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Pharmacognostic standerdization, HPTLC and *in-vitro* antioxidant studies of *Averrhoa carambola* L. leaves

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Key words

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ABSTRACT:

This paper deals with the detailed pharmacognostical evaluation, HPTLC studies and in-vitro *anti- oxidant activity of the leaves of A. carambola (Oxalidacea). The physicochemical, morphological and histological parameters studied in this study may be proposed as parameter to establish the authenticity of A. carambola. The microscopical evaluation defines the presence of anomocytic stomata , multicellular trichomes, calcium oxalate crystals, starch grains, spiral vessels and characterstic collenchymas and vascular bundles. Various leaf constants were obtained. In physico-chemical evaluation total ash, acid- insoluble ash, water soluble ash and extractive value using different solvent were studied. Fluorescence analysis and elemental ash analysis was attempted to identify fluorescence and various elements such as iron, calcium, sulphates, nitrates, carbonates, etc. in Averrhoa carambola leaves.*

Successive solvent extracts and crude extracts including ethanolic, hydro –alsoholic and water extract, were subjected to preliminary chemical test revealing the presence of carbohydrates, alkaloids, saponins, tannins, and cardiac glycosides. HPTLC studies were used to quantify gallic acid and quercetin content of A. carambola leaves. The concentration of gallic acid and quercetin in test sample was found to be 0.271 mg /g and 0.784 mg/g respectively. Anti- oxidant activity was carried out through model viz. DPPH assay and hydrogen peroxide assay and results were found positive.

INTRODUCTION

The use of plant as medicine is as old as human civilization. People of all ages in both developed and developing countries use plants in an attempt to cure various disease and to get relief from physical suffering. *A. carambola*l L. (Oxalidacea), known as star fruit is native form tropical and sub- tropical regions of Asia. It was introduced to Brazil in 1817. *A. carambola* has been used as an appetite stimulant, anti-diabetic, an anti- emetic, an anti- diarrhoeal, an antifebrile and treatment of eczema. Also,

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decoction of leaves has been used in diabetic treatment. Previous investigation on *A. carambola* have revealed the presence of steroids and triterpenes, cynidin- glycosides, o-glycosyl flavonoids and C- glycosyl flavones.

The species *A. carambola* presents hypoglycaemic, hypocholesterolemic, anti-microbial, anti-oxidant and anti- inflammatory effects [9].

In the present work, the pharmacognostical standardization have been perforfmed for the leaf of the plant alongwith in- vitro anti- oxidant activity viz. DPPH and hydrogen peroxide assay.

MATERIALS AND METHODS

Plant Material

The leaves of *A .carambola* L. (Oxalidacea) were collected from the herbal garden of Babu Banarasi Das University, Lucknow and identification was performed by Dr .S . Khatoon, scientist, Pharmacognosy and Ethnopharmacology division, National Botanical Research Institute, Lucknow. A dried specimen was deposited in herbarium (voucher specimen no. NBRI/ CIF/ 323/ 2012).

Macroscopical Characterization [3] [8]

Macroscopical studies of leaves was done by naked eye and shape, colour, odour, and taste of leaf was determined and reported.

Microscopical Characterization [3] [8]

For the investigation of structure of the leaf transeverse section was studied. Study included the preparation of slide for leaf lamina, epidermal peel, and leaf powder. Preparation of slide include dissection, cleaning, clearing, staining and mounting of specimen obtained from drug and observed under compound microscope.

Leaf Constant [8]

Leaf fragments were observed under microscope for presence and quantification of epidermal cell. Stomata (type and distribution), vien islet number and vein termination number. Stomatal index was calculated as the percentage of number of stomata present per number of epidermal cell and each stoma was counted as one.

Physico- chemical Parameters [3] [4]

The shade dried leaves of *A.carambola* was subjected for determination of physic-chemical parameters such as foreign organic matter, total ash, acid insoluble ash, water soluble ash, sulphated ash, loss on drying, and foaming index.

Determination of extractable matter was done by hot and cold extraction using different solvents. Solvents used for hot extraction were n-hexane, chloroform, ethyl acetate, ethanol and water while that used for cold extraction are ethanol and water.

2.6 *Estimation of Primary and Secondary Metabolites*

Gravimetric Method

Determination of Total Alkaloids [7]

5 gm of sample was weighed using a weighing balance and dispersed into 50 ml of 10% acetic acid solution in ethanol. The mixture was well shaken and then allowed to stand for about 4 hours before it is filtered. The filtrate was then evaporated to one quarter of its original volume on hot plate. Concentrated ammonium hydroxide was added drop wise in order to precipitate the alkaloids. A pre-weighed filter paper was used to filter off the precipitate and it was then washed with 1% ammonium hydroxide solution. The filter paper containing the precipitate was dried on an oven at 60^0C for 30 minutes, transferred into desiccator to cool and then reweighed until a constant weight was obtained. The constant weight was recorded. The weight of the alkaloid was determined by weight difference of the filter paper and expressed as a percentage of the sample weight analyzed. The experiment was repeated five times and reading recorded as the average of triplicates.

Determination of Total Saponins [7]

For the saponins determination, 5 g of each plant samples was weighed and was dispersed in 100 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55°C. The filtrate and the residue were re-extracted with another 100 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and about 30 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponin content was calculated in percentage.

Determination of Total Cardenolides [5]

Extract 0.25 g of drug (180 mesh) for 1 hour with 50 ml water at room temperature, with intermittent shaking. Add 5 ml of 15% w/v solution of lead acetate and shake. After few minutes add 7.5 ml of 4% w/v solution of sodium dihydrogen orthophosphate and filter. To 50 ml filtrate add 5 ml of 15% HCl. Heat under reflux on water bath for 1 hour and transfer to a separating funnel. Rinse the flask with two 5 ml quantities of water. Extract with chloroform (3 x 25). Dry the combined chloroform extract over anhydrous sodium sulphate.

Determination of Total Fibre by the Dutch method [8]

Weigh 2 gm of powdered drug in a beaker and add 50 ml of 10% v/v nitric acid. Heat to boil with constant stirring (till about 30 second after boiling starts). Strain through fine cotton cloth on a Buchner funnel. Give washing to residue with boiling water. Transfer residue from the cloth to a beaker. Add 50 ml of 2.5% v/v sodium hydroxide solution. Heat to boil, maintain at boiling point for 30 seconds, stirring constantly. Strain and wash with hot water as mentioned earlier. For quantitative determination, transfer the residue in a cleaned and dried crucible. Weigh the residue and determine percentage crude fiber.

Spectrophotometric method

Determination of total tannins content [16]

The principle behind this method is reduction of phosphotungstomolybdic acid in alkaline solution to produce blue colored complex with phenols. Standard tannic acid made by dissolving 100 mg in 100 ml distilled water (working standard 5:100 with water). Stock solution was made by dissolving 100 mg in 100 ml of water. The sample (0.1 ml) was made up to 10.0 ml. Standards were prepared by taking 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of stock and diluted up to 3.0 ml with distilled water. Folin denis reagent 0.5 ml, water 10.0 ml and 10.0 ml (0.01l) of 1N Na_2CO_3 were added and diluted up to 100.0 ml. The absorbance was measured at 515 nm, a calibration curve was plotted of concentration and absorbance. The concentration of the sample was extrapolated from the graph.

Concentration of phenols is given by concentration extrapolated from the graph × final volume/weight in g × dilution factor /volume aliquot × 1/1000 = c × 10/1 ×5/1 × 1/1000.

Determination of total flavonoids content [12]

Standardization of extracts was carried out based on the total flavonoids content of the extracts measured using aluminum chloride colorimetric assay. For this assay, 0.1 mg of extract was dissolved in 1 ml of methanol. The solution was then mixed with 0.1 ml of 10% (v/v) aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 mL of distilled water. After 30 minutes of incubation at room temperature, the absorbance reading of the reaction mixture was measured at 415 nm using spectrophotometer with methanol as blank. A standard curve was constructed using quercetin as the standard at the concentrations ranging from 50 to 200 ìg/ml. Total flavonoids content of the extracts was then compared with the standard curve, expressed in mg quercetin equivalents per 100 g dry weight (mg QE/100 g DW) and further calculated as % of total flavonoids content.

Determination of total sugars [2]

0.5 gram powdered material was homogenated in 80% ethanol with the help of Centrifuge at 2000 rpm for 15 minutes. The supernatant obtained is made upto known volume (generally up to 10 ml or depending on the expected concentration of sugar). Taken 0.2 ml aliquot, added 0.1 ml of 80% phenol and 5 ml conc. Sulphuric acid, then made the volume up to 10 ml with 80% ethanol, cooled in ice bath. Total sugar was calculated by using D-Fructose (mg/ml) as the standard whose reading was y=0.24x + 0.006, r^2= 0.998 at 490 nm using UV-1 Double beam spectrophotometer, where y was the absorbance and x the D- Fructose equivalent (mg/ml).

Elemental Ash Analysis [8]

Prepare ash of drug material, add 5ml of HCl and 5 ml of distilled water . shake well and then filtered.

