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Full Length Research Paper

Olea ferrugenia: A potential natural source of protection from oxidative stress

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The methanolic extract of *Olea ferrugenia* Royale was dissolved in distilled water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol successively. The phytochemical screening of all these organic fractions and remaining aqueous fraction was carried out and then the antioxidant potential of all studied fractions was evaluated by four *in vitro* methods: DPPH free radical scavenging activity, total antioxidant activity, FRAP assay and ferric thiocyanate assay. Total phenolics of these organic fractions were also determined. All the fractions showed significant antioxidant potential. The results revealed that ethyl acetate soluble fraction showed highest value of percentage inhibition of DPPH radical (92.96% ± 0.13), with IC_{50} value of (19.76 ± 0.12 µg/ml, relative to butylated hydroxytoluene (BHT), having IC_{50} of 12.1 ± 0.92 µg/mL. This fraction also exhibited highest FRAP value (714.45 ± 2.61 µg of trolox equivalents), highest total phenolic contents (416.4.59 ± 7.73 mg of gallic acid equivalents), as well as highest value of inhibition of lipid peroxidation (57.51% ± 1.56) when compared with other fractions. However, the chloroform soluble fraction showed highest total antioxidant activity (0.9101 ± 0.03) when compared with other fractions.

Key words: *Olea ferrugenia* Royale, DPPH assay, total antioxidant activity, FRAP value, total phenolics, inhibition of lipid peroxidation (%).

INTRODUCTION

Natural products based drugs have been used against various diseases since time immemorial and are a significant part of today's drugs. Folk-lore used herbs as therapeutics and medicaments. The importance of some plants has long been published but a large number of them remain unknown yet. So, it is essential to explore their uses and to conduct pharmacognostics and pharmacological studies to discover their therapeutic uses (Bhatti et al., 1998). In recent years, the use of biologically active compounds from natural sources is intensified, therefore, a broad field of bioactivity assays, isolation methods and spectroscopic studies has been developed. Plants are a rich source of natural antioxidants, some of them, e.g. tochopherols (vitamin E), ascorbic acid (vitamin C) and carotenoids are substances of major importance in human composition and food. The large scale accessibility of agricultural and industrial plant waste materials and their low cost makes them a striking source of natural antioxidants (Perez-Bonilla et al., 2006). Over the last years, researchers have become more and more interested in nutritional phenolic compounds because of their free radical scavenging activity and other potential beneficial effects on human health (Manach et al., 2004). Olive oil is the main fatty component of natural olive (*Olea europea* L.) and mediates positive effects on cardiovascular, metabolic, inflammatory and autoimmune diseases (Lastra et al., 2001).

Olives and their derivatives may be considered, as potential sources of natural anti-oxidants which could be used in food and pharmaceutical industries (Savarese et al., 2007). In recent years, the consumption of olive oil and olives has steadily increased. This trend is being fostered by the recognized nutritional value of

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Mediterranean diet (Sabitini and Marsilio, 2008). Olive and its oil consumption have been shown to be associated with a variety of health benefits including a lower incidence of heart disease and certain types of cancer. These beneficial effects have been attributed not only to a low saturated/ mono unsaturated fatty acid ratio, but also to other additional molecules present in minor concentration, particularly antioxidant phenol compounds (Perez-Jimenez et al., 2007). Olive leaves are being utilized as a traditional remedy for reducing fever, preventing/curing malaria and in preventing hypertension (Ranalli et al., 2006). A major bioactive compound, Oleuropein (OE) has been isolated from Olive tree exhibiting potent antiatherogenic, anticancer, anti-inflammatory and antimicrobial properties (Gikas et al., 2007). *O. ferrugenia* Royale (kao, Indian Olive), belongs to family Oleaceae comprising of 29 genera and 600 species (Baqar, 1969). It is a native broad leaved tree of sub-continent, distributed from 500 to 2000 m in sub-tropical, dry and moist temperate regions of Pakistan (Ahmad et al., 2009). This frost and drought resistant species has adopted 250 to 1000 mm/ annum precipitation and -10 to 40°C temperature, therefore it can easily be planted on wide areas of arid, semi-arid and dry temperate regions of Pakistan with minimum input. The water-shed, microclimatic and edaphic conditions of the area can be improved through plantation of this tree. Its fruit and wood may be used as fodder, oil and fuel (Komaki et al., 2003). Moreover its leaves have been used for medical purposes and recently introduced into Pharmacopeia Ph Eur 5 (Jensen et al., 2002). *O. ferrugenia* Royale fresh fruit in summer season are collected, dried and recommended to diabetic patient in winter season for reducing blood glucose level (Ahmad et al., 2009). Based on our knowledge about the plant under consideration, no comprehensive study on the antioxidant activities was carried out. Therefore, we illustrate the *in vitro* studies on various fractions and methanolic extract of *O. ferruginea* to asses its effectiveness for protection from oxidative stress.

