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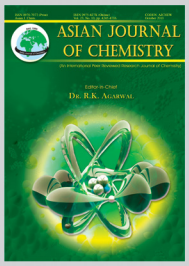

In Vitro Total Antioxidant and Antimicrobial Activities of Essential Oils from Leaves and Rind of *Aegle marmelos* L. Correa

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Aegle marmelos L. Correa is a pharmaceutically important plant which has been claimed to be effective against various diseases in the folk medicinal system of Indo-Pak subcontinent. A detailed study regarding total antioxidant capacity and radical scavenging and antimicrobial effects of the essential oils from leaves of *Aegle marmelos* L. Correa (AML) and *Aegle marmelos* L. rind (AMR) of this plant were investigated. Essential oils obtained from steam distillation process were subjected to 2,2'-azinobis(3-ethylbenzothiazoline-6-sulpohonic acid) (ABTS) radical cation decolorization, ferric reducing antioxidant power (FRAP), 2.2'-diphenyl-1-picrylhydrazil (DPPH) radical decolorization, total phenolic and total flavonoid contents, lipid peroxide inhibition using linoleic acid emulsion, superoxide anion radical scavenging and iron chelation activity assays. Total phenolic and total flavonoid contents values were found to be 18.078 and 13.310 mg/L gallic acid equivalent and 3.349 and 12.087 mg/L quercetin equivalent for AML and AMR, respectively. A linear correlation was observed between the per cent inhibition of ABTS radical cation and the amount of essential oils from leaves (R^2 = 0.9973) and rind (R^2 = 0.997). The per cent superoxide anion radical scavenging activity was found to be 47.37 and 2.50 for AML and AMR, respectively. The reducing power in terms of FRAP values were found to be 1.2 and 2.2 mM $FeSO_4$ for AML and AMR, respectively. Both AML and AMR exhibited good metal chelating activities, 62.75 and 78.67 for AML and AMR, respectively. The oils also showed antimicrobial and antifungal activities comparable to chloramphanicol. The data obtained from oils demonstrate the powerful antioxidative, radical scavenging and antimicrobial properties of the plant.

Key Words: Antioxidant potential, Radical scavenging, TEAC, *Aegle marmelos* L.

INTRODUCTION

The plant *Aegle marmelos* (Rutaceae), is found in many countries of South and South-eastern Asia as a medium sized tree. In the traditional Ayurvedic and Unani system of medicine it is used for the treatment of several diseases[1]. The plant is mainly known for its antidiabetic and hypoglycemic activities in human and animal systems[2-4]. Almost all the parts are used for various ailments. The decoction made from roots and bark is considered to be effective against malaria[5] and the ripe fruit is useful for diabetes[6,7], dyspepsia, constipation and body heating problems[8]. *A. marmelos* extract has been shown to attenuate CCl_4-mediated hepatic oxidative stress, toxicity, tumor promotion and subsequent cell proliferation response in Wistar rats[9]. The seed extracts of plant are known to exhibit significant activity against *Vibrio cholerae*, *Staphylococcus aureus* and *Escherichia coli*[10]. Radioprotective effects against ionizing radiations of extracts from *A. marmelos* have been demonstrated in animal model systems[11,12]. The isolated periplogenin from the leaves of *A. marmelos* could potentially inhibit doxorubicin-induced cardiovascular problems and hepatotoxicity in rats, where, its moderate dose was found to be the most effective[13]. Positive contraceptive effects on the reproductive organs of male rats of the aqueous and ethanolic extracts from the leaves of the plant have been reported[14,15]. Essential oils isolated from *A. marmelos* have shown promising antifungal activities against *Physalospora tucumanensis*, *Ceratocystis paradoxa*, *Sclerotium rolfsii*, *Curvularia lunata*, *Helminthosporium sacchari*, *Fusarium moniliforme* and *Cephalosporium sacchari*[16]. *A. marmelos* oil has been recommended as a plant-based antimicrobial in food protection over synthetic preservatives[17]. The decoction of the unripe fruit pulp of the plant, has been shown to affect the bacterial colonization to gut epithelium and production and action of certain enterotoxins[18]. The extracts of *Phyllanthus urinaria*, *Thevetia nerifolia*, *Jatropha gossypifolia*, *Saraca asoca*, *Tamarindus*

