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# Chromatography separation, $^{1}$H NMR analysis and bioautography screening of methanol extract of *Excoecaria agallocha* L. from Bhitarkanika, Orissa, India

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ABSTRACT

**Objective:** To separate the bioactive compounds from crude methanol extract of leaf of *Excoecaria agallocha* (*E. agallocha*), a mangrove plant from Bhitarkanika, Odisha, India. **Methods:** Chromatographic separation was carried out by paper, thin layer chromatography (TLC) and column chromatographic techniques. Bioautography of the column fractionated extract and TLC chromatogram were evaluated *in vitro* for antibacterial and DPPH free radical scavenging activity. The HPLC and $^{1}$H NMR spectroscopy of the extract was also carried out to detect the presence of compounds and possible functional groups in the crude plant extract. **Results:** Evaluation of solvent system for chromatographic separation revealed that *n*-butanol: acetic acid: water in the ratio of 4:1:1 was the most appropriate for the separation of phenolic compounds in the crude extract. The antibacterial bioautography screening of the TLC fractionated extract of *E. agallocha* showed positive activity against *Staphylococcus aureus* (MTCC-1141) and *Pseudomonas aeruginosa* (MTCC 1034). Evaluation of antioxidant properties of TLC fraction of *E. agallocha* showed 23.36% scavenging activity using DPPH assay at 40 $\mu$L concentration. HPLC chromatogram of the plant extract produced seven peaks for the leaf and two peaks for the stem/bark with the highest peaks at 2.563 and 2.493 minutes of retention time, respectively. The $^{1}$H NMR spectroscopic analysis of the plant extract revealed the presence of acyclic aliphatic and $\alpha$- mono substituted aliphatic group of compounds in the sample. **Conclusions:** Thus, it is apparent from the present study that methanol crude extract of *E. agallocha* is rich in aliphatic group of compounds which can be purified and characterized for possible pharmaceutical utilization.

## 1. Introduction

Mangrove plants are rich source of bioactive compounds. Many secondary metabolotes like alkaloids, phenolics, steroids, terpenoids have been characterized from mangrove plants which have much pharmacological importance[1]. Mangrove plants have been used in folklore medicines and the extracts from mangrove species have proven activity against human, animal and plant pathogens. Such activities could be attributed to the presence of biologically active antiviral, antibacterial and antifungal compounds[1,2].

*Excoecaria agallocha* (*E. agallocha*) L. is an important mangrove species in India. This plant is used as a traditional remedy for several diseases like epilepsy, conjunctivitis, dermatitis, haematuria, leprosy and toothache[1]. Potential medicinal value of this plant has been reported for antimicrobial, antioxidant, antiviral, antifungal and anticancer properties[3-7].

However, there is a need for isolation characterization, determination of bioactivity of the lead compound for its pharmaceutical exploitation. Analytical methods play important roles in the discovery, development and manufacture of pharmaceuticals[8]. Isolation of pharmacologically active constituents from the medicinal plant extracts remains a long and tedious process. The traditional methodology of studying natural products includes the fractionation of a complex mixture, separation and isolation of the individual components using liquid

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chromatography and structure elucidation using various spectroscopic methods[9,10]. Isolation and separation of bioactive compounds for further analysis are highly necessary. Further chemical screening of crude extracts is thus performed to allow localization and targeted isolation of new or useful types of constituents with potential activities which allows to know metabolites in extracts or at the earliest stages of separation and is thus economically very important. Analytical methods like the column chromatography; thin layer chromatography (TLC) and bioautography are the economical methods in the field of pharmacognosy. Chromatographic techniques are one of the simplest and cheapest methods for detecting plant constituents because these methods are easy to run, reproducible and requires little equipments[9,10]. HPLC is a valuable tool for reliable identification. It can provide chromatographic fingerprints that can be visualized and stored as electronic images. While $^1$H NMR spectroscopy has been employed for obtaining detailed structural information about the organic compounds present[8,11-15].

