

Extraction, Identification and Pharmacological Evaluation of Phyto-Active Compound in *Manilkara Hexandra* (Roxb.) Dubard Stem Bark

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Plants are made up of chemicals of differing nature produced by metabolism. These phytochemicals show varied biological activity. The present work was aimed to identify such active principle present in the stem bark of *Manilkara hexandra* (Roxb.)Dubard. Proximate, fluorescence, histochemical, mineral analysis was carried out for the plant material. From the preliminary analysis, the flavonoid rich extract was identified which was then subjected to using chromatographic methods for compound separation. Characterization of the separated compound was done by UV, FT-IR, and GC-MS studies. 7,9- DI-TERT-BUTYL-1-OXASPIRO[4.5] DECA-6,9-DIENE-2,8-DIONE was identified by GC-MS analysis. Antibacterial activity was carried and it shows high zone of inhibition, when concentration increases from 30 to 150 µg/ml. The separated flavanoid in future could be used to study its wide pharmacological applications as a lead compound in drug delivery.

Keywords: Fluorescence; Histochemical; *Manilkara Hexandra*; Mineral Analysis Proximate.

Phytochemistry is the study of phytochemicals, which are chemicals derived from plants. This study describes the structure of primary and secondary metabolic products found in plants such as leaves, vegetables and roots that have defense mechanism and protects from various diseases. The medicinal plants are useful for healing, curing of human disease because of the presence of phytochemical constituents¹. A plant containing beneficial phytochemicals are alkaloids, terpenoids, saponin, and phenolic compounds such as flavanoids, tannins and lignins,² found in plants which act as anti- inflammatory, anti-bacterial³, may supplement the needs of the human

body by acting as natural antioxidant⁴. These phytochemicals, which are part of a large and varied group of chemical compounds, also are responsible for the color, flavor, and odor of plant foods, such as blueberries dark hue, broccoli's bitter taste, and garlic's pungent odor. Thousands of phytochemicals have been identified, and researchers speculate that there are likely many more they haven't yet discovered in the foods we eat. Though the broadest groups of phytochemicals, such as flavonoids, isoflavones, or anthocyanidins, often are referred to as a homogenous group, the individual compounds within each group have different chemical structures, are metabolized

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differently by the body, and may have different health effects⁵. Research strongly suggests that consuming foods rich in phytochemicals provide health benefits, but not enough information exists to make specific recommendations for phytochemical intake⁶. Proximate and nutrient analyses of edible plant and vegetables play a crucial role in assessing their nutritional significance⁷. In general, 22 nutrients are present in each human body for their requirements such as Fe, Ca, Mg, K, and Zn⁸. The antimicrobial activities have shown that the higher plants represent a potential source of novel antibiotic prototypes⁹. This has forced scientist to search for new antimicrobial substances from various sources like the medicinal plants¹⁰. The stem bark of *Manilkara hexandra* was used to cure gastro intestinal problem, burning sensation, febrifuge, odontopathy, hallucination, dyspepsia, anthelmintic, astringent and tonic¹¹.

Thus the present work was aimed to find out phytochemicals present in *Manilkara hexandra* through, proximate, histochemical, fluorescence, mineral analysis, separation and identification by using the thin layer and column chromatography, characterizing the identified phytoconstituents using analytical techniques such as UV-Visible spectra, FT-IR and GC-MS and evaluation of their antimicrobial activity.

MATERIAL AND METHODS

Collection of the plant material

Manilkara hexandra (Roxb.) Dubard (Authentication Number : 0011) was collected on December to February (Winter) season from Jayakondam at Ariyalur District, Tamilnadu, India and authenticated by Rapinet Herbarium, St. Joseph's

Table 1. Phytochemical Screening for stem bark methanolic extract of this plant

S.No	Phytochemicals	Observations
1.	Alkaloids	++
2.	Flavonoids	++
3.	Terpenoids	+
4.	Saponin	++
5.	Steroids	++

+ = Good ++ = Very Good

College (Autonomous), Trichy. The stem bark was washed with running water, shade dried, and grained by pestle mortar.

Preparation of the plant extract

All the chemicals (AR) were acquired from Sigma Aldrich. The powdered stem bark material was defatted with hexane, petroleum ether, and ethyl acetate by cold percolation method for 3 days. This solution was extracted by utilizing Whatman No.1. filter paper and stored in refrigerator at 4° C.

