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PRELIMINARY PHYTOCHEMICAL ANALYSIS AND THIN LAYER CHROMATOGRAPHY OF THE EXTRACTS OF *EXCOECARIA AGALLOCHA* L.

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ABSTRACT: Purpose: The focus of the present paper was a preliminary phytochemical and TLC analysis of the various extracts (root, stem, and leaf) of the mangrove plant *Excoecaria agallocha* L. **Methods:** Phytochemical analysis was carried out using the standard phytochemical assays. TLC analysis of the petroleum ether and chloroform fraction of the leaves was carried out using the solvent system Toluene: Ethyl acetate (in 9:1 ratio) and Toluene: Ethyl acetate: Formic acid (in 5:5:0.2 ratio) for PE and chloroform fractions respectively. **Result and Conclusion:** The results of the preliminary phytochemical screening revealed the presence of various chemical compounds like alkaloids, glycosides, flavonoids, carbohydrates, anthraquinone, tannins, phenols, terpenoids, fixed oil, and fats. The organoleptic study revealed the specific nature of the plant parts used in the study. The behavior of the powdered plant parts in the presence of various chemicals was studied. TLC analysis of the petroleum ether and chloroform fraction of the leaves of *Excoecaria agallocha* L. revealed bands indicating the presence of various compounds that could act as potential antimicrobial agents.

INTRODUCTION: Plants are rich sources of a wide variety of secondary metabolites that are effective in controlling many of the microbial diseases [1, 2]. They contain tannins, alkaloids, terpenoids, flavonoids, *etc.* which bestow them with an undisputed position when it comes to the control of micro-organisms. Thus, they serve as a source of cheap and safe antimicrobial agents for the treatment of microbial infections [3].

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Mangroves are one of the prime members of the estuarine food chain as they are an integral part of the coastal plant population. They deserve special attention as they are unique, and are important to the overall development, maintenance, and well-being of their habitat. They produce a variety of metabolic products that are stimulated in response to the hostile situations that they are exposed to. Research on the antimicrobial effect of the secondary metabolites from mangroves reveals that they contain a wide variety of different chemical compounds that are effective as antimicrobial agents [4].

Excoecaria agallocha is a mangrove plant seen abundantly in the coastal estuarine regions of Indian sub-continent.

It possesses excellent antifungal and antifilarial activities [5]. The hydroalcoholic extract of the dried and ground bark of *E. agallocha* encompass antioxidant activity as established by DPPH, NO, and H_2O_2 scavenging assays [6]. Traditional medical practitioners use various parts of this plant for treating/curing a wide variety of ailments like ulcers, leprosy, paralysis, *etc.* Sores and stings from marine animals can be cured with the sap from this plant.

It could be used as purgative and emetic and also be used for curing rheumatism, dermatitis and conjunctivitis [7]. The potent anti-HIV, [8] anticancer, [9, 8] and antimicrobial [7, 10] effects of the various extracts are also reported. The wound healing properties of the latex of *E. agallocha* were extensively studied using an animal model and in man. It revealed that the wound healing properties were comparable to the standard medicine, furacin ointment [11].

FIG. 1: *EXCOECARIA AGALLOCHA* L. – PLANT PROFILE

The positive results of the antifungal studies conducted earlier [12] prompted us to explore the plant in detail. The present study was conducted to carry out a preliminary phytochemical analysis of the various solvent fractions extracted from different parts of *E. agallocha* L. and to check the TLC profile of the fractions. The results are represented in the present study.

MATERIALS AND METHODS:

Collection of Plant: Different parts (root, stem, and leaf) of the plant *E. agallocha* L. were collected from Wadakara, Calicut District, Kerala. The plant was identified and authenticated at CMPR, Kottakkal Arya Vaidya Sala, Kerala. The plant parts were shade dried and pulverized in a mechanical grinder to obtain moderately coarse powder and were subjected to aqueous extraction and further solvent fractionation in organic solvents in the increasing order of polarity.

Preparation of Extracts: About 5g each of the root, stem and leaf powders obtained were extracted separately with 400 ml double distilled water and refluxed for 6 h. The aqueous extract was then filtered and concentrated to dryness in a rotary evaporator under reduced pressure. The semi-solid extract of leaf, stem, and root thus obtained was re-dissolved in water and stored in a refrigerator until further use.

Pharmacognostic Studies: The organoleptic characters like color, taste, and odor can vary from plant to plant and can give clues on the nature of compounds present in the plant. The organoleptic studies of the powders of leaf, stem, and root of the plant were conducted.

