WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 6, 1365-1383. Research Article ISSN 2277– 7105

ISOLATION, CHARACTERISATION AND ANTI-ANGIOGENIC ACTIVITY OF MANGIFERIN FROM BANGANAPALLE VARIETY *MANGIFERA INDICA.L*

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Article Received on
26 March 2020,

Revised on 16 April 2020,
Accepted on 06 May 2020,

DOI: 10.20959/wjpr20206-17262

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ABSTRACT

This present work highlights the isolation, characterization, elucidation of isolated phytoconstituent and anti-angiogenic activity of Mangiferin from Banganapalle variety *Mangifera indica*. Linn. The sub-continent harbours more than 1000 mango cultivators and represents the biggest mango production in the world. The largest amount of mango plant production in india compare than other countries. Its require minimum quantity of water and surface area for growing plant. The whole plant parts were used in various diseases and rich amount of nutrients present in this plant. Mango trees were widely cultivated and easily available plant. The isolated phytoconstituents were characterized by means of spectral studies and subjected to determined its Anti- angiogenic potential by means of CAM Assay model in vivo method due to its transparency for studying vascular growth because. Angiogenesis is one of a factor for growing cancer disease so I carried my work through Anti-angiogenic activity finally results showing prevention of malignancy.

KEYWORDS: Mangiferin, Banganapalle variety, Anti-angiogenic activity, CAM Assay model.

INTRODUCTION

In India around 20,000 medicinal plant species have been recorded recently but more than 500 traditional communities use about 800 plant species for curing various diseases. Presently 80% of the world population depends on plant-derived medicine for primary health care for varied human diseases as it has no side effects. Plants are important sources

of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient. In the last century, nearly 121 pharmaceutical products were formulated based on the traditional awareness obtained from various sources. In connection one of the secondary metabolite Xanthone glycoside called mangiferin**.**

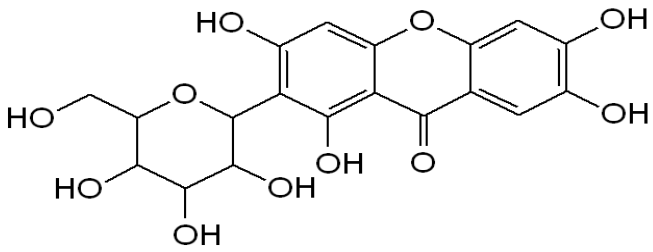


Fig-1

Mangiferin is an xanthone glycosides chemically it called as[1,3,6,7-tetrahydroxy-2-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]xanthen-9-one] or [(1S)-1,5-Anhydro-1-(1,3,6,7-tetrahydroxy-9-oxo-9H-xanthen-2-yl)-D-glucitol]

Molecular Formula: C19H18O11, Molecular Weight: 422.342g/mol. it is found in *Mangifera indica.*L, It is also found in the genera Salacia and Cyclopia. Among the group of Asplenium hybrids known as the "Appalachian Asplenium complex", mangiferin and isomangiferin are produced only by Asplenium montanum and its hybrid descendants. the total plant extract was used in variety of diseases from antiquity period. They are astringent, acrid, refrigerant, styptic, anti-syphilitic, vulnerary, anti-emetic, anti-inflammatory and constipating, haemorrhages, haemoptysis, haemorrhoids, wounds, ulcers, diarrhoea, leucorrhoea, menorrhagia, diabetes, heat burn and vomiting. etc.

The World Health Organization reported diabetes appears with projections to affect about 439 million adults by 2030, whereas cardiovascular diseases account for 30% of deaths annually and also cancer affect 30% of people in India. So, Cancer is one of the major health hazards in both already developed and developing countries. Because of high death rate associated with cancer and because of serious side effects of chemotherapy along with radiation therapy, many cancer patients turn to alternative methods of treatment. More than 50% of modern drugs in clinical use are of natural products. Cancer is the abnormal growth of cells in our bodies that can lead to death. It is a complex disease that is normally associated with a wide range of escalating effects both at the molecular and cellular

levels. It therefore appears unprobable that chemoprevention follows simple rules. The ancient saying **"Prevention is always better than cure"** is absolutely true in the case of malignancies **where a cure**, if ever possible, is associated with high cytotoxic loads followed by invasive procedures and also severe side effects of Anticancer & **Anti-Angiogenic drugs** which affect the normal cells and increasing deleterious effect. **ANGIOGENESIS -** The process of new blood vessel development from existing vessels, primarily venules. Critical in: Healing at sites of injury, Development of collateral circulations at sites of ischemia, Allow tumour‟s to increase in size beyond the constraints of their original blood supply. The behaviour of cancer cells infused into different regions of the same organ. One region was the iris with blood circulation; another was the anterior chamber without circulation. The cancer cells without blood circulation grew to 1–2 mm3 in diameter and then stopped, but grew beyond 2 mm3 when placed in an area where angiogenesis was possible. In the absence of vascular support, tumour‟s may become necrotic or even apoptotic Therefore, angiogenesis is an important factor in the progression of cancer.

I impressed and aim about my work, which is **"Prevention is always better than cure".** Angiogenesis is one of a factor for growing cancer disease so; I carried my work through Anti-angiogenic activity finally results prevention of malignancy. The **3R's** principle (Reduction, Refinement, and Replacement) was implemented that help to minimize harms to vertebrate animals used in science. Therefore we selected CAM assay model, our study due to its transparency for studying vascular growth. Therefore, there is need to develop value added products to enhance mango utilization and minimize losses.

MATERIALS AND METHODS

Plant Material and Extraction. Medicinal plants used in the present work were collected at Tamilnadu Agriculture University, Horticulture Department Madurai. The plants were identified by professor Dr.T.N.Balamohan, Ph.D., PDF., (TNAU), Madurai.

PREPARATION OF PLANT EXTRACT[1,2,4,6]

Preparation of ethanolic extract

The leaves of Mangifera indica (Var. Banganapalle) were collected, shade dried and powdered. The powdered plant material were defatted with petroleum ether (60-80ºC). The defatted powdered leaves 100g were extracted by maceration was made up at room temperature and the extraction solvent were changed every week for 3 weeks. The extract

was filtered and evaporated at 40ºC in vacuum until dryness to obtain the thickening semisolid mass of ethanolic extract.

Preparation of Aqueous extract

The leaves of Mangifera indica (Var. Banganapalle) were collected, shade dried and powdered. The powdered plant material were defatted with petroleum ether (60-80ºC). The defatted powdered leaves 100g were extracted by soxhelt with required quantity of aqueous solution for 21 hour concentrated under reduced pressure to yield semisolid mass.