Phytochemical screening of this plant

The phytochemicals present in methanolic extract was analyzed by using standard procedure¹².

Physical Characterizations

Physiochemical Parameters

The physiochemical factors were analyzed by using WHO guidelines and Indian Pharmacopeia for herbal ingredients¹³. These include total ash content, acid insoluble ash, water soluble ash, moisture content, sulfated ash, dry matter and pH.

Total ash

3g of the sample was weighed accurately in a silica crucible. Then, it was incinerated by using muffle furnace at a temperature 400-500°C. The carbon particle was dried till it turns white in color. The total ash was cooled and weighed.

$$\% \text{ Ash} = \frac{(\text{Weight of crucible+ash}) - \text{Weight of crucible}}{(\text{Weight of sample})} \times 100$$

Moisture content

3g of the sample was weighed in a crucible with lid. The sample was kept in hot air oven at 105°C and cooled by using desiccator. The

Table 2. Physiochemical parameters of stem bark of *Manilkara hexandra*

S.No	Proximate Tests	Stem bark
1	Total ash	5.89±0.15
2	Moisture content	6.25±0.07
3	Acid insoluble ash	0.35±0.65
4	Water soluble ash	3.76±0.12
5	Sulphated ash	7.47±0.09
6	Dry Matter	93.29±0.15
7	pH	5.71±0.19

Mean value ± standard deviation

successive results were obtained from the weight difference between before and after drying process.

$$\% \text{ Moisture} = \frac{((\text{Weight of sample+dish before drying}) - (\text{Weight of sample+dish after drying}))}{(\text{Weight of sample taken})} \times 100$$

Acid insoluble ash

The above ash was boiled with 25 ml of dilute HCl and filtered by using Whatman No.1 filter paper. After filtration, this paper was placed and dried in a crucible to get an acid insoluble ash. Before and after, weight of crucible was calculated.

Water soluble ash

The total ash (A) was boiled with 25 ml of distilled water in a crucible for 15 minutes at 450 °C. This portion was filtered and the insoluble portion (B) was collected. Again, this insoluble part was dried and weighed. Calculate the water soluble ash from subtraction of A-B.

Sulfated ash

The total ash was ignited with Con. H₂SO₄ at 550 °C to 650 °C for 30 minutes and is then cooled using a desiccator until it reaches a constant weight.

Dry matter

Dry matter of plant material consists of nutrition excluding the water content. It was obtained from percentage of moisture content¹⁴.

$$\% \text{ Dry matter} = 100 - \% \text{ Moisture}$$

pH

The stem bark powder was dissolved in distilled water and it was boiled for 25 minutes. Then, it was cooled and the filtrate was collected. Finally, the pH of the filtrate was noted by using a systronic pH meter.

Histochemical Analysis

The dried stem bark powder were kept on the slide and treated with 1-3 drops of newly prepared reagents such as phloroglucinal, concentrated HCl, dilute ammonia, sulfuric acid, mayer's reagent (Mercuric chloride, potassium iodide, and water), ferric chloride solution, liberman (acetic anhydride and H₂SO₄), and toluidine blue separately. The individual samples were prolonged and placed on stage in microscope to identify the phytoconstituents (Lignin, flavonoids, alkaloids, tannin, starch and steroids^{15,16}).

Fluorescence analysis

A pinch of stem bark powder was tested with hexane, chloroform, acetone, methanol, aqueous solvents and also added to chemical reagents such as sodium hydroxide in water, concentrated sulfuric acid, hydrochloric acid, nitric acid of one normality. The resultant specimens were examined under the presence of UV- Visible light of long and short wavelengths (365 – 254 nm)¹⁷.

