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Determination of total Phenolic and Flavanoid Contents in Traditionally used *Aegle Marmelos* Formulations by Spectrophotometric Method.

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ABSTRACT

In present investigation, methanolic extract of different marketed *Aegle marmelos* formulations (F_1, F_2, F_3, and F_4) screened for determination of total phenolic and flavanoid contents. Highest phenolic (6.145 ± 0.05 mg/kg) and flavonoid (8.134 ± 0.044 mg/kg) contents were noticed in formulation F_4. Free radical scavenging activity of all the studied formulations were evaluated by using DPPH (1,1-Diphenyl-2-picryl hydrazyl) method. Whereas, each formulation showed good scavenging of DPPH radical with IC_{50} values (F_1-2.185, F_2-2.216, F_3-2.243 and F_4-2.143 µg/ml), and is comparable to standard compounds (BHT, ascorbic acid and rutin).

Key-Words: *Aegle marmelos*, Total phenolic contents, Total flavanoid contents, DPPH

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INTRODUCTION

Aegle marmelos L. belongs to the family rutaceae, an important medicinal plant being used in folk therapy.[1] It is an old native of Indian tropical region and now cultivated throughout Southeast Asia and East Indian Archipelago[2]. It is a deciduous tree having profuse dimorphic branch; alternate, trifoliate, deep green leaves; membranous leaflets; large, sweet scented, greenish white flowers; and large, globose or ovoid or pyriform fruit[3] (Figure. 1). The plants are also used for treating anaemia, wound healing, high blood pressure, asthma, jaundice, and troubles during pregnancy, typhoid [4] and diabetes[5]. The edible portion contain water, protein, starch, fat, mineral salt, carotene, niacin, vitamin B1, vitamin B2, vitamin C, calcium and iron[6]. Fruit generally known as "*bael",* is widely consumed as '*sherbet*' (liquid fruit concentrate) and '*murabha*' (jam) [7]. Unripe fruit is highly recommended for diarrhoea and infusion of dried unripe fruit has been used as antidiarrhoeal and antidysentery agents[8]. Unripe fruits are used for making marmelle oil[9] and baelshut[10] which may be valuable for medicine. The ripe fresh fruits are eaten and its juice is used as soft drinks, for making candy, squash, pulp powder and nectar[11].Sweet drink (sherbet) prepared from the pulp of the bael fruits produce a soothing effect on the patients who have just recovered from bacillary dysentery[12, 13]. Some Ayurvedic medicine (Bilvadi churna, Bilva panchaka qwath and Bilva tailam), contained bael fruit as the principle ingredient[14]. The fruit of *A. marmelos* hold considerable amount of mucilage, pectin, marmelosin, marmelide, umbelliferone, quercetine, tannins, flavonoid and phenolic compounds and exhibited antioxidant activity [15, 16]. Flavonoids of fruit extract inhibit the release of autacoids and prostaglandins[17, 18]. It has been reported that the antioxidant activity might be due to the presence of phenolic compounds, such as phenolic acids, phenolic diterpine and flavonoids[19, 20]. The phenolic compounds act as primary antioxidant or free radical scavengers[21]. Naturally occurring product showed potent antioxidant activity other than vitamin C, E and carotenoid[22]. In addition, several synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroxy quinone and gallic acid esters have been reported to be carcinogenic[23]. Hence, strong limitation have been placed on their use and there is a trend to replace them with naturally occurring antioxidants[24, 25]. Furthermore, a study was design to evaluate *in vitro* antioxidant activity of umbelliferone and psoralen in a methanolic extract of *A. marmelos* fruit suggest that *A. marmelos* fruit is a potential source of natural antioxidants [26, 27]. Considering the therapeutic importance, it was planned to undertake the plant of the present study for its spectrophotometric determination of total phenolic and flavonoid contents in

methanolic extract of four *A. marmelos* formulation using Gallic acid and Quercetin as reference standard.

Figure 1: Fruits of *Aegle marmrlos*

MATERIAL AND METHODS

Chemicals and reagents

1, 1-Diphenyl-2-picryl hydrazyl (DPPH), rutin and ascorbic acid were purchased from Sigma–Aldrich (Bangalore, India). Tertbutyl-4-hydroxy toluene (BHT), gallic acid, Folin Ciocalteu reagent, and methanol were purchased from Merck Co. (Germany). All chemicals and reagents were of analytical grade. Formulations of *A. marmelos* were purchased from different Indian local markets.

Experimental

Preparation of sample

Accurately 5 gm of each formulation was weighted and sonicated for 30 min and extracted with methanol (3 × 50 ml) under reflux on water bath at 60^0C. The pooled extract of each sample was concentrated and transferred to 100 ml volumetric flask individually. It was used as working sample.

