European Journal of Medicinal Plants
4(6): 723-742, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Antioxidant and Antibacterial Activity of Field Grown and Tissue Cultured Root Callus of Mangrove Species

Chinnappan Ravinder Singh[1*], Kandasamy Kathiresan[1], Sekar Anandhan[1] and Kanagaraj Suganthi[1]

[1]Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608 502, Tamil Nadu, India.

Authors' contributions

All the authors contributed equally right from the design of the study to literature searches and analyses. All authors read and approved the final manuscript.

Original Research Article

Received 3rd October 2013
Accepted 12th December 2013
Published 3rd March 2014

ABSTRACT

Aim: The main aim of this study is to evaluate the anti-oxidant and antimicrobial activity of mangrove species and the development of the callus biomass.
Study Design: This is the first comparative evaluation report of field grown root and tissue cultured root callus of mangrove on anti-oxidant and anti-bacterial activity. *Acanthus ilicifolius, Calophyllum inophyllum* and *Excoecaria agallocha* were tested in this present investigation.
Place and Duration of Study: Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai 608 502, Tamil Nadu, India. Between February 2012 and July 2013
Methodology: In order to develop callus biomass, a number of growth hormones were supplemented with the MS medium. Following the callus development, anti-oxidant and anti-microbial activities were tested with field grown root and its tissue cultured root callus of *Acanthus ilicifolius, Calophyllum inophyllum* and *Excoecaria agallocha*. This was confirmed by different anti-oxidant, anti-bacterial and minimum inhibitory concentration assays.
Results: Maximum (89%) root callus biomass was obtained from *Acanthus ilicifolius* on

**Corresponding author: Email: chinnaravinder@yahoo.co.in;*

MS medium fortified with 0.3+0.3 mg/L of 2,4-D and KIN. 2,4-D 0.3mg/L and BAP 0.5 mg/L showed the maximum callus from both *Calophyllum inophyllum* (81%), *Excoecaria agallocha* (58%). In addition anti-oxidant and anti-bacterial effect of root and root callus of these three species were tested. In this study the root callus materials of all the three species showed the best anti-oxidant anti-bacterial activities.

Conclusion: The metabolites from mangroves are good remedy for number of health problems especially the enhanced level of metabolites through the tissue culture techniques. The present study confirmed the anti-oxidant and anti-bacterial effect of *Acanthus ilicifolius, Calophyllum inophyllum, Excoecaria agallocha* n root extract. This study will be a key to develop a new drug to achieve healthy life. especially the root callus materials showed the better activity when compared to the field grown plants.

Keywords: Callus biomass; Acanthus ilicifolius; Calophyllum inophyllum; Excoecaria agallocha; anti-microbial; resazurin method; anti-oxidant.

1. INTRODUCTION

The secondary metabolites constitute a source of bioactive substances and presently scientific interest has increased due to the search for new drugs of plant origin. Metabolites, with some novel chemical structures which belong to a diversity of chemical classes have been characterized from mangroves and mangrove associates. These include both primary and secondary metabolites [1]. The mangroves and their associated plants have various economic values and environmental functions [2,3] like fodder, firewoods, charcoal, timber, furniture, boat making, tannins for dying, among others and formation of detritus [4,5]. Apart from these applications, mangrove plants are known to possess medicinal values and have been used traditionally for ailment of various diseases by local inhabitants. Several mangrove species have also been found application as insecticides and pesticides and have recently attracted attention for pharmaceutical and other industries [6,3]. Besides, a number of mangrove plant secondary metabolites like gedunin, hydroquinone, xanthone, diterpenes, polyphenol etc. are currently used as anticancer agents in different cell lines, however, not much study has been undertaken [7-13].

Phytoconstituents are also important source of antioxidant and are capable to terminate the free radical chain reactions [14,15]. It is important to note that the mangroves are known to possess strong antioxidant properties due to the presence of several groups of polyphenols like anthocyanins, tannins, flavanones, isoflavones, resveratrol and ellagic acid [16,17]. There are also many reports describing the uses of mangrove plants for treatment of various disorders like headache, abdominal troubles, skin diseases among others [18,19,3,4]. These plants represent a great resource for detection of unique secondary metabolites which can supply information about the wide range of phytochemicals in nature and give knowledge about biological activities of plant compounds.

Similarly antimicrobial potential of different medicinal plants is being extensively studied all over the world but only a few studies have been carried out in a systematic manner [20]. Also the development of resistant strains of bacteria has increased the need for new antibiotics [21]. Several species of mangrove are proved for their bioactivities by inhibiting microbial growth [22]. Mangrove plants have been reported to have antimicrobial activity [23-26]. The antioxidant and antibacterial activities are attributed to bioactive polyphenols especially flavonoids which prevent people from many dreadful diseases [27]. Mangroves are rich source of polyphenols [28] beside this fact; the need of these products is high, so we

need to enhance the production of these specialized metabolites. The technique plant tissue culture is used not only for mass multiplication but also for production of high value secondary metabolites [29,30]. Growth regulators play a crucial role in secondary metabolite production *in vitro* condition. Also the *in vitro* raised plant cells serves as an effective antioxidant and antibacterial agent due to the presence of enhanced level of plant secondary metabolites [31]. Hence the present study has been designed to screen the anti-oxidant and anti-microbial potential of field grown and *in vitro* raised root callus biomass of *Acanthus ilicifolius, Calophyllum inophyllum, Excoecaria agallocha*. To the best of our knowledge this is the first comparative study report on field grown root and *in vitro*-derived root callus of mangrove species.

2. MATERIALS AND METHODS

2.1 Sample Collection and Extraction

Fresh roots of *Acanthus ilicifolius* (Acanthacae), *Calophyllum inophyllum* (Calophyllaceae), *Excoecaria agallocha* (Euphorbiaceae), known as mangroves were collected from Pichavaram mangrove forest and they are hereinafter referred as *A. ilicifolius*, *C. inophyllum* and *E. agallocha* respectively. The shade-dried root and *in vitro*-derived root callus samples of *A. ilicifolius, C. inophyllum,* and *E. agallocha* were extracted with methanol:chloroform in the ratio of 8:2 for 24 hours in a Soxhlet apparatus and this extracts were subsequently concentrated on a watch glass in an air draught condition for removing the chloroform [32].

2.2 Sterilization of Glassware

The most important step in tissue culture techniques is sterilization. All the glassware such as beakers, conical flasks, measuring cylinders, Petri dish and culture tubes were washed thoroughly with detergent (2% Teepol) in tap water, rinsed with double distilled water twice and dried in hot air oven at 30ºC prior to sterilization. Distilled water and other accessories such as forceps, blades and its holders, were autoclaved at 121ºC for 15 minutes at 15 lb/inch^2. After sterilization, they were kept in hot air oven until use.

2.3 Stock Solution and Media Preparation

The optimized MS (Murashige and Skoog medium [33]) medium was used for the present investigation. For the preparation of the basal MS medium, separate stock solution of macro nutrients, micro nutrients, iron supplements, vitamins and boric acid were prepared by dissolving required amount of chemicals in double distilled water and were stored at 4±1ºC. Individual growth regulators such as BA (benzyl adenine), kinetin, NAA (naphthalene acetic acid), IAA (indole acetic acid), IBA (Indole-3-butyric acid) and 2,4-D (2,4-dichlorophenoxy acetic acid) were prepared and kept at 4±1ºC.

