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Effect on anti-diabetic and antioxidant activities of flavonoid rich extract from the leaves of *Aegle marmelos*

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Abstract
The aim of this study was to evaluate the anti-diabetic and antioxidant, phytochemical composition of flavonoid rich extract from the leaves of *A. marmelos*. Phytochemicals were analyzed through chemical tests, thin layer chromatography (TLC). Flavonoid rich extract from the leaves of *A. marmelos* were evaluated for antidiabetic potential via the inhibition of amylase and glucosidase. *In vitro* antioxidant activity of the flavonoid rich extract from the leaves of *A. marmelos* was evaluated using ABTS, inhibition of lipid peroxidation and metal chelating. Flavonoid rich extract from the leaves of *A. marmelos* had the highest phenolic and flavonoid contents (71.32±2.34 µg GAE/g, 59.32±0.25 µg QE/g. *In vitro* antioxidant potential at a concentration of 100 µg/mL of flavonoid rich extract from the leaves of *A. marmelos*. The lowest EC_{50} (66.32 and 69.32 µg/mL) observed in the ABTS scavenging and inhibition of lipid peroxidation. Positive correlations were observed between total phenolics, antidiabetic and antioxidant potential of the flavonoid rich extract from the leaves of *A. marmelos*, indicating a significant contribution of flavonoid compounds.

Keywords: *A. marmelos*, antidiabetic, antioxidant

Introduction

Plants synthesize a vast range of organic compounds that are traditionally classified as primary and secondary metabolites although the precise boundaries between the two groups can in some instances be somewhat blurred. Siddha and Ayurveda systems of medicine provide good base for scientific exploration of medicinally important molecules from nature. Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against infections and degenerative diseases. The potential of the antioxidant constituents of plant materials for the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists and food manufacturers as consumers move toward functional foods with specific health effects (Lo liger, 1991). The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes (Shahidi *et al*., 1992). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury.

Flavonoids are an important class of natural products; particularly, they belong to a class of plant secondary metabolites having a polyphenolic structure, widely found in fruits, vegetables and certain beverages. They have miscellaneous favourable biochemical and antioxidant effects associated with various diseases such as cancer, Alzheimer's disease (AD), atherosclerosis, etc. (Lee *et al*., 2009) [7]. Flavonoids are associated with a broad spectrum of health-promoting effects and are an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. This is because of their antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme functions.

Aegle marmelos (L.) Correa (*A. marmelos*), commonly known as Bael belonging to the family Rutaceae, has been widely used in indigenous systems of Indian medicine due to its various medicinal properties. *A. marmelos* is native to Northern India, but widely found throughout the Indian Peninsula and in Ceylon. *A. marmelos* has been reported to contain several phytoconstituents mainly marmenol, marmin, marmelosin, marmelide, psoralen, alloimperatorin, rutaretin, scopoletin, aegelin, marmelin, fagarine, anhydromarmelin, limonene, â-phellandrene, betulinic acid, marmesin, imperatorin, marmelosin, luvangentin and auroptene. Extensive experimental and clinical studies prove that *Aegle marmelos* possesses antidiarrhoeal, antimicrobial, antiviral, radioprotective, anticancer, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility and anti-inflammatory properties, which help it to play role in prevention and treatment of many disease.

Materials and Methods

Plant material

The leaves of *Aegle marmelos* was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamilnadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-345/2021. The flowers were sufficiently air-dried in 5 days at the ambient room temperature, while the flower was cut into smaller pieces and air-dried in 7 days.

Phytochemical screening
The aqueous extract of *Aegle marmelos* leaves were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Aida *et al*., 2001; Hess *et al*., 1995).

