Original Article

Pharmacognostic Standardization and Chromatographic Fingerprint Analysis on Triterpenoidal Constituents of the Medicinally Important Plant *Artocarpus heterophyllus* by High-Performance Thin Layer Chromatography Technique

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Abstract

Background: *Artocarpus heterophyllus* commonly known as Kathal in Hindi and Jackfruit in English has a wide horizon of medicinal possessions. The plant is found in India and in its tropical regions. **Objective:** Although the plant and its extracts are renowned for its ethnic medicinal values diversely in India, yet organized data somewhere lack in reverse pharmacognostical approach of this plant that shows that plant have not been completely explored for its therapeutic potency. **Materials and Methods:** In the present study, the folklore potential of this plant has been explored by generating down its pharmacognostical standards along with measurement of its active therapeutic constituent ursolic acid and lupeol via. High-performance thin layer chromatography (HPTLC), evidence from organized data search says that ursolic acid and lupeol is ubiquitous to *A. heterophyllus*. The plant was also subjected to spectroscopic-based estimation of tannins (gallic acid and tannic acid) and flavonoids (quercetin and rutin). Antimicrobial testing was also performed. **Results:** Microscopic features revealed the presence of anomocytic type of stomata, collateral open type vascular bundle in which fascicular cambium is present, calcium oxalate crystal and covering trichomes were key features in leaves. Methanolic extract of leaves of the plant was subjected to HPTLC. HPTLC studies revealed that both ursolic acid and lupeol are present in appreciable amount. Plant showed good antibacterial activity which may be due to the high amount of tannins as the tannins has the ability to disintegrate the bacterial cell wall. **Conclusion:** The data generated could be significantly used as a reference for the authentication and quality control of *A. heterophyllus*.

Keywords: *Artocarpus heterophyllus, high-performance thin layer chromatography, lupeol, pharmacognostic, standardization, ursolic acid*

Introduction

Artocarpus heterophyllus belonging to family Moraceae is a constitutive part of common Indian diet and is freely available in Indian and neighboring continents, its medicinal properties are also mentioned in Ayurveda. It is commonly known as jackfruit in English, Kathal and Panas in Hindi, Kanthal in Bengali), Palaa in Tamil, Phanas in Gujrati and Marathi, and Chakka in Malayalam.[1] The term jackfruit is derived from the Portuguese word Jaca which, in turn, is adopted from the word "Chakka" of Malayalam-A regional Indian language.[2] This plant received a great level of scientific interest as they consist of therapeutically active secondary metabolites and is an economic source of food and widely used in traditional medicine.[3] Artocarpus species are used as traditional folk medicine in South-East Asia, Indonesia, Western part of Java and India.[4] The *A. heterophyllus* contains various chemical constituents as several flavones coloring matters, morin, dihydromorin, cynomacurin, and artocarpin. Triterpenic compounds such as cycloartenyl acetate, cycloartenone, and artocarpanone are also reported in the plant[5] other constituents such as β-sitosterol, ursolic acid, betulinic acid, and cycloartenone are ubiquitous throughout the plant.[6] Plant is integral part of the traditional system of medicine, it has been used conventionally, leaves are given in fever, boils, wounds, and

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skin ailments. The young fruits are acrid, astringent, and carminative, whereas ripen fruits are sweet, laxative, aphrodisiac, and also used as a brain tonic. The seeds are, reported to be diuretic, and constipating and wood is nervine tonic, antidiabetic, sedative and anticonvulsant.[7] *A. heterophyllus* is a plant that is ubiquitously distributed through India, but there is no organized literature available that may provide authentic information in the context of its therapeutic efficacy neither the justified standardization parameters are available for proper identification of the plant. Plants leaves are just used as fodder for animals due to its rich nutritional value, but the presence of secondary metabolites such as terpenoids and phenols make it a better choice as a medicinal plant rather than just being fodder for animals. The present study is an attempt to figure out the anticipated potential of this plant that has been validated by standardizing the plant in terms of its pharmacognostical standards along with measurement of its triterpenoidal constituent's ursolic acid and lupeol using high-performance thin layer chromatography (HPTLC) which is a quick analytical tool for qualitative as well as quantitative analysis of herbals. The HPTLC is more economic technique when compared to high-performance liquid chromatography (HPLC). HPTLC can run approximately 100 samples in parallel which cannot be done in HPLC where only one sample can be run at a time. Information from organized search of published literature remarks that ursolic acid and lupeol is widespread to this plant. Both of these constituents have a potent role in protecting the liver against several hepatic ailments, their antioxidant activity and ability to modulate the mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NF-κB) signaling pathway provide it outstanding hepatoprotective activity. It significantly decreases the activation of MAPK, which, in turn, inactivates the immunoregulatory transcription factor NF-κB,[8] that is responsible for regulating gene expression that encodes the products involved in tissue damage and inflammation;[9] this protects the liver against CCl4-induced oxidative stress and inflammation by the MAPK/NF-κB pathway.

Materials and Methods

Pharmacognostic studies

Collection and authentication

Fresh leaves and bark of *A. heterophyllus* were collected from the local garden of Lucknow in October 2014. Leaves were washed properly and air-dried. The collected plant material was authenticated from the National Institute of Science Communication and Information Resources (CSIR-NISCAIR) and voucher specimen number (Ref. No NISCAIR/RHMD/CONSULT/2015/2827/20-1) were submitted in LWG herbarium. The air-dried plant material was first washed with tap water, then again washed twice with double distilled water and then air dried. The air-dried specimen (leaves) were pulverized and sieved through 80# mesh size and stored in air-tight container at 25°C for future/further studies.

Macro and microscopic characteristics

The morphological characteristics of the specimen (leaves and bark) were studied and the photographs were taken. For microscopic studies, transverse section (TS) was preferred over longitudinal section. The fine sections of leaves were cut by free hand. The chlorophyll and the other pigments of the plant were removed by treating the sections with 5% potassium hydroxide and 20% chloral hydrate. Photographs of different magnifications were taken with Olympus Microscope, Model Olympus (India), attached to YOKO CCD Camera.

Quantitative microscopy

Quantitative microscopy of the leaf such as stomatal number, stomatal index, vein-islet, vein termination number, and palisade ratio were determined by using fresh leaves of the plant.[10,11]

Quantitative measurement

Dimensional figures of trichomes, vessels, stomata, and calcium oxalate crystal were measured by Psudo-coloring method by using Olympus Microscope, attached to YOKO CCD Camera.

