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Investigation of Antibacterial, Cytotoxic and Antioxidant Properties of the Mangrove Plant *Xylocarpus mekongensis*

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Abstract

The plant (*Xylocarpus mekongensis*) of the Sundarbans mangrove origin was evaluated for its antibacterial, cytotoxic and antioxidant properties using methanolic and chloroformic leaf, stem and bark extracts, respectively. The methanolic extracts contained higher amount of total phenolics, flavonoids, tannins than the chloroformic extracts and the result was in correlation with their ferric reducing power ability as well. However, the chloroformic bark extract contained more potent DPPH free radical scavenging activity than others. Antibacterial activity of the extracts was determined against both Gram-positive (*Micrococcus* and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Salmonella typhimurium* and *Salmonella paratyphi*) by disc diffusion assay and their zone of inhibitions (ZOI) were measured. Moreover, their minimum inhibitory concentrations (MIC) were determined by tube dilution method. Chloroformic bark and stem extracts showed strong inhibition to growth of *P. aeruginosa* (ZOI = 19 mm and MIC = 150 µg/ml) and *S. aureus* (ZOI = 19.5 mm and MIC = 250 µg/ml), respectively. All six extracts were subjected to brine shrimp lethality bioassay for possible measure of cytotoxicity. Concentration dependent increment in percentage mortality of brine Shrimp nauplii produced by the extracts indicated the presence of cytotoxic principles in these extractives. Therefore, *Xylocarpus mekongensis* showed antioxidant, antibacterial and cytotoxic activities.

Keywords

***Xylocarpus mekongensis*, Antioxidant, Antibacterial and Cytotoxicity**

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1. Introduction

The mangroves are a promising source of natural products as several bioactive compounds have been isolated [1] [2]. In this study, the mangrove plant *Xylocarpus mekongensis* has been chosen to scientifically validate the usage of the plant in traditional medicine or ethno medicine.

Xylocarpus mekongenesis (*X. mekongenesis*) (Lamk.) L.Mohan (Meliaceae) is commonly known as Passur. It is a glabrous, medium-sized tree that grows generally on the inter-tidal silty but consolidated clay or on the sandy or rocky bay distributed throughout the tropical and subtropical regions of Southeast Asia including the Sundarbans [3]. The plant has well developed aerial blunt end pneumatophores or root suckers and green coloured fruit of diameter generally not exceeding 15 cm [4].

The genus *Xylocarpus* consists of *Xylocarpus granatum*, *X. moluccensis*, *X. mekongensis*, *X. rumphii* that are ethnomedicinally important mangrove plants. Extracts of leaves, barks, pneumatophore and fruits of these plants have been reported for various ethnomedicinal uses such as fever, malaria, inflammation, dysentery, diarrhoea, cholera, abdominal problems, diabetes, elephantiasis, antimicrobials etc. In recent times, these plants are also reported for their antioxidant, anticancer, antidiabetic, antidyslipidemia, antimicrobials, antidiarrhoeal, antifilarial, antiulcer and cardiotonic properties [5]-[14] investigated the antibacterial and cytotoxic activities of *X. mekongensis* methanolic, ethyl acetate and chloroform bark extracts. However, the present study took into account three different types of plant materials including bark and in addition, also antioxidant activities from these extracts were determined.

The nature of the bioactivity analysis is permutative due to the uses of various plant parts and employing different solvent systems to extract the bioactive natural products. Therefore there remains the scope to analyze the bioactivity of *X. mekongensis*. Most of the previous studies on *X. mekongensis* were carried out with a single solvent system for extraction of plant materials while the present study employed both polar and non-polar solvent systems for sequential extraction of plant materials from three types of plant parts. The objective of this study was to evaluate the pharmacological basis of biological activity on *X. mekongensis* through antioxidant, antimicrobial and cytotoxic assay of chloroformic and methanolic leaf, stem and bark extracts, respectively.