MATERIALS AND METHODS

Plant Material

The plant *O. ferrugenia* Royale (voucher specimen no.) was collected from Kotly, Azad Kashmir in March 2009 and identified by Mr. Muhammad Ajaib (Taxonomist), Herbarium Department of Botany, GC University, Lahore. A voucher specimen (GC.Herb.Bot. 948) has been deposited in the herbarium of the same University.

Extraction and fractionation of antioxidants

The shade-dried ground whole plant (5.5 kg) was exhaustively extracted with methanol (12L × 4) at room temperature. The extract was evaporated to yield the crude methanolic residue (995 g), which was dissolved in distilled water (1.5L) and partitioned with *n*-hexane (1L × 4), chloroform (1L × 4), ethyl acetate (1L × 4) and *n*-butanol (1L × 4), respectively. These organic fractions were concentrated separately on rotary evaporator to get residues as 83, 90, 64 and 96 g, respectively. The remaining aqueous layer was also concentrated (180 g) in a similar way. For this study, the crude methanolic extract, organic fractions and the aqueous fraction thus obtained were used to evaluate their *in vitro* antioxidant potential and phytochemical screening.

Chemicals and standards

DPPH· (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), trolox, gallic acid, follin ciocalteu reagent and BHT (Butylated hydroxytoluene) were obtained from Sigma chemical company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

Phytochemical screening

The phytochemical components of plant extracts were screened by using standard procedure described by (Sofowara, 1993; Trease and Evans, 1989; Aylooa et al., 2008).

Test for reducing sugars (Fehling's test)

Fehling's solution (A and B) in a test tube was boiled with sample solutions (0.5 g of sample in 5 ml of water). Formation of red precipitates indicated the presence of sugars.

Test for terpenoids

Two methods were employed to check the presence of terpenoids. First (Salkowski test) ,Concentrated H_2S0_4 (3 ml) was carefully added in test tube containing 0.5 g of each of the extracts in 2 ml of chloroform to form a reddish brown coloration at the interface which indicated the presence of terpenoids. Second, TLC card having spots of samples was sprayed with ceric sulfate solution and heated further on a TLC heater. Appearance of brown color indicated terpenoids.

Test for flavonoids

Four methods were used for detection of flavonoids in plant extracts. First, dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was also added. A yellow coloration that disappeared on standing indicated the presence of flavonoids. Second, a few drops of 1% aluminium solution were added to sample solution. Yellow coloration indicated the presence of flavonoids. Third, TLC card having spots of samples was sprayed with Benedict's reagent. Green fluorescence in UV light indicated flavanoids. Lastly, TLC card having spots of samples was sprayed with Lead acetate which also showed Green fluorescence in UV light and indicated the presence of flavanoids.

Test for saponins

5 ml of distilled water was added in 0.5 g of extract in a test tube. The solution was shaken vigorously till the formation of a stable persistent froth. 3 drops of olive oil were added in the frothing and was shaken vigorously till the formation of an emulsion which indicated saponins.

Test for tannins

Mixture of 5 ml of n-butanol-HCl and 2 ml of sample solutions were warmed for 1 hr in a water bath. Appearance of red color indicated the presence of tannins.

Test for alkaloids

TLC card having spots of samples was sprayed with Draggendroff's reagent. Appearance of orange color indicated alkaloids.