Physiochemical parameters

Evaluation of the physicochemical parameters of the drugs is an important step in detecting adulteration or improper handling of drugs. It includes ash values (total ash, acid-insoluble ash, and water-soluble ash), extractive values (alcohol soluble, water soluble), and moisture content.[12]

Phytochemical screening

Preliminary phytochemical investigation of different extracts of leaves of *A. heterophyllus* was done by using several reagents assigned for the detection of several phytoconstituents such as alkaloids, glycosides, flavonoids, saponins, tannins, carbohydrates, steroids, and terpenoids.[13]

Quantification of carbohydrate

Two grams of the drug was dissolved in 80% of ethanol, the mixture was homogenated and centrifuged for 15 min at 2000 rpm. To the residue 16 ml of water was added and heated on water bath for 5 min, to this mixture 18 ml of perchloric acid was added and centrifuged for 15 min at 2000 rpm, supernatant was collected volume was made up to 10 ml with water., 0.1 ml of supernatant was taken to which 0.1 ml of phenol, 4.8 ml of water and 5 ml of concentration Sulfuric acid was added and absorbance was taken at 410 nm.[14]

Detection of phenols

Detection of quercetin and rutin (flavonoids)

One milliliter of standard (Quercetin/Rutin) of different concentrations (200, 400, 600, 800, and 1000 μg/ml) was taken into 10 ml volumetric flask to which 4 ml of distilled water and 0.3 ml of 5% sodium nitrite was added, after 5 min 0.3 ml of 10% $AlCl_3$ was incorporated into the resultant mixture after this at 6th min 2 ml of 1M NaOH was added. The volume of the final solution was made up to 10 ml with distilled water and absorbance was noted at 510 nm.

Sample solution

One milliliter of plant sample (200 μg/ml)**,** was taken into 10 ml volumetric flask to which 4 ml of distilled water and 0.3 ml of 5% sodium nitrite was added, after 5 min 0.3 ml of 10% $AlCl_3$ was poured to the mixture followed by addition 2 ml of 1 MNaOH of at the 6th min. The final volume was made up to 10 ml with distilled water and absorbance was taken at 510 nm.[15]

Detection of gallic acid and tannic acid (tannins)

The method is based on the oxidation of molecule containing an OH groups. The tannin and tannin-like compound reduce phosphotungustomolybdic acid in alkaline solution to produce a highly blue colored.

Follin Denis reagent

A total of 25 g of sodium tungstate and 5 g (5.31 ml) of phosphomolybidic acid was dissolved in 87.5 ml of water, to this 12.5 ml of phosphoric acid was added. Mixture was refluxed for 2 h and volume was made up to 250 ml.

Sodium carbonate solution

A total of 21.95 g of sodium carbonate was dissolved in 62.5 ml of water, kept overnight and then filtered with using glass wool.

Preparation of standard gallic acid solution

Five concentrations of working standard gallic acid were made from 1 μg/ml to 5 μg/ml. 1 ml from each aliquot was taken and to that 0.5 ml of Folin denis reagent and 1 ml of sodium carbonate solution was added and volume was made up to 10 ml. Absorbance was measured at absorption maxima 700 nm within 30 min of reaction against the blank.

Preparation of standard tannic acid

Six concentrations of working standard tannic were made from 5 μg/ml to 35 μg/ml.

One milliliter from each aliquot was taken and to that 0.5 ml of Folin denis reagent and 1 ml of sodium carbonate solution was added and volume was made up to 10 ml. Absorbance was measured at absorption maxima 700 nm within 30 min of reaction against the blank.

Extraction of tannin

Accurately weighed 0.5 g of the powdered material was transferred to a 250 ml conical flask. Add 75 mL water. Heat the flask gently and boil for 30 min. Centrifuged at 2000 rpm for 20 min and collect the supernatant in 100 mL volumetric flask and make up the volume. Transfer 1 mL of the sample extract (25 μg/ml) to a 100 mL volumetric flask containing 75 mL water. Add 5 mL of Folin-Denis reagent, 10 mL of sodium carbonate solution and dilute to 100 mL with water and Shaken well. Read the absorbance at 700 nm after 30 min.[16]

High-performance thin layer chromatography

Chemicals and reagents

HPTLC analyses were performed on Merck 10 cm × 10 cm HPTLC silica gel 60F254 (0.25 mm) plates. Ursolic acid and lupeol was supplied by Sigma, Aldrich, Germany. All the reagents used in the experiment were of analytical grade and were supplied by Merck, Darmstadt, Germany.

Preparation of standard solutions

Stock solutions of ursolic acid and lupeol were prepared by dissolving 0.1 mg/mL in methanol.

Sample preparation

The fresh leaves of *A. heterophyllus* were collected, thoroughly washed with water to remove all debris. The plant materials were shade dried and powdered by using the electric grinder at 60 mesh size. Extraction was performed by soxhlation method. First, the powdered plant material was defatted using soxhlet assembly with 250 mL of 98% petroleum ether for 24 h. This was followed by 48 h soxhlation of defatted powder by using 250 mL of methanol. The final methanolic fraction obtained was filtered through Whatman No. 1 filter paper. The filtrate obtained was concentrated under vacuum in a rotary evaporator at 40°C and stored at 4°C for further use. The dried extracts were dissolved in 98% methanol to obtain a stock solution of 10 mg/mL, which is used for application of spots on HPTLC plates.

Development of high performance thin layer chromatography fingerprinting of ursolic acid and lupeol

Instrumentation and chromatographic conditions

Spotting device – Linomat V automatic sample applicator; CAMAG (Muttenz, Switzerland), Syringe: 100 μL Hamilton (Bonaduz, Switzerland). Thin layer chromatography (TLC) chamber: glass twin trough chamber (20 cm × 10 cm × 4 cm); CAMAG. Densitometer: TLC Scanner 3 linked to winCATS software V.4.06 (CAMAG Scientific, Inc., 515 Cornelius Harnett Drive, Wilmington, NC 28401, USA); CAMAG. HPTLC plates: 10 cm × 10 cm, 0.2 mm thickness precoated with silica gel 60 F_{254}; E. Merck (Darmstadt, Germany). Experimental conditions: temperature,

25°C ± 2°C; relative humidity, 40%. Solvent system: toluene–ethyl acetate–formic acid (8:2:0.1). Detection wavelength: 500 nm. Visualization agent: Anisaldehyde-Sulphuric acid reagent. Slit dimension: 5.00 mm × 0.45 mm. Scanning speed: 10 mm/s and source of radiation: deuterium lamp.[17]

Method validation

The method was validated according to the ICH guidelines,[18,19] and the statistical analysis was performed using Excel 2000 (MS Office®).