*E. agallocha* is found abundantly in Bhitarkanika, the second largest mangrove forest of India. In the present study, attempt has been made to fractionate and isolate the biologically lead compound using different chromatographic techniques and development of suitable solvent system. Further characterization of the crude extracts was done by analytical HPLC followed by $^1$H NMR analysis. The bioautography of the fractionated compound were also evaluated for antioxidant and antimicrobial properties.

## 2. Materials and methods

### 2.1. Collection of plant material

Fresh, young and tender leaves of *E. agallocha* (L.) were collected from the mangrove forest of Bhitarkanika wildlife sanctuary which extends from $20^0$ 30′ to $20^0$ 50′ latitude and $86^0$ 30′ to $87^0$ 60′ E longitude. The specimens were identified at Department of Natural Products, Institute of Minerals and Materials Technology, Bhubaneswar (RRL-B), Orissa. India and voucher specimen (VS No. RRL-B 10, 002) was deposited.

### 2.2. Preparation of extracts

Plant samples were air dried for 15 days and then pulverized to fine powder using mortar and pestle. 25 g of powder samples was added to solvent acetone, methanol, ethanol and water 50 mL each and were left on a rotator shaker for 48 h. Extracts were then filtered and dried using a hot air oven at low temperature. Dried extracts were collected and stored in aliquots at 4℃ for further analysis[12].

### 2.3. Chromatographic characterization

#### 2.3.1. Paper chromatography

Paper chromatography was carried out as per standard procedure described by Sadasivam and Manickum[16]. Different solvent extracts of the plant samples were applied on chromatography paper and chromatogram was developed in eight different solvent systems[17].

#### 2.3.2. TLC

The TLC plates (precoated TLC plates Silica Gel G) were trimmed to strips and the position of the origin marked by a straight line. The methanol extract of *E. agallocha* was spotted on the origin and put in a lidded tank containing a solvent system. The procedure was followed with other strips and various solvent-solvent ratios (*n*-butanol: acetic acid: water in 12:3:5, 12:1:1, 10:1:1, and 4:1:1) until good resolution was noticed. The level of solvent in the tank was about 1 cm beneath the origin. The solvent travelled up the plate by capillary action till it reached the solvent front (also marked by a straight line across). The lid was lifted off and the strip dried before it was viewed by spraying with silver nitrate and iodine vapour and visualized under UV light[16, 17]. Catechol was used as reference standard. The retention factor (Rf) values of all the spots were determined by the following formula:

Retention factor = Distance traveled by the plant extract/ distance traveled by the solvent system.

#### 2.3.3. Column chromatography

The methanol extract of the leaf sample of *E. agallocha* was adsorbed onto silica gel by triturating in a mortar and left for about 10 hours to dry. The column (2 cm × 25 cm) was packed with a solution of silica gel with *n*-butanol using the wet slurry method. This involves preparing a solution of silica gel, with *n*-butanol in this case, in a beaker and subsequently adding this unto the column till it is about three-fourths filled. The solution was stirred for dispersal and quickly added to the column before the gel settles. This method was used to prevent the trapping of air bubbles. A ball of wool was pushed into the column to settle atop the packed silica gel. A substantial amount of *n*-butanol: acetic acid: water (4:1:1) was poured continuously into the column and allowed to drain but prevented from reaching the cotton wool. The quantity collected was poured back into the column. Periodically, a piece of rubber tubing was used to agitate the column to allow for the escape of trapped air bubbles. About 20 fractions are eluted and collected in dry glass bottles. The column fractions were again tested with TLC chromatogram and the Rf values were determined. The fractions with similar Rf value were combined together and kept for bioautography screening[18].

### 2.4. Antibacterial Screening

#### 2.4.1. Microbial strains used

Different microbial strains were used to evaluate the antimicrobial properties of the separated fractions of which, two were gram positive bacterial strains *i.e. Staphylococcus aureus* (*S. aureus*)(MTCC-1141) and *Bacillus subtilis* (*B. subtilis*) (MTCC-7461) and three were gram negative bacterial strains *i.e. Shigella flexneri* (*S. flexneri*)(lab isolated), *Pseudomonas aeruginosa* (*P. aeruginosa*)(MTCC-1034) and *Escherichia coli* (*E. coli*)(MTCC-1089). The strains are obtained form IMTECH, Chandigarh and were maintained on agar slants.

