Ceylon Journal of Science (*Bio. Sci.*) 40 (2): 147-155, 2011

Anti Reverse Transcriptase and Anticancer activity of stem ethanol extracts of *Excoecaria agallocha* (Euphorbiaceae)

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Accepted 05 October 2011

ABSTRACT

Excoecaria agallocha L. (Euphorbiaceae) is a mangrove plant widely used in folklore medicine for the treatment of several diseases. In the present study anti HIV and anticancer properties of active fraction of stem ethanol extracts of the plant were investigated. The fraction showed significant anti-reverse transcriptase activity in the enzyme-based direct binding assay which was as good as that of the standard synthetic inhibitor. Anticancer activity of the same fraction was determined using MTS in vitro assay. It showed potent cytotoxicity against pancreatic cancer cell lines Capan-1 and Miapaca-2 with IC_{50} values of 4 µg/ml and 7 µg/ml respectively. These results clearly indicate strong anti HIV and anticancer properties in stem extracts of *E. agallocha*.

Key words: mangrove, pancreatic cancer, activity guided fractionation, anti HIV properties

INTRODUCTION

Natural products, especially form plants, have been used for the treatment of various diseases for thousands of years. Countries such as Egypt, China, India and Greece have practiced use of plants as medicines from ancient times and an impressive number of modern drugs have been developed from them (Shoeb, 2006). Over the past decade, substantial progress has been made in defining strategies for the safe and effective treatment of human immunodeficiency virus (HIV) infection, the cause of acquired immunodeficiency syndrome (AIDS) (Pengsuparp *et al*., 1995). Because reverse transcriptase (RT) is required for early proviral DNA synthesis, inhibition of the RT-catalyzed polymerization of DNA from viral RNA inhibits virus replication. The only drugs approved for use in HIV-1 infection to date are azidothymidine (AZT), didanosine (dideoxyinosine or ddI), and zalcitabine (dideoxycytidine or ddC) (Pengsuparp *et al*., 1994). Although these compounds have been shown to benefit HIV infected individuals, there are toxic side effects associated with their use, and complete inhibition of viral replication is rarely achieved. Also, continuous therapy with these drugs leads to drug-resistant strains of the virus (Mekkawy *et al*., 2002). Natural products serve as one source of structurally novel chemicals and are expected to be fruitful for investigation as specific inhibitors of HIV RT. There are numerous reports available on isolation and characterization of bioactive compounds from plants having reverse transcriptase inhibiting activity (Pengsuparp *et al*., 1996; Sun *et al*., 1996; Lin *et al*., 1997).

Pancreatic cancer is considered to be one of the leading causes of cancer related deaths in developed countries and it is on the rise in developing countries like India. The disease is notoriously difficult to diagnose in its early stages. Conventional treatment using chemotherapy and radiation has only little effect in pancreatic cancer and the only potentially curative treatment is surgery (Michaud, 2004). Thus, there is an urgent need to develop new and effective strategies for the prevention and treatment for this form of cancer.

Plant derived compounds have also played an important role in the development of several clinically useful anticancer agents. These include vinblastine, vincristine, campothecin derivatives-topotecan and irinotecan, etoposide and paclitaxel (Cragg and Newman, 2005; Cochrane *et al*., 2008). The anticancer properties of these are related to the regulation of cancer-related

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gene expression, induction of apoptosis, cell cycle arrest and/or DNA fragmentation and inhibition of different cellular enzymes (Upur *et al*., 2008). Hence, screening and isolation of active components from the herbs possessing anticancer potential appears to be a promising way of discovering novel therapeutic compounds.

Mangroves are salt tolerant forest ecosystems found mainly in tropical and sub-tropical intertidal regions. These rich ecosystems provide a wide range of ecological and economic products and services, and also support a variety of other coastal and marine ecosystems. Today mangroves are found in about 30 countries in tropical subtropical regions covering an area of about 99,300 km^2 (Singh, 2000). Asia has the largest extent of mangroves (5.8 million hectares) accounting for some 38 % of the global mangrove area. Five of the ten countries with the largest extent of mangroves worldwide are found in this region (FAO, 2007).

