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EXTRACTION, ISOLATION AND ANTI-MICROBIAL SCREENING OF FLAVONOID CONSTITUENT FROM THE LEAVES OF MORINDA CITRIFOLIA L.

Chakravarthi K. S. S. and Suresh Kumar K.*

Professor and HOD, Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Kovai Estate, Kalapatti Main Road, Coimbatore - 641048, Tamil Nadu, India.

***Corresponding Author: Dr. Suresh Kumar K.**
Professor and HOD, Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Kovai Estate, Kalapatti Main Road, Coimbatore - 641048, Tamil Nadu, India.

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ABSTRACT

Natural products have numerous secondary metabolites that have their own pharmacological and biological activity. Different classes of phytoconstituents such as alkaloids, flavonoids, carbohydrates, tannins, steroids, terpenoids etc. are present. Flavonoids are the large group of phytonutrients of polyphenol class and generally flavonoids have 15 carbon skeleton structure. Isolation of a specific constituent from a plant extracts are still a tedious process which can be made simple by chromatographic process such as TLC. *Morinda citrifolia* L. was native to pacific societies and it comes under Rubiaceae family. It is commonly known as Noni. Noni is widely used for many pharmacological uses such as immunomodulatory, anti-cancer, anti-diabetic, gastric ulcer healing etc. In the present study an attempt has been made to identify the flavonoid constituent that is being present in the leaf of the plant by HPTLC. Further, the isolation of the same was performed using preparative TLC using solvent system of Chloroform: Ethyl acetate in the ratio 50:50. Confirmation of the isolate was done by HPTLC. The anti-microbial screening of the isolate was also performed by Disk diffusion and broth dilution method. The flavonoid constituent was isolated and anti-microbial screening was performed in 10 different test organisms. This strategy could be used to isolate constituents from the plant extracts by this we can get numerous informations.

KEYWORDS: Preparative TLC; *Morinda citrifolia*; Isolation; Anti-microbial screening.

INTRODUCTION

Natural products are mainly secondary metabolites obtained from plant and animal sources. Natural products are natural substances that have their own pharmacological and biological activity. They are used for the drug design.[1] Hippocrates one of the ancient authors, who described medicinal plants and listed about 400 different plant species of different medicinal activities. Natural products have been a fundamental piece of medicine for the old traditional medication. E. g. Chinese medicine, ayurvedic medicine, Egyptian medicine etc.[2] A single plant may be used for several conditions, such as fever, cancer, asthma, constipation, hypertension etc,. and the plants are applied in many sources as decoctions, tinctures, porridges, soups and administered in different ways including oral, nasal, topical, rectal etc.[3]

There are several different classes of phytoconstituents alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones, essential oils, steroids in which these secondary metabolites are responsible for the pharmacological and biological activity of the plant substances.

Morinda citrifolia commonly known as Noni, and it is native to Pacific societies and it comes under Rubiaceae family. This is most significantly used as a traditional source of medicine by the Pacific societies as it has numerous medicinal properties. Noni is well known for its environmental tolerance and it includes fire, flooding, wind and also in saline conditions. Noni can grow in acidic, alkaline and infertile soils and also in very dry to very wet areas. All the parts of the plant including root, leaves, trunks, bark and fruits have traditional as well as modern uses such as dyes, food and medicine, fire wood, tools etc.[4]

Morinda citrifolia is commonly known by many vernacular names worldwide including Indian mulberry, hog apple, mengkudu, gogu atoni, great morinda, jo ban, mona and kesengel, but the most widely used commercial name is noni. The plant produces a small tubular white flower which is perfect and has both male and female organs. The noni fruit grows upto a maximum of 10 cm long and 4 cm of breath. Oval in shape and contains many seeds. Some countries such as North America, Asia, Australia, Japan, Mexico has increased the cultivation on noni as it has numerous health benefits and also it has many commercial demand.[5] The pharmacological activities of Noni includes Anti-cancer activity, Hypotensive activity,

Cardiovascular activity, Estrogenic activity, Immunological activity, Anti-arthritic activity, Anti-inflammatory activity, Antidiabetic activity, Wound healing activity, Analgesic activity, Gastric ulcer healing activity, Memory enhancing activity, Anxiolytic and sedative activity.[6]

