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Isolation of Mangiferin from Flowering Buds of *Mangifera indica* L and its Evaluation of *in vitro* Antibacterial Activity

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ABSTRACT

Mango (*Mangifera indica L*) which belongs to the family of Anacardiaceae, is a rich source of biologically active compound mangiferin, which is a natural xanthone C-glucoside. Mangiferin, (1,3,6,7-tetrahydroxy xanthone-C2-b-D-glucoside), has been isolated from various parts of *Mangifera indica*. Mangiferin is a pharmacologically active phytochemical present in large amount in bark, fruits, roots and leaves of *Mangifera indica*. Mangiferin has been traditionally used in some parts of world as anti-inflammatory, antibacterial, analgesic, antipyretic, antioxidant, antitumor, antiviral, immunomodulatory, anthelminthic and in obesity treatment. The present work is aimed to isolate mangiferin by column chromatography from the ethanolic extract of flowering buds of *Mangifera indica L* and assess its antibacterial activity. The conclusive structure of the isolated compound was established using TLC, HPLC, UV/VIS, FTIR and NMR spectral analysis. *In vitro* antibacterial activity of the isolated mangiferin was studied on Gram positive and Gram negative bacteria by agar disc-diffusion techniques. The solutions of the isolated mangiferin showed activity with regard to various strains of two bacterial species: *Staphylococcus aureus* (Gram positive) and *Salmonella typhi* (Gram negative). The present study confirms the antibacterial effect of the isolated mangiferin which could be further processed for its development as an antibacterial agent.

INTRODUCTION

India has a rich heritage of traditional medicine. Materia medica of India provides a lot of information on the folklore practices and traditional aspects of therapeutically important natural products India has a rich heritage of traditional medicine which constitutes different components like Ayurveda, Siddha and Unani. The development of these traditional systems of medicines with the perspective of safety, efficacy, and quality will not only help to preserve the traditional heritage but also to rationalize the use of natural products in healthcare [1]. Unconventional medicine has become more popular in recent years [2]. Natural products have been our single most successful source of medicine.

The use of plant compounds for pharmaceutical purposes has gradually increased worldwide. The research and development thrust in the pharmaceutical sector is focused on the development of new innovative/indigenous plant-based drugs through the investigation of leads from the traditional system of medicine [3]. Each plant is like a chemical factory capable of synthesizing a limited number of highly complex and unusual chemical substances derived from plants that are considered as important drugs currently in use, while several other drugs are simple synthetic modifications of the natural products [4]. *Mangifera indica* L. (Anacardiaceae) is one of the most important and abundant tropical fruit in the world and India contributes to a major part of the world's mango production. In Indian delicacy, mango is considered as the king of all fruits. *Mangifera indica L.* (Anacardiaceae) is one of the most important tropical fruits in the world, as some 69 varieties of the species are known [5] and India contributes

major part of the world production. Mango is considered as the king of fruits in Indian delicacy. The tree (*Mangifera indica)* is indigenous to the Indian sub-continent and has been cultivated in India for over 4000 years. It is thought to have reached East Asia between the 4th and 5th century BC and cultivated in East Africa and thereafter in Brazil, West Indies, China, United States, Caribbean and Mexico.

Mango (*Mangifera indica* L.), the most important fruit of *Anacardiaceae* family, is a tropical fruit, comprising of high nutritional and medicinal value. It is reported that mango was first found in Indo-Burmese region, approximately 4000 years ago, but now it is being commercially grown in more than 87 countries [6]. Mangiferin ($C_{19}H_{18}O_{11}$), a glucoxanthone (1,3,6,7-tetrahydroxyxanthone-C 2-b-D-glucoside), is an active phytochemical that has been reported to be present in various parts of *Mangifera indica L* viz leaves[7], fruits [8], stem bark [9], heartwood [10] and roots [11]. Mangiferin is a natural *C*-glucoside xanthone (2-C-β-D-gluco-pyranosyl-1,3,6,7-tetrahydroxyxanthone; $C_{19}H_{18}O_{11}$; Molecular weight, 422.35; melting point, anhydrous 271°C), a polyphenol xanthone has been reported in various angiosperms and ferns [12]. The compound mangiferin is found in variety of plant families in varying concentration and it usually occurs as a glycoside. Some of the plant sources of mangiferin include: *Anemarrhena asphodeloides*, *Aphloia theiformis, Arrabidaea patellifera, Arrabidaea samydoides, Bersama abyssinica, Bombax ceiba, Bombax malabaricum, Cratoxylum cochinchinense, Cyclopia genistoides, Cyclopia subtarnata, Folium mangiferae, Folium pyrrosiae, Gentiana lutiae, Gentianella nitida, Hypericum perforatum, Mangifera indica, Mangifera odorata, Polygala hongkongensis, Phaleria cumingii, Phaleria macrocarpa, Pyrrosia gralla, Rhizoma anemarrhena, Salacia oblonga, Salacia reticulate, Swertia chirata, Swertia macrosperma, Swertia mussotii, Trichomanes reniforme, Zizyphus cambodiana.*

