INTRODUCTION

Plants have provided mankind with useful drugs for centuries. Despite the availability of different approaches for the discovery of therapeutics, natural products still remain as one of the best reservoirs of traditional and/or orthodox medicine. Infectious diseases are the leading cause of death worldwide. The emergence of multidrug resistant pathogens threatened the clinical efficacy of many existing antibiotics. This situation has been recognized globally as a serious concern and justifies further research to discover antimicrobial agents from natural origins including plant extracts.

Excoecaria agallocha (Euphorbiaceae) is a small mangrove tree found in tidal forests and swamps of the Sundarbans and Pichavaram Reserve Mangrove Forest. This plant has traditionally been used to treat sores and stings from marine creatures and ulcers as a Purgative, an emetic and the smoke of its bark oil been used to treat leprosy and paralysis. The bark oil has also been found effective against malarial condition inducing cancer. The interesting biological activities obtained by micropropagated plants of Excoecaria agallocha need to be further investigated to isolate active agents and to study their mechanism of action.

Key words: mangrove, antibacterial activity, antioxidant activity, phenol exudation and field grown plants.

MATERIALS AND METHODS

Explants of the (1-2 cm) of shoots tip and node were excised from the fresh growing tips of adult trees of Excoecaria agallocha from Pichavaram Mangrove Reserve Forest, Tamil Nadu. Explants transported at 4° C. Shoot tips and nodal segments (not longer than 1 cm) were cut from the young shoots. The explants were washed in tap water for 5 min, followed 10% Tween 20 (Liquid detergent; Hi media, India) for 5 min then surface sterilized with 70% (v/v) ethanol for 2 min and rinsed 3 times by sterile distilled water and then treated with one percent Methyl benzimidazol-2-ylcarbamate for 10 min. These explants were treated with 20% sodium tungstate and sodium carbonate solution to remove phenolic compounds from the explants and then finally the explant was washed with 0.1 % HgCl2 for 2 minutes followed by three times rinse were made with sterilized water.

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY BETWEEN MICROPROPAGATED AND FIELD GROWN PLANTS OF EXCOECARIA AGALLOCHA L.

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ABSTRACT

Excoecaria agallocha has long been used in folk medicine in many countries as antimicrobials and/or antioxidants. Eighty percent methanol extracts obtained from Excoecaria agallocha was evaluated for antibacterial activity using the disc diffusion assay, antiradical scavenging effects, reduced glutathione and phenolic. Best antibacterial activity as indicated by disc diffusion zone values was obtained by micropropagated Excoecaria agallocha against Staphylococcus aureus with a disc diffusion zone 25mm. Micropropagated and field grown plants inhibited growth of Klebsiella pneumoniae with zone values of 13mm and 11mm in diameter respectively. A remarkable DPPH scavenging effect was observed with micropropagated plants (IC50 of 10.2, 10.8 and 17.4 μg/ml, respectively). Ascorbic acid (AA) content observed in micropropagated plant leaf 18 mg/l while compared to field grown plant ascorbic acid content. Highest total phenolic contents were recorded in micropropagated plant leaf (207 and 205 mg/GAE/g). Our findings support the uses of the Excoecaria agallocha in traditional medicine. The interesting biological activities obtained by micropropagated plants of Excoecaria agallocha need to be further investigated to isolate active agents and to study their mechanism of action.
Shoot bud development from explant and culture conditions