indica, *Aegle marmelos*, *Acacia nilotica*, *Chlorophytum borivilianum*, *Mangifera indica*, *Woodfordia fruticosa* and *Phyllanthus emblica* showed antimicrobial activity in a range of 75-1200 μg/mL[19]. GC-MS analysis of the oil from *A. marmelos* found DL-limonene to be major component[17]. Several coumarins[20,21], anthraquinones[22], sterols[23], lignan-glucosides[24], protolimonoids[25] and alkaloid-amides[26,27] have been isolated from different parts of the plant. Different chemicals such as imperatorin, β-sitosterol, plumbagin, 1-methyl-2-(30-methyl-but-20-enyloxy)-anthraquinone, β-sitosterol glucoside, stigmasterol, vanillin and salicinonstituents including 2-isopropenyl-4-methyl-1-oxa-cyclopenta[β]anthracene-5,10-dione and (+)-4-(20-hydroxy-30-methylbut-30-enyloxy)-8*H*-[1,3]dioxolo[4,5-h]chromen-8-one, are isolated from the seeds of the plant[28]. Extracts from *A. marmelos* plants have been shown to possess significant antioxidative properties such as scavenging of ABTS and DPPH radicals and inhibition of lipid peroxidation and DNA damage[11,12].

The diverse antidisease activities claimed for *A. marmelos* extracts/oils coupled with the increasing demand for natural antioxidative compounds encouraged us to undertake a comprehensive study of the antioxidant and radical scavenging activities of the plant. The main focus of this study is to investigate and compare the total antioxidative capacity (TAC) and radical scavenging activities of extracts from leaves and seed coats of *A. marmeos*. TAC and radical scavenging activities were determined in terms of ferric reducing antioxidant power (FRAP) assay[29], ABTS, DPPH and superoxide anion radicals scavenging activities, total phenolic and total flavonoid contents and metal chelating activity. Further, the antimicrobial studies have also been carried out.

EXPERIMENTAL

All the chemicals used were of analytical reagent grade. The solvents used were obtained from E. Merck (Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), linoleic acid, sodium acetate, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), ABTS (2,2'-azino*bis*-(3-ethylbenzo-thiazoline)-6-sulphonic acid diammonium salt), gallic acid, quercetin, Tween 20 (polyoxyethylenesorbitan monolaurate), iron(II) sulphate, potassium thiocyanate, 1,1-diphenyl-2-picrylhydrazil (DPPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), Folin-Ciocalteau's reagent (FC reagent) were purchased from Fluka (Switzerland).

The plant material (leaves and rind) of *A. marmelos* was collected from Botanic Garden, GC University, Lahore, Pakistan. Water-cleaned and shade-dried plant material was subjected to steam distillation. The samples obtained were used either neat or in diluted form in various antioxidant and radical scavenging assays.

Total phenolic content: Total soluble phenolic compounds were determined following the method of Singleton *et al.*[30] using gallic acid as a standard phenolic. In brief, 40 μL of each sample and the standard was transferred into separate test tubes. To each test tube added 3.16 mL water and 200 μL of FC-reagent. The mixture was mixed well and left for 8 min and then added 600 μL of sodium carbonate solution with constant stirring. The solution was left at 40 ºC for 0.5 h and absorbance of each solution at 765 nm against the blank (without sample) was determined. A standard curve was graphed between absorbance and concentration. Total phenolic content of each sample was determined as milligrams of gallic acid equivalent (GAE) by using the straight line equation from the standard gallic acid graph.

Ferric reducing antioxidant power: Ferric reducing antioxidant power (FRAP) values were determined following the method of Benzie *et al.*[29]. Final results were expressed as FRAP values (mM $FeSO_4 \cdot 7H_2O$).

$ABTS^{\bullet+}$ scavenging activity: $ABTS^{\bullet+}$ scavenging activity was determined by following the method of Re *et al.*[31] with minor changes. For studying radical scavenging capacity of plant samples, the absorbance of ABTS radical cation solution was adjusted to an absorbance of 0.700 ± 0.020 at 734 nm (A_{734} nm) with PBS buffer (pH 7.4) and equilibrated at 30 ºC. After addition of 10 μL of neat or diluted sample to 2.99 mL of diluted $ABTS^{\bullet+}$ solution, the absorbance readings were taken after every 1 min for 8 min. The radical scavenging activity in terms of per cent inhibition (I_{734} nm) of absorbance was calculated by the following formula.

$$I_{734\,nm} = \left(1 - \frac{A_f}{A_0}\right) \times 100$$

where A_0 and A_f are the absorbance values of radical cation solution at time t = 0 and t = 8 min. after addition of sample/standard solution, respectively. For reference data, a standard curve of trolox was obtained by plotting concentration values and their respective per cent inhibition values. Each measurement was taken three times and in triplicate for both standards and samples.

