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Toxicity of selected mangroves species on brine shrimp (*Artemia salina*)

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Key words: Brine shrimp nauplii, Toxicity, Bio-pesticide, Potential utilization

Abstract

The study aimed to evaluate the toxicity of different mangroves species *Rhizophora apiculata, Rhizophora mucronata,* and *Bruguiera sexangula* against brine shrimp *(Artemia salina)* as a preliminary assessment of its potential adverse effects as an herbal medicine. Brine shrimp were chosen as test organisms due to their sensitivity to various substances, making them suitable indicators for toxicity testing. The mangrove samples were extracted and were prepared to different dilutions for this study: 1 µg/ml (T1), 10 µg/ml (T2), 100 µg/ml (T3), 1000µg/ ml (T4). The findings of the study showed that the lethality of the three mangrove extracts on brine shrimp nauplii was found to be concentration-dependent. The brine shrimp mortality increases as extract concentration increases with T4 (1000 µg/ml) consistently showed higher death percentage compared to lower treatments is advised that further research be done on the *Bruguiera sexangula* crude extract to determine the bioactive components that are responsible for both its toxicity and its biopesticide properties.

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Introduction

The utilization of herbal remedies has been an integral part of human healthcare practices for centuries. Herbal preparations derived from plants have gained popularity due to their perceived natural origins and potential therapeutic benefits. For the people who live in the mangroves, food and a vast range of traditional goods and artifacts are provided by the mangroves. Mangrove extracts and chemicals have been employed historically as insecticides and pesticides, primarily in folkloric medicine (such as bush medicine). But in addition to those already recognized in the folk pharmacopeia, the extraction of novel natural chemical compounds from mangroves is still in its infancy. It is desirable to have an understanding of the biological processes and/or chemical components of plants, not only to find new therapeutic medicines but also to reveal new sources of recognized biologically active substances (Bandaranayake, 2002).

Moreover, considering the increasing global demand for herbal products and the potential integration of mangrove-derived compounds into mainstream medicine, the evaluation of their toxicity gains even greater significance. Regulatory bodies and healthcare professionals require robust scientific evidence to ensure the safety and efficacy of herbal remedies before their widespread adoption. Toxicity assessments play a pivotal role in providing this evidence, aiding in the establishment of guidelines and regulations for the responsible use of herbal preparations (Ekor, 2013).

Brine shrimp (*Artemia* spp.*)* have long been recognized as model organisms for toxicity testing due to their sensitivity to various substances, including natural compounds. The inclusion of brine shrimp in toxicity studies allows for the preliminary evaluation of potential adverse effects and aids in the determination of safe dosage levels for the utilization of herbal compounds (Meyer *et al.*, 1982).

This study, therefore investigates the toxicity of selected mangrove species *Rhizophora apiculata, Rhizophora mucronata,* and *Bruguiera sexangula* against Brine Shrimp *(Artemia salina)*, unraveling crucial information about the toxicity of these herbal compounds. By systematically examining the effects of mangrove extracts on brine shrimp, we seek to identify any potential adverse reactions and determine appropriate dosage ranges for their safe administration. These findings will contribute to the broader understanding of the safety and efficacy of mangrove-derived compounds and facilitate evidence-based decision-making in their utilization as herbal remedies.

Materials and methods

Processing and preparation of the extracts

The plant samples were collected in the municipality of Buguey, Cagayan, wetlands on the north coast of Luzon, Philippines. The mangrove forest of the municipality covers an aggregate area of approximately 504 ha (Pasion *et al.*, 2015).

The young leaves of *Rhizophora apiculata, Rhizophora mucronata,* and *Bruguiera sexangula* were washed with tap water and air-dried for 15 days. The plant specimen was authenticated in the Plant Quarantine Facility, Department of Agriculture Region II, Cagayan Valley.

The dried plant samples were then extracted with 80% ethanol in order to produce the ethanolic extracts. With lowered pressure and a temperature below 55°C, the resultant extracts were further evaporated (Guevarra, 2005). An alternative dilution procedure developed by McLaughlin *et al.* (1998) was adopted in the preparation of the different dilutions of the plant extracts for Brine Shrimp Lethality Assay (BSLA) where 20 mg of each extract was dissolved in 2 mL of the solvent. The final concentrations are 1 mg/mL, 100 µg/mL, 100 µg/mL, 10 µg/mL, and 1 µg/mL. There were three replicates in each concentration.