Decontaminated explants with at least one axillary or terminal bud were placed in culture tubes with different PGRs combinations (Table 1) Murashige and Skoog16 (MS); Varatak and Shindikar17 (Modified Murashige and Skoog medium, MMS) and Llyod and Mucown18 (Woody Plant Medium, WPM). All the media were supplemented with 600 mg/l PVP for to mitigate exudates the phenolic compounds19. The Modified Murashige and Skoog medium consisted to MgSO4 (185 mg/l), KNO3 (950 mg/l). KH2PO4 (85 mg/l) and CaCl2 (440 mg/l) with the omission of NH4NO3 and supplemented with 30 g/l of sucrose, 8 g/l agar and pH was adjusted to 5.8. The three different medium was tried to culture the E. agallocha explants with different concentration of BAP (1.4, 3.9, 4.8, 5.4 and 7.4 µM) were used to estimate the suitable culture medium for E. agallocha (Table 1). Of the three medium, Modified Murashige and Skoog was found to induce the maximum growth in E. agallocha. Hence this medium was used throughout the experiment. Treatments consisted of three repetition one shoot was initiate culture tube within 10 ml of medium and 450 explants were used for the study. Culture chamber conditions for all experiment was 16 h photo-period (cool white lamps, Phillips Master LD 36 w, photosynthetic photo lux density 90 µmol m⁻² s⁻¹) and 24/19°C day/night temperature, respectively. The number of explants developed lateral and basal shoot and the length of the shoot and the physiological state of the main shoot were evaluated after 5 weeks. Based on the results from the multiplication cycle, the MMS medium was selected for further experiments. MMS medium was supplemented with BAP (1.4 to 7.4 µM) and combined with NAA (1.34 µM) (Table 2).

In the second experiment, the optimal concentration of BAP and NAA was estimated by using various concentrations of these growth regulating chemicals. Stem segments were vertically placed into each test tube for all multiplication treatments, nondestructive observations were performed after 4 and 6 wk cultures. Each experiment was repeated three times. Number of shoots per explants and shoot length were calculated (Table 2).

Rooting treatments and plant greenhouse establishment

Fifty microshoots were placed for elongation in half-strength MMS medium for root induction on 4 weeks with 1.34 µM of Naphthalene acetic acid (NAA), and cultured for 2 weeks. culture conditions 19°C C with 16h photoperiod20 following the root induction treatment, the shoots were transferred to root expression medium consisting of modified Murashige and Skoog (Half-strength of macronutrients) in PGRs free medium. The in vitro rooting experiment was set up in a complete randomized block design with three blocks per treatment and 15 shoots per block (total 45 shoots). After 6 weeks, data were recorded as percentage of shoots rooted, number of roots per shoot and mean root length. Percentage data were subjected to arcsin transformation before analysis of variance (ANOVA). Least significant-difference was at (P≤0.01) level.

In vitro rooted plantlets were removed from culture media and agar was washed from roots. After 5 wk, plantlets were transplanted into trays containing a peat-perlite mixture 1:1 (w/v) covered with bags to maintain a high relative humidity. The potted plantlets were kept in an unheated greenhouse with clear polyethylene plus 70% shade cloth. Greenhouse temperatures during the acclimation period were 16-22 °C during the day and 12-14 °C during the night. Ventilation of the plantlets was increased after 7 days by increasing size of the holes made in the polythene covering and after 3 wk the polythene cover was removed. After 5 weeks of acclimation individual plants were transferred to each plastic pot (diameter 9 cm) containing the same growing mixture and transferred to the shade net of 70% shade. Air temperature in the shade net was 18-22°C during the night and 25-28 °C during the day.

To investigate the activity of field grown plants, leaves of Excoecaria agallocha was collected from the Pichavaram mangrove Reserve forest, Tamil nadu, India. Voucher specimens (AUBOT 107Ex.) were deposited in the Department Herbarium. The collected materials were cleaned, dried in an oven at 60 °C, powdered and extracted using 80% methanol (20% water). The extracts were filtered using Whatman No. 1 filter paper. A rotary evaporator was used for removal of the solvent. Residues obtained were dried at room temperature prior to further tests.

Antibacterial Assay

The test organisms Bacillus cereus, Bacillus subtilis, Klebsiella pneumoniae, Proteus vulgaris, Staphylococcus aureus, and Salmonella typhi were collected from Department of Microbiology, Raja Sir Muthaiah Medical college, Annamalai University, Annamalai Nagar, Tamil Nadu, India. These pathogenic organisms were maintained aseptically by using nutrient agar medium. Antibacterial assay were carried out using the disk diffusion method described by Malakzadeh et al.21. 20 µl suspensions contained 10⁵ CFU/ml of bacterial cultures were spread on nutrient agar medium. The disk (6 mm in diameter) impregnated with 10, 20, 30, 40 and 50 mg of extracts were placed on the inoculated nutrient agar petridishes. Negative controls were prepared using the same solvents employed to dissolve the extracts of plant material. All the petridishes were incubated at 37°C for 18 hrs. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms in comparison to negative control and each assay was repeated thrice.