Kinetics of 2,2'-diphenyl-1-picrylhydrazyl radical scavenging: DPPH free radical scavenging activity of the plant oils was measured by using the method of Shimada *et al.*[32] DPPH solution (3 mL, 25 mg/L) in methanol was mixed with appropriate volumes of neat or diluted sample solutions. The reaction progress of the mixture was monitored at 517 nm over a time period of 0.5 h. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 517 nm as a function of time (min) was plotted for each sample.

Total antioxidant activity determination: Total antioxidant activity of the essential oils was determined according to the method employed by Mitsuda *et al.*[33]. In short, plant sample (100 L) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was mixed with 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The solution was incubated at 37 ºC in sealed bottles in dark. The solution without added extract was used as blank, while the solutions containing 100 μL (50 μg/20 μL) of trolox were used as positive control. At intervals of 24 h during incubation, 0.1 mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1 mL each of $FeCl_2$ (20 mM in 3.5 % HCl) and thiocyanate solution (30 %) to the ethanolic sample, the solution was stirred for 1 min. The absorption values of the solutions measured at 500 nm were taken as lipid peroxidation values.

Superoxide anion radical scavenging activity: Superoxide anion radical scavenging activity was determined using the method of Nikishimi *et al.*[34]. The PMS-NADH system was

used for production of superoxide radicals. The reaction mixture contained 100 µL sample, 200 µM NBT, 624 µM NADH and 80 µM PMS in 0.1M phosphate buffer (pH 7.4). After 2 min of incubation, absorbance was measured spectrophotometrically at 560 nm. The scavenging effect was calculated using the following formula:

$$\text{Scavenging}\,(\%) = \left(1 - \frac{A_s}{A_b}\right) \times 100$$

where A_s and A_b are the absorbances of sample and blank solutions at 560 nm, respectively.

Metal chelating activity: Metal (ferrous ion) chelation by plant samples was estimated according to the method employed by Dinis *et al.*[35]. A 100 µL sample was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by the addition of 5 mM ferrozine (0.2 mL) and total volume was adjusted to 4 mL with ethanol. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Chelating activity}\,(\%) = \left(\frac{(A_{\text{Control}} - A_{\text{Sample}})}{A_{\text{Control}}}\right) \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance in the presence of the sample of herbs/sample.

Antimicrobial activity: Metal (ferrous ion) chelation by plant samples was estimated by the method of Dinis *et al.*[35]. A 100 µL sample was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by the addition of 5 mM ferrozine (0.2 mL) and total volume was adjusted to 4 mL with ethanol. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Chelating activity}\,(\%) = \left(\frac{(A_{\text{Control}} - A_{\text{Sample}})}{A_{\text{Control}}}\right) \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance in the presence of the sample of herbs/sample.

RESULTS AND DISCUSSION

Per cent recovery, total phenolic conent and total flavonoid content: The per cent recovery, total phenolic content and total flavonoid content of the sample oils were calculated and are shown in Table-1. In comparison with *Aegle marmelos* L. rind (AMR) about 15 times greater per cent recovery of oil was found for AML sample. *Aegle marmelos* L. Correa (AML) oil of the plant was found richer in phenolic contents. On the contrary AMR oil demonstrated higher value of flavonoid contents. It is quite obvious that in spite of low per cent recoveries, the total phenolic content and total flavonoid content were quite good, showing richness of the oil samples with phenolic and flavonoid components. High total phenolic content and total flavonoid content indicated high antioxidant and radical scavenging capacities for both the samples.

TABLE-1
PER CENT YIELD, TOTAL PHENOLIC CONTENTS (TPC) AND TOTAL FLAVONOID CONTENTS (TFC) OF ESSENTIAL OILS FROM LEAVES AND RIND OF *Aegle marmelos*

Plant name	*Aegle marmelos* (leaves)	*Aegle marmelos* (rind)
Weight of specimen (Kg)	8.255	2.700
Weight of oil (g)	25.77	0.60
Recovery (%)	0.31	0.02
Colour of oil	Clear yellowish	Clear yellowish
TPC (mg/L GAE)	18.078	13.310
TFC (mg/L QE)	3.349	12.087

TPC = Total phenolic content; TFC = Total flavonoid content.

Polyphenolic compounds constitute a very important and diverse group of phytochemicals including phenols, phenol propanoids, lignins, flavonoids, stilbenes. These compounds perform diverse functions such as metal chelation, free radical scavenging and antioxidative actions[36].

ABTS radical scavenging capacity and relationship between TEAC and total phenolic content: ABTS radical cation produced as a result of reaction between ABTS and potassium persulfate in aqueous solution at physiological pH has considerable stability and sensitivity towards crude and specific antioxidants[37]. The reduction potential of ABTS radical cation is very similar to that of hydroxyl radical cation. So in test environment it may be taken as equivalent to hydroxyl radical produced in vivo during certain disorders and metabolic reactions. ABTS radical scavenging ability of the test samples were evaluated using ABTS radical cation decolorization assay.