The easiest source of mangiferin, however, is the mango plant (*Mangifera indica L).* Mangiferin is the major component (10%) of *Mangifera indica* (mango) belonging to the family Anacardiaceae, that grows mainly in tropical and sub-tropical regions and is widely used in folk medicines for various therapeutic indications [13]. Among the various polyphenolic compounds found in mango [14], mangiferin (C-2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone, also named C-glucosyl xanthone) is a distinct one. Mangiferin is a heat-stable molecule and a natural pharmacologically active phytochemical that has various biological activities.

Mangiferin, traditionally used by native inhabitants of Bolivia, Southern Guiana, the Antilles, Columbia, the Philippines, and India in the treatment of a number of diseases, has subsequently been proved to have a varied pharmacological activities such as antibacterial [15], antitumor, immunomodulatory, anti-HIV [16], anti-diabetic [17], anti-oxidative [18], anthelminthic and anti-allergic [19], anti-inflammatory activity [20], anti-viral [21], and macrophage-inducing activity [22].

Recently, the World Health Organization (WHO) has reported about 119 plant-derived pharmaceutical drugs, out of which 74% are used in modern medicine that are correlated directly with traditional uses as Herbal plant medicine. In the present scenario, about 80% of the world population presently uses herbal medicine for primary health care. The remaining 20 %, who mainly reside in developed countries, consume costly medicines [23].

Since ancient times, people have been exploring the nature, particularly plants, in search of new drugs. This has resulted in the use of a large number of medicinal plants with curative properties to treat various diseases [24]. In India, almost 95% of the prescriptions are plant based in the traditional systems of Unani, Ayurveda, Homeopathy and Siddha [25]. The study of plants continues principally for the discovery of novel secondary metabolites. Around 80% of products are of plant origin and their sales exceeded US $65 billion in 2003 [26].

Natural products from plants are of interest for the discovery of antimicrobial compounds. Today, the pace of antimicrobial discovery has slowed. During the 20-year period from 1983 to 2002, the FDA's (Food and Drug Administration) approval of new antimicrobial agents decreased by 56% [27].

Due to the report of increasing development of drug resistance in human pathogen as well as undesirable side effects of certain antimicrobial agents, it is essential to search for new agents that are better, cheaper and devoid of side effects for treating infectious diseases especially in developing countries. A wide variety of plant/natural products are used in the treatment of infections. Phyto constituents have been found to inhibit bacteria, fungi, viruses and pests [28]. Medicinal plants have their intrinsic ability to resist pathogenic microorganisms and this has led researchers to investigate their mechanisms of action and isolate the active compounds. This has enabled exploitation of medicinal plants for the treatment of microbial infections of both plants and humans by developing new antibacterial agents. Medicinal plant based antibacterial agents represent a vast untapped source of pharmaceuticals. So further exploration of plant antibacterial agents need to occur for treatment of infectious diseases both in plants and humans while simultaneously for mitigating many of the side effects that are often associated with synthetic antibacterial agents.

Plants possess limitless ability to synthesize aromatic secondary metabolites which include phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and Coumarins [29,30]. These compounds show antimicrobial effect and serve as plant defense mechanisms against pathogenic microorganisms.

The present study is aimed to isolate mangiferin from the flowering buds of *Mangifera indica* using different extracting solvents and the isolated mangiferin was identified by TLC, HPLC, UV/VIS, FTIR spectroscopy and NMR spectral analysis. The isolated compound was then screened for its *in vitro* antibacterial activity against Gram-positive and Gram-negative bacterial strains isolated from human infections.

MATERIALS AND METHODS

Plant Collection and Authentication

The flowering buds were collected from local trees of *Mangifera indica* of Sugandha, Hooghly, in the month of February, at morning. The plant material was authenticated at Botanical Survey of India, by Mr. V P Prasad on 23rd May 2014 and a voucher specimen of no-CNH/47/2014/Tech II/102 was then assigned, where a voucher specimen was preserved for further reference.

