Screening of antioxidant and antifilarial activity of leaf extracts of *Excoecaria agallocha* L.

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Abstract

Organic solvent extracts of leaf of *Excoecaria agallocha* L., a mangal species from Bhitarkanika, Orissa were evaluated for antioxidant and antifilarial bioassays. Antifilarial activity of the methanol extract revealed a dose dependent positive response as evident from induction of death in the developmental stages of a metazoan filarial parasite *Setaria digitata*. The aqueous extract showed DPPH (2, 2- diphenyl-1-picrylhydrazyl) radical scavenging activity, reducing power and hydrogen peroxide scavenging activity, which increased with increase in concentration of the extract. The phenol content varies from 2.215±0.049 to 1.109±0.049 % dry weight (DW) and ascorbic acid ranges from 0.851± 0.001 to 0.054±0.0002 % DW in different extracts respectively. The findings of the present study revealed that *Excoecaria agallocha* possesses a promising antioxidant property and antifilarial activity. This indicates that *Excoecaria agallocha* can be a potential source of agents that can be used not only for meeting the oxidative stress generated during chronic manifestation of lymphatic filariasis in human beings but also for blocking embryogenesis in filarial parasites which in turn can potentially affect their transmission and survival in host communities.

Keywords: Mangrove plant, *Excoecaria agallocha*, plant extract, antioxidant activity and antifilarial activity.

INTRODUCTION

Herbal drugs from medicinal plants constitute a major part in all traditional system of medicines. World Health Organization (WHO) has emphasized on the development and utilization of medicinal plant resources in the developing countries so as to extend the health care to maximum number of population in these countries (Goud *et al.*, 2005). Plant based antifilarials have enormous therapeutic potential as they serve the purpose with lesser side effects that are often associated with synthetic antifilarials. Lymphatic filariasis (LF) is a chronic and debilitating tropical disease, associated with clinical manifestations like lymphoedema, elephantiasis and / or Hydrocele. It is caused by vector borne helminthic parasites (e.g. *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*). In the absence of any preventive vaccine and effective chemotherapy an estimated 120 million people world over are silently suffering from this infectious disease. Further, being the inhabitant of areas endemic to lymphatic filariasis, about 1.3 billion people in more than 80 countries are at risk of infection from this disease (WHO 2006). Beginning with the widespread use of DEC for the treatment of LF in China during the 1970s, the mass treatment of human population with anthelmintic drugs known as mass drug administration (MDA), has been a major approach for controlling LF in developing countries (Hotez *et al.*, 2007). However, the success of MDA programs is marred by several factors including lack of any effective adulticidal drugs for the parasitic worms (Hotez *et al.*, 2008), adverse side reactions associated with the drug of choice for human filariasis - Diethylcarbamazine (DEC) (Haarbrink *et al.*, 1999), possibility of emergence of drug resistant parasites coupled with the lack of robust biomarkers for detection of resistance of helminthic parasites to the mainstay drugs of MDA programs (Hotez *et al.*, 2008) and chances of rapid reinfection of hosts by these parasites, post-treatment (Hotez *et al.*, 2008, Loukas *et al.*, 2005) etc. Due to these reasons it seems quite imperative to find out novel antifilarial drugs preferably from plant sources.

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Many bioactive and pharmacologically active substances have been isolated so far from terrestrial plants however, little is known about the therapeutic potential of mangrove plants. Mangroves are found in all most all continents in tropical and sub tropical regions of the world. Bhitarkanika wildlife sanctuary, Orissa, India extending between 20° 30' - 20° 50'N and 86° 30'- 87°E harbors a total of 32 true mangrove species and 29 associated species. *Excoecaria agallocha* L. (Euphorbiaceae) is a typical mangrove species found widely distributed in different parts of Bhitarkanika mangrove forest. In addition to its commercial use as timber and fuel its therapeutic potential has already been demonstrated in case of epilepsy, ulcers, conjunctivitis, dermatitis, tooth ache, leprosy etc. (Bandaranayake, 2002; Kirtikar et al., 1999; Jayaweera, 1980). A novel phorbol ester, an anti -HIV principle has also been isolated from the leaves and stem of this unique plant (Karalai et al., 1994).

In the present study we have investigated the antifilarial effect of the crude extracts of leaf of *Excoecaria agallocha* L in terms of its cytotoxic effect on the developmental stages of the bovine filarial parasite *S. digitata*. We have also evaluated the antioxidant effects of this extract with a view to assess its potential in alleviating the biological damage in the host, caused due to oxidative stress generated during chronic manifestations of filarial infections (Pal et al., 2006).