***In vitro* evaluation of yeast cell uptake of glucose**
Commercial baker's yeast was wash by repeated centrifugation (3,000×g, 5min) in distilled water until the supernatant fluid were clear and a 10% (v/v) suspension was prepared in distilled various concentration of flavonoid rich extract from the leaves of *A. marmelos* (25-100 µg/ml) were added to 1mL of glucose solution (25 mM) and further incubated for 10min at 37 °C. reaction was started by adding 100µl of yeast suspension, vortex and further at 37 °C at 60min, the tubes were centrifuged (2,500×g, 5min) and glucose was estimated in the supernatant (Cirillo, 1962) [3], metformin was taken as standard anti-diabetic drug used. The percentage of increase in glucose uptake by yeast cells was calculated using the following formula:

Inhibition of α-amylase enzyme
α-amylase (0.5 mg/ml) was mixed with the flavonoid rich extract from the leaves of *A. marmelos* at various concentrations (25-100 µg/ml) to which 1% of starch solution and 100 µl of 0.2 mm of phosphate buffer (pH -6.9) were added. The reaction was allowed to be carried out at 37°C for 5 min and terminated by addition of 2 ml of 3, 5-dinitrosalicylic acid reagent. The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath. α-amylase activity was determined by measuring color intensity at 540 nm in spectrophotometer.

Inhibition of α-glucosidases enzyme
The inhibitory activity was determined by incubating 1 ml of starch solution (2% w/v maltose) with 0.2 M tris buffer (pH 8) and various concentration of flavonoid rich extract from the leaves of *A. marmelos* (25-100 µg/ml). The reaction mixture was incubated at 37°C for 10 min. The reaction was initiated by adding 1 ml of α-glucosidase enzyme (1 U/ml) to it and incubation at 35°C for 40 min. Then the reaction was terminated by the addition of 2 ml of 6 N HCl. The intensity of the color was measured at 540 nm in spectrophotometer. The results were expressed as % inhibition using the formula:

$$\% \text{ inhibitory activity} = (Ac\text{-}As)/Ac \times 100$$

Where, Ac is the absorbance of the control and As is the absorbance of the sample.

ABTS (2, 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay
ABTS radical scavenging activity of flavonoid rich extract from the leaves of *A. marmelos* was followed by Re *et al*. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

$$\text{ABTS Scavenging Effect (\%)} = [(A_0 - A_1/A_0) \times 100]$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of flavonoid rich extract from the leaves of *A. marmelos*.

Inhibition of lipid peroxidation activity
Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Badmus *et al*. (2010). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of flavonoid rich extract from the leaves of *A. marmelos* flower in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.
Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Metal chelating activity
Metal chelating capacity of flavonoid rich extract from the leaves of *A. marmelos* was measured according to Dinis *et al*., (1994) [4]. 1 ml of different concentrations of flavonoid rich fraction was added to 0.05 ml of 2 mm ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mm Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The % inhibition of ferrozoine-Fe2+complex was calculated by following equation.

$$\% \text{ Inhibition of ferrozoine-Fe2+complex} = [(A_0\text{-}A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich extract from the leaves of *A. marmelos*.

Statistical analysis
Values were recorded as mean ± standard error of the mean. Statistical difference between the means was determined by one-way ANOVA followed by Duncan multiple range test.

Results and Discussion

Phytochemical screening

The phytochemical screening of the aqueous extract from the leaves of *A. marmelos* studied presently showed the presence of alkaloids, flavonoids, phenol, Terpenoids, glycosides, terpenoid and absence of glycosides, saponin (Table -1).

Table 1: Phytochemical screenings of aqueous extract from the leaves of *A. marmelos*

Sl. no.	Phytochemical constituents	Observation	Aqueous extract from the leaves of *A. marmelos*
1	Alkaloids		
	▪ -Dragendorff's test	Orange /red precipitate	+
	▪ -Mayers test	Cream pie ppt	+
2.	Flavonoids		
	▪ -Alkalai Reagent	Intense yellow colour	+
	▪ -Lead aceate test	Precipitate formed	+
3.	Glycosides		
	▪ -Keller-Killiani test	Pink colour (Ammonia layers)	-
4.	Tannin		
	▪ -$FeCl_3$ test	Blue-black colour	+
5.	Saponins		
	▪ -Frothing test	Foam	+
6.	Terpenoids		
	▪ -Salkowski test	Reddish brown colour ring formed in interface	-
7.	Polyphenols		
	▪ -Ferrozine test	Raddish blue	+
8.	Anthocyanin		
	▪ -Ammonia test	Pink color in ammonia layer	+