Precision

Repeatability of the sample application and measurement of peak area were carried out using nine determinants (3 concentrations/3 replicates) covering the specified range for the procedure (200, 400, and 600 ng per band of ursolic acid) and was expressed in terms of relative standard deviation (RSD). The intra- and inter-day variation for the determination of ursolic acid and lupeol was carried out in three different concentration levels of 200, 400, and 600 ng per band. Acceptance criteria for a procedure's repeatability or intermediate precision are based on the intended use of the analytical method.

Robustness of the method

By introducing small changes in the mobile-phase composition, mobile-phase volume, duration of mobile phase saturation, and activation of prewashed TLC plates with methanol, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of 200 ng per band of ursolic acid and the RSD and standard deviation (SD) of peak areas were calculated.

Limit of detection and limit of quantitation

To estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times and the signal-to-noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting the known concentrations of ursolic and lupeol acid until the average responses were approximately 3 or 10 times of the responses for six replicate determinations.

Recovery

The preanalyzed samples were spiked with extra 50%, 100%, and 150% of the standard ursolic acid lupeol and the mixtures were reanalyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the targeted analyte at different levels in the bark and leaves of the plant.

Ruggedness

Ursolic acid and lupeol solutions of concentration 200 ng per band were prepared and analyzed on day 0 and after 6, 12, 24, 48, and 72 h. Data were treated for %RSD to assess the ruggedness of the method.

Specificity

The specificity of the method was confirmed by analyzing the standard ursolic acid, lupeol and the extract. The band for ursolic acid and lupeol in the sample was confirmed by comparing the Rf values and spectra of the band with that of the standard. The peak purity of the ursolic acid and lupeol was assessed by comparing the spectra at three different levels, namely, peak start (S), peak apex (M), and peak end (E) positions of the band.

Calibration curve of ursolic acid and lupeol

Stock solutions of ursolic acid and lupeol (100 µg/ml) were prepared in HPLC grade methanol. Different volumes of stock solution were spotted on the TLC plate to obtain concentrations of 100-600 ng per band of ursolic acid and lupeol, respectively. The data of peak areas plotted against the corresponding concentrations were treated by least square regression analysis method validation.

Anti-microbial studies

Test micro-organisms

The various extracts of the powdered leaves of *A. heterophyllus* (L.) were subjected to antibacterial studies.

Following steps were involved in the study:

Step 1: Activation of microbes (first generation) – Inactive spores (formerly maintained <4°C) were added in the nutrient broth which was incubated for 36 h. The presence of turbidity indicates the growth of culture when compared with sterile.
Step 2: Preparation of "Microbial Culture" – The microbial preserves were cultured again in nutrient broth, few ml of microbial suspension was inoculated in 20 ml of nutrient broth and all were incubated at 37°C for 36 h.
Step 3: Preparation of "Main Colony" – The 36 h grown microbial culture of each test organism was used for streaking over nutrient agar plates and subjected to incubation at 37°C for 36 h.
Step 4: Preparation of "inoculum for antimicrobial activity" – From these isolated colonies loopful of pure microbial culture was dissolved in nutrient broth (50 ml) that was incubated at 37°C for 36 h. And then, this suspension was poured to 500 ml agar nutrient media. This nutrient agar culture media served as inoculums for determination of antimicrobial activities of the extract.
Step 5: Preparation of test sample – Test samples (all methanolic extracts) were dissolved in DMSO to get stock solution of the concentration of 1000 µg/ml. From this specific concentrations of 25, 50 and 100 µg/ml were prepared of each extract. DMSO was used as control.
Step 6: Standard preparation – Ciprofloxacin and fluconazole were used as standard antibiotics to give concentration of 25, 50, and 100 µg/ml, respectively.

Step 6: Zone of inhibition by (CUP PLATE METHOD) – Wells were made in the petriplates with the main colony in agar media. All concentrations of test samples, standards and control were poured into the wells, and all the plates were incubated for 37°C for 36 h after which the zone of inhibition was calculated.

Step 7: Evaluation of minimum inhibitory concentration (MIC) with different strains.[19] Nutrient agar and test sample poured in the form of wedge, second amount of agar is poured on to the wedge, the drug is allowed to diffuse and then the petriplate is allowed to dry. Streaking of organism is done, incubated overnight.[20,21]

Results

Morphology and microscopy

Leaf

Leaves are simple with the glossy ventral surface, having a slight odor and acrid taste, the leaf is dark green from dorsal side and light green from ventral side. Leaf has an average length of 10.65 cm and width of 4.8–6.5 cm with ovate shape, the margin is slightly serrate to entire while venation pattern is pinnate and the apex is cuspidate.

Transverse section of midrib and lamina

TS of the leaf shows a typical dorsiventral structure with slightly wavy upper and much wavier lower epidermis. Mesophyll shows the presence of a single layer of compacted elongated palisade cells which is followed by spongy parenchyma [Figures 1 and 2].

Vascular bundle

The central portion of the midrib is occupied by a prominent radial collateral open type of vascular bundle with xylem and phloem both of which are separated by fascicular cambium, in the center a small zone of pith is present that as slightly rounded cells [Figure 3].

Stomata

The lower surface of the leaf contains more number of anomocytic types of stomata than upper surface [Figure 4].

Extra feature

TS revealed that ground tissue (cells), as well as xylem cells, are parenchymatous in nature as it does not give pink color with saffranine. Phloem is scelenchymatous in nature (lignin present) as it gives pink color with safranin, oil glands are present in the lamina. Rosette forms of calcium oxalate crystals are present.

Powder characteristics

Powder microscopy showed xylem vessels, epidermal cells with anomocytic stomata, covering trichomes, rosette form of calcium oxalate crystals and starch grains [Figure 5].

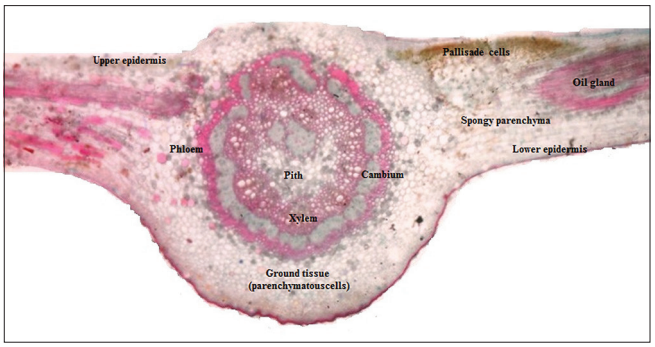


Figure 1: Transverse section of leaf

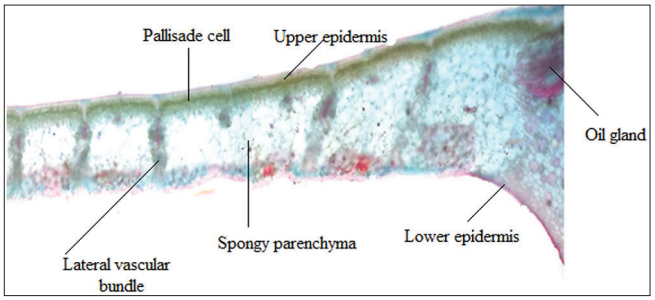


Figure 2: Transverse section of lamina showing mesophyll and lateral vascular bundle