- **Test for iron:** to the filtrate add ammonium thiocyanate, red ppt. is seen.
- **Test for calcium:** to the filtrate add ammonia solution+ potassium ferrocyanide , yellow ppt. is seen.
- **Test for chloride:** to the filtrate add silver nitrate, white ppt. is seen.
- **Test for sulphates:** to the filtrate add lead acetate, white ppt. is seen.
- **Test for carbonates:** to the filtrate add a solution of magnesium sulphate, white ppt. is seen.
- **Test for nitrates:** test with solution of ferrous sulphate yeild no brown colour but if sulphuric acid is added (slowly from the side of the test tube), a brown colour is produced at the junction of two liquid.

Phytochemical Analysis [8]

All the extracts were subjected to preliminary phytochemical screening for the detection of various chemical constituents. The presence or absence of various phyto constituents viz. triterpenoids, steroids, alkaloids, glycosides, flavonoids, sugar, tannins, etc. were detected by usual prescribed method.

Fluorescence Analysis [8]

The powdered drug along with crude ethanolic, hydro-alcoholic and aqueous extract were treated with different chemicals and was observed in day light and in UV (254 nm and 365 nm) observed fluorescence was reported.

Thin Layer Chromatography [6, 13, 14, 15]

10 mg per ml of *A.carambola* ethanolic leaf extract was dissolved in ethanol and used for TLC examination. TLC plates were prepared by using silica gel –G as adsorbent. 100 g silica gel –G was mixed with sufficient quantity of distilled water to make slurry. Slurry was immediately poured into a spreader and plates were prepared by spreading the slurry on glass plates of required size. Plates were allowed to dry for 1 hour and layer was fixed by drying at 110°c for 30 min. using a micropipette, about 10 plate µml of extracts were loaded gradually over and dried. The chromatograms were developed in glass chamber containing solvent system. The R_f value was obtained by using following formula:-

$R_{f=}$ Distance travelled by solute from the origin (cm)

Distance travelled by the solvent from the origin (cm)

High Performance Thin Layer Chromatography [1, 10]

Preparation of standard solution

Gallic acid and quercetin 1 mg was accurately weighed into a 10 ml volumetric flask and dissolved in ethanol and volume was make upto 10 ml. Further dilution was made with ethanol to obtain working standards of 20, 40, 60, 80 and 100 mg/ml each of gallic acid and quercetin respectively.

Preparation of sample solution

Dried powdered leaves were extracted in ethanol in soxhalet apparatus. Extracts were dried and concentrated under vacuum. 10 mg of dried ethanolic extract was dissolved in 10 ml of ethanol (concentration 1000 µg/ml).

Development of HPTLC techinique

The sample was spotted in the form of band with camg microlitre syringe (2µl) un a precoated silica gel plate 60 F_{254} (10 cm x 10 cm with 0.2 mm thickness, E.Merck) using CAMAG linomat 1v applicator. The plates were developed in a solvent system in CAMAG glass twin trough chamber previously saturated with solvent system for 30 minutes. The distance was 8 cm. subsequent to scanning TLC plates were air dried and scanning was performed on a camag TLC scanner operated by WINCATS software 4.03 version.

Stationary phase:	Silica gel plate 60 F_{254}
Mobile phase:	Benzene:pyridine:formic acid (7:1.8:1.5 v/v/v)
Standard:	Gallic acid (20, 40, 60, 80, and 100 µg/ml)
Sample:	Test solution (1000 µg/ml)
Scanning wavelength:	254 nm
Mode of scanning:	Absorption (illium)

Gallic acid estimation in test sample

Stationary phase:	Silica gel plate 60 F_{254}
Mobile phase:	Benzene :pyridine: formic acid (7:1.8:1.5 v/v/v)
Standard:	quercetin (20, 40, 60, 80, and 100 µg/ml)
Sample:	Test solution (1000 µg/ml)
Scanning wavelength:	366 nm
Mode of scanning:	Absorption (illium)

Quercetin estimation in test sample

Antioxidant Activity [11]

DPPH Assay

Preparation of Plant Extracts

The extracts was prepared by extracting 50g of powdered drug with 500 ml of ethanol in a Soxhlet apparatus. The extract was subjected to vacuum evaporation on a Buchi rota evaporator. 100 mg of extracts was taken, dissolved in ethanol and phosphate buffer respectively and final volume of flask was made up to 100 ml with same solvents. The final concentrations of solutions were 1000 ìg/ml which was used as stock solutions for further study. The different concentrations (20, 40, 60, 80, 100 ìg/ml) from stock solutions was prepared by diluting with ethanol.

Preparation of DPPH Solution

Solution of DPPH (0.1mM) in ethanol was prepared by dissolving 1.9 mg of DPPH in methanol and volume was made up to 100ml with ethanol. The solution was kept in darkness for 30 minutes to complete the reaction.

Determination of Anti-Oxidant Activity

1ml of DPPH solution was added to 1ml of different extracts and allowed to stand at room temperature for 30 min, and then absorbance was measured at 517 nm in a spectrophotometer. The percentage inhibition was measured by following formula:

$$\% \text{ inhibition} = (Ac-At) \times 100/Ac$$

Where Ac is the absorbance of control

At is the absorbance of test sample

Hydrogen Peroxide Assay

Preparation of Plant Extracts

The extracts was prepared by extracting 50g of powdered drug with 500ml of methanol in a Soxhlet apparatus. The extract was subjected to vacuum evaporation on a Buchi rota evaporator.

100 mg of extracts was taken, dissolved in ethanol and phosphate buffer respectively and final volume of flask was made up to 100 ml with same solvents. The final concentrations of solutions were 1000 ìg/ml which was used as stock solutions for further study. The different concentrations (20, 40, 60, 80, 100 ìg/ml) from stock solutions was prepared by diluting with phosphate buffer.

Preparation of Hydrogen Peroxide Solution

0.2M Potassium dihydrogen phosphate and 0.2M sodium hydroxide solutions were prepared as per the Indian Pharmacopoeia 1996 standards. 50 ml of Potassium dihydrogen phosphate solution was placed in a 200 ml volumetric flask and 39.1 ml of 0.2M sodium hydroxide solution was added in this and finally volume was made up to 200 ml with distilled water to prepare phosphate buffer (pH-7.4). 50 ml of phosphate buffer solution was taken and an equal amount of hydrogen peroxide was added in this to generate the free radicals and solution was kept a side to complete the reaction.

Determination of Anti-Oxidant Activity

1ml Extracts in distilled water was added to 0.6 ml of hydrogen peroxide solution and the absorbance was measured at 230 nm in a spectrophotometer. The percentage inhibition was measured by following formula.

% inhibition = (Ac-At) ×100/Ac

Where Ac is the absorbance of control

At is the absorbance of test sample

RESULTS

Macroscopical studies

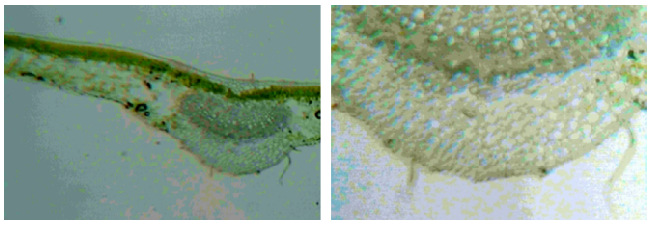
Leaves of *A .carambola* were 15-25 cm long present in 7-9 pairs, having pinnate venation, ovate to ovate lanceolate in shape, upper surface is smooth while lower surface is finely hairy, having acrid taste and characterstic odour. It is dark green on upper surface and light green on lower surface (Fig. 1a, b).

(a) (b)

Figure 1 (a) and (b) *Averrhoa carambola* leaves morphology

Microscopical studies

T.S. through midrib shows a single layered epidermis covered with striated cuticle (Fig. 2a, b). Epidermal cell are more or less rectangular or square in shape. Beneath the epidermal cells layer of chollenchymatous cell were present (Fig. 3). The central region of midrib is occupied by vascular tissue which are arch shaped showing showng phloem capping the xylem (Fig. 4). calcium oxalate crystals were large, single or in small groups (Fig. 5).