Mineral analysis

Ash was examined precisely using Gravimetric method by ASTM¹⁸. The stem bark of this plant was analyzed for organic content by the classical method of Walkey and Black¹⁹. Total Nitrogen estimated by the method of Micro Kjeldhal which were still used extensively in agricultural research²⁰. The Sulfur was measured using Gravimetric method²¹. Total Phosphorous was determined by Pemberton (1927) method²². For sodium, potassium, calcium and magnesium the flame intensity corresponding to the concentration of stock solution was noted using appropriate filters in Flame Photometry (Systronics mediflame 127) by the method of Stanford and English method²³. Total Zinc, Total

Table 3. Histochemical analysis for stem bark of *Manilkara hexandra*

S.No.	Test Reagents	Observation	Characterization	Stem bark
1.	Phloroglucinol + concentrate HCl	Pink	Lignin	++
2.	Dilute Ammonia + H ₂ SO ₄	Yellow	Flavonoids	++
3.	Mayers' Reagent	Reddish brown	Alkaloids	++
4.	FeCl ₃ Solution	Dark Blue to Black	Tannin	++
5.	Iodine	Blue	Starch grain	++
6.	Liberman (5 drops of acetic anhydride + 5 drops of H ₂ SO ₄)	Green	Steroids	+

Copper, Total Iron, Total Manganese, Total Boron, Total Molybdenum is analyzed by using Atomic Absorption Spectroscopy (Solar-AAS2-UK made). Perchloric-acid digestion method was used for elemental analysis²⁴.

Column chromatography

The column apparatus (15×4 cm) was washed with water and rinsed by using solvents. A small piece of cotton was inserted into the lower part of the column and is clamped to a stand. The silica gel (60-120 mesh) was activated in the oven at 120°C for 1 hour. The slurry of silica gel (100-

150g) was prepared, packed into the column and digested with petroleum ether (60°C-80°C). The ethyl acetate extract was mixed with the above slurry of silica gel and the following eluents were successively run out starting from hexane, chloroform, ethyl acetate, methanol in their increasing order of polarity. Finally, flavonoids were isolated using an eluent mixture in the ratio 7:3 (MeOH: Water) and the fraction were collected in test tubes²⁵.

Thin Layer Chromatography

The precoated TLC Silica gel 60 F₂₅₄ on aluminium sheets (20×20 cm), were purchased

Table 4. Observation of Fluorescence report for stem bark of *Manilkara hexandra*

S.No.	Chemicals	Visible Light	Short UV (254nm)	Long UV (365 nm)
1	Plant powder (PP)	Brown	Black	Black
2	Plant powder with water	Dark Green	Dark Green	Brown
3	PP with Hexane	Reddish Brown	Dark Brown	Dark Brown
4	PP with Chloroform	Black	Black	Black
5	PP with Methanol	Brown	Black	Black
6	PP with Acetone	Brown	Black	Black
7	PP with 1 N Sodium hydroxide in water	Brown	Green	Reddish Brown
8	PP with 1 N Hydrochloric acid	Light Green	Blackish Green	Dark Brown
9	PP with 1 N Sulphuric acid	Dark Green	Blackish Green	Dark Brown
10	PP with 1 N Nitric acid	Dark Green	Blackish Green	Dark Brown

Table 5. Minerals consist in the stem bark of *Manilkara hexandra*

S.No	Name of the parameter	Obtained value	Benefits of the element
1	Organic Carbon (%)	0.91	To regulate the pH and acid balance ²⁹ .
2	Total Sodium (%)	0.16	To control muscle contraction and involved in blood plasma, nervous system, digestive processes.
3	Total Potassium (%)	4.54	To maintain the body fluid and solve the renal problems ³⁰ .
4	Total Phosphorus (%)	0.32	To filter the impurities of kidney, refresh of tissues and cells in the body.
5	Total Magnesium (%)	4.31	To use in dietary disorders.
6	Total Calcium (%)	6.57	To apply in teeth, bones, and nerves ³¹ .
7	Total Nitrogen (%)	1.80	To use in the digestion process
8	Total Sulfur (%)	0.44	To synthesized protein and involved in enzyme reactions ³² .
9	Total Copper (ppm)	0.05	It involves in a metabolic process and also maintains the strength blood vessels ³³ .
10	Total Zinc (ppm)	1.65	It acts as a boosting agent of geriatric patients ³⁴ .
11	Total Boron (ppm)	0.042	To improve bone and apply in wound healing,
12	Total Molybdenum (ppm)	0.02	To retard the degradation of teeth enamel ³⁵ .
13	Total Manganese (ppm)	10.41	To use in Dietary recommendations
14	Total Iron (ppm)	129.51	To increase the hemoglobin and develop the immune system ³⁶ .