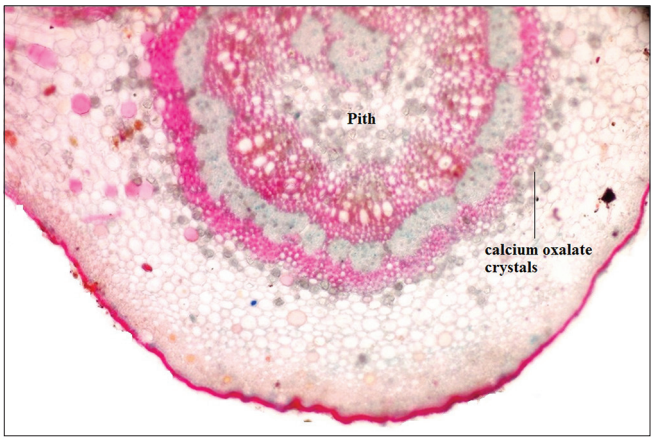


Figure 3: Transverse section of midrib showing calcium oxalate crystal and centrally located pith

Quantitative microscopy, quantitative measurement, physiochemical parameters, phytochemical screening, detection of carbohydrate content, and phenolic content

These standardization parameters were performed as per the guidelines of Ayurvedic Pharmacopoeia of India. The preliminary phytochemical investigation revealed the presence of carbohydrates, proteins, amino acid, tannins, flavonoids sterols, and terpenoids. The results of quantitative microscopy and dimensional measurements are depicted in Tables 1, 2 and Figure 6, whereas the results of phytochemical screening are shown in Table 3. The Physiochemical Properties of Ursolic acid and Lupeol are given in Tables 4 and 5 respectively. The physiochemical analysis is presented in Figure 7. Plants nutritional

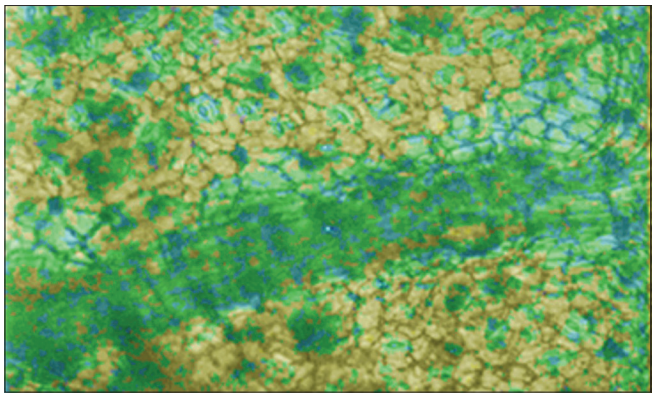


Figure 4: Lower surface of leaf showing anomocytic stomata

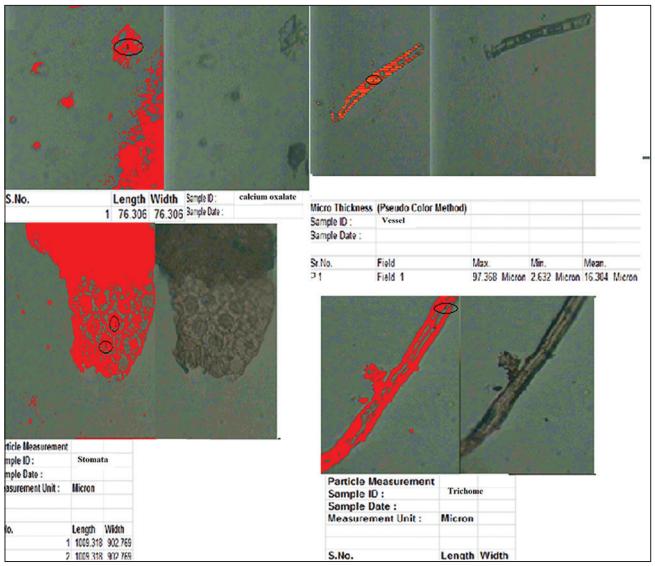


Figure 6: Quantitative microscopy and dimensional measurements of anatomical characteristic of leaf

value represented by carbohydrate content was found with the help of calibration curve which was linear with regression equation Y = 0.0338× +0.1794 and coefficient of correlation (R^2 = 0.9853), the carbohydrate content was found to be 780 µg/ml [Figure 8]. Total phenolic content was found in terms of Tannins and flavonoids. The calibration curve for gallic acid was linear in the range of 1–5 µg/ml. From the regression equation Y = 0.1466× +0.2098; R^2 = 0.9922 [Figure 9], concentration of gallic acid was found to be 1.65 µg/ml. The calibration curve for Tannic acid was linear in the range of 5–35 µg/ml. From the regression equation Y = 0.050× +0.063; R^2 = 0.987 [Figure 10], concentration of tannic acid in was found to be 0.7 µg/ml. The calibration curve for Rutin was linear in the range of 200–1000 µg/ml with the regression equation Y = 0.0771× +0.1221, R^2 = 0.9897 [Figure 11], the concentration of Rutin found was 2.98 µg/ml, the calibration curve for Quercetin was in the range of 20–100 µg/ml, with regression equation Y = 0.044× +0.0172; R^2 = 0.9817 [Figure 12] concentration of quercetin was found to be 13.59 µg/ml.