Preparation of Microwave Assisted Extraction

The leaves of *Mangifera indica* (Var.Banganapalle) were collected, shade dried and powdered. The powdered plant material were defatted with petroleum ether (60-80ºC). Then equipped with one 1000 mL container collocated in the space with mayor radiation, Around 100g of defatted leaves powder was put into extraction vessel with required quantity of aqueous solution added and processed under different MAE situations. The irradiation time was the most important parameter on the recovery of *Mangiferin* and the power of microwave 900 W, extraction time of 15 min and a solvent volume maintained under different MAE situation. The extract was filtered and evaporated at 40ºC in vacuum until dryness to obtain the thickening semisolid mass of aqueous extract. (With these parameters the maximum *Mangiferin* yield was 63.22%).

PHYTOCHEMICAL STUDIES

The qualitative chemical test for various phytoconstituents were carried out using the leaf extract of *Mangifera indica* L. [Kokate CK, 2005, Agarwal, 2007,].

ISOLATION OF MANGIFERIN

The extracted semisolid mass were resuspended in 50ml of 50% ethanol then partitioned with 100 ml of Dichloromethane for four times. The aqueous ethanolic phases which were hydrolysed by reflux with 2N Sulphuric acid at pH 3 for an hour with continuous stirring. After cooled to room temperature, it was partitioned with 100ml of ethyl acetate for 3 times. Subsequently, the combined ethyl acetate layer was dried at 40ºC using a vaccum rotary evaporator. The dried ethyl acetate fraction was dissolved in ethanol and left in a refrigerator (4-8ºC) over night. After that the precipitate came out and was isolated by filteration. For recrystallization, the precipitate was dissolved in 70% of aqueous ethanolic solution and left in a refrigerator (4-8ºC) over night. Finally the pale yellow needle

shaped crystals of Mangiferin were isolated and dried.

CHROMATOGRAPHY[3,5]

Chromatography comprises a group of methods for separation of mixture of components into individual compound. Its depends on the differential affinities of the solutes between two immiscible phases. One of the phases is fixed bed of large surface area called the stationary phase, while the other fluid, which moves through or over the surface of the fixed phase called the mobile phase. if the stationary phase is a solid, the process is called as adsorption chromatography and if the stationary phase is a liquid, its termed as partition chromatography.

The following various types of chromatography are used for separation of mixture of components into individual compounds.

- Paper chromatography **(PC)**
- Thin layer chromatography **(TLC)**
- Column chromatography **(CC)**
- Gas chromatography **(GC)**
- High performance liquid chromatography **(HPLC)**
- High performance thin layer chromatography**(HPTLC)**

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is an simple technique to adopt for separation and identification of organic compounds. The principle involved is adsorption. The solute competes with the solvent for the surface sites on the adsorption. Depending on the distribution coefficient. The compounds are distributed on the surface of the adsorbent. The compound which is readily soluble but not strongly adsorbed, moves up along with the solvent and that not soluble but more strongly adsorbed move up less readily leading to the separation of compounds.

MELTING POINT

The melting point of a substance is the temperature at which the solid phase converts to the liquid phase less than 1 atmosphere of pressure. The melting point is one of a number of physical properties of a substance that is useful for characterizing (describing) and identifying the substance. To measure the melting point of a substance, its necessary somehow to gradually heat a small sample of the substance while monitoring its temperature

with a digital meter.

Determination of the Melting Point using a Mel-Temp Apparatus

Place a very small quantity of the solid on a watch glass, and use a stirring rod to grind the solid to a powder. Use a spatula to gather the powder into a small pile. Stick the open end of melting point capillary into the pile to a depth about 1mm, then invert the capillary and tap the sealed end on the table to encourage the solid to drop to the bottom. The height of solid in the melting point capillary should be not more than 1-2mm. Loaded capillary is placed into one of the 3 sample wells of the Mel-Temp. Procedure continues until the first discoloration of the sample occurs. Noting temperatures at which changes occur.

Identification of compounds present in the extract of plant leaves by HPTLC analysis

High performance thin layer chromatography (HPTLC) is a modern adaptation of TLC with improved versatility, separation efficiency and detection limits. HPTLC is a useful tool for identification of plant extract because each plant species produces a distinct chromatogram, with unique marker compounds used for the plant identification. It is used as a quality control tool since comparison of chromatograms of different lots can demonstrate the similarities and differences between the test samples and their standard chemical markers. HPTLC fingerprint analysis is used for rapid identity check, for monitoring purity of drugs, for detection of adulterants, for determining whether a material is derived from a defined botanical species and also to know whether constituents were clearly characterized.

HPTLC SPECIFICATIONS

Instrument used	**CAMAG TLC Scanner 3**
Software	winCATS Planar Chromatography Manager
Sample application	Linomat 5
Detection	at 254nm in TLC Scanner 3
Stationary phase	HPTLC plates silica gel 60 F 254
Sample preparation	100mg per ml of sample was prepared in ethanol
Mobile phase	Ethyl acetate: Formic acid: Methanol
Sample solution	5µL
Standard solution	5µL
Drying device	Oven
Temperature	60°C
Volume	10.0ml
Scanning speed	20mm/s
Sample concentration	100mg in 1ml
Time	5minutes
Wavelength	254 nm

Lamp	D2&W
Measurement type	Remission
Measurement mode	Absorption

And then Structural Elucidation of Isolated Mangiferin was determined by spectroscopical instrumental methods like UV-Visible, IR, NMR and Mass spectroscopy.

PHARMACOLOGICAL ACTIVITY METHOD

ANTI-ANGIOGENESIS TEST CHORIOALLANTOIC MEMBRANE ASSAY.[7,8,9]

PREPARATION OF ISOLATED DRUG CONCENTRATION OF SOLUTION

The 10mg (0.01gm) of isolated Mangiferin was dissolved in 1000 ml distilled water. Keeped this solution as **Test stock drug solution** and this solution containing 10µg/ml. from this stock solution 1ml (10µg/ml), 1.5ml (15µg/ml), 2ml (20µg/ml) were used for further application.

PREPARATION OF STANDARD DRUG SOLUTION

The 10mg (0.01gm) of Itraconazole was dissolved in 1000 ml distilled water. Keep this solution as stock **standard drug solution** and this solution containing 10µg/ml. from this stock 1ml (10µg/ml), 1.5ml(15µg/ml), 2ml(20µg/ml) were used for further application.