(a) (b)

Figure 2: (a) and (b) Transeverse section of *Averrhoa carambola* leaves through midrib

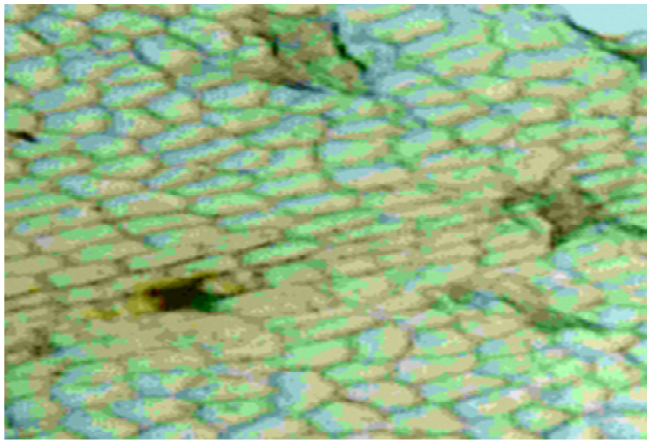


Figure 3: Region below epidermal cells showing palisade cell, made by paranchymatous cell

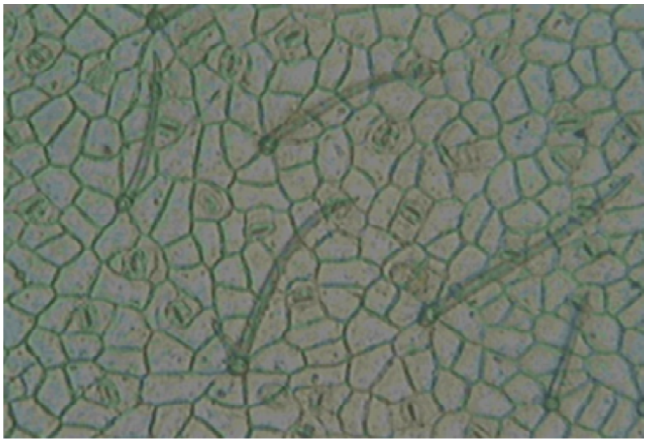


Figure 4: Anomocytic stomata and multi- cellular trichomes (mesophyll region).

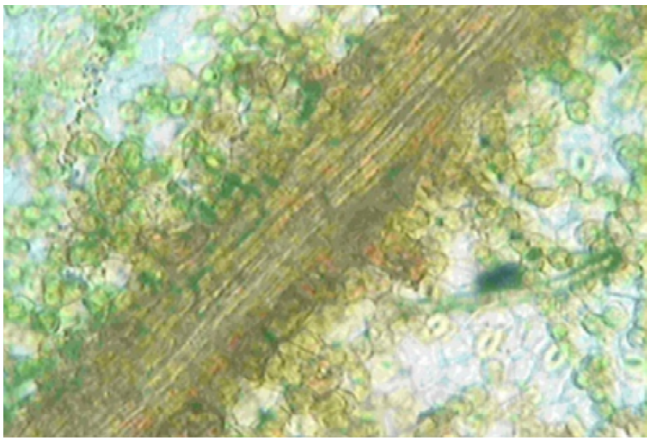


Figure 5: Vascular bundles

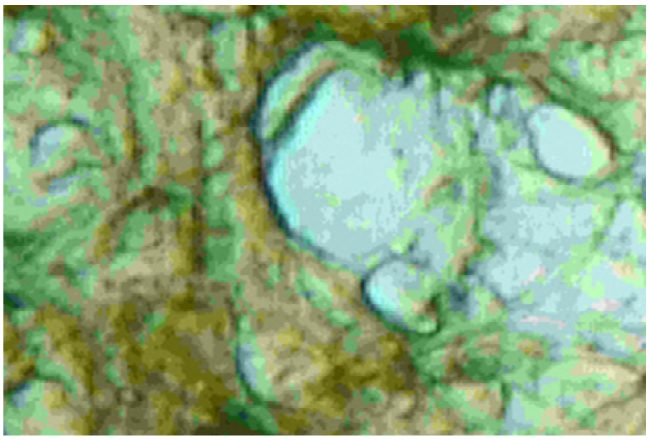


Figure 6: Calcium oxalate crystals

Microscopical studies of powder reveals the presence of large and single calcium oxalate crystals (Fig. 6&7). starch grain resembles those of rice starch, were polyhedral and ovate (Fig. 8). spiral vessels are present in spring like fashion (fig 3.3). fibres are unicellular, flattened, twisted, having large lumen and waxy cuticle. Xylem vessels are also visible. Cells containing oil globules were stained with sudan red II appear red in colour. Cells containing tannins were stained bluish-green with ferric chloride (Fig. 9-14). The result of quantitative microscopy of leaf of *A.carambola* are presented in Table 1.