**MATERIALS AND METHODS**

**Collection of sample**

Fresh young and tender Leaves of *Excoecaria agallocha* L. were collected randomly from a healthy plant at a height of 1-1.5 meter above the ground from the mangrove forests of Bhitarkanika wildlife sanctuary which extends from 20° 30'-20° 50' N and 86° 30'- 87° 6’ E. The specimen was identified at Department of Natural Products, Institute of Minerals and Materials Technology, Bhubaneswar, Orissa. India and a voucher specimen (No.-10, 002) was deposited in the herbarium of Institute of Minerals and Materials Technology.

**Preparation of plant extracts**

The leaves of the plant were shade dried for 15 days and then pulverized into fine powder using pestle and mortar. Twenty five grams of fine powder was added to a soxhlet apparatus along with a solvent (acetone/ ethanol/ methanol and aqueous) for extraction of chemicals. The liquid extracts were evaporated to dryness by vacuum distillation and stored at 4°C for further analysis (Mohanta et al., 2007). Percentage yield was calculated from the dry extract powder.

**Assays for antioxidant activity of the crude extracts of *E. agallocha* L.**

The antioxidant activity of the crude extracts of *E. agallocha* was evaluated using following assays such as Scavenging of DPPH radical, Evaluation of the reducing power, Scavenging of hydrogen peroxide, Estimation of Phenol content and Measurement of ascorbic acid content.

**Scavenging of DPPH radical**

The scavenging effect of crude aqueous extract of *E. agallocha* was determined by following standard methods (Hatano et al., 1988). Briefly, 2.0ml of 0.1 mM DPPH (2- 2' diphenyl-1-picrylhydrazyl) solution (in methanol) was added to the test tube containing 0.1 ml aliquote of aqueous plant extract and standard BHT (Butylated hydroxytoluene) (50-100µg/ ml). The mixture was vortexed for 1 minute and kept at room temperature for 30 minute in the dark. The absorbance of all the sample solutions was measured at 517nm. The percentage scavenging effect was calculated from the following equation.

\[
\text{% scavenging} = \frac{A_0 - A_1}{A_0} \times 100
\]

Where \( A_0 \) = Absorbance of control
\( A_1 \) = Absorbance of test sample

**Reducing Power**

The reducing power of aqueous plant extract was determined by the method of Oyaizu (Oyaizu, 1986). Different concentrations of plant extract (2.2, 4.4 and 6.6 mg/ml) in distilled water were mixed with potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50°C for 30 minutes. Aliquots of trichloroacetic acid (2.5ml, 10%) were added to the mixture, which was then centrifuged for 10 minutes at 2000 rpm. The upper layer of solution (2.5ml) was mixed with FeCl₃ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing power.

**Scavenging of hydrogen peroxide**

The ability of plant extract to scavenge hydrogen peroxide (H₂O₂) was determined according to standard method with minor modifications (Gulcin et al., 2003). Briefly, plant extracts and standard BHT (1.06, 2.05, 3.30 and 1.2 mg/ml respectively) in distilled water were added to hydrogen peroxide solution (2.9 ml, 20 m/M). Then the mixture was incubated at room temperature for 30 minutes and absorbance of hydrogen peroxide was measured at 240 nm. The percentage of scavenging was calculated from control value.
Total Phenol content

Total phenolic compounds were determined with Folin Ciocalteau’s Phenol reagent (FCP) according to the method of Slinkard and Singleton (1977) with minor modifications. About 10 µl of the plant extracts were taken and to it 2.0 ml of 2% sodium carbonate was added. It was mixed by vortexing vigorously upto 3 minute. Then about 0.1 ml of 50% of FCP was added to it and was kept for 30 minute at room temperature in dark. The absorbance of all the extracts was measured at 700 nm using spectrophotometer (SYSTRONICS-114). Phenolic content was expressed as guaicol equivalents % dry weight.

Ascorbic acid content

The ascorbic acid content was estimated following the methods of Swain & Tripathy (2003) with slight modifications. About 0.1 ml of the crude plant extracts was mixed with 2.0 ml of 2% ammonium molybdate, 2.0 ml of 5N sulphric acid and 2.0 ml of 1.5 X 10⁻⁴ M disodium hydrogen phosphate and boiled in boiling waterbath for 20 minute. It was cooled and the absorbance was measured at 660 nm using spectrophotometer (SYSTRONICS-114). The results were expressed in % dry weight.

