ISSN 2347-4289

Antitumor And Growth Effector Screen Of Leaf Extracts Of Selected Mangroves Of Bhitarkanika, Odisha

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ABSTRACT: Mangroves are known to possess medicinal properties and this has been established by research over years. The screen of antitumor and growth effector potential of few mangrove plants of Bhitarkanika natural reserve Odisha, helps to identify the total therapeutic potential of these plant extracts due to net composition of several primary and secondary metabolites ranging from carbohydrates, lipids, amino acids to that of aliphatic alcohols and pheromones. Radish seed phytotoxicity assay and Zone of inhibition study against *Agrobacterium tumefaciens* and potato disc assay show a distinct interference of plant extracts against tumor formation and bacterial growth, thereby attributing to its antitumor and growth inhibitor properties. This screen helps in identifying these potential plant types that can be used for medicinal purposes against specific bacterias and microbes and also be used as a nutrient supplement for fighting against the free oxide radicals.

Keywords : Antioxidant, Tumor, Germination, Hormones, Carbohydrates, Lipids, and Phytotoxicity.

1 INTRODUCTION

Mangroves are intertidal productive forested wetland constrained to the tropical and subtropical estuarine zones, serving as a nursery, feeding and spawning ground for commercial finfishes and shellfishes. Habitats of mangrove plants are commonly known as mangrove swamps, tidal forests, or mangals. These vascular halophytic plants constitute a vital component of marine flora and have significant ecological and socio-economic values. For centuries, mangroves have been traditionally used for food (fruits and nectar) feed and medicinal purposes in different parts of the world. They are well known to produce natural metabolites with diverse biological activities such as antibacterial, antiviral, antidiarrhoeal, antifeedant, insecticidal and cytotoxic activity [1],[3]. However, during the last decade screening of mangroves for bioactive compounds, has received high interest as a potential bio resource for novel drug leads. Until now, more than 200 bioactive metabolites have been isolated from true mangroves of tropical and subtropical populations. According to their chemical structure, most of the isolated compounds belong to steroids, triterpenes, saponins, flavonoids, alkaloids, tannins and phenolics with a wide range of therapeutic possibilities. Mangrove plants have been found to contain active chemical constituents in parts like stem, leaves and bark, which acts as a definite curing physiological response in the treatment of various ailments in human and other animals [2]. Knowledge about medicinal plants has been gathered through trial and error based upon speculations and superstitions. Medicinal plants are primary source of health care throughout the world for thousand of years. In the middle of 20th century, researchers preferred to use synthetic medicines over natural medicines for curing various diseases. However due to emergence of various side effects of synthetic drugs, trend to use medicinal plants to cure various diseases is becoming popular. Natural products from medicinal plants are known to be effective, inexpensive and least injurious with none or much reduced side effects as compared to synthetic medicines [3]. Therefore there has been a continued effort of investigating medicinal plants, which are commonly used, by public and derived from folklore or anecdotal information. Dubick 1986 reported that the medical use of herbs is deeply rooted in human history and folklore. Effective anti-cancer drugs such as vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan, isolated from plant provide evidence, that plants could be a source of novel cancer chemotherapeutic agents. Also mangrove plants are known be to rich in classes of polyphenols conferring them allopathic potential. Therefore, there is a need to investigate plant molecules or potential phytotoxicity, antibacterial and cytotoxic effects. Plants contain thousands of biologically active molecules. For their investigation, it is important to have the suitable biological assays[1],[4]. Initial screening for antitumor potential by using Agrobacterium *tumefaciens* (crown gall tumor formation on potato discs) could be used as a fairly rapid, inexpensive and reliable prescreen for antitumor agent. The potato disc bioassay also serves as an efficient alternative to extensive animal testing in the search for new anticancer drugs. Mangroves can be classified into three broad categories. True mangroves are mainly restricted to intertidal areas between the high water levels of neap and spring tides. Plant species from true mangroves belong to at least 20 different families. About 80 species of true mangrove trees/shrubs are recognized, of which 50–60 species make a significant contribution to the structure of mangrove forests. Minor species of mangroves are different in their ability to form conspicuous elements of vegetation, rarely forming a pure community. The mangal associates are salinity tolerant plant species, which are not found exclusively in the proximity of mangroves and may occur only in transitional vegetation, landwards and seawards. However, they do interact with true mangroves. Mangroves (mangroves, mangrove minors and mangal associates) are highly productive ecosystem with various important economic and environmental functions. The uses of mangroves are often quoted in scientific and popular articles fall into two major categories: Firstly the indirect use of the mangrove ecosystem are in the form of vital ecological functions such as control of coastal erosion and protection of coastal land, stabilization of sediment, natural purification of coastal water from pollution. Secondly, the economic benefits which are many and varied [3],[4],[5]. Apart from prawn