DPPH radical scavenging activity

The potential antioxidant activity of the studied plant materials were assessed on the basis of the free radical scavenging activity of the Excoecaria agallocha extracts against the stable 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical according to the method of Duh and Yen 22. The extracts (50 to 250 µg) were added to a methanolic solution (1 ml) of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Obtained absorbance value was then converted into the percentage of radical scavenging activity using the following equation: Radical scavenging activity ( %)=100-[(AS/AC)×100] where AS: absorbance of the sample; AC: absorbance of the negative control (ethanol). For IC50 determination, the extracts were serially diluted to six different concentrations (from 250 to 7.81 µg/ml). IC50 values were obtained from the graph of radical scavenging percentage against log concentration, at 50% of radical scavenging.

Total Phenols

The phenolic content was determined calorimetrically using Follin-Ciocalteu (FC) method of Gulcin et al 23. A sample
Ascorbic acid (AA) content was assayed as described by Omay et al. 

RESULTS AND DISCUSSION

The sample was prepared 1 mg/ml using ethanol. The same solvent was used to prepare gallic acid standard solutions. Briefly, 25 μl of Folin–Ciocalteu's reagent (50% v/v) was added to 10 μl of 1 mg/ml (w/v) sample. After 5 min incubation at room temperature, 25 μl of 20% (w/v) sodium carbonate was added. One hundred forty microliters of water was then added to yield a final volume of 200 μl per well. Water was used as blank (negative control). After 30 min of incubation the absorbance were read at 760 nm. All assays were carried out in triplicate. Gallic acid was used as standard and results were expressed as mg gallic acid equivalent per gram sample.

Ascorbic acid content

Ascorbic acid (AA) content was assayed as described by Omay et al., with BA (1.4 to 7.4 μM) and NAA (1.34 μM) combinations. Adventitious roots were induced directly from the shoot base without intervening callus phase in all media. Thus, research on adventitious root formation is highly important from the practical point of view. Hence, adventitious root production in isolated micro cuttings of *E. agallocha* was achieved in the presence of various auxins (IAA, IBA and NAA) in MMS medium. Exogenous auxins are often used in a number of plant species to promote *in vitro* rooting of *in vitro* raised microshoots. Overall, Presence of auxins 1.5 mg/l and 2.0 mg/l in medium exhibited better rhizogenesis. However, the significant frequency (86 ± 0.9%) of root formation, number (5.9 ± 0.61) of roots with length (3.8 ± 0.51) was observed on MMS medium supplemented with 5.02 μM of IAA. Rooting frequency was increased gradually and reached maximum percentage after 11 weeks of root culture. There are other reports of IAA being effective in stimulation adventitious such as Quraishi and Mishra. In figure 5 showed that equal molar concentrations IAA and IBA was induced maximum rooting response and the similar results were reported in *Rauvolfia tetraphylla*. In *in vitro* raised well rooted plants were uprooted from the test tubes, transferred to green house for hardening which were covered by holes polythene covers, then followed by potting and finally plants were transferred to picharavaram mangrove forest we observed that 86% of survival.

**Antibacterial activity**

Secondary metabolites are usually not distributed uniformly within the whole plant 28. Some are restricted to specific organ and may be synthesized in small quantities. Accumulation also depends very much on the seasons, on the developmental stage of the plants, and on surrounding biotic and abiotic factors. Besides, the overexploitation and anthropogenic factors also result in the depletion of natural plant resources.

