows the hydrogen peroxide scavenging ability of methanolic, ethanolic, aqueous and ethyl acetate *P. orientalis* extracts in comparison to that of ascorbic acid. The *P. orientalis* extracts were capable to scavenge hydrogen peroxide in a concentration-dependent manner. These results showed that both methanol and ethanol extracts produced strong hydrogen peroxide scavenging, which close to that of AA. According to figure 4, as the concentration of standard and the extracts increases, H_2O_2 removal rate increases. The hydrogen peroxide scavenging effect of four *P. orientalis* extracts and AA as reference compound (IC_{50}) decreases in the following order: T-H_2O> T-ethyl acetate>T- ethanol>T-methanol>AA (Table 1).

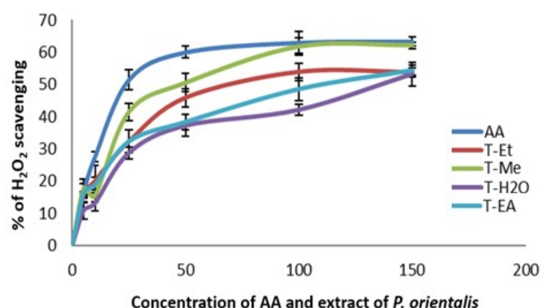

Fig. 4. Hydrogen peroxide scavenging activities of the ethanol, methanol, water and ethyl acetate extracts of *P. orientalis* in comparison to ascorbic acid. For abbreviation refer to table 1.

Correlation study

Polyphenols have been proposed as the main antioxidant compounds of extracts of *Thuja orientalis* (Xu *et al*., 2009). Hence, we examined the possible correlation between the total polyphenolic and flavonoid contents and the antioxidant potential of the extracts. The results showed that when all the extracts were examined, there was not a strong difference of correlations between total polyphenols and DPPH$^{\cdot}$ scavenging activity. The lack of this difference may be explained by the following: (i) antioxidant activity is attributed to specific polyphenols present in the extracts (ii) apart from polyphenols, antioxidant activity is due to other phytochemical compounds, and (iii) there is a synergism between polyphenols and other phytochemical compounds. However, there were some notable exceptions from this observation as for example, the methanolic extract of *P. orientalis* had both high polyphenol and flavonoid contents and DPPH radical scavenging activity. Polyphenols and flavonoids found in plant extracts are considered as main bioactive compounds with antioxidant activity. Thus, correlation coefficient (r) was calculated in order to estimate the correlation between the total polyphenol content and DPPH radical scavenging activity. This r value was the Pearson coefficient between DPPH radical scavenging activity and total phenol and flavonoid content. The aqueous extract of *P. orientalis* had low total polyphenol and flavonoid levels and week DPPH$^{\cdot}$ deactivation capacity. Among extracts from *P. orientalis* the methanolic extract had the highest antioxidant activity in DPPH and H_2O_2 assays with correlation coefficient; r =0.77; 0.73 respectively and $p<0.05$.

Table 2 shows the values that present a significant direct correlation between total phenol content and total flavonoid content with DPPH free radical scavenging activity in all the extracts ($r >0.7$).

Previous phytochemical investigation of this plant resulted in the isolation of many chemical constituents such as flavonoid, terpenes, and phenolic compounds. Sesquiterpenoids and diterpenoids from the heartwood, mono and sesquiterpenoids in essential oils of different parts of the plant, flavonoids from leaves, two monolignol derivatives from pollens, and some long chain aliphatic compounds were previously reported as chemical constituents of this valuable plant (Asili *et al.,* 2007).

According to Xu et al, several bioflavonoids and flavonoid glycosides from the fresh fruits of *P. orientalis* were isolated and estimated their antioxidant and elastase inhibitory activities (Xu *et al.,* 2009).

Table 2. The correlation between total polyphenolic content (TPC) and total flavonoid content (TFC) versus IC_{50} values of DPPH scavenging activity of the extracts of *P. orientalis*

Extracts of *T.orientalis*	r (TPC vs. DPPH)	r (TFC vs. DPPH)
T-Me	0.77	0.73
T-Et	0.87	0.81
T-EA	0.86	0.80
T-H_2O	0.92	0.77

(r = correlation coefficient; $p<0.05$) For abbreviation refer to Table 1.

In conclusion, the results of the present study indicated that *P. orientalis* collected from Gorgan may be helpful in preventing or stopping the progress of various oxidative stress-related diseases. The extraction in methanol and ethanol as solvents are more effective for extraction of flavonoid and phenolic compounds, and resulted the higher antioxidant activity. Finally, further research is necessary to separate the component of these extracted samples and then evaluate the antioxidant activity of each component using several different methods.

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