Test for cardiac glycosides (Keller-Killiyani test)

0.5 g of extract was diluted with 5 ml water. 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added drop wise in the diluted extracts.

This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Antioxidant assays

The following antioxidant assays were performed on various extracts of the plant under study.

DPPH radical scavenging activity

The DPPH radical scavenging activities of various samples of this plant were examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the method of (Lee and Shibamoto, 2001). Briefly, various amounts of the extracts (1000, 500, 250, 125, 60, 30, 15 and 8 µg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 1 h. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity. The percent of DPPH decoloration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = A_{control} - A_{sample} / A_{control} \times 100$$

Each sample was assayed in triplicate and mean values were calculated.

Total Antioxidant Activity by Phosphomolybdenum Method

The total antioxidant activities of various samples were evaluated by phosphomolybdenum complex formation method (Prieto et al., 1999). Briefly, 500 µg/mL of each extract was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution.

The vials were capped and incubated in water bath at 95°C for 90 min. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT).

All determinations were assayed in triplicate and mean values were calculated.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was done according to the method of (Benzie and Strain, 1996) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $CH_3COONa.3H_2O$ and 16 mL CH_3COOH), pH 3.6, 10 mM TPTZ (2, 4, 6-Tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. The solutions of plant samples and that of trolox were formed in methanol (250 µg/mL). 10 mL of each of sample solution and BHT solution were taken in separate test tubes and 2990 mL of FRAP solution was added in each to make total volume up to 3 mL. The plant extracts were allowed to react with FRAP solution in the dark for 30 min. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm.

The FRAP values were determined as micromoles of trolox equivalents per mL of volatile oil by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE mM/mL.

Total phenolic contents

Total phenolics of various samples were determined by the method of Makkar et al. (1993). 0.1 mL (0.5 mg/mL) of sample was combined with 2.8 mL of 10% Na_2CO_3 and 0.1 mL of 2N Folin-ciocalteu reagent. After 40 min absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. The standard curve was linear between 50 to 400 mg/mL of gallic acid. Results were expressed in GAE mg/mL.

Ferric thiocyanate (FTC) Assay

The antioxidant activities of various samples on inhibition of linoleic acid peroxidation were assayed by thiocyanate method (Valentao et al., 2002). The 0.1 mL of each of sample solution (0.5 mg/ mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40°C. The mixture without sample was used as control. The 0.1 mL of the mixture was taken and mixed with 5.0 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature. Precisely 3 min. after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation (IP %) [IP% = {1-(abs. of sample) / (abs. of control)} × 100]. The antioxidant activity of BHT was assayed for comparison as reference standard.

Statistical analysis

All the measurements were done in triplicate and statistical analysis was performed by Microsoft excel 2007. Results are presented as average ± SEM.

Table 1. Phytochemical constituents of *Olea ferrugenia* Royale.

Test	*n*-hexane soluble fraction	Chloroform soluble fraction	Ethyl acetate soluble fraction	*n*-butanol soluble fraction	Remaining aqueous fraction
Alkaloids	-	+	+	++	+
Terpenoids	+++	+++	++	++	+
Saponins	-	+	+	+	++
Tannins	-	+	+	+	+
Sugars	-	-	+	+++	++
Phenolics	-	+++	+++	++	+
Flavanoids	-	+++	+++	++	+
Cardiac glycosides	+	++	++	+++	+

+ shows presence and – shows absence.

RESULTS AND DISCUSSION

The phytochemical screening was performed on all fractions of the plant under study and results are shown in (Table 1). It was observed from the results that chloroform, ethylacetate and n-butanol soluble fractions carried flavanoids and phenolics, as well as alkaloids which were absent in n-hexane fraction. Cardiac glycosides were present in all fractions but chloroform, ethylacetate and n-butanol soluble fraction showed them in higher concentration and n-hexane soluble fraction carried them in least amount. Tannins and sugars were present in all fractions except in n-hexane soluble fraction whereas n-butanol soluble fraction contained sugars in appreciable amount. Most of the terpenes were present in n-hexane soluble fraction and lesser in all other fractions. Saponins were also present in all fractions in smaller amount except in n-hexane soluble fraction. The remaining aqueous layer indicated the presence of phenolics, flavanoids, cardiac glycosides, tannins, saponins and alkaloids which are significantly biologically active components.

