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Evaluation of antioxidant, anti-hemolytic, cytotoxic effects and anti-bacterial activity of selected mangrove plants (*Bruguiera gymnorrhiza* and *Heritiera littoralis*) in Bangladesh

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Abstract

Objectives: In this present study, antioxidant, anti-hemolytic, cytotoxic and anti-bacterial activities derived from methanol extracts of *Bruguiera gymnorrhiza* and *Heritiera littoralis* were investigated in order to determine their medicinal activities.

Materials and methods: Folin-Ciocalteu reagent method and aluminum chloride methods were used to determine the mangroves' total phenolics and total flavonoid content, respectively. Antioxidant capacity was assessed via the following methods: 1,1-diphenyl-2-picryl hydroxyl (DPPH), 43 mM H₂O₂, Fe²⁺ quenching assay, and anti-hemolytic activity. Brine shrimp (*Artemia salina* L.) lethality assay was also carried out to determine the cytotoxic potential of the mangroves along with anti-bacterial activity test using five Gram-negative and another two Gram-positive bacterial strains.

Results: The mangroves yielded 58.917 ± 0.601 and 36.625 ± 0.551 mg Gallic acid equivalent (GAE)/g sample and 76.417 ± 0.19 and 113.637 ± 0.17 mg quercetin equivalent (QE)/g sample in *B. gymnorrhiza* and *H. littoralis*, respectively. Methanol extracts of both mangroves exhibited high radical scavenging activity against DPPH, H₂O₂ and Fe²⁺ radicals. The reductive capacity of the extracts increased with increasing concentrations of samples, and the extracts inhibited H₂O₂ induced hemolysis in human red blood cells (RBCs). Antioxidant properties were found to be moderately weaker than that of the reference standard, L-ascorbic acid (AA), and Gallic acid (GA). Further, brine shrimp (*Artemia salina* L.) lethality assay revealed significant cytotoxicity (241.4 and 272.6 µg/mL, respectively). Methanol extracts could also inhibit the growth of pathogenic bacterial strains.

Conclusion: This study showed that the crude methanol extract of selected mangrove plants possesses free radical scavenging, anti-hemolytic, cytotoxic and anti-bacterial activity. The experimented plant has the potential to be used as a traditional medicine and replace synthetic drugs. Further studies are necessary to isolate active compounds responsible for the overall antioxidant activity of the crude extracts.

Keywords: Antioxidant, Free radical scavenging, Red blood cell hemolysis, Cytotoxicity, Anti-bacterial, Mangrove plants, Bangladesh

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Introduction

The term mangrove usually describes the assemblage of woody trees and shrubs which mainly grow in coastal saline habitats in the world's tropical and subtropical regions. Plants are an important source of potentially useful phytochemicals for the development of new chemotherapeutic agents [1]. Extracts and phytochemicals of several mangrove plant species are used extensively in folkloric medicine mostly to treat a number of diseases, for example microbial infections, cardiovascular diseases, and cancer. These compounds are also being currently used as pesticides (insecticides, herbicides, and fungicides) in agriculture [2]. According to one study, about 80% of present-day drugs are either directly or indirectly obtained from medicinal plants [3]. In most of the world's developing countries, natural plants are deemed a precious source of natural wealth and have been incorporated in several healthcare programs. Mangrove forests are distributed among 112 countries and the global coverage of these forests is approximately 18 million hectares [4]. Leaves of mangrove plant species are rich in phenols and flavonoids that serve as ultraviolet screening compounds. Due to their significant constitution of tannin, phenol, flavonoid and other antioxidant substances, they are commonly used as folk medicines against several diseases [2].

Research on plants' antioxidant properties has garnered much importance in recent years in the field of medicine as well as in the food industry. Inherited consumption of oxygen leads to the generation of a series of reactive oxygen species (ROS) during cell growth [5]. ROS consist of superoxide anion radicals (O_2^-), hydroxyl radicals (OH^*) and non-free radical species like hydrogen peroxide (H_2O_2) and singlet oxygen (1O_2) [6]. Such oxidative species initiate peroxidation of membrane lipids, leading to the accumulation of lipid peroxides and are also responsible for aging and cell membrane disintegration, membrane protein damage, DNA mutations, and various other diseases. If scavenging by cellular constituents does not occur effectively on ROS then it may lead to diseased conditions. ROS, however, also represent an important anti-microbial host defense mechanism of plants and animals against pathogens, e.g. a defense mechanism against *Salmonella enterica* and *Staphylococcus aureus* [7, 8]. Consequently, it is important to maintain a balanced state of ROS in order to maintain a host defense mechanism while using plant crude extracts for therapeutic purposes.

The most important components of medicinal plants are phenolics, tannins, flavonoids and alkaloids [9]. Both edible and non-edible plants consist of phenolic compounds and they have been reported to create different biological outcomes. Phenols and flavonoids exhibit a preventive role in the development of cancer and cardiac diseases [10]. At present, the antioxidants that are widely

available comprise butylated hydroxyanisole (BHA), butylated hydroxytoluene ((BHT), propyl gallate and *tert*-butyl hydroquinone. Of these four commercially available antioxidants, BHA and BHT have aroused suspicions of being responsible for pancreatic damage and carcinogenesis. This situation has led to the growing interest in natural antioxidants [11–14]. Despite being the main constituent of several medicinal properties, plants are also noted for their toxicity [15]. In such cases, brine shrimp (*Artemia salina* L.) cytotoxicity assays have been conducted to determine the pharmacological activity and toxicity of medicinal plants [16]. The cytotoxicity assay based on brine shrimp lethality was considered an essential part of preliminary toxicity assessment [17].

Due to the saline nature of mangrove soil, mangrove plants usually grow differently, subsequently resulting in variations in their vegetation, species composition, and structure in the global, regional and local contexts [18, 19]. The soil of the mangrove forest acts as a reservoir of carbon that interacts with the atmosphere, storing about three times the biomass that makes up the vegetation and structure of mangroves [20]. The pH of mangrove soil significantly affects the composition of inhabitant species, primarily due to the change in availability of both essential elements such as Phosphorus (P), as well as non-essential elements [21, 22]. Analyses have detected significant correlations between the variation in soil structure with soil physico-chemical characteristics and particularly with differences in salinity levels [23]; soil NO_3^- , especially the nitrate to phosphate ratio, soil moisture content and soil temperature [24]. Most investigations of nutrient limitations to mangrove swamps have focused on macronutrients N and P, which most likely limit the structure and productivity of mangroves [25]. In mangrove soils, N was considered the primary nutrient that affects species composition and forest structure, although more recent analysis found that N and P influence structure and composition in approximately equal proportions [26]. A more recent analysis of salt stress on *Schizonepeta tenuifolia* Briq, showed that salt stress results in reduced plants' dry biomass. Further, the phytochemical analysis showed that the antioxidant content, including phenolics and flavonoids, increased at low (25 mM) or moderate (50 mM) levels. However, it declined at severe (75 and 100 mM) levels of salinity [27]. For this reason, it can be assumed that mangrove soils saline conditions yield some major effects on plants' physiology and biosynthesis.