MS medium [33] was prepared with all the stock solutions in appropriate proportions and the final volume was made up to required quantity by adding double distilled water, in which, sucrose 3% (W/V) was added to the medium as a source of carbon. Various concentrations and combinations of growth regulators were added to the medium before adjusting the pH to 5.8 using 1.0 N NaOH or 1.0 N HCl and gelled with 0.8% agar by melting the agar in a boiling water bath. Then the media were distributed in phyta jars and autoclaved. The autoclaved media were kept in inoculation chamber until use.

2.4 Surface Sterilization

The young root explants of *A. ilicifolius, C. inophyllum, E. agallocha* were immersed in water immediately after collection. The root samples were washed with tap water to remove epiphytes and other unwanted external matters. And the explants were surface sterilized with different surface sterilizing agents. Therefore the explants were disinfected with a detergent solution (2% Teepol, Reckill and Colman, India) for 5 min. Then the explants were washed in 0.1% mercuric chloride for 1.5 min followed by 70% ethanol for 45 seconds.

2.5 Aseptic Transfer of Explants

Aseptic transfer of tissue was done in a laminar air-flow hood. The interior was swabbed with 95% ethanol before inoculation. The autoclaved equipments were flame sterilized three times before using them for tissue transfer using 95% ethanol taken in a coupling jar. After sterilization, all the explants were cut into small pieces (1.0 to 1.5 cm long) and were individually placed on MS medium supplemented with various concentration and combinations of cytokinins and auxins, 3.0% sucrose and 0.8% agar.

2.6 Biochemical Assays

2.6.1 Determination of total phenol content

Total phenolic content of sample extracts were estimated by Folin-Ciocalteu's method [34]. Extracted samples (10 mg) were dissolved in 10 mL of distilled water. An aliquot of 100 µL of appropriate dilution of the samples were shaken for 1 min with 500 µL of the Folin-Ciocalteu reagent freshly prepared in our laboratory and 6 mL of distilled water. The mixture was briefly shaken and 2 mL of 15% (by mass per volume) sodium carbonate was added and the mixture was shaken again for 30 sec. Finally, the solution was brought up to 10 mL with distilled water. After 2 h of reaction at ambient temperature, the absorbance was measured at 750 nm. Gallic acid was used as standard; the total phenolic content of the samples were expressed as Gallic acid equivalents (GAE).

2.6.2 Total antioxidant activity

The total antioxidant capacities (TAC) of the crude extracts were evaluated by the method of Prieto, et al. [35]. 1ml sample was prepared in different concentrations (50 µl, 125 µl, 250 µl and 500 µl) with 3 ml of reagent solution 0.6 M sulphuric acid, 28 mM sulphuric acid and 4 mM ammonium molybdate. Reaction mixture was incubated at 95ºC for 90 minutes in a water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid of extract (mg/g).

2.6.3 DPPH-Free radical scavenging assay

Radical scavenging activities of the fractions were assessed using the DPPH (1,2-diphenyl-1-picrylhydrazyl) free radical method, adapted from Duan, et al. [36]. Briefly, 100 µl of various concentration of the phenol (50, 125, 250 and 500 µg/ml) was mixed with 2900 µl DPPH solution (120 µM) in methanol and incubated in darkness at 37ºC for 30 minutes and its absorbance was read at 517 nm. DPPH radical scavenging activity was expressed as butylated hydroxyl toluene (BHT).

$$\% \text{ of inhibition activity} = (A_B - A_s)/ A_B \times 100$$

2.6.4 Nitric oxide radical inhibition assay

Nitric oxide radical inhibition assay was performed based on the method of Govindarajan et al. [37] and Badami et al. [38]. 3 ml of reaction mixture containing 2 ml of sodium nitroprusside (10mM), 0.5 ml phosphate buffer saline and 0.5 ml (50, 125, 250 and 500 µg/ml) of extract solution was incubated at 25ºC for 150 min. After incubation, 1 ml of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) was added to 0.5 ml of reaction mixture for 5 min to complete diazolization. Then 1 ml naphthyl ethylene diamine dihydrochloride (NEDD) was added and allowed to stand for 30 min at 25ºC. A pink colored chromatophore formed was measured at 540 nm. Nitric oxide radical scavenging activity is expressed as butylated hydroxyl toluene (BHT/g).

2.6.5 Hydrogen peroxide radical inhibition assay

Hydrogen peroxide radical inhibition activity of samples was measured according to the method of Govindarajan et al. [37]. Extract was rapidly mixed with 2 ml of 10 mM phosphate buffer (0.1 M, pH 7.4) hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer after incubation for 10 min at 37ºC against a blank without hydrogen peroxide (H_2O_2). The radical scavenging activity is expressed as Gallic acid equivalents (GAE/g).

$$\% \text{ of scavenged activity} = (A_o - A_1)/ A_o \times 100$$

2.6.6 H_2O_2 inhibition effect

The H_2O_2 inhibition effect of plant extracts were determined by spectrophotometer [39]. One milliliter (2.6 and 10 mg/ ml) of sample, 3.4 ml of 0.1 M phosphate buffer (pH 7.4) and 0.6 ml of 43 mM H_2O_2 were mixed and after 60 min the absorbance of mixture was measured at 230 nm. Control solutions without H_2O_2 were prepared for each sample concentration. To determine the H_2O_2 (mM) concentration that was not involved in the reaction, a linear regression equation was used. 3.4 ml of phosphate buffer was added to 0.6 ml of 10,15, 25, 43 mM of H_2O_2 at 230 nm. Regression equation formulae were obtained by the graphic of standard curve of absorbance vs different concentrations of (+)-Catechin.

$$A\ (230) = 0.0125 \times C\ (H_2O_2, \text{mM}) + 0.0873\ (R^2 = 0.9783)$$

(+)-Catechin was used as the reference antioxidant. The equation used is as follows:

$$H_2O_2 \text{ inhibition capacity } (\%) = [1-(H_2O_2 \text{ conc. of sample}/H_2O_2 \text{ conc. of control})] \times 100$$

2.6.7 Measurement of reducing power

The method described by Oyaizu [40] was used to determine the reducing power of the extracts. 1ml of sample containing different concentrations of phenol (20-100 µl) was mixed with 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml $KFe(CN)_6$ (potassium ferric cyanide) 1% were added. The mixture was incubated in a water bath at 50ºC for 20 min. After incubation, 10% TCA (2.5 ml) was added and the resulting mixture was centrifuged at 3000×g for 10 min. Supernatant (2.5 ml) was added to 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ solution. The absorbance was measured at 700 nm. Increased absorbance

indicates reducing power. Concentration of reducing power is expressed as Gallic acid equivalents (GAE) in mg/g of extract.

