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Phytochemical Analysis, Estimation of Total Phenolic, Flavonoid and In-vitro Anti-oxidant Profile of Hydroalcoholic Extract of *Mangifera Indica* Leaves

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A B S T R A C T

Mango, which is popularly referred to as king of fruits, botanically named as *Mangifera indica* which as multiple benefits for the whole plant according to traditional knowledge as well as literature search. The present study is aimed to prepare hydroalcoholic extract of the shade dried leaves and it should be subjected for preliminary phytochemical screening to know about the secondary metabolites present in the extract and further subjected for determination of Total Phenolic and Total Flavonoid and In-vitro anti-oxidant activity using DPPH scavenging assay.

Keywords: Mangifera indica, hydro alcohol, phenol, flavonoid, DPPH assay

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

The medicinal plants are considered as a rich source of ingredients which can be used in drug development and synthesis. Besides that, these plants play a critical role in the development of human culture around the world. Moreover, some plants are considered as an important source of nutrition and as a result these plants are recommended for their therapeutic values. It has been estimated that about 13,000 species of plants have been employed for at least a century as traditional medicines by various cultures around the world. A list of over 20,000

medicinal plants has been published, and very likely a much larger number of plants[1].

Plant Profile

Taxonomical Classification

Kingdom: plantae, order: spindles, Family: Anacardiaceous, genus: *Mangifera*, species: indica.

Synonyms

Greek: Manko, Mango,**English**: Mango,**Hindi**: am (or**)**ambi, **Telugu**: Mamudi, **Tamil**: Mampalam.

Fig.1 Whole plant of Mango tree

Mangifera indica is a large evergreen tree, with a dome - shaped crown. It belongs to the family anacardiaceous. It is found all over the tropical regions of the world. It is used as a medicinal plant [2]. *Mangifera indica* leaves are alternative, simple, lethargy, oblong-lanceolate, curved upwards from the midrib and sometimes with edges a little wavy [3]. Anomocytic type of stomata is present, which are bordered by a varying number of cells and not different from the epidermis,they are small in size and presented only on the lower surface of the leaf[4].

Chemical constituents

Mangifera indica mainly consists Mangifera, phenolic compounds, benzophenones, flavonoids, carotenoids, quercetin, isoquercetin, ascorbic acid, and tocopherols. Terpenoids, carbohydrates, sterols, carotenoids, vitamins, fatty acids, amino acids[5]. Mangiferin is the main contributor of most of the biological activities of *Mangiferaindic*a's extract. The mango leaves contain high number of phenolic compounds such as benzoic acid, p-hydroxy benzoic acid, pyrogallol, vanillic acid, syringic acid, ferulic acid, Gallo catechin, protocatechuic acid, manindicin A&B[6].

Uses

Mangifera being a polyphenolic antioxidant and a glucosyl xanthone, has a strong antioxidant, anti-lipid peroxidation, immunoglobulin, cardiotonic, hypotensive, wound healing, anti-degenerative and anti-diabetic activity, denitrifies, antiseptic, astringent, diaphoretic, stomachic, vermifuge,tonic, laxative and diuretic and to treat diarrhoea, dysentery, anaemia, asthma, bronchitis, cough, hypertension, insomnia, rheumatism, toothache, leucorrhoea, haemorrhage and piles[7].

Phenolic compounds have the capability to quench lipid peroxidation, prevent DNA oxidative damage, scavenge free radicals and prevent inhibition of cell communications of all which are precursors to degenerative diseases. Free radicals cause depletion of the immune system anti-oxidant, change in gene expression and induce abnormal protein resulting in degenerative diseases and ageing. Use of pure isolated compounds has been found to be less effective than the use of crude mixtures from the particular mango leaves suggesting that polyphenols are important for maximum antioxidant activity[8].

Pharmacological properties reported on *Mangifera indica*

Anti-oxidant activity

The antioxidant activity of the fractions and isolated compounds from the leaves was studied using DPPH radical scavenging assay 70% hydroalcoholic extract. The fraction of ethyl acetate and n-butanol of mango leaves exhibited pronounced anti-oxidant activities, as they were subjected to isolation and purification of flavonoids in mango leaves. Most flavonoids have antioxidant activity based on analysis of structure activity relationship of flavonoids, the presence of catechol substitution in structure possessing a strong antioxidant activity and induce damage to biological molecule, including proteins, lipids and DNA with the activity are beta -carotene, vitamin-c and vitamin -E. The antioxidant is used for both chemo prevention and treatment purpose[9].