Excoecaria agallocha L. (Euphorbiaceae) is a small mangrove tree found extensively on seashores and edge-mangroves throughout tropical Africa, Asia, and northwest Australia. The plant has been used in traditional medicine against rheumatism, leprosy, epilepsy, paralysis, conjunctivitis and dermatitis (Jayaweera, 1980; UNESCO, 1981; Prakash *et al*., 1983; Wiriyachitra *et al*., 1985; Bandarnayake, 1998; 2002; Ghani, 2003) and for as a dart and fish poison (Ohigashi *et al*., 1974). The plant is reported to have anti HIV (Erickson *et al*., 1995), antinoceptive (Subhan *et al*., 2008a), anti microbial (Subhan *et al*., 2008b; Vadlapudi *et al*., 2009; Ravikumar *et al*., 2010), anti oxidant (Subhan *et al*., 2008c), anti-ulcer (Thirunavukkarasu *et al*., 2009) and anti filarial properties (Patra *et al*., 2009). Clinical trials carried out on this plant suggest that the plant possesses anti-bacterial, anti-HIV, anti-cancer and anti-viral properties (Morgany *et al*., 1999; Subhan *et al*., 2008a). Hence, the present study was carried out for the assessment of its anti reverse transcriptase and anti-cancer activity and comparing the same with standard drugs.

MATERIALS AND METHODS

Preparation of plant extract

Stems of *E. agallocha* were collected near Ratnagiri (Latitude 17.072/ longitude 73.668) coast from the state of Maharashtra, India. Care was taken to select only healthy, fresh, growing stem parts of about 10-15 mm diameter. Very young, delicate twigs and very hard and old stem parts were avoided. The plant material was identified and authenticated by Dr. B.L. Jadhav of the Dept. of Life Sciences University of Mumbai, an expert taxonomist of mangroves. The collected plant material was brought to the laboratory, washed thoroughly under running tap water in order to remove dirt, and other contaminants from the sample. Stems of *E. agallocha* were then oven dried at 40°C to reduce the moisture content, ground thoroughly in to a powder with the help of mortar and pestle. The powder was carefully sieved through muslin cloth. The stem powder (10 g) was used to prepare hot extracts of *E. agallocha* using soxhlet extraction method with ethanol (200 ml) as solvent. The extract was evaporated on a rotary evaporator to remove the solvent completely and reduce the volume of 20 ml (50% w/v). The resultant dark reddish green gummy mass was then stored in airtight bottles and kept at 4°C till further use.

Fractionation

Stem ethanol extract of *E. agallocha* that showed significant antioxidant and antibacterial activity previously (Subhan *et al*., 2008b; Subhan *et al*., 2008c) was subjected to bioactivity guided fractionation study. Fig.1 gives the flow chart employed for fractionation of stem ethanol extract. Soxhlet ethanol stem extract was evaporated to dryness in a Rotary evaporator and the residues collected were serially dissolved in different solvents ranging from non-polar to polar (petroleum ether, chloroform, benzene, ethyl acetate, acetone, ethanol and methanol) with centrifugation at 3000 rpm and filtration at intervals. The selection of these solvents was according to the Eluotropic series suggested by Trappe (Kirchner, 1978). The fractions obtained were designated as fraction ‘a’ to fraction ‘g’ from non-polar to polar respectively. All the fractions were checked for possible antimicrobial activity on the test organisms and only fractions exhibiting potent bioactivity was subjected to anti reverse transcriptase activity and anticancer activity assays.

Test microorganisms and media

Seven standard human pathogens comprising five bacteria and two fungi were procured from the National Chemical laboratory (NCL), Pune, India. The organisms were maintained on slants of their respective media as indicated in Table 1 and stored at 4°C with periodic sub-culturing.

