Regular Article

Journal of Medicinal Herbs and Ethnomedicine 2015, 1: 97-102
http://scienceflora.org/journals/index.php/jmhe/
doi:10.5455/jmhe.2015.09.022

Studies on *in vitro* antioxidant activities of nine different fruit tree leaves collected from Mediterranean region of Turkey

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Received: 16-09-2015
Accepted: 25-09-2015
Published: 28-09-2015

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ABSTRACT

To investigate antioxidant of the methanolic and water extracts obtained from nine different fruit tree leaves (avocado, walnut, mulberry, fig, carob, lemon, pomegranate, grape, and loquat) collected from Mediterranean region of Turkey. The antioxidant activities were evaluated with different *in vitro* antioxidant assays including phosphomolybdate assay, free radical scavenging assays (•OH, NO, and O_2^-), β-carotene/linoleic acid test system, and ferric reducing power. The contents of saponin and tannin in extracts were also determined. The present study suggests that the extracts were good radical scavengers. These results suggest that the extracts examined should be beneficial as a source of natural agents for the food industry and pharmacological applications.

KEY WORDS: Antioxidant activity, fruit tree leaves, saponin, tannin, Turkey

INTRODUCTION

Free radicals, especially reactive oxygen species, play role in the development many chronic diseases such as diabetes, atherosclerosis, and cancer (Basaga, 1990). Free radicals are capable of attacking the healthy cells of the body, causing them to lose their structure (Cadenas and Davies, 2000). Antioxidants are used in prevention and treatment of free radical-related diseases (Aruoma and Cuppette, 1997). Therefore, synthetic antioxidant compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were commonly used to protect against free radicals in the food industry. However, these compounds have been reported to cause a variety of adverse health effects (Ito *et al.*, 1986). Researches have focused on new antioxidant sources of natural origin. Vegetables and fruits are rich source of natural antioxidants (Percival, 1998). High intake of vegetables and fruits is consistently associated with lower incidence rates of certain types of cancer and cardiovascular diseases (Steinmetz and Potter, 1996; Toor *et al.*, 2006). The protective effects of vegetables and fruits have been attributed to their high antioxidant contents (Solomon *et al.*, 2006).

Some pharmacological and biological properties of the fruit tree leaves used in this study have been reported in the literature. For example, Loquat (*Eriobotrya japonica* Lindl.) fruit and leaf are often used Chine herbal medicine for treating cough, asthma (Wang *et al.*, 2003). *Vitis vinifera* leaves are possess astringent and hemostatic activities, which can be used in the treatment of diarrhea, hemorrhage (Felicio *et al.*, 2001). However, in the previously published papers, there is only little information about antioxidant of the leaf samples (Owolabi *et al.*, 2010; Hsouna *et al.*, 2011). The main objective of this work was to investigate antioxidant of two different solvents (methanol and water extracts) obtained from fruit tree leaves collected from Mediterranean region of Turkey.

MATERIALS AND METHODS

Plant Materials and Preparation of the Extracts

The leaves of *Persea americana* Mill. (avocado), *Juglans regia* L. (walnut), *Morus alba* L. (mulberry), *Ficus carica* L. (fig), *Ceratonia siliqua* L. (carob), *Citrus limon* L. (lemon), *Punica granatum* L. (Pomegranate), *V. vinifera* L. (grape), *and E. japonica* (Thunb.) Lindl. (loquat) were collected from Mediterranean Region of Turkey.

The aerial plant materials were dried at the room temperature. The dried plant materials were ground to a fine powder using a laboratory mill. 15 g of powdered plant were mixed with 250 ml methanol and extracted in a Soxhlet apparatus for 6-8 h. The extracts concentrated under vacuum at 40°C by a rotary evaporator. To obtain water extracts, air dried powdered plants were boiled with 250 ml of distilled water for 30 min. The aqueous extracts were filtered and lyophilized (−80°C, 48 h). Extracts were stored at +4°C in the dark until use.