fisheries, many other species of economic importance are associated with mangroves; these include crabs, shrimp, oysters, lobsters and fish. Traditionally, the mangroves have been exploited for firewood and charcoal and their uses include construction of dwellings, furniture, boats and fishing gear and production of tannins and for dying and leather production. Mangroves provide food and a wide variety of traditional products and artifacts for mangrove dwellers. The mangrove leaves are useful contributors to the nutrient system of the mangrove environment. It is known that mangrove leaves contain sufficient amounts of minerals, vitamins and amino acids, which are essential for their growth, and nourishment of marine organisms and livestock. Mangrove foliage plays an important role in the formation of detritus, which is utilized by several estuarine and marine detritovorous organisms and mangrove leaves make a superior fodder due to their high salt and iodine content [1]. Two basic factors justify the study of the chemical constituents of mangrove plants. There are modifications or alterations in other physiological processes such as carbohydrate metabolism or polyphenol synthesis and due to these reasons; they may have chemical compounds, which protect them from these destructive elements. The second reason is that numerous mangrove plants are been used in folklore medicine, and recently, extracts from mangroves and mangrove-dependent species have proven activity against human, animal and plant but only limited investigations have been carried out to identify the metabolites responsible for their bioactivities. In Odisha, among three mangrove zones i.e. mangroves of the Mahanadi Delta, Balasore coast and the Bhitarkanika mangrove zone, the last one is the most important due to its largeststretch and unique biodiversity.It is also considered as the third largest mangrove zone of the country [4]. Cancer is now one of the ten leading causes of deaths in India. It is characterized by the deregulate growth and proliferation of abnormal cells bypassing apoptosis that invade and disrupt surrounding tissues. Some conventional systems such as surgery, chemotherapy, radiation therapy immunotherapy, monoclonal antibody therapy or other methods are being used for Cancer treatment [1],[2],[5]. Most of the agents have been revealed as mutagenic or carcinogenic and are highly toxic not only for cancer but also for normal cells. Crown gall is a plant neoplastic disease induced by the gram-negative bacterium *A. tumefaciens* [4]. *A.tumefaciens* cause series plant infections with more than 60 dicotyledonous families and many gymnosperms lead to great. The tumor formation starts when bacterial cell transfer part of the Ti (tumor-inducing) plasmid to the infected plant cell genome. The Ti-plasmid leads to rapid multiplication of plant cell without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers. The potato disc assay demonstrates the inhibition of tumor formation on potato discs; plant extracts that inhibit these plant tumors have a high predictability of showing activity against the P388 (3PS) leukemia in mice [2].Thus the validity of potato disc bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals [5]. Chemical Classes derieved from Mangrove Plants: Metabolites, some with novel chemical structures and belonging to a diversity of chemical classes, have been characterized from mangroves and mangal associates. Aliphatic alcohols and acids, amino acids and alkaloids, carbohydrates, carotenoids, hydrocarbons, free fatty acids including polyunsaturated fatty acids (PUFAs), lipids, pheromones, phorbol esters, phenolics, and related compounds, steroids, triterpenes, and their glycosides, tannins, other terpenes and related compounds, are among these classes. Among the latest additions are an array of substances from gums and glues to alkaloids and saponins and other substances of interest to modern industry and medicine. Chemicals such as amino acids, carbohydrates and proteins, are products of primary metabolism and are vital for the maintenance of life processes, while others like alkaloids, phenolics, steroids, terpenoids, are products of secondary metabolism and have toxicological, pharmacological and ecological importance.