2. Materials and Methods

2.1. Chemicals

Folin-Ciocalteu (FC) reagent, aluminium chloride, sodium hydroxide, sodium carbonate, and sodium nitrite were purchased from Merck Specialities Private Limited, Mumbai, India. Ferric Chloride, Ethanol, Methanol, Chloroform were bought from Merck KGaA, Germany. Potassium ferricyanide was purchased from UNI-CHEM chemical reagents, China. Gallic acid, 2, 2-diphenyl-1-picryl hydrazyl (DPPH) and Quercetin were brought from Sigma-Aldrich Chemicals Pvt. Ltd. (Germany). Nutrient agar was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India) and the rest of the chemicals and solvents used were of analytical grade.

2.2. Collection of Plant Material

The plant sample of *X. mekongensis* was collected from Dhangmaree, Chadpai range, East zone of the Sundarbans, Khulna, Bangladesh on 16th December, 2011 and collected plant samples were sent to Bangladesh National Herbarium, Dhaka, Bangladesh for taxonomical identification. A voucher specimen (Accession No.: DACB-35372) was also deposited.

2.3. Extraction

The plant materials (bark, stem and leaf) were separated from each other and then cleaned by gentle washing with distilled water followed by air drying for several weeks. The dried material was ground into coarse powder with a motorized plant grinder (capacitor start motor, Wuhu Motor Factory, China). The powder was kept in a dry, cool and dark place in a suitable airtight container until analysis commenced. The dried material was ground into coarse powder with a motorized plant grinder (capacitor start motor, Wuhu Motor Factory, China). The powder was kept in a dry, cool and dark place in a suitable airtight container until analysis commenced. About 120 gm of powdered leaf, 160 gm of powdered stem and bark each was soaked into 440 mL and 500 ml petroleum ether respectively, in three clean, flat-bottomed glass containers for a period of 5 days with occasional stirring and shaking. It was then filtered and after this first filtration, the remaining residues (approx. 115 gm

powdered leaf, 157 gm powdered stem and 155 gm bark) were soaked into 400, 470 and 450 ml chloroform respectively, kept for a period of 6 days and then filtered; then final remaining residues (113 gm of powdered leaf, 155 gm of powdered stem and 150 gm of powdered bark) were soaked into 390, 450 and 450 ml methanol respectively, kept for a period of 6 days with occasional stirring and shaking and then filtered. Coarse plant material was separated from the mixture by pouring through a clean cloth filter. These extracts were passed through filter paper, and the filtrates were evaporated, yielding the chloroformic and methanolic extracts, respectively.

2.4. Screening of Antioxidant Activity

2.4.1. Measurement of DPPH Free Radical Scavenging Activity

The free radical scavenging property of extracts were analyzed by 1, 2-diphenyl 1-picryl hydrazyl (DPPH) assay developed by Brand-Williams *et al.*, [15]. In determining DPPH free radical scavenging activity, different concentrations (1.75, 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml) of the extracts and the positive control (Quercetin) were prepared in ethanol. Then 2 mL of 0.004% DPPH solution was added in test tube of different extracts. The test tubes were allowed to stand at dark for 30 min to complete the reaction and then absorbance was recorded at 517 nm [16]. The decrease in absorbance with blank was also measured. Control was prepared in the same way as the sample except addition of sample or standard. Percent scavenging activity was calculated using the formula: scavenging activity = $(A_0 - A_1)/A_0 \times 100\%$, where A_0 is the absorbance of control, and A_1 is the absorbance of sample or standard. The experiment was carried out in triplicate.

2.4.2. Determination of Reducing Power

The reducing power of the extract was evaluated according to the method of Oyaizu (1986) [17]. According to this method different concentrations of extracts (25, 50, 100, 200 and 400 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50˚C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Blank was prepared same as sample without addition of extract or standard. Quercetin was used as standard. Reducing power of the extract was compared with standard quercetin by drawing curve plotting absorbance against concentration.