Determination of Total Saponins

The total saponin content of the extract was determined by the vanillin-sulfuric acid method (Aktumsek *et al*., 2013). These extracts were reacted with vanillin (8%) and sulfuric acid (72%). The mixture was incubated at 60°C for 10 min. Then, the mixture was cooled for another 15 min, followed by absorbance measurement at 538 nm. Quillaja saponin was used as a standard, and the content of total saponins was expressed as Quillaja equivalents (QAE g/mg extract).

Total Condensed Tannin Content

The total condensed tannin content was determined by the vanillin method Bekir *et al*. (2013) with slight modification. The sample solution (0.5 ml) was mixed with vanillin reagent (1.5 ml, 1% in 7 M H_2SO_4) in an ice bath and then mixed well. Similarly, a blank was prepared by adding sample solution (0.5 ml) to 7 M H_2SO_4 (1.5 ml). The sample and blank absorbances were read at 500 nm after 15 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The total condensed tannin content was expressed as equivalents of (+)-catechin according to the equation that was obtained from the standard (+)-catechin graph.

Nitric oxide (NO) Radical Scavenging Activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generated NO, which was measured by the Griess reaction (Srivastava and Shivanandappa, 2011) Sample solution (0.5 ml) was mixed with sodium nitroprusside (0.5 ml, 5 mM) in phosphate buffer (0.2 M, pH 7.4) and incubated for 150 min at room temperature. Similarly, a blank was prepared by adding sample solution (0.5 ml) to phosphate buffer (0.5 ml). The incubated sample was added to dilute Griess reagent (1 ml, 1:1) and allowed standing for 30 min. The sample and blank absorbances were read at 548 nm. The absorbance of the blank was subtracted from that of the sample and the NO radical scavenging activity was expressed as equivalents of trolox according to the equation that was obtained from the standard trolox graph. The results were reported as percentage inhibition and calculated according to:

$$I(\%) = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

Where, A_0 is the absorbance of the control, A_1 is the absorbance of the extract/standard.

Superoxide Anion (O_2^-) Radical Scavenging Activity

The superoxide anion radical scavenging activity was followed in the riboflavin-light-nitroblue tetrazolium (NBT) system (Dasgupta and De, 2004) with slight modification. Sample solution (0.25 ml) was added to reaction mixture containing riboflavin (0.1 ml, 0.1 mg/ml), ethylenediaminetetraacetic acid (EDTA) (0.1 ml, 12 mM) and NBT (0.05 ml, 1 mg/ml), phosphate buffer (1 ml, 50 mM, pH 7.8), and 1-butanol (0.5 ml). The reaction mixture was illuminated for 10 min at room temperature, and the sample absorbance was read at 560 nm. The unilluminated reaction mixture was used as a blank. The absorbance of the blank was subtracted from that of the sample and the superoxide radical scavenging activity was expressed as equivalents of trolox according to the equation that was obtained from the standard trolox graph. The results were reported as percentage inhibition and calculated according to Equation (1).

Hydroxyl (•OH) Radical Scavenging Activity

The hydroxyl radical scavenging activity was measured by the method described by Halliwell *et al*. (1987) with slight modification. Ascorbic acid (1 mM, 0.1 ml) was added to premixed reaction mixture containing 10 mM deoxyribose (0.28 ml), 50 mM phosphate buffer (0.41 ml, pH 7.4), 10 mM ferric chloride (0.01 ml), 10 mM hydrogen peroxide (0.1 ml), 1 mM EDTA (0.1 ml) and sample solution (0.25 ml), and then incubated for 12 h at 37°°C. Similarly, a blank was prepared by adding sample solution (0.25 ml) to the reaction mixture (1 ml) without ferric chloride. Afterward, the incubated sample was mixed with 0.75 ml trichloroacetic acid (2.8% w/v) and 0.75 ml thiobarbituric acid reagent (1% w/v, in 50 mM NaOH), followed by heating at 100°°C for 1 h and subsequent cooling to room temperature. The sample and blank absorbances were read at 532 nm. The absorbance of the blank was subtracted from that of the sample, and the hydroxyl radical scavenging activity was expressed as equivalents of mannitol according to the equation that was obtained from the standard mannitol graph. The results were reported as percentage inhibition and calculated according to Equation (1).