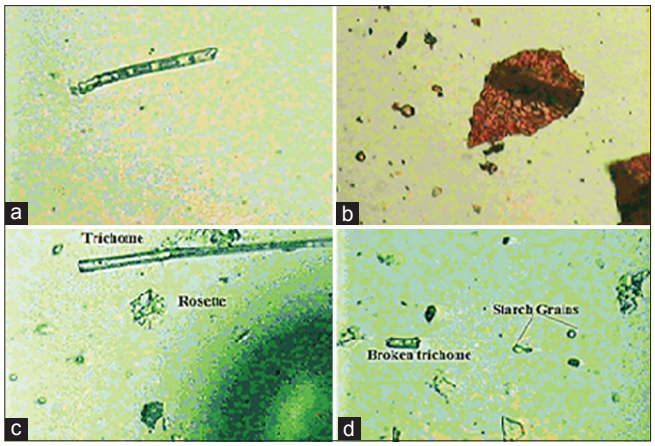


Figure 5: Powder microscopy of leaf a) Xylem Vessel at 4X magnificatio; b) Epidermal cells showing Anomocytic stomata at 4X magnification; c) Rossette type of calcium oxalate crystal and covering trichome at 4X magnification; d) Broken trichome and starch grains at 4X magnification

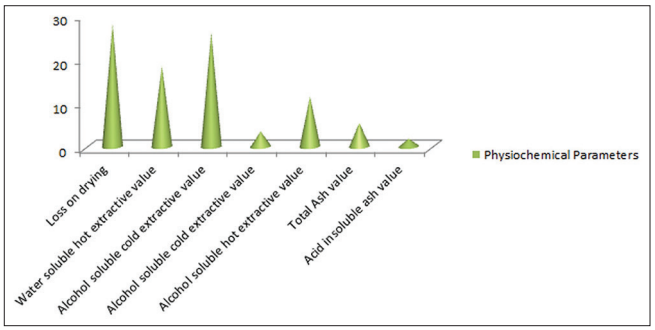


Figure 7: Physicochemical parameters of leaf

Table 1: Quantitative microscopy *Artocarpus heterophyllus* leaf

Parameters	Result
Stomatal number	15.6
Stomatal index	25.7
Vein islet number	15.4
Vein termination number	30.7

Table 2: Dimensional measurement of microscopic parameters

Parameter (10X)	Dimensions (micron)
Stomata	
Length	1009.318
Breadth	902.769
Trichome thickness (leaves)	48.756
Xylem vessel thickness (leaves)	16.304
Calcium oxalate crystal rossete (leaves)	
Length	76.306
Width	76.306

High-performance thin layer chromatography and validation

In this study, several solvent systems were used for the estimation of this triterpenoid and were investigated to

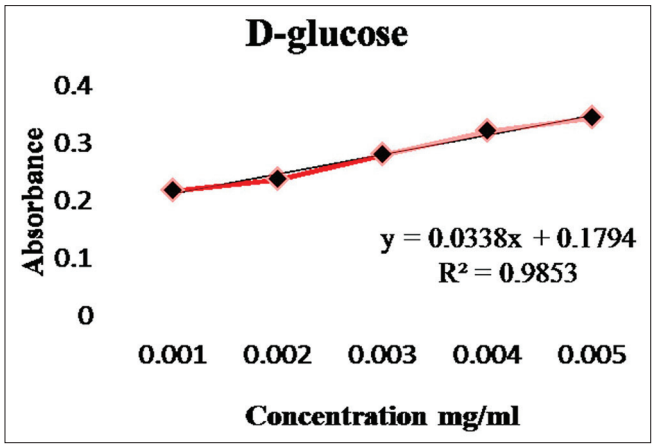


Figure 8: Calibration curve for the carbohydrate estimation by ultraviolet-spectrophotometery

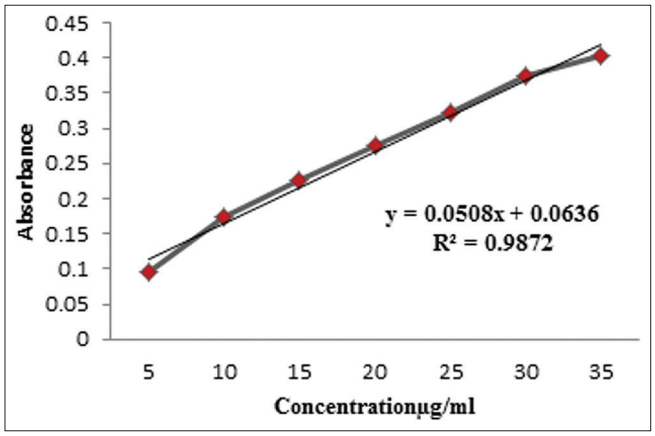


Figure 10: Calibration curve for the estimation of tannic acid by ultraviolet spectrophotometery

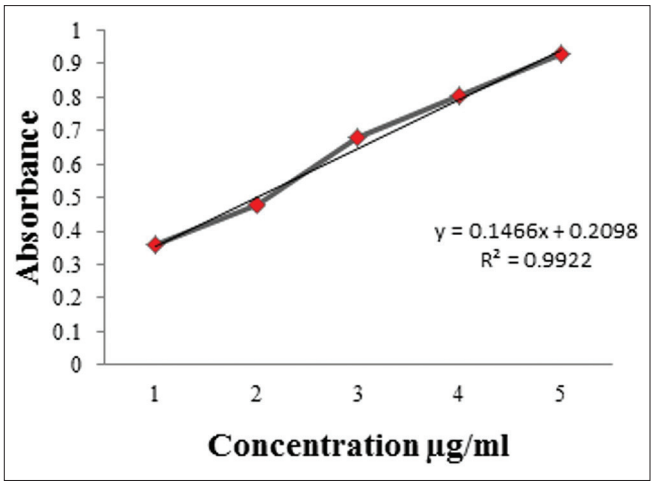


Figure 9: Calibration curve for the estimation of gallic acid by spectrophotometery

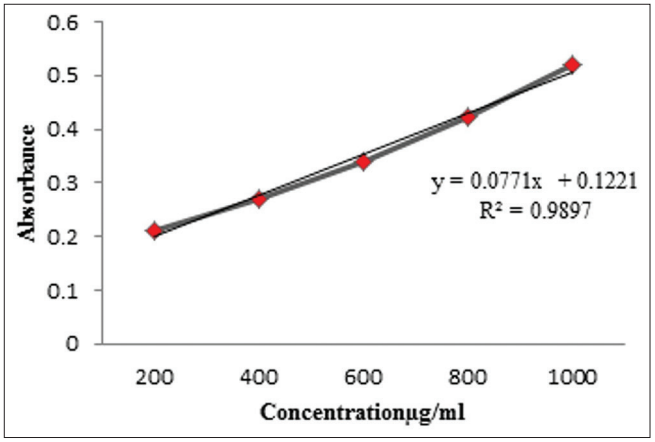


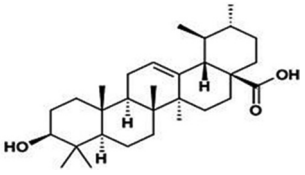
Figure 11: Calibration curve for the estimation of rutin by ultraviolet spectrophotometery

Table 3: Phytochemical screening of *Artocarpus heterophyllus* leaf

Test	n-hexane	Methanolic extract	Aqueous extract
Carbohydrate	–	++	–
Alkaloids	–	–	++
Proteins and amino acids	–	+	+
Flavonoids	–	++	–
Tannins	–	++	+
Saponins	–	–	–
Steroids	++	–	–
Terpenoids	+	++	–