2.4.3. Determination of Total Phenolic, Flavonoid, Tannin Content

Total phenolic content of the extracts was determined by using Folin-Ciocalteau assay [18] where extract or standard solution (25 to 250 µg/ml) of 1 ml was added to distilled water (9 ml), and then 1 mL of FC reagent (10 times diluted with distilled water). After 5 min, 10 ml 7% Na_2CO_3 was added to the mixture, kept for 30 minutes and then the absorbance was measured at 750 nm using UV spectrophotometer. The percentage of total phenolics was calculated from the calibration curve of gallic acid plotted by using the similar procedure as the extracts and expressed as mg gallic acid equivalent (GAE)/g dried plant material.

Total flavonoid content of the extracts was determined by using an aluminium chloride colorometric assay [18] where extract or standard solution (25 to 400 µg/ml) of 1 ml was added to distilled water (5 ml); 0.3 ml 5% $NaNO_2$ then added to the mixture followed by addition of 0.6 ml 10% $AlCl_3$ and 2 ml 1M NaOH after 5 min. Then absorbance was measured at 510 nm; percentage of total flavonoids was calculated from the calibration curve of quercetin plotted by using the similar procedure as the extracts and expressed as mg quercetin equivalent (QE)/g dried plant material.

Total tannins content in plant extract was determined by using Folin-Denis method as described by Polshettiwar *et al.* 2007 [19]. Extract solution (1 ml of 100 µg/ml) was mixed with 7.5 ml distilled water and 0.5 ml FC reagent. After 5 min, 1 ml of 35% sodium carbonate was added and the final volume was adjusted to 10 ml with distilled water. The mixture was allowed at room temperature for 30 minutes and absorbance was measured at 725 nm using spectrophotometer. Gallic acid was used as standard for calibration curve.

2.5. Determination of Antibacterial Activity

2.5.1. Screening for Antibacterial Activity

Seven strains of microorganisms were tested in this study. Two Gram-positive bacteria include *Micrococcus*

spp., *S. aereus* (ATCC 25923) and five Gram-negative bacteria include *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 27833), *S. typhimurum* (ATCC 13311), *V. cholerae* and *S. paratyphi*. These strains were collected from the Microbiology Laboratory, Khulna University, Bangladesh, as pure cultures were used. The bacterial isolates were cultivated in nutrient broth at 37˚C for 24 hours.

Antibacterial activity of *X. mekongensis* extracts was tested by disc diffusion method [20]. Bacterial strains were maintained on the nutrient agar medium. The sterile filter paper discs were prepared by adding desired concentration (250 and 500 µg/disc) of extracts on the disc with the help of a micropipette. Standard Erythromycin disc (10 µg/disc), discs containing extracts and control discs were then impregnated, incubated overnight at 37˚C, checked for the zone of inhibitions and then diameters of inhibition zone were measured in millimeters (mm). Each measurement was carried out in triplicate.

2.5.2. Determination of MIC Values

The extracts that showed antimicrobial activity in disc diffusion were later tested to determine the MIC value for each bacterial sample by the tube dilution method from [21] with minor modification. Briefly, a stock solution of 20 mg/ml was prepared and was serially diluted to obtain various ranges of concentrations between 100 and 1000 µg/ml. From stock solution, different concentrations were prepared by adding different volume into sterile test tubes containing 2.0 ml of nutrient broth. Then 10 µl of freshly grown inoculums (from 4 hr culture, 5×10^6 cfu/ml) was added to each test tube. A set of test tubes containing broth alone was used as control. All the test tubes and control were then incubated at 37˚C for 24 h. After incubation, the MIC of each sample was calculated by measuring the optical density (OD) using spectrophotometer at 700 nm.