from Merck. The isolated fractions were spotted by using a capillary tube on the cut plate (Stationary phase) (6.5×3.5). The spotted strip were dried and immersed into the developing eluents (Mobile phase) of system (n-Butanol, acetic acid and water (4:1:5)). Then, it was covered with a lid. Finally the four fraction plate was dried and retention factor of

that chromatogram was noted using the following formula²⁶,

$$\text{Rf} = \frac{\text{(Distance of the solute travelled from the origin)}}{\text{(Distance of the solvents travelled from the origin)}} \times 100$$

Confirmatory tests for flavonoid

1. The presence of flavonoid in the fractions was confirmed by sprinkling NH_3 .
2. The presence of flavonoid was also confirmed by adding NaOH.

Characterization

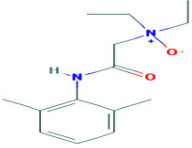
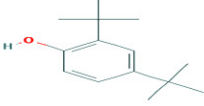
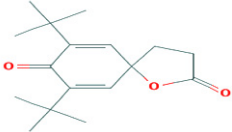


UV-Visible Spectral Analysis

Lambda 35 UV-Visible spectrophotometer which is a PC controlled, double beam scanning spectrophotometer is a useful analytical technique to identify some functional groups in molecules thus aiding in structural elucidation in bio-molecules. It covers the wavelength range 200 nm to 800 nm with a setting accuracy of ± 1 nm.

Table 6. Rf value of flavonoids

S. No	Fractions	Rf	Compound
1.	Petroleum ether	0.62	Chalcones
		0.82	Kaempferol
2.	Ethyl acetate	0.03	Unidentified
		0.51	Apegenin
		0.89	Flavonones
3.	Methanol	0.03	Unidentified
		0.68	Chalcones
4.	Methanol: Water	0.96	Flavonoids (Quercetin derivatives)

Table 7. GC-MS report of isolated compound from TLC

S. No.	Molecular weight	Compound name	Structure	Formula
1.	250	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine		$\text{C}_{14}\text{H}_{22}\text{N}_2\text{O}_2$
2.	206	2,4-bis(1,1-dimethylethyl)		$\text{C}_{14}\text{H}_{22}\text{O}$
3.	276	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione (flavonoid)		$\text{C}_{17}\text{H}_{24}\text{O}_3$
4.	256	n-Hexadecanoic acid		$\text{C}_{16}\text{H}_{32}\text{O}_2$
5.	284	Octadecanoic acid		$\text{C}_{18}\text{H}_{36}\text{O}_2$

FT-IR spectral analysis

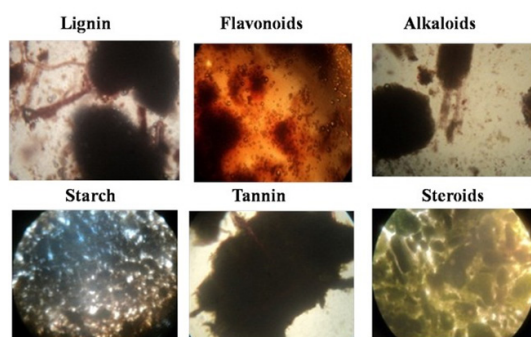
IR spectra of plant substance can be taken in an automatic recording IR spectrophotometer either in solution (in chloroform or carbon tetrachloride 1-5%) as a mull with nujol oil or in the solid state mixed with potassium bromide. In the latter case a thin disc is prepared under anhydrous condition from a powder containing about 1mg of material and 10 to 100mg KBr using mould and press. The range of measurement is from 4000 to 667cm⁻¹ and the spectrum takes about three minutes to be recorded .