2 Materials and Methods:

2.1 Collection of sample:

Fresh plant parts as indicated in the table below were collected from the mangrove forest of Bhitarkanika wild life sanctuary which extends from $20^0 30^{'}$ to $20^0 50^{"}$ Latitude and $86^0 30^{"}$ to $87^0 60^{'}$E longitude.

PLANT	PART
Phoenix paludosa	Leaf
Avicennia alba	Leaf
Heritiera fomes	Stem, Leaf
Excoecaria agallocha	Stem, Bark, Leaf
Sonneratia apetala	Bark
Suaeda maritima	Stem, Leaf

2.2 Preparation of plant extracts (Methanolic and Aqueous):

Different plant parts were collected and rinsed well with tap and distilled water (DW) and kept under shade still drying. Dried material was coarsely powdered using mortar and pestle followed by oven dry and further reduced to powder using an electric blender and stored in air tight glass container.50gm powder each was then dissolved in methanol and DW by allowing to sediment at room temperature (27-30°C) with occasional shaking at 250rpm. This step was repeated several times for 7 days till the extraction was complete followed by filtration using whatman no.1 filter paper and Teton cloth respectively. The filtered extracts were concentrated by evaporation at 70-90^0C.The concentrated filtrates were then transferred into glass beaker and dried into semisolid material using water bath.50mg of each semisolid extract was then dissolved in 1 ml DMSO to prepare 50 mg/ml concentration of methanol and aqueous extract of the plant parts. All the extracted materials were preserved at -4°C.

2.3 Agrobacterium strains:

Three *A. tumefaciens* strains namely from Institute of Microbial Technology Chandigarh, India, using standard procedure was used during antitumor, antibacterial and phytotoxicity assay.

2.4 Bacterial Culture Preparation:

Agrobacterium strains were cultured on agar medium with the following composition: 1 gm/l Beef extract + 2 gm/l yeast extract + 5 gm/l Peptone + 5 gm/l Nacl Single colony was transferred into broth and incubated at 30°C for 48hours. Six to seven loops of bacterial suspensions (1.0×10 cfu) were transferred

into sterilized phosphate buffer saline and this was used during antitumor assay as inoculums.

2.5 Radish Seed Phytotoxicity Assay:
For Root Length Determination: Whatman No. 1 filter paper kept on Petri dish and 5 ml plant methanolic extracts (1000ppm) was added separately. Filter paper was dried at room temperature for reducing extra solvent. 5 ml DDW was added and then 20 radish seeds were placed on Petri dishes followed by tightly sealed and incubation at 23±2°C. Root length was measured each day upto 5 days. Only DDW containing Petri dish was used as control. Each experiment was carried out in three times. A graph of root length vs. no of days was plotted for each extract separately in comparison to the root length of the control to study the phytotoxic effect. For Seed Germination Determination: This method involves use of 40 radish seeds per plate. Germinated seeds were counted after every day up to 5 days. Each experiment was carried out in three times. A graph of $_{percentage}$ of seed germination vs. no of days was plotted for each extract and control to study the phytotoxic effect.

2.6 Antibacterial assay against *A tumefaciens*:
Petri dishes supplied with a deep NB medium, were inoculated with 1% of test bacterium. Four wells were punched out of the agar, by using a clean sterile 6mm cork borer. The base of each hole was sealed with a drop of melted sterile water agar (15 g agar per liter H_2O) using sterile Pasteur pipette. For each plant extract 50-mg/ml concentrations was used to determine their antimicrobial effect on the *A. tumefaciens*. Each extract was pipetted into one well, then the plates were incubated at 28^0C for 24hr. After the incubation period, radius of the inhibition zone was measured and the images were photographed.