Preliminary Phytochemical Screening: Further, for the pharmacognostic studies (a preliminary phytochemical analysis), an aqueous extract of leaf was prepared to know the nature of secondary metabolites and compounds present in it. The details of the screening are as follows:

Test for Carbohydrates:

Molisch's Test: A small quantity (3ml) of the extract was dissolved in 4 ml distilled water and filtered. The filtrate was subjected to Molisch's test. Formation of the reddish-brown ring indicates the presence of carbohydrates.

Fehling's Test: A small quantity (3ml) of the extract was dissolved in 4 ml distilled water and filtered. The filtrate was treated with Fehling's solution. Brown color development indicates the presence of carbohydrates.

Test for Phenols:

Phosphomolybdic Acid Test: The extract was spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the spot and was exposed to ammonia vapors. The blue coloration of the spot is the confirmation for the presence of phenols.

Test for Flavonoids:
Shinoda Test: To 2-3 ml of the extract, a piece of magnesium ribbon and 1 ml of concentrated hydrochloric acid were added. Formation of pink red or red color is indicative of the presence of flavonoids.

Lead Acetate Test: To 5 ml of the extract, 1ml of lead acetate solution was added. A flocculent white precipitate formed indicates the presence of flavonoids.

Test for Tannins:
Braemer's Test: To 2-3 ml of the extract, 10% alcoholic ferric chloride solution was added. Dark blue or greenish-grey coloration of the solution indicates the presence of tannins in the drug.

Test for Steroids/ Terpenoids:
Liebermann-Burchard Test: To 1ml of the extract, 1ml of chloroform, 2-3ml of acetic anhydride and 2-3 drops of concentrated sulphuric acid were added.

Green coloration of the solution indicates the presence of steroids, and the presence of terpenoids is indicated by dark pink or red coloration of the solution.

Test for Alkaloids:
Dragendorff's Test: A drop of the extract was spotted on a small piece of precoated TLC plate, and the plate was sprayed with a modified Dragendorff's reagent. If alkaloids are present in the sample, the spot will change to orange color.

Hager's Test: The extract was treated with a few drops of Hager's reagent. Yellow coloration indicates the presence of alkaloids.

Wagner's Test: The extract was treated with a few drops of Wagner's reagent. A reddish-brown precipitate will be formed if the solution contains alkaloids.

Test for Glycosides:
Legal's Test: 0.1ml of the extract was dissolved in 2ml of pyridine. To this solution, 2 ml of sodium nitroprusside solution was added.

This solution was made alkaline with sodium hydroxide. A pink to red colored solution indicated the presence of glycosides.

Test for Saponins:
Foam Test: 1ml of the extract was diluted with distilled water and made up to 20 ml. The solution was shaken vigorously in a graduated cylinder for about 15 min. A one cm layer of foam formed confirms the presence of saponins in the sample.

Test for Anthraquinones:
Borntrager's test: About 5 ml of the extract was heated with 10% ferric chloride solution and 1ml of concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. This extract was further extracted with strong ammonia. A pink or deep red coloration of the aqueous layer indicates the presence of anthraquinones.

Test for Amino Acids:
Ninhydrin Test: A small quantity of the extract was dissolved in a few ml of distilled water, and one ml of ninhydrin reagent was added to it. Development of a blue color indicates the presence of amino acids.

Test for Fixed Oils and Fats: A small quantity of the petroleum ether (PE) extract was pressed between two filter papers. Oil stains on the paper indicate the presence of fixed oils and fats.

Solvent Fractionation: The dried aqueous extract (about 4.5 g) of each plant part was refluxed with 100 ml petroleum ether for 6 h. In short, a quantity of 25 ml of the individual extract was taken in a separating funnel with an equal quantity of the solvent (PE) and kept undisturbed for some time. The organic layer was collected, and the process repeated for further fractionation till the whole extract (100 ml) was fractionated. Thus, the PE fraction of root, stem, and leaf was obtained.

The extract was then concentrated to dryness in a rota vapour. The whole process was repeated to obtain the chloroform, ethyl acetate and n-butanol fractions of root, stem, and leaf. The various solvent extracts thus prepared were re-extracted with DMSO and aliquoted and stored in 4 °C till further use.

