Dry and ripe fruit of *Aegle marmelos*. L: A potent source of antioxidant, lipoxygenase inhibitors and free radical scavenger

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Abstract: The antioxidant, lipoxygenase inhibitory activities and free radical scavenging capacity of the crude extract, aqueous and some organic fractions of dry and ripe fruit of *Aegle marmelos.* L were studied to understand the protective and therapeutic role for the use of the fruit as a remedy in different ailments. All the tested fractions and extracts showed to possess significant antioxidant, free radical scavenging capacity and lipoxygenase inhibitory potential. However, chloroform and aqueous fractions showed significant ability to quench radicals, to reduce ferric chloride and to inhibit soyabean lipoxygenase. Their antioxidant and lipoxygenase inhibition was estimated by IC_{50} values, for antioxidant ranging from 88-65% activity at concentration of 5-0.15µg/mL and similarly for lipoxygenase inhibition ranging from 89-69% at various concentrations of 5-0.15µg/mL, in chloroform and aqueous fractions respectively. The scavenger molecules in the dry and ripe fruit of *A. marmelos* may attribute to therapeutic and protective effect during different progressive stages of ailments.

Keywords: *A. marmelos,* dry and ripe fruit, lipoxygenase inhibition, antioxidant, free radical scavenging capacity.

INTRODUCTION

Antioxidants are the organic compounds, which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Free radical are molecular species, containing one or more unpaired electrons and are highly reactive. They are formed by regular metabolism during oxygen utilization, to burn food for energy (Fleschin *et al.,* 2000). Their development occurs endlessly within the body as a result of both enzymatic and non-enzymatic reactions. These reactive oxygen species (ROS) are dangerous for vital cell components like fatty acids, Proteins, Nucleic acids and carbohydrates.

Antioxidants are competent for stabilizing or neutralizing free radicals before they bother cell. They are firm enough to provide electron to rampage free radicals and counteract them, thus dropping their capacity to harm cells. It is well known that the plants having more powerful antioxidants properties have great potential for lipoxygenase inhibition. Antioxidants can be synthetic or can be natural. Most of natural antioxidants are polyphenols. These phenolics have redox property due to which they can act as antioxidant agent and also behave as reducing agents, free radical scavengers and quenchers of singlet oxygen. The synthetic antioxidants have many side effects therefore replacement by new and natural compounds are the focus of current research. The Pakistani flora contains numerous medicinal plants whose biological and chemical properties are incompletely known. Antioxidant and radical scavenging properties of plants are subject to intensive research. The aim of the present study was to screen the antioxidant activities of some commonly available plants and fruits.

Several medicinal plants in Pakistani flora, which possess Pharmacological properties are partly identified. One such medicinal plant used since ancient time is *Aeglemarmelos* (L.) commonly known as Bael (Rutaceae). Its vast medicinal, pharmacological and antimicrobial properties were reported and well explained in Ayurveda Indian traditional medicine and greatest medicinal value, however, has been attributed to its fruit (Reddy *et al.,* 2010; Dhuley 2003; Brijesh *et al.,* 2009; Jagetia and Venkatesh 2005; Prince and Rajadurai 2005; Rani and Khullar 2004; Baliga *et al.,* 2010).

Through in vitro and in vivo studies it is pointed out that *A. marmelos* is a major source of accepted antioxidants, which might be supportive in controlling the progress of various oxidative stresses in the body thus have a good potential to use in various progressive inflammatory diseases, to use as radio-protective and as a protective agent in hepatic and heart disorders (Jagetia *et al.,* 2004; Jagetia *et al.,* 2003). *A. marmelos* fruit extract exerts its protective effect by decreasing thiobarbituric acid reactive substances and elevating antioxidants status in isoproterenol treated rats (Krushna *et al.,* 2012). It is generally believed that even short-term endurance exercise results in a rapid increase in myocardial (MnSOD) activity (Golbidi and Laher 2011), which plays