Bruguiera gymnorrhiza (*B. gymnorrhiza* (L.) Lamk), also known as the large-leaved mangrove is one of the most prominent and widespread plant species in the Pacific region. It, mostly grows in the intertidal regions ranging from Southeast Asian coastlines to Japan's Ryukyu Island [28]. *Heritiera littoralis* (locally known as "Sundari" in Bangladesh) is another mangrove plants species

with an appreciable economic value. Leaves, stems, and seeds of *H. littoralis* are widely used in the treatment of diarrhea, dysentery, stomach aches and traditionally for controlling mosquitoes [2, 29–31]. In Bangladesh, this plant species is highly abundant in the southern region of Sundarbans, the largest mangrove forest of Southeast Asia. It is a mangrove area in the delta formed by the confluence of the Ganges, Brahmaputra and Meghna Rivers in the Bay of Bengal. Its geographical location is Longitude: 22.4281° N, Latitude: 89.5888° E. Several studies have suggested the medicinal uses of *B. gymnorrhiza*. The fruits have been used for the treatment of diarrhea, shingles and eye diseases [29, 32, 33]. The bark has also been used as an astringent treatment of diarrhea and malaria [32, 33]. The roots and leaves have been used to treat burn [33]. The largest amount of crude extract has been produced from *B. gymnorrhiza* fruits by extraction with methanol, which was 7.85%. This crude extract also resulted in significant antioxidant activity [34]. *H. littoralis*, on the other hand, has resulted in significant biochemical compounds of flavonoid and terpenoid family as well as cinnamoylglucosylflavonoid, antimycobacterial and antioxidant properties [30, 35–39]. Despite the fact that antimicrobial, antioxidant, anticancer, antihyperglycemic and antinociceptive effects of *H. fomes* have already been reported, only a few studies were carried out on *H. littoralis* [40–44].

The present study was done using methanol extracts of Bangladeshi endogenous *B. gymnorrhiza* and *H. littoralis* leaves to evaluate the antioxidant potential, bioactive compounds as free radical scavengers, antimicrobial and cytotoxic effects of the crude extract. It commenced with the collection and processing of samples, extraction, and biochemical assay. Reported here in great detail are the bioactive, antioxidative constituents as well as antioxidant, antimicrobial and cytotoxicity of crude extracts of two large-leaved mangrove species (*B. gymnorrhiza* and *H. littoralis*).

Materials and methods

Plant material collection and preparation

Fresh leaves of *H. littoralis* and *B. gymnorrhiza* were collected on 20th July 2018 from Sundarbans, Bangladesh (geographical location is Longitude: 22.4281° N, Latitude: 89.5888° E). The plants were botanically identified and confirmed by the Bangladesh National Herbarium. First, the leaves were dried in the shade for 10 days at room temperature. Then the air-dried leaves of both plants were cut into small pieces and turned into crude powder using a mechanical chopper. The crude powder was stored in appropriate containers and kept in the cold and dark to prevent fungal contamination.

Extract preparation

For the preparation of methanol extracts, about 300 g of air-dried powder of both plants were put into a flat

bottomed glass jars and soaked in 1000 mL methanol (95%). They were then sealed and kept for 72 h in a dark room and subjected to occasional shaking and stirring. The mixtures were filtered coarsely using Whatman filter paper. Followed by the organic filtrates obtained being evaporated utilizing a water bath at 98 °C with an electric fan until they were completely dried. The crude extracts of both *H. littoralis* and *B. gymnorrhiza* weighed about 3.9 g and 6.8 g, respectively.

Total phenolic content assay

Polyphenols in the plant extracts react with redox reagents to form blue complex chromogens that can be detected by visible-light spectrophotometry [45]. In our study, we followed a previous method with a slight modification to determine the total phenolic content of crude extracts [46]. Gallic acid solution was diluted to five different concentrations (31.25–500 µg/mL). The extract solution was prepared at a concentration of 500 µg/mL. Extract solutions were later added with 5 mL of 10% Folin-Ciocalteu reagent (Scharlab S.L., Spain) followed by 4 mL of 7% sodium carbonate (Emplura, Merk, India). Absorbance was measured at 750 nm wavelength after 20 min of incubation. The standard calibration curve of Gallic acid was plotted.

$$\text{Total Phenolic Content TPC, A} \\ = (C \cdot V) / M \text{ (mg/gm)}$$

Here, A is the phenolic assay, C is the Gallic acid equivalent concentration, V is the volume of the extract solution used and M is the weight of the extract used.

Total flavonoid content assay

Total flavonoid content assay for both plants was carried out with modifications based on previous methods [47, 48]. Quercetin was used as a standard by dissolving in D₂O and diluted to different concentrations (31.25–1000 µg/mL). Similarly, 1 mg/mL crude extract solutions were prepared. In each test tube, 0.5 mL of standard solutions were poured and made up to 1 mL by adding D₂O. Later the diluted solutions were mixed with 4 mL of distilled water, 0.3 mL of 5% NaNO₂ and further added with 0.3 mL of 10% AlCl₃ after an incubation time lasting approximately 5 min. Then, 2 mL of 1 mol/L NaOH solution were added followed by added distilled water to bring the final volume to 10 mL. This mixture was incubated at room temperature for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the results were expressed as mg

quercetin equivalent (QE)/g of extract. The standard calibration curve of quercetin was plotted.

$$\begin{aligned} \text{Total Phenolic Content TPC, A} \\ = (C \cdot V) / M \text{ (mg/gm)} \end{aligned}$$

Here, A is the flavonoid assay, C is the quercetin acid equivalent concentration, V is the volume of the extract solution used and M is the weight of the extract used.