The leaves are prepared for the crude extracts by being washed in water, cut into smaller pieces, crushed with a mortar and pestle, and weighed.

Thereafter, the extract would filter in cheesecloth to remove particulate matter. Stock solution is diluted with distilled water to 1 mg/mL, 10 µg/mL, 100 µg/mL, 10 µg/mL and 1 mg/mL concentrations. Fresh extract was prepared for each experiment.

The leaves were cleaned with water, chopped into smaller pieces, and weighed for the preparation of aqueous extracts. Forty grams of fresh plant material was mixed with 200ml of distilled water in a beaker (20% stock solution) and boiled for 15 minutes then cooled at room temperature for 10mins. Thereafter, the extract was filtered in cheesecloth to remove particulate matter. The stock solution was diluted with distilled water to 1 µg /mL, 10 µg/mL, 100 µg/mL, and 1 mg/mL concentrations. The fresh extract was prepared for each experiment.

Test sample preparation for brine shrimp bioassay

The test sample was prepared following Baravalia *et al.* (2012) were dissolved in Dimethyl sulfoxide to acquire the stock solution of 1mg/ml concentration. The final concentration of Dimethyl sulfoxide in the assay volume was kept at 2% to prevent possible false effects originating from Dimethyl sulfoxide toxicity. Pure Dimethyl sulfoxide and artificial seawater were used as negative control and potassium dichromate was used as the reference standard for the toxicity assay.

Toxicity testing

Following the brine shrimp lethality assay, the brine shrimps were hatched, and the 10 nauplii were subjected to various doses of the plant extract. After 24 hours, the number of survivors was counted and the mortality percentage was computed. The absence of regulated forward motion for the course of a 30-second observation period is the bioassay's definition of mortality. Following Quazi *et al.* (2017), the percentage of nauplii lethality for each concentration and control was computed.

For each tube, count the number of dead and number of live nauplii, and determine the % mortality.

%Mortality = {(Number of dead nauplii)/ (Number of dead nauplii + Number of live nauplii)}×100

Statistical analysis

The data gathered were analyzed using (ANOVA) and Duncan's Multiple Range Test to test the significant difference of the treatment and control set-up. Graphs were generated to facilitate better presentation, visualization, and interpretation of data. The LD_{50} were calculated using probit analysis.

Results and discussion

Percentage of mortality of A. salina against mangrove extracts

In this study, after 24 hours of observation, all shrimps survived in the control. it was shown that the percentage of the mortality rate of *Artemia salina* exhibits the highest percentage of mortality in the concentration of the three different extracts.

The Fig. 1 shows the percentage mortality of nauplii treated in different extracts of *Rhizophora apiculata* for 24 hours. Among the three extracts, crude extracts exhibit the highest percentage of mortality (33.33%) using 1mg/ml which is comparable to the positive control (potassium dichromate) of the same concentration. On the other hand, aqueous and ethanol extracts with the least concentration (1 µg/ml) had a 6.66 % mortality effect on the nauplii, while the negative control garnered zero mortality percentage.

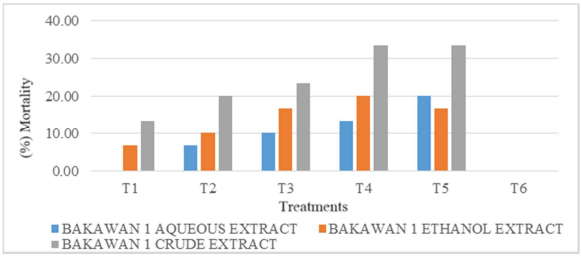

Fig. 1. Percent mortality of nauplii in *Rhizophora apiculata* (Aqueous, ethanol and crude extract)

Legend: T1= 1 µg/ml, T2= 10 µg/ml, T3= 100 µg/ml, T4= 1 mg/ ml (1000 µg/ml), T5= Positive control (potassium dichromate), T6= Negative control (DMSO: Dimethyl sulfoxide)

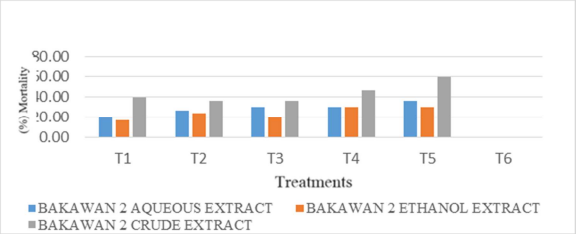

Fig. 2. Percent mortality of nauplii in *Rhizophora mucronata* (Aqueous, ethanol and crude extract)
Legend: T1= 1 µg/ml, T2= 10 µg/ml, T3= 100 µg/ml, T4= 1 mg/ ml (1000 µg/ml), T5= Positive control (potassium dichromate), T6= Negative control