+ Positive result; - Negative result

The partial characterization of flavonoid rich extract from the leaves of *a. marmelos* by tlc

The flavonoid rich extract from the leaves of *A. marmelos* loaded on Pre-coated TLC plates (60F 254 Merck) and developed with a solvent system of hexane, ethyl acetate and acetic acid in the ratio of 10:5:0.5. The developed plate was viewed under UV 240nm and 360nm. The Rf value of compounds were shown in Table-1.

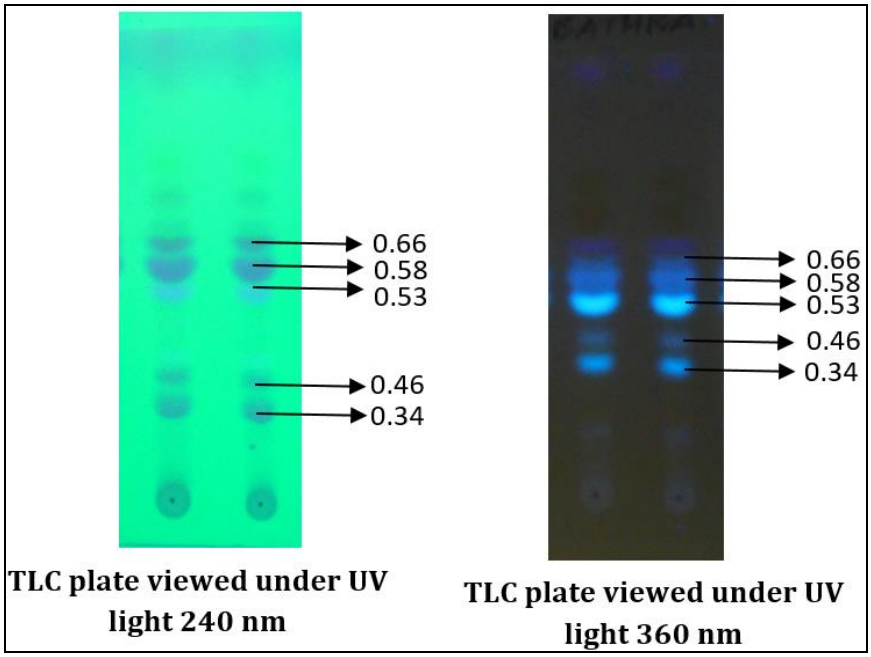

Fig 1: The partial characterization of flavonoid rich extract from the leaves of *A. marmelos* TLC

Glucose uptake in yeast cells

The flavonoid rich extract from the leaves of *A. marmelos* were subjected to *in vitro* glucose uptake examine engaging yeast as model. The percentage of glucose uptake in yeast cells by the flavonoid rich extract was compared with standard drug diclofenac sodium (Fig-2). Flavonoid rich extract from the leaves of *A. marmelos* exhibited highest percentage of glucose uptake 83.32%, which was almost near to the standard 81.23% at 100 µg/ml Concentration. Plants are the major source for discovering new compounds with medicinal value for drug development (Sunila *et al*., 2012) [13]. Accordingly, these results encourage further studies on extracts and identify particular active chemical compounds responsible for the specific biological activity in order to standardize the plant preparation for maximum therapeutic benefit to treat diabetes.