Flavonoids are a huge group of mixtures incorporated by plants that have a typical chemical structure. Flavonoids are poly-phenolic compounds having 15 carbon atoms and two benzene rings joined by a straight three carbon chain which might be additionally separated into subclasses. Flavonoids comprise perhaps the most trademark classes of mixtures in higher plants, where they can be effectively perceived as bloom colors in angiosperms. In any case, their event isn't limited to flowers just, yet incorporate all the parts of a plant. Flavonoids are additionally known to assume a significant part in offering protection to the plant species, for example, rotenone, which is an isoflavonoid, act as an effective insectiside. The impact of isoflavonoids on human is likewise widely explored particularly in the prevention of cancer and specifically hormone dependent cancer, for example, breast cancer.[7-11]

MATERIAL AND METHODS

Collection of samples

Fresh leaves was collected from Eden Nursery and Biotech Pvt. Ltd., Mettupalayam of Coimbatore district, Tamil Nadu, India. The authentication was carried out in Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. The leaf samples were cleaned under running tap water, properly shadow dried and then dehydrated in hot air oven at a temperature which is not exceeding 50ºC. The materials are coarsely ground by the help of a grinder and the powder was passed through the sieve no.60. The powder was then stored in a tightly closed container so that it is not exposed to the atmospheric moisture.

Preparation of extracts

After de-fatting the leaf powder with Petroleum ether the Chloroform, Ethyl acetate, Methanol and Water extract was prepared by the method of cold maceration by adding 250gm of leaf powder to thrice its volume quantity of solvent. The contents were occasionally shaken for a period of three days and the extract was filtered through Whatman number 1 filter paper. The extract was then concentrated in water bath in which the temperature not exceeding 45ºC and the residue was collected. The residue was then dissolved in methanol and used for further analysis.

Screening of flavonoids

The initial screening of the flavonoid was carried out by the basic qualitative test for flavonoids, in which a few ml of the extract was mixed with 5 ml of Conc. H_2SO_4 and few magnesium turnings.[12] Further, HPTLC for the extract was carried out with the solvent system selected as Toulene: Ethyl acetate: Formic acid: Methanol (3:6:1.6:0.4) to identify all the constituents that present in the extract. Quercetin was placed as a flavonoidal standard. In the HPTLC screening a thin strip of TLC aluminium plate of 5 X 10 cm was taken and 10µl volume of extract was applied by Linomat 5 sample applicator. The plate was then kept for development in the chromatographic chamber containing 10ml of the prepared solvent system. After development the plate was air dried and examined under UV chamber at 366 nm.

Preparative TLC of the extract

From the successful identification of compounds in HPTLC, preparative TLC was performed with manually prepared TLC glass plates of 20 X 20 cm with Silica gel G (Passing 350 mesh; SDFCL, Mumbai) and was activated at 110ºC for 30 minutes. The solvent system used was Ethyl acteate: Chloroform (50:50) for the proper separation of the required band. The sample was spotted and kept for development in the twin trough chromatographic chamber containing 70ml of prepared solvent system. After development the plate was air dried and examined under UV chamber at 366 nm.

Isolation of flavonoid constituent

The developed plate was then scale marked from 0 to 20 cm and placed under UV chamber at 366 nm. The bands were then marked on the scale. The marked area was then scrapped along with silica with spatula and collected in Erylenmayer flask. The same solvent system Ethyl acetate: Chloroform (50:50) was used for the extraction of constituent from the silica. The content was then concentrated by evaporating the solvent at room temperature and the content was marked and stored.[13,14,15]

Confirmation of isolate

The isolate was then chromatographed with a basic flavonoidal compound Quercetin in CAMAG Linomat 5 HPTLC instrument with Toulene: Ethyl acetate: Formic acid: Methanol (3:6:1.6:0.4) as solvent system and silica gel coated, self indicator Aluminium TLC plates 10X10 cm as stationary phase. The developing chamber was 10X10 twin trough chromatographic chamber containing 10 ml of the solvent system. After development the plate was air dried and examined under UV chamber at 366 nm. Further confirmation was done by UV-VIS spectrophotometer (Shimadzu 1800) in range of 800-200 nm for single peak.

Anti-microbial screening of isolate[16,17]

The anti-microbial screening of the isolate was performed by Disk diffusion method. A required quantity of nutrient agar was prepared and sterilized by autoclaving at 15 lb pressure, 121ºc for 15 minutes. The sterile media and sterile petridish was transferred to aseptic room and the media was poured into the petridish. The media was allowed to solidify at room temperature. Gram positive standardized inoculums of *Bacillus subtilis*, *Staphylococcus albus*, *Bacillus lintus*,

Micrococcus luteus, *Streptococcus aureus* and gram negative standardized inoculums of *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella Paratyphi*, *Vibrio cholera*, *Klebsiella pneumonia* were spreaded over the surface of the plate using non-absorbent sterile cotton swab. The isolate solution impregnated discs (10µg/disc) and standard ciprofloxacin (10µg/disc) were placed on the inoculated agar medium using sterilized forceps. All the plates were placed for incubation at 37ºC for 24 hours. After incubation time, the zone of incubation of sample and standard was measured in mm.