Fig. 1 shows the dose dependent per cent inhibition of ABTS radical cation by the two samples. A significant correlation (R^2 = 0.9973 and 0.997 for AML and AMR, respectively) was found between per cent inhibition and amount of the sample.

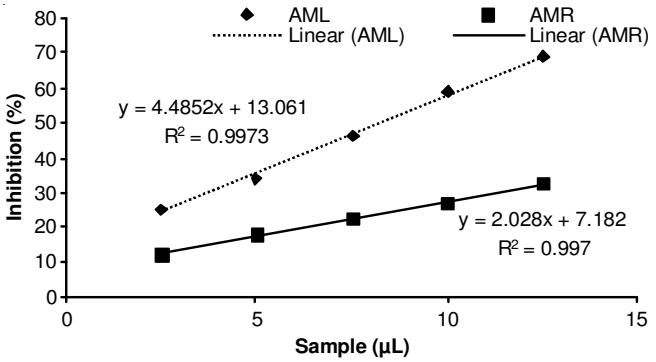

Fig. 1. Comparison of dose dependent per cent inhibition of ABTS radical cation by essential oils from leaves and rind of *Aegle marmelos*

Due to health-promoting effects of antioxidants a general recommendation to the consumer is to increase the intake of foods rich in antioxidant compounds (*e.g.*, polyphenols, flavonoids)[38-45]. Phenolic compounds, especially flavonoids and anthocyanins, are very important antioxidants because of their natural origin and their ability to act as efficient free radical scavengers[45-47]. Small berries have been reported to be rich sources of phenolic compounds such as phenolic acids as well as anthocyanins, proanthocyanidins and other flavonoids, which display potential health promoting effects[48-54].

Total phenolic contents in terms of gallic acid equivalents of all the extracts were determined using Folin-Ciocalteu's method. The extracts showed high GAE values. The amount of total phenolics for AML and AMR samples were found to be 18.078 and 13.31 mg/L gallic acid equivalent, respectively. Alike TPC, high TFC values were recorded for both AML and AMR samples. The TFC values were found to be equal to 3.349 and 12.087 mg/L quercetin equivalent for AML and AMR samples, respectively. High values of TPC and TFC obtained for both the samples demonstrated presence of various phenolic acids and flavonoid components in these samples. It is also evident from the data that ABTS radical cation decolorization assay is more linearly related to TPC. Attempts have been made to derive a relationship between the phenolic contents and antioxidant activity. Controversial results have been obtained regarding a linear relationship between TPC and antioxidant activity[55,56].

The present study showed a relatively good relationship between TPC and antioxidant activity determined through ABTS radical cation decolorization assay and FRAP assay. Non-acquisition of absolutely linear relationship between TPC and the two assays may be due to different response of different phenolics in Folin-Ciocalteau reagent[57,58], difference in the pH of the medium of assays and the reduction potential of the oxidized species. Furthermore the antioxidant activity strongly depends upon the chemical structure of phenolic compounds. Therefore no definite quantitative relationship could be obtained for general application to all the plant extracts.

DPPH, lipid peroxyl and superoxide anion radicals scavenging activities: DPPH and lipid peroxide free radicals have been used to evaluate reducing properties and to assess free radicals chain breaking abilities of phyto-chemicals. Fig. 2 demonstrates the kinetics of DPPH radicals scavenging by AML and AMR. Both the extracts showed time dependant quenching of DPPH radicals. AML sample was found to be a better quencher of DPPH radicals than AMR extract. The absorbance continued to decrease with almost a uniform gradient throughout the time span of 0.5 h showing the presence of a good amount of slow reacting antioxidant components in both the mixtures.