DPPH radical scavenging assay

The DPPH scavenging activity of both plant extracts was measured according to previous methods with a few modifications [49]. Sample extract and L-ascorbic acid (standard) were weighed and dissolved in 95% methanol to make a homogenous stock solution with the highest concentration i.e. 100 µg/mL, with the help of vortex. Then aliquots of 5 concentrations (3.12, 6.25, 12.5, 25, 50 and 100 µg/mL) were created through a serial dilution technique both for plant extract and L-ascorbic acid (Research lab, India), which served as a positive control. DPPH (Sigma-Aldrich, Germany) was also weighed and dissolved in 95% methanol to make 0.2 mM solution. 2 mL of 0.2 mM DPPH solution was placed by micropipette into each test tube containing 2 mL of sample solution. The final volume of the solution was 4 mL. Then the test tube was retained in a dark environment for 30 min to complete the reaction. A blank test tube was also filled with DPPH with an equal volume of 95% methanol. The absorbance of each test tube was determined by UV spectrophotometer at 517 nm.

Percentage of inhibition was calculated as-

$$\% \text{inhibition} = \{(A_o - A_s) / A_o\} * 100$$

Here, A_o is the absorbance of positive control and A_s is the absorbance of the sample.

The IC₅₀ value is the concentration of the sample required to scavenge 50% of DPPH free radical and we calculated this from the plot of % inhibition against the log concentration of sample extract.

Peroxide radical scavenging assay

In this study, we used a prior method [50] with some modifications for our experiments. A solution of 43 mM H₂O₂ was prepared in 0.1 M phosphate buffer (pH 7.4). Both extract and standard solution were prepared at four different concentrations (7.8125, 15.625, 31.25 and 62.5 µg/mL). Sample solutions were dissolved in 3.4 mL of phosphate buffer and 0.6 mL of H₂O₂ solution (43 mM) was added. Absorbance was measured at 230 nm by UV spectrophotometer. A blank was prepared using a sodium phosphate buffer without H₂O₂. The percentage of H₂O₂ scavenging was calculated using the following equation:

$$\% \text{inhibition} = \{(A_o - A_s) / A_o\} * 100$$

Here, A_o is the absorbance of positive control and A_s is the absorbance of the sample.

The IC₅₀ value is the concentration of the sample required to scavenge 50% of H₂O₂ free radicals and we calculated this from the plot of % inhibition against the log concentration of sample extract.

Ferric reducing antioxidant potential (FRAP) assay

Ferric reducing activity of both plant extracts was measured according to a previously documented method [51]. Crude extracts (100–500 µg) were added to 300 µl of distilled water followed by 3 mL of FRAP reagent. The FRAP reagent was prepared by adding 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mmol/L HCl and 20 mmol/L FeCl₃·6H₂O at a ratio of 10:1:1. The absorbance was read at 593 nm after 4 min incubation at ambient temperature against a blank of distilled water. Results were expressed in mmol Fe (II)/g of a sample using FeSO₄·7H₂O standard curve.

Anti-hemolytic activity assay

The anti-hemolytic potential of both extracts was determined by the spectrophotometric procedure as described previously with few modifications [52]. Ten milliliters of blood from a healthy person was collected in EDTA vials (10%) and centrifuged for 10 min at 3000 rpm. The supernatant was removed and the pellet was washed thrice with 0.2 M PBS (pH 7.4) and centrifuged for 10 min at 300 rpm before re-suspending in saline solution (0.9% NaCl). 0.4 mL of extracts (125–1000 µg/mL in PBS) were dispensed to 0.4 mL of erythrocyte suspension and incubated at 37 °C for 5 min. Later, 0.2 mL of H₂O₂ (0.82 M, PBS) solution was added to the reaction mixture for inducing the membrane lipids' oxidative degeneration. The samples were again incubated at a temperature of 37 °C at 120 rpm for 3 h. After incubation, the samples were subsequently centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant was noted at 540 nm. Relative hemolysis was assessed in comparison with the hemolysis in H₂O₂ treated negative control. In this experiment, L-ascorbic acid was used as the standard (31.5–1000 µg/mL) and the blank solution was prepared by adding 0.2 M PBS and 0.82 M H₂O₂. Approval for this experiment was obtained from the Ethical Research Committee, Jashore University of Science and Technology, Jashore, Bangladesh.

Antibacterial activity assay

The antibacterial activity of each plant extract was evaluated using seven bacterial strains, of which five strains were Gram-negative (*Escherichia coli*, *Escherichia coli* O157: H7, *Salmonella enterica* subsp. *enterica*; serotype:

Typhi", [query: all this is worded correctly?] *Enterobacter cloacae* and *Salmonella enterica*) and the other two were Gram-positive (*Streptomyces aureus*, *Listeria monocytogenes*). All these strains were collected from the Microbiology Department laboratory, Jashore University of Science and Technology. The inoculums were prepared using a nutrient broth medium, and Mueller Hinton (MH) was used to grow the bacteria. Bacterial strains were gently swabbed over MH agar media by pouring 100 μ l of LB broth culture. Four holes were made in each petri dish by using the Bohrer method. And then four different doses of plant extract solution (50, 100, 200 and 400 mg/mL) were poured onto the petris dishes. After incubation lasting 24 h at 37 °C, the diameter zone of inhibition (mm) was measured against the test organisms. The antimicrobial activity of four antibiotics COL²⁵ (Colistin Sulfate-25 μ g/disc), NA³⁰ (Nalidixic acid-30 μ g/disc), C³⁰ (Chloramphenicol-30 μ g/disc) and E¹⁵ (Erythromycin-15 μ g/disc) was measured against three bacteria *E. coli*, *E. coli* O157:H7 and *S. aureus*. Another four antibiotics AZM³⁰ (Azithromycin-30 μ g/disc), CAZ³⁰ (Ceftazimide-30 μ g/disc), VA³⁰ (Vancomycin-30 μ g/disc) and AMP²⁵ (Ampicillin-25 μ g/disc) were measured against four bacteria, namely *S. typhi*, *E. cloacae*, *L. monocytogenes*, and *S. enterica*. These were also assayed as positive control.

Brine shrimp lethality assay

The previous method was followed to determine brine shrimp lethality assay of both plant extracts [53]. Eggs of brine shrimp were hatched in an aquarium using 1 L of 1 M sodium chloride brine solution (pH 8.5). The eggs were incubated for 48 h under fluorescent light and after hatching the nauplii were transferred to test tubes. Ten couples were transferred to each tube. 1.5 mL NaCl solution was added to each test tube. Plant extract solutions at five different concentrations (62.5, 125, 250, 500 and 1000 μ g/mL) were used for this assay. Test tubes later had 1 mL of NaCl solution added to them. 1% DMSO served as the negative control and after 12 h of incubation a count of the dead nauplii was done. LC₅₀ value is the concentration of the sample required to kill 50% of the brine shrimp population and this was calculated from the plot of % inhibition against the log concentration of sample extract. According to Meyer et al., a LC₅₀ value of less than 1 mg/mL is considered toxic while a LC₅₀ value greater than 1 mg/mL is deemed to be non-toxic [54].