Evaluation of Antifilarial activity of the plant extracts

Isolation of intrauterine developmental stages of filarial parasite S. digitata.

Adult female filarial worms Setaria digitata were collected from the peritoneum of cattle, slaughtered at a nearby abattoir in sterile Hank’s Balanced Salt Solution (HBSS) medium (Sigma H 2387-1L) and transported to the laboratory in a sterile container. The medium containing 1% glucose (Sigma G 7528), Penicillin 100 units/ml, Streptomycin 100µg/ml (Sigma P 4333), and Amphotericin - B 0.25µg/ml (Sigma A 2942) was buffered with NaHCO₃ (Sigma S 5761). About 5 to 7 worms were taken in a petridish, washed three times in medium, dissected into small pieces in 10 ml of medium under sterile conditions and incubated at 37°C for 30 minutes to allow the release of intra uterine developmental stages (eggs and microfilariae) into the medium. The in vitro released developmental stages were harvested into sterile 15 ml centrifuge tubes and washed three times by centrifuging at 300g for 10 minutes each with medium and the final pellet was suspended in 1ml of RPMI-1640 medium (Sigma R 8005) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma F 2442).

In vitro culture and treatment of the developmental stages of S. digitata

After counting the number of developmental stages of S. digitata in the above preparation under light microscope, the suspension was diluted with RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS) to get a final suspension of 1×10⁴ developmental stages /ml. From this suspension 1 ml each was dispensed into individual wells of a 24 well tissue culture plate. One set of wells was taken as untreated control where only medium containing 10% FBS was added. Other sets of wells were subjected to treatment with crude methanolic extracts of Exococaria agallocha L. at a concentration range of 10-100µg/ml for 24 hr at 37°C in a 5% CO₂ incubator. After the said incubation motility of the microfilariae was scored in each well, under an inverted microscope and then viability was scored under light microscope by trypan’s blue staining.

Evaluation of cytotoxicity of E. agallocha extracts on developmental stages of S. digitata

In the present study the cytotoxic effects of crude methanolic extracts of Exococaria agallocha L. on the intrauterine developmental stages of S.digitata in culture was scored in terms of the loss of motility of actively motile developmental stages (e.g. microfilariae-Mf) under microscopy, loss of membrane integrity by Trypan’s blue staining and fragmentation of chromosomal DNA by TUNEL staining using fluorescent microscopy.

Trypan’s blue dye is known to be excluded by viable cells while nonviable cells with lost membrane integrity, take up the dye and thus they appear blue in colour. Approximately 1x10⁶ developmental stages were suspended in 1ml of PBS. Then 0.1% (w/v) of Trypan’s blue and the suspension of developmental stages were loaded in to the hemocytometer in the ratio of 1:1 and the numbers of unstained (viable) and stained (dead) cells were counted under light microscope. Hundred developmental stages were counted and the number of Trypan’s blue positive developmental stages was taken as the percentage of death.

Fragmentation of chromosomal DNA was detected by TUNEL staining. Briefly, the treated developmental stages were harvested from culture, fixed with 1% para formaldehyde followed by washing with PBS and storage in 70% ethanol at -20°C. After 18 hrs of storage the developmental stages were subjected to TUNEL staining using the APO Direct kit supplied by BD biosciences (BD biosciences-556381) and analyzed by a fluorescent microscope (Leica).
Statistical analysis

Paired comparison were conducted using paired t – test, and all data are presented as mean value ± SD. Differences were considered significant at 95% confidence levels. All statistical analysis was performed with Graph Pad Prism, version 5.01 (Graph Pad Software).

RESULTS

Antioxidant properties

The percentage yield of the methanol extract is 5.05 % and the aqueous extract is 3.75 %. The antioxidant study of the aqueous plant extract of *Excoecaria agallocha* was evaluated using DPPH scavenging assay, H$_2$O$_2$ scavenging assay and reducing assay. DPPH radical scavenging activity (%) of the aqueous extract is shown in Fig. 1 [Supplementary data]. It shows significantly higher activity (82.66 %), which increases with increase in concentration. The scavenging capacity of aqueous extract of plant on hydrogen peroxide is shown in Fig. 2 [Supplementary data]. These results showed that the extract has H$_2$O$_2$ scavenging activity. The reducing capacity of aqueous extract of plant is shown in Fig. 3 [Supplementary data]. The result suggested that the extract has reducing power, which increased with increasing amount of concentration.