#### 2.4.2. Agar well diffusion method

Antimicrobial screening of the separated plant fractions were carried out by agar well diffusion method against standard test microorganisms as described by Patra *et al*[12]. Nutrient agar (NA) plates were prepared as per manufacturer instructions. Overnight nutrient broth culture of the test organisms was seeded over the NA plates using sterile cotton swab so as to make lawn culture. Wells of 6 mm diameter were punched over the agar plates using sterile gel puncher (cork borer). The bottom of the well was sealed by pouring 2 μL of molten NA into the scooped well by the sterile micropipette. 20 μL of extract were poured into the wells. The plates were incubated at 37℃ for 24 h. The zone of the clearance around each well after the incubation period, confirms the antimicrobial activity of the respective extract. Each experiment was carried out in triplicates. The clear zones formed around each well were indicative of the antibacterial activity of the column fractions.

For TLC plate bioautography, the chromatogram developed by TLC were placed in a sterile bioassay petri dish and an inoculum of different bacterial strains containing CFU/mL in molten nutrient agar was distributed over the plates. After solidification of the medium, the sterile bioassay petri dishes with the TLC plate were incubated for 24 hours at 37℃. Clear zones around the TLC plates are recorded[19].

### 2.5. Determination of antioxidant properties

#### 2.5.1. Screening of antioxidant activity with TLC bioautography method

Separated compounds on TLC plates were sprayed with 1,1diphenyl 2 picrylhydrazl (methanolic DPPH) to detect the presence of antioxidant active compound. It gives a orange spot or a deep yellow coloured spot on a purple background[20].

#### 2.5.2. DPPH free radical scavenging assay

The antioxidant potential of the phenolic compound (spot A) of the TLC chromatogram of methanol extract of leaf of *E. agallocha* was determined by modified method of Patra *et al*[13]. The spot A was scraped from the TLC plate and dissolved in methanol and allowed to stand for 10 minutes. Then the supernatant was used for the experiment as the plant extract. DPPH solution was added to the plant extract, mixed well, vortex and kept at room temperature in dark 30 min. Absorbance was measured at 517 nm against an appropriate blank. The percentage scavenging effect of the plant extract (spot A) against methanolic DPPH was considered as the antioxidant activity of the separated spot.

Percentage scavenging assay = $(A_o-A_1)$ / A $A_o$ Where A $A_o$ =Absorbance of control; $A_1$=Absorbance of test sample.

#### 2.5.3. High performance liquid chromatography (HPLC)

Analysis of methanol extracts of leaf of *E. agallocha* was performed by HPLC. The HPLC system consists of LC-20AT prominence liquid chromatograph pump and SPD-20A prominence UV-Vis detector and Rheodyne type injector fitted with 20 μL capacity fixed loop all from Shimadzu Corporation, Japan. The column used was Phenomenex luna 5 μ $C_{18}$ (2) 100A (250 mm × 4.6 mm) at ambient temperature. The output signals were monitored and processed using spinchrom CFR software. The solvent system optimized for the analysis was methanol: acetonitrile: water in the ratio 25:35:40. The flow rate was 1 mL/minute and detection wave length was set at 232 nm. The run time of the method was 10 minute and all analytes were separated within the run time.

### 2.6. NMR analysis

$^{1}$H NMR spectra of methanol extract of *E. agallocha* was recorded on a NMR-400 MHz (with multi-nuclei analysis from $^{1}$H, $^{19}$F to $^{15}$N) and chemical shifts were recorded as δ values. The result graph was compared with the reference chart and possible functional group present in the plant were determined[21].