PROCEDURE

1. Structurally similar (size, shape, egg shell thickness) and three numbers of unfertilised chicken eggs were collected.
2. The collected eggs washed with tap water and then cleaned with using 0.1% Benzalkonium Bromide
3. Cleaned eggs were wiped with sterilised cotton cloth then separated by three groups each groups containing one eggs. There are named by Sample, Standard, controlled.
4. The above mentioned three groups of eggs were incubated at 37.5 °C in 85% humidity for 2- 3days for embryo formation
5. These 3 day embryos are located by handling and its position is marked by marker on the shell. Then the marked location was wiped by using Nacl solution
6. A hole is drilled in the small end of the egg to allow 1 ml of albumin was removed (by using syringe with needle) to lower the embryo so that its extra- embryonic membrane will not stick to the shell.
7. A window is cut in the shell with rotating carborundum disc, not to be break the whole

shell, and then vascular zone was easy to be identified on the CAM.

8. Ringer's solution is used to wash away the shell dust and to moisten the shell membrane vascular zone was to be identified on the CAM.
9. The prepared drug solution was directly applied by using syringe {1 ml (10µg/ml), 1.5 ml (15 µg/ml), 2 ml (20µg/ml)} and adhere to the vascular zone.in the same manner standard drugs (Itraconazole) was applied as that of test drug solution.
10. Upon sealing the openings with sterile flexible paraffin film, the eggs were further incubated for indicated periods.
11. After the completion of incubated period, the controlled egg was observed and then the sample and standard eggs clamped and raised by ophthalmic forceps, the CAM was observed and the blood vessels were viewed, photographed and quantified by counting the number of blood vessel branch points.

RESULTS

ISOLATION OF MANGIFERIN

The Ethanolic extract yield of isolated mangiferin from 100g of mangifera indica leaves was found to be 3.12g.

The Aqueous soxhlet extract yield of isolated mangiferin from 100g of mangifera indica leaves was found to be 2.32g.

The Aqueous Micro Wave irradition extract yield of isolated mangiferin from 100g of mangifera indica leaves was found to be 2.83g.

TLC OF THE ISOLATED MANGIFERIN

Adsorbent: Precoated silica gel-60 F/254

Mobile phase: ethyl acetate formic acid, glacial acetic acid, water. 100:11:!1:26

Sample preparation: 0.05% w/v of sample were prepared and 10µl applied on TLC Plate.

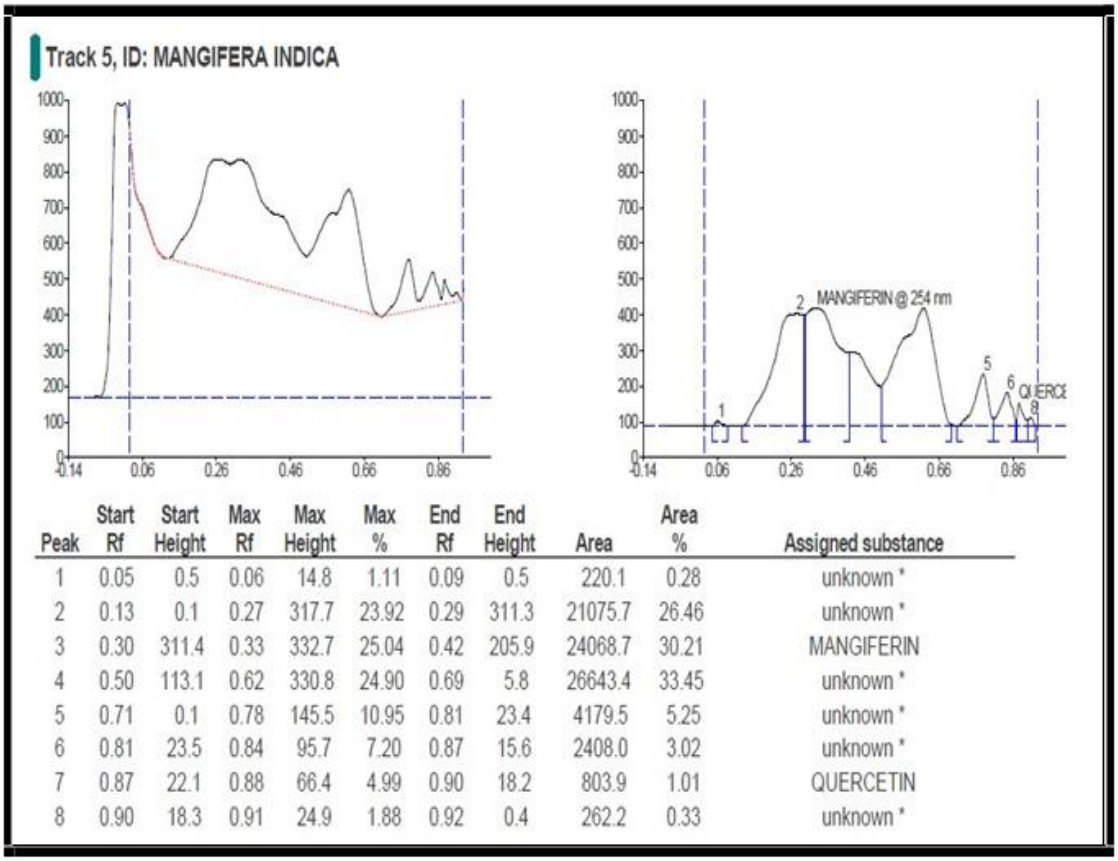
Detector: ferric chloride reagent spray

Rf value: 0.49.

HPTLC CHROMATOGRAM OF THE COMPOUND

A Camag (Muttenz, Switzerland) HPTLC system including a Lino-mat V sample applicator, a Camag twin-trough plate developmentchamber, Camag TLC Scanner 3 and WinCATS integration soft-ware was used. Aluminum backed HPTLC plates 10 cm × 10 cm with0.2 mm layers of silica gel 60F254(E. Merck, Darmstadt, Germany),pre-washed with methanol, were used (Agrawal et al., 2013). Thelength of the chromatogram run was 8 cm. Subsequent to chro-matographic development, TLC plates were dried in air with the help of a TLC plate dryer.