The Fig. 2 shows the percentage mortality of nauplii treated to different extracts of *Rhizophora mucronata* for 24 hours. Among the three extracts, crude extracts exhibit the highest percentage of mortality (46.66%) using 1mg/ml on the nauplii which has a lesser effect compared to the positive control that obtained 60.00 % of mortality effect on the nauplii. On the other hand, ethanol extracts with the least concentration (1 µg/ml) has 16.66% mortality effect on the nauplii, while the negative control garnered zero mortality percentage.

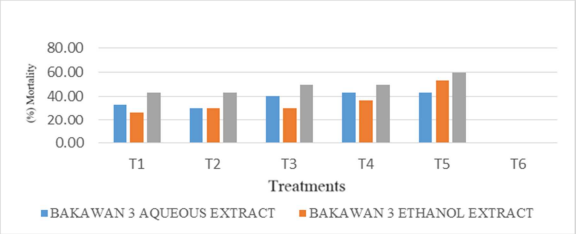

Fig. 3. Percent mortality of nauplii in *Bruguiera sexangula* (Aqueous, ethanol and crude extract)
Legend: T1= 1 µg/ml , T2= 10 µg/ml, T3= 100 µg/ml, T4= 1 mg/ ml (1000 µg/ml), T5= Positive control (potassium dichromate), T6= Negative control (DMSO: Dimethyl sulfoxide)

The Fig. 3 shows the percentage mortality of nauplii treated to different extracts of *Bruguiera sexangula* for 24 hours. Among the three extracts, crude extracts with a concentration of 100 µg/ml and 1mg/ml both exhibits the highest percentage of mortality that reaches up to 50.00 % which has lesser effect compared to the positive control that obtained 60.00 % of mortality effect on the nauplii. On the other hand, ethanol extracts with the least concentration (1 µg/ml) has 26.66% of mortality effect on the nauplii, while the negative control garnered zero mortality percentage.

Comparison of three mangrove species extracts in terms of mortality rate of brine shrimps

Based on the test conducted by Anderson, 1991 the extract which is toxic in the Brine Shrimp Lethality test means that it is also active against tumor cells/cancer cells (Anderson *et al.*, 1991). Because of its great accuracy, the Brine Shrimp Lethality test is a typical test for subsequent testing. This is the easiest toxicity test, because *A. salina* is sensitive to various chemical compounds and can be used widely. This method is thought to be beneficial for determining the toxicity of chemical compounds in extracts (Solis *et al.*, 1993). *Bruguiera sexangula* crude extract has toxic properties as a crude extract; however, this is the most basic stage, and further evaluation of the partitioned crude extract with polar and nonpolar solvents such as n-hexane and ethyl acetate, as well as filtering more complex compounds such as toxic properties of pure compounds to the direct test of extract toxicity (Ginting *et al.*, 2021).

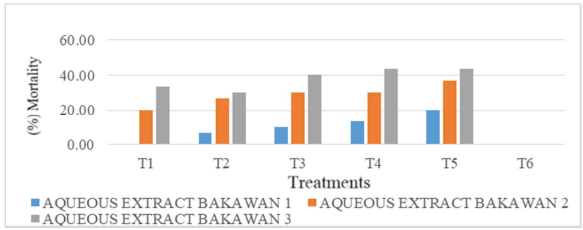

Fig. 4. Percent mortality of nauplii in 3 species of bakawan aqueous extract
Legend: T1= 1 µg/ml, T2= 10 µg/ml, T3= 100 µg/ml, T4= 1 mg/ ml (1000 µg/ml), T5= Positive control (potassium dichromate), T6= Negative control (DMSO: Dimethyl sulfoxide)

The crude extract stage, when the extract often contains a variety of several toxicity components, which might make selective toxicity findings difficult. The crude extract was the most harmful based on each mangrove species' extract.