GC-MS Analysis

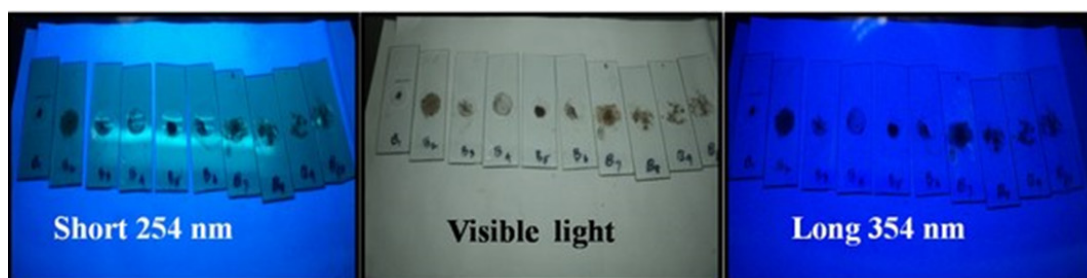
The GC-MS analysis was performed on a combined GC-MS instrument (ITQ 900 Model of Thermo Fisher Scientific make) using a HP-5 fused silica gel capillary column. The method to perform the analysis was designed for both GC and MS. 1 ¼L aliquot of the sample was injected into the column using a PTV (Programmed Temperature Vaporizing) injector whose temperature was set at 275°C. The GC program was initiated by a column temperature set at 60°C for 5 min, increased to 300°C at a rate of 8 C/min, held for 10 min. Helium

Table 8. Antibacterial report for isolated flavonoid fraction

Plant extracts	Concentrations (µg/ml)	Organisms/Zone of inhibition (mm)		
		<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
Extracts	30	0	0	0
	50	0	0	0
	90	0	0	0
	120	7	6	6
	150	8	7	6
Methanol	10 µl/disc	0	0	0

**Fig. 1.** Image of histochemical analysis

was used as the carrier gas (1.5 mL/min). The mass spectrometer was operated in EI (Electron ionization) mode with mass source set at 200°C. The chromatogram and spectrum of the peaks were visualized. The particular compounds present in the samples were identified by matching their mass spectral fragmentation patterns of the respective peaks in the chromatogram with those stored in the National Institute of Standards And Technology Mass Spectral database (NIST-MS, 1998) library²⁷.

**Fig. 2.** Fluorescence photos for stem bark of *Manilkara hexandra*

Antibacterial assay**Collection of test organisms**

To examine the antibacterial activity of methanol extract, two gram positive bacterial strains viz., *Staphylococcus aureus* (MTCC 25923) and *Bacillus subtilis* (MTCC 2451) respectively. One gram negative bacterial strain *Escherichia coli* (MTCC 25922) were prepared as test organisms. All the strains were procured from the Microbial Type Culture and Collection (MTCC) at Chandigarh, India. Bacterial strains were cultivated at 37°C and maintained on nutrient agar (Difco, USA) slant at for 4°C.

Screening of Antibacterial Activities**Antibacterial activity of methanol extracts (disc diffusion method)**

Antibacterial activity of methanol: water (7:3) extract was determined using the disc diffusion method. The petri dishes (diameter 60

mm) were prepared with Muller Hinton Agar and inoculated with test organisms. Sterile disc of six millimeter width was impregnated with 10 μ l of sample A at various concentrations of 30-150 μ g/ml respectively. Prepared discs were placed onto the top layer of the agar plates and left for 30 minutes at room temperature for compound diffusion. Negative control was prepared using the respective solvent. The dishes were incubated for 24 h at 37°C and the zone of inhibition was recorded in millimetres²⁸.

RESULT AND DISCUSSIONS**Physical characterizations****Phytochemical Screening**

The plant extract was subjected to phytochemical screening using standard procedures. The result reveals the presence of

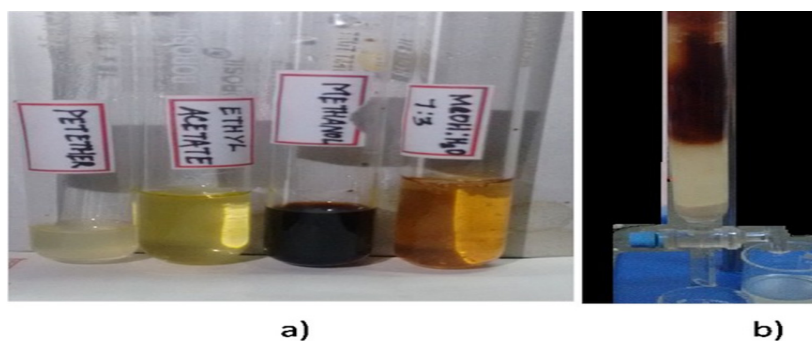


Fig. 3. a) Collection of fractions (petroleum ether, ethyl acetate, methanol and methanol:water) b) Separation process in column