Bioassay of fractions of *E. agallocha* (*In vitro* antimicrobial assay)

All the fractions of *E. agallocha* stem extract were evaluated for the bioassay test against the test organisms by Agar cup method (Spooner and Sykes, 1972). To 20ml of the sterile nutrient medium, 0.6 ml of test culture was added and poured into the plate, slowly shaken to mix the culture and medium thoroughly and were kept on a plane surface. After solidification four wells were made with the help of a flame sterilized stainless steel cork borer (8 mm). In to one of the wells, 80 µl of solvent was added as negative control while a similar quantity of standard antibiotic Ampicillin and Kanamycin were added as positive control at a concentration of 10mg/ml. Into the other two wells, 80 µl fractions of stem extract were loaded using a micropipette and kept for diffusion at 4°C for 20 min, and the plates were incubated at required temperature for 24 h.

Anti reverse transcriptase activity assay

Ethanol fraction of stem extracts was obtained through activity-guided fractionation and was tested for possible inhibition of HIV reverse transcriptase. The assays were carried out at Haffkine Research Institute, Parel, Mumbai according to the method of Pengsuparp *et al.*, (1994). The median inhibitory concentration was calculated from a linearly regressed dose response plot of percent control activity vs. concentration of compound, utilizing at least five concentrations of the test compound. Results were compared with a standard drug *i.e.* AZT.

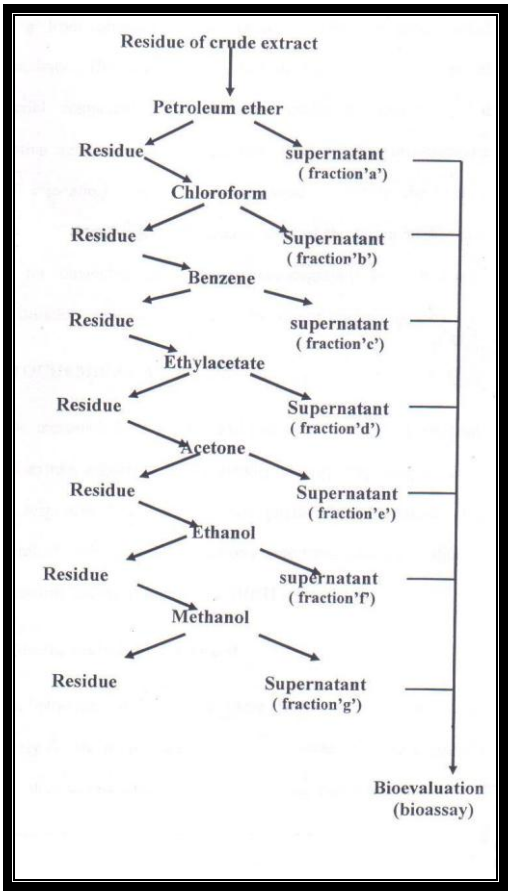

Figure 1. Flow chart for fractionation of stem ethanol extract of *E. agallocha*.

Table 1. Microorganisms selected for the study and media used.

Sr. No.	Microorganism	Type	Source	Media	Incubation temperature
1	*Aspergillus fischeri*	Fungi	NCIM 517	Potato Dextrose agar	RT
2	*Candia albicans*	Fungi	NCIM 3471	Potato Dextrose agar	RT
3	*Escherichia coli*	Gram - ve Bacteria	NCIM 2576	Nutrient agar	37° C
4	*Klebsiella pneumoniae*	Gram - ve Bacteria	NCIM 5082	Nutrient agar	37° C
5	*Staphylococcus aureus*	Gram + ve Bacteria	NCIM 5021	Nutrient agar	37° C
6	*Bacillus cereus*	Gram + ve bacteria	NCIM 2322	Nutrient agar	RT
7	*Salmonella typhimurium*	Gram – ve Bacteria	NCIM 2501	Nutrient agar	37° C

