Original Article:

The Phytochemical Screening and Anti-cancer activity of Ethanolic Extracts of Selected Mangrove Plants

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ABSTRACT

Background: Mangrove ecosystems have been recognized to include a wide range of secondary metabolites, which are biochemically distinct, resulting in a diverse range of natural compounds with unique bioactivity. They have active metabolites with new chemical structures from a variety of chemical classes.

Objectives: This study was undertaken to evaluate the phytochemical screening and cytotoxicity of four major mangrove plants (*Excoecaria agallocha* L., *Acrostichum aureum* L., *Aegiceras corniculatum* L., and *Avicennia officinalis* L.).

Methods: This experimental study was held in the Biochemistry and Molecular Biology Laboratory of Khulna University, Bangladesh, in 2016. At the first phytochemical screening of the selected plants was observed. Then, the bioactivity as preliminary cytotoxic activity was performed using brine shrimp lethality (BSL) bioassay where a significant 50% Lethal Concentration (LC_{50}) was exerted using polar solvent (ethanol) extract of different plant parts (leaf, bark, and stem). Then, Resazurin Cell Viability Assay was performed only for ethanolic leaf and bark extracts of *E. agallocha* using four standard bacteria (*Escherichia coli* ATCC 8739, *Salmonella typhi* ATCC 6539, *Salmonella paratyphi* ATCC 9150, and *Staphylococcus aureus* ATCC 25923).

Results: The experimental findings showed significantly strong LC_{50} by ethanolic leaf and bark extracts of *E. agallocha* and other plants, like *A. corniculatum*, *A. aureum*, and *A. officinalis* showed moderate and negligible cytotoxicity, respectively. Then, the experimental findings showed significantly ($P \leq 0.05$) strong IC_{50} by ethanolic leaf and bark extracts of *E. agallocha*.

Conclusion: The screens employed in this present study are preliminary and advanced assays are needed to verify and reveal further this bioactivity present in those plants, particularly *E. agallocha*.

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Introduction

Natural products derived from various sources (plants, microorganisms, marine organisms, vertebrates, and invertebrates) have been used for treating and preventing human diseases from ancient times across the world [1, 2]. Despite significant scientific and technological advancements in combinatorial chemistry, medicines generated from natural products continue to play an important role in drug discovery [3]. Biologically active compounds known as phytochemicals (derived from plants) provide disease-related targets with selected ligands, affect disease-related pathways and eventually, alter the biological network from disease to healthy status [4, 5]. Natural products and associated medications are purportedly utilized to treat 87% of all classified human diseases, including antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressive agents [6]. More than 28% of new chemical entities introduced into the market are derived from natural products [7]. Mangrove plants are biochemically distinct, producing a diverse range of natural compounds with unique bioactivity, and have long been utilized in traditional medicine [8]. In the recent decade, identifying medicinal plants with considerable cytotoxic potential beneficial for the development of cancer therapies has become increasingly important, and research in this subject is expanding. More than 1000 plant species with anti-cancer potential have been found. Many medicinal plants in Bangladesh have long been thought to have cytotoxic and anti-cancer qualities. The traditional and medicinal properties of plants in this study are shown in Table 1. This study was done to assess phytochemical screening and preliminary cytotoxic activities of four selected plants, including E. *agallocha*, A. *corniculatum*, A. *aureum*, and A. *officinalis*.

Materials and Methods

Collection of the sample

Excoecaria agallocha L., *Aegiceras corniculatum* L., *Acrostichum aureum* L., and *Avicennia officinalis* L. were selected for the present research (herbarium image is shown in Figure 1). These plants were collected from Hodda substation, Nolian forest, East zone of the Sundarbans, Khulna, Bangladesh on the 11th of January 2016. Then, collected plant samples were sent to Bangladesh National Herbarium, Dhaka, Bangladesh for taxonomical identification. A voucher specimen for *E. agallocha* L. (Accession No.: DACB-35375), *A. corniculatum* L. (Accession No.: DACB-35376), *A. aureum* L. (Accession No.: DACB-35377), and *A. officinalis* L. (Accession No.: DACB-35378) was also deposited. The experimental study was held in the Biochemistry and Molecular Biology Laboratory of Khulna University, Bangladesh, in 2016.

