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Antioxidant Activity and Phytochemical Composition of Aqueous Extract of *Markhamia lutea* (Benth) K. Schum. Leaves

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ABSTRACT

In the present study *Markhamia lutea* (Benth) K. Schum., a Bignoniaceae member was evaluated for its physicochemical properties, phytochemical constituents and antioxidant activity with a view to develop a herbal drug for treating / preventing oxidative stress mediated diseases. Physicochemical properties (total ash, acid soluble ash, water soluble ash and extractive values) and mineral composition were analyzed in the leaf material of *M. lutea*. Aqueous extract was prepared and its phytochemical profiles were screened and presence of flavonoids was identified through LC-MS/MS analysis. Further, antioxidant activity was evaluated using *in vitro* assays like ferric reducing power, phosphomolybdate reducing power, radical scavenging against DPPH, superoxide and hydrogen peroxide. Results of physicochemical analysis and phytochemical screening revealed total ash (8.88%), acid soluble ash (0.05%) and water soluble ash (1.36%) contents and extractive values (1.88-3.16%), calcium (80.4%), potassium (10.7%) and iron (1.5%). Phytochemical screening revealed that the aqueous extract contains alkaloids, flavonoids, phenolic compounds, glycosides and carbohydrates. LC-ESI-MS/MS analysis revealed the presence of flavonoids such as rutin, pectolinarin, chrysoeriol, curcumin, kaempferol-3-glucoside-3-rhamnoside, isorhamnetin-3-galactoside-6-rhamnoside and 3-hydroxy-3,4-dimethoxy flavones in the aqueous extract. Aqueous extract showed dose-dependent ferric reducing power (1.03 Abs at 700 nm) and phosphomolybdate reducing power (270 Ascorbic acid equivalent antioxidant activity) at a concentration of 1 mg/ml. It also showed maximum level of scavenging of DPPH (71.27%) followed by hydrogen peroxide (55.09%) and superoxide radicals (43.66%). *M. lutea* leaf could be explored as a potential source of antioxidant compounds to prevent various oxidative stress-induced diseases.

Introduction

Various systems of medicine namely Ayurveda, Siddha and Uunani were evolved using plants as the major components in their drug formulations. Plants synthesize a wide range of phytochemicals, which have an important therapeutic and preventive role against diseases and thus forming a base for drug formulations. Even-though many plants have been investigated for their medicinal efficacy, there is always a need for natural and novel source of drug to prevent / cure oxidative stress which causes several chronic diseases such as cancer, diabetes, cardiovascular diseases etc. Various species belonging to the family of Bignoniaceae have been claimed to have high medicinal importance and many potential drugs were derived from Bignoniaceae members to manage anti-cancer, anti-pyretic and analgesic conditions. Unexplored members of Bignoniaceae are good sources for investigation and for developing new herbal drugs to manage various diseases and disorders. Some of the members of Bignoniaceae family, *Markhamia acuminata*, *M. caudafelina*, *M. obtusifolia* have been evaluated for antioxidant activity [1].

Markhamia lutea (Benth). K. Schum (Bignoniaceae), commonly known as Nile trumpet, has its origin in South Western Uganda and also found in India. This plant is characterised by its broad leaves and bright yellow flowers which born in axillary inflorescence. Traditionally the root powder is consumed as tea which cures diarrheal and leaves are used as anti-parasitic [2]. Antiviral activity mainly against respiratory syncytial virus, anti-leishmanial and anti-trypanosomal activities have been reported [3, 4]. Phenyl propanoid glycosides (luteoside A - C, verbascoside and isoverbascoside), have been identified in the roots, while cycloartane triterpenoids, musambins A–C and glycoside derivatives, musambiosides A–C, phaeophorbide A, Beta-sitosterol, pentacyclic triterpenes and arjunic acid were identified in the leaves of *M. Lutea* [1, 3 – 6].

In our previous work, we have identified terpenoid musambin from *M. lutea* bark and evaluated its antioxidant and anticancer activities [7]. However, there is no report on antioxidant property of leaf samples of this plant and hence this present study investigates the physicochemical, phytochemical and antioxidant properties of the aqueous extract of *M. lutea* leaves.