NCIM: National Collection of Industrial Microorgansims, NCL, Pune, India. RT- Room Temperature

Anticancer activity on pancreatic cancer cell lines

Cell lines and culture maintenance conditions

The human pancreatic cancer cell lines Miapaca-2, PANC-1, Capan-1 and BxPC-3 were purchased from the American Type Culture Collection (MD, USA). All the chemicals and media were purchased from Sigma, USA except for FBS which was from Hyclone, USA. Miapaca-2 and BxPC-3 were grown in RPMI-1640 culture medium, Panc-1 in Dulbecco's Modified Eagle's Medium, Capan-1 in Iscove's Modified Dulbecco's Medium. Each medium was supplemented with 10% heat inactivated FBS, 2% L-glutamine (200 mM) and 1% penicillin-streptomycin. Cells were grown as a monolayer culture at 37°C in a humidified 5% CO_2 incubator.

***In vitro* cytotoxicity assay (MTS assay)**

Cells were seeded at a density of 3×10^4 cells per well in 96-well plates. The following day, 20µl of culture medium (negative control) or medium containing the test compound was added to the wells. Each concentration was plated in triplicate. 48 h of drug exposure, the effect on the cell viability was measured using MTS cell proliferation assay (Cory *et al*., 1991). 20 µl of non- radioactive MTS reagent (Promega, USA) was added to each well. Cells were incubated further to allow for colour development before the absorbance values were read at 490 nm using a microplate reader. The percentage cytotoxicity based on control treatment (untreated- negative control) was calculated and plotted against fraction concentrations. The concentration of *E. agallocha* fraction inducing 50% inhibition of cancer cells (IC_{50} values) was calculated from the cytotoxicity curves.

Chemical characterization of bioactive fraction

Fourier Transform Infra Red Spectroscopy (FT-IR)

Bioactive fraction was subjected to crystallization and these crystals were analysed to detect the functional groups by IR spectroscopy. The analysis was carried out at IIT, Mumbai using Fourier Transform Infra Red Spectroscopy (Perkin Elmer Spectrum One spectrometer). A known amount of powdered sample (1-2%) was mixed with a weighed amount of powdered KBr and the mixture was subjected to a pressure of several tones in die, to produce a highly transparent disc, which was inserted into the spectrophotometer. Scan range was 450-4000 cm^{-1}.

High Performance Thin layer Chromatography (HPTLC)

Bioactive fraction was also screened for the presence of phytoconstituents by HPTLC to detect the principle class of secondary metabolite attributed to the activity. 20 µl of active fraction was loaded in duplicates on a ready made fluorescent pre-coated silica gel-G aluminum (supplied by MERCK) HPTLC plates and developed using appropriate solvent systems as described by Wagner and Bladt (1996). The analysis was done on Linomat-5 supplied by CAMAG in the Anchrom, R & D laboratory, Mulund, Mumbai. The resultant chromatograms were illuminated at UV-254 or UV-366 nm or 560/580 nm for the characteristic quenching or fluorescence respectively for the particular class of bioactive compounds. The plates were derivatized and heated if necessary on a HPTLC heater for the detection of the compounds.

RESULTS

Fraction ‘f’, *i.e.*, the ethanol fraction of crude stem ethanol extract of *E. agallocha* showed most potent antibacterial activity (Table 2) in the bioassay and hence was tested for its anti reverse transcriptase and anticancer activity. This fraction showed 33.7 % inhibition in the anti reverse transcriptase assay which was as good as that of the standard synthetic inhibitor AZT (35.5%) (Table 3).