In this experimental study deals with compared with antibacterial activity of *in vitro* raised plants and wild plants. Among the various types of solvent extract used for extraction from the plant parts and only methanolic extract showed zone of inhibition for the selected test organisms. The methanolic extracts of leaf- derived callus revealed strong antibacterial activity against all the test microorganisms as compared to *in vitro* raised and field grown plants (Table. 3). Negative controls prepared using the same solvents employed to dissolve the extracts of plant material showed no response of zone of inhibition (data not presented). In order to test for the presence of superior chemical constituents of methanolic callus extract as compared to other explant sources, isoflavonoids content were estimated. The higher antibacterial activity of methanolic callus extract revealed significantly the highest content of *Excocariotoxins* (0.522±0.04 mg/l DW) in comparison with wild plant (0.321±0.02 mg/l DW). These
results indicate that the leaf derived callus with superior chemical constituents (preferably *Excoecaria*toxins) may be used as a substitute to root biomass of naturally grown *Excoecaria agallocha* in the preparation of pharmaceutical preparation.

The antioxidant

The antioxidant activity of the field and micropropagated plant leaves of *Excoecaria agallocha* was determined using the DPPH scavenging assay. The radical scavenging activity of the extracts (250 μg/ml) is expressed as percentage inhibition and IC₅₀ values. Most of the tested micropropagated plant leaves extracts showed scavenging activity above 80% with IC₅₀ values ranging between 10.2 and 128 μg/ml (Graph.1). The lowest IC₅₀ values were obtained by field grown plant leaves. Total phenolic contents of the tested *Excoecaria* extracts were determined using the Folin–Ciocalteau colorimetric method by manipulation of the regression equation of gallic acid calibration curve (y=1.509x+0.077, r² =0.9779). The amount of phenolics per each extract was expressed as gallic acid equivalent (mg GAE/g dry extract). Results obtained from the assay expressed as means ± standard deviation of triplicate analyses were made. Highest total phenolic contents were recorded for micropropagated plant leaves. Phenolic compounds are commonly encountered in the plant kingdom, and they have been reported to have multiple biological effects, including antioxidant activity 30, 32. In the current study, the phenolic content of *in vitro* propagated leaves and native leaves extract. Our results showed that *in vitro* propagated leaves exhibited a higher total phenolic content than the Ciocalteau method. Antioxidant activity of both the extracts showed a dose dependent response in terms of phenolic content. Even the higher antioxidant activity of *in vitro* propagated leaves correlated with the increased total phenolic content (graph.3). The results clearly demonstrated that the antioxidant activity of both the extracts and the higher activity in the *in vitro* propagated leaves may be attributed to the total phenolic content. The lowest amount was obtained by field grown plant leaves. With the exception of extracts from micropropagated plant leaves total phenolic content correlated with DPPH radical scavenging activity.

The non-enzymatic antioxidant, ascorbic acid content varied with micropropagated plant leaf 18 mg/l plant* in Excoecaria agallocha* plants when compared to field grown plant Ascorbic acid content (graph. 2). This may indicate the involvement of some phenolic compounds in the antioxidant activity observed by the extracts. On the other hand lack of correlation between antioxidant capacity and total phenolic contents observed may suggest the presence of non-phenolic compounds with possible antiradical effects in these extracts. A similar observation was also previously reported 33. The non-enzymatic antioxidant content plays major roles in maintaining the balance between free radical production and elimination 32. In plants the Ascorbic acid – glutathione cycle has been shown to be of great importance in free – radical scavenging and in multiplication stress reactions. Being the major antioxidant species in plants contents in ascorbic acid, vary in different subcellular compartments, according to the intensity of the stress 33. The total phenol recorded for field grown plant leaves was relatively low when compared to the previous report of Kumaran and Karunakaran 34. This may be due to the drying process used. Some phenolic compounds decompose rapidly in direct sunlight or relevant temperature 35. The drying process in some cases may result in a depletion of naturally occurring antioxidant and phenols in plants materials as most of these agents are unstable. However, these processes cause little or no changes in other cases 36.

**REFERENCES**


35. Lim, Y.Y., Martijaya, J., 2007. Antioxidant properties of Phyllanthus amarus extracts as affected by different drying method. LWT-Food Science and Technology 40, 1664–1669.