Estimation Total Phenolic Content (TPC)

Total phenol of all the extracts was measured at 765 nm by Folin Ciocalteu reagent (Dissolve 10 g sodium tungstate and 2.5 g sodium molybdate in 70 ml water. Add 5 ml 85% phosphoric acid and 10 ml concentrated hydrochloric acid. Reflux for 10 hr. Add 15 g lithium sulfate, 5 ml water and 1 drop bromine. Reflux for 15min. Cool to room temperature and bring to 100 ml with water) [28]. The dilute methanolic extract (0.5 ml of 1:10 g/ml) and gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 v/v diluted with distilled water) and aqueous sodium carbonate (4 ml, 1 M). The mixture was allowed to stand for 15 min and the total phenol was determined by spectrophotometer at 765 nm. The standard curve was

prepared using 0, 50, 100, 150, 200, 250, 300 and 350 µg/ml solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg/kg of dry mass), which is a common reference compound.

Estimation Total Flavonoid Content (TFC)

Flavonoid was estimated according to the aluminium chloride colorimetric technique[29]. Each extract (0.5 ml of 1:10 g/ml) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 415 nm with a double beam UV Visible spectrophotometer, SHEMADZU (Japan). The calibration curve was plotted by preparing the quercetin solution at concentrations 0, 20, 40, 60, 80, and100 µg/ml in methanol.

Free radical scavenging activity by DPPH assay

The free radical scavenging activity of different formulations were measured in terms of hydrogen donating or free radical scavenging ability using the stable 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH)[30]. Various concentration of methanolic extract (0.5 ml) were mixed in a test tube with a mixture of 2.5 ml of methanol and 75 µM DPPH (stable free radical), absorbance was recorded at 517 nm. The reaction mixture was set aside in the dark at room temperature for 90 min. Data were recorded in triplicate. BHT, rutin and ascorbic acid were used as standard. IC_{50} value is the concentration of sample required to scavenge 50% of DPPH free radicals. The radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = \{[Ab-Aa]/Ab\} \times 100$$

where Ab is the absorption of the blank sample and Aa is the absorption of the extract

Statistical analysis

The statistical significance between antioxidant activity value of the extracts was evaluated by using *ANOVA* followed by a least significant difference (LSD) test at $P = 0.05$ to compare means.

RESULTS AND DISCUSSION

Total phenolic contents were measured by Folin Ciocalteu reagent in terms of gallic acid, (standard curve equation: $y = 0.004x + 0.003$, $r^2 = 0.991$). Similarly, total flavonoid contenst measured by aluminium chloride colorimetric technique in term of quercetin (standard curve equation: $y = 0.005x + 0.007$, $r^2 = 0.999$). Total phenol in methanolic extract of different formulation of *A. marmelos* were found to be F_1 (4.241 ± 0.017 mg /kg), F_2 (1.153 ± 0.002

mg/kg), F_3 (1.054 ± 0.004 mg/kg) and F_4 (6.145 ± 0.055 mg/kg) respectively. The maximum flavonoid were observed in F_4 (8.134 ± 0.044 mg/kg) followed by the F_1 (6.351 ± 0.001 mg/kg) formulation (Table 1).The result of the present study showed that methanolic extract of F_4 formulation contained highest amount of phenolic and flavonoid compounds; exhibited the greatest antioxidant activity. High scavenging property of F_4 formulation may be due to the hydroxyl groups existing in the phenolic compounds. A potent scavenger of free radicals may serve as a possible preventative intervention for the disease[31].The presence of flavonoids and tannins in all plants is likely to be responsible for the free radical scavenging effects. Phenolics and flavonoids are a major group of compounds that act as primary antioxidants or free radical scavengers.

Table 1. Total phenolic and flavonoid contents in different formulation of *A. marmelos*

Formulation code	Total Phenolic contents (mg/kg)	Total Flavonoid content (mg/kg)
F_1	4.241 ± 0.017	6.351 ± 0.001
F_2	1.153 ± 0.002	1.837 ± 0.005
F_3	1.054 ± 0.004	1.921 ± 0.001
F_4	6.145 ± 0.055	8.134 ± 0.044

Means ± SE of 3 replicates from two experiments. Result are no significantly different at P = 0.05 level according to LSD test. F_1 = Formulation1 F_2 = Formulation2, F_3 = Formulation3, F_4 = Formulation4

Table 2. Comparison of DPPH free radical inhibitory concentration of the formulation and those of BHT, ascorbic acid and rutin

Formulation code and standard	Concentration (µg/ml)	% inhibition
F_1	4.5	63.14 ± 0.04
F_2	4.5	52.163 ± 0.20
F_3	4.5	44.37 ± 0.05
F_4	4.5	67.58 ± 0.03
BHT	15	65.09 ± 0.22
Ascorbic acid	15	54.12 ± 0.01
Rutin	15	72.686 ± 0.56