β-carotene-Linoleic Acid Assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius *et al.*, 1998). A stock solution of b-carotene-linoleic acid mixture was prepared as following: 0.5 mg b-carotene was dissolved in 1 ml of chloroform (high-performance liquid chromatography grade). 25 µl linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml of oxygenated distilled water was added with vigorous shaking; 2.5 ml of this reaction mixture was dispersed to test tubes, and 0.35 ml of the methanolic extract (2 mg/ml) were added and the emulsion system was incubated for up to 2 h at 50°°C. The same procedure was repeated with the positive control BHT, BHA, and a blank. After this incubation period, the absorbance of the mixtures was measured at 490 nm. Measurement of absorbance was continued until the color of b-carotene disappeared. The bleaching rate (R) of b-carotene was calculated according to Equation (2).

$$R = \ln (a/b)/t \quad (2)$$

Where, ln = natural log, a = absorbance at time 0, b = absorbance at time t (120 min). The antioxidant activity (*AA*) was calculated in terms of percent inhibition relative to the control using Equation (3).

$$AA = ([R_{Control} - R_{Sample}]/R_{Control}) \times 100 \quad (3)$$

Anti-oxidative activities of the extracts were compared with those of BHT and BHA at 2.0 mg/ml.

Reducing Power Activity (Iron [III] to iron [II] Reduction)

The ferric reducing power method applied with slight modifications of the method of Oyaizu (1986). Various concentrations of the extracts (2.5 ml) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 2.5 ml of 10% trichloroacetic acid was added, 2.5 ml of the reaction mixture was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride. The solution absorbance was measured at 700 nm. The reducing power of samples increased with the absorbance value.

RESULTS AND DISCUSSION

Total bioactive compounds of extracts tested were calculated with spectrophotometric methods, and the results are presented in Table 1. Saponins are a very diverse and large group of natural compounds widely distributed in the plant kingdom (Güçlü-Ustündag and Mazza, 2007). Saponins possess a wide range of biological properties such as antioxidant, neuroprotective, cytotoxic, anti-allergic, hypocholesterolemic (Hostettman and Marston, 1995; Lacaille-Dubois and Wagner, 1996; Francis *et al.*, 2002). The total saponin content of each extract were calculated as QAE. For the total saponin content of the methanol extracts the following activity orders were found: Avocado > loquat > fig > grape > lemon > carob > walnut > mulberry > pomegranate (Table 1).

Tannins exhibit remarkably wide range of biological properties such as antioxidant (Muir, 1995; Amarowicz *et al.*, 2000a; Amarowicz *et al.*, 2000b; Amarowicz and Troszynska, 2003), anti-carcinogenic, anti-mutagenic, and antimicrobial (Katiyar and Mukhtar, 1996; Chung *et al.*, 1998; Harborne and Williams, 2000; Amarowicz *et al.*, 2000c). Vannilin-HCl assay was used for the assay of tannins in extracts and the results obtained were expressed as catechin equivalents. According to the data reported, the highest total tannins content was obtained in methanol extract of avocado (25.24 µg CEs/mg). Methanol extracts can be ranked in the following order: Avocado (25.24 µg CEs/mg) > loquat (19.90 µg CEs/mg) > grape (6.88 µg CEs/mg) > walnut (5.37 µg CEs/mg) > carob (4.83 µg CEs/mg) > pomegranate (1.01 µg CEs/mg). Aqueous extracts decreases in the following order: Avocado (21.27 µg CEs/mg) > loquat

Table 1: Phytochemical content of methanol and water extracts of nine leaves

Samples	Saponin[a] (mg QE/g)	Tannin[b] (µg CE/mg)
Methanol		
Avocado	411.44±75.19*	25.24±0.23
Walnut	141.29±15.37	5.37±0.11
Mulberry	107.75±0.33	nd
Fig	193.75±4.63	nd
Carob	156.13±6.94	04.83±0.16
Lemon	167.46±3.14	0.03±0.26
Pomegranate	97.47±10.25	1.01±0.16
Grape	193.29±22.80	6.88±0.62
Loquat	355.47±4.63	19.90±0.41
Water		
Avocado	162.67±9.58	21.27±0.23
Walnut	117.68±10.08	3.62±0.07
Mulberry	70.59±5.29	3.73±0.02
Fig	84.50±1.16	2.94±0.32
Carob	91.74±2.81	11.16±0.85
Lemon	80.88±0.33	0.95±0.07
Pomegranate	46.64±2.48	nd
Grape	88.94±3.47	7.94±0.14
Loquat	145.73±30.57	16.85±0.37