2.7 Antitumor potato disc assay:
Agrobacterium media preparation: 100 ml of LB media in a 250 ml flask was autoclaved for 12 minutes. The medium was allowed to cool followed by inoculation of one loop of *A.tumefaciens* from the storage culture on the agar slant, using sterile techniques. The flask was placed for 48 hours on a shaker at 28^0C at 200 rpm. Potato Disc preparation: Russet potatoes (*Solanumtuberosum L.*) were disinfected by scrubbing under running water with a brush, then immersing in 10% exalin for 20 mins. Potatoes were removed from detergent, blotted on sterile tissue papers and then immersed in 10% NaICO for 20 mins followed by washing with distilled water and blotting on tissue paper for surface sterilization. The same process was repeated using 1% $HgCl_2$. A flat surface potato without skin of height 2mm and diameter 8mm were made with the help of cork borer from the disinfected section using a sterile cork borer (10 mm).

2.8 Plant Extract Inoculum preparation:
The methanolic plant extract inoculum was used for antitumor activity test as: 50 µl test extract + 75 µl Distilled Water (DW) + 125 µl *A. tumefaciens* culture.50 µl of DMSO in place of test extract was used as negative control.

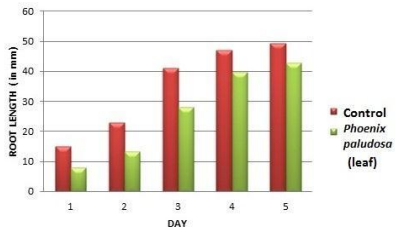
2.9 Anti-Tumor Assay:
The prepared potato discs were placed in a 24-well culture plate containing 1.5% water agar. A 50 µl of appropriate inoculum (test and negative) are placed on the surface of each potato disc. The plates were sealed with parafilm and incubated at 28°C in dark for 21 days. After 21 days discs are stained with Lugol's solutions (10% KI, 5% I_2) and tumors are counted under a microscope and photographed.Tumor inhibition was calculated by using following formula,

% of tumor inhibition = 100 – (ns / nc) x 100

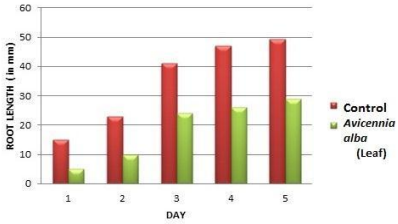
Where, ns = number of tumors in sample; nc = number of tumors in control.

3 Results and Discussion:

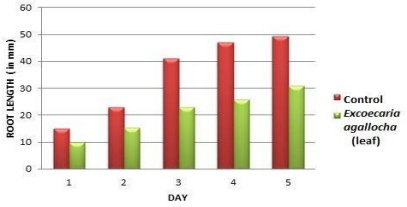
3.1 Root Elongation:



(a)



(b)



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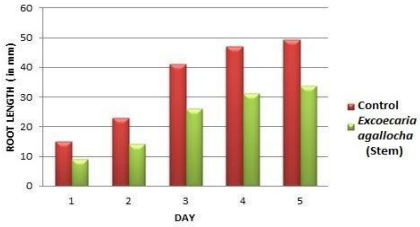