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.1.0 (Graph Pad Software, Inc., USA).

Variations between and within groups have been analyzed for statistical significance by standard parametric and nonparametric tests, as appropriate. Linear regression analysis was also used to calculate the IC₅₀ values. $P < 0.05$ was taken as a level of statistical significance in all tests. The results were expressed as mean \pm SEM (Standard Error of Mean).

Results

Total phenolic content (TPC) of both plants' methanol extract was extrapolated from the calibration curve (Fig. 1a). They were 36.625 ± 0.551 and 58.917 ± 0.601 mg Gallic acid equivalents/g dry plant material in *H. littoralis* and *B. gymnorrhiza*, respectively (Table 1).

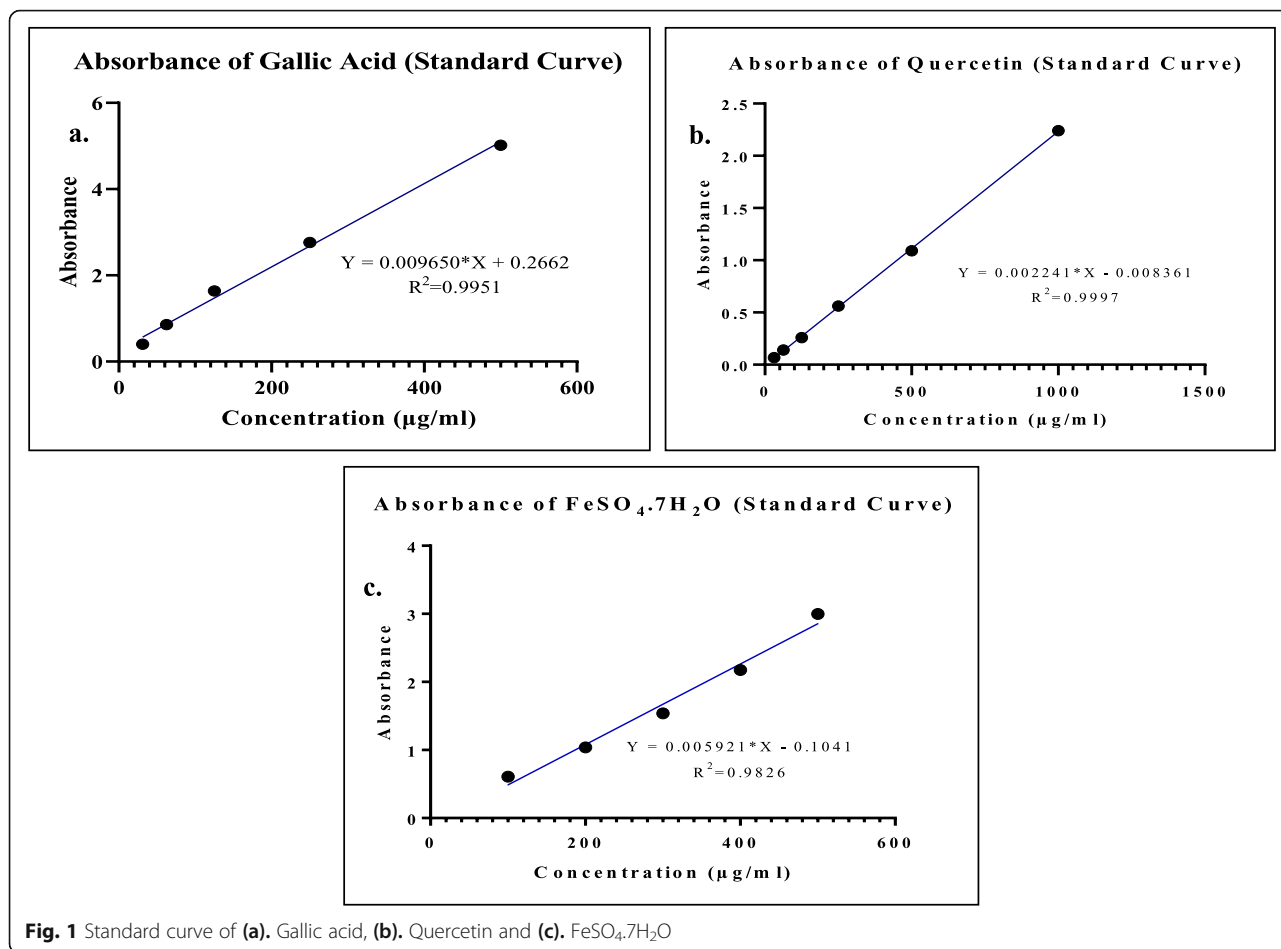
Total flavonoid contents were extrapolated from the linear equation of quercetin standard curve (Fig. 1 b). TFC obtained for both methanol extracts of *H. littoralis* and *B. mycorrhizae* are 114.52 ± 0.339 and 77.21 ± 0.016 mg quercetin equivalent/g extract (Table 1).

The methanol extracts of *B. gymnorrhiza* and *H. littoralis* exhibited a concentration-dependent DPPH scavenging activity (Fig. 2a) with values for IC₅₀ being 113.79 ± 0.168 μ g/mL and 121.23 ± 0.321 μ g/mL, respectively (Table 1). The DPPH scavenging activity was less than that of L-ascorbic acid (IC₅₀ 35.7 ± 0.46 μ g/mL), which is similar to what Hwang et al. (2007) reported and the value was 30.06 ± 0.42 μ g/mL [55].

The ability of *B. gymnorrhiza* and *H. littoralis* to scavenge hydrogen peroxide is summarized in Table 1. *B. gymnorrhiza* leaf extract exhibited a concentration-dependent H₂O₂ scavenging activity (Fig. 2b) with IC₅₀ of 112.91 ± 0.164 μ g/mL. Similarly, *H. littoralis* leaves exhibited 50% inhibition at a concentration of 140.32 ± 0.301 μ g/mL. The IC₅₀ value of L-ascorbic acid was 112.91 ± 0.164 μ g/mL.

Ferric reducing antioxidant capacity was calculated from the linear equation obtained from the standard curve of FeSO₄·7H₂O (Fig. 1c). In this study, methanol extracts of *B. gymnorrhiza* demonstrated a greater FRAP value (193.97 ± 0.508 mmol equivalent of Fe (II)/gram sample) than *H. littoralis* (101.11 ± 0.418 mmol equivalent of Fe (II)/gram sample) (Table 1). On the other hand, FRAP value for standard L-ascorbic acid was found to be 36.78 ± 0.263 mmol, which was equivalent to the Fe (II)/gram sample.