-: Absent, +: Present, ++: Strongly present

Table 4: Physicochemical properties of ursolic acid

Physicochemical Property	Description
Molecular weight	456.711 g/mol
Physical description	Solid, platelets from alcohol
Melting point	284°C
Solubility	One part dissolves in 88 parts methanol, 178 alcohol (35 boiling alcohol), 140 ether, 388 chloroform, 1675 carbon disulfide. Moderately soluble in acetone. Soluble in hot glacial acetic acid and in 2% alcoholic NaOH. Insoluble in petroleum ether
Vapor pressure	3.49×10-14 mm Hg at 25°C (est)
Chemical structure	

evaluate the combinatorial separation of these compounds in a single solvent system and between different components of the extract. Among the different solvents systems investigated, a mobile phase consisting of toluene: ethyl acetate: formic acid in the ratio of 8: 2: 0.1 v/v/v demonstrated good resolution between other peaks of the extract. The procedure for the separation and determination of different compounds in methanolic fraction of *A. heterophyllus* leaves using HPTLC-densitometry is reported at six point calibration

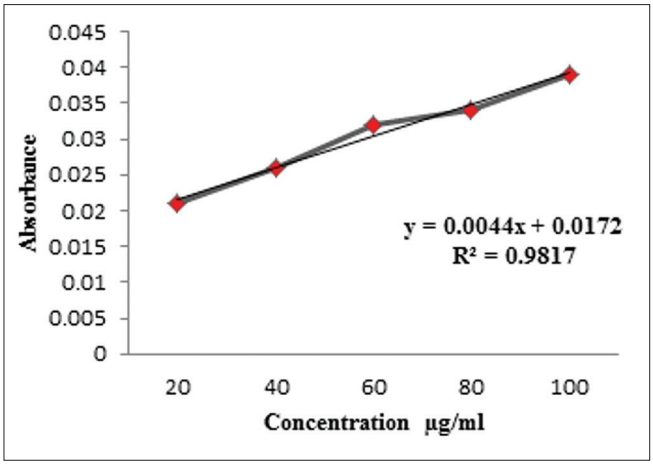


Figure 12: Calibration curve for the estimation of quercetin by ultraviolet spectrophotometery

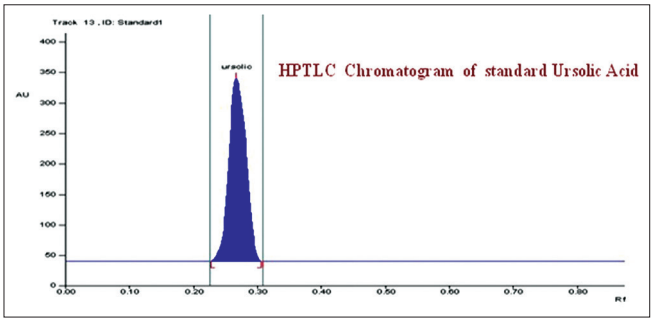
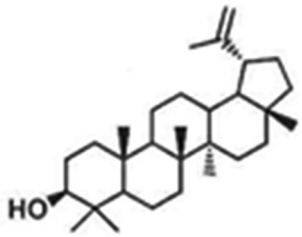


Figure 14: Densiometric chromatogram of working standard ursolic acid

Table 5: Physicochemical properties of lupeol

Physicochemical properties	Description
Molecular weight	426.729 g/mol
Physical Description	Needles from alcohol
Solubility	Very soluble in ethanol, acetone, chloroform, freely soluble in ether, benzene, petroleum ether, warm alcohol. Practically insoluble in dilute acid and alkalies
Vapor pressure	5.03×10-11 mm Hg at 25°C (est)
Chemical structure	

curves in which amount of ursolic acid and lupeol in the leaves of *A. heterophyllus* were found to be 0.024% and 0.025%**.** The Rf value for ursolic was found to be 0.68 ± 0.01 and that of lupeol was 0.46 ± 0.01. HPTLC chromatogram and densitograms were obtained from standard compounds and methanolic fractions [Figures 13-15], separation of all bands of plant samples and standard is shown in Figure 16. Both targeted compounds were identified by

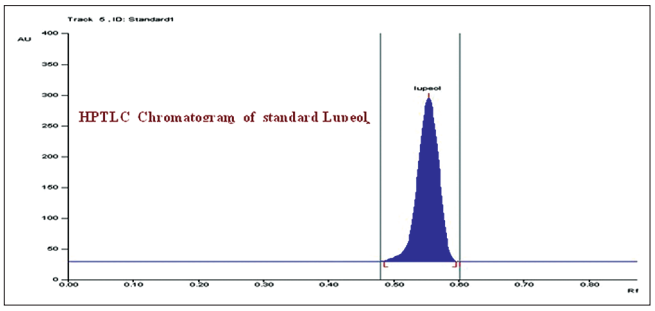


Figure 13: Densiometric chromatogram of working standard of Lupeol

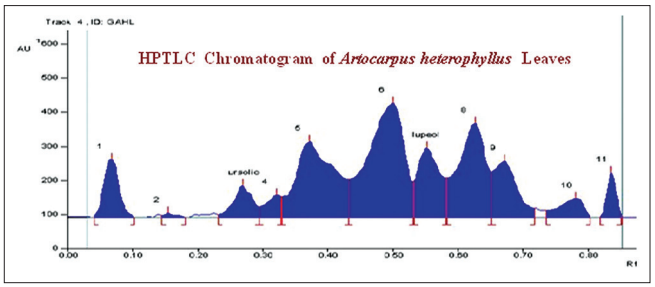


Figure 15: Densiometric chromatogram of *Artocarpus heterophyllus* leaf

Table 6: Summary of validation parameters

Parameters	Lupeol	Ursolic
Rf	0.46±0.01	0.68±0.01
Linearity range	100-600	100-600
Regression equation	y=18.847*x+29.620	y=21.577*x-1617.168
R^2	0.996	0.998
Slope	18.847	21.577
Intercept	29.620	1617.168
LOD (ng)	40	35
LOQ (ng)	100	100
Scanning (nm)	500	500

LOD: Limit of detection, LOQ: Limit of quantitation, Rf: Retention factor

retention factor (Rf), peak purity 3D spectra and overlay ultraviolet-spectrum [Figures 17-19]. Summary of validation parameter is given in Table 6. Precision studies have been performed by analyzing intra- and inter-day variation for the determination of these triterpenoidal compounds which was carried out at three different concentration levels of 200, 400, and 600 ng per band; mean percentage intraday RSD values were found to be 0.54, and 0.51 for lupeol and ursolic acid, respectively, while inter-day analysis showed mean percentage RSD values of 1.47 and 1.37 for lupeol and ursolic acid, respectively, which shows good precision [Table 7]. For recovery studies, preanalyzed samples of *A. heterophyllus* was spiked with extra 50, 100, and 150% of the standard compounds and the mixtures were reanalyzed which shows a good recovery ranging from 97.14% to 100.05% for lupeol and 97.96%–99.20% for ursolic acid. The experimental data are expressed as mean percentages of recovered analytes, SD and RSD is also presented in Table 8.