Mangifera indica L. Total Extract Chromatogram:10µ/ml



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.5	0.06	14.8	1.11	0.09	0.5	220.1	0.28	unknown *
2	0.13	0.1	0.27	317.7	23.92	0.29	311.3	21075.7	26.46	unknown *
3	0.30	311.4	0.33	332.7	25.04	0.42	205.9	24068.7	30.21	MANGIFERIN
4	0.50	113.1	0.62	330.8	24.90	0.69	5.8	26643.4	33.45	unknown *
5	0.71	0.1	0.78	145.5	10.95	0.81	23.4	4179.5	5.25	unknown *
6	0.81	23.5	0.84	95.7	7.20	0.87	15.6	2408.0	3.02	unknown *
7	0.87	22.1	0.88	66.4	4.99	0.90	18.2	803.9	1.01	QUERCETIN
8	0.90	18.3	0.91	24.9	1.88	0.92	0.4	262.2	0.33	unknown *

Sample concentration

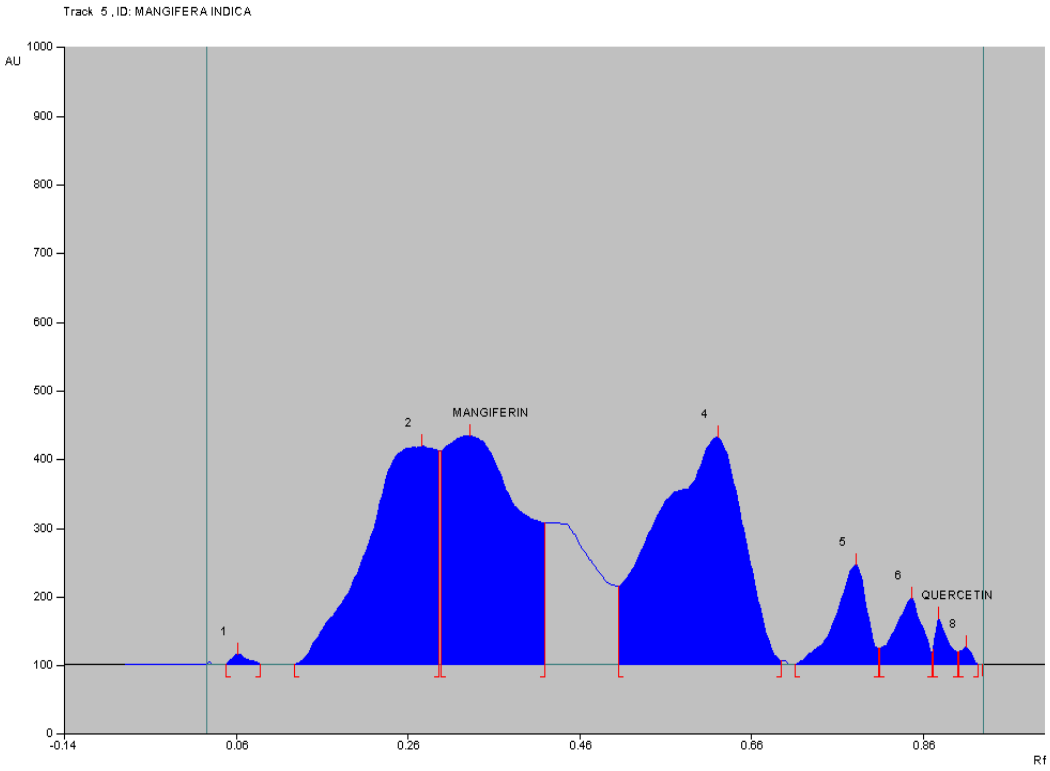
100mg in 1ml 100,000 mg in1000 µl

Therefore1 µl contains 100 µg of sample of extract

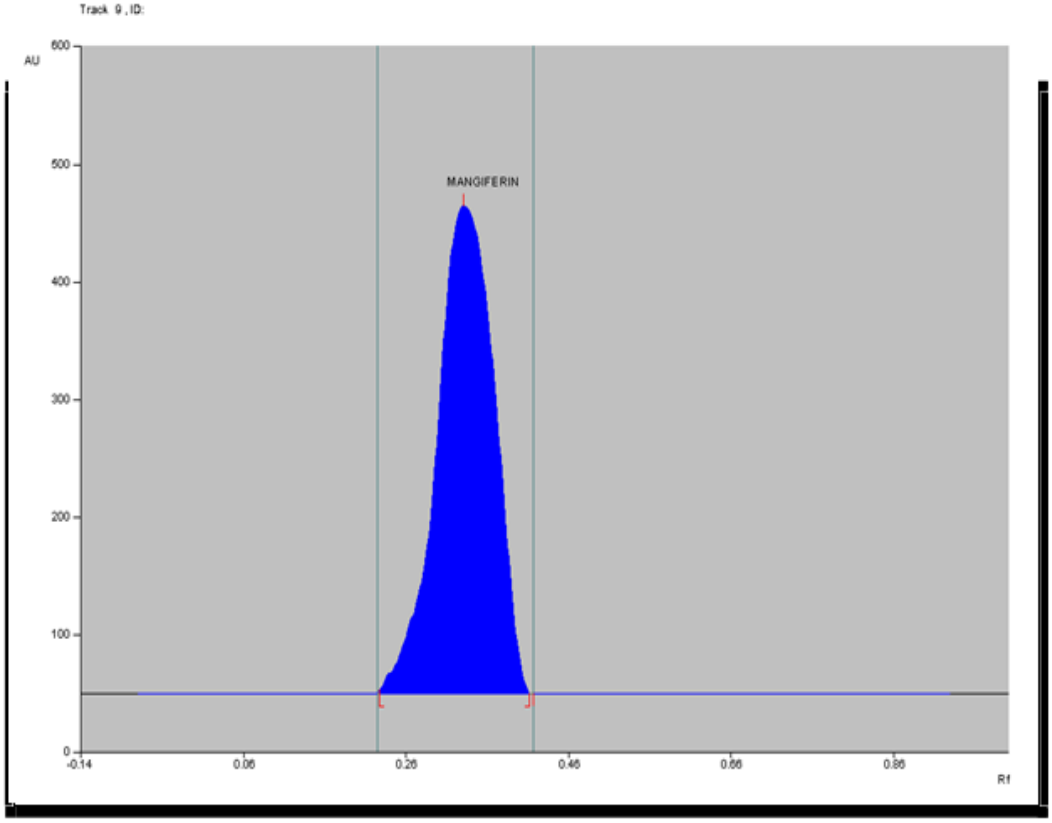
For 5 µl of standard contains area of 28356.2 for 5 µg of std.

But, 10 µl of sample contains area of 24068.7 for 1000 µg of extract.