The anti-hemolytic activity of both methanol extracts was obtained from a concentration (125–1000 μ g/mL) dependent assay. The absorbance of *B. gymnorrhiza* treated samples ranged from 0.72–1.18 which is close to the absorbance obtained from samples treated with L-ascorbic (0.71–1.41). On the other hand, *H. littoralis* showed slightly low absorbance on a concentration-dependent assay (0.61–1.02). According to these results, *B. gymnorrhiza* and



H. littoralis were reported to have IC₅₀ values of 311.28 ± 5.48 and 526.90 ± 25.85 µg/mL, respectively. L-ascorbic acid indicated an IC₅₀ value of 282.14 ± 6.96 µg/mL. The results are summarized in Table 2.

The antibacterial properties of methanol extract of *B. gymnorrhiza* and *H. littoralis* have shown activity against both Gram-positive and Gram-negative bacteria, with MICs of 10.3–20.1 mg/mL and 6.8–17.6 mg/mL, respectively (Table 3). *B. gymnorrhiza* has demonstrated the most activity against *E. cloacae* (20.1 mg/mL) and *E. coli O157:H7* (18.8 mg/mL) (Fig. 3a), while *H. littoralis* was highly lethal against *S. enterica* (17.6 mg/mL) (Fig. 3b). The positive control

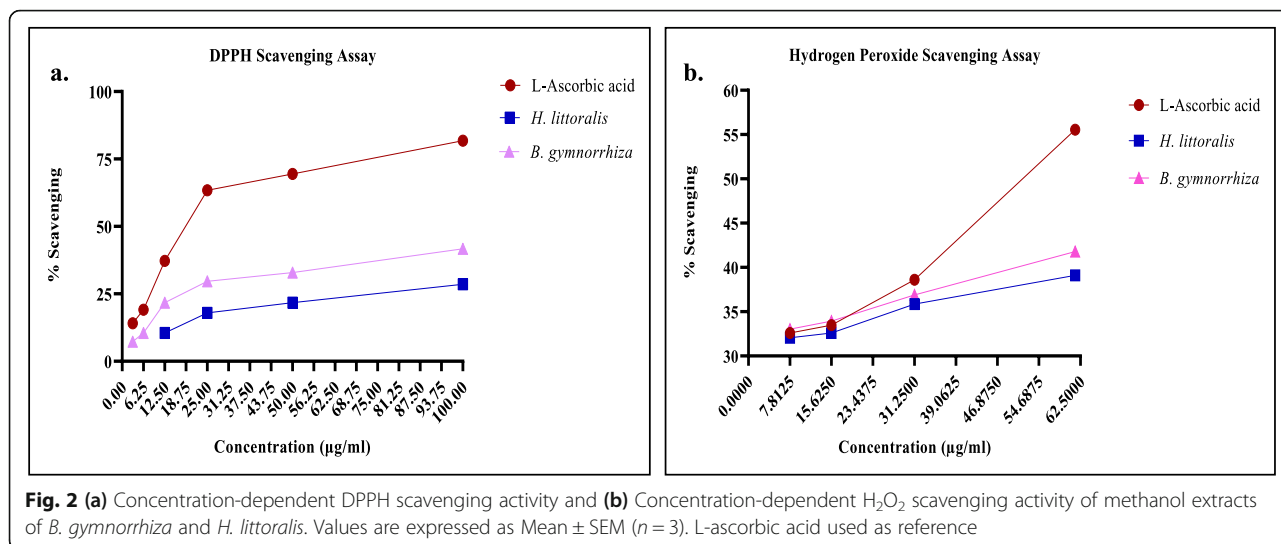
groups, on the other hand, showed growth inhibition against susceptible bacterial strains and were significant compared to the examined extracts. However, no inhibition zone was found in the negative control group.

Brine shrimp lethality assay of both plants extract was carried out in a concentration-dependent manner ranging from 62.5–1000 µg/mL (Fig. 4). The lethality concentration (LC₅₀) were within the 216.3–343.1 µg/mL range (LogLC₅₀ = 2.335–2.535 µM, R² = 0.9451) and 211.5–276.5 µg/mL (LogLC₅₀ = 2.325–2.442 µM, R² = 0.9789) for both *H. littoralis* and *B. gymnorrhiza*, respectively (Table 4).

Table 1 Antioxidant properties of methanol leaf extract of selected mangrove plant species

Tested materials	Total phenolic content ^a	Total Flavonoid content ^b	DPPH scavenging capacity IC ₅₀ (µg/mL)	H ₂ O ₂ scavenging capacity IC ₅₀ (µg/mL)	FRAP value ^c
<i>B. gymnorrhiza</i>	58.917 ± 0.601	77.21 ± 0.016	113.79 ± 0.168	112.91 ± 0.164	193.97 ± 0.508
<i>H. littoralis</i>	36.625 ± 0.551	114.52 ± 0.339	121.23 ± 0.321	140.32 ± 0.301	101.11 ± 0.418
L-ascorbic acid	–	–	35.7 ± 0.46	51.80 ± 0.143	36.78 ± 0.263

^amg gallic acid equivalent (GAE)/g sample, ^bmg quercetin equivalent (QE)/g sample, ^cmmol Fe(II) equivalent/g sample. Values are mean ± SEM (n = 3), Statistical significance p < 0.05



Discussion

Plants consist of a large group of phenolic compounds, such as simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids. Because of their physiological functions such as free radical scavenging, anti-mutagenic, anti-carcinogenic and anti-inflammatory effects, phenolic compounds of all classes have attracted much scientific attention [56–58]. According to Pietta (2000) and Soobrattee (2005), the antioxidant activity of phenol compounds largely occurs due to their redox properties which make them act as reducing agents, hydrogen donors, singlet oxygen quenchers and as well as potential metal chelators [59, 60]. The hydroxyl group of phenolics and their derivatives mostly react with reactive oxygen and reactive nitrogen species in a termination reaction, which breaks the cycle of a generation of new radicals [61–65]. The interaction of the hydroxyl groups of phenolics with the π-electrons of the benzene ring gives the molecules special properties and allows them to generate free radicals where each radical is stabilized by delocalization. Formation of these relatively long-lived radicals is able to modify the radical-mediated oxidation process [66]. In this study, a considerably moderate level of phenolics was observed in the methanol extracts of *B. gymnorrhiza* and *H. littoralis* (Table 1).