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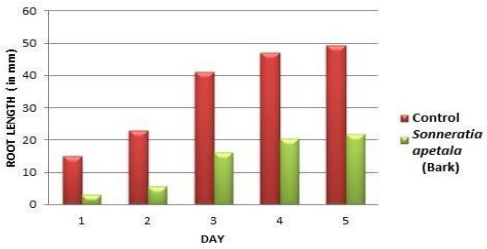
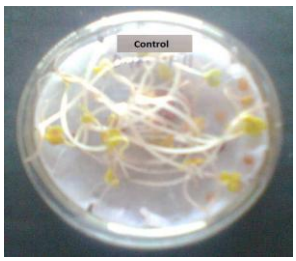
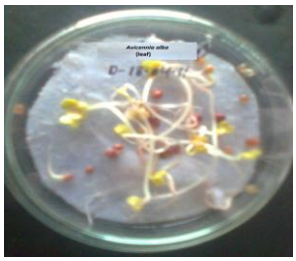


(e)

Fig.1 (a-e): images of effect on root elongation of different plant extracts with a time scale from Day 1-5.



(a)

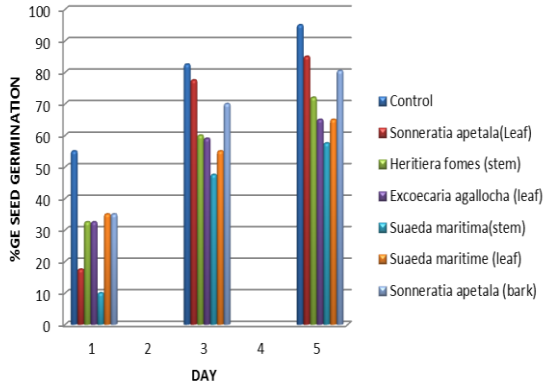


(b)

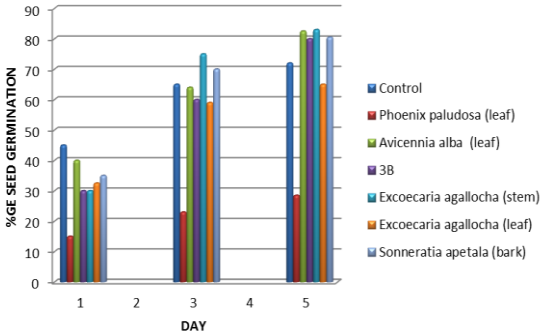
Fig.2 Radish seed phytotoxicity in terms of root length for *Avicennia alba.* Leaf.

The elongation of root is a significant marker of developmental stage of a plant and continues throughout the lifetime with differential rates depending on the system requirements. The bark extracts of *Sonneratia apetala* (fig.1.e) shows a distinct inhibition of root elongation in terms of branch and root hair formation, and main tube elongation. The suppression is around 50% in comparison to the control and most significant in relation to other plant extracts being studied (fig.1-2). Leaf extracts of *Avicennia alba,* also shows a similar suppression effect. The gradient of root length suppression however varies over time over a range of 5 days and most significance results are for fourth and fifth day of observation, indicating the cross talk between the metabolites of plant extracts and metabolic by products of seed germination. However the extracts of *Phoenix paludosa* (leaf) and *Excoecaria agallocha* (leaf, stem) show relatively lesser effects on root elongation indicating lower phytotoxicity in comparison to *Sonneratia apetala* and *Avicennia alba*. Phytotoxicity of plant extracts on root elongation thereby marks an inhib itory action on growth potential.

3.2 Seed Germination:



(a)



(b)

Fig.3.Effect of plant extract on seed germination (a & b).

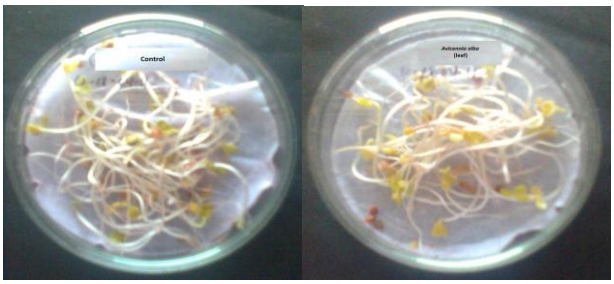


Fig.4 Radish seed phytotoxicity in terms of % seed germination for *Avicennia alba* leaf.