2.7 Antibacterial assay using resazurin method

A versatile microtitre plate bioassay for quick and sensitive determination of antibacterial activity was followed to screen antibacterial property of the plant extracts by the method of Langfield et al. [41] and Sarker et al. [42]. This assay was also used to determine minimum inhibitory concentrations of methanolic extracts of roots of *A. ilicifolius*, *C. inophyllum, E. agallocha* and their respective calli. Since conventional methods like well diffusion or agar well-diffusion method are time consuming and require significant quantities of the test materials [43], this microtitre plate assay has increased sensitivity for small quantities of extract, ability to distinguish between bacteriostatic and bactericidal effects and quantitative determination of minimal inhibitory concentrations (MIC). The use of a colorimetric indicator eliminates the need for a spectrophotometric plate reader and avoids the ambiguity associated with visual comparison or measurement of growth inhibition rings on agar plates.

2.7.1 Preparation of bacterial cultures

Ten species of clinical pathogens were isolated from different clinical samples received from the Department of Microbiology, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamilnadu, India (*Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Enterobacter aerogens*, *Pseudomonas aeruginosa*, *Serratia marsescens*, *Proteus vulgaris*, *Staphylococcus aureus*, *Shigella dysenteriae Bacillus subtilis* and *Salmonella typhi*) were labeled as P1 to P10 as given in the above order that was used for this assay. A single colony of each bacterial species was transferred to a 5 ml of trypticase soy broth and placed in an incubator at 35ºC for overnight. The overnight cultures of bacterial species were spun down at 4000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 20 ml of MHB so as to obtain a final concentration of 5×10^6 cfu/mL. Thus the test bacterial cultures were prepared for antibacterial assay.

2.7.2 Culture Media and Antibiotics

Trypticase soy broth (TSB) was used to prepare overnight culture. Mueller Hinton broth (1x MHB and 3.3x MHB strength) was used for dilution and screening assay. Ciprofloxacin (for P1 to P6 1mg/ml each), ampicillin/dicloxacillin (for P7 1mg/ml), nalidixic acid (for P8 1mg/ml) and streptomycin (for P9 and P10 1mg/ml) were used as standard drug for this antibacterial study.

2.7.3 Preparation of resazurin solution

Resazurin dye 270 mg (Sigma, India) was dissolved in 40 mL of sterile double distilled water to prepare resazurin solution. To prepare homogenous solution, a vortex mixer was used for proper and efficient mixing of resazurin.

2.7.4 Preparation of test sample

Methanol extracted roots of *A. ilicifolius*, *C. inophyllum* and *E. agallocha* and their respective calli were dissolved in methanol (10 mg/ml since it is a crude extract whereas 1 mg/ml is recommended for purified compounds) and were labeled as AI-R, AI-R/C CI-R, CI-R/C, EA-R and EA-R/C for further use.

2.7.5 Preparation of the microtitre plates

The antibacterial activity of methanolic extracts of roots of *A. ilicifolius*, *C. inophyllum* and *E. agallocha* and their respective calli was estimated in a microtitre plate based method by using resazurin assay [42]. Test samples were dissolved in methanol and were run at these concentrations. Sterile 96-well microtitre plates were used for this assay (0.3 ml volume, TARSONS) and were labeled.

All wells in column wise, except the first row, were filled with MHB (100 µl). Test sample (200 µl) was added to the first row of all the columns and serial two-fold dilutions were made down in such a way that each well had 100 µl of the test material in serially descending concentrations. Serial dilutions were performed using a multichannel pipette. To each well 20 µl of resazurin indicator solution was added. Using a pipette 60 µl of 3.3 × strength broths was added to each well to ensure that the final volume was single strength of the MHB. Finally, 20 µl of bacterial suspension was added to each well to achieve a concentration of 5×10^5 cfu/ml and the final volume was made up to 200 µl per well.

Each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. This assay was carried out with a total of 8 plates with controls.

(i) four columns with antibiotics, indicator dye and broth (ciprofloxacin**,** ampicillin/ dicloxacillin, nalidixic acid and streptomycin respectively in serial dilution (labeled as C.Ab1 to C.Ab4) (Sterility control for antibiotics)),
(ii) six well with test compounds in broth with indicator dye dilution (labeled as C.AI-R, C.AI-R/C C.CI-R, C.CI-R/C, C.EA-R and C.EA-R/C) (Sterility control for test compound),
(iii) ten wells with pathogens and broth incorporated with indicator dye alone (labeled as C.P1 to C.P10)
(iv) ten columns with serially diluted respective antibiotics in broth incorporated with indicator dye and added with ten different clinical pathogens (labeled as C.Ab1+P1, C.Ab1+P2, C.Ab1+P3, C.Ab1+P4, C.Ab1+P5, C.Ab1+P6, C.Ab2+P7, C.Ab3+P8, C.Ab4+P9, C.Ab4+P10),
(v) ten columns per test compound were added with ten different clinical pathogens in serially diluted test compound in broth added with indicator dye, totally 60 columns for six test compounds (AI-R, AI-R/C CI-R, CI-R/C, EA-R and EA-R/C)

The plates were prepared in triplicate, and placed in an incubator set at 37ºC for 18–24 h. The colour change was then assessed visually. The colour retaining property of the sample recorded as positive. Any colour changes from dark blue/purple to pink (signifying live growth) or colour less were recorded as negative. The MIC was determined as the lowest test compound concentration at which no pink color (signifying no growth) appeared. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

3. RESULTS

This study mainly focused on the investigation of the free radical scavenging and anti-bacterial activity of field grown and *in vitro*-derived root callus of *A. ilicifolius, C. inophyllum* and *E. agallocha*. In order to increase the secondary metabolites several growth hormones were tested, among total hormones tested 0.3 mg/l 2,4-D and 0.5 mg/l of BA showed the

maximum callus biomass in all the species *A. ilicifolius* (89±0.6), *C. inophyllum* (81±0.0) and *E. agallocha* (58±0.6). The dried biomass of this root and root callus extracts of these species have been used for the further studies (Table 1).

Table 1. Effect of different concentrations of auxins and cytokinins on root callus biomass production in *A. ilicifolius, C. inophyllum* and *E. agallocha*

Growth hormones(mg/l)	% of callus response in *A. ilicifolius*	% of callus response in *C. inophyllum*	% of callus response in *E. agallocha*
NAA			
0.1	11±0.1	22±0.2	12±0.0
0.3	20±0.3	28±0.1	15±0.3
0.5	27±0.2	21±0.3	16±0.1
IAA			
0.1	10±0.1	14±0.1	21±0.2
0.3	14±0.5	11±0.2	22±0.0
0.5	17±0.2	30±0.5	22±0.5
2-4-D			
0.1	25±0.2	29±0.4	31±0.2
0.3	48±0.1	31±0.0	33±0.4
0.5	69±0.7	47±0.6	35±0.3
NAA+KIN			
0.5+0.1	11±0.2	12±0.3	35±0.1
0.5+0.3	29±0.6	26±0.2	32±0.3
0.5+0.5	51±0.2	28±0.4	25±0.3
NAA+BAP			
0.5+0.1	21±0.8	19±0.1	29±0.5
0.5+0.3	37±0.7	30±0.2	23±0.0
0.5+0.5	52±0.5	31±0.5	25±0.7
IAA+KIN			
0.5+0.1	07±0.3	15±0.1	10±0.1
0.5+0.3	12±0.2	19±0.1	10±.0.6
0.5+0.5	21±0.2	17±0.4	11±0.1
IAA+BAP			
0.5+0.1	25±0.7	16±0.4	22±0.4
0.5+0.3	36±0.6	51±0.1	24±0.3
0.5+0.5	39±0.5	42±0.0	26±0.5
2,4-D+KIN			
0.3+0.1	20±0.2	17±0.6	34±0.4
0.3+0.3	49±0.0	43±0.9	33±0.2
0.3+0.5	70±0.8	51±0.1	36±0.3
2-4-D+BAP			
0.3+0.1	39±0.3	31±0.3	44±0.3
0.3+0.3	57±0.1	59±0.2	47±0.0
0.3+0.5	89±0.6	81±0.0	58±0.6

Note: Data are expressed as fresh weight of callus, 50 explants were taken for each experiment. Each experiment was repeated five times.