Extraction

The plant elements (bark, stem, and leaf) were separated from one another and washed in distilled water before being air-dried for many weeks. A motorized plant grinder (capacitor start motor, Wuhu Motor Factory, China) was used to crush the dried material into a coarse powder. The powder was maintained in an air-tight container in a dry, cool, and dark location until analysis began.

For *E. agallocha*, about 311 g powder from leaves and 75 g powder from bark were taken into different clean, flat-bottomed glass jars and soaked into 750 mL and 300 mL ethanol (70%), respectively.

For *A. aureum*, about 328 g of powder from leaves was taken into clean, flat-bottomed glass jars and soaked into 1050 mL ethanol (70%).

For *A. corniculatum*, about 301 g powder from leaves and 461 g powder from bark were taken into different clean, flat-bottomed glass jars and soaked into 950 mL and 1000 mL ethanol (70%) respectively.

For *A. officinalis*, about 365 g powder from leaves and 465 g powder from stem were taken into different clean, flat-bottomed glass jars and soaked into 900 mL and 1500 mL ethanol (70%), respectively.

They were then enclosed and kept in a dim environment for five days accompanied by occasional shaking and stirring. By passing the mixture through a clean cloth filter, coarse plant material was removed. Ethanol is a polar solvent; thus, polar compounds were filtrated.

Evaporation and storage

Filtrates obtained from different solvent systems were evaporated at room temperature with an electric fan until they were completely dried. The crude (2.3%) extracts were weighed and kept in a refrigerator for further experiments.

Phytochemical Analysis

The content of alkaloids, saponin, tannins, steroids, flavonoids, anthraquinone, and glycosides in the extracts was determined using techniques found in the literature [14, 15, 16].

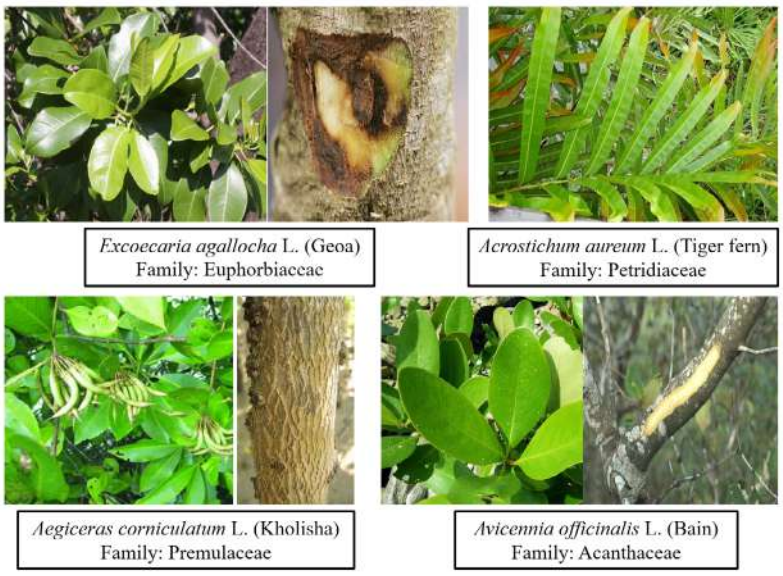

Figure 1. Herbarium image of *E. agallocha* L., *A. corniculatum* L., *A. aureum* L., and *A. officinalis* L.

Test for flavonoids

To assess flavonoids, 2 ml of extract solution was treated with 1.5 mL of 50% methanol solution. The solution was warmed before adding metal magnesium. In this solution, a few drops of concentrated hydrochloric acid were added and red color was observed for flavonoids and an orange color for flavones.