[a]Total saponins was expressed as Quillaja equivelant, [b]Total condense tanin was expresses as catechin. *Values expressed are means±SD. nd: Not detected, SD: Standard deviation

(16.85 µg CEs/mg) > carob (11.16 µg CEs/mg) > grape (7.94 µg CEs/mg) > mulberry (3.73 µg CEs/mg) > walnut (3.62 µg CEs/mg) > fig (2.94 µg CEs/mg).

β-carotene/linoleic acid assay, the degree of linoleic acid oxidation was determined by measuring oxidation products of linoleic acid which simultaneously attacked β-carotene. In the assay, maximum activity was observed in methanol extract in loquat (93.86%). The present study indicated that the methanol extracts displayed higher activity than the water extracts (except for carob). Two methanolic extracts (carob and loquat) demonstrated more inhibitory activity potential than the standard inhibitors (BHA and BHT). Percentage inhibition capacity of water extracts can be ranked in the following order: Pomegranate > avocado > carob > grape > loquat > walnut > fig > mulberry > lemon. Unlike the water extracts, methanol extract in the following order: Loquat > grape > avocado > lemon > pomegranate > walnut > fig > mulberry > carob (Table 2).

Free radical scavenging activity of methanolic and aqueous extracts of leaves was measured using the different radical scavenging assays. Hydroxyl radical scavenging activity was determined by measuring the inhibition of the degradation of deoxyribose by the free radicals generated by the fenton reaction. The hydroxyl radical scavenging activity of extracts compared with the mannitol as standard. The strongest activity was obtained in the methanolic extract of pomegranate (Table 2). The results of the assay showed that this sample had greater anti-oxidative potential than mannitol, which is a potent OH scavenger. The hydroxyl radical scavenging activity not detected in grape and fig. Considerable hydroxyl radical scavenging activity was found in methanol extracts of lemon and water extracts of lemon, 65.38%, and 34.99%, respectively. These findings were in contrast to the previous report by Muthiah *et al*. (2012) who found that no activity of *C. limon* leaves on hydroxyl radical.

Table 2: Free radical scavenging and inhibition of linoleic acid oxidation activities of nine leaves

Samples	•OH[a]	NO[b]	O_2^-	Inhibition[d]
Methanol				
Avocado	38.94±0.68*	60.70±0.57	98.18±1.84	89.26±2.58
Walnut	62.98±7.48	65.85±0.99	86.29±2.00	84.62±2.85
Mulberry	67.98±5.44	58.14±0.10	10.59±0.14	79.14±2.63
Fig	nd	52.46±0.33	5.66±2.67	80.60±0.19
Carob	60.10±2.04	71.80±0.67	148.68±3.56	76.18±1.65
Lemon	65.38±12.24	60.16±0.38	44.48±0.67	88.51±0.48
Pomegranate	75.00±8.61	72.95±0.19	172.89±0.44	87.24±5.27
Grape	nd	70.90±5.47	84.56±4.45	92.93±1.64
Loquat	64.90±4.76	78.63±0.33	82.51±2.89	93.86±0.64
Water				
Avocado	50.89±2.16	53.06±0.52	155.68±3.01	78.98±9.52
Walnut	73.79±2.16	56.56±1.86	138.10±1.01	67.64±2.93
Mulberry	64.63±2.16	48.96±1.95	109.86±4.22	44.55±2.90
Fig	nd	33.28±0.62	97.44±1.33	55.27±0.97
Carob	59.29±1.80	54.14±1.28	187.03±1.78	78.52±0.55
Lemon	34.99±0.90	49.43±4.62	52.49±1.91	40.09±0.51
Pomegranate	60.05±1.80	61.07±0.81	186.40±0.44	79.34±10.89
Grape	nd	55.08±0.33	100.90±5.06	75.61±7.95
Loquat	51.27±3.42	58.92±1.09	108.92±4.22	73.44±0.93
Mannitol	62.98±0.18	-	-	-
BHA	-	61.64±2.19	-	90.43±0.32
BHT	-	16.35±4.85	-	91.99±1.77