Chemical Screening: TLC is a very convenient and simple way of screening plants [13] and can be successfully employed in the target-directed isolation of active compounds. The TLC employed in the current study is only a preliminary one to

detect the compounds. Further studies are on track to isolate the compound based on the present results.

TLC Profiling of Petroleum Ether Fraction:
Test Solution: The crude aqueous leaf extract was refluxed with 100ml petroleum ether for 6 h. It was filtered and concentrated under reduced pressure. The residue was dissolved in 10 ml of petroleum ether, and the extract was used for TLC profiling. The solvent system used was Toluene: Ethyl acetate in 9:1 ratio (standardized by trials). Silica gel 60 F_{254} plates (E. Merck) of uniform thickness of 0.2 mm was used as the stationary phase.

Procedure: 10 µl of the test solution was applied twice on a pre-coated TLC plate and developed in the solvent system in a twin trough chamber to a height of 8 cm. The plate was visualized in visible, and UV light (254 nm and 365 nm) and again in visible light and 365 nm after spraying with anisaldehyde-sulphuric acid reagent [14] and the colors and R_f values of each band were recorded according to the formula

Retention factor (R_f) = Distance traveled by the plant extract/distance traveled by the solvent front.

TLC Profiling of Chloroform Fraction: After the petroleum ether extraction, the remaining material was dried and again refluxed with 100 ml chloroform for 6 h. It was then filtered and concentrated under reduced pressure. The residue was dissolved in 10 ml of chloroform, and this fraction was used for TLC profiling. The solvent system used was Toluene: Ethyl acetate: Formic acid (in 5:5:0.2 ratio obtained and standardized by trials). Silica gel 60 F_{254} plates (E. Merck) of uniform thickness of 0.2 mm was used as the stationary phase. The further procedure followed for visualization was the same as described above for PE fraction.

RESULTS: The results of the various analyses carried out in the study are represented below.

Organoleptic Study Table 1 and 2: The color, taste, and odor of the powders of the various plant parts and their behavior on treatment with various chemicals were studied, and the observations are shown in **Table 1** and **Table 2**. Further, when the powders of different plant parts were treated with various chemicals like $FeCl_3$, NaOH, KOH, H_2O, HCl, NaOH + H_2O, ethanol, HNO_3, and H_2SO_4, *etc.* they showed varying shades of green and brown. The results of the study are depicted in **Table 2**.

Preliminary Phytochemical Analysis Table 3: Pharmaceutical preparations derived from natural sources often contain compounds that contribute to the antimicrobial defense system and apparently will account for the protection against degenerative diseases. In the present study, preliminary phytochemical analysis of the aqueous leaf extract of *E. agallocha* L. was carried out to detect the active constituents such as alkaloids, anthra-quinone, amino acids, carbohydrates, flavonoids, glycosides, saponins, steroids/terpenoids, tannins, phenols, and fixed oils and fats. Almost all major groups of phytochemicals were found to be present in the sample as evidenced by the various chemical analyses as detailed out in **Table 3**.

TABLE 1: ORGANOLEPTIC STUDY OF THE PLANT PARTS OF *EXCOECARIA AGALLOCHA*

S. no.	Plant part	Color	Taste	Odor
1	Leaf	Green	Bitter	Pleasant
2	Stem	Brown	Bitter	Pungent
3	Root	Light brown	Sour	Pungent

The color, taste and odor of the leaf, stem and root revealed typical nature of the plant part as expected. Stem and root exhibited pungent smell and were bitter/sour when tasted.

TABLE 2: COLOR OF THE PLANT PART ON TREATING WITH DIFFERENT CHEMICALS

Chemical added to the powder	Color of the plant part			Chemical added to the powder	Color of the plant part		
	Leaf	Stem	Root		Leaf	Stem	Root
2% $FeCl_3$	Green	Light brown	Brown	NaOH + HCl	Green	Brown	Brown
10% NaOH	Green	Light brown	Brown	Ethanol	Green	Light brown	Brown
5% KOH	Green	Light brown	Brown	Nitric acid	Green	Brown	Brown
Water shake	Green	Light brown	Brown	H_2SO_4	Green	Brown	Brown
HCl	Green	Brown	Brown	Iodine	Green	Light brown	Brown

The plant parts showed different colors when various chemicals were added to the powdered plant part.

TABLE 3: PRELIMINARY PHYTOCHEMICAL SCREENING OF THE AQUEOUS LEAF EXTRACT OF *EXCOECARIA AGALLOCHA* L.