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an important role in preventing cells from oxidative stress and inhibiting tumorigenicity (Oberley and Buettner 1979). Phytochemical screening of *A. marmelos* discovered the occurrence of alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids, and steroids (Sharmila and Devi 2011). Scientific literature reflected that almost all parts of this plant except dry and ripe fruit, were used to isolate several active principles like marmelosin, marmelide, Luvangetin, aurapten, psoralen and tannin etc (Kamalakkannan and Prince 2003; Maity *et al.*, 2009). The present studies are focused to explore the total phenolic content, DPPH- radical scavenging and lipoxygnease inhibition activity in crude extract, aqueous and organic fractions (ethyl acetate, chloroform, butanol) of dry and ripe fruit of *A. marmelos.*

MATERIALS AND METHOD

Chemicals

1,1-diphenyl-2-picryl-hydrazil (DPPH) & all solvents were procured from Roche Diagnostics, Mannheim, Germany. DMSO, potassium hexacyanoferrate [$K_3Fe(CN)_6$], F-C reagent, sodium carbonate (Na_2CO_3), butylatedhydroxyanisol (BHA), Ferric chloride, trichloroacetic acid (TCA) were purchased from MP Biomedicals (France). Lipoxygenase sodium dihydrogen phosphate, linoleic acid, tween 20, baiclein were purchased from Sigma.

Plant material

The dry and ripe fruit of *A. marmelos* was procured from neighboring market of Karachi**,** Pakistan and recognized by Dr. Beena Naqvi, taxonomist, PCSIR-labs Karachi, the specimen voucher no: Am 0012/2010 was preserved in the herbarium of the pharmacology lab of PCSIR-labs.

Preparation of plant extract

The dry and ripe fruit of *A. Marmelos* (3.50Kg) was cleaned of dust particle and other adulterants by manual examination and picking and then thickly grounded. The coarse milled stuff was covered with 7Lmethanol:water (70:30) mixture at room temperature (23-25°C) for 72 hrs with intermittent shaking. It was first filtered through muslin cloth and then through Whatman No.1 filter paper, thrice; filtrate was pooled and then concentrated under reduced pressure at 40°C on rotary evaporator. The resulting dark brown colored semi solid mass (13.26%) was named as crude extract and stored at 4°C until use. Half of the crude extract was used for further fractionation. For preparation of different fractions crude extract was mixed with 100ml of water and 200 ml of ethyl acetate in a separating funnel and left for 24 hours. Next day aqueous and ethyl acetate layers were collected separately. Ethyl acetate layer was evaporated to obtain ethyl acetate fraction while aqueous layer was again treated with 200mL of chloroform and then 200mLof butanol in the similar manner as mentioned above to obtain chloroform, butanol and aqueous soluble fractions. The crude extract and fractions were stored at 4°C in air tight bottles for further studies (Yaqeen *et al.*, 2013).

Estimation of total phenolics

Total phenolic content was evaluated by Folin-Ciocalteu (FC) reagent according to the method described by Hazra *et al.,* (2008) with variation. Briefly, the plant extract (0.1mL) was mixed with 0.75ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22ºC, then 0.06% Na_2CO_3 solution was added. After incubation at 22ºC for 90min, the absorbance was measured at 725nm. The phenolic content was evaluated from a gallic acid standard curve**.**

DPPH radical scavenging assay

The free radical scavenging activity was measured by method describing by Iqbal *et al*., (2007) using 1,1-diphenyl-2-picryl-hydrazil (DPPH) as free radical. The solution of DPPH of 0.3μM was prepared in ethanol. Each sample (5µL) (of different percentages) was mixed with 95µL of DPPH solution in ethanol, incubated at 37ºC for 30min. The absorbance at 515 nm was measured by ELISA plate reader (Spectramax plus 384 Molecular Device, USA) and percent radical scavenging activity was determined. BHA is used as standard.

DPPH scavenging effect (%) = Ac **–** As/Ac × 100
where Ac = Absorbance of control (DMSO treated); As = Absorbance of sample**.**

Total reduction capability

Total reduction capability was estimated according to the method of Iqbal *et al.,* (2007) with some modification. Test solution (100μL) from extract and each fraction with various concentrations was mixed with 250μL of 0.2M phosphate buffer (pH 6.6) then 250μL solution of 1% potassium ferricyanide [$K_3 Fe (CN)_6$] was added. The mixture was incubated at 50°C for 20 min then 250 μL trichloroacetic acid (10%) was added and centrifuged for 10min at 3000rpm. 250μL from upper layer was separated and mixed with equal volume of DMSO/ methanol. 50μL of 0.1% Ferric chloride [$Fe Cl_3$] was added and the absorbance was measured at 700nm by using spectrophotometer (Specord 2000, Germany). Reduction capability was calculated in terms of percentage with respect to reference standard (BHA).