Test for alkaloids

In a boiling water bath, the extract of each solvent's crude dry powder was evaporated to dryness. The residues were dissolved in 2N hydrochloric acid. The filtrate was divided into three equal amounts after the mixture was filtered. One portion was treated with a few drops of Mayer'sreagent, one portion was treated with an equal amount of Dragondroff's reagent, and the third portion was treated with an equal amount of Wagner's reagent, respectively. The presence of different alkaloids was indicated by the creamish, orange, and brown precipitate.

Test for saponins

In a test tube, about 0.5 g of plant extract was mixed with water and then heated to boiling. The appearance of frothing was taken as early evidence of the saponin's presence.

Test for tannins

In a test tube, 0.5 g of extract was added to 10 mL of water and filtered. A few drops of 0.1% ferric chloride were added, and the coloring was checked for brownish green or blue-black.

Test for anthraquinones

In a dry test tube, 0.5 g of extract was mixed with 5 mL chloroform and agitated for 5 minutes. The extract was filtered, and the filtrate was agitated with a 10% ammonia solution in an equal volume. The presence of anthraquinones is indicated by a pink-violet or red color in the ammonical layer.

Test for steroids

To assess steroids, 2 mL of acetic anhydride was added to 0.5 g of methanol extract of each sample with 2 mL sulphuric acid. In certain samples, the color changed from violet to blue or green, indicating the presence of steroids.

Test for proteins

To 2 mL of protein solution, 1mL of 40% NaOH solution and 1 to 2 drops of 1% $CuSO_4$ solution were added. The existence of peptide linkage was shown by a violet color.

Test for cardiac glycosides

To assess cardiac glycosides, 0.2 g of extract was dissolved in 1 mL of glacial acetic acid containing one drop of ferric chloride solution. This was then under-layered with 1 mL of concentrated sulphuric acid. The presence of a deoxysugar characteristic of cardioids was shown by a brown ring produced in contact.

Evaluation of cytotoxic activity

The attribute of being hazardous to cells is known as cytotoxicity. Anti-cancer drug development takes up a significant amount of current pharmacological research. Chemoprevention is a very promising cancer prevention method and some plants have been used in traditional medicine. It is now widely acknowledged as one of the most important sources of cancer chemopreventive drug development. There are various in vitro and in vivo methods to evaluate cytotoxicity either as pure compounds or as plant extracts. The most commonly used in vivo methods for evaluation of the cytotoxic activity of natural compounds derived from medicinal plants are given below:

Evaluation of brine shrimp lethality assay

The most practical test species for toxicity investigations are brine shrimp (Artemia salina). The cytotoxicity activity of substances can be predicted using a brine shrimp lethality bioassay. The technique is easily manageable and requires a small amount of test material. Once the active compounds have been isolated, this strategy aims to provide a front-line screen that can be backed up by more specific and more expensive bioassays. This is a simple bioassay for determining the bioactivity of plant extracts, which in most cases corresponds fairly well with cytotoxic and anti-tumor effects. The lethality of the ethanolic extracts to brine shrimp was determined on A. salina after 24 hours of exposure to the samples. At different concentrations, test samples showed different mortality rates, and the mortality rate of brine shrimp was shown to increase as the concentration of the sample rose. The 50% lethal concentration (LC_{50}) values of the plant extracts were obtained by a plot of the percentage of the shrimp nauplii killed against the concentrations of the extracts. As a positive control, vincristine sulfate was utilized.

Preparation of Seawater (Brine)

Sixty grams of sea salt (pure NaCl 20 g and table salt 40 g) was precisely weighed, dissolved in distilled water to produce one litter, and filtered to obtain a clear solution.

Preparation of stock solution

Sixteen milligrams of dried ethanolic extracts of *E. agallocha*, *A. corniculatum*, *A. aureum*, and *A. officinalis* were taken in 25 mL individual volumetric flask and 25 mL seawater, and one drop of Tween-80 was added. This solution had a concentration of 0.64 g/L and was used as a stock solution.