Minimum Inhibitory Concentration (MIC) of isolate:
MIC was performed by broth dilution method. Test organism that shows zone of inhibition in disk diffusion method was selected for MIC.

The standardized MIC tubes were labeled 1 to 8 and 1 ml of nutrient broth solution was added to each of the tubes. The MIC tubes were sterilized in autoclave at 15 lb pressure, 121ºc for 15 minutes and transferred to aseptic room. One ml of isolate solution was aseptically added to the first tube, mixed well and 1ml was serially transfered up to tube 7. Eighth tube acts as control. A loop full of standardized test organisms were inoculated to all tubes including control, mixed and incubated for 24 hours at 37ºC. The highest dilution of isolate showing no turbidity was recorded.

RESULTS AND DISCUSSION

Screening of flavonoids

The initial screening with basic preliminary test for flavoloids was performed on the extracts and the results are given in the Table 1. The Rf values that are obtained in the HPTLC analysis are given in the Table 2. The HPTLC plate, extract chromatogram and standard chromatogram are given in the Figures 1, 2 and 3 respectively. The Rf value of basic flavonoid quercetin matches with the Rf value of a eluent in the ethyl acetate extract. That specific compound was found to be strong blue fluorescent under UV 366 nm and that compound was selected for isolation.

Table 1: Phytochemical investigation by chemical tests of extracts.

S. No	Name of the test	Pet.ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract	Water
1.	Test for Alkaloids	-	-	-	-	-
2.	Test for carbohydrates	-	-	+	+	+
3.	Test for proteins	-	-	+	+	-
5.	Test for glycosides	-	-	-	+	+
6.	Test for Flavonoids	-	-	+	-	-
7.	Test for Tannins	-	-	-	+	+
8.	Test for Triterpenoids/steroids	+	+	+	-	-
9.	Test for fixed oils	+	+	-	-	-
10	Test for saponins	-	+	-	-	-

Table 2: HPTLC analysis of ethyl acetate extracts and their Rf values.

Peak	Rf value	Standard		
		Quercetin	Rutin	Gallic acid
1	0.00			
2	0.22			
3	0.32			
4	0.45			
5	0.61	**0.80**	0.15	0.70
6	0.65			
7	0.74			
8	**0.81**			
9	0.90			

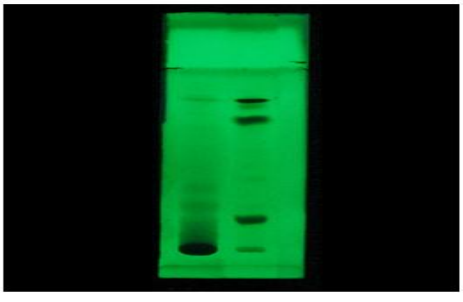

Fig. 1(a): Measured at UV 254nm.

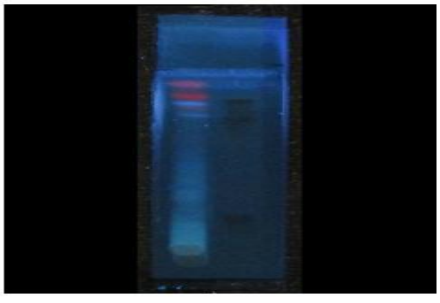

Fig. 1(b): Measured at UV 366nm.

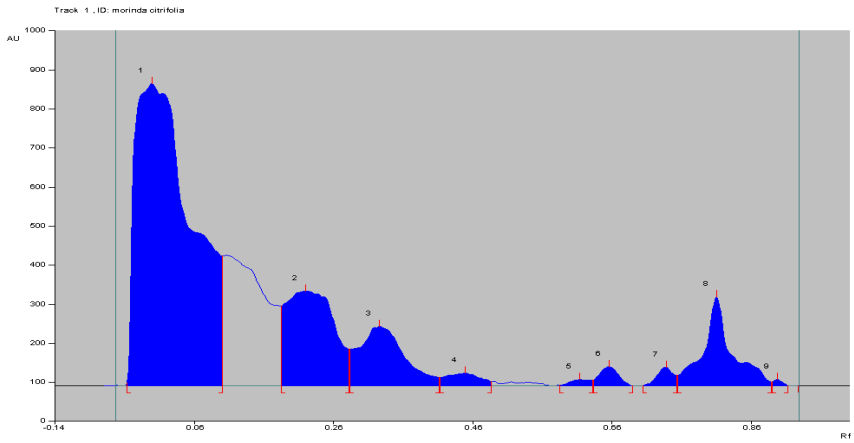

Fig. 2: HPTLC chromatogram of *Morinda citrifolia* ethyl acetate leaf extract.