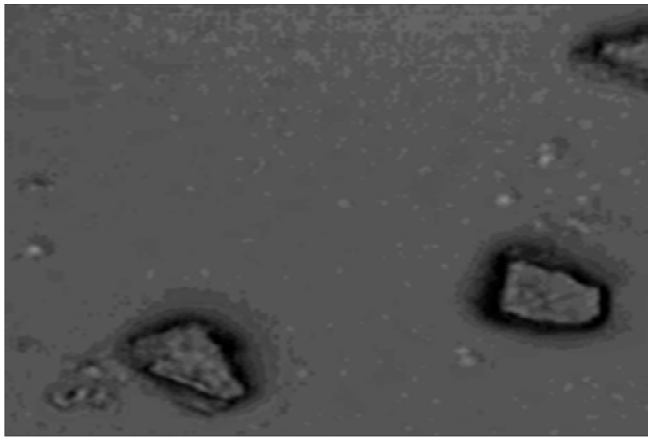


Figure 3.1 Calcium oxalate crystals

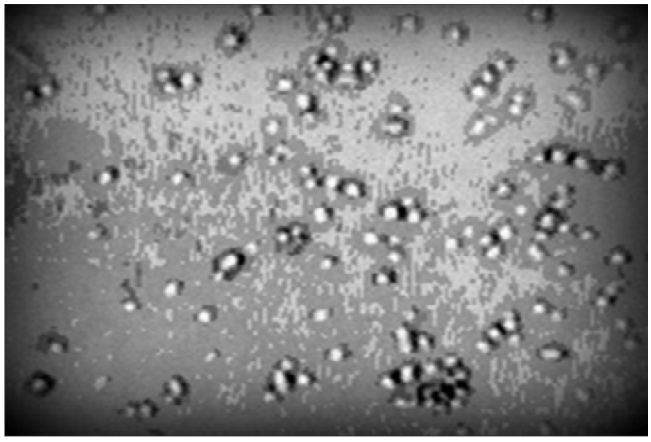


Figure 8: Starch grains

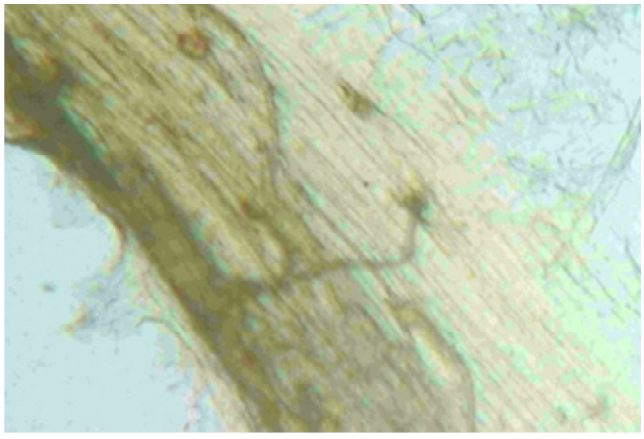


Figure 9: Xylem vessels

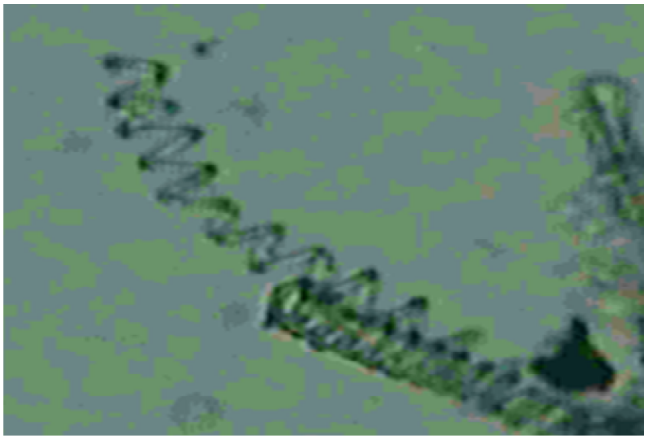


Figure 10: Spiral vessels

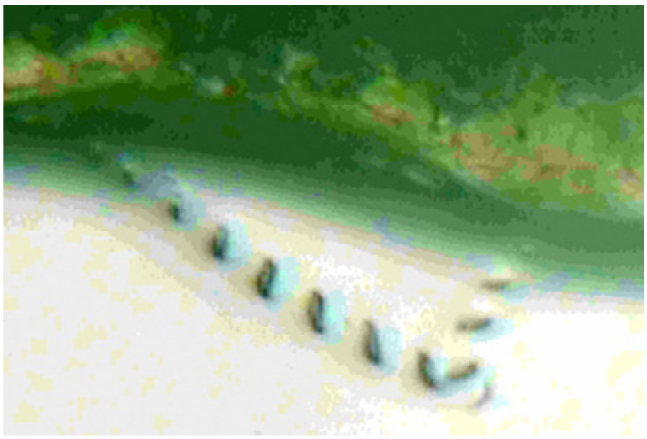


Figure 11: Spiral vessels

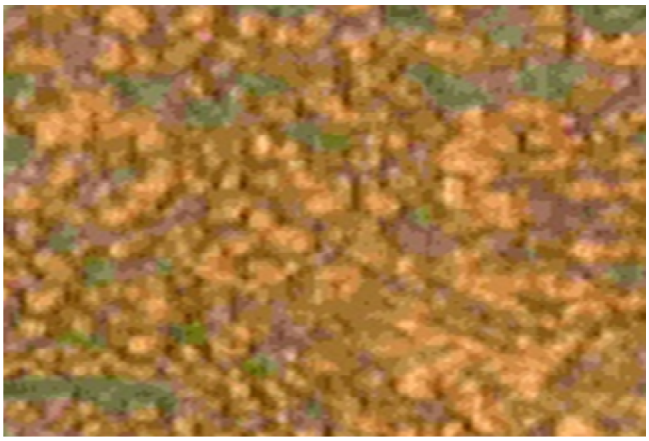


Figure 12: Cells containing oil globules

Figure 13: Cells containing tannins

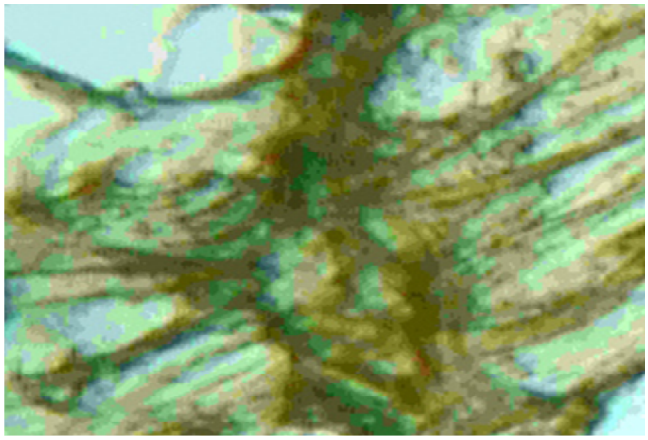


Figure 14: Fibres

Table 1: Leaf constants

Parameters	**Values [AVG ± SD] n = 4**
Stomatal size (in micron)	
Upper epidermis Lower epidermis	110.44 ± 0.6 117.42 ± 0.5
Stomatal index (per mm^2)	
Upper epidermis Lower epidermis	24.58 ± 0.2 22.05 ± 0.2
Vein islet number (per mm^2)	17.25 ± 0.2
Vein termination number (per mm^2)	24.00± 0.2

Physic-chemical properties

The physic-chemical character of powdered drug of leaves of *A.carambola* such as foreign matter, total ash, acid insoluble ash, water soluble ash, sulphated ash, loss on drying, foaming index and extractive value by hot and cold maceration using various solvents are presented in Table 2.

Estimation the of primary and secondary metabolite

The phytochemical quantitative composition of *A.carambola* by gravimetric method including

Table 2: Physico-chemical parameters

Standard Parameters	Values [%(W/W) ± SD] n= 3
Foreign matter	0.5± 0.05
Total ash	7.73 ± 0.1
Acid-insoluble ash	0.15 ± 0.05
Water soluble ash	3.28 ± 0.07
Sulphated ash	10.33 ± 0.5
Loss on drying	1.86 ± 0.002
Foaming index	0.5 ± 0.04
Extractive values by cold maceration	
n-hexane soluble extractive	60.95 ± 0.03
Chloroform soluble extractive	51.84 ± 0.05
Ethyl acetate soluble extractive	61.99 ± 0.01
Ethanol soluble extractive	59.11 ± 0.01
Water soluble extractive	58.77 ± 0.01
Extractive values by hot maceration	
Ethanol soluble extractive	23.46 ± 0.4
Water soluble extractive	27.84 ± 0.1

estimation of total alkaloids, total saponins, total cardenolides and total fibers are shown in Table 3. Spectrophptometric estimation of total tannins, total flavonoids and total sugar are enumerated in Table 4-6 respectively along with standard calliberation curve of gallic acid (Fig. 15), quercetin (Fig. 15) and fructose (Fig. 16).

Table 3: Estimation of primary and secondary metabolites by gravimetric method

S.no.	Primary/secondary metabolites	Values [%(W/W) ± SD] n=3
1.	Total Alkaloids	5.23 ± 0.05
2.	Total Saponins	1.23 ± 0.05
3.	Total Cadenolides	2.13 ± 0.2
4.	Total Fibres	5.16 ± 0.02

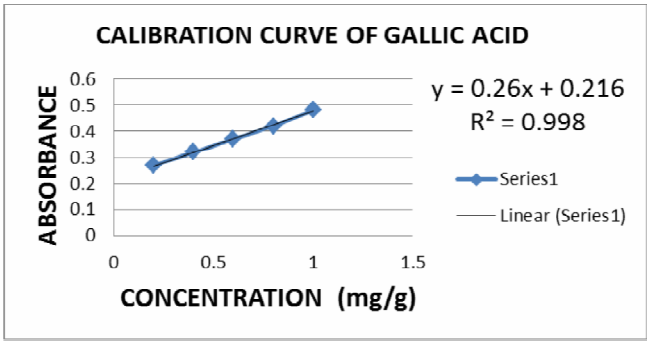


Figure 15: Calibration curve of gallic acid

Table 4: Result of gallic acid content in *Averrhoa carambola* leaves.

Parameter	Values [mg/100g]
Average	55.3
Range	30 - 106

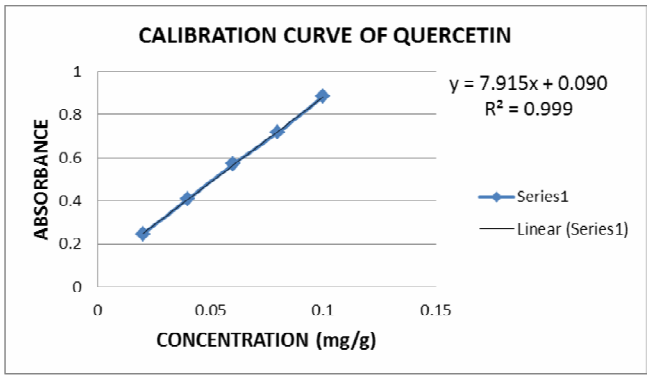


Figure 16: Calibration curve of quercetin

Table 5: Result of quercetin content in *Averrhoa carambola* leaves.

Parameter	Values [mg/100g]
Average	1.3
Range	1.2 – 1.5

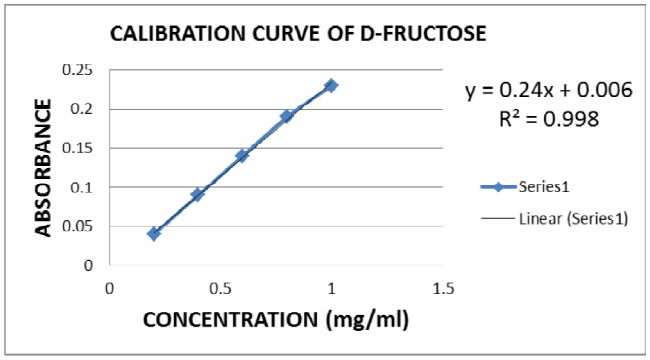


Figure 17: Calibration curve of D-fructose

Table 6: Result of D-fructose content in *Averrhoa carambola* leaves.

Parameter	Values [mg/100g]
Average	1440
Range	1400 - 1460

Elemental ash analysis

Detection of various elements such as iron , calcium chloride, sulphates, carbonates and nitrates were conducted on ash of leaves of *A.carambola* . Result was enumerated in Table 7

Table 7: Elemental ash analysis

S. No.	Tests	Result
1.	Test for Iron	+
2.	Test for Calcium	+
3.	Test for Chloride	+
4.	Test for Sulphates	+
5.	Test for Carbonates	-
6.	Test for Nitrates	-

Extraction and percentage yield

After extraction with different solvents, the residues were dried and measured. Percentage yield from successive solvent extraction are enumerated in Table 8 and % yield of crude extract is enumerated in Table 9.

Table 8: Percentage yield of extract obtained from successive solvent extraction

S. No.	Extracts	Values [%(W/W) ± SD] n=3
1.	n-hexane	5.51 ± 0.05
2.	Chloroform	2.41 ± 0.05
3.	Ethyl acetate	4.34 ± 0.05
4.	Ethanol	14.32 ± 0.05
5.	Water	6.18 ± 0.05

Table 9: Percentage yield of crude extract obtained from various solvents

S. No.	Extracts	Values [%(W/W) ± SD] n=3
1.	Methanol	35.42 ± 0.04
2.	Hydro - alcoholic	26.68 ± 0.05
3.	Water	20.60 ± 0.05

Determination of fluorescence analysis

The fluorescence analysis of powdered drug of *A.carambola* in various solvents and chemical reagent was performed under day light and UV (254 nm and 365 nm). Fluorescence analysis of crude ethanolic, crude hydro-alcoholic and crude water extract is tabulated in Tables 10-13.