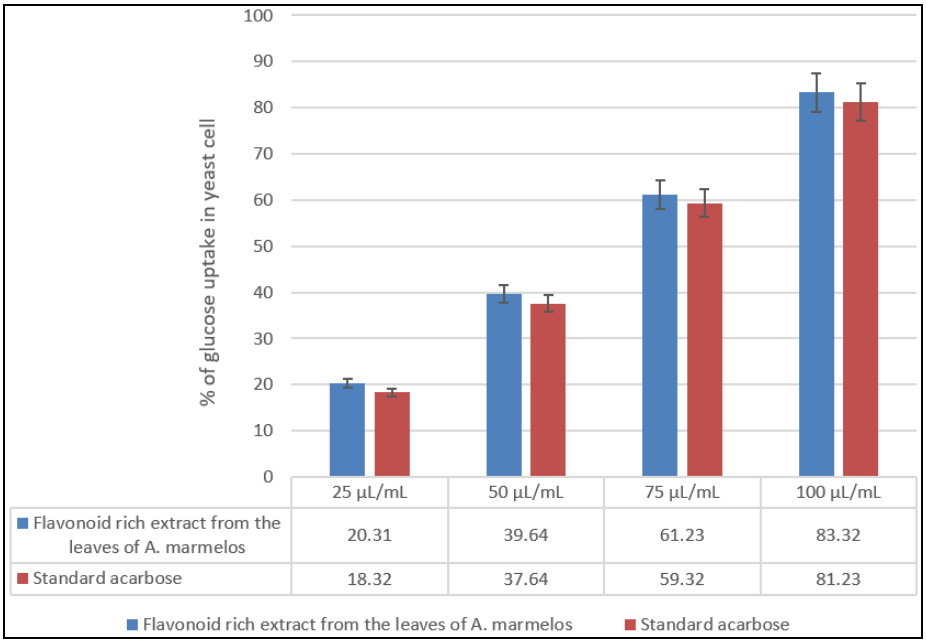

Fig 2: Glucose uptake in yeast cells by flavonoid rich extract from the leaves of *A. marmelos*

Inhibition of α-amylase activity

Flavonoid rich extract from the leaves of *A. marmelos* produced 74.56% inhibition of α-amylase activity at 100 µg/mL concentrations and its EC_{50} was found to be 63.21 µg/mL. The standard drug acarbose exhibited 70.12% inhibition of α-amylase activity at 100 µg/mL and its EC_{50} for acarbose was found to be 68.23 µg/mL (Fig-3). Currently, available drugs in this category are acarbose and miglitol, which competitively inhibit α-amylase enzymes. But these drugs have common side effects such as flatulence and abdominal bloating (Liu *et al*., 2004) [10]. New drugs or formulations which are devoid of the above side effects will improve the compliance in type 2 diabetic patients. The present study results clearly demonstrated that flavonoid rich extract from the leaves of *A. marmelos* possesses potent pancreatic *α*-amylase inhibition which confirmed that *in vitro* antidiabetic action.

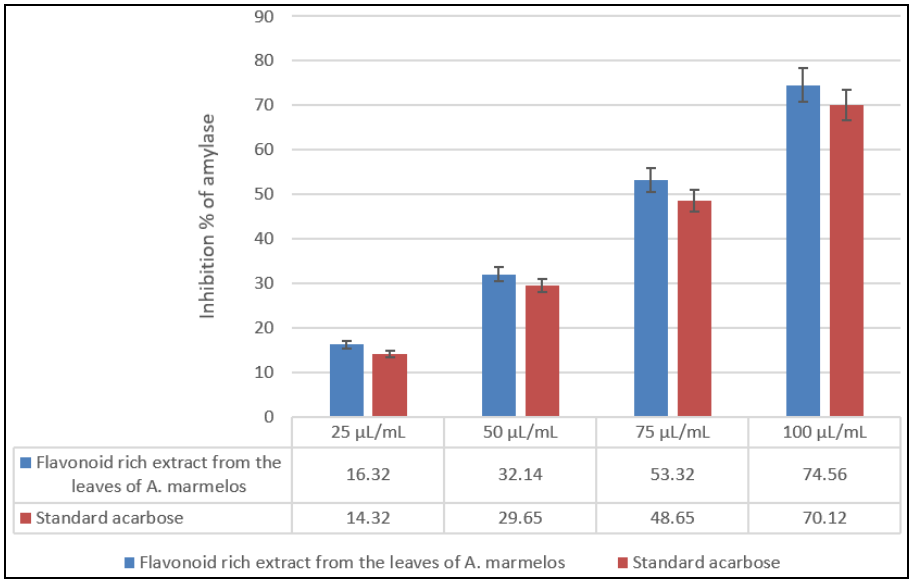

Fig 3: Inhibition of α-amylase activity by flavonoid rich extract from the leaves of *A. marmelos*