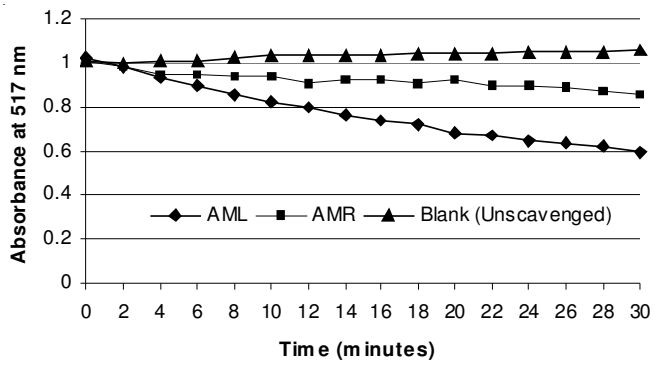

Fig. 2. Kinetics of DPPH radical scavenging by AML and and AMR extracts

In living organisms, unsaturated fatty acids present on biomembranes are more susceptible to free radicals attack which results in lipid peroxidation, decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins leading to cell inactivation[59]. Many disorders like hyperglycaemia have been ascribed to development of oxidative stress due to increased lipid peroxide production[60]. Lipid peroxidation values of the extracts were found using linoleic acid emulsion system. Linoleic acid after its aerial oxidation to peroxyl radicals converts ferrous to ferric form. The extent of conversion is assessed through iron(III) complex with thiocyanate, spectrophotometrically. The antioxidative components in proportionate to their amount halt this conversion by trapping peroxyl radicals. Fig. 3 shows that both the samples had considerable resistance to lipid peroxidation which is quite comparable with that of Trolox (10 μM).

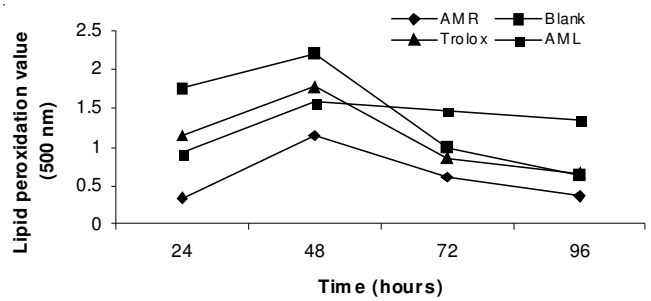

Fig. 3.

Superoxide (SO) anion radical is one of the important ROS which is produced first after oxygen is taken inside the body. The subsequent dismutation of SO leads to the formation of other injurious ROS. So the capacity of samples to scavenge ROS can play a crucial role in determining the overall strength of antioxidant activity. The per cent superoxide anion radical scavenging activity was found to be 47.37 and 2.50 % for AML and AMR samples, respectively.

Reducing and metal chelating activities: FRAP assay was employed to estimate the ferric reducing activity of the samples using $FeSO_4$ as the standard reducing agent. At low pH, reduction of ferric tripyridyl triazine (Fe(III) TPTZ) complex to intense blue coloured ferrous form can be examined by measuring the change in absorption at 593 nm. Being a non-specific reaction any half reaction that has lower redox potential, under the test conditions, than that of Fe(III)/Fe(II) reaction, will convert Fe(III) to Fe(II). The change in absorbance is therefore, reflects cumulative reducing power of the electron donating antioxidants present in the reaction mixture. The FRAP values were found to be 1.2 and 2.2 mM $FeSO_4$ for AML and AMR, respectively. The metal chelating activity was found by determining the chelating activity of the sample with ferrous ion in the presence of ferrozine (a ferrous chelating agent). In the presence of chelating components of the sample the formation of Fe(II)-ferrozine complex, which may be monitored at 562 nm spectrophotometrically is halted. The per cent inhibition of the complex formation was found to be 62.75 and 78.67 for AML and AMR, respectively.

Antibacterial and antifungal activity: Agar-well diffusion method was performed to calculate zone of inhibition for both the samples. The results obtained (Table-2) showed that

TABLE-2
ANTIMICROBIAL AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM LEAVES AND RIND OF *A. marmelos*

Plant part/standard	Zone of inhibition (cm)												
	B. subtilis	*S. aureus*	*S. typhimorium*	*E. coli*	*P. aeruginosa*	*E. aerogenes*	*S. cococus*	*M. roseus*	*A. niger*	*P. chrysogenum*	*S. cerevisiae*	*R. oligosporons*	*R. minuta*
AML*	1.4	1.2	1.2	2.8	1.4	2.4	1.9	1.6	2.0	1.9	2.6	1.8	2.0
AMR*	1.8	2.0	2.2	2.1	1.6	2.0	1.8	1.8	2.6	2.4	1.8	1.6	2.0
Chloromphenicol	1.0	1.2	2.4	0.8	2.0	1.6	1.8	2.0	2.5	2.0	2.1	2.0	2.6

*100 µL of each oil was used in the experiment. The concentration of chloromphanicol was 1 mg/mL.

both the samples have powerful antimicrobial activities comparable to chloromphanicol.

Conclusion

The data presented here show that *A. marmelos* extracts have great antioxidant and radical scavenging activity and thus may be used as a good source of natural antioxidants. The *in vivo* efficacy of *A. marmelos* oils against diabetes mellitus or other degenerative diseases may be partially attributed to radical scavenging and antioxidant activity of the plant.

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