## 3. Results

In order to obtain some information on the active components present in crude extract of *E. agallocha*, a important medicinal mangrove plant from Bhitarkanika, Orissa (India) and to characterize it, different types of chromatographic techniques *viz*. paper chromatography, TLC, column chromatography along with HPLC and $^{1}$H NMR studies were undertaken. Prior to the chromatographic separation, proper solvent system was determined.

**Table 1.**
Selection of solvent system for chromatographic separation.

| Solvent System | Ratio | *E. agallocha* | | | |
|---|---|---|---|---|---|
| | | A | E | M | Aq |
| acetic acid: chloroform | 9:1 | – | – | – | – |
| chloroform: methanol | 9:1 | – | √ | √ | – |
| *n*-butanol: acetic acid: water | 10:1:1 | – | – | √ | – |
| toluene: ethyl acetate: acetic acid | 5:5:1 | √ | – | – | – |
| *n*-butanol: acetic acid: water | 12:3:5 | – | – | √ | √ |
| *n*-butanol: acetic acid: water | 12:1:1 | √ | √ | √ | √ |
| ethyl acetate: acetic acid: formic acid: water | 10:1:1:2 | – | – | – | – |
| *n*-butanol: acetic acid: water | 4:1:1 | √ | √ | √ | √ |

A= acetone extract, E= ethanol extract, M= methanol extract and Aq= aqueous extract.

**Table 2.**
Paper chromatography of solvent extracts of leaf of *E. agallocha*.

| Solvent system | Plant extracts | No. of bands | Rf value |
|---|---|---|---|
| *n*-butanol: acetic acid: water (4:1:1) | Acetone | 2 | A=0.121, B=0.963 |
| | Ethanol | 2 | A=0.121, B=0.963 |
| | Methanol | 3 | A=0.117,B=0.724,C=0.941 |
| | Aqueous | 2 | A=0.117, B=0.292 |

**Table 3.**
TLC of methanol extract of leaf of *E. agallocha* in different ratios of solvent system.

| Solvent system | No. of Bands | Rf value |
|---|---|---|
| *n*-butanol:acetic acid :water (12:3:5) | 2 | A= 0.371, B=0.571 |
| *n*-butanol:acetic acid:water (10:1:1) | – | – |
| *n*-butanol:acetic acid:water (12:1:1) | 6 | A=0.072, B=0.176, C=0.235, D=0.294, E=0.435, F=0.623 |
| *n*-butanol:acetic acid: water (4:1:1) | 2 | A=0.176, B=0.294 |

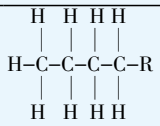
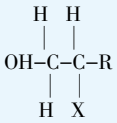
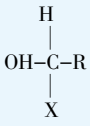
**Table 4.**
TLC of methanol extract of column chromatography fractions of leaf of *E. agallocha*.

| Solvent system | Column fractions | No. of bands | Rf value |
|---|---|---|---|
| *n*-butanol: acetic acid: water (4:1:1) | 1 | 1 | A=0. 335 |
| | 2 | No bands | – |
| | 3 | 1 | A=0.187 |
| | 4 | 2 | A=0.1,B=0.312 |
| | 5 | 2 | A=0.1 B=0.312 |
| | 6 | No bands | – |
| | 7 | No bands | – |
| | 8 | No bands | – |
| | 9 | No bands | – |
| | 10 | 1 | A=0.152 |
| | 11-20 | No bands | – |

Eight solvent systems taking different polar and non polar solvents such as water, methanol, acetic acid, chloroform, *n*-butanol, toluene, ethyl acetate and formic acid in varying ratios were used to determine the appropriate solvent system for a particular extract for separation of compounds by paper chromatography (Table 1). The result showed that *n*-butanol: acetic acid: water in the ratio 4:1:1 was most appropriate solvent system for all the plant extracts. Further, paper chromatography separation and Rf value determination of the crude extracts using this solvent system were undertaken (Table 2). Among the four extracts, better separation of the active compounds was achieved in case of the methanol extract by the solvent system and was used for further characterization study.