Extraction of Mangiferin

The flowering buds of *Mangifera indica* were first cut into small pieces, and then they were dried at room temperature and further coarsely powdered. The coarsely powdered flowering buds of *M. indica* were extracted exhaustively with petroleum ether (60-80°C) in soxhlet apparatus for 56 hours to remove any fatty matter. Defatted powdered flowering buds were extracted by using soxhlet apparatus with required quantity of ethanol (95%) as solvent for 21 hours and concentrated under reduced pressure to yield a semisolid mass [31].

The semisolid mass was defatted repeatedly and finally dissolved in ethanol at room temperature. The ethanolic extract was further concentrated under reduced pressure which yielded a yellow amorphous powder.

Isolation of Mangiferin [32]

The dried alcoholic extract was adsorbed on silica gel (60-120 mesh) and chromatographed over silica gel column packed in petroleum ether (60-80°C). The column was eluted with chloroform: acetone: formic acid (8: 1.5: 0.5), which gave mangiferin as a pale yellow amorphous powder. This upon crystallization using ethanol produce pale yellow needle shaped mangiferin crystals.

Lastly, the pale yellow needle-shaped crystals of mangiferin were isolated and dried.

The isolated compound was further characterized using TLC, HPLC, UV/VIS, FTIR and NMR spectrophotometer. The melting point of the isolated compound was also determined.

Identification of the Isolated Mangiferin

Thin layer chromatography [33,34]

Adsorbent: Pre-coated and pre-activated TLC plates (silica gel GF 254) were used for the analysis.

Mobile phase used: Separation was best achieved using chloroform: acetone: formic acid (8: 1.5: 0.5) as mobile phase.

Sample preparation: 0.05% w/v of sample (mangiferin) was prepared in ethanol and 10 µl was applied on the TLC plate.

Detection: Ammonia vapour was used as spraying agent. The TLC plate was kept in UV chamber for 10 minutes and R_f value was observed at 366 nm.

High Performance Liquid Chromatography [35,36]

The determination of mangiferin was achieved by reversed-phase high performance liquid chromatography (RP-HPLC). Analytical determination was conducted by RP-HPLC (Agilent 1260 Infinity) with ultraviolet detection at 278 nm.

The chromatographic separation was made on Hypersil™ ODS C_{18} analytical column (4.6 × 100 mm; 3.5 µm) using chloroform: acetone: formic acid (8: 1.5: 0.5) as the mobile phase, at a flow rate of 1 mL min^{-1} and at room temperature. The injection volume was 10 µL, and the column temperature was 25°C. The detection was achieved at 278 nm.

Chloroform, acetone, formic acid were of HPLC grade which were procured from Merck (Darmstadt, Germany) and were used without further purification.

Five (5) mg of the isolated mangiferin crystals were dissolved in 10 ml methanol, it was filtered and injected six times in an HPLC column (ODS C_{18}) and its retention time was determined and was compared with the reference standard.

Ultraviolet Spectroscopy [37]

One (1) mg of the isolated mangiferin crystals were dissolved in methanol and the maximum wavelength of absorption were determined by UV-VIS spectrophotometer (UV-1800-240V SHIMADZU). Scanning of the isolated compound was performed at the wavelength range of 200-400 nm and was compared with reference standard.

Fourier Transform Infrared Spectroscopy [38]

One (1) mg of the isolated mangiferin crystals were measured using potassium-bromide (KBr) pellet method in FTIR spectrometer (Bruker-Alpha).

IR data of isolated compound was compared with the reference standard of mangiferin.

Nuclear Magnetic Resonance Spectroscopy [39]

NMR spectra of the isolated mangiferin crystals were obtained on Bruker avance II-400 MHz, spectrometer using TMS as internal reference.

In Vitro Antibacterial Evaluation [40,41]

Preparation: Peptone, sodium chloride, beef extract were weighed as required and poured in a conical flask. Water was added to it. The pH of the preparation was measured and agar was added to it. Then volume was made up. It was then sterilized by autoclaving at 121°C, 15 psi for 15 minutes **(Table 1)**.

Table 1. Composition of nutrient agar media [42].

Ingredient	Quantity in %w/v
Peptone	1
Sodium chloride	0.5
Beef extract	0.5
Agar	1.5
Water	q.s to 100ml
pH	7.2-7.4

Preparation: Peptone, beef extract, sodium chloride were weighed as required and poured in a conical flask. Water was added to it; pH was measured and adjusted with N/10 sodium hydroxide. The volume was made up. It was then sterilized by autoclaving at 121°C, 15 psi for 15 minutes **(Table 2)**.