Table 1. Shoot induction response of Excoecaria agallocha L on MMS medium with various combinations of NAA and BAP

<table>
<thead>
<tr>
<th>Growth regulators (µM)</th>
<th>Shoot initiation per explant (%)</th>
<th>Shoot length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA</td>
<td>BAP</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.4</td>
<td>46±0.6</td>
</tr>
<tr>
<td>0.4</td>
<td>0.8</td>
<td>55±0.7</td>
</tr>
<tr>
<td>0.2</td>
<td>0.4</td>
<td>50±0.4</td>
</tr>
<tr>
<td>0.1</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
<tr>
<td>0.05</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
<tr>
<td>0.02</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
<tr>
<td>0.01</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
<tr>
<td>0.005</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
<tr>
<td>0.002</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
<tr>
<td>0.001</td>
<td>0.4</td>
<td>61±0.6</td>
</tr>
</tbody>
</table>

Table 2. Rooting response in E. agallocha on half-strength MMS medium with various concentration of auxins

<table>
<thead>
<tr>
<th>Growth regulators (µM/L)</th>
<th>% Response</th>
<th>Mean No. of roots ± Mean</th>
<th>Mean root length ± Mean (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>1.47</td>
<td>2.0 ± 0.31</td>
<td>1.6 ± 0.18</td>
</tr>
<tr>
<td>IAA</td>
<td>2.85</td>
<td>2.9 ± 0.90</td>
<td>2.4 ± 0.41</td>
</tr>
<tr>
<td>NAA</td>
<td>4.23</td>
<td>4.6 ± 0.41</td>
<td>3.4 ± 0.44</td>
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<tr>
<td>-</td>
<td>5.61</td>
<td>5.9 ± 0.90</td>
<td>1.8 ± 0.28</td>
</tr>
<tr>
<td>-</td>
<td>3.38</td>
<td>6.9 ±1.12</td>
<td>3.1 ± 0.34</td>
</tr>
<tr>
<td>-</td>
<td>5.02</td>
<td>5.9 ±0.62</td>
<td>3.8 ± 0.51</td>
</tr>
<tr>
<td>-</td>
<td>6.6</td>
<td>7.2 ±0.51</td>
<td>3.2 ± 0.46</td>
</tr>
<tr>
<td>-</td>
<td>1.26</td>
<td>1.2 ±0.61</td>
<td>1.1 ±0.12</td>
</tr>
<tr>
<td>-</td>
<td>2.53</td>
<td>18.9 ±1.2</td>
<td>1.6 ±0.36</td>
</tr>
<tr>
<td>-</td>
<td>3.80</td>
<td>38.6 ±0.9</td>
<td>2.6 ±0.86</td>
</tr>
<tr>
<td>-</td>
<td>5.07</td>
<td>26.8 ±1.2</td>
<td>2.1 ±0.36</td>
</tr>
</tbody>
</table>

Table 3. Antibacterial activity of Methanolic extracts of plant and callus of Excoecaria agallocha (measured in terms of diameter of zone of inhibition (mm) around the disk impregnated with extract).

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>In vitro raised plants extracts (mg/disk)</th>
<th>Callus extract (mg/disk)</th>
<th>Wild plant extract (mg/disk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>10 20 30 40 50</td>
<td>10 20 30 40 50</td>
<td>10 20 30 40 50</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>- - 4 6 7</td>
<td>- - 9 10 12 13 15</td>
<td>- - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>- - 8 10 12 13 21 22 24 26</td>
<td>- - 9 11 13 13 14 - 22</td>
<td>26 29 32</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>- - - - 8 10 9 11 12 21 22 24 26 27 28 29</td>
<td>- - - - - - - - - - - - -</td>
<td>- - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>- - - - 8 11 12 21 22 24 26 27 28 29 30</td>
<td>- - - - - - - - - - - - -</td>
<td>- - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>- - - - - - - - - - - - 6 8 10 7 8 6 5 4 3</td>
<td>- - - - - - - - - - - - -</td>
<td>- - - - - - - - - - - - - -</td>
</tr>
</tbody>
</table>

The antibacterial activity was tested based on disk diffusion assay (Malekzad et al. 2001)
Fig. 1: Phenols exudation
Fig. 2: Shoot initiation
Fig. 3: Shoot elongation
Fig. 4: Root initiation
Fig. 5: Uprooted microshoots
Fig. 6: Hardening
Fig. 7: Potting
Fig. 8: Field performance

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