With a view to select out the appropriate proportion of solvent for proper separation of the compounds in methanol extract, *n*-butanol: acetic acid: water in different ratios was evaluated in TLC as given in Table 3. Two clear and distinct spots/bands on spraying silver nitrate were visualized in the solvent system at ratio 4:1:1 with Rf 0.176 and 0.294 as against other three ratios of the solvent system which gave unclear spots with varying Rf values (Table 3 and Figure 1). On comparing this Rf value with the reference standard (catechol) in the TLC plate, the presence of phenolic compounds in the extract was conformed as blue spots on spraying silver nitrate along with presence of brown spot for other compound(s) (Table 3 and Figure 1). The bioautography of the separated fractions in TLC were studied for presence

**Table 6.**
Possible functional groups and their structures present in the methanol extract of leaf of *E. agallocha* as analyzed by $^{1}$H NMR.

| Peak in PPM | Possible type of group of compound | Possible structure |
|---|---|---|
| 0–2 | Alipathic alicyclic compounds |  |
| 1–2 | β –Substituted alipathic compounds |  |
| 2–5 | α –Mono substituted alipathic compounds |  |

'R'= elongation of chain; 'X'= attachment of any functional group.

of antimicrobial and antioxidant activities. Both the spots showed selective antimicrobial and antioxidant activities (Figure 2A). Out of two spots (Rf 0.176 and 0.294), the spot with phenolic compound showed positive antimicrobial activity against one (*P. aeruginosa*) out of five strains used while the other spot (unknown compound) showed positive activity against two strains (*S. aureus* and *P. aeruginosa*) (Figure 2A). Both the spots (Rf 0.176 and 0.294) for phenolic and unknown compound showed positive antioxidant activity on spraying DPPH solution to the TLC plates. It gave a deep yellow coloured spot on a purple background. The spot A (blue colour, Rf 0.176) of the TLC chromatogram of the *E. agallocha* showed potent DPPH radical scavenging activity of 0.53% and 23.36% at 20 μL and 40 μL concentrations, respectively.

**Table 5.**
Antimicrobial bioautography screening of column fractions of methanol extracts of leaf of *E. agallocha* against pathogenic strains.

| Bacterial strains | Column fractions | | | | |
|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F10 |
| *S. aureus* (MTTC–1141) | + | – | – | – | + |
| *S. flexneri* (lab isolated) | – | – | – | + | + |
| *P. aeruginosa* (MTCC–1034) | – | – | – | – | + |
| *B. subtilis* (MTCC–7461) | + | – | + | + | + |
| *E. coli* (MTCC–1089) | – | – | – | – | + |

Subsequently the extract was fractionated by column chromatography and the different fractions are checked by TLC for the presence of active compounds following similar Rf values as determined before (Table 4). Out of 20 fractions, five fractions showed clear spots/bands with different Rf values in the TLC plate. These spots were again checked for antimicrobial and antioxidant activity following bioautography method. The bioautography results obtained in this experiment (Table 5 and Figure 2B) coincides with the result obtained earlier with respect to different Rf values as obtained in the previous experiment (Table 3, Figure 2A). These column fractions of *E. agallocha* showed antibacterial activity against *B. subtilis* (4/5) followed by *S. flexneri* (2/5), *S. aureus* (2/5), *P. aeruginosa* (1/5) and *E. coli* (1/5) (Table 5). Antioxidant bioautography assay of separated fractions were also carried out by DPPH assay. In this assay spots/bands with antioxidant activity reduces the stable radical DPPH to the pale yellow coloured diphenylpicrylhydrazyl on a violet background within few minutes on spraying with DPPH solution.