2.6. Screening of Cytotoxic Activity

Brine shrimp lethality bioassay was carried out according to [22] to investigate the cytotoxicity test while vincristine sulphate was used as positive control [23] [24]. The eggs of the brine shrimp, *Artemia salina*, and sea water were collected from BRAC prawn hatchery, Sreeghat, Bagerhat, Bangladesh. Followed by 24 h hatching, eggs matured and these were then called nauplii. *X. mekongensis* extracts were dissolved in DMSO and was added in test tubes in such a way that each tube contained 4 mL of sea water with different concentrations of extracts ranging from 5 to 320 µg/ml. The final volume for each test tube was adjusted to 10 mL with artificial sea water and 10 living nauplii were introduced into each tube. After observing test tubes in the subsequent 24 hours, the number of survived nauplii was recorded [25]. The percentage of dead nauplii in the test and standard group was established by linear correlation when logarithm concentration versus percentage of mortality was plotted and LC_{50} value was calculated.

2.7. Statistical Analysis

The results were expressed as means ± standard deviation (SD). *P* values < 0.05 were considered as the level of significance. The correlation statistical analysis was performed for observing the correlation among different type of assay data of antioxidant activity. Regression analysis was conducted for analyzing the data obtained from brine shrimp lethality bioassay to study the relationship between different samples and vincristine sulphate. The statistical analysis was carried out using GraphPad Prism Version 6.01 (GraphPad Software, Inc., USA).

3. Results and Discussion

The antioxidant capacity expressed as IC_{50} value, %increase in reducing power, total phenolic content (mgGAE/g), total flavonoid content (mg QE/g), total tannin content (mgGAE/g)of all extracts are shown in **Table 1** ($P <$ 0.05). The test of free radical scavenging properties of plant extracts was carried out on the basis of dose dependent scavenging of DPPH free radicals which is apparent in **Figure 1**. Among all the extracts, scavenging activity was highest in chloroformic bark (IC_{50}~25.94 µg/ml) than others. Additionally, the methanolic bark and stem extracts of *X. mekongensis* also showed good inhibition with IC_{50} values 35.842 µg/ml and 50.71 µg/ml respectively in comparison with quercetin (IC_{50}~7.65 µg/ml) as a standard. In the determination of reducing power, higher absorbance of the reaction mixture indicates higher reductive potential [26]. The reducing powers of various solvent extracts from *X. mekongensis* are shown in **Figure 2**. In addition, **Table 1** exhibits that all extracts of *X. mekongensis* displays percent (%) increment of the reducing power activity. The methanolic and chloro-

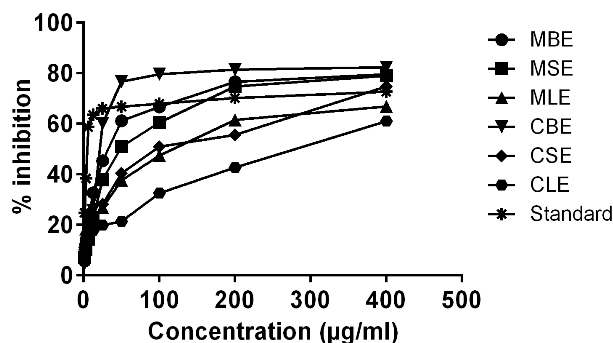

Figure 1. DPPH free radical scavenging activity of different extracts of *X. mekongensis* and quercetin (MBE-methanolic bark extract, MSE-methanolic stem extract, MLE-methanolic leaf extract, CBE-chloroformic bark extract, CSE-chloroformic stem extract, CLE-chloroformic leaf extract).