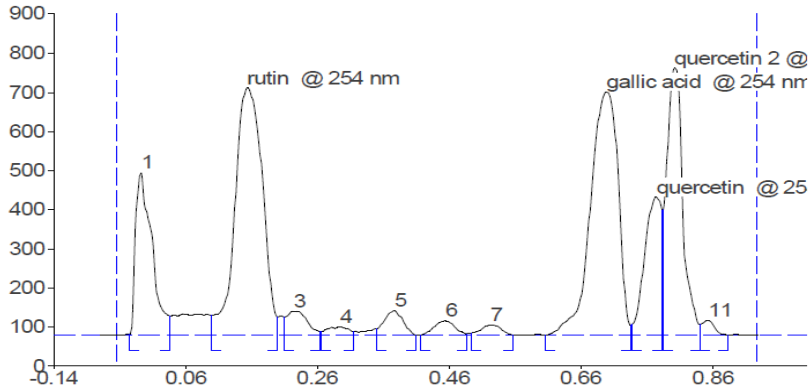

Fig. 3: HPTLC chromatogram of standard quercetin, Rutin and Gallic acid.

Preparative TLC of the extract

The preparative TLC is one of the cost effective, reliable method for the isolation of compounds from herbal extracts. The solvent system used for the isolation of strong blue fluorescent compound was Chloroform and Ethyl acetate in the ratio of 50:50. The solvent system was selected by trial and error. The preparative TLC plate under UV 366 nm was displayed in the Figure 4. The crude extract revealed five bands of light red, dark band, bright blue fluorescent, light orange and bright red color with Rf value of 0.11, 0.21, 0.56, 0.72 and 0.94 respectively in chloroform and ethyl acetate solvent system (50:50). The dark blue fluorescent band of Rf 0.56 was selected for isolation.

Fig. 4: Preparative TLC plate under UV 366 nm.

Isolation of flavonoid constituent

The dark blue fluorescent band was scrapped from the plate and the isolate was extracted with same solvent system chloroform and ethyl acetate in the ratio 50:50. The contents were placed in the magnetic stirrer for

about 30 minutes and filtered using whatman number 1 filter paper and concentrated.

Confirmation of isolate

The isolate solution was then again analysed in HPTLC with quercetin (Fig. 5) as standard and the Rf values were compared. The Rf values are given in the Table 3. The purity of the isolate was also confirmed by obtaining single Rf value. The UV-VIS spectrophotometer also showed single peak in the complete wavelength range of 800-200 nm. The λ_{max} obtained was 404 nm in UV-VIS spectrophotometer.

Table 3: Comparision of Isolate and Standard quercetin Rf value.

Rf value of isolate	Rf value of standard quercetin
0.84	0.85

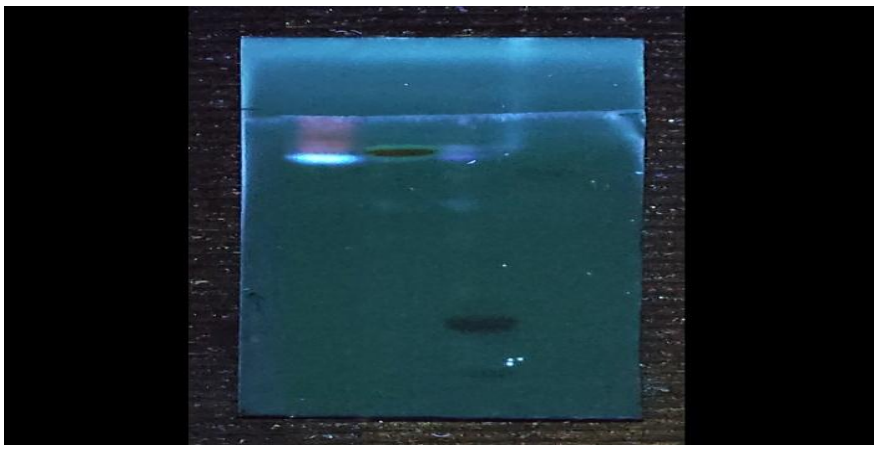

Fig. 5: HPTLC plate of Isolate and Standard quercetin under UV 366 nm.