Hatching of the Brine Shrimp

The glass jar was filled with seawater, shrimp eggs were placed on one side of the jar, and the top of the jar was covered with a net. The shrimps were allowed to hatch and mature as nauplii (larvae) for two days. The hatched shrimps were collected and transferred for bioassay.

Application of test solution and control on Brine shrimp nauplii to the test tube

Seven test tubes were taken, with each one correctly marked to indicate the 5 mL volume. Then, with the help of the micropipette, 5mL of seawater was added to each of the test tubes. Then, specific volumes (10, 20, 40, 80, 160, 320, and 640 µg/mL) of sample concentration were added to the test tubes trough serial dilution method to get (5, 10, 20, 40, 80, 160, and 320 µg/mL) final concentration with a micropipette. In this study, vincristine sulfate (VS) was utilized as a positive control. For standard test, VS (1, 2, 4, 8, 16, 32, and 64 µg/mL) was added to the test tubes trough serial dilution method to get final concentration (0.5, 1, 2, 4, 8, 16, and 32 µg/mL) with a micropipette. Finally, 10 alive brine shrimp nauplii were placed inside each test tube with a pasture pipette.

Counting of nauplii

The test tubes were checked after 24 hours, the number of surviving nauplii in each test tube was counted, and the results were recorded. The percentage of brine shrimp nauplii lethality at each concentration for each sample was computed. Percent mortality of the samples was calculated using the following equation:

CellTiter-Blue® Cell Viability Assay

The CellTiter-Blue® Cell Viability Assay provides a consistent approach for counting viable cells. It measures the metabolic capacity of cells, which is a sign of cell viability, using the indicator dye resazurin. Viable cells retain the ability to reduce resazurin into resorufin. Nonviable cells lose metabolic capacity quickly, do not diminish the indicator dye, and do not produce an absorbent signal. A buffered solution containing highly purified resazurin is used in the CellTiter-Blue® Reagent. Resazurin is a dark blue substance with limited intrinsic absorbency until it is converted to resorufin, a pink substance with a high absorbency (570 nm).

Micro-Organisms The cytotoxicity activity of crude plant extract was tested using four bacterial strains (Table 2). Preparation of bacterial culture Using aseptic techniques,

Table 1. Traditional and medicinal properties of plants used in the study

No.	Plant	Local Name	Medicinal properties	References
1	*E. agallocha* L.	Geoa, Gneoa or Genwa	Rheumatism, leprosy, epilepsy, paralysis, conjunctivitis, dermatitis, fish poison, hematuria, and toothache	[9, 10]
2	*A. corniculatum* L.	Kholisha or Kharsi	Asthma, diabetes, and rheumatism	[11]
3	*A. aureum* L.	Tiger fern, mangrove fern	Rheumatism, wound healing, boils, bleeding	[12]
4	*A. officinalis* L.	Bain	Boils, tumors, aphrodisiac, smallpox sores, skin afflictions, especially scabies, and snakebite	[13]

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a small portion of fresh bacteria was transferred into a test tube containing nutrient broth and incubated for 4-6h at 37° C (until the growth reached the log phase).

Preparation of Test Sample Ethanolic extract of *E. agallocha* (0.4 mg) was accurately measured by using the electronic balance and taken into screw-capped vials (vials were nullified on the electronic balance by auto-zero). Then, 5mL of distilled water was added to the vial to get the sample of 400 µg/mL.

