PHYTOCHEMICAL STUDIES AND EVALUATION OF ANTIOXIDANT POTENTIAL OF VARIOUS EXTRACTS OF *Aegle marmelos* BARK.

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Summary

Natural antioxidants fight against free radicals and keep the oxidative stress state in balance, thus preventing degenerative diseases and aging symptoms. The aim of the current study is to evaluate antioxidant activity of ethanol, ethyl acetate, and aqueous extracts from bark of *Aegle marmelos. Aegle marmelos* is commonly known as beal in India. It is a spiny tree belongs to the family of Rutaceae. The leaves, bark, roots, seeds, and fruits of *Aegle marmelos* are edible. The medicinal properties of this plant have been described in Ayurveda. The bark was collected, shade dried and coarsely powdered. The powder was successively extracted with ethanol, ethyl acetate and water using cold percolation method. The extracts were concentrated by distilling the solvent and air dried. The phytochemical screening of the crude bark extracts proved the presence of alkaloids, flavonoids, tannins, glycosides, phenols, sterols and terpenoids. The antioxidant activity of ethanol, ethyl acetate, and aqueous extracts were determined using three complement assays: DPPH, Phosphomolybdate, Thiocyanate method. The result obtained in the present study indicates that the bark of *Aegle marmelos* is a potential source of a natural antioxidant.

Keywords: *Aegle marmelos*, Antioxidant activity, DPPH, Alkaloids and Flavonoids

Introduction

Plant and plant products are being used as a source of medicine since long time. Among the most important constituent of edible plant produce, low molecular weight antioxidants are the most important species. It is known that consumption of fruits and vegetables is essential for normal health of human being. Vegetarian diet can reduce the risk of cancer, atherosclerosis, *etc*. *Aegle marmelos* has been used in Ayurveda, the ancient Indian system of medicine. It has been used for treatment of several disorders such as common cold, scurvy, cancer and heart diseases. It is believed that the major constituents responsible for these activities is vitamin C. Ascorbic acid shows antioxidant, anti-inflammatory and antimutagenic properties. It is a very effective free-radical scavenger. The ascorbic acid alone and that overall effect is due to other polyphenols such as ellagic acid, gallic acid and tannins. It is in fact reported that autoxidation of ascorbic acid, such properties remain interesting and useful task particularly for finding new source of natural antioxidant, functional food and neutraceuticals including polyphenolic such as flavonoid, tannin, proanthocyanidin[1].

Polyphenols are a group of highly hydroxylated phenolic compounds present in the extractive fraction of several plant materials. Polyphenols in plant include hydroxycoumarins, hydroxycinnamate derivatives, flavanoids, flavonones, flavones, anthocyanins, tannins, hydroxystilbenes, aurones *etc.* Polyphenols are well documented to have microbicide activities against a huge number of pathogenic bacteria. Flavonoids and Phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic *etc.*[2]

Aegle marmelos is a sacred tree, dedicated to lord Shiva**.** The offering of beal leaves is a compulsory ritual of the worship of lord Shiva in the hills. This importance seems largely due to its medicinal properties. All parts of this, *viz.,* root, trunk, fruit, and seeds are used for curing one human aliment or another [3]

Aegle marmelos is considered to be very auspicious and sacred to Hindus as its leaves and wood are used as essential items of “Pooja samagri For worship of “ Lord Shiva” for this reason it is also called “ Shivadurme ”. Since ancient time, its leaves are offered to “Lord shiva” and “ Parvathi ”. It is considered as an “Emblem of fertility’. Because of spiritual use, it is often found in the garden of temples [4]. The bark of *Aegle marmelos* are useful in intermittent fever, mental disorders, pericarditis, angina pectoris, hypoglycaemic activity stop pain the abdomen, palpitation of the heart, and allay urinary troubles and also the roots of this plant are one of the ingredients of dashamula (10 roots), a medicine commonly used by Ayurvedic practitioners [5].

Materials and Method

Collection of plant materials and extracts preparation

The fresh plant material belongs to the *Aegle marmelos* were collected from Thanjavur District, Tamil Nadu, India. The bark of the *Aegle marmelos* was shade dried for four days. The dried samples were powdered with electrical blender and made into coarse powder and stored in airtight container at room temperarure. Approximately 500gm of the sample were taken and extracted using cold percolation method with various organic solvent such as ethanol, water and ethyl acetate. The extractions were carried out for approximately 48hrs. Extracts were vacuum evaporated, dried and stored in an air tight container for further analysis [6].