Preliminary phytochemical screening

Phytochemical analysis showed the presence of carbohydrates, alkaloids, tannins, flavonoids, saponis and glycosides in different extracts obtained from successive solvent extraction (Table 14) and from crude extracts (Table 15).

Table 10: Fluorescence analysis of leaf powder

S. No.	Treatment	Visible	Short U.V. 254 nm	Long U.V. 365 nm
1.	Powder	Dark Green	Black	Light Green
2.	Powder +1N NaOH (aq.)	Light Green	Black	Dark Green
3.	Powder +1N NaOH (alc.)	Dark Green	Grey	Light Green
4.	Powder +1N HCl	Light Brown	Grey	Light Green
5.	Powder +50% H_2SO_4	Light Green	Black	Dark Green
6.	Powder +Conc. H_2SO_4	Dark Brown	Black	Black
7.	Powder +Picric acid	Yellowish Green	Brown	Light Green
8.	Powder +Acetic acid	Dark Brown	Black	Dark Green
9.	Powder +5% $FeCl_3$	Dark Green	Black	Dark Green
10.	Powder +5% Iodine	Light Green	Grey	Light Green
11.	Powder +50% HCl	Light Green	Grey	Light Green
12.	Powder +Ammonia	Light Brown	Grey	Light Green
13.	Powder +1N KOH (aq.)	Greenish Brown	Grey	Light Green
14.	Powder +1N KOH (alc.)	Dark Brown	Black	Dark Green
15.	Powder +Methanol	Dark Green	Grey	Light Green
16.	Powder +5% KOH	Yellowish Green	Brown	Light Green
17.	Powder +5% NaOH	Yellowish Green	Black	Light Green

Table 11: Fluorescence analysis of crude ethanolic extract

S. No.	Treatment	Visible	Short U.V. 254 nm	Long U.V. 365nm
1.	Extract	Light Brown	Light Green	Black
2.	Extract +1N NaOH (aq.)	Light Brown	Yellowish Green	Black
3.	Extract +1N NaOH (alc.)	Yellowish Green	Yellowish Green	Grey
4.	Extract +1N HCl	Light Green	Light Green	Grey
5.	Extract+50% H_2SO_4	Light Green	Yellowish Green	Black
6.	Extract +Conc. H_2SO_4	Brown	Dark Green	Black

7.	Extract +Picric acid	Yellowish Green	Yellowish Green	Black
8.	Extract +Acetic acid	Light Green	Light Green	Black
9.	Extract +5% $FeCl_3$	Dark Brown	Dark Brown	Black
10.	Extract +5% Iodine	Light Brown	Light Green	Black
11.	Powder +50% HCl	Light Green	Light Green	Grey
12.	Extract +Ammonia	Light Green	Light Green	Grey
13.	Extract +1N KOH (aq.)	Yellowish Green	Yellowish Green	Black
14.	Extract +1N KOH (alc.)	Yellowish Green	Yellowish Green	Black
15.	Extract +Methanol	Light Brown	Light Green	Black
16.	Extract +5% KOH	Yellowish Green	Yellowish Green	Black
17.	Extract +5% NaOH	Yellowish Green	Yellowish Green	Grey

Table 12: Fluorescence analysis of crude hydro-alcoholic extract

S. No.	Treatment	Visible	Short U.V. 254 nm	Long U.V. 365nm
1.	Extract	Light orange	Light Yellow	Grey
2.	Extract +1N NaOH (aq.)	None	Light Yellow	Grey
3.	Extract +1N NaOH (alc.)	None	None	Grey
4.	Extract +1N HCl	None	None	Grey
5.	Extract+50% H_2SO_4	None	Light Yellow	Grey
6.	Extract +Conc. H_2SO_4	Light Brown	Light Yellow	Grey
7.	Extract +Picric acid	Yellow	Yellow	Black
8.	Extract +Acetic acid	None	None	Grey
9.	Extract +5% $FeCl_3$	Light Green	Light Green	Black
10.	Extract +5% Iodine	Light Green	Light Green	Grey
11.	Powder +50% HCl	Light Brown	None	None
12.	Extract +Ammonia	Light Brown	None	None
13.	Extract +1N KOH (aq.)	Light Brown	Light Yellow	Grey
14.	Extract +1N KOH (alc.)	Light Brown	Light Yellow	Grey
15.	Extract +Methanol	Light Brown	None	Grey
16.	Extract +5% KOH	Light Brown	None	Grey
17.	Extract +5% NaOH	Light Brown	Light Green	Grey

Table 13: Fluorescence analysis of crude water extract

S. No.	Treatment	Visible	Short U.V. 254 nm	Long U.V. 365nm
1.	Extract	Light Brown	None	Grey
2.	Extract +1N NaOH (aq.)	None	None	None
3.	Extract +1N NaOH (alc.)	None	None	None
4.	Extract +1N HCl	None	None	None
5.	Extract+50% H_2SO_4	None	None	None
6.	Extract +Conc. H_2SO_4	None	None	Grey
7.	Extract +Picric acid	Yellow	Yellow	Black
8.	Extract +Acetic acid	None	None	None
9.	Extract +5% $FeCl_3$	Light Green	Light Yellow	Black
10.	Extract +5% Iodine	Light Green	Light Yellow	Grey
11.	Powder +50% HCl	Light Brown	None	None
12.	Extract +Ammonia	Light Brown	None	Grey
13.	Extract +1N KOH (aq.)	Light Brown	None	Grey
14.	Extract +1N KOH (alc.)	None	None	None
15.	Extract +Methanol	None	None	Grey
16.	Extract +5% KOH	Light Brown	None	Grey
17.	Extract +5% NaOH	None	None	None

Table 14: Phytochemical screening of successive solvent extract

Class of primary/ secondary metabolites	Extracts				
	n-hexane	Chloro-form	Ethyl acetate	Ethanol	Water
Carbohydrate	-ve	+ve	-ve	+ve	+ve
Protein	-ve	-ve	-ve	-ve	-ve
Amino acid	-ve	-ve	-ve	-ve	-ve
Steroids	+ve	+ve	+ve	-ve	-ve
Flavonoids	-ve	+ve	+ve	+ve	-ve
Tannins	+ve	+ve	+ve	+ve	+ve
Alkaloids	-ve	+ve	-ve	+ve	+ve
Glycosides	+ve	-ve	-ve	+ve	+ve
Saponins	-ve	-ve	-ve	+ve	+ve
Anthraquinones	-ve	-ve	-ve	+ve	-ve
Fats and oils	+ve	-ve	-ve	-ve	-ve

Table 15: Phytochemical Screening of crude extract

Class of primary/ secondary metabolites	Extracts		
	Ethanol	Hydro alcoholic	Water
Carbohydrate	+ve	+ve	+ve
Protein	-ve	-ve	-ve
Amino acid	-ve	-ve	-ve
Steroids	+ve	-ve	+ve
Flavonoids	+ve	-ve	-ve
Tannins	+ve	+ve	+ve
Alkaloids	+ve	+ve	+ve
Glycosides	+ve	-ve	-ve
Saponins	+ve	+ve	+ve
Anthraquinones	+ve	+ve	-ve
Fats and oils	-ve	-ve	-ve

Figure 20: TLC plate of flavonoids in visible light

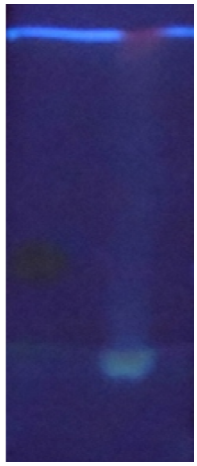


Figure 21: TLC plate of flavonoids in UV 366 nm

TLC examination

Ethanolic extract was subjected to TLC studies and important lead from the same are presented in Table 16; Fig 18-27.