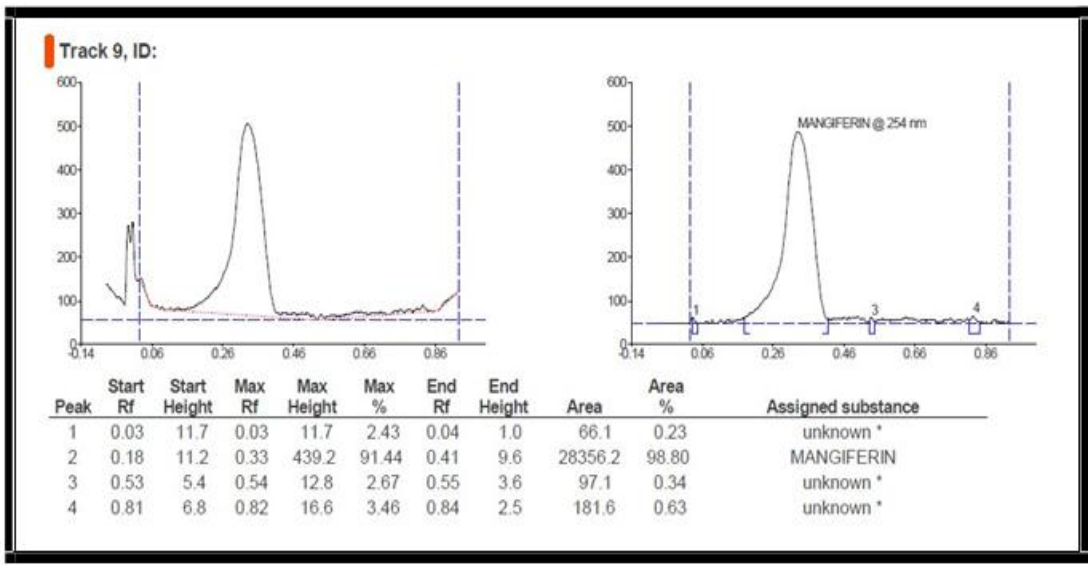
Mangifera indica L. Extract Chromatogram



STANDARD MANGIFERIN CHROMATOGRAM

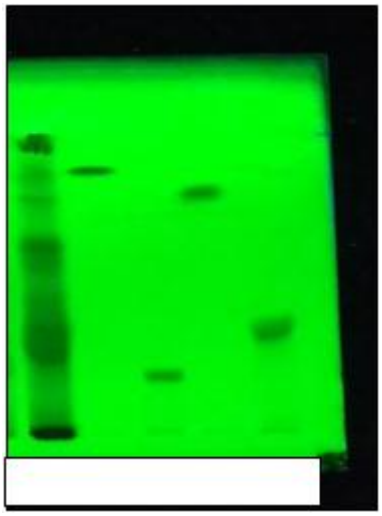


Standard Mangiferin Chromatogram



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.03	11.7	0.03	11.7	2.43	0.04	1.0	66.1	0.23	unknown *
2	0.18	11.2	0.33	439.2	91.44	0.41	9.6	28356.2	98.80	MANGIFERIN
3	0.53	5.4	0.54	12.8	2.67	0.55	3.6	97.1	0.34	unknown *
4	0.81	6.8	0.82	16.6	3.46	0.84	2.5	181.6	0.63	unknown *

Standard concentration: 1000µg in 1000 µl of methanol Therefore 1 µg in 1 µl Volume applied of standard 5 µg in 5 µl.



1 2 3 4 5

1. Mangifera leaf extract 2. Quercetin

3. Rutin 4. Galli acid 5. Mangiferin

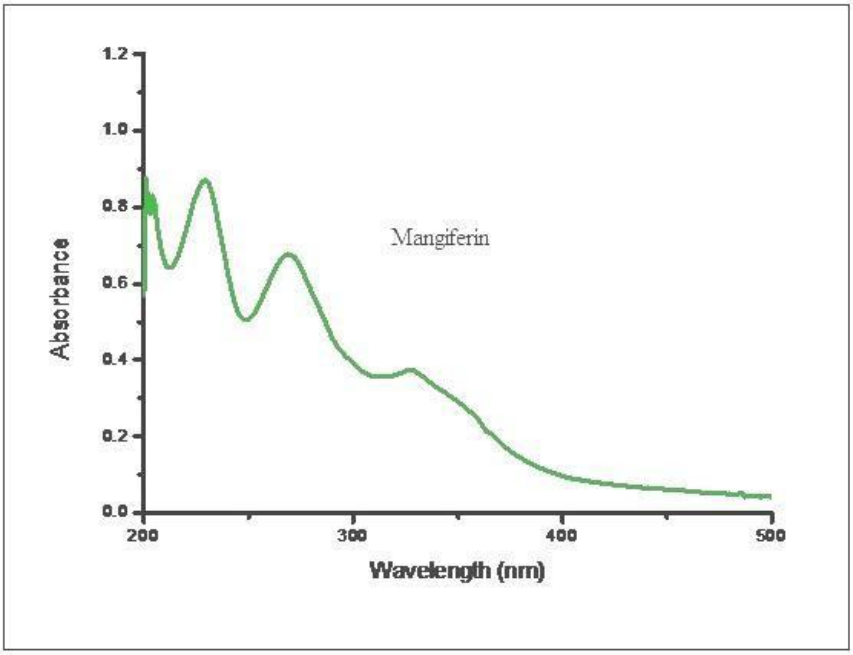
MELTING POINT

The melting point of isolated *Mangiferin* was found to be 269-270^{0}C.

STRUCTURAL ELUCIDATION REPORT OF ISOLATED MANGIFERIN

1. Ultra violet spectroscopy analysis of the compound

About 5µM of compound was dissolved in methanol and analyzed using UV-Visible spectroscopy in the range of 200-400 nm. This study shows a peaks at region 230, 270 and 330 nm 215nm which corresponds to n-π* and π-π* transition.



UV spectrum of the compound

The result clearly indicates that the isolated compound has moiety which was probably due to the presence of flavonoids, phenolic compounds, and organic acids.

2. FT-IR analysis of the isolated compound

The FT-IR spectral analysis was done for the above isolated compound in orderto find the functional groups (Fig-IR) A broad intense peak was obtained at 3,367 cm^{-1} represents phenol O-H stretch bonding interaction, the most intense bands appear in the 1,670 cm^{-1} region usually involving a combination of the C=O stretching, aromatic C=C stretching (1,624 cm^{-1}), and aliphatic C–H stretching(2918,2849cm^{-1})vibrations.