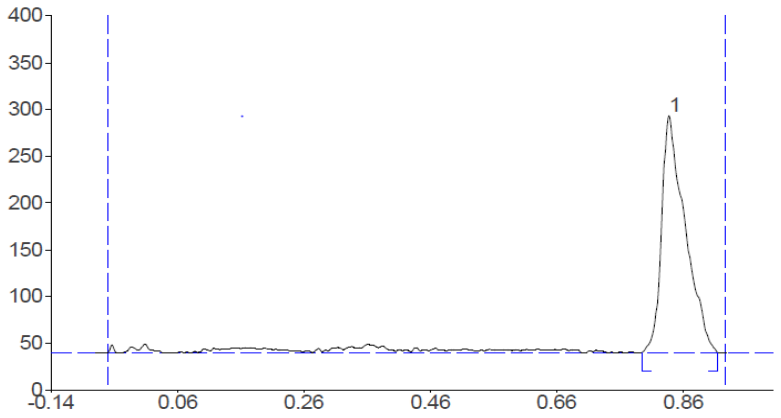

Fig. 6: HPTLC chromatogram of isolate.

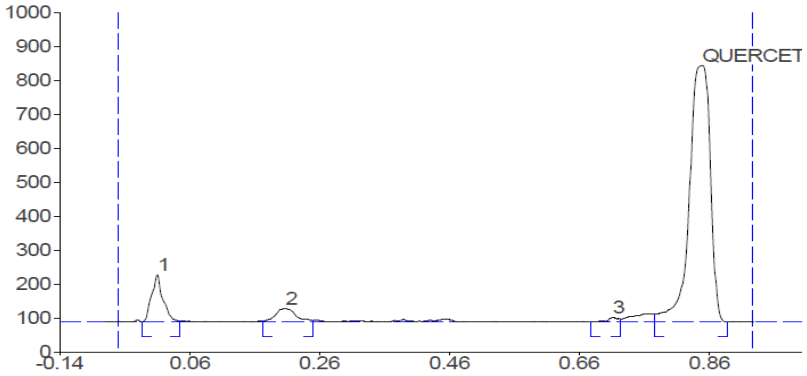

Fig. 7: HPTLC chromatogram of standard quercetin.

Anti-microbial screening of isolate:
The results of anti-microbial screening of isolate by disk diffusion method was performed and displayed in the Table 4. The maximum inhibition was recorded against *Escherichia coli* in 15mm.

Table 4: ZOI of isolate in different test organism by disk diffusion method.

S. No	Micro-organisms	Zone of Inhibition (in mm)	
		Isolate (10µl/disc)	Std*(10µg/disc)
1	*Bacillus subtilis*	-	37
2	*Staphylococcus albus*	8	30
3	*Bacillus lintus*	8	40
4	*Micrococcus luteus*	-	30
5	*Streptococcus aureus*	8	30
6	*Escherichia coli*	15	37
7	*Pseudomonas aeruginosa*	-	35
8	*Salmonella paratyphi*	-	30
9	*Vibrio cholera*	10	32
10	*Klebsiella pneumonia*	-	33

*Ciprofloxacin

Minimum Inhibitory Concentration (MIC) of isolate
The MIC was performed by broth dilution method and the results are given in the table.

Table 5: Minimum Inhibitory Concentration (MIC) of isolate.

S. No.	Micro-organisms	MIC values (mg/ml)
1	*Staphylococcus albus*	6.25
2	*Bacillus lintus*	12.5
3	*Streptococcus aureus*	12.5
4	*Escherichia coli*	12.5
5	*Vibrio cholera*	12.5

CONCLUSION
From the above methodology, it tends to be obviously clear that the plant *Morinda citrifolia* contains the flavonoids. The above expressed method is simple in the isolation of compounds from the extract since the isolation of compounds from the herbal extracts were difficult one. This technique, being an exceptionally very simple one, and is efficient for the isolation of compounds from the herbal extracts. Likewise the solvent system, standardized for the separation of flavonoids, establishes to be reasonable for the separation, where the clear separation of compounds is a very difficult task faced during the isolation of compounds from herbal extracts. Anti-microbial activity of the isolate was compared with the standard antibiotic with test organisms. As a result it is sure that the isolate can inhibit the growth of these micro-organism, thereby preventing various diseases such as skin infections etc. Thus, *Morinda citrifolia* leaf extracts provides safe, effective and every day practical solution, leaving no toxins, providing clean and safe atmosphere. The overall results provide basic information for the use of extract/isolate against infectious organisms.

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