Hepatoprotective activity

The *Mangifera indica* can be used to ease gripping (or) colicky pains and relieves dyspepsia (or) indigestion and expel wind [10].the aqueous extract of leaves of an m. indicavariety successfully inhibited CCL4-induced hepatocellular toxicity in albino rats. Results were further confirmed by analysing lipid profiles, high density lipoproteins and malondialdehyde levels [11].in rats,m. indica leaves extract [300-600 mg /kg] for 2 weeks, significantly reducing the elevated levels of biochemical markers. The hepatoprotective activity of the extract could be attributed to its antioxidant activity. The methanol, ethanol and aqueous extract of Mangifera indica having more potential activity against Alternaria alternate at 6.25 mg/ml concentration [12].

Anti-ulcer and anti-obesity activity

The gastro protective effect of *Mangifera indica* leaf decoction was evaluated in different gastric ulcer inducing ethanol and non-steroidal anti-inflammatory drugs in mice and rats. Which results in a significant decrease of gastric lesions[13]. The Mangiferin affords gastroprotection against gastric injury induced by ethanol and indomethacin

most possibly through the anti-secretory and antioxidant mechanism of action. The reported activity of the MI leaf extracts against gastric clostridium tetani which causes many deaths around the world. Ether and ethanolic extract of Mangifera indica leaves extract showed anti- clostridium tetani activity with an MIC of 6.25 and 12.5 mg/ml respectively (14). Methanolic extract of Mangifera indica leaves can reduce the cholesterol lowering effects in albino rats. A significant decrease in plasma cholesterol levels has been observed in rat administered with cholesterol in study results of these studies conducted with aqueous extract and ethanolic extract of mango leaves have shown promising hyperlipidaemic rat models. Aqueous extract of Mangifera indica leaves significantly decreased the total serum cholesterol, triglycerides, low density lipoprotein and very low-density lipoproteins and increased high density lipoproteins in rats[15].

2. Material & Methods

2.1 Chemicals

Ethanol, Methanol, 10% NaOH, Dil. HCL, Benedict's reagent, Fehling's reagent, Iodine, Biuret reagent, Chloroform, Alpha-glucosidase, PNPG [para nitrophenyl-alpha-glucopyranoside], Phosphate buffer, Bovine albumin serum, Folin-Ciocalteu reagent, Sodium carbonate, Gallic acid, Potassium acetate, Aluminium chloride, Rutin, 2,2-Diphenyl-2-hydrazyl (DPPH), Ascorbic acid, Sodium acetate, Glacial acetic acid, 2,3,5-triphenyltetrazolium chloride (TPTZ), Hydrochloric acid and Ferric chloride.

2.2 Collection & Authentication

The leaves of the Mangifera indica collected from nearby places of Tirupati, Andhra Pradesh andthe plant specimen was authenticated by Dr.K. Madhava Chetty, Assistant professor. Botanical survey of medicinal plant unit, Sri Venkateswara University, Tirupati, AP.

Fig: 2 Mango leaves

Fig: 3 Leaves were cutpieces and kept for shade drying

2.3 preparation of 80% hydroalcoholic extract

The plant materials were collected, washed and dried under shade. After complete drying the plant materials were powdered in a mixer to obtain a coarse powder and then passed through a60mesh sieve and stored in an airtight container. The shade dried powdered is used for extraction. A weighed quantity of air-dried powdered drug of Mangifera indica are taken and kept for maceration respectively by 80% hydroalcoholic extraction. The extracts were evaporated to dryness in a rotary flash evaporator at a temperature not exceeding 60^{o}C. Phytochemical tests were carried out by following standard procedures.

2.4 Phytochemical analysis:

Chemical tests were carried out on the plant extract using standard procedures to identify the constituent molecules as described by sofowora and co-workers. Hydroalcoholic extract of *Mangifera indica* leaves **(HAEMIL)**are prepared. Then phytochemical analysis was carried out on plant extract.