The hydroxyl group of polyphenols is responsible for the scavenging of free radicals which also allows rapid screening of antioxidant activity. According to the results documented by Sur et al. (2016), the amount of phenolic content found in hydro-methanol extract of *B. gymnorrhiza* leaves was smaller than the phenolic content found in this study (2.34 ± 0.039 µg Gallic acid equivalent/mg extract). Meanwhile the results of Nurjanah et al. (2016) reported 30.07 mg GAE/g extract in methanol extract of *B. gymnorrhiza* [67, 68]. On the other hand, this is the first report on the antioxidant activity of *H. littoralis*. Chemical structures of the total phenolic compounds are determined by the level of solvent polarity [69]. Polar solvents are highly capable of attracting phenolic compounds and the outcome is high phenolic activity. Ethanol has a higher polarity than methanol. Results reported by Haq et al. (2011) also showed higher phenolic content in ethanol extract when compared to methanol and chloroform [70].

The results of total flavonoid content (TFC) assay reported the highest presence of flavonols in *H. littoralis* compared to *B. gymnorrhiza* extract (Table 1). Flavonoids may have an additive effect on the endogenous scavenging compounds, which are subsequently depleted due to increased production of reactive oxygen species during an injury [71]. These compounds play an important role as plants secondary metabolites (flavones,

Table 2 Anti-hemolytic activity of methanol leaf extracts of selected mangrove plant species

Tested materials	Positive control (A)	Negative control (B)	Optical density at 540 nm at concentration (µg/mL)				IC ₅₀ (µg/mL)
			125	250	500	1000	
<i>B. gymnorrhiza</i>	1.45 ± 0.005	0.11 ± 0.001	0.72 ± 0.01	0.82 ± 0.01	0.94 ± 0.01	1.18 ± 0.01	311.29 ± 5.48**
<i>H. littoralis</i>	1.45 ± 0.005	0.11 ± 0.001	0.61 ± 0.01	0.72 ± 0.02	0.88 ± 0.02	1.02 ± 0.01	526.90 ± 25.85**
L-ascorbic acid	1.45 ± 0.005	0.11 ± 0.001	0.71 ± 0.02	0.76 ± 0.01	1.08 ± 0.01	1.41 ± 0.01	282.14 ± 6.96**

All values represent mean ± SEM (n = 4), Statistical significance (p < 0.005) is the same for values in the same column (*). **A;** phosphate buffer saline treatment results in 0% hemolysis, **B;** hydrogen peroxide replaced extract to serve as a negative control, treatment results with 100% hemolysis

Table 3 In vitro activity of methanol extracts of *B. gymnorrhiza* and *H. littoralis* leaves against some opportunistic pathogens

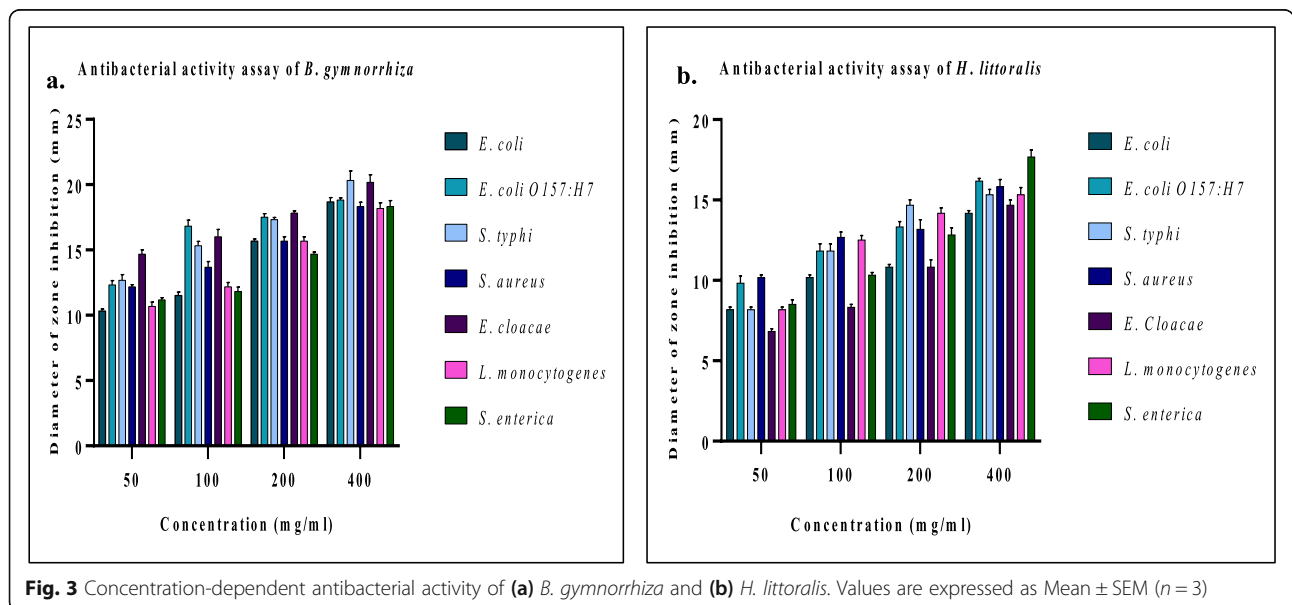
Diameter of the zone of inhibition (mm)								
Tested materials	Concentration (mg/mL)	EO ₁	EO ₂	EO ₃	EO ₄	EO ₅	EO ₆	EO ₇
<i>B. gymnorrhiza</i>	50	10.3 ± 0.17	12.3 ± 0.33	12.6 ± 0.44	12.1 ± 0.17	14.6 ± 0.33	10.6 ± 0.33	11.1 ± 0.17
	100	11.5 ± 0.29	16.8 ± 0.44	15.3 ± 0.33	13.6 ± 0.44	16.0 ± 0.58	12.1 ± 0.33	11.8 ± 0.33
	200	15.6 ± 0.17	17.5 ± 0.29	17.3 ± 0.17	15.6 ± 0.33	17.8 ± 0.17	15.6 ± 0.33	14.1 ± 0.17
	400	18.6 ± 0.33	18.8 ± 0.17	20.3 ± 0.72	18.3 ± 0.33	20.1 ± 0.60	18.1 ± 0.44	18.3 ± 0.44
<i>H. littoralis</i>	50	8.1 ± 0.17	9.8 ± 0.44	8.1 ± 0.17	10.1 ± 0.17	6.8 ± 0.17	8.1 ± 0.17	8.5 ± 0.29
	100	10.1 ± 0.17	11.8 ± 0.44	11.8 ± 0.44	12.6 ± 0.33	8.3 ± 0.17	12.5 ± 0.29	10.3 ± 0.17
	200	10.8 ± 0.17	13.3 ± 0.33	14.6 ± 0.33	13.1 ± 0.60	10.8 ± 0.44	14.1 ± 0.33	12.8 ± 0.44
	400	14.1 ± 0.17	16.1 ± 0.17	15.3 ± 0.33	15.8 ± 0.44	14.6 ± 0.33	15.3 ± 0.44	17.6 ± 0.44
NA ³⁰	–	20.3 ± 0.17	23.1 ± 0.17	–	23.6 ± 0.17	–	–	–
C ³⁰	–	31.0 ± 0.50	27.8 ± 0.44	–	29.3 ± 0.33	–	–	–
AZM ³⁰	–	–	–	16.1 ± 0.17	–	35.8 ± 0.44	17.0 ± 0.58	16.6 ± 0.33
CAZ ³⁰	–	–	–	17.6 ± 0.33	–	19.8 ± 0.17	21.1 ± 0.60	18.1 ± 0.44
VA ³⁰	–	–	–	–	–	16.3 ± 0.67	–	–
AMP ³⁵	–	–	–	23.1 ± 0.60	–	15.3 ± 0.17	13.8 ± 0.44	21.1 ± 0.60
Negative control (methanol)	–	–	–	–	–	–	–	–