3.1 Total Phenolic Content and Total Antioxidant Activity

Among all assayed extracts, *E. agallocha* root callus (90.32%) showed high phenol content followed by field grown root extract (82.10%), *C. inophyllum* root and root callus showed the 85.06% and 89.63% and *A. ilicifolius* root and root callus 77.13% and 78.64%. Similar results obtained for total anti-oxidant activity. The maximum activity was recorded in

E. agallocha root callus (71.02%), for field grown root (71.02%). In *C. inophyllum* root callus showed (69.47%) in root alone (66.97%). *A. ilicifolius* showed similar activity in both root and root callus (71.02%) (Fig. 1a and 1b).

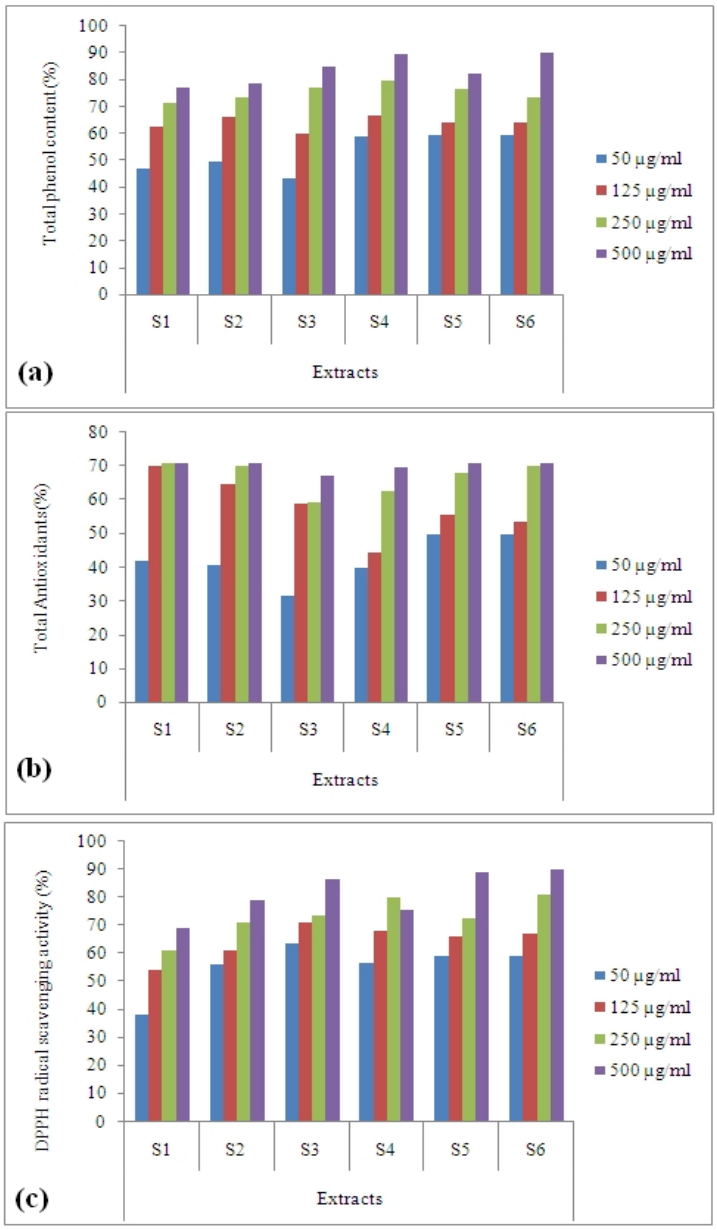

Fig. 1. (a) Total phenol content, (b) Total antioxidants and (c) DPPH radical scavenging activity. Note: S1=AI-R, S2=AI-R/C, S3=CI-R, S4=CI-R/C, S5=EA-R and S6=EA-R/C

3.2 DPPH Free Radical Scavenging Method

Of these extracts, the root callus extract of *A. ilicifolius* (71.24%), *C. inophyllum* (80.21%) and *E. agallocha* (81.25%) showed maximum free radical scavenging activity compared to field grown root extracts (Fig. 1c).

3.3 Nitric Oxide Neutralizing Method

Among the total extracts tested, the root callus extract of *E. agallocha* (82.31%) showed maximum NOº neutralising capacity when compared to root extract (67.24%), similarly *C. inophyllum* root callus extract (81.24%) and root extract (74.25%) and minimum activity has been observed from *A. ilicifolius* root callus (74.25%), root extract (71.31%) as shown in Fig. 2a. As per the overall estimation all the root callus extracts showed a significant activity.

3.4 Reducing Power Assay and Hydrogen Peroxide Radical Inhibition Assay

Of these extracts, the root and root callus extract of *E. agallocha* (79.68 and 86.54%) showed maximum reducing capacity when compared to *A. ilicifolius* (71.65 and 71.35%), *C. inophyllum* (76.23 and 73.25%). From these all extracts, root callus extract showed the better activity. Similarly the root callus of *A. ilicifolius* (74.26%), *C. inophyllum* (81.24%) and *E. agallocha* (82.31%) showed the better Hydrogen peroxide radical inhibition activity. And the root extract of *E. agallocha* (67.24%), C. *inophyllum* (74.25%) and *A. ilicifolius* (71.31%) showed the minimum inhibition activity (Fig. 2b and 2c and Table 2).

3.5 Antibacterial Activity

At the beginning of the reaction all the wells were in blue colour. After 24 hrs of the incubation, some of the wells turned into pink colour indicating the bacterial growth. The extracts of root-callus of *E. agallocha, C. inophyllum* and *A. ilicifolius* were found to have higher antibacterial activity than their root extracts as evident by the colour change. The extracts of root-callus of *E. agallocha* were found to have higher antibacterial activity than the extracts of root-callus of *C. inophyllum* and *A. ilicifolius* as evident by the colour change. All the bacterial pathogens showed more sensitivity to the extracts of root-callus than their normal root extracts.

Using the microtitre plate bioassay we found that a crude methanolic root-callus extract of *E. agallocha, C. inophyllum* and *A. ilicifolius* inhibited growth of all the ten clinical pathogens at concentrations of less than 350 µg/ml whereas the crude methanolic root extract of the same plant species which is more than 600 µg/ml.