Preparation of Resazurin Dye Solution (RDS)

Resazurin dye (300 mg) was dissolved in 40 mL of sterile water. The solution was homogenized using a vortex mixer. Percent mortality of the samples was calculated by the following equation:

Application of test solution

Seven test tubes were taken. Then, with the help of a micropipette, 1000 µL of nutrient broth was added to each of the test tubes. Then, specific volumes (6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL) of sample concentration were added to the test tubes trough serial dilution method to get (3.0625, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) final concentration with a micropipette. In this study, VS was utilized as a positive control. Then, with the help of the micropipette, 100 µL of resazurin solution was added to each of the test tubes. Then, with the help of the micropipette, 100 µL of bacterial suspension was added to each of the test tubes. For standard test, VS concentration (0.3625, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg/mL) were added to the test tubes trough serial dilution method to get final concentration (0.3625, 0.625, 1.25, 2.5, 5, 10, and 20 µg/mL) with a micropipette.

Materials and Method

After 24 hours, the test tubes were observed. At first, vortexing was done, then the optical density (OD) of the bacterial solutions was measured at 570 nm. The percentage Inhibition of Concentration (IC50) was calculated at each concentration for each sample.

Statistical analysis

One-way ANOVA was carried out using SPSS software v. 16. Here, P-values ≤0.05 were considered to determine the significant difference between the test sample and the control group.

Results

Phytochemical Screening

Preliminary phytochemical screening revealed that solvent extracts include the majority of phytochemicals, such as flavonoids, alkaloids, steroids, tannins, saponins, and others (Table 3).

Table 2. Four standard bacteria given below used in the study

Name	Types
E. coli ATCC 8739	Gram-Negative
S. typhi ATCC 6539	Gram-Negative
S. paratyphi ATCC 9150	Gram-Negative
S. aureus ATCC 25923	Gram-Positive

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Table 3. Phytochemical analysis of the ethanol extract of *E. agallocha* L., *A. aureum* L., *A. corniculatum* L., and *A. officinalis* L.

Phytochemical Constituens	*E. agallocha* L.	*A. officinalis* L.	*A. corniculatum* L.	*A. aureum* L.
Flavonoids	+	+	_	_
Alkaloids	+	+	_	_
Saponins	+	+	+	+
Tannins	+	_	_	_
Anthraquinones	_	_	_	_
Steroids	+	+	_	+
Proteins	_	_	_	+
Glycosides	_	_	+	_

(Key: '+' = Present, '-' = Absent).

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Brine shrimp lethality assay

Comparative analysis

The concentration, at which 50% mortality occurs is called the LC_{50} value. The comparative analysis of the values of the plant extracts and control is given below in Figure 2. Comparative analysis of the plant extracts and control is shown in Table 4. From the graph (Figure 3), it was observed that ethanolic leaf and bark extract of *E. agallocha* showed the highest activity on brine shrimp nauplii. On the other hand, ethanolic leaf and bark extract of *A. corniculatum* showed moderate cytotoxic activity. At last, the leaf extract of *A. aureum* and leaf and stem of *A. officinalis* showed low cytotoxic activity compared to positive control VS.

CellTiter-Blue® Cell Viability Assay

The results of the study shown in Figure 4 showed that *E. agallocha* L. possessed a broad spectrum of activity against a panel of microorganisms. *E. agallocha* bark extract showed higher toxicity than *E. agallocha* leaf extract on selected bacterial species.

Discussion

The organic solvent extracts showed the presence of many phytochemicals, which are summarized in Table 3. The presence of such phytochemicals may be correlated with the fact that the solvent extracts have maximum anti-cancer activity. Several phenolic substances contained in plant cells, such as tannins, are effective inhibitors of plant-pathogen hydrolytic enzymes. Antifungal effects are also found in other substances, such as sapo-

Table 4. Comparative analysis of the plant extracts and control

Sample	LC_{50} (µg/mL)
Vincristine sulfate (VS)	0.405±0.002
Leaf extract of *E. agallocha* L.	6.26±0.03
Bark extract of *E. agallocha* L.	2.27±0.06
Leaf extract of *A. aureum* L.	19.95±0.05
Leaf extract of *A. corniculatum* L.	9.26±0.02
Bark extract of *A. corniculatum* L.	11.74±0.06
Leaf extract of *A. officinalis* L.	21.37±0.05
Stem extract of *A. officinalis* L.	16.98±0.03

Here, LC_{50}: 50% Lethal Concentration.