The radical scavenging activity of the studied samples was evaluated using a methanolic solution of the ‘‘stable’’ free radical, DPPH (Ferreira et al., 2006). A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. The purple color generally fades when an antioxidant is present in the medium. Thus, antioxidant molecules quenched DPPH free radicals (that is, by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and converted them to a colorless product (that is, 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), which resulted in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract (Table 2). The IC_{50} values in µg/mL (Table 3) of different samples were calculated in the present study and the highest radical scavenging activity, was shown by ethyl acetate soluble fraction (19.76 ± 0.12), followed by the order of chloroform soluble fraction (60.09 ± 2.91), n-butanol soluble fraction (66.74 ± 1.66), n-hexane fraction (140.21 ± 1.65), methanolic extract (173.53 ± 0.25) and by water fraction (226.19 ± 7.21).

The total antioxidant capacity of the *O. ferrugenia* Royale extract and fractions was measured spectrophotometrically by Phosphomolybednum method, in which the reduction of Mo (VI) to Mo (V) took place by various fractions of plant which was detected at 695 nm by spectrophotometer due to the formation of green phosphate Mo (V) compounds (Miladi and Damak, 2008). The highest total antioxidant capacity (Table 3) is in the order of chloroform soluble fraction (0.9101 ± 0.03), n-hexane (0.8987 ± 0.02), crude methanolic extract (0.6766 ± 0.00), ethyl acetate soluble fraction (0.4034 ± 0.01), n-butanol soluble fraction (0.3326 ± 0.01) and lowest by water fraction (0.2603 ± 0.02).

The reducing power of various samples was observed by FRAP assay in which, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of different samples. This assay utilized antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess (Benzie and Strain, 1997). At low pH, reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue color) can be monitored by measuring the change in absorption at 593 nm. The reaction is non-specific, in that any half reaction that has lower redox potential, under reaction conditions, than that of ferric ferrous half reaction, will drive the ferrous (Fe III to Fe II) ion formation. The change in absorbance is therefore, directly related to the combined or “total” reducing power of the electron donating antioxidants present in the reaction mixture. The presence of reducers (that is, antioxidants) causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl’s Prussian blue at 595 nm can be monitored by the Fe^{2+}concentration (Isabel et

Table 2. Free radical scavenging activity of various fractions of *Olea ferrugenia* Royale using 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH).

S/N	Sample	Concentration in assay (µg/4 ml)	Age scavenging of DPPH radical (%) ± S.E.M[a)]
1	Methanolic extract	500	81.13±0.10
		250	63.05±0.34
		125	47.55±0.23
		60	33.18±0.11
2	Hexane soluble fraction	1000	83.68±0.08
		500	68.83±0.41
		250	56.53±0.37
		125	45.43±0.26
3	Chloroform soluble fraction		85.37±0.10
		250	67.88±0.55
		125	54.14±1.15
		60	44.81±0.57
		30	32.81±0.29
4	Ethyl acetate soluble fraction	60	92.96±0.13
		30	73.68±0.07
		15	40.77±0.04
		8	32.57±0.31
5	n-butanol soluble fraction	250	90.80±0.16
		125	68.29±0.71
		60	52.54±0.37
		30	35.16±0.36
6	Water fraction	500	74.10±0.43
		250	56.26±0.25
		125	44.46±0.46
		60	29.42±1.07
7	BHT [b)]	60	91.25±0.13
		30	75.56±0.07
		15	42.67±0.04
		8	23.57±0.31

(a). Standard mean error of three assays. (b). standard antioxidant.

al., 2007). The present study showed that the extract and fractions of *O. ferrugenia* showed potent antioxidant power with FRAP values in µM/mL (Table 2). The ethyl acetate soluble fraction exhibited highest FRAP value of (714.45 ± 2.61), followed by crude methanolic extract (685.93 ± 4.54), n-butanol soluble fraction (606.66 ± 6.57), chloroform soluble fraction (340.53 ± 10.29), n-hexane fraction (311.91 ± 11.86) while the lowest value was shown by aqueous fraction (180.56 ± 7.88).