The phenol content in acetone, ethanol, methanol and aqueous extracts was found out to be 2.215±0.049, 1.625±0.006, 1.405±0.006, 1.109±0.049 % DW tissue respectively (Table 1 [Supplementary data]). The leaf extract of *E. agallocha* are good sources of ascorbic acids and the result is summarized in Table 1.

Cytotoxic effect of crude extracts of *Excoecaria agallocha* L. on the intrauterine developmental stages of *S. digitata*

Treatment with crude methanolic extract at concentrations ranging from10-100µg/ml resulted in the dose dependent inhibition of motility in Mfs (Fig. 4). Following cessation of motility of Mfs, when we scored the viability of developmental stages by live cell impermeant dye Trypan’s blue, about 30%, 75% and 90% of the developmental stages were found to be dead (Fig. 5 [Supplementary data]) after 24hr treatment with 10, 50 and 100µg/ml of concentration of crude methanolic extracts of *Excoecaria agallocha* L. in three independent experiments indicating the cytotoxic effect of the extract on the developmental stages of *S. digitata*. Further, when we subjected the developmental stages to TUNEL staining, most of the treated developmental stages exhibited TUNEL positivity under fluorescent microscope (Fig. 6) indicating that the death of the developmental stages involves fragmentation of chromosomal DNA.

![Figure 4: Dose dependent reduction in motility of mfs of S. digitata after 24 hours treatment with the crude extract (methanol) of Excoecaria agallocha.](image)

![Figure 6: Demonstration of fragmentation of chromosomal DNA in intrauterine developmental stages of S. digitata by TUNEL staining after 24 hr in vitro treatment with 50 µg/ml Excoecaria agallocha L. The fixed and permeabilised embryonic stages of S. digitata were subjected to TUNEL staining. A, B: Fluorescent image of Untreated control (B) and treated (A) developmental stages of S. digitata after TUNEL staining.](image)
Antioxidant and antifilarial activity of leaf extracts of Excoecaria agallocha L.

DISCUSSION

Antioxidant properties

Mangroves usually grow in estuarine swamps with high salinity, high temperature, low nutrition and high radiation. They survive in high environmental stress as they have unique properties to combat stress. Exposure to these stress situations results in production of reactive oxygen species (ROS) in these plants. In order to mitigate the adverse consequences of this stress mediated ROS generation, the mangrove plants are known to produce anti oxidant enzymes (Das et al., 2001) and various defense compounds including polyphenols like tannins (Naskar et al., 1995). Phenolics are traditionally considered as the defense compound that protects the plant from the herbivorous animals but the main role of these secondary metabolites in mangrove plants is to protect the leaves from photo damage by acting as antioxidants (Banerjee et al., 2008).

DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle et al., 1996) and is a useful reagent for investigating the free radical scavenging activities of compounds (Duan et al., 2006). Our results show high DPPH scavenging activity. Many researchers have reported positive correlation between free radical scavenging activity and total phenolic contents, which also matches with our findings. Hydrogen peroxide itself is not very reactive, but it can give highly reactive species OH~ through Fenton reaction (Halliwell, 1989). Earlier reports suggested that H2O2 could induce DNA break in the intact cell and purified DNA (Imlay et al., 1988). Thus, removal of H2O2 is crucial for medicinal importance. The reducing capacity of a compound may serve as indicator of its potential antioxidant capacity (Meir et al., 1995). The antioxidant capacity of compounds has been attributed to various factors such as prevention of chain reaction, chelating metals, reductive capacity and radical scavenging activity etc. (Diplock, 1997; Yildirim et al., 2001).

Phenolic compounds are commonly found in plants and have been reported to possess several biological activities including a strong antioxidant activity (Duh et al., 1999 Chandini et al., 2008). The antioxidant capacity of phenolic extracts is very often attributed to their radical scavenging ability mediated by hydroxyl groups (Hatano et al., 1980). It is suggested that polyphenolic compounds also have inhibitory effects on mutagenesis and carcinogenesis in human beings (Gulcin et al., 2003). In our studied plant part we found higher amount of phenolic compounds indicating its strong therapeutic potential as an anti oxidant.