STANDARD FT-IR SPECTRUM

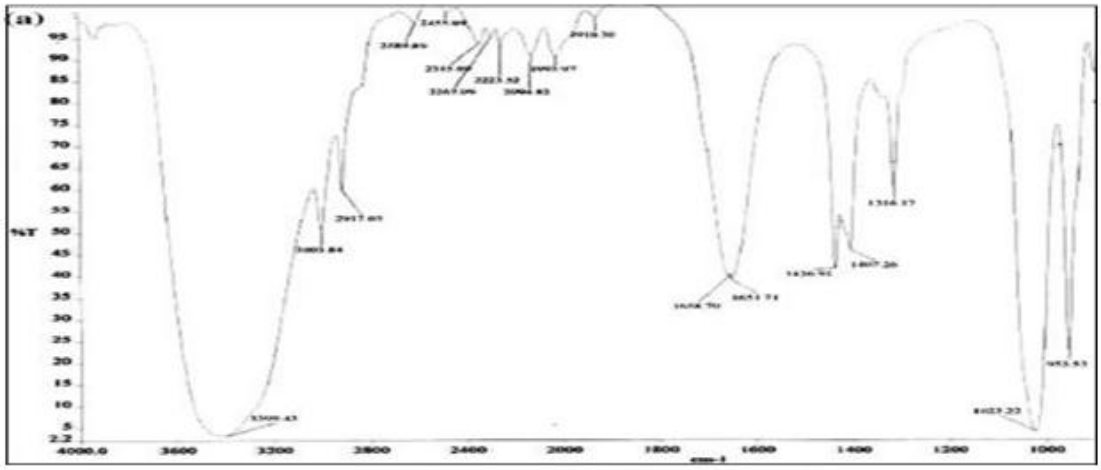
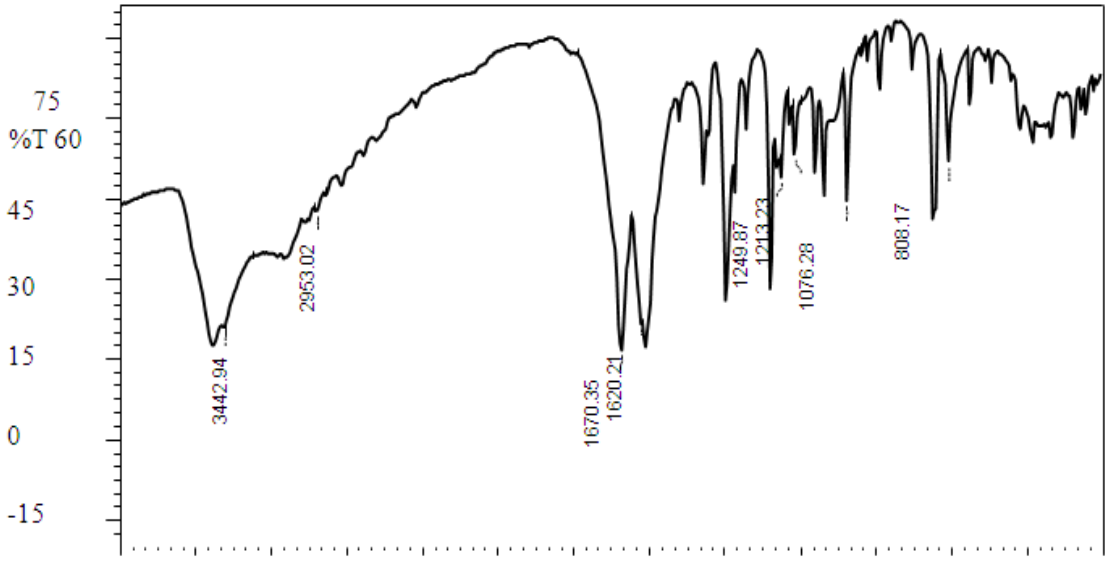


Table 5. Interpretation of IR spectra of the isolated sample.

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

INTERPRETATION OF IR-SPECTRA OF THE ISOLATED SAMPLE



4000	3200	2400	1800	1400	1000	800	600	400
mangiferin						1/cm		

Table -1

S.NO	ABSORBANCE (CM^{-1})	GROUPS
1	3442.94	Phenol O-H Stretching
2	2953.02	Aliphatic C-H stretching
3	1670.35	Keto C=O stretching
4	1620.21	Aromatic C=C stretching
5	1213.23	Ar-O-Ar ether C-O-C stretching
6	1076.28	RCH2OH O-H stretching

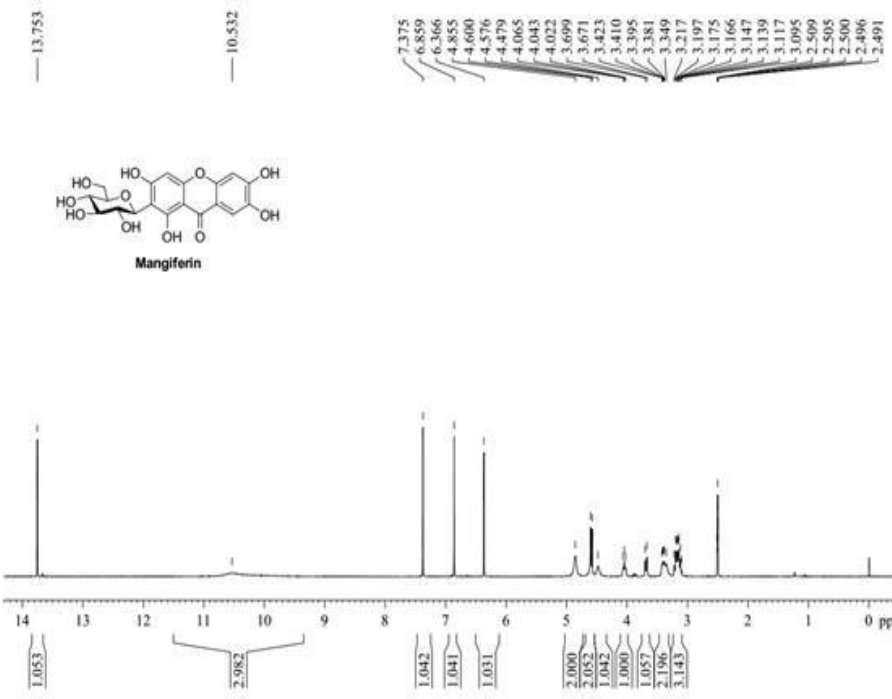
3. Nuclear magnetic resonance spectroscopy analysis of the compound

The Compound was also characterized by NMR analyses.