Detection of Carbohydrates

1. Molisch'stest: To 2 ml of the ethanolic extract, add 1ml of α-naphthol solution and it is concentrated with sulphuric acid through the sides of the test tube. Appearance of purple or reddish violet colour junction between the two liquids confirms the presence of carbohydrates.
2. Fehling's test: to 1ml of the ethanolic extract, an equal quantity of Fehling's solution A and B were added. Upon heating, a brick red precipitate confirms the presence of carbohydrates.
3. Benedict's test: to 5 ml of the benedict's reagent, add 1 ml of the ethanolic extract solution and boil it for 2 min and then cool it. formation of red precipitate shows the presence of carbohydrates.

Detection of Alkaloids

1. Dragendroff's test: to 1 ml of the ethanolic extract, 1ml Dragendroff's reagent is added. An orange red precipitate indicates that alkaloids are present in the extract.
2. Wagner's test: to 1 ml of the ethanolic extract, 2ml of Wagner's reagent was added. Appearance of a reddish-brown precipitate specifies the presence of alkaloids.
3. Hager's test: to 1 ml of the ethanolic extract, add 3ml of Hager's reagent. Appearance of yellow precipitate indicates the presence of alkaloids.
4. Mayer's test: to 1ml of the ethanolic extract, add 2 ml of Mayer's reagent. A dull white or creamy precipitate specifies the presence of alkaloids.

Detection of Proteins and Free Amino Acids

1. Biuret test: to 1 ml of the extract, add 1 ml of 40% sodium hydroxide solution and 2 drops of 1 %

copper sulphate solution. Formation of violet colour indicates the presence of proteins.

2. Xanthoproteic test: to 1 ml of the extract add 1ml of concentrated nitric acid. A white precipitate is formed, it is boiled and cooled. Then 20% of sodium hydroxide or ammonia is added. Orange colour indicates the presence of proteins.
3. Lead acetate test: to 1ml of the extract, 1ml of lead acetate solution is added. Formation of dull white precipitate indicates the presence of proteins.
4. Ninhydrin test: add 2 drops of freshly prepared 0.2%of ninhydrin reagent to the extract solution and heat. Development of blue colour indicates the presence of proteins.

Detection of Tannins and Phenolics:

To 1 ml of the extract, add ferric chloride solution. Formation of a dark blue or greenish black colour product shows the presence of tannins. To the extract, add potassium dichromate solution. Formation of a precipitate shows the presence of tannins and phenolics.

Detection of Flavonoids

Shinoda's test: to 1 ml of the extract, add magnesium turnings and 1-2 drops of concentrated hydrochloric acid. Formation of red colour shows the presence of flavonoids.

Detection of Triterpenoids

Dissolve two or three granules in metal in 2ml thionyl chloride solution. Then add 1 ml of the extract into the test tube. The formation of a pink colour indicates the presence of triterpenoids.

Detection of Steroids

1. Liebermann Burchard test: dissolve the extract in 2 ml of chloroform in a dry test tube. Add 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid. The solution becomes red, then blue and finally bluish green indicates the presence of steroids.
2. Salkowski test: dissolve the extract in chloroform and equal volumes of concentrated sulphuric acid. Formation of bluish red to cherry red colour in chloroform layer and green fluorescence in the acid layer represent the steroid components in the tested extract.
3. Liebermann's reaction: mix 3 ml of extract with 3 ml acetic anhydride, heat and cool, and a few drops of concentrated sulphuric acid, blue colour appears.

Detection of Saponins

About 1 ml of extract is diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. A 1 cm layer of foam indicates the presence of saponins.

Detection of Fixed Oils

1. **Spot test:** press a small quantity of extract between two filter papers. Oil strains on paper indicate the presence of fixed oils.
2. Saponification test: to 1 ml of the extract, add few drops of 0.5 N alcoholic potassium hydroxide along with a drop of phenolphthalein. Heat the mixture in a water bath for 1-2 hours. The formation of soap or partial neutralisation indicates the presence of fixed oils.

Detection of Glycosides

1. Legal test: dissolve the extract in pyridine and sodium nitroprusside solution to make it alkaline. The formation of pink red to red colour shows the presence of glycosides.
2. Bal jet's test: to 1 ml of the test extract, add 1ml sodium picrate solution and transformation of yellow to orange colour reveals the presence of glycosides.
3. Bontrager's reagent: to 1 ml of extract solution, add a few ml of dilute sulphuric acid. Boil, filter and extract the filtrate with chloroform. The chloroform layer is treated with 1 ml of ammonia. The formation of red colour shows the presence of anthraquinone glycosides.
4. **Keller Kilian test:** Dissolve the extract in acetic acid containing traces of ferric chloride and transfer to a test tube containing sulphuric acid. At the junction, formation of a reddish-brown colour, which gradually becomes blue, confirms the presence of glycosides.