Inhibition of *α*-glucosidase activity

Flavonoid rich extract from the leaves of *A. marmelos* exhibited 79.32% inhibition of *α*-glucosidase activity at 100 *µ*g/mL concentration and its EC_{50} was found to be 58.32 *µ*g/mL. The standard drug acarbose produced 72.31% inhibitory effect on *α*-glucosidase activity at 100 *µ*g/mL concentrations, and its EC_{50} was found to be 62.34 *µ*g/mL. Insulin resistance in peripheral tissues such as liver, skeletal muscle, and adipose tissue is commonly observed. The occurrence of cardiovascular diseases in type 2 diabetic patients mainly due to insulin resistance mediated hyperglycemia and dyslipidemia. Drug which diminishes insulin resistance will effectively control hyperglycemia, normalize lipid metabolism in type 2 diabetes, and hence it will prevent the diabetes-mediated cardiovascular complications (Reaven *et al*., 2004).

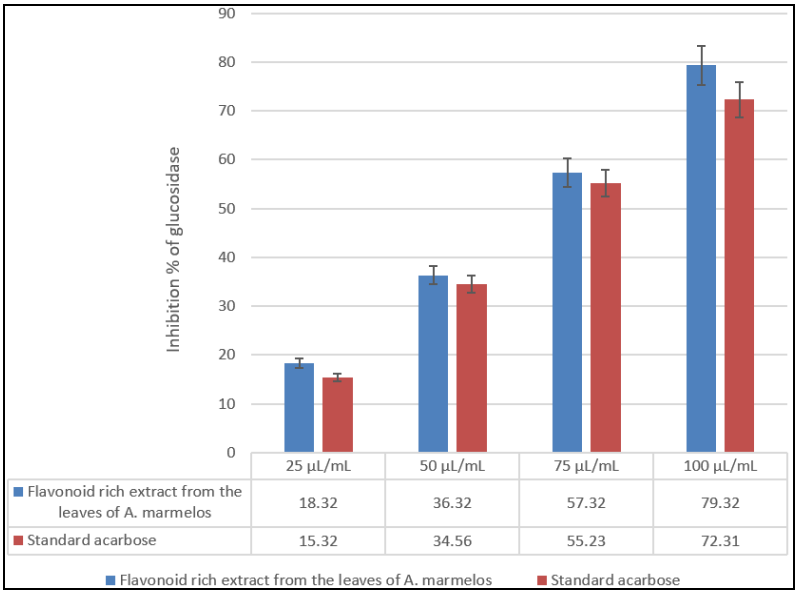

Fig 5: Inhibition of *α*-glucosidase activity by flavonoid rich extract from the leaves of *A. marmelos*

Free radical-scavenging ability using abts assay

The radical scavenging ability was measured by ABTS assay as per given in table 4. The inhibition percentage of the ABTS radical activity was assessed on average and high free radical-scavenging values were found in flavonoid rich extract from the leaves of *A. marmelos.* In ABTS assay, inhibition percentage was high in flavonoid rich extract from the leaves of *A. marmelos* 77.32% with EC_{50} value 66.32 µl/ml. The pure ascorbic acid was lower activity (Table-2). Nevertheless, in present study, it is showed that these activities were mainly due to flavonoids compounds. It is known that vitamin C (ascorbic acid) and carotenoids are chief source of discrepancy of antioxidant/ antiradical activities in plant materials. Phenolic compounds are responsible for the antioxidant activity of vegetables and medicinal plants. Although in recent years the antioxidant analysis of medicinal plants has been extensively researched worldwide, very few studies have been carried out to assess medicinal plants and vegetables grown in India and consumed locally or exported to several countries (Leopoldini *et al*., 2006) [8].