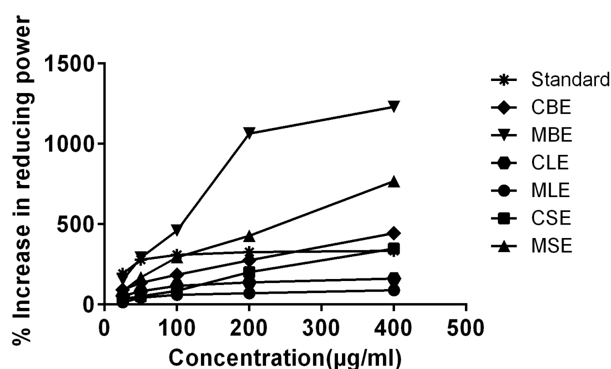

Figure 2. Reducing power of different types extracts of *X. mekongensis.*

Table 1. Activities related to antioxidant properties of different types of extracts from *X. mekongensis.*

Type of plant extracts	DPPH IC_{50} (μg/ml)	% Increasing range of reducing power (range)	TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg GAE/g)
CLE	492.66	54.7 - 161.9	16.5 ± 0.002	42 ± 0.004	0.6 ± 0.006
CSE	96.281	**34.23 - 347.65**	31 ± 0.003	78 ± 0.003	9.2 ± 0.011
CBE	**25.94**	**92.11 - 444.8**	27.5 ± 0.006	76 ± 0.007	40.2 ± 0.010
MLE	113.25	13.93 - 89.77	26 ± 0.002	77 ± 0.004	4.6 ± 0.011
MSE	50.71	**82.72 - 767.79**	82.5 ± 0.002	185 ± 0.007	28.6 ± 0.009
MBE	35.842	**154.36 - 1230.03**	**137.5 ± 0.009**	**200 ± 0.006**	**73 ± 0.009**
Standard (Quercetin)	7.65	191.69 - 334.55	X	X	X

N.B: CLE = Chloroformic leaf extract, MLE = Methanolic leaf extract, CSE = Chloroformic stem extract, MSE = Methanolic stem extract, CBE = Chloroformic bark extract, MBE = Methanolic bark extract, TPC = Total phenolic content, TFC = Total flavonoids content, TTC = Total tannin content, GAE = Gallic acid equivalent, QE = Quercetin equivalent, IC_{50} = 50% Inhibition concentration.

formic bark and stem extracts of have shown to exhibit better percent (%) increase in reducing power than quercetin. However, initially the percentage of increase in reducing power of all the extracts with minute concentration were relatively lower that the standard trend. Along with the increase of the concentration gradient, methanolic bark extract (MBE) and methanolic leaf extract (MLE) as well as chloroformic bark extract (CBE) supersede and the chloroformic stem extract (CSE) reaches the range of the standard's trend at their highest concentration. Chloroformic leaf extract (CLE) and MLE remain steady and lower than the standard's capacity regardless the intensiveness of the concentration.

Phenolic compounds exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals. Total phenolic content of the different extracts of *X. mekongensis* was solvent dependent and expressed as mg GAE/g (**Figure 3**). The total phenol content (TPC) determined in CLE, CSE and CBE as well as MLE, methanolic stem extracts (MSE) and MBE have been shown in **Table 1**, respectively. Among the six extracts of *X. mekongensis*, the content of phenolics can be ranked as MBE > MSE > CSE > CBE > MLE > CLE.

The content of flavonoids was expressed as QE/g of extracts while MBE (200 mg QE/g) contained the highest level of TFC among all extracts, and the order of the flavonoid richness resembles in the order of MBE > MSE > CSE > MLE > CBE > CLE. Total Tannin content of the extracts was shown in **Table 1** where the MBE possesses highest total tannin content (73 mg GAE/g). From **Table 2** it can be viewed that, total phenolic, flavonoid and tannin content of different extracts of *X. mekongensis* are correlated with each other and is statistically significant at $P < 0.05$. Most importantly the novel outcome of this research work is the finding of richness and potency of phenolic, flavonoid and tanin contents as well as DPPH assay of the bark and stem extracts of *X. mekongensis*. Reducing power assay indicated good antioxidant property with the stronger reducing capability of $FeCl_3$ as well. In reference to the literature, polyphenols or flavonoids are important group of pharmacologically active compounds that are considered to be the most active antioxidant derivatives in plants [27] which is notably remarkable in the experimentation of *X. mekongensis*.

