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Antioxidant and antiproloferative activity of methanolic and ethanolic extract of *Excoecarica Agallocha* leaves on human breast cancer cell lines MCF-7

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ABSTRACT

A comparative free radical scavenging and antiproliferative potential of methanolic and ethanolic extracts of leaves of *Excoecaria agallocha* (MEEA, EEEA) were investigated. The free radicals such as superoxide, nitric oxide and hydrogen peroxide scavenging activity were evaluated using standard protocols. Tryptan blue assay was performed to evaluate the antiproliferative activity. Quantitative estimation phenols and flavonoids were also done. The EEEA extract exhibited significant antiproliferative and antioxidant activity than the MEEA extract which was comparable to the positive drug doxorubicin and ascorbic acid used in the present study which may be due to the presence of phenols and flavanoids. The antioxidant and anticancer activity observed in the present study may be due to the presence of phytochemicals along with the phenols and flavanoids.

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INTRODUCTION

Cancer is the second leading cause of death worldwide after cardiovascular and infectious diseases (Sianipar *et al.*, 2018). The World Health Organization, have estimated that there may be 21.4 million cases of cancer and 13.2 million deaths from cancer annually by 2030 (Society, 2011). Genetic based molecular modification, which includes transformation, uncontrolled proliferation, deregulation of apoptosis invasion, metastasis and angiogenesis are the characteristic features of cancer (Fimognari *et al.*, 2011). During the biochemical reactions living cells continuously produces free radicals like superoxide, hydroxyl, hydrogen peroxide and nitric oxide radicals which are cumulatively called as Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) as the result of oxidative metabolic reactions which get scavenged with the help of endogenous antioxidant defence mechanisms. However, excess production of these radicals is implicated in the carcinogenesis, coronary heart disease and various other health problems (Kim *et al.*, 2010). These ROS and RNS can cause not only DNA damage but also enhance cellular growth and their survival and migration and becomes responsible for the tumour initiation by creating genetic variation as well as sustain subsequent tumour progression.

All over the world, a wide variety of phyto chemicals are being experimented for the potential drug for cancer chemotherapy as they have the ability to combat with the oxidative stress-induced damage, a primary pathology involved in cancer initiation, propagation and promotion.

So plant products play a significant role in cancer prevention and treatment (Muchtaridi and Wijaya, 2017). *Excoecaria agallocha* L is a small mangrove tree found abundant in Pichavaram coastal

mangrove forest, India (Satyavani *et al.*, 2015). *Excoecaria agallocha* L. belongs to *Euphorbiaceae* family is commonly called a "blinding tree" which can cause temporary blindness due to the milky sap present in the tree. The sap present in the plant causes skin blisters and irritation. Clinical trials conducted by , (Peter and Sivasothi, 1999) have reported that the plant possesses anticancer, antiviral antibacterial and anti-HIV properties. The present study was aimed to evaluate, the antioxidant and antiproliferative effect of the ethanolic (EEEA) and methanolic (MEEA) extracts of leaves of *Excoecaria agallocha* on human breast cancer MCF-7 cell lines.

MATERIALS AND METHODS

Chemicals

The chemicals such as nitro blue tetrazolium (NBT) phenazine methosulphate, nicotineamide adenine dinucleotide, Folin–Ciocalteu's reagent, Gallic acid, Catechin and Vitamin E were obtained from Hi media laboratories Pvt, Ltd. The other chemicals used in the study were of analytical grade.

Collection and preparation of methanolic and Ethanolic leaf extracts of *Excoecaria allogacha*

The mangrove plant *Excoecaria allogacha* leaves were collected from Pichavaram mangrove forest, Southeast coast of Tamil Nadu, India. Plant species identification was made in Herbaria of Centre of Advanced Study in Marine Biology, Annamalai University, Tamil Nadu, India. The collected leaves were washed in tap water and dried in the shade and powdered using a blender. The ethanolic extract and methanolic extract of leaves of *Excoecaria allogacha* (EEAE and MEAE)

Were prepared in the Soxhlet apparatus using 90% ethanol and methanol. Using the rotaflash evaporator, the excess solvent was removed from the filtered extract and yield was calculated. The yield was found to be 2.12%w/w for EEEA and 1.89% for MEEA. The extracts were in 4^0C until study.

Assay of Free radical scavenging activities of MEEA and EEEA

The free radical scavenging activities of MEEA and EEEA extracts were determined using well-established methods. The process of Robak and Gryglewski analyzed the superoxide scavenging activity of the plant extracts (Robak and Gryglewski, 1988). The purpose of (Marcocci *et al.*, 1994), was followed for estimating the scavenging potential of nitric oxide by the plant extracts. The H_2O_2 scavenging ability of the plant extracts was calculated by (Ruch *et al.*, 1989).