The maximum antibacterial effect was noted with root-callus extract which are in the range between 0.039µg/ml and 0.35µg/ml (equivalent to the extracts of root callus concentrations of 39µg/ml and 350µg/ml) for all the three root-callus extracts against all the ten clinical pathogens tested. The results are shown in Table 3.

Table 2. Anti-oxidant potential of root ant root derives callus extract of *A. ilicifolius, C. inophyllum* and *E. agallocha*

Parameters	Conc. (µg/ml)	% scavenging activity of S-1	% scavenging activity of S-2	% scavenging activity of S-3	% scavenging activity of S-4	% scavenging activity of S-5	% scavenging activity of S-6
Total phenol	50	47.1	49.56	43.21	58.64	59.24	59.21
	125	62.32	66.21	60.08	66.76	64.31	64.21
	250	71.28	73.32	76.88	79.64	76.32	73.2
	500	77.13	78.64	85.06	89.63	82.1	90.32
Total antioxidant	50	41.74	40.49	31.46	39.92	49.62	49.84
	125	70.09	64.48	58.87	44.54	55.45	53.58
	250	70.71	70.09	59.19	62.61	67.91	70.09
	500	71.02	71.02	66.97	69.47	70.71	71.02
DPPH radical scavenging activity	50	38.28	56.32	63.71	56.77	59.34	59.24
	125	54.16	61.02	71.23	68.22	66.24	67.21
	250	61.23	71.24	73.69	80.21	72.39	81.25
	500	69.35	79.23	86.45	75.78	89.21	90.21
NO radical scavenging activity	50	43.47	52.17	38.26	56.25	38.21	54.98
	125	52.21	55.65	47.94	67.32	41.35	69.54
	250	61.23	68.21	69.24	78.24	56.95	76.35
	500	71.31	74.26	74.25	81.24	67.24	82.31
H_2O_2radical scavenging activity	50	36.24	39.21	40.21	44.21	40.21	36.78
	125	40.21	42.23	49.35	49.65	51.24	53.62
	250	51.24	57.38	67.34	65.34	68.54	69.54
	500	69.29	67.54	76.59	73.54	74.35	79.34
Total reducing power	50	54.21	60.23	49.37	54.23	56.24	58.64
	125	64.32	64.35	57.35	67.24	69.25	69.83
	250	69.35	69.35	65.34	68.67	71.98	77.65
	500	71.65	71.35	76.23	73.25	79.68	86.54

Note: S1- root extract of A. ilicifolius, S2- root callus extract of A. ilicifolius, S3- root extract of C. inophyllum, S4-root callus extract of C. inophyllum, S5-root extract of E. agallocha, S6-root callus extract of E. agallocha.

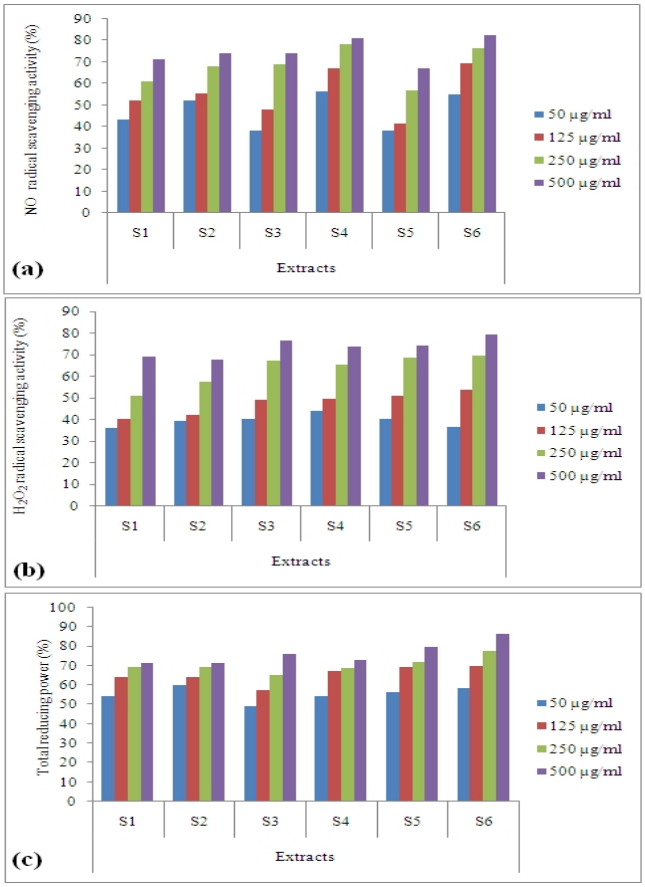

Fig. 2. (a) NO radical scavenging activity, (b) H_2O_2 radical scavenging activity and (c) Total reducing power. Note: S1=AI-R, S2=AI-R/C, S3=CI-R, S4=CI-R/C, S5=EA-R and S6=EA-R/C

Table 3. Shows minimum inhibitory concentration (MIC) value for extracts against ten different clinical pathogens

Pathogen	MIC (µg/ml)					
	AI-R	AI-R/C	CI-R	CI-R/C	EA-R	EA-R/C
Aeromonas hydrophila	39	39	78	20	156	20
Klebsiella pneumonia	313	156	313	20	313	39
Enterobacter aerogens	78	20	156	78	156	78
Pseudomonas aeruginosa	78	78	313	20	313	78
Serratia marsescens	313	156	625	156	625	78
Proteus vulgaris	313	156	625	156	313	78
Staphylococcus aureus	78	20	78	39	156	20
Shigella dysentriae	625	313	625	156	313	78
Bacillus subtilis	156	78	78	20	78	39
Salmonella typhi	78	20	156	20	78	39

Note: AI-R - root extract of A. ilicifolius, AI-R/C - root callus extract of A. ilicifolius, CI-R - root extract of C. inophyllum, CI-R/C - root callus extract of C. inophyllum, EA-R -root extract of E. agallocha, EA-R/C - root callus extract of E. agallocha.

Fig. 3 shows comparative analysis between normal root extracts of *A. ilicifolius, C. inophyllum, E. agallocha* and their root-callus extracts against ten different clinical pathogens.