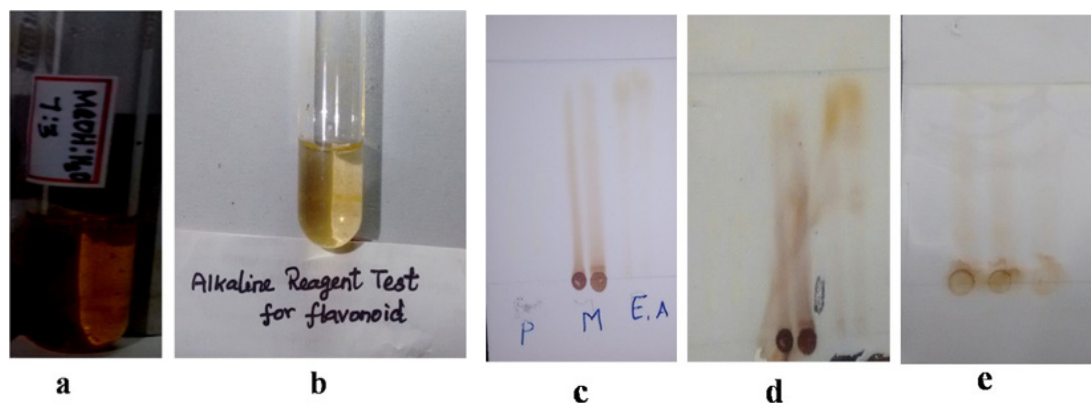


Fig. 4. a) Addition of NaOH b) Addition of Hcl c) Before spraying ammonia d) After spraying ammonia e) Methanol: water (7:3)

alkaloids, flavonoids, terpenoids, saponin, and steroids as shown in Table 1.

Physiochemical parameters

Today essential chemical evaluation of physiochemical parameters such as total ash, moisture, acid insoluble ash, water soluble ash, sulfated ash, dry matter and pH is needed for the detection of adulterants and get a fix on drugs. The physiochemical parameters reported in the Table 2 shows the high presence of total ash (5.84 ± 0.15) in this plant indicates the presence of diverse minerals. Acid insoluble ash value (0.35 ± 0.65),

indicates the evidence for presence of silica and the sulfated ash (7.47 ± 0.09) reveals that the presence inorganic substance in organic materials. The water soluble ash (3.76 ± 0.12) determines the quantity of inorganic substances present in the stem bark of *Manilkara hexandra*. The pH of the sample is found to be 5.71.

Histochemical Analysis

By observing the variation of intensity in color viz; lignin (pink), flavonoids (yellow), alkaloid (reddish brown), steroid (green), tannins (black) and starch (blue) by histochemical analysis