Determination of Total phenol and Flavonoids in MEEA and EEEA extract

The total phenols present in the MEEA and EEEA extract were estimated based on the method followed by (Slinkard and Singleton, 1977) and were expressed as gallic acid equivalents. The quantitative analysis of total Flavonoids present in these extracts was performed according to the method of (Zhishen *et al.*, 1999).

MCF-7 Human breast cancer cell line procurement and Maintenance

The MCF-7 (Human breast carcinoma) cell line was obtained from National Centre for Cell Sciences (NCCS), Pune, India and initially cultured in DMEM (Dulbecco's Modified Eagle Medium)and further subcultured in DMEM cell culture medium by providing the supplements such as L-glutamine, sodium bicarbonate,10% FBS with antibiotic solution containing Streptomycin (100μg/ml) and Penicillin (100U/ml) under appropriate cell culture conditions. Confluent monolayer cells of two days old were trypsinized and suspended in 10% growth medium. The antiproliferative activity of the EEEA and MEEA extracts were studied using cell suspension ($5x10^4$cells/well) seeded in 96 well tissue culture plates and incubated in 5% humidified CO_2 incubator for 24 hours.

Effect of EEEA and MEEA extracts on MCF-7 Human breast cancer cell viability

To the previously incubated, 96 well seeded MCF-7 cell lines, different concentrations EEEA and MEEA extracts were added and incubated further for 72 hrs in the same conditions as stated earlier to analyze the effect of these extracts on the viability of MCF-7 (human breast cancer cells)in the presence of the extracts. The Doxorubicin served as a positive control, and negative control was MCF-7 cells without any treatment. At the end of incubation, the treated cells were diluted with an equal volume of growth medium and stained with trypan blue (0.4 % in PBS) dye for 3 mints, and the number of viable and non-viable cells were counted under the microscope using heamocytometer (Haldar *et al.*, 2010). Percentage viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

Statistical Analysis

Statistical analysis was carried out using Statistica/Macsoftware (Prism, USA). All Experimental data were performed in triplicates and expressed as mean ± SEM, followed by ANOVA and Dunnet's "t" test. P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Assay of Free radical scavenging activities of MEEA and EEEA

Superoxide scavenging activity

The Superoxide scavenging activity of EEEA, MEEA and the positive control ascorbic acid are depicted in Table 1. In the present study, both the extracts showed the inhibitory activity of superoxide, the EEEA had a better scavenging potential than the MEEA. At higher concentrations of 800 and 1000 μgms/ml, the ethanolic extract of *Excoecaria allogacha* showed inhibitory activity which was compared to the positive control ascorbic acid

Nitric oxide scavenging activity

Figure 1 shows the nitric oxide scavenging activity of the EEEA, MEEA and the positive control Ascorbic acid. All the three extracts scavenged the nitric acid in the concentration

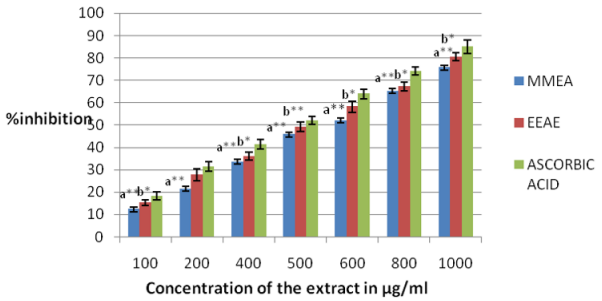

Figure 1: Nitric oxide scavenging activity of different plant extracts Excoecaria allogacha

Dependent manner. However the ethanolic extract of *Excoecaria allogacha* showed an enhanced scavenging activity when comparable to the methanolic extract.

Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide Scavenging activity of the MECP, EECP and the positive control ascorbic acid was shown in the Table 2. The EEEA showed the higher inhibitory activity of

Hydrogen peroxide radicals than the MEEA. Though the percentage hydrogen peroxide inhibitory activity was found to be low when compared to superoxide and nitric oxide scavenging activities, starting from 100 μgms the extract exhibited radical scavenging activity which was almost similar with the positive control ascorbic acid used in the present study.

Total Phenolic and Flavonoid contents

Table 3 shows the total phenolic and flavonoid contents of EEEA and MEEA extracts, which were determined and expressed in terms of gallic acid and catechin equivalents. The antiproliferative and antioxidant activity of these plant extract may be probably due to these secondary metabolites.