Separation and identification of phytochemicals of selected medicinal plants

The presence of bioactive secondary metabolites from the leaves of *Aegle marmelos are* qualitatively analysed by thin layer chromatography.

TLC plate preparation

Solid phase of silica gel are kept in hot air oven in 100^{o}C for 20 minutes. Silica powder was mixed with petroleum ether and makes slurry. 20x 20 cm TLC glass plates covered with that slurry and allowed to air dried. After drying the plates were kept in hot air oven in 72^{o}C for 15min. After developing the plates the condensed filtrate was spotted using capillary tube. The different spots were separated using a different solvent mixture act as mobile phase. It was given below

(a) TLC study of alkaloids

The extracted leaves of *Aegle marmelos a*re wetted with a half diluted NH_4OH and lixiviated with EtOH Ac for 24hrs at RT. The organic phase is separated from the acidified filtrate and basified with NH_4OH (pH 11-12). It is extracted with chloroform, condensed by evaporation and used for chromatography. The alkaloid spots were separated using the solvent mixture chloroform and methanol (15:1). The colour and hR_f value of the separated alkaloids were recorded both under Ultra Violet (UV 254nm) and visible light after spraying with Dragendorff's reagent.

(b)TLC study of flavanoides

One gram powdered leaves are extracted with 10ml methanol on water bath (60^oC/5min). The filtrate was condensed by evaporation, added a mixture of water and EtOHAc (10:1mL), and mixed thoroughly. The EtOHAc phase thus retained is used for chromatography. The flavonoid spots were separated using chloroform and methanol (19:1) solvent mixture. The colour and hRf value of these spots were recorded under ultraviolet (UV_{254nm}) light.

(c) TLC study of glycosides

The powdered leaves are extracted with 70% EtOH on rotary shaker (180 thaws/min) for 10hr. 70% lead acetate is added to the filtrate and centrifuged at 5000rpm/10min. the Supernatant was further centrifuged by adding 6.3% Na_2Co_3 at 10000 rpm/10min. the retained supernatant was dried, redissolved in chloroform and used for chromatography. The glycosides were separated using EtOAc-MeOH-H_2O (80:10:10) solvent mixture. The colour and hRf values of these spots were recorded by observing under ultraviolet (UV_{254nm}).

(d) TLC study of phenols

The powdered leaves are lixiviated in methanol on rotary shaker (180 thaws/min) for 24h. The condensed filtrate was used for chromatography. The phenols were separated using chloroform and methanol (27:0.3) solvent mixture. The colour and hRf values of these phenols were recorded under visible light after spraying the plates with Folin-Ciocalteu's reagents heating at 80^oC/10min.

(e) TLC study of saponins

Two grams of powdered leaves are extracted with 10ml 70% EtOH by refluxing for 10min. The filtrate was condensed, enriched with saturated n-BuOH, and thoroughly mixed. The butanol was retained, condensed and used for chromatography. The saponins were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and hR_f values of these spots were recorded by exposing chromatogram to the iodine vapours.

(f) TLC study of sterols

Two grams of powdered leaves are extracted with 10ml methanol in water bath (80^oC/15min). The condensed filtrate is used for chromatography. The sterols were separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The colour and hRf values of these spots were

recorded under visible light after spraying the plates with anisaldehyde -sulphuric acid reagent and heating (100^{o}C/6min).

INVITRO ANTIOXIDANT ASSAYS

DPPH RADICAL SCAVENGING ASSAY

The free radical scavenging activity of the extracted samples from *Aegle marmelos* were quantatively assessed using DPPH radical method adopted by spectrophotometry. Extracts that exhibited strong antioxidant capacity using the DPPH Method proposed by [7](Chaturvedi *et al.,*2004). Breifly, 0.1mM solution of DPPH in methanol was prepared and 0.1ml of this solution was added to 0.5ml of samples in various concentration. After 30 minutes, the absorbance was measured at 510nm. The DPPH radical - scavenging activity was calculated.

PHOSPHOMOLYBDATE METHOD

The total antioxidant capacity of the trace fractions was determined by phosphomolybdate method using α-tocopherol as the standard [8]. An aliquot of 0.1ml of the fractions (100µg) solution was combined with 0.1ml of reagent (0.6 M Sulphuric acid mM sodium phosphate and 4mM Ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. after the samples had cooled to room temperature , the absorbance was measured at 695nm against the blank using spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as µg equivalents of α-tocopherol by using the standard tocopherol graph.