Figure 18: TLC plate of tannins in visible light

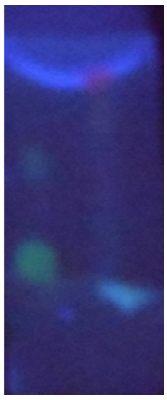


Figure 19: TLC plate of tannins in UV 366 nm

Figure 22: TLC plate of cardiac glycoside

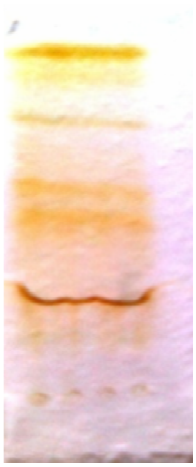


Figure 23: TLC plate of Anthraquinones

Table 16: TLC examination of crude ethanolic extract

Groups	Solvent system	Detecting agent/ UV	No. of spot	Rf value	Fig. no.
Tannins	Benzene: pyridine: formic acid (7:1.8:1.5)	Visible	1	0.27	7.22
		UV-366 nm	1	0.27	7.23
Flavonoids	Benzene: pyridine: formic acid (7: 1.8:1.5)	Visible	1	0.35	7.24
		UV-366 nm	1	0.35	7.25
Cardiac glycoside	Ethyl Acetate: Methanol:Water (5:1:4)	iodine vapour	3	0.21, 0.34, 0.61	7.26
Anthraquinones	Ethyl Acetate :Ethanol: Water (3:1:2)	iodine vapour	5	0.35, 0.54, 0.6, 0.77, 0.92	7.27
Saponins	Chloroform: Methnol : Water (6.7:2.8:0.5)	iodine vapour	4	0.26, 0.38, 0.49, 0.63	7.28
Steroids	Toluene: Chloroform (4: 3.5)	Visible	1	0.37	7.29
Alkaloids	n-butanol: ethyl acetate: formic acid: water (2:4:2:2)	iodine vapour	3	0.23, 0.46, 0.68	7.30
carbohydrates	n-butanol: acetic acid: ether: water (3:1.5:0.5:3)	Visible	1	0.22	7.31

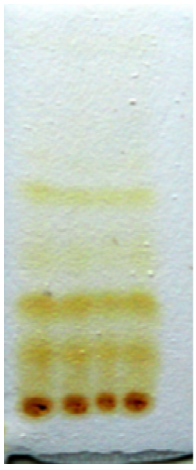


Figure 24: TLC plate of saponins

Figure 25: TLC plate of steroids

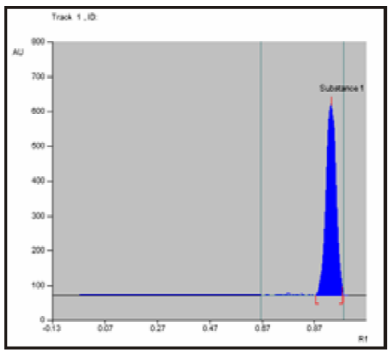
Figure 26: TLC plate of alkaloids

Figure 27: TLC plate of carbohydrates

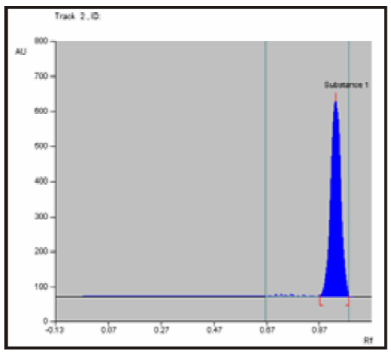
High Performance Thin Layer Chromatography

Determination of gallic acid in Averrhoa carambola leaves

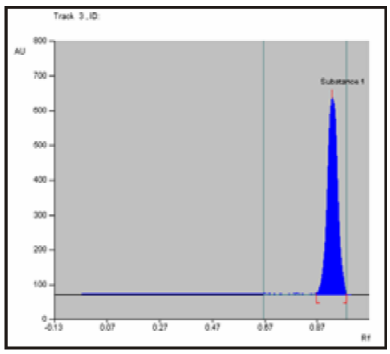
Under chromatographic conditions described above the Rf value of gallic acid was about 0.97. The chromatograms of standard gallic acid is shown in Figure 28 (a-e) and that of gallic acid in test sample was shown in figure 28(f). The 3D spectrum of all tracks scanned at 270 nm is shown in figure 29. Spectral comparison of standard gallic acid with gallic acid content in test sample was



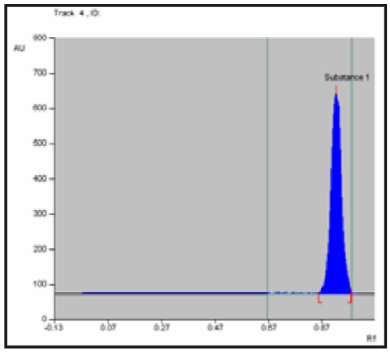
(a) Standard Gallic acid of conc. (20 µg / ml)



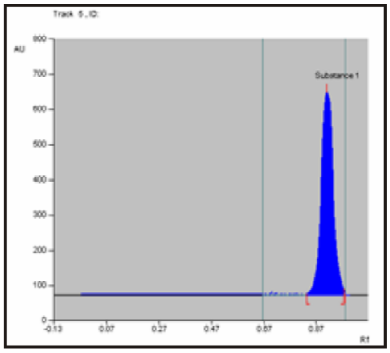
(b) Standard Gallic acid of conc. (40 µg / ml)



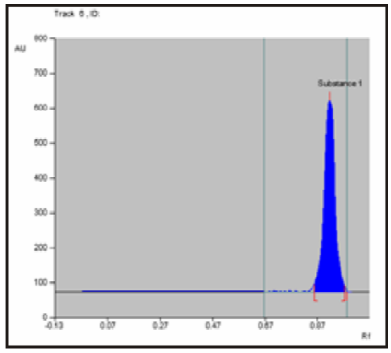
(c) Standard Gallic acid of conc. (60 µg / ml)



(d) Standard Gallic acid of conc. (80 µg / ml)



(e) Standard Gallic acid of conc. (100 µg / ml)



(f) Gallic acid present in test sample (conc. 1000µg / ml)

Figure 28: A typical HPTLC chromatogram for standard gallic acid and test sample.

shown in Fig. 30. The area under the curve (AUC) for various concentration was enumerated in Table 17. The calibration curve was linear in the range of 20- 100 (ìg ml^{-1}), as illustrated in Fig. 31. From regression equation, y = 1093x + 16612, the concentration in test sample i.e. leaves was estimated to be about 3.17 ìg /ml. the estimated value on per gram basis of drug was about 0.317 mg/g.

Table 17: Area under curve values for different concentrations of working standards of gallic acid

S.No.	Concentrations of working standard gallic acid of (µg ml^{-1})	Area under curve (AUC)
Track 1	20	18400.0
Track 2	40	18632.4
Track 3	60	19346.0
Track 4	80	19793.0
Track 5	100	23286.3

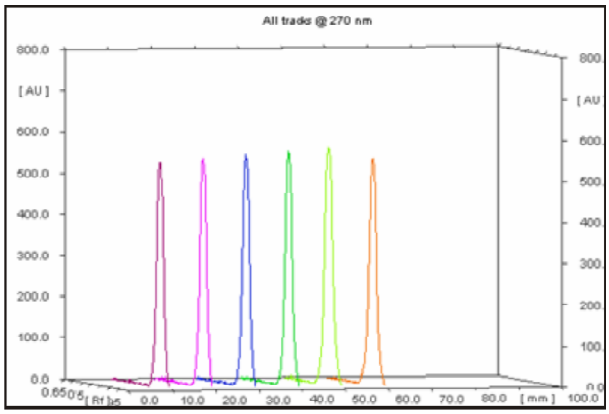


Figure 29: 3D display of standard gallic acid tracks (1-5) and test track (6) at 270 nm

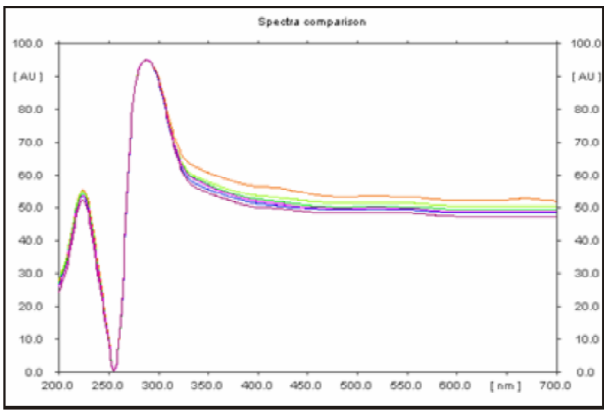


Figure 30: Spectral comparison of all standard gallic acid tracks 1-5 and test track 6 at 270 nm.

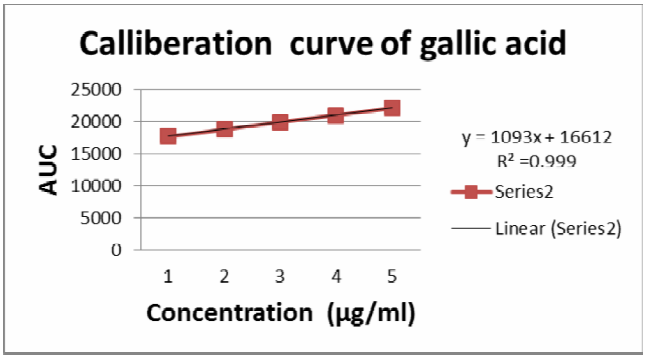


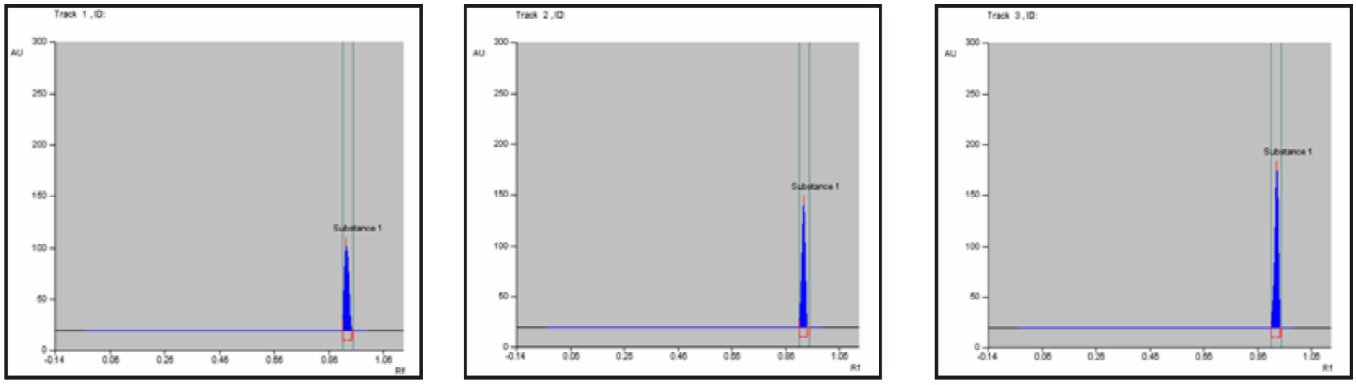
Figure 31: Calibration curve for standard Gallic acid