Materials and Methods

Plant material

Leaves of *M. lutea* were collected from Benguluru and is authenticated by Dr. N. Ravichandran, Botanist, School of Chemical and Biotechnology, SASTRA University and a herbarium specimen is deposited at CARISM. The leaves were shade-dried, chopped and powdered and then used for the evaluation of physicochemical properties and mineral composition.

Physicochemical characterization

The total ash content was determined by taking 0.5 g of the powdered leaf sample in a clean dry crucible and incinerated at 450˚C for 3 h. The crucible was then cooled and weighed. After obtaining two consecutive

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constant weight values, the percentage of ash was calculated using the formula (weight of ash/weight of sample x 100). The acid insoluble and water soluble ash contents were analyzed from the residue of total ash [8]. Percentage of various metals and minerals present in the selected plant material was determined by X-Ray Fluorescence Spectrometer (S8 Tiger, Bruker).

Preparation of aqueous extract

Powdered sample (2 g) was macerated in water (40 ml) for 48 h and filtered. The filtrate was directly used for the quantification of total phenols and flavonoids and the level of phytochemicals in the plant material was calculated. Then the extract was dried on water bath at 60°C and then re-dissolved in water at 1 mg/mL ratio and used for antioxidant experiments.

Phytochemical analysis

The phytochemical screening was done using standard protocols [9] to identify the presence of various phytoconstituents in the aqueous extract like alkaloids, flavonoids, phenols, glycosides, quinines, saponins, sterols, carbohydrates and proteins.

Total phenolic content

Extract (50 µL) was taken with 250 µl of Folin-Ciocalteu reagent and 4.7 mL of sodium carbonate (2.2%) and mixed well [10]. The reaction mixture was incubated at 37°C for 30 min and the absorbance was measured at 725 nm. The total phenol content was calculated using the standard curve prepared with gallic acid (R^2 = 0.994, y = 0.0012 x + 0.1093) and expressed as gallic acid equivalent (GAE).

For LC-MS analysis crude aqueous extract was weighed and re-dissolved in water to get final concentration of 1 mg/mL and then filtered using 0.45 µm syringe filter and analyzed using liquid chromatography coupled to mass spectrometer (LC/ESI/MS/MS, MicroTOF-Q II, Bruker, Germany). Solution (50 µL) was injected for liquid chromatography separations in a C18 reverse phase column (120 Å, 2.1 x 150 mm, Acclaim 120). UV detector was set arbitrarily at 260 nm. A discontinuous gradient elution at a flow rate of 0.2 mL/min was performed using mobile phase A (Acetonitrile) and mobile phase B (MilliQ Water) acidified with acetic acid (1%). The gradient started from 95% of B for 10 min, followed by achieving 90% B in 1 min, to 60% B in the next 9 min, next 10 min B reaches 80%, next 10 min to reach 40% B, 5 min to reach 0% B and was maintained for another 10 min until the run ended. Mass spectrometer with ESI ionization at negative mode equipped with HyStar 3.2 software was optimized to detect the exact mass and mass fragmentation pattern of each eluted compound. Total ion current (TIC) spectra were acquired and elaborated using the HyStar software Data Analysis module. MS/MS experiments were carried out by means of Auto scanning mode, where the mass spectrometer software made a choice in real time about the selection of ion to fragment based on the intensity of each peaks with a threshold set above 1500 absolute counts. Optimized parameters consisted in collision energy 10 eV, focusing potential of 350 voltage per peak, transfer time of 800 µs, pre-pulse storage of 5 µs the instrument was operated in the negative ion mode with a capillary voltage of 3.5 KV, capillary temperature was 280ºC, sheath gas (N2) flow rate was 6 mL/min and the data were acquired in the AutoMSn scanning modes with the scan range of 50 – 1500 *m/z*.

Antioxidant assays

The antioxidant activity of aqueous extract was evaluated in terms of ferric reducing power [11], phosphomolybdate reducing power [12], DPPH radical scavenging activity [13], hydrogen peroxide inhibition [14] and superoxide radical scavenging activity [15].