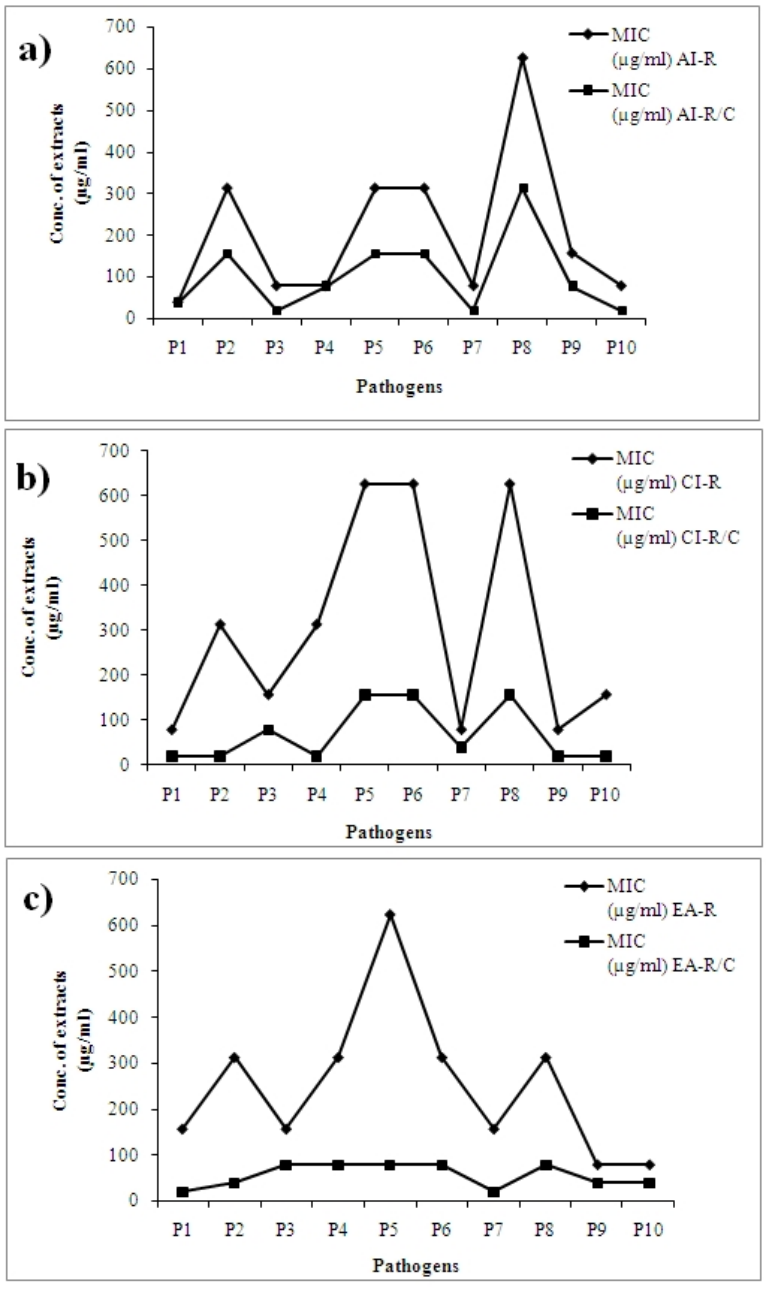

Fig. 3. Comparative analysis between normal root extracts of *A. ilicifolius, C. inophyllum* and *E. agallocha* and their root-callus extracts against ten different clinical pathogens.

3.6 Minimum Inhibitory Concentration

The antibacterial activities of root and root callus extract of *A. ilicifolius, C. inophyllum* and *E. agallocha* were tested against clinical pathogens such as *A. hydrophila*, *K. pneumoniae*, *E. aerogens*, *P. aeruginosa*, *S. marsescens*, *P. vulgaris*, *S. aureus*, *S. dysenteriae, B. subtilis* and *S. typhi* using microtitre plate bioassay.

The minimum inhibitory concentration (MIC) of AI-R was 0.039 mg/ml against *A. hydrophila,* 0.313 mg/ml against *K. pneumoniae, S. marsescens* and *P. vulgaris* 0.078 mg/ml against *E. aerogens*, *P. aeruginosa, S. aureus* and *S. typhi,* 0.625 mg/ml against *S. dysenteriae* and 0.156 mg/ml against *B. subtilis* (Fig. 4a). AI-R/C was 0.039 mg/ml against *A. hydrophila,* 0.156 mg/ml against *K. pneumonia, S. marsescens* and *P. vulgaris,* 0.078 mg/ml against *P. aeruginosa* and *B. subtilis,* 0.02 mg/ml against *E. aerogens, S. aureus* and *S. typhi,* 0.313 mg/ml against *S. dysenteriae* (Fig. 4b).

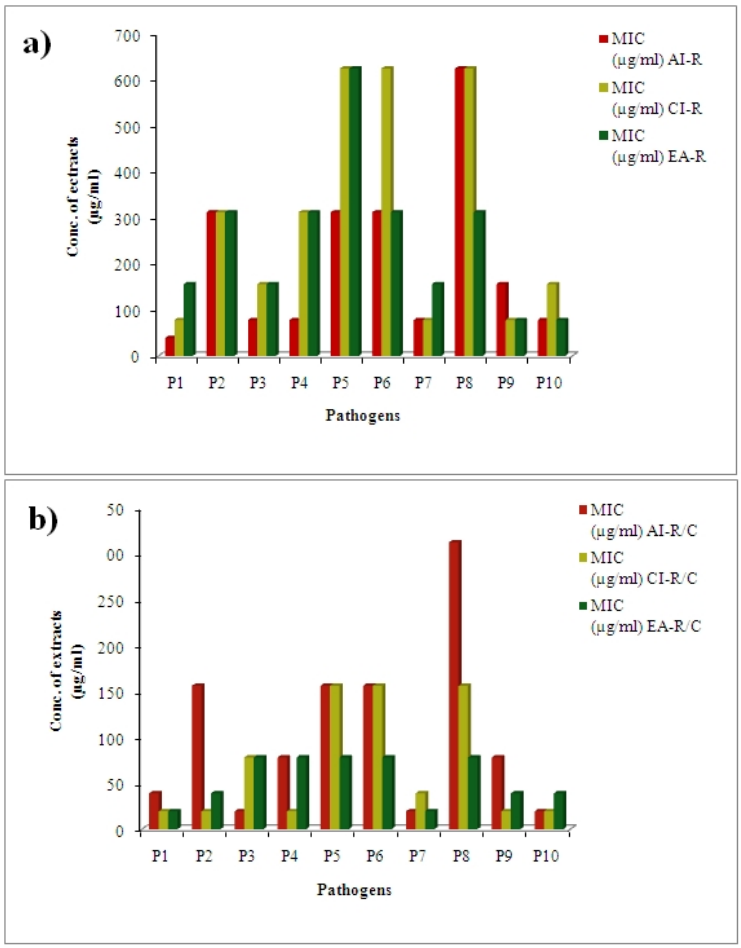

Fig. 4. (a) Shows comparison between root extracts of *A. ilicifolius, C. inophyllum, E. agallocha* against ten different clinical pathogens. (b) Shows comparison between root-callus extracts of *A. ilicifolius, C. inophyllum, E. agallocha* against ten different clinical pathogens.

CI-R was 0.078 mg/ml against *A. hydrophila, S. aureus* and *B. subtilis,* 0.313 mg/ml against *K. pneumonia* and *P. aeruginosa,* 0.156 mg/ml against *E. aerogens* and *S. typhi,* 0.625 mg/ml against *S. marsescens*, *P. vulgaris* and *S. dysenteriae* (Fig. 4a). CI-R/C was 0.02 mg/ml against *A. hydrophila, K. pneumoniae, B. subtilis, P. aeruginosa* and *S. typhi,* 0.078 mg/ml against *E. aerogens,* 0.156 mg/ml against *S. marsescens*, *P. vulgaris* and *S. dysenteriae,* 0.039 mg/ml against *S. aureus* (Fig. 4b).