The antibacterial activities of extracts were evaluated by the diameters of the inhibition zone around the disc and MIC; these diameters and MIC values are reported in **Table 3**. The average zone of inhibition produced by the plant extracts was observed at a concentration of 500µg/disc. The MIC ranged from 150 to 1000 µg/ml. For the interpretation of antibacterial assay results, we scale of measurement adopted was as zone of inhibition of >15 mm as strongly inhibitory, 10 - 15 mm as moderately inhibitory, and <10 mm as not inhibitory, according to Carović-Stanko *et al.* (2010) [28].

CBE and CSE were strongly active against *P. aeruginosa* (ZOI = 19 mm and MIC = 150 µg/ml), *S. aureus* (ZOI = 19.5 mm and MIC = 250 µg/ml), respectively with high zone of inhibition and the lowest MIC values. MSE was also strongly active against *S. aureus* (ZOI = 15.75 mm and MIC = 300 µg/ml) with high zone of inhibition and the lowest MIC value. However, MBE showed no activity against *E. coli* and *S. typhimurum*. CLE and MSE also possessed no activity against *S. paratyphi* and *E. coli* respectively. Analyzing the results of the antimicrobial activity of *X. mekongensis* extract, it was found that chloroformic extracts exhibited higher antimicrobial activity compared to methanolic extracts. Some of the phytochemical compounds e.g. glycoside, saponin, tannin, flavonoids, terpenoid, alkaloids, have previously been reported to have antimicrobial activity [29] [30]. These compounds become usually available in the extractives in the presence of non-polar solvents.

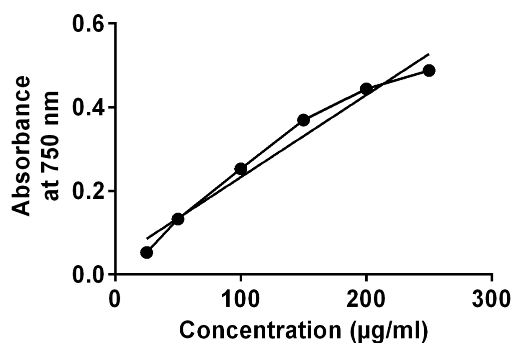

Figure 3. Standard calibration curve of gallic acid to determine total phenolic content of *X. mekongensis*.

Table 2. Correlation of total phenolic, flavonoid and tannin contents among the extracts of *X. mekongensis*.

Compared parameters for correlation analysis	Level of Significance at $P < 0.05$	R^2
Phenol vs. Flavonoid	**	0.8998
Phenol vs. Tannin	*	0.7257
Flavonoid vs. Tannin	*	0.5814

In the brine shrimp lethality bioassay, the result of cytotoxic potential of extracts in terms of percent (%) mortality of brine shrimps is presented in **Figure 4**. The degree of lethality was directly proportional to the concentration of the extracts. The LC_{50} (50% lethal concentration) values of different extracts were recorded and compared to positive control (vincristine sulphate, VS, LC_{50}~26.68 µg/ml) with regression analysis in **Table 4**.

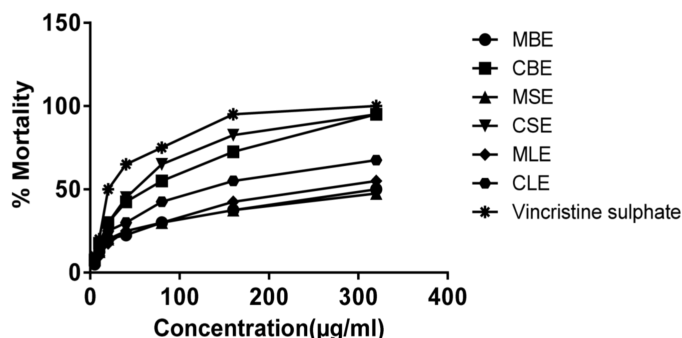

Figure 4. Brine shrimp lethality bioassay of different extracts of *X. mekongensis* with standard vincristine sulfate (c).