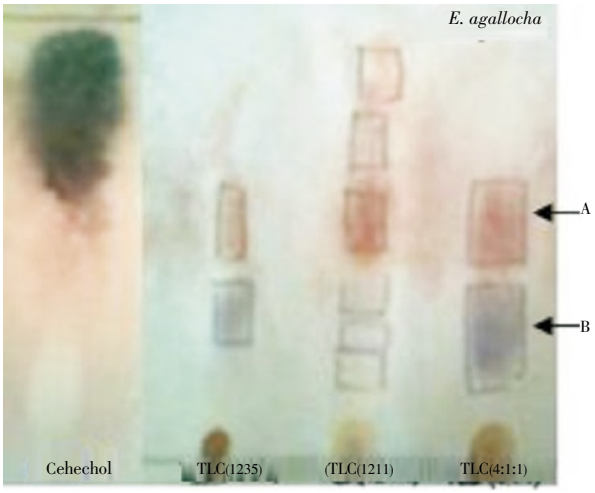



**Figure 1.** TLC of methanol extracts of leaf of *E. agallocha*.
A:Unknown compound(brown spot); B:Phenolic compound(Bhue/Black spot).

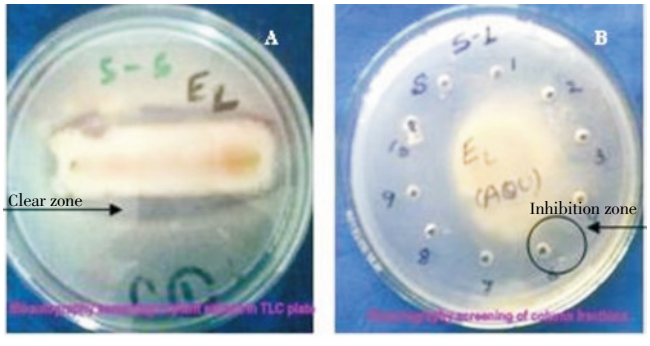



**Figure 2.** Bioautography screening of different fractions of methanol extracts of leaf of *E. agallocha*.
A= Bioautography screening of TLC plate against pathogenic strain,
B= Bioautography screening of column fractions.

Further analytical HPLC and $^1$H NMR spectroscopy was undertaken to assess the various components present in the extract and to find out the functional group present in the crude extract. HPLC analysis of methanol extract of leaf of *E. agallocha* was carried out with the mobile phase methanol: acetonitrile: water in the ratio 25:35:40 gave a total of 7 peaks at retention time 0.320, 2.563, 3.737, 5.647, 6.340, 8.407 and 9.740 (Figure 3). The highest peak was seen at the retention time 2.563 minute. $^1$H NMR analysis of the same methanol extract of leaf of *E. agallocha* showed a number of peaks in between $\delta$ 0 to 2 and $\delta$ 3 to 5. Both the group of peaks may be acyclic aliphatic compounds and $\alpha$- mono substituted aliphatic group of possible chemical compounds respectively as conformed by the standard chart of Silverstein *et al*[21] (Table 6).

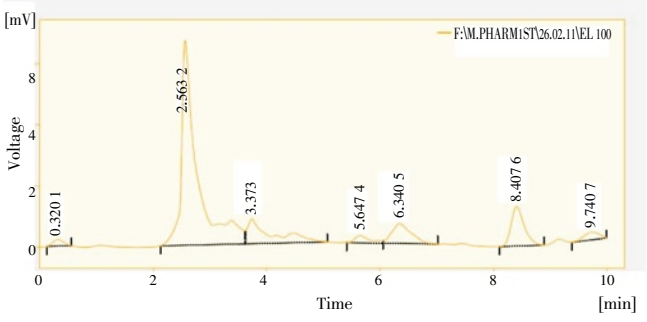



**Figure 3.** HPLC analysis of methanol extract of leaf of *E. agallocha*.

## 4. Discussion

Plants produce a diverse range of bioactive molecules making them rich source of different types of medicines. Various techniques are employed for their investigation which includes bioassays for chemical screening and their evaluation for presence of biological activities. Isolation of pure pharmaceutically active constituents from plants remains a long tedious process. Chemical screening is performed to target isolation of new or useful type of constituents having potential activities. This procedure enables recognition of known metabolites in extracts in the earliest stages of separation and thus economically very important. To characterize the bioactive compounds several techniques were used among which chromatographic techniques were extensively used.