Results and Discussion

The physicochemical characterisation parameters studied include the determination of total ash, acid soluble and water insoluble ash and extractive values of *M. lutea* leaf and the data obtained are presented in Table 1. The total ash content of *M. lutea* leaf material was found to be 8.88%, which comprised of mostly water soluble ash (1.36%) as compared to acid insoluble ash (0.05%). Ash is the inorganic residue remaining after the water removal and the organic matter being removed by heating in the presence of oxidizing agents, thus provides a measure of the total amount of minerals present and these tests are mainly performed to assess the quality of the herbal drug. When considering the extractive values, both aqueous (3.16%) and ethyl acetate (3.11%) gave higher extractive values, followed by chloroform (2.29%) and petroleum ether (1.88%) (Table 1). This result indicates the presence of highly polar compounds in larger proportion in *M. lutea* leaf sample. The results of metal and mineral composition of *M. lutea* leaf are presented in the Table 2.

Ash test provides the total number of minerals excluding the organic matter and water present within the material whereas metal and mineral determination gives the amount of specific minerals present within the material. Acid insoluble ash measures the amount of silica present, especially as sand and siliceous earth. Extractive value determines the amount of active constituents extracted with solvents from a given amount of herbal material.

The metal and mineral composition of *M. lutea* leaf showed the presence of high level of calcium (80.4%), which is followed by potassium (10.79%) and iron (1.5%) (Table 2). Calcium is an integral part of bones, skeletal muscles and enamel of teeth and also essential for blood coagulation and activation of digestive enzymes [16]. Potassium plays a vital role as electrolyte, it helps in maintaining body fluids homeostasis and reduces blood pressure and is important for signal transduction [17]. Iron plays a vital role in oxygen transport as a constituent of RBC [18]. Deficiency of iron leads to anaemia, which is a major public health problem among women in many developing countries like India. Hence, consumption of *M. lutea* leaf material could provide a nutritionally rich essential supplement enriched with minerals such as calcium, potassium and iron.

Phytochemical constituents of the plant are shown in Table 4. It revealed the presence of alkaloids, flavonoids, phenolic compounds, glycosides and carbohydrates in the aqueous extract of *M. lutea* leaf. Further, occurrence of various flavonoids such as kaempferol-3-glucoside-3-rhamnoside, isorhamnetin-3-galactoside-6-rhamnoside, rutin, pectolinarin, chrysoeriol, curcumin and 3-hydroxy-3,4-dimethoxy flavone were identified in the aqueous extract by LC-ESI-MS/MS analysis (Table 5, Figure 2 and Supplementary File).

Alkaloids from plants play important role as antibiotics, analgesic and nervous stimulant while phenols are known for their anti-oxidant and anti-inflammatory activities along with cancer prevention potential [19]. Glycosides are integral part of cardiac drugs, laxatives and analgesics and sterols play vital role in cancer prevention [20]. Flavnoids have many medicinal properties such as anti-oxidant, anticancer and anti-ageing [21]. The pharmacological activities of phenolics include anti-diabetic, anti-fungal, and anti-proliferative properties. Total phenols in the aqueous extract of *M. lutea* leaf was found to be 407.83 mg GAE/100 g sample (Table 1), which could attribute to the health benefits exhibited by this plant as previously stated.

LC-ESI-MS/MS results were compared with the mass spectra data bank and the phytoconstituents were identified as kaempferol-3-glucoside-3-rhamnoside, isorhamnetin-3-galactoside-6-rhamnoside, rutin, pectolinarin, chrysoeriol, curcumin and 3-hydroxy-3,4-dimethoxy flavone in the aqueous extract (Table 5 and Figure 2).