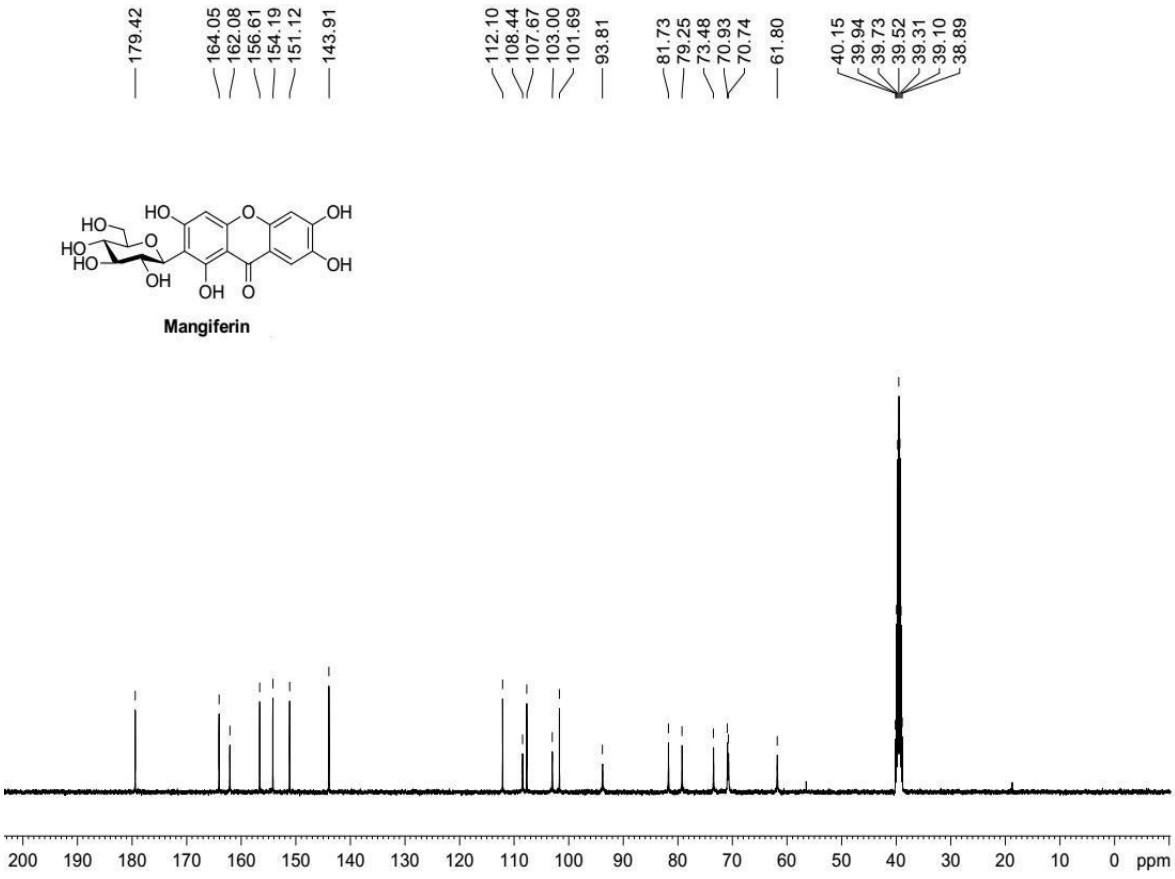
^{1}H NMR (DMSO, 400 MHz): 13.80 (l-OH), 6.40 (lH, s, H-4), 6.86 (1H, s, H-6), 7.41 (s, H-8), 4.60 (d, H-1') 4.03 (t, H-2), 79.0, 70.6, 81.5 (m, H-3', H-4', H-5'), 3.40, 3.60 (dd, H-6').

^{13}C NMR(DMSO, 400MHz): 161.7 (C-1),107.5 (C-2), 163.8 (C-3), 93.3 (C-4), 102.4 (C-5),150.9 (C-6), 143.9 (C-7), 107.8 (C-8), 111.4 (8a), 154.6 (8b) 179.0 (CO), 73.1 (C-1'), 70.3 (C-2'), 79.0, 81.5 (C-3', C-5'), 70.6 (C-4').

^{1}H NMR SPECTRA OF MANGIFERIN



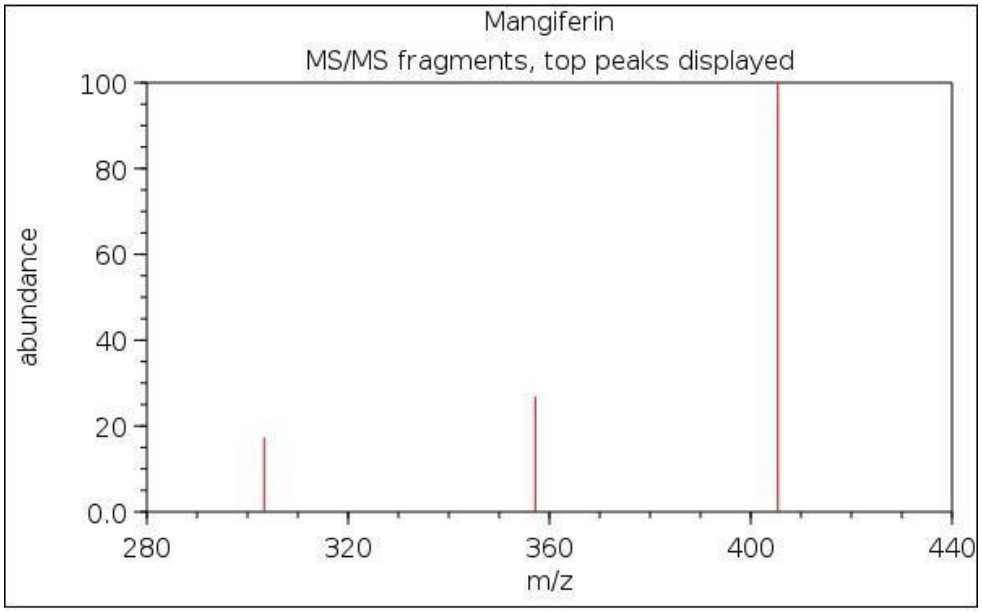
13C NMR OF MANGIFERIN



Position	^{13}C	^{1}H, m, *J* (Hz)
1	161.7	13.80 (1-OH)
2	107.5	-
3	163.8	-
4	93.3	6.40, s
4a	156.2	-
4b	101.2	-
5	102.4	6.86, s
6	150.9	-
7	143.9	-
8	107.8	7.41, s
8a	111.4	-
8b	154.6	-
CO	179.0	-
1´	73.1	4.60, d, 8.3
2´	70.3	4.03, t, 9.5
3´	79.0	3.16, m,
4´	70.6	3.16, m
5´	81.5	3.16, m
6´	61.4	3.40, dd, 11.0, 2.1 3.60, dd, 11.0, 4.6

4. LC-MS Analysis of the compound

The molecular mass of the isolated compound was tested by GC-MS, gave a molecular ion peak at m/z 422.33 g/mol $[M-H]^-$ with the molecular formula $C_{19}H_{18}O_{11}$.