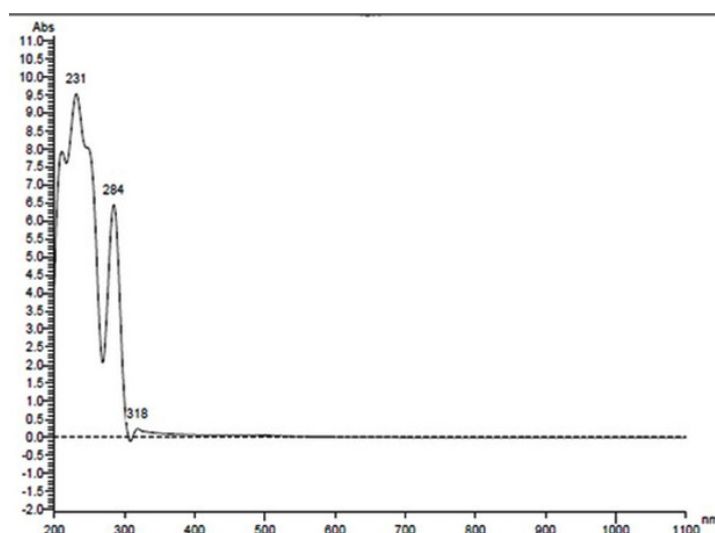


Fig. 5. UV- Visible spectroscopy for methanol: water fractions

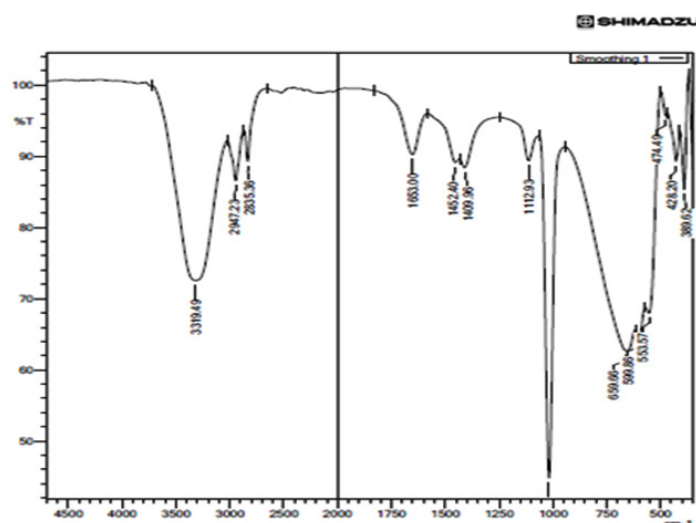


Fig. 6. FT-IR spectrum of isolated flavonoid fraction on stem bark of *Manilkara hexandra*

it is possible to categorize the quantitative presence of these metabolites in the stem bark as shown in Table 3 and Figure 1.

Fluorescence analysis of stem bark of *Manilkara hexandra*

Fluorescence analysis is carried out to confirm the presence of secondary metabolites by examining under the presence of UV-Visible light of long and short wavelengths (365-254 nm). Their results are as mentioned in Table 4 and Figure 2.

Mineral analysis

The impact of minerals plays an important role in our body. This report declares that the iron, calcium, magnesium, potassium, zinc, nitrogen were present in rich and other elements were present in trace amounts as shown in Table 5.

Identification and Separation of Phytocomponents

Chromatographic Analysis

Column Chromatography

Column chromatography is commonly utilized for the purification of bioactive compounds. In this work, the methanolic stem bark extract of *Manilkara hexandra* was eluted with low polar to high polar eluents. The fractions eluted from petroleum ether, ethyl acetate, methanol and methanol: water (7:3) was collected as shown in Figure 3a, and b.

Retention factor (R_f)

The compounds identified from various solvent fractions (pet. ether, ethyl acetate, methanol, and methanol:water) spotted on TLC plate are