The effects of this fraction of *E. agallocha* on the growth of various human pancreatic cell lines were tested under in vitro conditions using different concentrations (1, 3, 10, 30 and 100 µg/ml) and cell survival after 48 h of treatment is given in Table 4. The fraction offered a high degree of inhibition over the growth of two cell lines- Capan-1 and Miapaca-2, which was significantly more than that on the other two cell lines- PANC-1, and BxPC-3 (Figure 2). The IC_{50} values were calculated to be 4 µg/ml and 7 µg/ml for Capan-1 and Miapaca-2 respectively (Table 5). The inhibition was found to be dose dependent with greater inhibition at the highest concentration (100µg/ml). The cytotoxic activity was compared with the effect of flavopiridol (positive control), which showed significant cytotoxic activity on all the four cell lines with IC_{50} values in the range of 0.03 to 0.11 µg/ml.

Table 2. Activity guided fractionation of soxhlet stem ethanol extract of *E. agallocha*.

Organism	**Pet. Ether Fract. 'a'**	**Chloroform Fract. 'b'**	**Benzene Fract. 'c'**	**Acetone Fract. 'd'**	**Ethyl acetate Fract. 'e'**	**Ethanol Fract. 'f'**	**Methanol Fract. 'g'**
E. coli	-	-	-	+	++	+++	++
B. cereus	-	-	-	++	++	+++	++
Candida albicans	-	-	-	++	++	+++	++
Aspergillus fischeri				+	+	++	+
Klebsiella pneumoniae	-	-	-	+	+	++	++
S. aureus	-	-	-	+	+	++	++
Salmonella typhimurium	-	-	-	+	+	++	++

\+ Mild activity, ++ Moderate activity, +++ Strong activity, - No activity

Table 3. Anti-reverse transcriptase activity of ethanol fraction of stem ethanol extract of *E. agallocha*.

Sample	**% Reverse transcriptase (RT) inhibition**
Bioactive fraction Stem ethanol extract of *E. agallocha*	33.7
AZT drug (8 µg/ml) (positive control)	35.5
Ethanol (negative control)	NIL

Table 4. Cytotoxic activity of ethanol fraction of stem ethanol extracts of different Concentrations of *E. agallocha* on human pancreatic cancer cell lines after 48 h of treatment.

Conc. of extract (µg/ml)	**% growth inhibition**			
	Cell line			
	Capan- 1	**Miapaca- 2**	**PANC- 1**	**BxPC-3**
1	0	8	0	0
3	0	19	0	0
10	58	59	0	0
30	73	73	0	0
100	81	79	35	36

Table 5. Mean IC_{50} values of ethanol fraction of stem ethanol extract of *E. agallocha* in human pancreatic cancer cell lines.

Cell line	**Mean IC_{50} (µg/ml)**	
	***E. agallocha* ethanol extract**	**Flavopiridol**
Capan-1	4	0.08
Miapaca	7	0.04
PANC-1	> 100	0.03
BxPC-3	> 100	0.11

(Significant dose effects were observed in two cancer cell lines Capan-1 and Miapaca-2 compared to PANC-1 and BxPC-3, ($P < 0.005$) with a lower cell viability observed at the highest treatment concentrations)