Kaempferol rhamnosides from *Hibiscus cannabinus* were reported to exhibit anti-inflammatory activity [22]. Similarly, Tatsimo *et al*. [23] reported the antimicrobial and antioxidant activities of Kaempferol rhamnosides in *Bryophyllum pinnatum*. Isorhamnetin is an O-methylated flavonol, which is also reported in apple extract [24] and *Ginkgo biloba* [25] and exhibited neuronal differentiation in cell line model. Rutin is reported to demonstrate broad range of biological activities such as antimicrobial, anti-inflammatory, antioxidant, neuroprotective, antiviral, anti-ulcer and anti-cancer activities [26]. Pectolinarin is a flavnone heteroside reported to be present in Bignoneaceae family members with anti-oxidant, anti-inflammatory and hepatoprotective activities [27]. Quercetin (3,5,7,3´,4´-pentahydroxy flavone) is one of the most abundant bioflavonoids mainly present in edible fruits and vegetables. Because of its potent antioxidant and metal ion chelating capacity, quercetin has been reported to be effective against inflammation, atherosclerosis, bleeding, allergy and swellings. In addition, epidemiological data suggests that quercetin is associated with reduced risk of certain types of cancers [28]. Chrysoeriol is a flavonoid and its antioxidant and antimicrobial properties were studied in *Capsicum frutescence* [29]. Curcumin is a hydrophobic polyphenol mainly present in the rhizome of turmeric and possess antioxidant, anti-inflammatory, anti-microbial, anti-carcinogenic, hepatoprotective, nephroprotective and cardioprotective activities [30]. 3-Hydroxy-3,4-dimethoxy flavones is a natural anticancer drug and its cytotoxic and wound healing properties have been reported [31]. Thus, presence of various flavonoids in the aqueous extract might attribute to the various medicinal properties including antioxidant activity of *M. lutea*.

The aqueous extract of *M. lutea* leaf revealed remarkable antioxidant activity in terms of ferric ion reducing power, phosphomolybdate reducing

Table 1: Physicochemical properties of *Markhamia lutea* leaf powder.

Physicochemical properties	Value (%)
Test for identity, purity and strength	
a) Total ash	8.88 ± 0.12
b) Acid insoluble ash	0.05 ± 0.01
c) Water soluble ash	1.36 ± 0.01
Extractive values	
a) Petroleum ether	1.88 ± 0.01
b) Chloroform	2.29 ± 0.29
c) Ethyl acetate	3.11 ± 0.59
d) Aqueous	3.16 ± 0.05
Total phenols (mg GAE/ 100 g sample)	407.83 ± 0.24

The values are mean ± standard deviation of triplicate determinations

Table 2: Metal and mineral composition of *Markhamia lutea* leaf powder.

Metals and minerals	Content (%)
Calcium (Ca)	80.48 ± 0.36
Potassium (K)	10.79 ± 0.06
Silicone (Si)	1.74 ± 0.03
Chloride (Cl)	1.58 ± 0.02
Iron (Fe)	1.50 ± 0.02
Aluminium (Al)	0.97 ± 0.01
Sulphur (S)	0.71 ± 0.04
Magnesium (Mg)	0.60 ± 0.01
Phosphorous (P)	0.58 ± 0.02
Sodium (Na)	0.17 ± 0.01
Zinc (Zn)	0.13 ± 0.01
Strontium (Sr)	0.12 ± 0.00
Molybdenum (Mo)	0.11 ± 0.01
Titanium (Ti)	0.09 ± 0.01
Tin (Sn)	0.09 ± 0.01
Copper (Cu)	0.07 ± 0.01

The values are mean ± standard deviation of triplicate determinations

Table 3: Reducing power of aqueous extract of *Markhamia lutea* leaf powder.

Concentration of the extract (µg/ml)	Ferric reducing power	Phosphomolybdenum reducing power (Ascorbic acid equivalent activity)
1000	1.03	290
500	0.564	108
250	0.223	66
125	0.1295	50
62.5	0.112	11.75
31.25	0.1045	3.25

Table 4: Phytochemical screening of *Markhamia lutea* leaf extracts.

S. No.	Phytoconstituents	Aqueous extract
1	Alkaloids	+
2	Flavonoids	+
3	Phenolic compounds	+
4	Glycosides	+
5	Quinones	-
6	Saponins	-
7	Sterols	-
8	Carbohydrates	+
9	Proteins	-

The symbol + / - indicates presence / absence of the phytoconstituent.