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Table 5. Comparison table for four standard bacteria

Bacteria	VS (positive control) (µg/mL)±SE	Bark extract (µg/mL)±SE	Leaf extract (µg/mL)±SE
E. coli ATCC 8739	0.206±0.002	12.07±0.03	19.45±0.05
S. typhi ATCC 6539	0.1615±0.14	8.76±0. 38	16.7168±0.47
S. paratyphi ATCC 9150	0.1624±0.24	9.72±0.51	31.3019±0.63
S. aureus ATCC 25923	0.467±0.18	10.35±1.76	16.7021±1.81

Here, VS: Vincristine Sulfate, and SE: Standard Error.

nins. Many plants release phenolic compounds, which are harmful to microbial pathogens [17]. Flavonoids, on the other hand, are powerful antioxidants and free radical scavengers that are water-soluble, which prevent oxidative cell damage and have strong anti-cancer activity, and were found in *E. agallocha* and *A. officinalis*. Pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents for analgesic, antispasmodic, and antibacterial effects [18], which were found in *E. agallocha* and *A. officinalis*. Saponins have also been found to be effective antifungal agents. Tannins have been shown to inhibit microorganism proliferation by precipitating microbial protein and rendering nutritious proteins inaccessible to them [19] and were only found in *E. agallocha*. Steroids were found to be present in *E. agallocha* and *A. officinalis*. It has been found that the investigated plant contained steroidal compounds. It should be noted that steroidal compounds are of importance and interest in pharmacy due to their relationship with such compounds as sex hormones [20] and were found in *E. agallocha*, *A. officinalis*, and *A. aureum*. As a result, the compound discovered could be responsible for plant extracts' anti-cancer effect.

As shown in Table 4, ethanolic leaf and bark extracts of *E. agallocha* revealed much closer LC_{50} values compared to a positive control (VS). Where the percent mortality of bark extract of *E. agallocha* is much nearer to the standard and both leave and bark extracts had potential anti-cancer properties. *E. agallocha* exhibited significant anti-tumor-promoting activity [21]. Ethanolic leaf and bark extracts of *A. corniculatum* revealed higher LC_{50} values compared to a positive control (VS). Where, the percent mortality of leaf extract of *A. corniculatum*