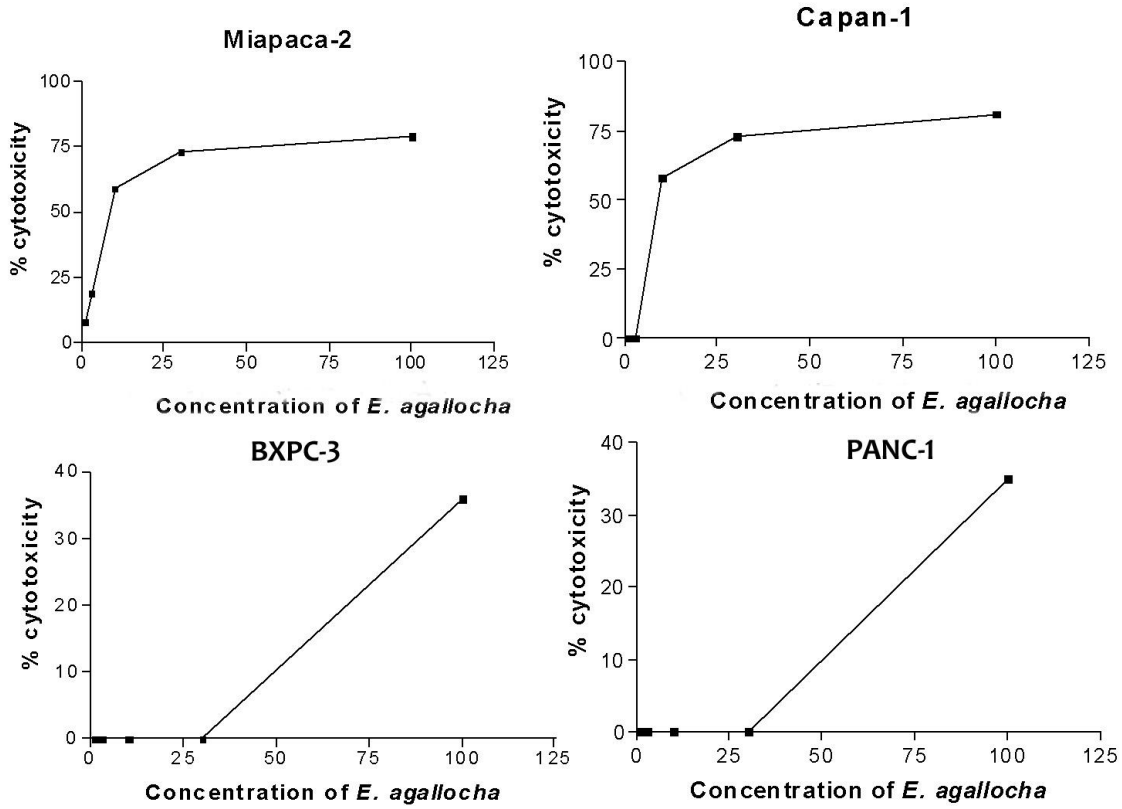

Figure 2. Growth inhibitory effect of ethanol fraction of stem ethanol extract of *E. agallocha* on human pancreatic cancer cell lines.

Infra red spectrum of bioactive fraction analyzed for detecting functional groups showed the presence of a peak at 3331.59 cm^{-1} indicating presence of bonded -OH (hydroxyl) group containing compounds in it. The spectrum also showed the presence of two humps at 2927.7 cm^{-1} and 2098.45 cm^{-1} indicating - CH Stretching. It further showed a hump 1618.01 cm^{-1} indicating compounds with C=C Stretching. The spectrum then showed two humps at 1515.22 cm^{-1} and 1404.89 cm^{-1} indicating Methyl C – H Stretching. Infra red spectrum of methanol fraction A finally showed series of humps between 1240.15 cm^{-1} to 639.50 cm^{-1} indicating compounds with C – C Stretching.

Bioactive stem fraction of *E. agallocha* was analyzed for the presence of all the ten major classes of bioactive compounds viz. Alkaloids, anthraglycosides, arbutin, bitter drugs, cardiac glycosides, coumarins, essential oils, flavonoids, saponins and valeportraites. The fraction showed presence of seven peaks in case of cardiac glycosides and three peaks in case of saponins. Any other class of phytoconstituents could not be detected in the bioactive fraction.

DISCUSSION

Mangrove designates an intertidal wetland ecosystem which proliferates luxuriantly in the intertidal area of low lying coasts and river estuaries, deltas, backwaters and lagoons throughout the low lying tropical and sub-tropical latitudes. Mangrove plants can produce metabolites and toxins that are unique to these plants and there have been certain examples in the recent years, which suggest that they may be a source of novel compounds. These are used clinically to develop a variety of drugs for the treatment of diseases.