Table 2. Composition of nutrient broth media [42].

Ingredient	Quantity in %w/v
Peptone	1
Sodium chloride	0.5
Beef extract	0.5
Water	q.s to 100ml
pH	7.2-7.4

Preparation [44]: Six test tubes were labeled as standard 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 in amounts as shown in table above, a 1% w/v solution of anhydrous barium chloride and a dilute 1% v/v solution of pure sulphuric acid were added. Using 0.5 McFarland standards, a visual comparison of the turbidity of a bacterial suspension with the turbidity of the McFarland Standard was performed. The turbidity of the test suspension was then adjusted until it matches the standard **(Table 3)**.

Table 3. Composition of McFarland standard preparations [43].

Preparation of McFarland standard			
Standard tube numbers	**Barium chloride 1% (ml)**	**Sulphuric acid 1% (ml)**	**Corresponding bacterial concentration (million/ml)**
0.5	0.05	9.95	150
1.0	0.1	9.9	300
2.0	0.2	9.8	600
3.0	0.3	9.7	900
4.0	0.4	9.6	1200
5.0	0.5	9.5	1500

Agar Well Diffusion Method [45,46]

In vitro antibacterial activity was determined by agar well diffusion method according to National Committee for Clinical Laboratory standards (NCCLS). Various strains of two bacterial species: - *Staphylococcus aureus (*Gram positive) and *Salmonella typhi* (Gram negative) were used.

The nutrient agar plates were prepared by pouring 15 ml of molten media in sterile petridishes. The plates were allowed to solidify for 5 minutes and standardised inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 minutes. The isolated mangiferin crystals were subjected to serial dilution as follows: 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml. With the help of a sterile cork borer, wells (6-8 mm in diameter) were dug on the middle of the agar plates. 0.5 ml of the prepared sample solution was then introduced into the appropriately labeled bored agar well. Each plate also had a control where only sterile water was added.

The plates were incubated at 37°C for 24 hours [47]. After incubation the diameter of inhibitory zones formed around each well were measured in mm and recorded.

RESULTS

Chromatographic Data

HPLC chromatogram: Following **Figure 1** shows the LC chromatogram of the isolated mangiferin obtained from the extract of the flowering buds of *Mangifera indica* **(Table 4)**.

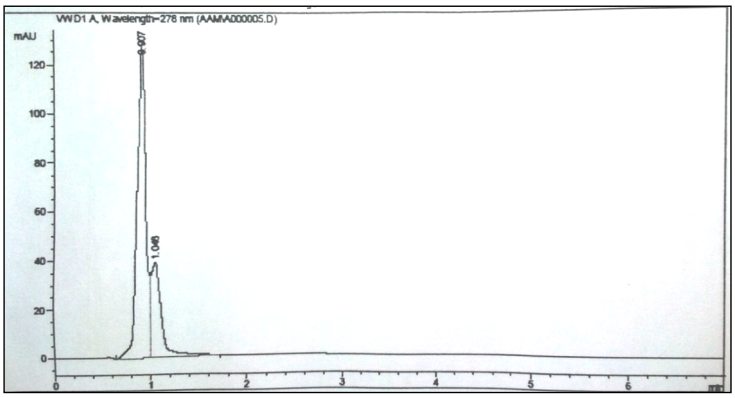

Figure 1. LC chromatogram of the isolated mangiferin obtained from the extract of the flowering buds of *Mangifera indica.*

Table 4. R_f values of the isolated *mangiferin*.

Observations	R_f	Average
01	0.79	0.8
02	0.82	
03	0.78	

Melting point: Melting point of isolated mangiferin was 269°C.

Ultraviolet spectroscopy: The UV spectrums of isolated mangiferin and reference standard in methanol showed two major peaks which were as follows: 278 nm and 215 nm.

FT-IR Spectral Data

IR data of isolated compound was compared with the reference standard, and from the spectra we can say that the isolated compound from the ethanolic extract of flowering buds of *Mangifera indica* might be mangiferin. The structure of mangiferin is in **Figure 2 and Table 5.**

NMR data: The NMR data **(δ ppm)** of the isolated mangiferin obtained as: 13.81 (ArOH intermolecularly bonded, 1H), 7.9 (ArOH, 3H), 6.82 (Ar-H, 1H), 6.36 (Ar-H, 1H), 7.4 (ArH, 1H), 2.5 (-C-OH, 4H), 3.7 (-CH-O-, 2H), 3.3 (-CH-O-, 2H), 3.5 (-CH-, 3H).