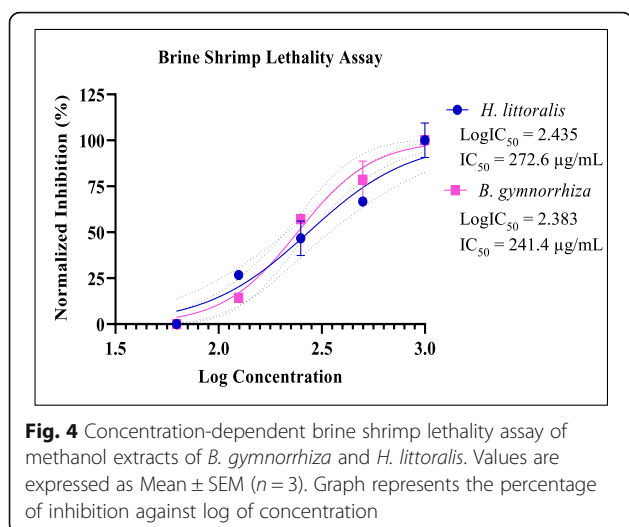
All the data are means of three replicates (n = 3) ± SEM, **EO = Experimental Organism; EO₁ = *E. coli*, EO₂ = *E. coli* O157:H7, EO₃ = *S. typhi*, EO₄ = *S. aureus*, EO₅ = *E. cloacae*, EO₆ = *L. monocytogenes*, EO₇ = *S. enterica*. COL²⁵ and E¹⁵ showed no zone of inhibition in this experiment

flavanols, and condensed tannins). Their antioxidant activity depends on the concentration of free OH groups, especially 3-OH. Flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of free radicals. The high reactivity of the hydroxyl group of the flavonoids inactivates the free radicals [72]. It has both in vitro and in vivo antioxidant activity characteristics [73, 74]. Sur et al. (2016) reported 5.20 ± 0.115 µg quercetin equivalent/mg hydro-methanol extract of *B.*

gymnorrhiza which is significantly smaller than our study's finding. The smaller quantity might have resulted due to the higher concentration of free OH⁻ radicals of hydro-methanol extract in the previous study [67]. There are no previous reports of the flavonoid profile of *H. littoralis*.

DPPH scavenging assay is one of the best-known, accurate, and frequently employed methods which we used in this analysis. As shown in Table 1, the DPPH





quenching capacity was found to be greater in *B. gymnorrhiza* than *H. littoralis*. One study, however, reported by Haq et al. (2011) showed slightly higher DPPH scavenging activity in ethanol extract of *B. gymnorrhiza* [70]. The difference may be caused by the presence of non-polar compounds (fats and oils). Similarly, another study reported 96.8% radical scavenging at 100 µg/mL of ethanol extract of *H. littoralis* leaves, which is approximately 3.5 times higher than the result obtained in our study [39]. Furthermore, the results confirmed a significant correlation between total phenolic content and DPPH scavenging capacity. Sun and Ho (2005) reported a significant correlation between total phenolics and scavenging ability of buckwheat extracts on DPPH radicals [75]. It can be inferred from the results that both of our plants have stable DPPH radical scavenging potential by antioxidants compounds present in the extracts. However, L-ascorbic acid showed the highest DPPH radicals scavenging with IC₅₀ of 35.7 ± 0.46 µg/mL.

Hydroxyl radical (OH[•]) is the most reactive free radical which has the capacity to conjugate with nucleotides in DNA and cause strand breakage that may lead to carcinogenesis, mutagenesis, and cytotoxicity [76]. Of the two mangrove species, *B. gymnorrhiza* resulted in comparatively better H₂O₂ scavenging activity than *H. littoralis* (Table 1). According to the results, both plants have the potential to be cytotoxic and this means generating hydroxyl radicals in cells [77]. H₂O₂ scavenging results from the phenolic contents of the extracts which are able to donate electrons and thus form water [78]. These

results also showed a significant relationship between H₂O₂ scavenging capacity and available total phenolic content. However, the IC₅₀ value of L-ascorbic acid was significantly better than both of the plant extracts, which may suggest a methanol extracts engaged in only weak antioxidant activity compared to the standard.

The FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe₃⁺-TPTZ) complex and produces a colored ferrous tripyridyltriazine (Fe₂⁺-TPTZ) [51]. Ferric reducing antioxidant properties are associated with the presence of compounds that cleave the free radical chain by donating a hydrogen atom [79, 80]. The reducing properties are mostly linked with the presence of compounds which exert their action by breaking the free radical chain by releasing a hydrogen atom [79]. In the present study as shown in Fig. 3 b, the absorbance of Fe₂SO₄.7H₂O standard curve clearly increased, due to the formation of the Fe²⁺-TPTZ complex with an increasing concentration. Highest Fe²⁺ quenching concentration was reported in *B. gymnorrhiza* extract. Results of the FRAP assay of both plants were significantly higher than the FRAP value of L-ascorbic acid (36.78 ± 0.263 mmol equivalent of Fe (II)/gram sample). They show a strong relationship between total phenolic content and FRAP assay which is similar to the findings reported by Benzie and Szeto [81]. The redox potential of phenolic compounds allows them to act as hydrogen donors, reducing agents and singlet oxygen quenchers [80]. Such redox potential has played an important role in the determination of antioxidant potential. To date, no report on FRAP assay found for any of our experimental plants has been published. Consequently, it can be inferred from the results (Table 1), that both of our plants have higher Fe²⁺ quenching potential than the available commercial antioxidants.