S. no.	Test for	Test name	Reagents	Reaction	Inference*
1	Alkaloids	Dragendorff's test	Bismuth nitrate, glacial acetic acid, potassium iodide	Orange coloration	+++
		Hager's test	Picric acid	Yellow precipitate	-
		Wagner's test	Iodine in water	Reddish-brown precipitate	+++
2	Antraquinone	Borntrager's test	$FeCl_3$ + conc. HCl+ diethyl ether + strong ammonia	Deep red or pink color of aqueous layer	++
3	Amino acids	Ninhydrin test	Ninhydrin in n- Butanol	Blue color	-
4	Carbohydrates	Molisch's test	α-naphthol in 95% alcohol	Reddish brown ring	+++
		Fehling's test	Copper sulphate+ potassium sodium tartarate+NaOH	Reddish-brown precipitate	-
5	Flavonoids	Shinoda test	Magnesium ribbon + Conc. HCl	Pink or red color	+
		Lead acetate test	Lead acetate	Flocculent white precipitate	++
6	Glycosides	Legal's test	Pyridine + sodium nitro prusside +NaOH	Pink to red coloration	+++
7	Saponin	Foam test	Distilled water	A 1cm layer of foam	++
8	Steroids/ Terpenoids	Liebermann-Burchard test	Chloroform + acetic anhydride +conc. H_2SO_4	Dark green (Steroids) Dark pink/ green (Terpenoids)	+++
9	Tannins	Braemer's test	10% alcoholic ferric chloride	Dark blue or greenish grey	+++
10	Phenols	Phospho-molybdic acid test	Phospho-molybdic acid + ammonia	Blue color	+++
11	Fixed oils and fats		Extract pressed between filter paper	Oil stain develops	+++

* - absent, + trace, ++ present, +++ abundant

TLC Analysis of the PE and Chloroform Fractions of the Leaf Extract: The Thin Layer Chromatographic analysis of the PE and chloroform fractions of the leaf extract was carried out as explained. The chromatogram revealed 8 bands under UV and 6 bands in visible light corresponding to various compounds present in the petroleum ether fraction of leaf extract. Similarly, 8 bands were seen under UV and 9 bands in visible light in the chloroform fraction of leaf extract **Fig. 2** and **3**.