Cell viability assay

The trypan blue assay is performed to determine the antiproliferative activity of the *Excoecaria allogacha* extracts and was depicted in the Figure 2. The plant extract treatment significantly.

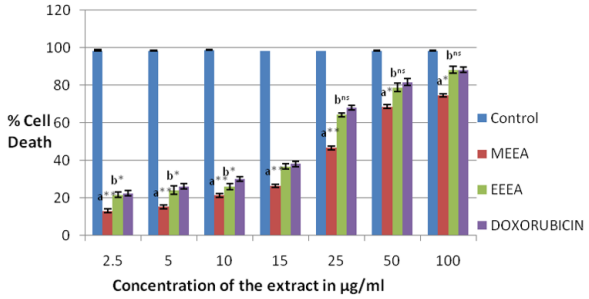

Figure 2: Trypan blue assay of different plant extracts Excoecaria allogacha

increased the death of MCF-7 cells when compared to untreated control cells. As observed in other assays, the ethanolic extract of *Excoecaria allogacha* leaves exhibited increased anticancer activity than the methanolic extract of *Excoecaria allogacha* leaves in terms of percentage cell death. At concentrations starting from 15μgms/ml, the anticancer activity was almost similar to that of doxorubicin the positive control used in the present study.

Free radicals or reactive oxygen species (ROS) are produced endogenously from various biochemical reactions which can trigger lipid peroxidation process involved in the pathogenesis of cancer, cardiovascular disease, diabetes and ageing (Halliwell *et al.*, 1992; Remacle *et al.*, 1995; Bhatt *et al.*, 2013). These Reactive oxygen species (ROS) includes O_2^-, H_2O_2 and hydroxyl radicals readily oxidizes the various biomolecular structures such as carbohydrates, proteins, fatty acids and DNA leading to cellular degeneration and their death. Great attention is now being provided to medicinal plants due to their easy availability and cost-effectiveness. They possess a wide variety of biomolecules with antioxidant potential, which can act individually or synergistically to combat the oxidative stress-related health issues (Konishi *et al.*, 2000).

In the present investigation, the free radical scavenging potential of the *Excoecaria agallocha* methanolic and ethanolic extracts were studied. The antiproliferative activity of these plant extracts was also evaluated in the human breast cancer MCF-7 cell lines. The ethanolic extract was more

Table 1: Superoxide scavenging activity of different plant extracts Excoecaria allogacha

Concentration in μgms / ml	Inhibitory activity of MEEA (%)	Inhibitory activity of EEEA (%)	Inhibitory activity of Ascorbic acid (%)
100	12.31±2.16a*	16.20 ± 3.72b^{ns}	18.68±4.31
200	23.43±1.38a*	25.51 ± 2.43b*	28.20±2.30
400	33.14±4.41a*	35.15 ± 3.69b*	39.10±2.71
500	42.12±3.16a*	46.10 ± 3.00b*	51.14±3.15
600	55.62±1.69a**	64.18 ± 2.10b^{ns}	65.30±4.95
800	61.20±3.17a**	80.23 ± 4.64b^{ns}	81.80±1.72
1000	72.21±2.47a**	89.12 ± 3.41b^{ns}	89.40±4.84

Superoxide scavenging activity of MEEA, EEEA and Ascorbic acid. Each value represents the mean ± SEM (n = 3). Comparison between a- MEEA vs Ascorbic acid and b-EEEA vs Ascorbic acid .*p<0.05, * *p<0.01, NS–Not Significant

Table 2: Hydrogen peroxide scavenging activity of different plant extracts Excoecaria allogacha

Concentration (μg/ml)	Inhibitory activity of MECP (%)	Inhibitory activity of EECP (%)	Inhibitory activity of Ascorbic acid (%)
100	14.10±2.74a**	19.42±2.70 b^{ns}	20.30±2.42
200	19.82±3.18a**	23.95 ± 2.83 b^{ns}	24.34 ± 1.79
400	22.78 ±1.91a**	26.98 ± 2.73 b^{ns}	27.12 ± 1.53
500	26.26 ±2.62a**	34.70 ± 2.19 b^{ns}	35.96 ± 1.50
600	35.57 ±3.26a**	51.66 ± 2.27 b^{ns}	52.92 ± 1.98
800	52.79 ± 2.53a**	69.23 ± 4.00 b*	71.75 ± 4.56
1000	72.73 ± 4.27a**	77.75±3.33 b *	82.15±4.23

Hydrogenperoxide scavenging activity of MEEA, EEEA and Ascorbic acid. Each value represents the mean ± SEM (n = 3). Comparison between a- MEE Avs Ascorbic acid and b-EEEA vs Ascorbic acid. *p<0.05, **p<0.01, NS–Not Significant