EA-R was 0.156 mg/ml against *A. hydrophila, E. aerogens, and S. aureus,* 0.313 mg/ml against *K. pneumonia, P. aeruginosa, P. vulgaris* and *S. dysenteriae,* 0.625 mg/ml against *S. marsescens*, 0.078 mg/ml against *S. typhi,* and *B. subtilis* (Fig. 4a). EA-R/C was 0.02 mg/ml against *A. hydrophila* and *S. aureus,* 0.039 mg/ml against *K. pneumoniae, B. subtilis* and *S. typhi,* 0.078 mg/ml against *E. aerogens*, *P. aeruginosa*, *S. marsescens*, *P. vulgaris* and *S. dysenteriae* (Fig. 4b).

4. DISCUSSION

Mangroves have many bioactivities such as antioxidant, antibacterial and anticancer due to the presence of numerous phytochemicals [43-46]. The application of antioxidants in pharmacology is important to improve the current treatments for various diseases. Plants are endowed with free radical scavenging molecules, which are rich in antioxidant activity [47]. The root and root callus extracts of *A. ilicifolius*, *C. inophyllum* and *E. agallocha* were analysed for their antioxidant properties. An earlier study has also reported that the biochemical assays such as total antioxidant and DPPH scavenging potential are particularly suitable for the evaluation of antioxidant activity of crude extracts [48]. The remarkable free radical inhibition activity was already stated for *A. ilicifolius, C. inophyllum* and *E. agallocha* plant parts using DPPH assay methods [49].

4.1 Total Phenolic Content and Total Antioxidant Activity

Phytochemical content of mangroves shows excellent antioxidant activities [50]. The phenolic compounds are unique, produced by plants as secondary metabolites. The significance of this plant phytochemical is their maximum antioxidant activity due to the presence of hydroxyl group that serves as hydrogen donor terminating the disease causing free radical chain. The root and root-callus extract of *A. ilicifolius, C. inophyllum* and *E. agallocha* were analysed for their total phenolic content and total anti-oxidant activity. Root-callus extract of all the three species showed the best activity when compare to field grown root. There is a positive correlation between phytochemical content and its antioxidant activities, as already reported [51].

4.2 DPPH Free Radical Scavenging Method

Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging and other degenerative diseases in human [52]. The *in vivo* generations of free radicals are done by addition of DPPH that acts as source of free radicals. The extracts having hydrogen donor group will scavenge this free radicals by binding to them, hence in the present investigation the extracts *A. ilicifolius, C. inophyllum* and *E. agallocha* were tested for their free radical scavenging activity. Similar reports on tissue cultured callus of coastal species *Ipomea aquatica* has showed hyper antioxidant activities which are evident by DPPH assay method [53].

4.3 Nitric Oxide Neutralizing Method

The NOº on reaction with oxygen gives ONOO- which can react and denature the biomolecules present in our body leads to disease progression. Hence the NOº can be scavenged by hydrogen donating extracts by binding with them will be an effective antioxidant. This test was made in the present investigation for the mangrove extracts of *A. ilicifolius, C. inophyllum* and *E. agallocha*. There was no record on nitric acid assay with tissue cultured callus materials.

4.4 Reducing Power Assay and Hydrogen Peroxide Radical Inhibition Assay

Many degenerative diseases are induced due to the formation of peroxides and free radical chain reactions in human system. An effective antioxidant will serve as reductones. Reductones are molecules responsible for the reduction of peroxides and free radical chain reactions by donating hydrogen atoms. Therefore, in the present investigation, the reducing capacity and hydrogen peroxide radical inhibition of root and root callus extract of *A. ilicifolius, C. inophyllum* and *E. agallocha* were tested. Tissue culture-derived callus of coastal species *Ipomea aquatica* and *Citrullus colocynthis* also has been shown to have hyper antioxidant activities [53,54].

4.5 Anti-bacterial Activity

The results showed that the root-callus extracts of *A. ilicifolius, C. inophyllum, E. agallocha* showed elevated antimicrobial effect against all the pathogens when compared to root extract alone. This increased antibacterial activity of callus could be attributed to the effect of growth hormones 2-4-D (0.3mg/l) and BAP (0.5mg/l) of tissue culture media that might have influenced the synthesis of the phytochemicals. This result is in accordance with the previous work with *Premna serratifolia* and *Ipomea aquatica* which have displayed higher antimicrobial activity with leaf callus tissue than intact leaf tissue [53,55, 56].

5. CONCLUSION

The study has revealed the potential bioactivities of *A. ilicifolius, C. inophyllum* and *E. agallocha* which showed significant antioxidant and antimicrobial activities which could be successfully elevated by *in vitro* callus culture method. This deserves further research on *in vitro* callus culture method on mangroves.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

The authors are thankful to the authority of Annamalai University for providing facilities and Dr. C. Ravinder Singh to University Grants Commission, Government of India, New Delhi for "UGC-Dr.D.S. Kothari Post-Doctoral Fellowship" (Grant No. BL/11-12/0037).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Osbourne AE. Preformed antimicrobial compounds and plant defense against fungal attack. Plant cell. 1996;8:1821-1831.
2. Kathiresan K, Qasim SZ. Biodiversity in mangrove ecosystems. Hindustan publishers, New Delhi. 2005;251. (ISBN: 8170750792).
3. Bandaranayake WM. Bioactivities, bioactive compounds and chemical constituents of mangrove plants. Wetlands Ecology and Management. 2002;10(6):421-452.
4. Pattanaik C, Reddy CS, Dhal NK, Das R. Utilization of Mangrove forest in Bhitarkanika wildlife Sanctuary Orissa. Indian J Trad Knowledge. 2008;7(4):598-603.
5. Upadhyay VP, Mishra PK, Sahu JR. Distribution of mangrove species with Bhitarkanika National Park in Orissa, India. Tres for Life J. 2008;3(4):001-005.
6. Bandaranayake WM. Traditional and medicinal use of Mangrove. Mangrove and Salt Marshes. 1998;2:133-148.
7. Konishi T, Konoshima T, Fujiwara Y, Kiyosawa S. Exocoecarins D, E and K from *Exocoecaria agallocha.* J Nat Prod. 2000;63:344-346.
8. Konishi T, Takasaki M, Tokuda H, Kiyosawa S, Konoshima T. Anti-tumor promoting activity of diterpenes from *Excoecaria agallocha.* Biol Pharm Bull. 1998;21:993-996.
9. Konoshima T, Konishi T, Takasaki M, Yamazoe K, Tokuda H. Anti-tumor promoting activity of the diterpene from *Excoecaria agallocha II.* Biol Pharm Bull. 2001;24:1440-1442.
10. Scheck AC, Perry K, Hank NC, Clark WD. Anticancer activity of extracts derived from the mature roots of *Scutellaria baicalensis* on human malignant brain tumor cells. Biomed Centr Complement Alternat Med. 2006;6:27-31.
11. Uddin SJ, Nahar L, Shilpi JA, Shoeb M, Borkowski T, Gibbons S, et al. Gedunin, a limonoid from *Xylocarpus granatum*, inhibits the growth of $CaCo_2$ colon cancer cell line *in-vitro*. Phytotherapy Res. 2007;21(8):757-761.
12. Xu M, Cui J, Fu H, Proksch P, Lin W, Li M. Embelin derivatives and their derivatives and their anticancer activity microtubule disassembly. Planta Medica. 2005;71:944-948.
13. Uddin MJ, Mondal D, Morris CA, Lopez T, Diebold U, Gonzalez RD. An *in-vitro* controlled release study of valproic acid encapsulated in a titania ceramic matrix. Appl Surf Sci. 2011;257:7920-7927.
14. Cody V, Middleton E, Harborne JB. Plant Flavonoids in Biology and Medicine-Biochemical, pharmacological and structure-activity relationships, Alan R. Liss, New York; 1986.