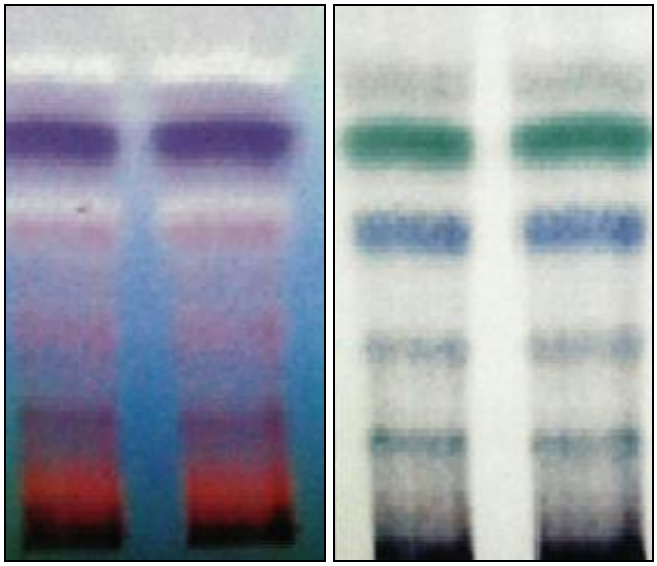

FIG. 2: TLC PROFILE OF THE PETROLEUM ETHER FRACTION OF LEAF AFTER DERIVATI-ZATION

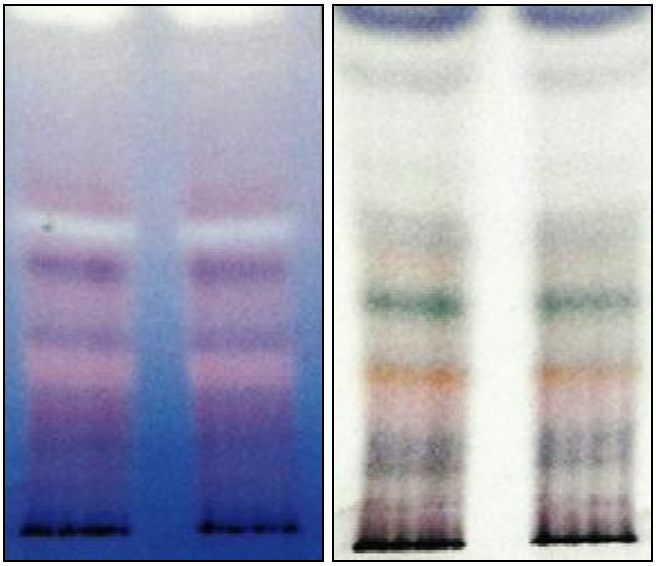

FIG. 3: TLC PROFILE OF THE CHLOROFORM FRACTION OF LEAF AFTER DERIVATIZATION

Most of the compounds were visualized after derivatization on spraying with the anisaldehyde-sulphuric acid reagent.

The R_f values calculated for each compound in petroleum ether extract and chloroform extract are depicted in **Table 4**.

TABLE 4: R_f VALUES CALCULATED FROM TLC PROFILE OF THE LEAF EXTRACT OF *E. AGALLOCHA* (AFTER DERIVATIZATION)

Petroleum ether fraction				Chloroform fraction			
Under UV $_{365}$		Under visible light		Under UV $_{365}$		Under visible light	
Color	**R_f**	**Color**	**R_f**	**Color**	**R_f**	**Color**	**R_f**
Brown	0.038	Brown	0.071	Pink	0.157	Brown	0.027
Red	0.076	Dark green	0.178	Pink	0.263	Pink	0.055
Brown	0.192	Blue	0.321	Blue	0.289	Brown	0.138
Pink	0.307	Dark blue	0.500	Blue	0.394	Pink	0.222
Pink	0.461	Green	0.642	White	0.473	Orange	0.277
White	0.500	Light green	0.714	Pink	0.526	Green	0.377
Violet	0.615			Light orange	0.789	Pink	0.444
Light blue	0.692			White	0.868	Brown	0.500
						Blue	0.666

DISCUSSION: In the present investigation, the aqueous extract of *Excoecaria agallocha* was tested for the presence of phytochemicals using various chemical reagents. It was found that the extract contained saponins, alkaloids, anthraquinone, carbohydrates, flavonoids, glycosides, steroids, tannins, phenols, and fixed oils at various levels. Their presence was further strengthened by TLC profiling of leaf extract, which showed different bands representing various constituents. This was very well in line with the TLC autobiography and phytochemical screening of the leaf and stem extracts of *Ixora coccinea* L. which showed the presence of compounds belonging to terpenoid, flavonoid, coumarin, alkaloid and phenolic groups [15]. Many of the compounds present in the sample could act as potential antimicrobial agents. Further studies are warranted to identify each of these compounds.

Excoecaria agallocha is a well-studied a mangrove plant, with reports on its chemical constituents [16]. Investigations on the presence of metabolites from the plant revealed the presence of diterpenoids, [17, 18] triterpenoids, [19, 20] flavonoids, [21] glucosides and polyphenols [22, 23].

The phytochemical studies on the leaf and stem bark of *Drimys angustifolia* demonstrated that both extracts contain flavonoids, saponins, glycosilated triterpenoids, fixed acids, cyanogenic glycosides, quinones, tannins, xanthone and steroidal glycones [24]. Similar studies in *Mentha piperita* [25] showed the presence of alkaloids, flavonoids, steroids, tannins, and phenols.

The present work corroborated very well with that of other researchers [26]. The phytochemical analysis of the crude aqueous stem bark extract of *Musanga cecropioides* showed that it contained saponins, flavonoids, alkaloids, tannins, phlobatannins, glycosides, reducing sugars and anthraquinones [27]. Phytochemical screening of plant parts revealed the presence of alkaloids, saponins, tannins, flavonoids, terpenoids, coumarins, quinines, cardiac glycosides, xantho proteins, glycosides, steroids, phenols, resins, and carboxylic acid groups in varying concentrations [28, 29].

CONCLUSION: To conclude, it was understood from the present study that the extract of *Excoecaria agallocha* contained many phytochemicals as revealed by phytochemical studies and TLC analysis. This study was only a preliminary one as the mere presence of any compound is not sufficient a reason for the discovery of potent new drugs. The combination of biological and chemical screening will provide important information about the plant constituents.

Further studies are underway to find the effect of these extracts on various bacterial strains. Also, the growth inhibitory effect of the extract on various human cancer cells lines are also being attempted with special emphasis on the mechanism of action of the extract/compound.

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CONFLICT OF INTEREST: Nil

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