Lipoxygenase inhibition assay

Inhibition of Lipoxygenase activity was calculated by the spectrophotometric method developed by Tappe l1962. Briefly describing 160µL sodium phosphate buffer (pH 8.0, 100mM) and 10μL solution of test (extract and fractions) of various percentages were added in each well labeled as test. 20μL of lipoxygenase (LOX) solution (130 units per well) was added, mixed and incubated for 10min at 25°C**.** The reaction was then started by the addition of 10µL substrate solution (linoleic acid 0.5mM, 0.12% tween 20) after 15min the absorbance was measured at 234nm by ELISA plate reader (Spectramax plus 384 Molecular Device, USA) Baiclein was used as positive control.

IC_{50} determination

The IC_{50} values were determined by EZ-Fit, Enzyme kinetics Program (Perrella Scientific In., Amhherset, USA).

RESULTS

Estimation of total phenolics in extract and fractions

Phenolic content revealed that crude extract has highest phenolic content followed by ethyl acetate fraction. Phenolic compounds behave as key constituents because of their hydroxyl groups, which bestow scavenging ability (table. 1).

Table 1: Phenolic content of extracts and various fractions of *A. marmelos* L

S. No	Fraction	Phenolic content (mgGAE/g)
1	Crude extract	367
2	Aqueous	115.3
3	Ethyle acetate	65
4	Chloroform	244.9
5	Butanol	123.4

Total reduction capability

Reducing Power of a compound may provide a significant indicator of its potential antioxidant activity as illustrated in fig. 1, that ferrous was reduced to ferric due to the presence of *A. marmelos* extract and fractions along with the reference compound BHA to measure the reductive capability. This result indicates that these fractions and extract also have good reducing power.

DPPH radical scavenging

In the present study antioxidant potential is studied by the inhibition of DPPH free radical and ferric chloride reducing power assay. In both assays, the antioxidant capacity of dry and ripe fruit crude extract and subsequent fractions of *A. marmelos* showed promising results. table 2 shows IC_{50} values of crude extract as 61.4µg/mL, chloroform and aqueous 67.4 and 55.5µg/mL respectively which are well comparable with standard (BHA) which showed IC_{50} as 44.2µg/mL. While butanol and ethyl acetate posses less antioxidant potential as compared to other fractions but, however the fruit has good antioxidant activities.

Lipoxygenase inhibition

Results in fig. 2 showed that all fractions along with crude extract appeared to be the prominent inhibitor of soybean lipoxygenase. The effect of various fractions and extract on lipoxygenase inhibition challenge was very profound.

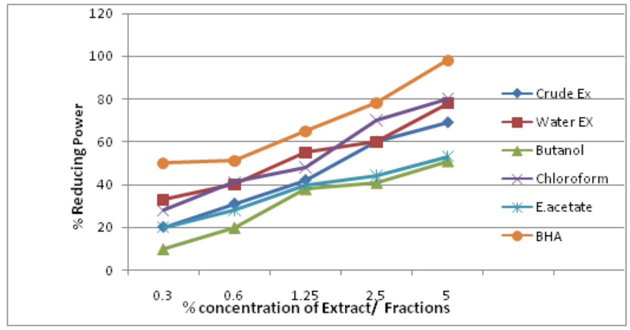

Fig. 1: Dose dependant Reduction Capability of extracts and various fractions of *A. marmelos* L (Capital or normal letter)

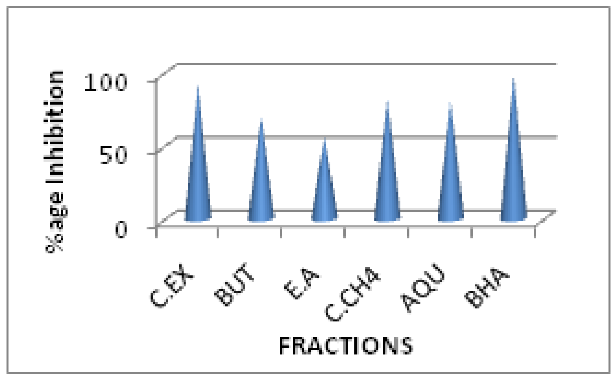

Fig. 2: Lipoxygenase inhibitory potentials of extracts and various fractions of *A. marmelos* L.