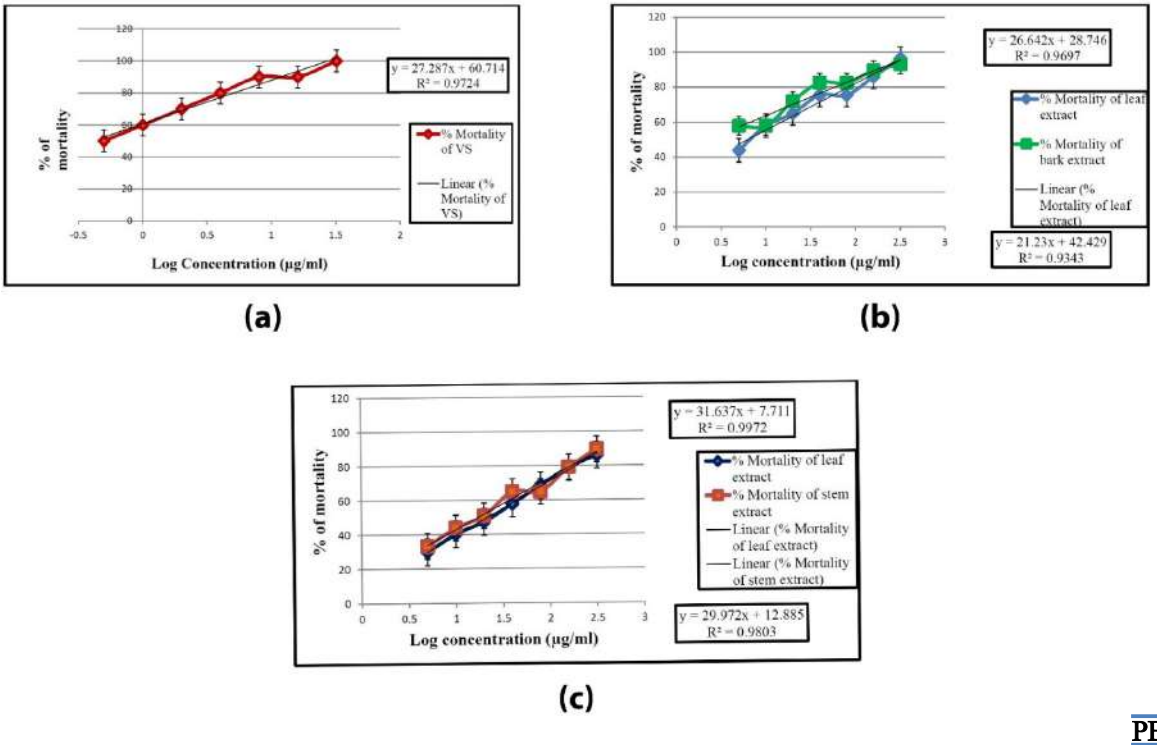

Figure 2. The plot of logarithmic concentration versus percent mortality after 24 hr of exposure to (a) vincristine sulfate (Positive control), (b) both *E. agallocha* leaf and bark extracts, and both *A. officinalis* leaf and bark extracts

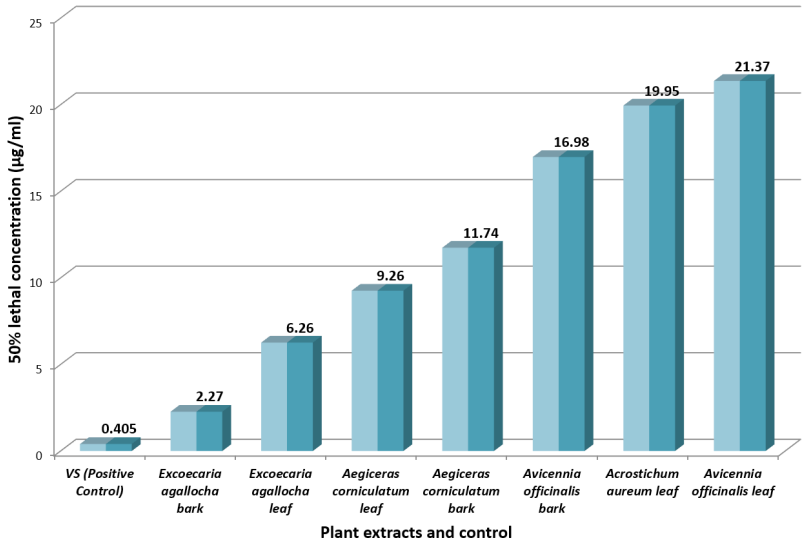

Figure 3. Comparison of the LC_{50} values of different plant extracts and positive control
Here: P≤0.05; thus, there was a significant difference between the test sample and control group.

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is much nearer to the standard but both leaf and bark extracts could be a potential anti-cancer agent. *A. corniculatum* leaf extract was discovered to have potent anti-nauplii action in brine shrimp. As a result, the extract may contain anti-cancer, antibacterial, or pesticidal compounds, based on the positive reaction achieved in this experiment [22]. The percent mortality of the extract of *A. aureum* is higher than the standard but this extract could be a potential source having anti-cancer activity. *A. aureum* exhibited significant anti-cancer activity [23]. Ethanolic leaf and stem extracts of *A. officinalis* revealed much higher LC_{50} values compared to a positive control (VS). Where, the percent mortality of leaf and stem extracts of *A. officinalis* showed low cytotoxicity against brine shrimp nauplii.