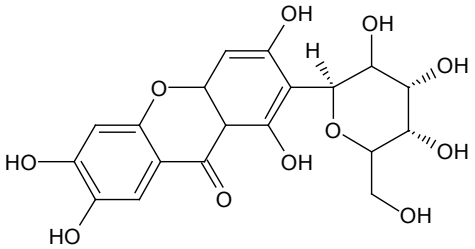

Figure 2. The structure of mangiferin matches with the interpretation of IR spectra.

Table 5. Interpretation of IR spectra of the isolated sample.

S. No	Absorbance(cm^{-1})	Groups
1	3367	Phenol O-H stretch
2	2918.38, 2849.99	Aliphatic C-H stretch
3	1670	Keto C=O stretch
4	1624.26	Aromatic C=C ring stretch
5	1253.98	Ar-O-Ar ether C-O-C stretch
6	1199.30	C-O stretch
7	1051.52	RCH2OH O-H stretch
8	828.41	Tetra substituted aromatic bending

Anti-bacterial Activity

Antibacterial activity of the isolated mangiferin against Gram-positive and Gram-negative bacteria is given in **Table 6**.

Table 6. Antibacterial activity of the isolated mangiferin against Gram-positive and Gram-negative bacteria.

Name of bacteria	Diameter of zone of inhibition (mm) at different concentration (mg/ml)				Diameter (mm) of zone of inhibition of control
	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	
Staphylococcus aureus NCTC 29137	13	11	-	-	-

Staphylococcus aureus 8531	17	14	12	11	-
Staphylococcus aureus ATCC 29157	16	14	13	11	-
Staphylococcus aureus ML 152	14	13	12	11	-
Staphylococcus aureus ML 267	13	12	11	-	-
Staphylococcus aureus ML 366	14	13	12	11	-
Staphylococcus aureus ML 145	14	12	11	-	-
Staphylococcus aureus ATCC 25923	13	12	11	-	-
Staphylococcus aureus ML 276	13	12.5	11	-	-
Salmonella typhi E860	13	12	-	-	-
Salmonella typhi C2114	13	11	11	-	-
Salmonella citreus	15	13	11	11	-
Salmonella typhi C7087	13	12	-	-	-
Salmonella typhi E856	14	12	11	-	-
Salmonella typhi G1846	13	11	-	-	-
Salmonella typhi DT 7327	13	11	-	-	-
Salmonella typhi A2467	14	12	11	-	-

DISCUSSION AND CONCLUSION

In the present study, the flowering buds of *Mangifera indica* was first defatted with petroleum ether (60-80°C) prior to extraction with 95% ethanol. Followed by this, the extract was chromatographed over silica gel and eluted with chloroform: acetone: formic acid (8:1.5:0.5) to obtain mangiferin as a pale yellow needle shaped crystals.

The isolated mangiferin crystals were characterized by R_f, melting point, HPLC and UV, FRIR and NMR spectral analysis. The isolated mangiferin obtained from the ethanolic extract of flowering buds of *Mangifera indica* showed identical TLC chromatography, HPLC and UV, FTIR and NMR spectrum to reference standard mangiferin. The absorbed maxima 278 nm and 215 nm of isolated mangiferin crystals is closely related to that of reported reference standard UV spectral data. Mangiferin was also confirmed by proton NMR signals.

The available literature on structure elucidation of the isolated mangiferin reveals the conclusive structure of mangiferin ($C_{19}H_{18}O_{11}$) can be established as glucoxanthone (1,3,6,7-tetrahydroxyxanthone-C 2-b-D-glucoside).

In vitro antibacterial activity of the isolated mangiferin was evaluated by using various strains of two bacterial species: *Staphylococcus aureus* (gram-positive) and *Salmonella typhi* (gram-negative). The solution of the isolated mangiferin was found to exert promising antibacterial activity against gram-positive and gram-negative bacteria. The present study found a very promising and readily available source (mango, *Mangifera indica)* for treating infections caused by bacteria. This is particularly significant because drug resistance to human pathogen has been increased not only in the developing countries but throughout the world due to indiscriminate use of antibiotics [48]. So from the present screening it could be concluded that mangiferin possess antibacterial activity and may be processed further for the development of an antibacterial agents.

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