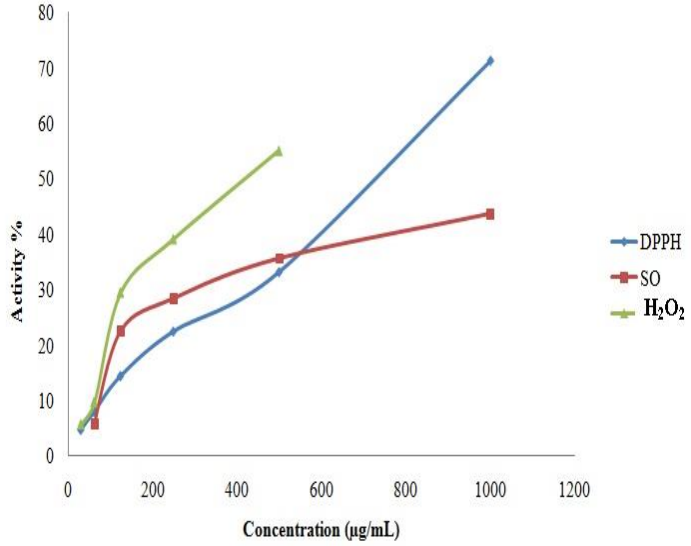


Figure 1: Free radical scavenging activity of aqueous extract of *Markhamia lutea* leaf.

power and radical scavenging activity against DPPH (71.27%), hydrogen peroxide (55.08%) and superoxide (40%) (Table 3 and Figure 1). The aqueous leaf extract showed dose-dependent reducing power against ferric ion and phosphomolybdate (Table 3). This reducing power of aqueous extract could be attributed to the presence of phenolic compounds, especially flavonoids as revealed by the LC-MS profile. Due to spontaneous and fast release of protons by the phenolic molecules present in aqueous extract, the ferric ion is reduced to ferrous ion in iron reducing assay and reduction of Molybdenum (VI) to Molybdenum (V) in Phosphomolybdate assay.

The DPPH radical scavenging activity of *M. lutea* was comparable to other Bignoniaceae family members such as *M. acuminata* (83%) and *Tecoma stans* (77%) and even higher than that of *Kigelia pinnata* (45%) and *Jacaranda mimosifolia* (51%) [1, 32 – 34]. These results indicate that the aqueous extract is more effective against synthetic free radical compared to the biological free radical (superoxide) and its intermediate (hydrogen peroxide). However, by scavenging both superoxide and hydrogen peroxide, which are sources for the generation of highly reactive and biologically significant hydroxyl radicals, the aqueous extract could prevent the oxidative damage caused by highly reactive and dangerous free radicals and ultimately protects from oxidative-stress mediated diseases like cancer, atherosclerosis, cardio vascular diseases etc.

Conclusion

The present study also revealed the presence of nutritionally important minerals such as calcium, potassium and iron in the leaf of *M. lutea*. The LC-MS analysis revealed the presence of certain flavonoids such as rutin, pectolinarin, quercetin, chrysoeriol, curcumin, kaempferol-3-glucoside-3''rhamnoside, isorhamnetin-3-galactoside-6-rhamnoside, 3-hydroxy3,4-dimethoxy flavones, which might be responsible for the antioxidant activity observed in this study. For the first time, the antioxidant activity of *M. lutea* leaf was evaluated and the results indicated that it could be a potential antioxidant to prevent free radicals-induced oxidative stress. Further, in depth *in vivo* studies could be useful in developing a new bioactive molecule from this plant.

Conflict of interest

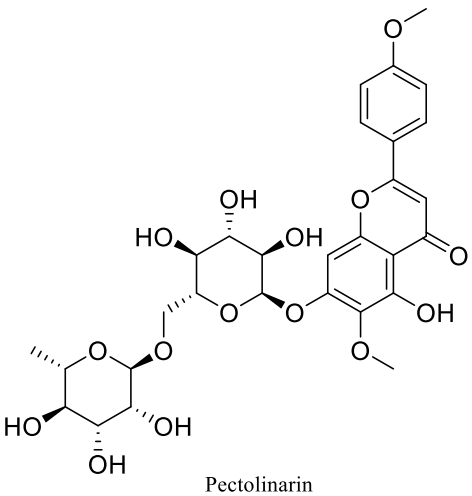
The authors declare no conflict of interest.