Different polar and non polar solvents such as water, methanol, acetic acid, chloroform, *n*-butanol, toluene, ethyl acetate and formic acid in different combinations were used to carry out TLC. Except *n*-butanol, acetic acid and water no solvent system gave successful results in chromatographic separation of the components. The successful separation of biomolecules by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient (K) for each target compound[22].

TLC is the simplest and cheapest method for detecting plant constituents because the method is easy to run, reproducible and requires little equipment. However, for efficient separation of metabolites, good selectivity and sensitivity of detection, together with the capability of providing on-line structural information, hyphenated HPLC techniques are preferred. They play an important role as an analytical supporting the work of phytochemists for the efficient localization and rapid characterization of natural products. It posses a wide range of solvent system and spraying reagent. The choice of solvents used was also influenced by several factors like toxicity of solvents, solubility, reactivity of compounds used in the bioassays and reagents used. They are also easy, cost efficient and sensitive technique[15,16]. TLC chromatogram of the plant extracts showed presence of phenolic compounds. Phenolic compounds such as tannins and flavonoids posses diverse biological properties such as anti-inflammatory, anti bacterial and antioxidant activities[23].

The bioautography screening (antimicrobial and antioxidant) properties of the chromatographic separated fractions of leaf of *E. agallocha* were carried out by standard procedures and the results showed that the fractions possess strong antimicrobial and antioxidant properties. The bioactivity properties of the plant extracts may be due to the presence of various secondary metabolites such as alkaloids, flavonoids, phenols, Saponins *etc*. The antimicrobial properties of the plants may be attributed to the presence of some active compounds in all organic extracts such as lipophiles and phenolic compounds. These results are in accordance with the previous results on related plants regarding gram positive and gram negative bacteria[12,13]. The result of the antioxidant screening reveals that the plant extract exhibit strong free radical scavenging activity which may be due to the presence of phenolic compounds since phenols have been known to scavenge free radical with multiple biological activities[23-25]. Bioautography is a very convenient and simple way of testing plant extracts and pure substances for their effects on both human pathogenic and plant pathogenic microorganisms. It can be employed in the target directed isolation of active constituents. Screening programmes for biologically active natural products requires the right bioassay[15,16]. Detection of compounds with the desired activity in complex plant extracts depends on the reliability and sensitivity of the test system used. Bioassays are also essential for monitoring the required effects throughout activity- guided fractionation: all fractions are tested and those continuing to exhibit activity are carried through further isolation and purification until the active monosubstances are obtained.

According to Springfield *et al*[26], HPLC fingerprinting is the best way for chemical characterization. HPLC profile differentiation is one such important and powerful procedure which has often employed for this purpose. Each and every metabolite has a specific role and functions in harmony with other metabolites within the organizational framework of cells in the defence mechanism of the plants[14]. The $^1$H NMR analysis of the methanol extract of *E. agallocha* showed presence of alipathic group of compounds[21] which might be responsible for the bioactive properties of the plant extract. Indeed, NMR spectroscopy is by far the most powerful spectroscopic techniques for obtaining detailed structural information about organic compounds in solution[15]. The data generated from the experiment have provided the chemical basis for bioactivity and the therapeutic use of *E. agallocha* for various aliments. Such properties of this

plant can be attributed to its adaptation in environment stress condition and presence of phenolic compounds since phenolics have been known to scavenge free radicals with various biological effects including antimicrobial properties. Our study demonstrates that *n*-butanol: acetic acid: water is an effective solvent system for separation of phenolic compounds from mangrove plants however further separation of phenolic compounds has not been possible in the present study. More research work should be devoted in this direction to determine the kind of phenolic compounds present in the extract however, determination of biological activities of phenolic compounds and their possible presence of functional groups by $^{1}$H NMR analysis has given good information to work further for its purification and detection of the compound to exploit its pharmaceutical purposes.

## Conflict of interest statement

We declare that we have no conflict of interest.

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