As shown in Table 5, *S. paratyphi* ATCC 9150 and *E. agallocha* bark extracts showed higher toxicity against *E. agallocha* leaf extract. In *E. coli* ATCC 8739, positive control showed higher toxicity than other bacteria. In the case of *E. coli* ATCC 8739, *E. agallocha* bark extract showed higher toxicity against *E. agallocha* leaf extract. In *S. typhi* ATCC 6539, *E. agallocha* bark extract showed higher toxicity against *E. agallocha* leaf extract. In *S. typhi* ATCC 6539, *E. agallocha* bark extract showed higher toxicity than other bacteria. Therefore, *E. agallocha* bark extract showed higher toxicity than *E. agallocha* leaf extract.

As a result, we may conclude that the solvent fractions' cytotoxic activity was promising, indicating the presence of powerful bioactive chemicals and possibly anti-tumor

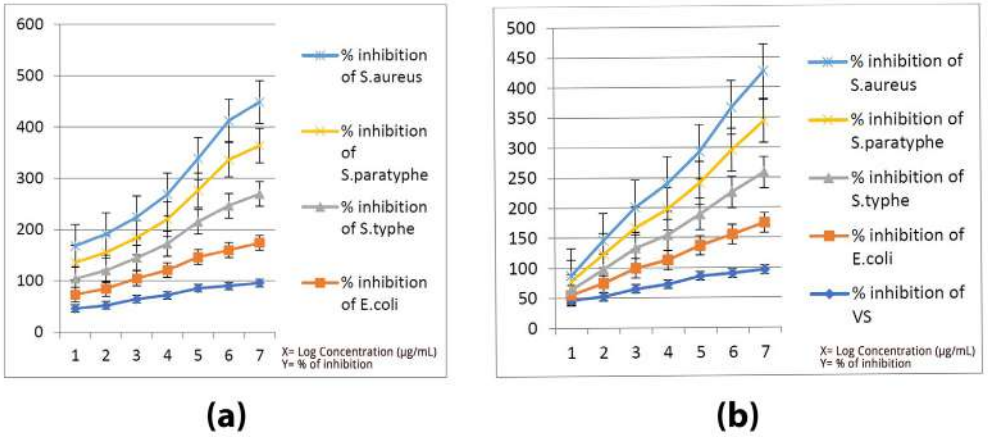

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Figure 4. Plot of logarithmic concentration of vincristine sulfate in four standards bacteria in resazurin cell viability assay of (a) *E. agallocha* bark extract, and (b) *E. agallocha* leaf extract. Here, X= Log Concentration (µg/mL) and Y= % of Inhibition.

and anti-cancer properties. Further research is required in the future in order to find novel bioactive compounds.

Conclusion

Further research is needed to fractionate the extract, identify the bioactive components, and evaluate the biological activity in in vitro and in vivo experiments. Medicine, nutraceuticals, agrochemicals, and, last but not least, cosmetics could all benefit from such bioactive compounds. In the future, research is needed in the hopes of discovering novel bioactive compounds, and endeavors to develop any feasible drug in Bangladesh should be promoted.

Ethical Considerations

Compliance with ethical guidelines

This article is a meta-analysis with no human or animal sample. There were no ethical considerations to be considered in this research.

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The paper was extracted from the BSc. thesis. Dissertation or extracted from a research project of the first author, Biotechnology and Genetic Engineering discipline, Khulna University.

Authors' contributions

Conceptualization, literature search, experimental studies, data acquisition, data analysis, statistical analysis, and manuscript preparation: Sharmin Sultana; Sample collection, herbarium preparation, data acquisition, data analysis, statistical analysis, and manuscript preparation: Rana Biswas; Definition of intellectual content, data analysis, manuscript preparation, manuscript editing, and manuscript review: Kazi Mohammed Didarul Islam.

Conflict of interest

The authors declare no conflicts of interest.

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