Table 2: Free radical-scavenging ability using ABTS assay of flavonoid rich extract from the leaves of *A. marmelos*

Different concentration of extract	ABTS assay	
	Flavonoid rich extract from the leaves of *A. marmelos*	Standard Vitamin-C
25 µl/ml	21.23±0.58	17.32±2.46
50 µl/ml	40.12±2.78	36.32±1.69
75 µl/ml	57.32±1.78	55.32±0.23
100 µl/ml	77.32±0.25	72.31±1.46
EC_{50} value	66.32	73.21

[a] Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

Inhibition of lipid peroxidation

Flavonoid rich extract from the leaves of *A. marmelos* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in flavonoid rich extract from the leaves of *A. marmelos* 74.32% with EC_{50} value 69.32 µl/ml and lowest inhibition percentage ascorbic acid 67.32% with EC_{50} 78.32 (Table-3). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of flavonoid compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals. The major polyphenolic constituents of Rutaceae family plants, flavonols such as quercetin and kaempferol, and anthocyanidins, show a greater efficacy as antioxidants on a mole for mole basis than the antioxidant nutrients vitamin C, vitamin E and carotenoids (Liu *et al*., 2008) [9].

Table 3: Inhibition of lipid peroxidation activity of flavonoid rich extract from the leaves of *A. marmelos*

Different concentration of extract	Lipid peroxidation inhibition percentage	
	Flavonoid rich extract from the leaves of *A. marmelos*	Standard Vitamin-C
25 µl/ml	17.32±2.45	15.32±0.89
50 µl/ml	33.32±1.36	30.34±1.49
75 µl/ml	52.34±2.47	45.32±0.56
100 µl/ml	74.32±0.28	67.32±2.46
EC_{50} value	69.32	78.32

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Metal chelating activity

Iron is essential for life because it is required for oxygen transport, respiration and activity of many enzymes. However, iron is an extremely reactive metal and will catalyze oxidative changes in lipid, protein, and other cellular components. In addition, liposome peroxidation and oxidative damage of protein model systems are induced by a Fenton reaction in which ferrous ions catalyze the conversion of hydrogen peroxide to hydroxyl radical with the production of ferric ion. Although metal chelating agents are not antioxidants, they play a vital role in the stabilization

of fatty acids against rancidity. The flavonoid rich extract from the leaves of *A. marmelos* displayed the maximum metal chelating activity was 81.23% than Vitamin-C 75.32%. It was already reported that chelating agents which form ó bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, there by stabilizing the oxidized form of the metal ion (Kumar *et al*., 2013) [6].

Table 4: Metal chelating activity of the flavonoid rich extract from the leaves of *A. marmelos*

Different concentration of extract	Percentage of nitric oxide radical scavenging activity	
	Flavonoid rich extract from the leaves of *A. marmelos*	Standard Vitamin-C
25 µl/ml	23.64±1.47	20.31±2.34
50 µl/ml	46.32±2.36	41.32±0.89
75 µl/ml	65.31±1.45	62.34±1.59
100 µl/ml	81.23±0.78	75.32±2.14
EC_{50} value	54.32	65.21

[a] Results are expressed as percentage of metal chelating activity with respect to control. Each value represents the mean+SD of three experiments.

Conclusion

These results of phytochemical screening and TLC profile can be used as pharmacognostical tool for the identification of novel drugs from leaves of *A. marmelos.* Based on the results obtained from different *in vitro* anti-diabetic and antioxidant acticities, there is significant difference in anti-diabetic activity of flavonoid rich fraction evaluated. Flavonoid rich fraction of *A. marmelos* leaves has shown significant anti-diabetic activity and antioxidant compared to standard. The result also demonstrated that flavonoid rich fraction of *A. marmelos* plant can be exploited to discover the bioactive natural products which may serve in the development of new pharmaceuticals.

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