The leaf extracts of *Phoenix paludosa*, shows drastic inhibition on seed germination in contrast to the other plant extracts and control used. Most other plant extracts show negligible suppression effect (fig.3-4). The effects are significant mostly for the earlier days of germination, i.e. means till third day and by the later days (day 4 and 5) the effect is almost nullified and the germination rate is same as that of control. The plant extracts used in the experiment interefers with normal seed germination metabolism and in the sue course of time this extract gets diluted and degraded by the by products of germination process. *Heritiera fomes* shows around 20% reduction in seed germination and other distinct negative effect is seen for *Suaeda maritima* with approx. 35% seed germination inhibition. The secondary metabolites of plant products is known to

be a source of nutrition for microbes and thereby possibly the degradation of plant extracts over a longer time period indicates, a same kind of extract degradation by microbes. *Phoenix paludosa* leaf shows a 30% reduction in seed germination and more acutely in the late days of germination (4-5 days after seed implantation). Seed germination apart from root elongation also, is a very important indicatrix of growth potential and thus showing higher inhibitory effect on growth by *Phoenix paludosa, Heritiera fomes* and *Suaeda maritima* in comparison to *Avicennia alba, Excoecaria agallocha* and Phoenix paludosa.

3.3 Zone of Inhibition against *A.tumafaciens*:

Fig.5 Zone of Inhibition induced by plant extracts

ZONE OF INHIBITION (in mm)

Plant Extract	Methanolic medium	Aqueous medium
Phoenixpaludosa (Leaf)	×	×
Avicennia alba (Leaf)	20	17
Excoecaria agallocha (Stem)	×	12
Excoecaria agallocha (Leaf)	×	×
Sonneratia apetala (Bark)	×	×
Sonneratia apetala (Leaf)	16	16
Heritiera fomes (Stem)	×	×
Heritiera fomes(Leaf)	×	×
Sonneratia apetala(Leaf)	16	16
Suaeda maritima	×	×
Suaeda maritime	23	×

The zone of inhibition for the growth of tumefaciens is developed due to the effect of different plant extracts (fig.5) and noted in the table given above. The inhibition is distinct mainly in case of extracts of *Avicennia alba* (leaf) and *Sonneratia apetala* (Leaf). The results congruents to the results of suppression of root elongation and seed germination. The antitumor activity of the medicinal plant extracts is evaluated in terms of percentage of crown gall tumor inhibition by the following formula:

% of tumor inhibition = 100 – (Average no. of tumors of extract / Average no. of tumors of control) × 100

This tumor inhibition index at various extract concentrations is statistically analyzed with ANOVA and a plot of comparative variance in tumor inhibition is obtained.

4 Conclusions:

Of the selected mangrove plants *Avicennia alba* (leaf) and *Sonneratia apetala* (Leaf/Bark/Stem) show most distinctive properties of negative growth regulators and antitumor mediators. The plant extracts used range from primary metabolites (those important for life processes) to those mediating several metabolic processes assisting sustenance (secondary metabolites). As in case of root elongation and seed germination, both being developmental processes for plants, the steady level of homeostasis is very vital. The use of plant extracts with chemical constituents, cross talks with these developmental processes in general in a suppressive way. The antitumor and growth inhibition potential of these mangrove plants originate from the wide range of chemical components including aliphatic alcohols, lipids, carbohydrates and pheromones. The major hormones involved in root elongation and seed germination involve auxin and gibberellin, which are known to be the major interactive compounds with mangrove extracts and thereby showing effects. So the mangrove trees of Bhitarkanika show a high antitumor potential in terms of the phenotypic effects and also chemical similarity. Further use of these medicinal plants, can help in designing several drugs targeting bacteria and other microbes and also being used as biofertiliser or biopesticide for agricultural purposes. Further research using molecular characterization and chemical component analysis can help us know the exact chemical involved involved in this process.

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