Thus, the compound has molecular weight of 422.33 g/mol. Melting point is 270°C. From the results obtained from the above spectral studies, the molecular formula is confirmed as $C_{18}H_{19}O_{11}$. The observed results were well in accordance with previously reported literatures for the above compound.

The data proves the presence of the structure of the compound and it's known as **MANGIFERIN.**

ANTI-ANGIOGENIC ACTIVITY

Aqueous and ethanolic extract of Mangiferin from Mangifera indica are potent anti-angiogenic effect and have demonstrated in chick embryo.

Various assays are used to screen natural health products for anti-angiogenic activity.

PHARMACOLOGICAL ACTIVITY

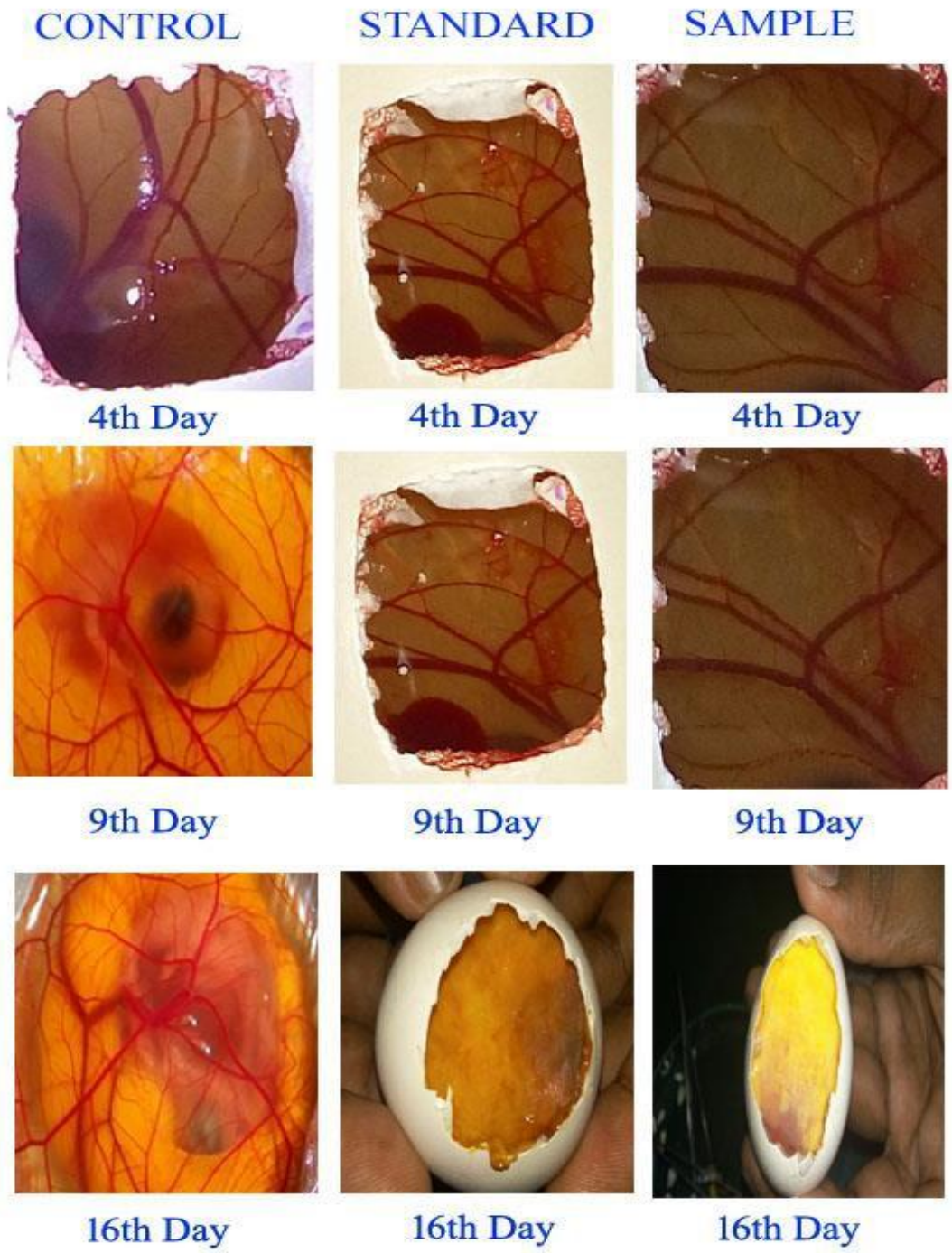


Fig-2

Mangiferin that inhibit the process of new blood vessels formation from existing vessels. These results shows that Mangiferin demonstrated Anti-Angiogenic activities and these properties could be pharmacological relevant for its use of treatment of some Tumors where neovascularization results essentially for the development of the malignance process.

Observation of blood vessels on CAM

Table -2

S.No	DAYS	DOSES	NO. OF BRANCHES(Mean±SD)		
			CONTROL (without drug)	STD (Itraconazole)	TEST (Mangiferin)
1	1	10 µg/ml	26.4±1.14	25.6±1.14	26.8±1.30
2	4	15 µg/ml	45.2±0.83	21.6±1.14	19.8±0.83
3	7	20 µg/ml	94.6±1.14	3.8±0.44	1.4±0.54

ACTIVITY REPORT

Table-3

S.NO	EGGS	INFERENCE
1	CONTROL	Gradually increasing the branches of blood vessels
2	STD	Gradually reducing the branches of blood vessels
3	TEST	Significantly reducing the branches of blood vessels

DISCUSSION

The xanthone glycoside of Mangiferin (1,3,6,7 tetrahydroxy xanthone-2 glycopyranoside) reported to have pharmacological activities including anti- angogenic activity was isolated and identified by melting point, TLC. The IR data showed relevant bands for the functional groups present in the isolated compound. The ^{1}H NMR and ^{13}C NMR also showed relevant proton peaks for the isolated compound. The molecular weight of the compounds were done by MASS Spectroscopy.

The **3R's** principle (Reduction, Refinement, and Replacement) was implemented that help to minimize harms to vertebrate animals used in science. Therefore we selected CAM assay model, our study due to its transparency for studying vascular growth. Further studies to be feasible it is vital to find economical and ecologically acceptable ways of producing mangiferin. and it has to be made on *Mangifera indica* helps to develop therapeutic agent which are multi targeted to fight against several diseases.

"Prevention is always better than cure" is absolutely true in the case of our study. Angiogenesis is one of a factor for growing cancer disease so I carried my work through Anti-angiogenic activity finally results showing prevention of malignancy. Therefore, there is need to develop value added products to enhance mango utilization and minimize losses.

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