Table 7: Intra-day and inter day precisions

Standard markers	Concentration (ng/band)	Intraday		Interday	
		Percentage RSD	Mean RSD	Percentage RSD	Mean RSD
Lupeol	200	0.53	0.54	1.69	1.47
	400	0.62		1.53	
	600	0.47		1.19	
Ursolic acid	200	0.68	0.51	1.32	1.37
	400	0.42		1.73	
	600	0.44		1.07	

RSD: Relative standard deviation

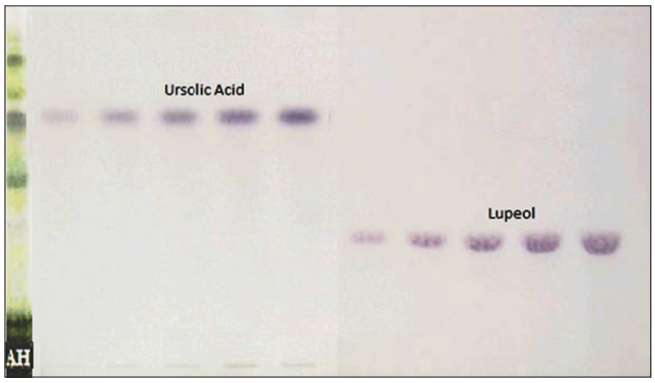


Figure 16: High performance thin layer chromatography plate showing bands of *Artocarpus heterophyllus* leaves, ursolic acid and lupeol

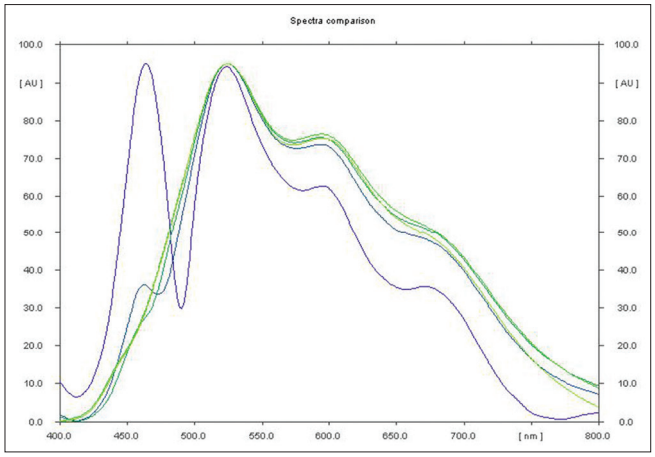


Figure 18: Spectral comparison of lupeol

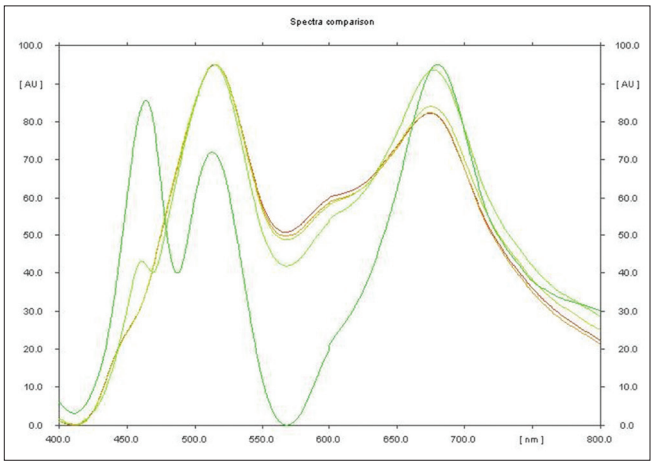


Figure 17: Spectral comparison of ursolic acid

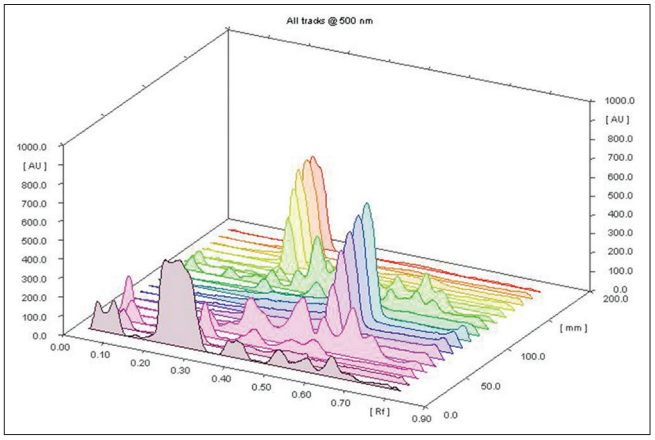


Figure 19: Three-dimensional spectra of all peaks at 500 nm

Antimicrobial activity

Zone of inhibition of test and standard substances are given in Tables 9 and 10 respectively and Figure 20, whereas minimum inhibitory concentration (MIC) for different strains is shown in Table 11, respectively. Different microbial stains used for the antimicrobial study are given in Table 12.

Discussion

Artocarpus hetrophyllus is one of the most common distributed plants in tropical regions of India; this plant has been part of ethnic culture since ancient time to treat several disorders and health issues, despite of high therapeutic possessions the plant is yet not much highlighted for its medicinal values factually it is highly demanded in food sectors. Pharmacognostic standardization was essential as oodles of uncertainty prevail when it comes to authentic information regarding the plant; also there was no justified pharmacognostic or anatomical work reported for the plant. Large number of phytoconstituents present in the plant gives it high medicinal value, the result from preliminary phytochemical screening as well as literature survey narrates that the plant has abundant amount of ursolic acid and lupeol thus it was thought worthwhile to subject the plant for quantitative analysis of ursolic acid and lupeol: the triterpenoids, ursolic acid, and lupeol are pronounced

antihepatotoxic agent,[22] thus by detecting the presence and amount of these active constituents in leaf can pave a pathway for its utilization in several hepatic ailments, the physiochemical properties of the plant is given in Tables 5 and 6 This plant somewhere lacks in the standardizing parameters that can act as a quality control tool for the better identification of the plant which is the primary step for better research of herbals. An attempt has been made to pinpoint the morpho-microscopic parameters that can be utilitarian in authentication of the plant. In this background certain reliable exemplar in TS of the leaf can be the presence of collateral open type vascular bundle that in which xylem and phloem are separated by cambium, TS of the leaf also shows that xylem and ground tissue is parenchymatous in nature while bast tissue is sclerenchymatous [Figure 1]. Cells of ground tissue contains calcium oxalate crystals whose type was very well confirmed in powder microscopy, the sui generis remarks of the leaf powder is the presence of starch grains, rosette type of calcium oxalate crystals, anomocytic stomata and sheathing trichomes [Figure 5]. HPTLC was accomplished to generate fingerprint profile of *A. heterophyllus* to detect and quantify the amount of ursolic acid and lupeol in leaves of