Table 3. Summary of results related to antibacterial properties of *X. mekongensis* extracts.

Name of the bacteria	Assay Parameters	CLE	MLE	CSE	MSE	CBE	MBE	Ery
E. coli	MIC (µg/ml)	600	1000	400	NA	400	NA	nd
	ZOI (mm)	8.25 ± 0.5	7.5 ± 0.577	10.25 ± 0.5	-	10.75 ± 0.5	-	15.5
P. aeruginosa	MIC (µg/ml)	1000	>1000	350	>1000	150	250	nd
	ZOI (mm)	7.75 ± 0.5	7.25 ± 0.5	15 ± 0.816	6.25 ± 0.5	**19 ± 0.816**	14.5 ± 0.577	24
V. cholerae	MIC (µg/ml)	350	350	350	350	340	320	nd
	ZOI (mm)	12.25 ± 0.5	12.5 ± 0.577	14 ± 0.816	11.75 ± 0.5	13 ± 0.816	13 ± 0.816	29
S. typhi	MIC(µg/ml)	340	600	350	300	380	NA	nd
	ZOI (mm)	12.5 ± 0.577	8.25 ± 0.5	14.75 ± 0.957	14.5 ± 0.577	11 ± 0.816	-	25
S. paratyphi	MIC (µg/ml)	NA	600	400	350	370	370	nd
	ZOI (mm)	-	8.75 ± 0.5	11.5 ± 0.577	12.25 ± 0.5	12.75 ± 0.957	10.25 ± 0.5	21
Micrococcus	MIC (µg/ml)	400	470	300	350	430	370	nd
	ZOI (mm)	10 ± 0.816	9.75 ± 0.5	9.75 ± 0.5	11.25 ± 0.957	9.25 ± 0.5	10.75 ± 0.5	24
S. aureus	MIC(µg/ml)	400	>1000	250	300	200	450	nd
	ZOI (mm)	11 ± 0.816	7.5 ± 0.577	**19.5 ± 0.577**	**15.75 ± 0.957**	14.75 ± 0.5	9.25 ± 0.5	23

N.B.: MIC = Minimal inhibitory concentration (µg/ml), ZOI = Zone of inhibition (mm) at 500 µg/disc, Ery = Erythromycin, NA = Not ACTIVE, nd = Not determined, (-) = No zone of inhibition.

Table 4. Brine-shrimp bioassay of different extracts from *X. mekongensis*.

Types of extracts	LC_{50} (µg/ml)	Regression equation	R^2
CBE	49.29	$y = 47.16x - 29.83$	0.982
MBE	465.08	$y = 23.13x - 11.70$	0.975
CLE	117.13	$y = 32.92x - 18.10$	0.984
MLE	298.79	$y = 26.99x - 16.81$	0.973
CSE	42.65	$y = 51.31x - 33.63$	0.99
MSE	549.48	$y = 21.35x - 8.498$	0.987
VS	26.68	$y = 52.79x - 25.29$	0.972

N.B.: CLE = Chloroformic leaf extract, MLE = Methanolic leaf extract, CSE = Chloroformic stem extract, MSE = Methanolic stem extract CBE = Chlorofornic bark extract, MBE = Methanolic bark extract, VS = Vincristine sulphate.

Percent mortality of CBE and CSE showed comparatively nearer value to the standard, which indicated that these extracts could be potential candidates for determination of antitumor and anticancer properties. The crude extracts resulting in LC_{50} values less than 250 μg/ml are considered significantly active [25].

4. Conclusion

Based on the results of this experimental study, it can be suggested that the extracts of *X. mekongensis* possess antioxidant, antibacterial, and cytotoxic properties. Further investigation is necessary to fractionate the extract, to identify the bioactive compounds and should be evaluated *in vitro* and *in vivo* studies for the biological activity. Such bioactive compounds have the potential to be developed into medicine, nutraceuticals and agrochemicals and last but not least, cosmetics.

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