Natural ascorbic acid is vital for the body performance (Okwu, 2004). Lack of ascorbic acid impairs the normal formation of intercellular substances throughout the body, including collagen, bone matrix and tooth dentine, a striking pathological change resulting from this defect is the weakening of the endothelial wall of the capillaries due to a reduction in the amount of intercellular substances. Therefore the clinical manifestations of scurvy hemorrhage from mucous membrane of the mouth and gastrointestinal tract, anemia, pains in the joints can be related to the association of ascorbic acid and normal connective tissue metabolism (Okwu, 2004; Hunt et al., 1980). The later function of ascorbic acid also accounts for its requirement for normal wound healing. The ascorbic acid is also known to possess a significant anti oxidant activity (Patra et al., 2001). In the present study the leaf extracts of Excoecaria agallocha L. was found to contain appreciable amount of ascorbic acid (Table 1) which further corroborates the plant’s therapeutic potential as an anti oxidant.

Cytotoxic effect of crude extracts of Excoecaria agallocha L on the intrauterine developmental stages of S. digitata

Among the intrauterine developmental stages the Mfs are the actively motile stages of the parasite. We considered motility as a visually recognized energy dependent process and changes in motility of Mfs as an indicator of alteration in their metabolism. (Das et al., 2001). Hence, when the motility of the Mfs stops completely upon treatment in culture it indicates that there is a severe compromise in the energy generating process, which will ultimately affect the viability of the parasites (Das et al., 2001; Mukherjee et al., 2002). Induction of death in the intra uterine developmental stages of filarial parasite S. digitata, in terms of reduction of motility of Mfs (Fig. 4) and loss of membrane integrity of both Mfs and eggs (Fig. 5) by the methanolic extracts of Excoecaria agallocha L was found to be statistically significant even at the lowest concentration of the extracts used (10µg/ml) in the current study, indicating its potential anti filarial activity. Findings of the present study further, revealed that the cytotoxic effects of crude extracts of Excoecaria agallocha L on the developmental stages of S. digitata involve fragmentation of chromosomal DNA. Although this fragmentation of chromosomal DNA is considered as an important event in cell death (Fink and Cookson, 2005), its detection in dead cells doesn’t specifically indicate the underlying mechanism of death (Fink and Cookson, 2005). This fact is supported by reports in literature saying degradation of chromosomal DNA is a terminal event that occurs during many forms of cell death including apoptosis, pyroptosis (Fink and Cookson, 2007) and programmed necrosis (Edinger and Thompson, 2004). Further, apoptotic death may occur without oligonucleosomal DNA fragmentation (Kroemer et al., 2009). Hence, further studies are
needed to confirm the precise mechanism of death of the developmental stages of \textit{S. digitata} observed in this study.

It is an established fact that chemical mediators elaborated during any infection by the inflammatory cells may generate oxidative stress (Kumar et al., 2004) in the hosts. Increase in oxidative stress and lipid peroxidation products in circulation during several pathological states has already been reported in literature in case of, atherosclerosis, diabetes, asthma, cancer, sepsis and autoimmune disorders (Kumar et al., 2004) and also during parasitic infections including malaria (Erel et al., 1997), leishmaniasis (Biswas et al., 1997), bacterial infections (Koedel and Pfister, 1999; Kwiatkowska et al., 1999) and helminth infections like Schistosomiasis (Gharib et al., 1999) lymphatic filariasis (Pal et al., 2006) etc. One of the major targets of this oxidative stress is the polyunsaturated fatty acid of membrane phospholipids. Their unstable reactive double bonds make them susceptible to oxidative attack that leads to a chain reaction of lipid peroxidation (Lee et al., 2004). Lipid peroxidation in turn is known to produce many reactive species including conjugated dienes, malondialdehyde (MDA), lipid hydroperoxides among others (Halliwell and Chirico 1993). When unmetabolized, the lipid hydroperoxides break down to yield long chain aldehydes, which in turn can react with proteins and nucleic acids to cause biological damage (Barret, 1991). However, compounds with antioxidant activity can significantly reduce this oxidative damage in the host. Hence, in the present study we have evaluated the antioxidant activity along with the antifilarial activity of the crude extracts of \textit{Excoecaria agallocha} \textit{L}.

The result of the present study revealed that \textit{Excoecaria agallocha} possesses a promising antioxidant property and antifilarial activity. This indicates that \textit{Excoecaria agallocha} can be a potential source of agents that can be used not only for meeting the oxidative stress generated in hosts during various chronic disease manifestations in man including lymphatic filariasis but also for blocking embryogenesis in filarial parasites which in turn can potentially affect their transmission and survival in host communities.

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