Table 8: Recovery analysis of lupeol and ursolic acid

Standards	Amount added (%)	Amount recovered (%) mean	SD	RSD
Lupeol	50	97.62	1.61	1.65
	100	99.05	1.14	1.15
	150	97.80	1.30	1.32
Ursolic acid	50	99.31	1.27	1.28
	100	101.07	1.15	1.14
	150	97.92	1.00	1.02

SD: Standard deviation, RSD: Relative standard deviation

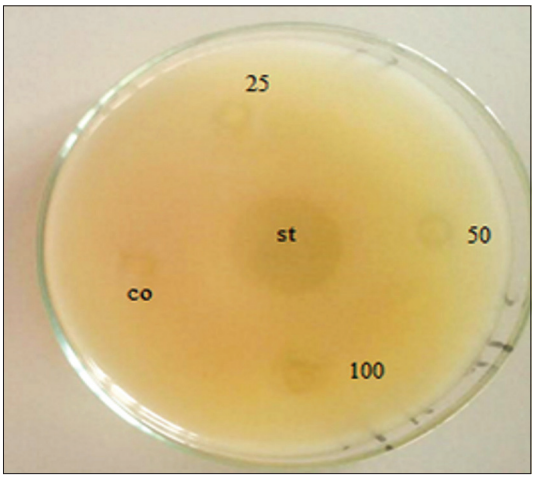


Figure 20: Antimicrobial activity of *Artocarpus heterophyllus* at different concentations

Table 9: Zone of inhibition by compounds at various concentration against different strains

Zone of inhibition by compounds at various concentration against different strains (mm)						
Test compounds	Concentration (µg/ml)	Gram negative strains		Fungal strain	Gram positive strains	
		Escherichia coli	*Pseudomonas aeruginosa*	*Aspergillus tubingensis*	*Bacillus subtilis*	*Staphylococcus aureus*
Artocarpus heterophyllus leaf	25	Nil	Nil	2.8±0.04	3.5±0.03	3.6±0.10
	50	1.9±0.06	1.8±0.05	3.2±0.03	4.5±0.10	4.4±0.11
	100	2.8±0.04	3.1±0.02	3.6±0.10	5.5±0.12	5.7±0.17

Table 10: Zone of inhibition by standard at various concentration against different strains

Zone of inhibition by standard compounds at various concentration against different strains (mm)						
Standard compound	Concentration (µg/ml)	Gram negative strains		Fungal strains	Gram positive strains	
		Escherichia coli	*Pseudomonas aeruginosa*	*Aspergillus tubingensis*	*Bacillus subtilis*	*Staphylococcus aureus*
Ciprofloxacin	25	10.3±0.40	10.6±0.42	Nil	9.6±0.57	9.9±0.59
	50	10.8±0.44	11.3±0.53	Nil	10.2±0.36	10.4±0.39
	100	11.1±0.52	11.6±0.58	Nil	10.9±0.46	10.8±0.44
Fluconazole	25	Nil	Nil	10.6±0.55	Nil	Nil
	50	Nil	Nil	11.5±0.56	Nil	Nil
	100	Nil	Nil	12.1±0.61	Nil	Nil

Table 11: Evaluation for minimum inhibitory concentration with different strains

Evaluation for MIC with different strains (µg/ml)					
Test compounds	Gram-negative strains		Fungal strain	Gram positive strains	
	Escherichia coli	*Pseudomonas aeruginosa*	*Aspergillus tubingensis*	*Bacillus subtilis*	*Staphylococcus aureus*
Artocarpus heterophyllus (leaf)	0.45±0.15	0.43±0.08	0.44±0.05	0.36±0.07	0.33±0.07

MIC: Minimum inhibitory concentration

Table 12: Different microbial stains used for antimicrobial study

Name of strains	Sources	MTCC number of strains
Staphylococcus aureus	IMTECH Chandigarh	1430
Escherichia coli	IMTECH Chandigarh	1573
Bacillus subtilis	IIMTECH Chandigarh	441
Pseudomonas aeruginosa	IIMTECH Chandigarh	2453
Aspergillus tubingensis	IIMTECH Chandigarh	117

MTCC: Microbial type culture collection, IMTECH: Institute of microbial technology

the plant, the results revealed that it is present in substantial amount, both of these biomarkers has corroborated itself in fortifying the liver against several adverse conditions. Furthermore, quantitative analysis of various constituents such as gallic acid, tannic acid, rutin, and lupeol hits the antioxidant potential of the plant also the plant can be further explored for its antihepatotoxic potentials. Plant possess good antibacterial activity [Figure 20] this may be due to the presence of tannins such as gallic acid and tannic acid, the literature says that tannins have the ability to disintegrate the bacterial colony, which may be due to the interference with the bacterial cell wall.[23,24] Data generated in this study is factual and verifiable in terms of standardization of this crude drug and also demands the attention pharmacologist to explore this plant in the field of medicine based research and development.

Conclusion

The author tried to bring out every necessary detail on macro-miroscopic characters of this plant. Phytochemical screening of leaf revealed the presence of several phytoconstituents such as flavonoids, tannins, terpenoids, and sterols which in itself focus that this plant can be the core of several pharmacological activities. HPTLC fingerprinting of *A. heterophyllus*, showed that it contains pronounced amount of ursolic acid and lupeol. Thus, this analytical result pave pathway for this plant to establish itself as an antihepatotoxic agent as speculated by author. The present study is an attempt to figure out primary needs required to generate technical or scientific standards so as to justify this herbal drug for further research work and also to keep a check on double sword (intentional and unintentional) adulteration, this study is an attempt to lay down the standards which could be used as the necessary parameters for the identification and authentication of plant *A. heterophyllus*. Quantification of good amount of ursolic acid and lupeol in this plant will provide extra emphasis to get explored as a potent hepatoprotective plant as both of these constituents possess hepatoprotective potential. Antimicrobial activity of the plant will help in combating the secondary infections due to liver dysfunctioning. Thus, this plant has the potential to treat several diseases along with the prevention of secondary infections caused by liver failure.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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