2.5 Total flavonoid content: Total flavonoid content of the sample was determined by using methods described by faud, A. Flwi, A., and zulkifly, with slight modifications. A stock rutin standard solution of 10 mg/ml was prepared. 10µg/ml, 20µg/ml, 30µg/ml, 40 µg/ml with different concentrations of sample extracts. 1ml of sample from the stock solution was mixed with 3 ml of methanol, 0.2 ml of 1 M potassium acetate and 0.2mL of 10% aluminium chloride 5.6mL of distilled water was added. The solution was incubated for 30 minutes at room temperature. Two hundred microlitres of sample extracts were transferred into a 96- well plate from the centrifuge tube and the absorbance was measured at 415 nm using a spectrophotometer. The result was expressed as mg of rutin equivalent per gram of sample.

2.6 Total phenol content: Total phenolic content of *Mangifera*sp. Leaf extracts were analyzed using the folin-ciocalteu colorimetric method described by *Gao et.al. (2000)*. About 0.02mL of 2 mg/mL extract solution was mixed with 0.2mL folin -ciocalteu reagent and 2mL of distilled water. After 3 minutes, 1mL of sodium carbonate was added. The mixture was re- incubated for 20 minutes at room temperature. Then, the absorbance was measured at 765nm using a spectrophotometer. The total phenolic content was calculated from the gallic acid standard curve. A stock gallic acid standard Solution of 1 mg/mL was prepared by dissolving gallic acid in distilled water. The result was expressed as mg gallic acid equivalent per gram of extract[26].

In-vitro antioxidant and free radical scavenging activity

DPPH free radical scavenging activity was evaluated using the method described by *Blois.* Various concentration of *HAEMIL* and ascorbic acid (standard) ranging (2-10µg/ml) were mixed with 1 ml of freshly prepared 0.3mM DPPH ethanol solution and 2 ml of 0.1M acetate buffer. Incubated at room temperature for 30mins and the absorbance of resulting solution were then measured calorimetrically at 517 nm. DPPH solution (1.0 ml, 0.3Mm)treated with 1 ml of ethanol, served as negative control. Ascorbic acid was used as positive control under the same assay conditions. Samples were prepared in triplicates for each analysis and the mean of absorbance was obtained. Higher absorbance indicates lower free radical scavenging activity. The percentage of DPPH scavenging activity of extract was calculated from decrease in absorbance in comparison of with control by using formula[27].

3. Results and Discussion

Preliminary phytochemical investigation studies on mango leaves contain a rich source of chemical constituents and those are identified by performing by identification test. The present studies of mango leaves indicate the presence of phenols, flavonoids, alkaloids, glycosides, terpenoids but the absence of saponins, the present study reveals the presence of phenols and flavonoids are responsible for the anti-oxidant radical scavenging activity. This traditional plant is rich in phenols and flavonoids as the absorbance increases the concentration of phenolic compounds also increases and this result were equivalent with standard gallic acid mg/g. The concentration of total flavonoids were expressed as mg/g of rutin equivalents. Maximum absorption depends on the concentration of flavonoid content in the extract.

Table 1: Preliminary Screening

S.No	CHEMICAL TEST	OBSERVATION	INFERENCE
TEST FOR CARBOHYDRATES			
1.	Molisch’s test	+	present
2.	Fehling’s test	-	absent
3.	Iodine test	+	present
4.	Benedict’s test	+	present
TEST FOR PROTEINS AND AMINO ACIDS			
1.	Xanthoproteic test	+	present
2.	Biuret test	+	present
TEST FOR ALKALOIDS			
1.	Dragendorff's test	+	present
2.	Mayer’s test	-	absent
3.	Iodine test	-	absent
TEST FOR TANNINS			
1.	10% NaOH	-	absent
TEST FOR PHENOLS			
1.	Ferric chloride’s test	+	present
2.	Potassium dichromate’s test	+	present
3.	Iodine test	-	absent

TEST FOR FLAVONOIDS			
1.	Alkaline reagent test	+	present
TEST FOR SAPONINS			
1.	Foam test	-	absent

Total flavonoid content

The total flavonoid content in samples of mangiferin sp. Leaves. The studies conducted by rutin as standard. The concentration of total flavonoid content is expressed in mg/gm of rutin equivalent. Maximum absorption depends on the concentration of flavonoids and the results were shown in Table :2