Current therapy for human immunodeficiency virus (HIV) infection relies primarily on the administration of anti-retroviral nucleoside analogues, either alone or in

combination with HIV-protease inhibitors. Although nucleoside analogues, such as AZT have been approved for clinical use in HIV-1 infection, there are substantial toxic side effects associated with their use, and complete inhibition of viral replication is not achieved. Recently, significant progress has been made towards the development of natural and synthetic agents that can directly inhibit HIV replication or its essential enzymes. Natural product RT inhibitors, such as benzophenanthridine (Ono *et al*., 1988) and protoberberine (Sethi, 1979), alkaloids, flavonoids (Sethi, 1983; Spedding *et al*., 1989), a variety of other compounds with phenolic hydroxy groups (Ono *et al*., 1990; Kakiuchi *et al*., 1985) and certain antibiotics (Nakane *et al*., 1991), were found to inhibit HIV-1 RT (as well as HIV-2 RT) with similar potency. Previous studies by Premanathan *et al*., (1996) showed that mangrove species such as *Rhizophora mucronata, Rhizophora apiculata, Ceriops decandra, Rhizophora apiculata, Rhizophora lamarckii* exhibit in vitro anti HIV activity. In this study, we revealed the potent anti HIV activity of the active fraction of stem ethanol extract of *E. agallocha*, which showed significant anti reverse transcriptase activity as potent as the standard approved drug AZT.

Several reports are available on the anticancer activity of compounds isolated from different mangrove species. Mangroves species such as *Acanthus illicifolius, Bruguiera sexangula, Morinda citrifolia, Terminalia catappa, Ecteinacidia turbindate* have been shown to produce compounds that show strong activity against a variety of carcinomas, melanomas and lymphomas (Bandarnayake, 2002). Extracts of *B. sexangula* bark were active against two tumors, Sarcoma 180 and Lewis Lung Carcinoma (Loder and Russell, 1969). Jongsuvat *et al.,* (1981) also found that the extracts of *A. illicifolius* were not toxic to experimental mice but displayed significant anti leukemic activity. However, anticancer activity of *E. agallocha* has not been explored sufficiently. Subhan *et al.,* (2008a, 2008c) had indicated that ethanolic stem extract of *E. agallocha* has potent antioxidant and antibacterial activity. In the present study stem ethanol extract was subjected to activity-guided fractionation and its bioactive fraction (ethanol fraction) was tested for its anticancer and anti reverse transcriptase activity. The greatest cytotoxic effect of this fraction was observed on two out of four pancreatic cancer cell lines-Capan-1 and Miapaca-2. The cytotoxic activity increased in a dose-dependent manner after 48 h of exposure. The IC_{50} values calculated by plotting cytotoxicity curves were 4 µg/ml and 7 µg/ml respectively which were < 20 µg/ml and hence the extract can be considered as 'active' according to National Cancer Institute's guidelines (Boyd, 1997).

In conclusion, activity guided ethanol fraction of stem ethanol extract of *E. agallocha* has significant anti reverse transcriptase activity. It also exhibits concentration-dependent anticancer activity on human pancreatic cancer cell lines. HPTLC fingerprinting indicated presence of only couple of phytochemical groups *viz*. cardiac glycosides and saponins in the bioactive fraction indicating that these groups must be responsible for attributing the activity. Further molecular studies are underway to elucidate the mechanism(s) of action of these extracts on cancer cells. Thus, the stem extracts of *E. agallocha* has the potential to be developed as an anti HIV and anticancer drug.

ACKNOWLEDGEMENTS

Authors are indebted to Dr. U. A. Jadhav of University Department of Life Sciences for her encouragement and authorities at Haffkine Research Institute, Indian Institute of Technology (IIT) and Anchrom R & D, Mumbai for providing facilities for reverse-transcriptase inhibition as say, FT-IR analysis and HPTLC analysis respectively.

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