Erythrocytes are the most abundant cells in the human body with their own replicative biological and morphological characteristics. The hemoglobins and polyunsaturated fatty acids (PUFA) mainly target the erythrocytes due to their redox-active oxygen transportation feature. As a result, hemolysis of erythrocyte membrane lipids and proteins are mutilated by oxidation. Such mutilation is accompanied by a number of factors such as deficiencies in erythrocyte antioxidant coordination, radiation, high quantity of transition metals, oxidative drugs, and hemoglobinopathies, etc. [78, 82]. Hemolysis occurs at a higher rate when erythrocytes are exposed to toxins like hydrogen peroxide [83]. In this experiment, we aimed at

Table 4 Cytotoxic activity of methanol leaf extract of selected mangrove plant species on brine shrimp nauplii

Tested plants	Concentration tested (µg/mL)	LogLC ₅₀ (µM)	LC ₅₀ (µg/mL)	95% confidence interval
<i>B. gymnorrhiza</i>	62.5, 125, 250, 500, 1000	2.383	241.4	211.5–276.5
<i>H. littoralis</i>	62.5, 125, 250, 500, 1000	2.435	272.6	216.3–343.1

determining whether *B. gymnorrhiza* and *H. littoralis* prevented oxidative damage to the erythrocyte membrane or not. According to the results, *B. gymnorrhiza* exhibited higher anti-hemolytic activity with an IC_{50} value of 311.28 ± 5.48 $\mu\text{g/mL}$ while *H. littoralis* exhibited less significant activity with an IC_{50} value of 526.38 ± 25.85 $\mu\text{g/mL}$ (Table 2). The IC_{50} value of L-ascorbic acid is 282.14 ± 6.96 $\mu\text{g/mL}$. In all cases, lysis of erythrocytes declined when an increase in the concentration of extracts and standards occurred. *B. gymnorrhiza* showed an effective hemolysis prevention activity. Furthermore, the results show a strong connection between total phenolic content and anti-hemolytic assay.

The obtained results suggest that both *B. gymnorrhiza* and *H. littoralis* are a potential source of broad-spectrum antibacterial agents (Table 3). A more recent study on the antibacterial activity of n-hexane extract of *B. gymnorrhiza* leaf showed a significant level of inhibition against a large number of opportunistic bacterial species compared to the aqueous extract [84]. The antibacterial activity of the methanol extracts may be attributed to their total flavonoid content (TFC), which has been reported to be involved in inhibition of nucleic acid biosynthesis and also a number of other metabolic processes of microorganisms [85]. Flavonoids are also known to influence the permeability of both natural and synthetic membranes which might be a strong basis of the often mentioned bacteriocidal and antimycotic activity of flavonoids and propolis [86, 87].

Flavonoids are also known for their inhibitory activity against enzymes such as ATPase, phospholipase A_2 , prostaglandin cyclooxygenase and lipoxygenase [87]. One study has reported the ability of flavonoids and its derivatives to inhibit spore germination of plant pathogens [88]. Ohemeng et al. (1984) screened 14 derivatives of flavonoid for their inhibitory activity against *Escherichia coli* DNA gyrase, and for antibacterial activity against *Staphylococcus epidermidis*, *S. aureus*, *E. coli*, *Salmonella typhimurium* and *Stenotrophomonas maltophilia* [89]. The phenolic compounds with a C3 side chain also result in antimicrobial activity by lowering the level of oxidation [90]. The partially hydrophobic nature of polyphenols means they exhibit microbial lethality by inhibiting microbial hydrolytic enzymes (protease) or by inactivating microbial adhesins, cell envelop transport proteins and non-specific interaction with carbohydrates [91]. The polarity of methanol might have contributed to the activity of polyphenols thus resulting in microbial inhibition.

In the brine shrimp lethality assay, both plants showed a significant level of cytotoxicity (Table 4). The results of our experimental plants are significantly lower than the cytotoxicity of Vincristine sulfate ($LC_{50} = 0.91$ $\mu\text{g/mL}$) reported by Ullah et al. [92]. According to Meyer et al., crude extracts of plants are considered toxic (active) if

they have a LC_{50} value less than 1000 $\mu\text{g/mL}$ [54]. Brine shrimp lethality assay permits a larger number of samples and dilutions by using a smaller quantity of extracts within a shorter time than using the original test vials [93]. Furthermore, it has been established that those crude extracts which exhibit significant cytotoxicity against *A. salina* larvae can be further recommended as a guide for pesticidal and anti-tumor compound detection. This is due to its simplicity, low cost and significant correlation with human solid tumor cell lines [84]. The in vitro antioxidant activity displayed by our experimental plants is a primary indicator of in vivo anti-tumor activity. However, there is a wide range of phytochemicals capable of exhibiting non-specific cytotoxic effects against *A. salina* larvae. On the other hand, anti-bacterial reported in this study represented a higher inhibitory zone at 500 mg/mL which is significantly higher than the LC_{50} value obtained from the cytotoxicity assay. Thus, further studies by isolating pure compounds via GC-MS are required to identify the toxic compounds responsible for the resulting cytotoxicity.

Conclusion

It can be suggested here that methanol extracts of *B. gymnorrhiza* and *H. littoralis* leave exert a stabilizing effect on reactive oxidative species (ROS), inhibiting pathogenic bacterial strains and promoting cytotoxicity. In most cases, *B. gymnorrhiza* resulted in the highest yield of phenolic compounds than *H. littoralis*, which in contrast yielded higher flavonols. *B. gymnorrhiza* was more potent than *H. littoralis* in terms of antioxidant potential. However, the methanol extracts did not significantly reduce the free radicals under in vitro conditions. Both species have shown significant Fe^{2+} quenching, antibacterial and anti-hemolytic activity on human red blood cells (RBCs). Furthermore, this study paves the way to search for more effective antimicrobial compounds by isolating pure compounds and thereby developing novel antibiotic drugs against opportunistic bacterial species. Moreover, hepatoprotective, anti-hyperglycemic, anti-nociceptive, analgesic and anti-tumorigenic compounds from both of these mangrove species and can be isolated and administered for therapeutic approaches in vivo.

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Author' contributions

Md. AK and Md. AI conducted all the experiments. Mst. SS and SB carried out the antibacterial activity assay. Md. MI and Md. SR helped in the collection of plant material and executing of the experiments. Md. AK wrote the manuscript. Dr. NH has supervised the entire experiment and manuscript preparation. MJH, KKM, and Md. MR revised the manuscript and worked to improve the quality of the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

This study was approved by the Ethical Research Committee of Jashore University of Science and Technology, Jashore-7408, Bangladesh.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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