Table 3: Total Phenolic and Flavonoid contents of MEEA and EEEA Extracts

Extracts	Total phenolic content (mg/g)	Total flavonoid content (mg/g)
EEEA	29.76±2.50	36.17±1.30
MEEA	23.12±2.23	22.56±2.76

Values are expressed in mean ± SEM (n = 3)Total phenolic content was expressed as mg gallic acid. Equivalents/g dried extract. Total flavonoid content was expressed as mg catechin equivalent/g dried extract

effective in scavenging these radicals than the methanolic extracts. The Superoxide is a dangerous molecule since it acts as a precursor for other free radicals such as hydroxyl and hydrogen peroxide which is a reduced form of oxygen by one electron. All together, these radicals have the ability to initiate the lipid peroxidation process leading to tissue injury. The superoxide radicals were considerably scavenged by the extracts of *Excoecaria agallocha* in our present study. Several researchers have reported the presence of monoterpenoids, diterpenoids, triterpenoids, tannins, flavonoids, alkaloids and steroids in the leaves of the plant (Wang *et al.*, 2012; Parihar and Hemnani, 2003; Halliwell *et al.*, 1991; Zou *et al.*, 2006). These compounds may act either individually or in a cumulative manner might be responsible for the scavenging effect of the superoxide observed in the present study.

The nitric oxides, as well as reactive nitrogen species, are produced in some metabolic reactions along with reactive oxygen species and as stated by Hemnani and Parihar [He], these nitrogen species damage the DNA, create oligo nuleosomal fragments, by nitration of tyrosine residues destroy proteins and causes lipid peroxidation. In the present investigation, significant scavenging of the nitric oxide was observed by both the plant extracts. The quantitative estimation of phytochemicals also revealed the presence of considerable amounts of phenols and flavonoids. It is an accepted fact that most of our Indian plants contain significant quantities of secondary metabolites

in particular phenols and flavonoids which contribute sufficiently towards the antioxidant activity. The extent of nitric oxide scavenging activity is maybe due to these phenols and flavonoids. (Zou *et al.*, 2006), have reported the presence of Excogallochaols A-D, Sesquiterpenes, Secoatisane diterpenoids, Agallochaols G-J and Phorbol ester in the leaves of *Excoecaria agallocha*.

The removal of hydrogen peroxide is considered as an essential antioxidant system since in biological systems, in the presence O_3 and transition metal ions it initiates the Fenton reaction and becomes an oxidizing agent (Mahakunakorn *et al.*, 2004). This H_2O_2 induces DNA damage, alter the intracellular homeostasis of calcium ions, thereby increasing intracellular ATP permeability and affects the cells. The plant extract scavenged the nitric oxide radicals considerably indicating the protective nature.

The antiproliferative nature of the *Excoecaria agallocha* extracts was assessed by trypan blue assay in the MCF-7 cell lines. The plant extracts significantly causes the death of the MCF-7 cells, which was comparable with the positive drug doxorubicin**.** (Satyavani *et al.*, 2015), have reported the presence of phytoconstituents such as dodecanediol, L-alanine-4-nitroanilide, benzene methanol, 1,1-diethoxyundecane, hexadecane, Metaraminol, 1,2-benzenediol, tetradecane, hexadecane, benzyl alcohol, benzenemethanol, 4-trifluoroacet benzyl alcohol, L-alanine -4-nitroanilide, alanine, 2,6-Octadiene-4, undecane, Pentanoic acid, hydroxybenzenepropanoic acid, diethyl methylphosphonate, acridine, trifluroacetic acid, triethyl (pentafluorophenyl)silane, Ngainone, N-1-Adanantyl-p-methylbenzalimine, pentachlorophenol, Isohumulone, Octadecanoic acid, decane, diethylphthalate, benzamide, pentanenitrile, diacetate, clivorine and 1,2,5-trimethylphyyole in the petroleum and hexane extracts of leaves of *Excoecaria agallocha* . The presence of these compounds was expected in the ethanolic extract also were responsible for the antioxidant and antiproliferative activity observed in the present study.

CONCLUSION

It can be concluded that owing to the presence of significant phytoconstituents especially diterpenes, triterpenoids and diterpenoids. The *Excoecaria agallocha* may be developed into a potential drug for treating the breast cancer as well as oxidative stress-related diseases. However, proper preclinical and clinical investigations have to be conducted to prove the cytotoxic nature of this drug. Added to that it can also be given in the form of adjuvant therapy in cancer chemotherapy to reduce the oxidative stress-related conditions this occurs in cancer chemotherapy.

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