DISCUSSION

The aim of the present study was to screen indigenous plants, especially which are not commonly used, to identify antioxidant ability and enzyme inhibition capacity. Most bioactive food constituents are derived from plants; this study is therefore an essential research tool to further elucidate the potential health effects of phytochemical antioxidants in diet. Due to unpleasant effects of synthetic drugs, which are commonly used against oxidative damage, it is wise and advisable to use herbal antioxidants as food supplements and traditional medicines. Therefore, globally interest is developed towards green medicines because herbs are good source of many vital nutrients required to run life healthy and smoothly (Ahmed *et al.*, 2011; Rahman *et al.*, 2013). The results of this study confirmed that crude extract has highest phenolic content followed by ethyl acetate fraction (table. 1) and it is well reported that majority of natural antioxidants are poly phenol in nature which confer their scavenging ability (Wojdylo *et al.*, 2007).

The results of total reduction capability (fig 1) illustrate that Fe^{3+} was transformed to Fe^{2+} in the presence of *A.*

Table 2: IC_{50} values of extracts and fractions of dry and ripe fruit of *Aeglemarmelos* L.

S. No	Extract/fraction	DPPH Scavenging IC_{50} µg/ml± SD	Lipoxygenase Inhibition IC_{50} µg/ml± SD
1	Crude extract	61.4± 0.12	99.3±0.84
2	Aqueous extract	55.5±0.40	101.8±1.2
3	Ethyl Acetate	110.7±0.71	227.3±1.89
4	Chloroform	67.4±0.63	102.4±0.95
5	Butanol	93.6±0.59	207±3.5
6	BHA	44.6±0.05	----
7	Baicalein	---	22.6±0.02

marmelos extract and fractions to quantify the reductive capability. This result exhibited that these fractions and extract also have good reducing power (fig. 1). In both assays, the antioxidant capacity of dry and ripe fruit crude extract and subsequent fractions of *A. marmelos* exhibited promising results. The results of DPPH radical scavenging activity showed IC_{50} values of crude extract as 61.4µg/mL, chloroform and aqueous 67.4 and 55.5µg/mL respectively, which are well comparable with standard (BHA) which showed IC_{50} as 44.2µg/mL. While butanol and ethyl acetate possessless antioxidant potential as compared to other fractions (table. 2).

Lipoxygenase inhibition activity showed that all fractions along with crude extract appeared to be the prominent inhibitor of soybean lipoxygenase. The effect of various fractions and extract on lipoxygenase inhibition was very profound (fig. 2). It is evident from IC_{50} values in table. 2 that crude extract, aqueous and chloroform fractions have good lipoxygenase inhibition potential (99, 101 and 102 µg/mL) respectively.

The important role of the 5-lipoxygenase pathway in carcinogenesis is vital as inhibition of the 5-lipoxygenase pathways clearly has chemo preventive effects on various cancers, (Chen X *et al.*, 2006). We observed in our study that all fractions and crude extract of *A. marmelos* strongly inhibit lipoxyganase and have potential of scavenging of DPPH radical, this can be assumed that the components present in fractions and extract may block the cascade process of arachidonic acid metabolism by inhibiting lipoxygenase and may serve as strong scavenger of free radicals to save cellular organs.

CONCLUSION

The results suggests that the extract and different fractions of dry and ripe fruit of *A. marmelos* L exhibits significant antioxidant and free radical scavenging activities/capacity which might be associated with the presence of main phytochemicals like flavonoids, phenols and tannins. This fruit also has good reducing power and as expected all these extract and fractions have good enough lipoxygenase inhibitory potential. Therefore the presence of the scavenger molecules in the dry and ripe fruit of *A. marmelos* may attribute health benefits by providing the protective effects during the progressive stages of different acute and chronic disorders.

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