The antioxidant activity of plant materials correlated well with the phenolic content. The phenols concentration in the different extracts is expressed as milligram of gallic acid equivalents (GAEs) per gram of extract (Skerget et al., 2005). Amongst different fractions (Table 2), ethyl acetate soluble fraction showed the highest phenolic content (416.4 ± 7.73), followed by n-butanol soluble fraction (413.1 ± 8.02), aqueous fraction (371.04 ± 15.63), chloroform soluble fraction (349.6 ± 4.12), methanolic extract (336.2 ± 1.6) and lowest phenolic contents were found in n-hexane soluble fraction (315 ± 3.01).

The Ferric thiocynate method (Kim and Kim, 2010), determines the amount of peroxide produced at the beginning stage of Linoleic acid emulsion during incubation, which reacted with ferrous chloride to form ferric chloride which further formed ferric thiocynate (red pigment) upon reaction with ammonium thiocynate (Table 2). The ethyl acetate soluble fraction showed potent lipid

Table 3. IC_{50} total phenolics, total antioxidant activity, FRAP values and inhibition of lipid peroxidation values of different *Olea ferrugenia* fractions.

Sample	IC_{50} of DPPH assay (µg/ml) ± S.E.M[a)]	Total antioxidant activity ± S.E.M[a)]	FRAP value TE (µM/ml) ± S.E.M[a)]	Total phenolics (GAE mg/g of extract) ± S.E.M[a)]	Inhibition of lipid peroxidation (%) ± S.E.M[a)]
Crude methanolic extract	173.53 ± 0.25	0.6766 ± 0.00	685.93 ± 4.54	336.2 ± 1.6	50.18 ± 0.34
n-hexane soluble fraction	140.21 ±1.65	0.8987 ± 0.02	311.91 ± 11.86	315 ± 3.01	49.20 ± 0.18
Chloroform soluble fraction	60.09 ± 2.91	0.9101± 0.03	340.53 ± 10.29	349.6 ± 4.12	44.75 ± 1.09
Ethyl acetate soluble fraction	19.76 ± 0.12	0.4034 ± 0.01	714.45 ± 2.61	416.4 ± 7.73	57.51 ± 1.56
n-Butanol soluble fraction	66.74 ± 1.66	0.3326 ± 0.01	606.66 ± 6.57	413.1 ± 8.02	50.38 ± 0.31
Water fraction	226.19 ± 7.21	0.2603 ± 0.02	180.56 ± 7.88	371.04 ± 15.63	43.92 ± 0.84
BHT[b)]	12.1 ± 0.92	1.2186 ± 0.07		-	62.91 ± 0.60

(a). Standard mean error of three assays (b). Standard antioxidant.

peroxidation inhibitory potential (57.51 ± 1.56), followed by n-butanol soluble fraction (50.38 ± 0.31), aqueous fraction (43.92 ± 0.84), chloroform (44.75 ± 1.09), n-hexane (49.20 ± 0.18) and crude methanolic extract (50.18 ± 0.34) compared to standard BHT (62.91 ± 0.60).

Conclusion

The results of phytochemical screening suggested that the claimed medicinal importance of the plant is due to the presence of large number of phenolics and flavanoids, alkaloids, cardiac glycosides, saponins, tannins and reducing sugars which are all biologically active components. The chloroform, ethylacetate and *n*-butanol soluble fractions contained most of them while n-hexane fraction showed least activity due to the absence of these components.

By the evaluation of the antioxidant activities of the crude methanolic extract and various fractions of *O. ferrugenia* Royale by above discussed methods, it was revealed that ethyl acetate soluble fraction showed highest DPPH radical scavenging activity, FRAP value, inhibition of lipid peroxidation as well as total phenolic contents. The chloroform soluble fraction exhibited highest total antioxidant activity as compared to other fractions. So, it was generally concluded that the medium-polar fractions of this plant contain strong antioxidants and are valuable resources of natural bioactive materials which may find their use to prevent the deterioration of food by inhibition of lipid oxidation and improve the health-promoting properties when they are added.

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