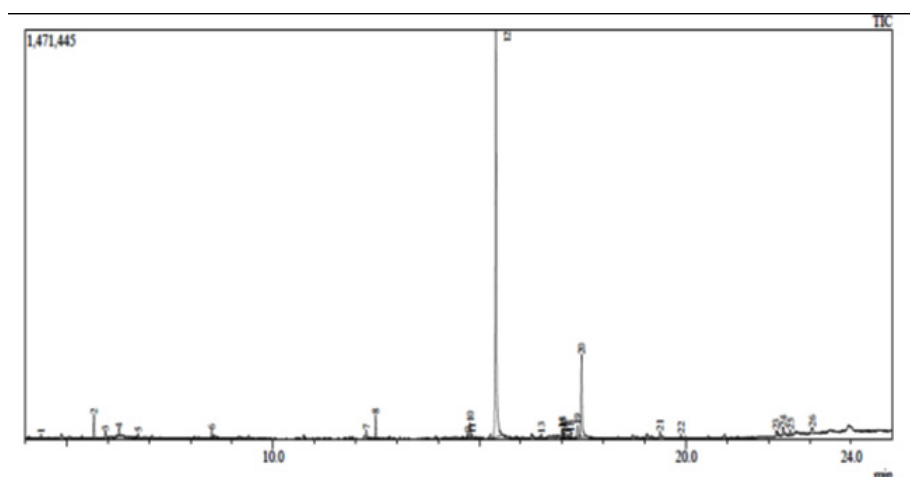


Fig. 7. GC-MS report for isolated flavonoid fraction

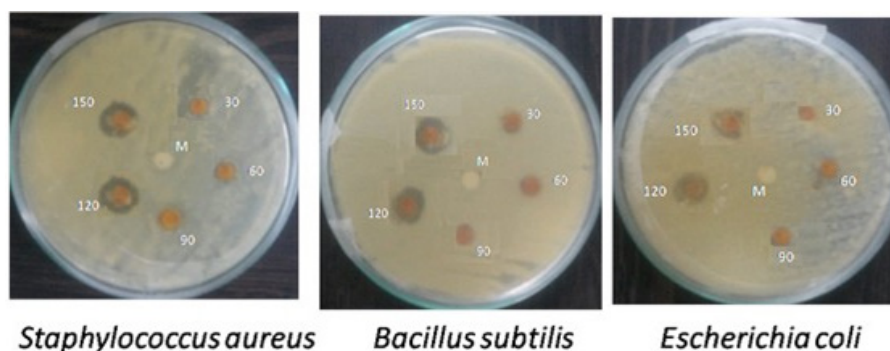


Fig. 8. Antibacterial report for isolated flavonoid fraction

shown in Table 6. The Rf values obtained were compared with literature data and it is inferred that most of them are flavonoids in nature.

Thin Layer Chromatography

The methanol: water (7:3) fraction is spotted on a TLC plate to find out retention factor (Rf) using the solvent system, n- butanol:Acetic acid:Water (4:1:5). The methanol: water (7:3) gave single spot at (Rf = 0.96), which indicates that it might be due to the presence of the flavonoid. The presence of flavonoid is further confirmed qualitatively from the following analysis.

i. It gave yellow color on reaction with NaOH, which decolorized on the addition of HCl (Alkaline reagent test) Figure 4a, and b.

ii. The compound turned from greenish yellow to deep yellow by spraying ammonia for above fractions. (Figure 4c,d and e)

Spectral analysis

UV- Visible spectroscopy

The UV – Visible spectra suggested that the absorbance of a compound at $\lambda_{\text{max}} = 318 \text{ nm}$, 284 nm and 231 nm is a flavonoid (quercetin), as shown in Figure 5.

FT-Infrared spectroscopy

From the occurrence of peaks at 3319 cm^{-1} , 2947-2835 cm^{-1} , 1653 cm^{-1} , 1452 cm^{-1} , 1409 cm^{-1} , 1112 cm^{-1} , 1030 cm^{-1} at 3319 cm^{-1} suggested and further confirmed the presence of the flavonoid as shown in Figure 6.

GC-MS analysis

The GC-MS spectra reveals that the presence of flavonoid compound is 7,9-DI-TERT-BUTYL-1-OXASPIRO[4.5]DECA-6,9-DIENE-2,8-DIONE. The flavonoid compound appears at 14 minutes, (RT). Along with this, five major compounds were obtained and it was identified from NIST and WILEY as shown in Table 7 and Figure 7.

Antibacterial activity

The results of the antibacterial activity of methanol extracts were tested against pathogens by disk diffusion method are shown in Table 8 and Figure 8. The sample showed growth inhibitory activity against *Staphylococcus aureus* (8 mm) at concentration 150 $\mu\text{g/ml}$ and against *Bacillus subtilis* (7 mm). At concentration 120 $\mu\text{g/ml}$, the sample exhibited the antibacterial activity against, *Staphylococcus aureus* (7 mm). However, the sample showed better inhibitory actions against

pathogens at a concentration 90, 120 and 150 $\mu\text{g/ml}$ than at lower concentration. As the concentration of extracts increased from 30-150 $\mu\text{g/ml}$, the inhibitory actions of the plant extracts increased towards all the strains used in this study.

CONCLUSION

From the present investigation the following conclusion can be drawn. Biologically active phytochemicals such as alkaloids, flavonoids, saponins and steroids are present in the methanolic extract of *Manilkara hexandra* by carrying out phytochemical analysis. Histochemical and Fluorescent analysis further confirms their presence. Separation and identification of the isolated compound done by using chromatographic techniques such as Column chromatography and Thin layer chromatography using the subsequent eluting method made ease to separate active flavanoids (quercetin derivative) from the methanol:water fraction confirmed by GC-MS analysis also. This was further confirmed using spectral tools such as UV-Visible spectroscopy and FT-IR. Good antimicrobial activity was also found in the separated compound with increase in the concentrations. Thus, this study is only a preliminary study pertaining to the occurrence of certain properties of *Manilkara hexandra*. An in-depth study will provide a concrete base for all the phytochemical functions exhibited by this plant used in traditional medicine.

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