Authors' Declaration

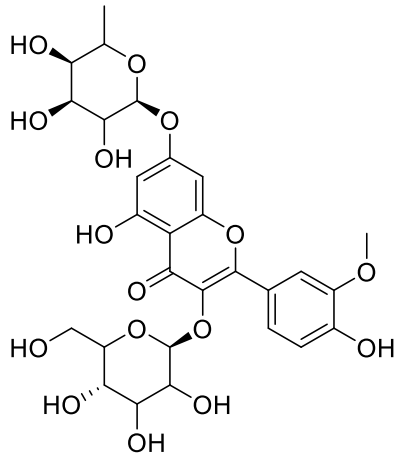
The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Table 5: LC-MS of aqueous extract of *Markhamia lutea* leaf sample.

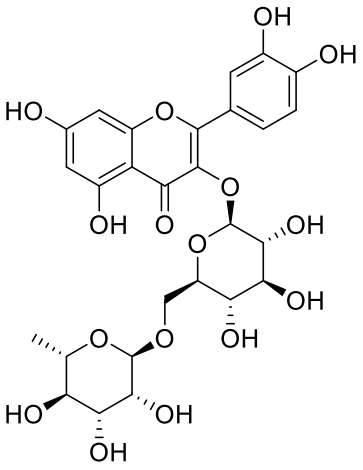
Retention time (min)	Identified Compound	[M-H]	MS/MS (Fragment Ion)
2.6-2.7	Kaempferol-3-Glucoside-3''-Rhamnoside	593.1	227, 255, 284, 285
2.9-3.2	Isorhamnetin-3-galactoside-6''-rhamnoside	623.2	243, 255, 271, 285, 299, 314, 315
3.3-3.5	Rutin	609.1	151, 191, 227, 255, 271, 300
3.3-3.6	Pectolinarin	621.1	299, 313
4.8-4.9	Chrysoeriol	299	133, 151, 183, 227, 255, 283
20.4-20.5	Curcumin	367.1	134, 149, 158
42.7-43.1	3-Hydroxy-3',4'-Dimethoxyflavone	297.1	119, 183



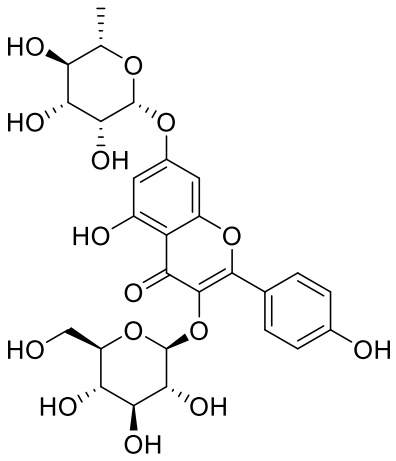
Pectolinarin



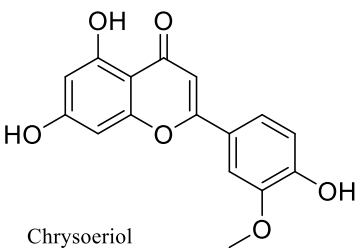
Isorhamnetin-3-galactoside-7-rhamnoside



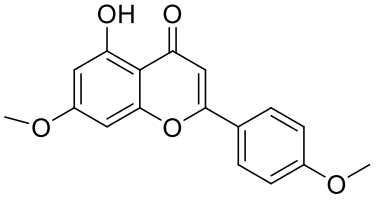
Rutin



Kaempfero-3-glucoside-7-rhamnoside



Chrysoeriol



3-hydroxy-3',4'-dimethoxyflavone

Figure 2. Flavonoids identified in the aqueous extract of *Markhamia lutea* leaf

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