THIOCYANATE METHOD

The peroxy radical scavenging activity was determined by thiocyanate method using α-tocopherol (50-800 µg / ml) in 0.5ml of distilled water was mixed with 2.5ml of 0.02 M Linolic acid emulsion (in 0.04 M Phospho buffer pH 7.0) and 2ml phosphate buffer (0.04 M pH 7) in a test tube and incubated in darkness at 37°C.At intervals during incubation the amount of peroxide formed was determined by reading the absorbance of the red colour developed at 500nm by the addition of 0.1ml of 30% ammonium thiocyanate solution and 0.1 ml of 20mM ferrous chloride in 3.5% hydrochloride acid to the reaction mixture. The percentage scavenging activity was calculated and the IC 50 values of the fractions were compared with the standard α-tocopherol [9].

Results and Discussion

The phytoconstituents of various extracts of bark of *Aegle marmelos*, when subjected to qualitative analysis for alkaloids, glycosides, flavonoids, phytosteroids, tannins, phenolic compounds, cardiac glycosides, saponins, gums, mucilages, coumarins, carbohydrates, proteins, free amino acids and sulphur. All the tested phytochemicals are found to be present in the various extracts of the bark of *Aegle marmelos* except gums and mucilages in ethanolic and ethyl acetate extracts (Table.1).

Table-2 Shows the antioxidant potential of various extracts *Aegle marmelos* at different concentration. The result shows the maximum DPPH scavenging activity of ethyl acetate extract of *Aegle marmelos* appeared to be as potent as standard

vitamin C with a maximum inhibition of 80.16% at 250µg/ml which is comparable to 77.92% for Vitamin C at the same concentration. Followed by aqueous (70.30%) with a maximum inhibition then Vitamin C. Ethanol extract shows least potent effect of (62.03%) when compared to that of standard.

The free radical scavenging activity of the Ethyl acetate extract was also evaluated through its ability to quench the synthetic radical 1,1-diphenyl-2-picryl-hydrazyl(DPPH).The phenolic compounds or secondary metabolites, constitute a wide and complex assay of phytochemical that exhibit antioxidant action and consequently a beneficial physiological effect.[10].The key role of phenolic compounds as scavenger of free radicals is emphasized in several reports. Phenols are important components of plants. They were reported to eliminate radical due to their hydroxyl groups, and they contribute directly to antioxidant effect of system. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity [11].

The antioxidant activity of phenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donars, and singlet oxygen quencher. In addition, they have a metal chelating potential. Flavanoids and polyphenolic compounds are responsible for health care because of their antioxidant and anticancer properties. Bio-active compounds found in plants that are known to possess antioxidant activity [12].

DPPH is a reactively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidant towards the stable free radical DPPH. From the present results, it may be postulated the *Aegle marmelos* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principle [13].

The Antioxidant activity of the fractions of *Aegle marmelos* was determined by the thiocyanate method and compared with the standard α-tocopherol. The absorbance decreased with the increasing concentration of the fractions, which indicate that the fractions could effectively decrease the amount of formed peroxides. The total antioxidant activity of the extracts such as ethyl acetate, aqueous, ethanol (98.66>85.28>81.71) and the of the standard α-tocopherol 75.79%. The fractions shows good antioxidant activity at higher concentration (250 µg/ml). The amount of formed peroxides was measured by the thiocyanate method. The fractions were incubated with linoleic emulsion in dark at 37°C and the amount of peroxides was determined spectrophotometrically by measuring the absorbance at 500nm. A decrease in absorbance indicated the antioxidant activity of the fractions which might be due to the inactivation of the free radicals and the presence of flavonoid like phytochemicals.

Fig 1: DPPH Radical Scavenging activity of various extracts of *Aegle marmelos*

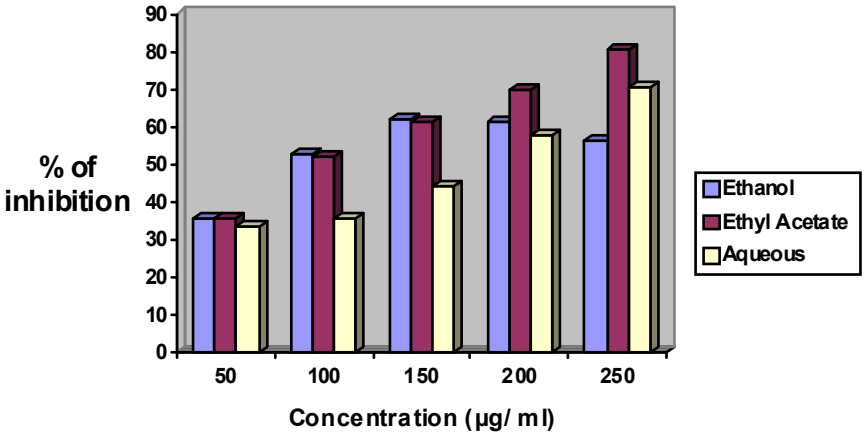

Fig 2 : Antioxidant activity of various extracts of *Aegle marmelos* towards Thiocyanate