Determination of quercetin in *Averrhoa carambola* leaves

Under chromatographic conditions described above the Rf value of gallic acid was about 0.95. The chromatograms of standard quercetin is shown in Figure 32 (a-e) and that of quercetin in test sample was shown in Figure 32 (f). The 3D spectrum of all tracks scanned at 366 nm was shown in Figure 33. Spectral comparison of standard gallic acid with gallic acid content in test sample is shown in Figure 36. The area under the curve (AUC) for various concentration was enumerated in Table 18. The calibration curve was linear in the range of 20- 100 (µg ml^{-1}), as illustrated in Figure 37. From regression equation, y = 190.4x + 909.3, the concentration in test sample i.e leaves was estimated to be about 7.84 µg/ml. the estimated value on per gram basis of drug was about 0.784 mg/g.

Table 18: Area under curve values for different concentrations of working standards of quercetin

S.No.	Concentrations of working standard quercetin (µg ml^{-1})	Area under curve (AUC)
Track 1	20	1080.5
Track 2	40	1255.7
Track 3	60	1555.3
Track 4	80	1702.9
Track 5	100	1809.2



(a) Standard Quercetin of conc. (20 µg / ml) (b) Standard Quercetin of conc. (40 µg / ml) (c) Standard Quercetin of conc. (60 µg / ml)

(d) Standard Quercetin of conc. (80 µg / ml) (e) Standard Quercetin of conc. (100 µg / ml) (f) Quercetin present in test sample (conc. 1000µg / ml)

Figure 32: A typical HPTLC chromatogram for standard quercetin and test sample.

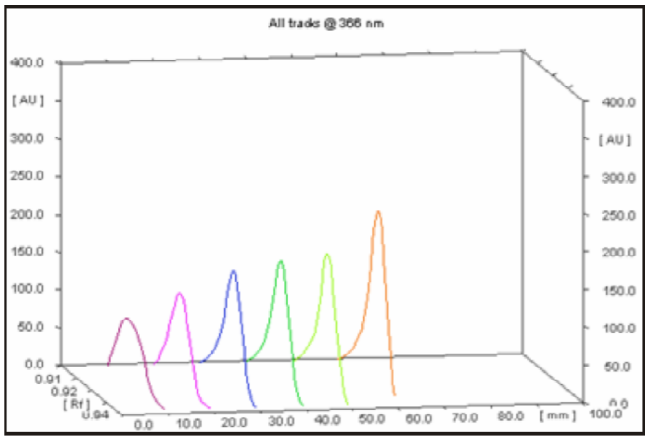


Figure 33: 3D display of standard quercetin tracks (1-5) and test track (6) at 366 nm

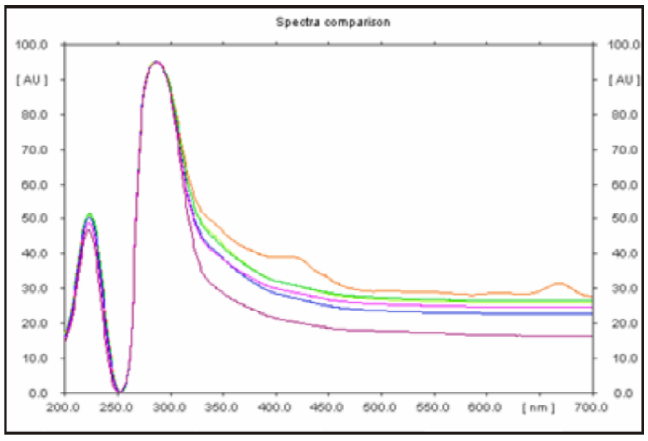


Figure 34: Spectral comparison of all standard quercetin tracks 1-5 and test track 6 at 366 nm.

Antioxidant activity

The anti-oxidant activity of ethanolic, hydro-alcoholic activity and water extract was expressed as IC50 value. Which was obtain through DPPH assay (table 19) and hydrogen peroxide assay(table 20). Percentage inhibition curve for ethanolic extract, hydro-alcoholic extract and water extract for DPPH assay are shown in Figs 36,37 and 38 respectively. Percentage inhibition curve for ethanolic extract, hydro-alcoholic extract and water extract for hydrogen peroxide assay were shown in Figs 39, 40 and 41 respectively.

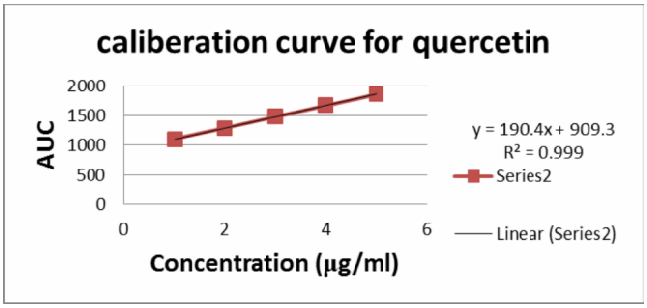


Figure 35: Calibration curve for standard Quercetin

Table 19: Concentration and % inhibition of different extracts for DPPH assay

S.No.	Concentration (µg /ml)	% Inhibition		
		Ethanol	Hydro-alcoholic	Water
1	20	72.80	50.57	68.35
2	40	74.71	54.62	73.26
3	60	76.87	58.67	79.33
4	80	78.90	61.12	85.26
5	100	80.34	64.16	91.32
IC_{50}value		43.10	82.8	54.38

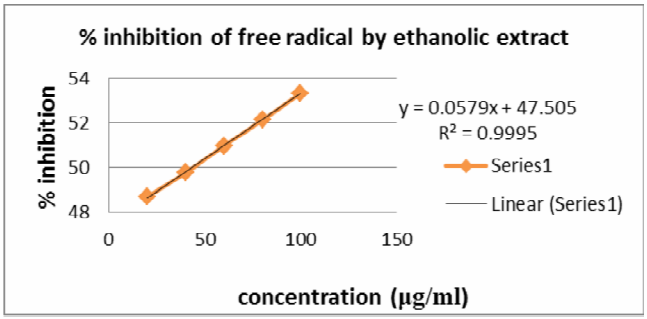


Figure 36: % inhibition curve of ethanolic extract (DPPH assay)

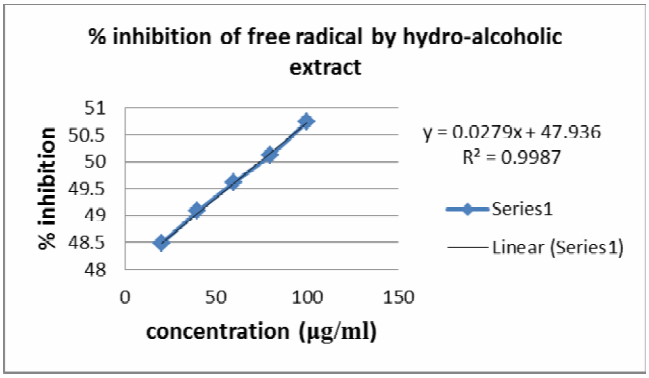


Figure 37: % inhibition curve of hydro- alcoholic extract (DPPH assay)

DISCUSSION

WHO emphasize on exploring the therapeutic potential of medicinal plant which were economical

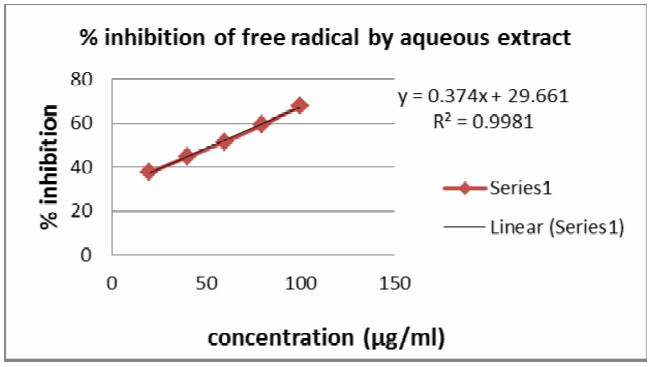


Figure 38: % inhibition curve of water extract (DPPH assay)