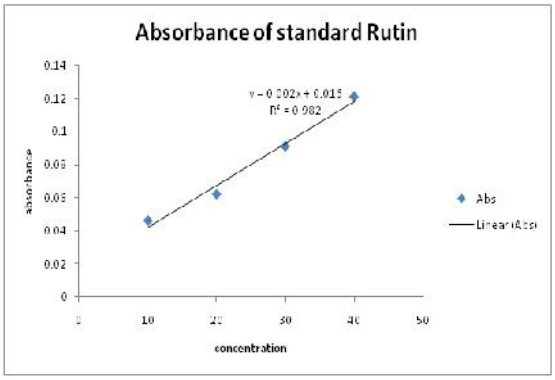

Fig 4: calibration curve of Rutin showing linearity over concentration range from 10-40µg/ml.

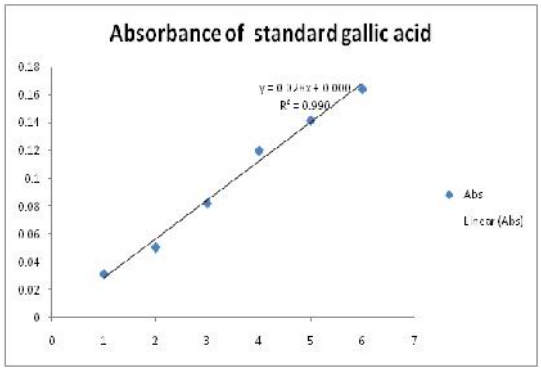

Fig: 5 Calibration curve of gallic acid showing linearity over concentration range of 10-50 µg/ml.

3.3 Total Phenolic content: The total phenolic content of hydroalcoholic extract of *Mangifera indica* leaves were expressed in term of gallic acid equivalent**.** The total phenolic content in the extract of *M. indica* leaves were 54.65%and the results were shown in Table: 2

Table1: Percentage of total phenolic and flavonoid content by hydroalcoholic extract of Mangifera indica leaves

Plant extract	Total phenolic content (%)	Total Flavonoid content (%)
Hydroalcoholic extract of Mangifera indica	1.38 ± 0.080	4.8 ± 0.55

In-vitro antioxidant and free radical scavenging activity of *HAEMI:* Radial scavenging activity of extract was observed from decrease in absorbance of DPPH with increase in concentration. Absorbance value of *HAEMIL* had shown as 76.17, 77.28, 78.12, 80.74and 82.09 and standard ascorbic activity exhibited as 21.90, 43.57, 64.2, 71.7 and 79.24 at 2, 4, 6, 8,10 µg/ml respectively. *HAEMIL* at a concentration of 2-10 µg/ml inhibited production of DPPH radical by 50-80% and showed significance scavenging effect on DPPH radical compared to standard ascorbic acid which exhibited 40-85% inhibition and were shown in table 3 & 4.

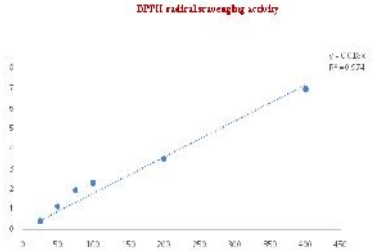

Figure 6

Table: 3 Standard ascorbic acid values

Ascorbic acid(standard) Concentration	Mean ± S. D
2µg/ml	20.93 ± 1.29
4µg/ml	43.02 ± 1.01
6µg/ml	65.8 ± 2.39
8µg/ml	71.7 ± 3.45
10µg/ml	79.30 ± 1.85

*values are from three replicates.

Table: 4 Effect of *Mangifera indica* on DPPH

Concentration of extract	Mean ± S. D
20µg/ml	76.20 ± 1.85
40µg/ml	77.32 ± 1.98
60µg/ml	78.15 ± 1.34
80µg/ml	79.85 ± 0.38
100µg/ml	79.15 ± 0.47

*values are from three replicates.

4. Conclusion

The present study reveals that hydroalcoholic extract of Mangifera indica shows potent inhibition activity at different concentrations when compared with standard ascorbic acid and it has showed significant effect at 60µg/ml of extract with 10µg/ml. It is revealed that at a low concentration with higher amounts of phenols and flavonoids and inhibition effect is considered to be potent for further investigation.

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