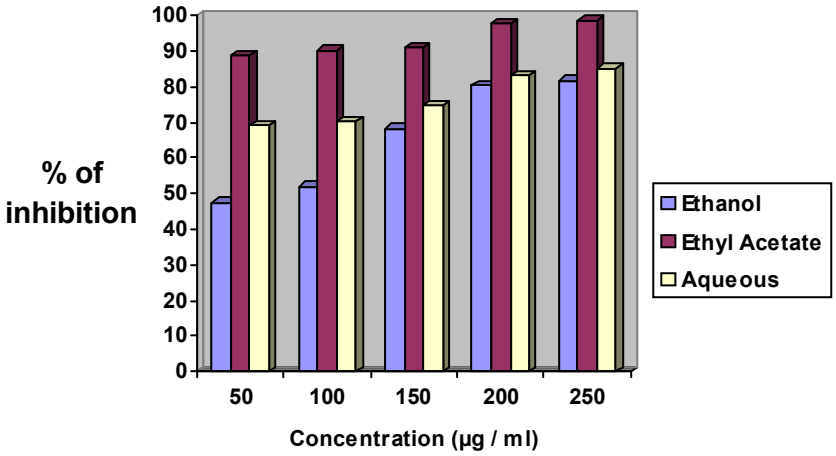

Fig 3 : Antioxidant activity of Aqueous Extract of *Aegle marmelos* towards Phosphomolybdate

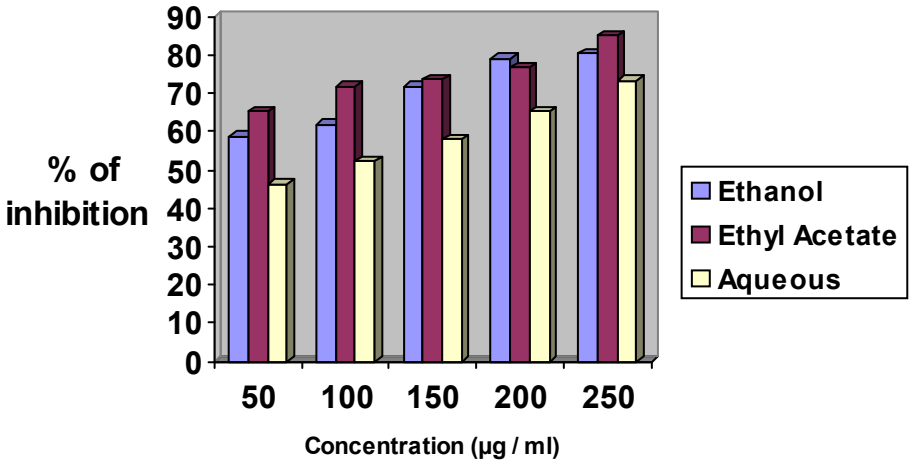

Table 1 Showed the Phytochemical Constituents of *Aegle marmelos*

S.NO	Phytochemical Constituents	Inference
1.	Alkaloids	+
2.	Flavonoids	+
3.	Tannins	+
4.	Phenols	+
5.	Glycosides	+
6.	Steroids	+
7.	Terpenoids	+
8.	Carbohydrates	+
9.	Proteins	+
10.	Amino acids	+

TABLE 2: Antioxidant activity of various extracts of *Aegle marmelos* towards DPPH, PHOSPHOMOLYBDATE, THIOCYANATE ASSAYS

S.No.	*Aegle marmelos* Extracts	Concentration (µg/ml)	% of inhibition		
			DPPH	PM	TC
1	Ethanol	20	35.15	47.51	58.75
		40	52.53	51.91	61.84
		60	62.03	68.21	71.77
		80	61.37	80.31	79.21
		100	56.39	81.71	80.39
2	Ethyl Acetate	20	35.4	88.91	65. 32
		40	52.06	90.31	71.84
		60	60.90	91.08	73.61
		80	69.83	97.96	76.84
		100	80.16	98.66	85.19
3	Aqueous	20	33.36	69.17	46.31
		40	35.62	70.38	52.36
		60	43.89	74.77	57.96
		80	57.70	83.18	65.39
		100	70.30	85.28	73.48

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