Table 20: Concentration and % inhibition of different extracts for Hydrogen Peroxide assay

S.no.	Concentration (µg /ml)	% Inhibition		
		Ethanol	Hydro-alcoholic	Water
1	20	48.71	48.48	37.55
2	40	49.77	49.08	44.61
3	60	50.98	49.62	51.51
4	80	52.12	50.12	59.18
5	100	53.33	50.75	67.67
IC_{50}value		43.10	107	54.54

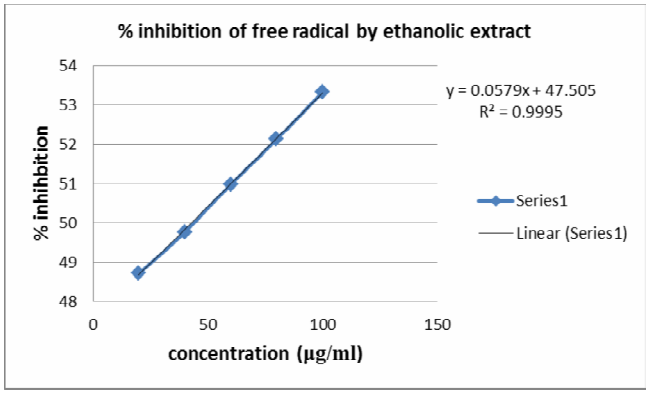


Figure 39: % inhibition curve of ethanolic extract (Hydrogen Peroxide assay)

and easily available and would help to support the economy of developing countries. This fact makes it necessary to investigate about various pharmacognostic parameters, phyto-constituent and their pharmacological activity present in the leaves of *A.carambola.*

Macroscopy study of leaves indicated that its colour, odour and taste may be an important characterstic feature for identifying the plant. In microscopical studies , transeverse section through

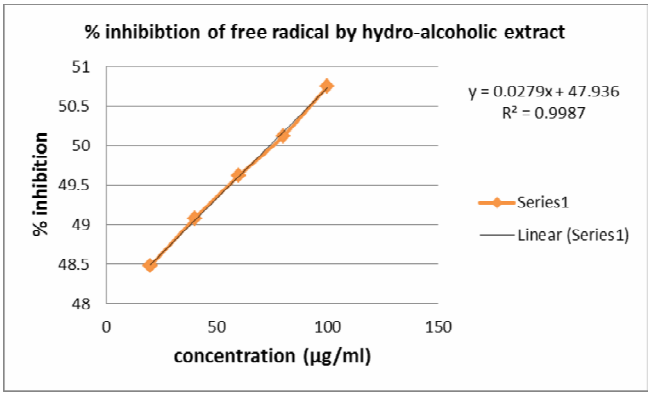


Figure 40: % inhibition curve of hydro-alcoholic extract (Hydrogen Peroxide assay)

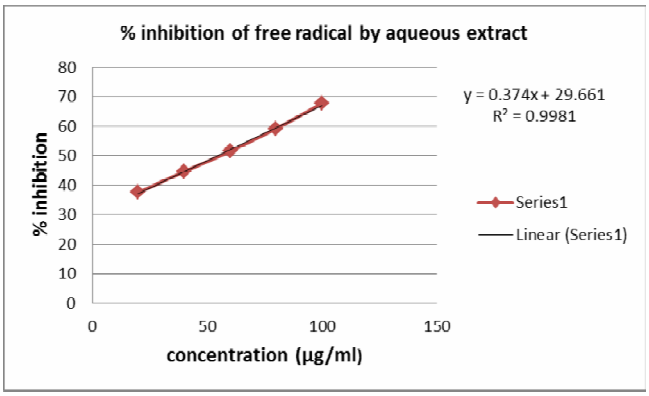


Figure 41: % inhibition curve of water extract (Hydrogen Peroxide assay)

midrib of leaf show the presence of rectangular to square epidermal cell. Vascular bundle show the presence of xylem and phloem, anomocytic stomata, multicellular trichomes, and calcium oxalate crystals. Powder microscopy reveals the presence of calcium oxalate crystals, starch grain, xylem vessels, spiral vessels, unicellular fibres, oil globules and tannins containing cells. In determination of leaf constant stomatal size of upper and lower epidermis, stomatal index of upper and lower epidermis, vein-isleet and vein termination number was calculated. Sulphated ash was almost ten times of acid-insoluble ash and three times of water soluble ash. Ethyl acetate soluble extractive value obtained from cold maceration was slightly higher than n-hexane and ethanol soluble extractive value. Water and ethanol soluble extractive value obtained by hot maceration was much less than that obtained by cold maceration. In estimation of primary and secondary metabolite by gravimetric method total alkaloid and total fiber content are almost five times of total saponin content and approximately double of total cardenolide content. In spectrophotometric determination sugar content was found to be highest followed by tannins and flavonoids content. Presence of iron, calcium, chlorine, and sulphur was found in elemental ash analysis while carbonates and nitrates are absent. Percentage yield of ethanolic extract was found to be four times of ethyl acetate and eight times of chloroform extract obtained by successive solvent extraction. Crude ethanolic and water extract has much higher percentage yield as compared to successive solvent extract.

Fluorescence studies was subjected on powdered drug, crude ethanolic, hydro-alcoholic and water extract.

Detailed phytochemical investigation on said plant was attempted by means of qualitative chemical test on various extracts, which showed the presence of tannins, alkaloids, flavonoids, cardiac glycosides, and saponins. Results from phytochemical tests was verified by subjecting extracts to Thin Layer Chromatographic studies. Anti-oxidant activity was carried out on crude ethanolic, hydro-alcoholic and water extract using in-vitro methods viz. DPPH assay and Hydrogen Peroxide assay. IC_{50} value was calculated for both assay. In both DPPH assay and Hydrogen Peroxide assay maximum IC_{50} value was shown by hydro-alcoholic extract.

Hence, it was inferred that finding revealed in the present study would be beneficial in establishing the correct identity of the plant, also the plant seems promising to carry out further studies to fully explore its therapeutic potential.

REFERENCES

1. Ahmad H, Mishra A, Gupta R, Saraf SA. 2011. Determination of gallic acid in *Michelia champaca* L. (Champa) leaves and stem bark by HPTLC. *Der Pharmacia Lettre* **3:** 307-317.

2. Anon. The Ayurvedic Pharmacopoeia of India, Govt. of India, Ministry of Health and Family

Welfare Dept, AYUSH, P 230.

3. Anon. 1991. Guidelines for the Assessment of Herbal Medicines, Document No.WHO/ TRM/91,4, World Health Organization, Geneva, P 22- 33.
4. Anon. 1996. Indian Pharmacopoeia, The Controller of Publication, New Delhi, Vol 2, P A-47.
5. Gupta AK, Tondon N, Sharma M. 2005. Quality Standards Of Indian Medicinal Plants, Indian Council Of Medical Research, New Delhi, Vol 2, P 44.
6. Harborne JB 2005. Phytochemical Methods. 3rd ed, Chapman and Hall, London, P 49-244.
7. Hussain I, Ullah R, Khurram M, Ullah N. 2011. Phytocemical analysis of selected medicinal plant. *Afr J Biotechnol* **10:**7487-7492.
8. Khandelwal KR. 2006. Practical Pharmacognosy- Techniques and Experiments.Nirali Prakashan, Pune, India, P 132- 161.
9. Moresco HH, Queiroz GS, Pizzolatti GM. 2012. Chemical constituents and evaluation of the toxic and antioxidant activities of *Averrhoa Carambola* Leaves. *Rev Bras Farmacogn* **22:** 319-324.
10. Prabhu K, Karar PK, Hemlatha S, Ponnuduraik. 2011. A preliminary chromatographic detection of phenolic compounds from ethanolic stem extracts of *Viburnum* Linn. Species by TLC and PC. *Der Pharmacia Sinica* **2:** 74-80.
11. Singh R, Lawania RD, Mishra A, Gupta R. 2010. Role of *Cordia Dichotoma* seeds and leaves extract in degenerative disorders. *Int J Pharm Sci Rev Res* **2:** 21-24.
12. Tang LK, Ling LPK, Koh RY, Chye SM. 2012. Screening of anti-dengue activity in methanolic extracts of medicinal plants. *Complement Altern Med* **12:** 3.
13. Wagner H, Blat S, Zgainski EM .1984. Plant Drug Analysis, Springer-Verlag, Berlin Heidelberg, New York, P 93-94.
14. Wagner H, Blat S, Zgainski EM .1996. Plant Drug Analysis, Springer-Verlag, Berlin Heidelberg, New York, P 196.
15. Wagner H, Blat S, Zgainski EM .1996. Plant Drug Analysis, Springer-Verlag, Berlin Heidelberg, New York, P 306.
16. Wetwitayaklung P, Charoenteeraboon J, Limmatvapirat C. 2012. Antioxidant activities of some Thai and exotic fruits cultivated in Thailand. *Res J Pharm Biol Chem Sci* **3:** 12-21.