Means ± SE of 3 replicates from two experiments. Result are no significantly different at P = 0.05 level according to LSD test. F_1 = Formulation1 F_2 =Formulation2, F_3 = Formulation3, F_4 = Formulation4

Free radical scavenging activity by DPPH

The formulations were tested for free radical scavenging activity by DPPH radical. In (Table 2) result shows the comparison of DPPH free radical inhibitory concentration of different formulation extracts and those of BHT, ascorbic acid and rutin. The % inhibition of F_1 (63.14 ± 0.04), F_2 (52.163 ± 0.20), F_3 (44.37 ± 0.05) and F_4 (67.583 ± 0.03) in comparison to BHT (65.09

± 0.22), ascorbic acid (54.12 ± 0.01) and rutin (72.686 ± 0.56) were observed respectively. The IC_{50} (µg/ ml) values of formulation for free radical scavenging activity by DPPH radical shown in Figure 2. IC_{50} value of standard compounds; BHT, ascorbic acid and rutin were 18.726 µg/ml, 29.338 µg/ml, 12.231µg/ml respectively. The radical scavenging activity in different extracts decreased in the following order $F_4 > F_3 > F_2 > F_1$. F_4 show the higher antioxidant activity due to lower IC_{50} values with comparison to F_3, F_2 and F_1.The free radical scavenging effect of F_4 at 4.5µg/ml concentration was comparable to BHT, ascorbic acid and rutin at15µg/ml (Table 2).

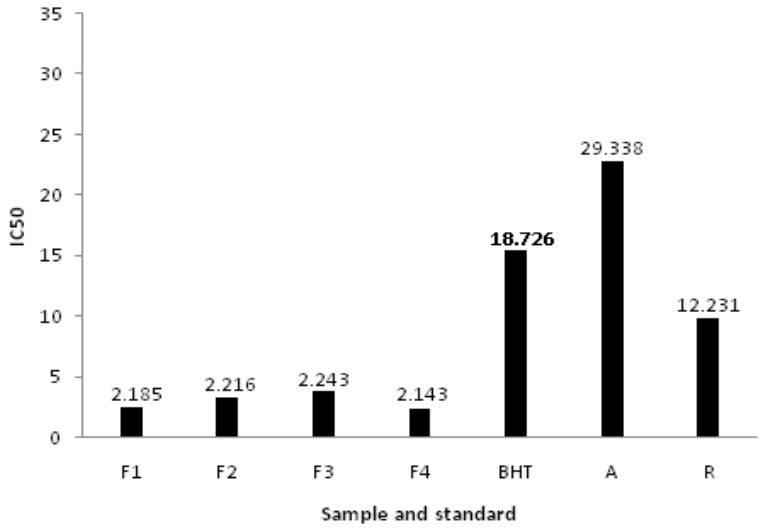

Figure 2. IC_{50} (µg/ml) values of formulations and standard for free radical scavenging activity by DPPH free radical.

Lower IC_{50} value indicates higher antioxidant activity. F_1 = Formulation1 F_2 = Formulation2, F_3 = Formulation3, F_4 = Formulation4, BHT = Butylated hydroxyl toluene, A = Ascorbic acid and R = Rutin

The DPPH test provides information on the reactivity of the test compounds with a stable free radical. DPPH gives a strong absorption band at 517nm in visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extract. This study suggests that these formulations possess antioxidant activities which can counteract the oxidative damage. Natural antioxidants (umbelliferone and quercetin) strengthen the endogenous antioxidant defences from reactive oxygen species (ROS) and restore optimal balance by neutralizing the reactive species. In this context, *A. marmelose* can rightly be mentioned as a plant of considerable interest. Flavonoids

and phenolics present in the plant extract are reported to inhibit release of autacoids and prostaglandins, thereby inhibit motility and secretion[32, 33].

The study on medicinal plants to reveal the mechanism of action and to justify their claims by traditional healers being in continuation over the years. Free radicals quenching constituents posses the ability to reduce the oxidative damage associated with many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS[34,35]. Antioxidants through their scavenging power are useful for the management of these diseases[36].

CONCLUSION

In present investigation traditionally used formulations of *A. marmelos* showed good antioxidant property due to the presence of polyphenolic compounds. The high potential of phenolics to scavenge free radicals may be due to many phenolic hydroxyl groups they posses[37,38]. Flavonoids show significant antioxidant action on human health and fitness, act through scavenging or chelating process[39, 40]. Phenolics and flavonoids, which hold hydroxyls group are responsible for protection against free radicals by inhibiting DPPH radical[41,42].

The present study has verified that remedial plants could be good source of antioxidant substances.

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