15. Oluwaseum AA, Ganiyu O. Antioxidant properties of methanolic extracts of mistletoes (*Viscum album*) from cocoa and cashew trees in Nigeria. Afr J Biotechnol. 2008;7:3138-3142.
16. Espin JC, Garcia-Conesa MT, Thomas-Barberan FA. Nutraceuticals: Facts and fiction. Phytochem. 2007;68:2986-3008.
17. Ksouri R, Falleh H, Megdiche W, Trabelsi N, Mhamdi B, Chaieb K, et al. Antioxidant and antimicrobial activities of the edible medicinal halophyte *Tamarix gallica* L. and related polyphenolic constituents. Food and Chemical Toxicol. 2009;47:2083-2091.
18. Choudhury BP. Socioeconomic aspect of Bhitarkanika wildlife Sanctuary in the state of Orissa. Orissa Review. 1987;43(9):45-47.
19. Kathiresan K. A review of studies on Pichavaram mangroves, southeast India, Hydrobiologia. 2000;430:185-205.
20. Arora DS, Kaur GJ, Kaur H. Antibacterial activity of tea and coffee: Their extracts and preparations. Int J Food Prop. 2009;12:286-294.
21. Eloff JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica. 1998;64:711-713.
22. Miki T, Sakaki T, Shibata M, Inukai Y, Hirosue H, Ikema Y, et al. Soxhlet extraction of mangrove and biological activities of extracts. 1994;53:3347-3352.
23. Scalbert A. Antimicrobial properties of tannins. Phytochem. 1991;30:3875-3883.
24. Ravikumar S, Kathiresan K. Influence of tannins, amino acids and sugar on fungi of marine halophytes. Mahasagar. 1993;26(1):21-25.
25. Fennel CW, Lindsey KL, McGaw JL, Stafford GI, Elgorashi EE, Grace MO, et al. Assessing African medicinal plants for efficacy and safety. Pharmacological screening and Toxicology. J. of Ethnopharmacol. 2004;94:205-217.
26. Ravikumar S, Gnanadesigan M, Suganthi P, Ramalakshmi A. Antibacterial potential of chosen mangrove plants against isolated Urinary tract infectious bacterial pathogens. Int J Med and Medical Sci. 2010;2(3):94-99.
27. Middleton EJr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. Pharmacol Rev. 2000;52:673-751.
28. Cushnie TPT, Andrew J. Lamb antimicrobial activity of flavonoids *Int J Antimicrobial Agents.* 2005;26:342-356.
29. Alfermann AW, Petersen M. Natural products formation by plant cell biotechnology. Plant Cell Tissue Organ Cult. 1995;43:199-205.
30. Dornenburg H, Knorr D. Challenges and opportunities for metabolite production from plant cell and tissue cultures. Food Technol. 1997;51:47-54.
31. Amid A, Johan NN, Jamal P, Zain WNWM. Observation of antioxidant activity of leaves, callus and suspension culture of *Justicia gendarusa.* Afr J Biotechnol. 2011;10(81):18653-18656.
32. Harborne JB. Phytochemical Methods. Chapman and Hall, London; 1998.
33. Murashige T, Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant. 1962;15:473-497.
34. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. Methods on Enzymology. 1999;299:152-178.

35. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999;269:337-341.
36. Duan XJ, Zhang WW, Li XM, Wang BG. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. Food Chem. 2006;95:37-43.
37. Govindarajan R, Rastogi S, Vijayakumar M, Rawat AKS, Shirwaikar A, Mehrotra S, et al. Studies on antioxidant activities of *Desmodium gangetium.* Biological and Pharmaceutical Bulletin. 2003;26:1424-1427.
38. Badami S, Rai RS, Suresh B. Antioxidant activity of *Aporosa lindleyana* root. J Ethnopharmacol. 2005;37(35):1-5.
39. Ruch RJ, Cheng SJ, Launig JF. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989;10:1003-1008.
40. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Jpn J Nutr. 1986;44:307-315.
41. Langfield RD, Scarano FJ, Heitzman ME, Kondo M, Hammond GB, Neto CC. Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant *Peperomia galioides.* J Ethnopharmacol. 2004;94:279-281.
42. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods. 2007;42(4):321-324.
43. Faulkner DJ. Marine natural products. Natural Product Reports. 2000;17:7-55.
44. Banerjee D, Chakrabarti S, Hazra AK, Banerjee S, Ray J, Mukherjee B. Antioxidant activity and total phenolics of some mangroves in Sundarbans. Afr J Biotechnol. 2008;7(6):805-810.
45. Premanathan M, Chandra K, Bajpai SK, Kathiresan K. A survey of some Indian marine plants for antiviral activity. Bot Mar. 1992;35:321-324.
46. Boopathy NS, Kathiresan K. Anticancer Drugs from Marine Flora*:* An Overview. J Oncol. 2011;18:201-320.
47. Zheng W, Wang YS. Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem. 2001;49(11):5165-5170.
48. Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signaling. Curr Med Chem. 2004;11:1163-1182.
49. Vadlapudi V, Naidu CK. Evaluation of Antioxidant potential of selected Mangrove Plants. J Pharm Res. 2009;2(11):1742-1745.
50. Zhang WZ, Venardos K, Chin-Dusting J, Kaye DM. Adverse effects of cigarette smoke on NO bioavailability: role of arginine metabolism and oxidative stress. Hypertension. 2006;48:278-285.
51. Bendini A, Cerretani L, Pizzolante L, Gallina-Toschi T, Guzzo F, Cedolo F, et al. Phenol content related to antioxidant and antimicrobial activity of *Passiflora* spp. extracts. Eur Food Res Technol. 2006;223:102–109.
52. Percival M. Antioxidants. Advanced Nutrition Publications, Inc., Nut. 1996;03:10-35.
53. Prasad NK, Prasad MS, Shivamurthy GR, Aradhya SM. Callus induction from *Ipomoea aquatica* Forsk. Leaf and its antioxidant activity. Indian J Biotechnol. 2006;5:107-111.

54. El-baz FK, Mohamed AA, Ali SI. Callus formation, phenolics content and related antioxidant activities in tissue culture of a medicinal plant colocynth (*Citrullus colocynthis*). Nova Biotechnologica. 2010;10(2):79-94.
55. Ravinder Singh C. Antimicrobial effect of Callus and Natural plant extracts of *Premna serratifolia* L. Int J Pharm Biomed Res. 2011;2(1):17-20.
56. Drummond AJ. Waigh RD. Recent Research Developments in Phytochemistry 2000;4:143–152.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=433&id=13&aid=3879