[a]•OH radical scavenging activity (%), [b]NO radical scavenging activity (%). [c]superoxide anion radical scavenging activity (mgTEs/g extract) [d]Inhibition activity of linoleic acid oxidation (%). *Values expressed are means±SD. nd (not detected). BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene, SD: Standard deviation

All extracts exhibited effective NO scavenging activity (Table 2). Methanol extracts demonstrated higher scavenging activity than water extracts. The methanol extract of Loquat (78.63%) displayed the highest NO scavenging activity followed by pomegranate (72.95%) > carob (71.80%) > grape (70.90%). Furthermore, these extracts showed higher scavenging activity compared to BHA and BHT.

Superoxide anion scavenging activities were tested by NBT method and the results are expressed as trolox equivalents (mgTEs/g extract). The values obtained for water extracts were in the order of carob > pomegranate > avocado > walnut. The observed differential scavenging activities of the methanol and aqueous extracts against superoxide could be due to the presence of different level of phenolics. This situation was supported by other works (Dasgupta and De, 2004; Sahreen *et al*., 2010) (Table 2).

Anti-oxidative activity has been proposed to be related to reducing power. Therefore, in order to assess the electron-donating power of the methanolic and water extracts, its ability to reduce iron (III) was investigated. All extracts were tested for their ability to reduce the Fe^{+3}/ferricyanide complex to the ferrous (Fe^{+2}) form. All the extracts showed reducing activity in the amount dependent manner. From Figure 1, generally, the reducing activity of both methanol extracts and water extracts decreases in the following order: Pomegranate > carob > avocado.

CONCLUSION

The results obtained in this work have considerable value with respect to the antioxidant of methanol and water extracts of nine different fruit tree leaves samples. These samples contained high levels of antioxidant components including tannins and saponins. Consequently, our results suggested that the extract can be utilized as an effective natural source of antioxidant agents. In the future, it will

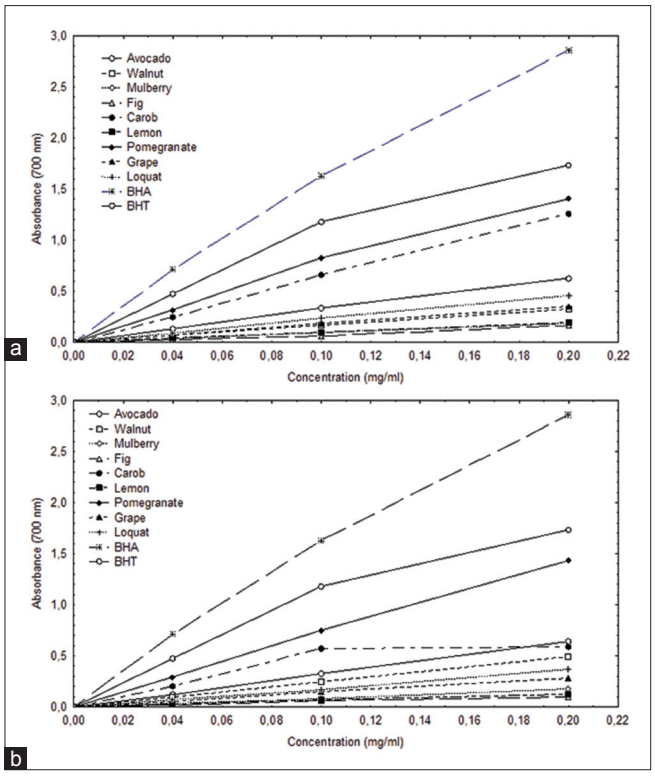

Figure 1: Ferric reducing power of methanol (a) and water extracts (b) from nine fruit leaves

be very interesting to see the results *in vivo* studies on biological effects of these leaf extracts.

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