The aqueous extracts of the three plants tested showed different brine shrimp larvicidal activity (Fig. 4). The highest mortalities (43.33 %) were observed at concentration of 1 mg/ ml. Based on the results, the brine shrimp lethality of the three plant extracts were found to be concentration- dependent. The observed lethality of the *Bruguiera sexangular* aqueous extract obtained the same percentage of mortality compared to the positive control to brine shrimp indicated the presence potent totoxic. In support, *Bruguiera sexangular* bark extracts are active against two tumors, Sarcoma 180 and Lewis Lung Carcinoma (Loder and Russell, 1969 cited by Mitra *et al.*, 2021).

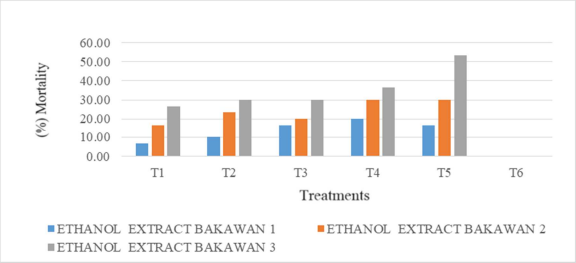

Fig. 5. Percent mortality of nauplii in 3 species of bakawan ethanol extract

Legend: T1= 1 µg/ml, T2= 10 µg/ml, T3= 100 µg/ml, T4= 1 mg/ ml (1000 µg/ml), T5= Positive control (potassium dichromate), T6= Negative control (DMSO: Dimethyl sulfoxide)

The ethanol extracts of the three plants tested showed different brine shrimp larvicidal activity (Fig. 5). The highest mortalities (36.66 %) were observed at concentration of 1 mg in *Bruguiera sexangular* ethanol extract. The observed lethality of the *Bruguiera sexangular* ethanol extract obtained lesser percentage of mortality compared to the positive control to brine shrimp. On the other hand, ethanol extracts with the least concentration (1 µg/ml) has 6.66% of mortality effect on the nauplii, while the negative control garnering zero mortality percentage.

The crude extracts of the three plants tested showed different brine shrimp larvicidal activity (Fig. 6). The highest mortalities (50.00 %) were observed at concentration of 1 mg in ethanol *Bruguiera sexangular* crude extract. The observed lethality of the *Bruguiera sexangular* crude extract obtained lesser percentage of mortality compared to the positive control to brine shrimp. On the other hand, crude extracts with the least concentration (1 µg/ml) has 13.33% of mortality effect on the nauplii, while the negative control garnering zero mortality percentage.

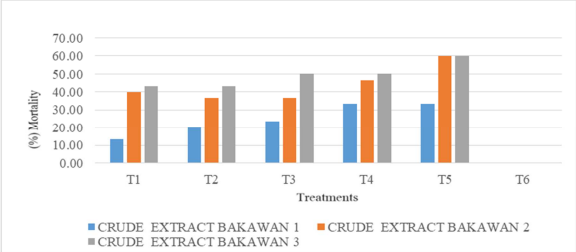

Fig. 6. Percent mortality of nauplii in 3 species of bakawan crude extract

Legend: T1= 1 µg/ml , T2= 10 µg/ml, T3= 100 µg/ml, T4= 1 mg/ ml (1000 µg/ml), T5= Positive control (potassium dichromate), T6= Negative control (DMSO: Dimethyl sulfoxide)

Main effect of extracts

Toxicity testing plays an essential role in identifying the potential adverse effects caused by chemicals (Gupta, 2022). The toxicity of extracts from various plant sections was tested in this study. Therefore, bioassay of brine shrimp (*Artemia salina*) is useful for isolating bioactive chemicals from plant preparations (Sam, 1993). With the use of Brine Shrimp Bioassay method, a factorial completely randomized design with a total of 54 mangrove extracts which includes; crude extract, ethanolic extract and aqueous extract. Extracts were randomly assigned for treatments.

Table 1. Main effect of extracts

Extract	Mean
Rhizophora apiculata	6.2963[a]
Rhizophora mucronata	5.4630[b]
Bruguiera sexangula	5.0556[b]

Note: Means with the same letter are not significantly different. N=54

Table 1. shows compared to the three species regardless of concentration *Rhizophora apiculata* with a mean of 6.2963 is significantly different from *Rhizophora mucronata* with a mean of 5.4630 and *Bruguiera sexangula* with a mean of 5.0556.

Main effect of different concentrations

Toxicity testing plays an essential role in identifying the potential adverse effects caused by chemicals (Gupta, 2022). The toxicity of extracts from various plant sections was tested in this study. Therefore, bioassay of brine shrimp (*Artemia salina*) is useful for isolating bioactive chemicals from plant preparations (Sam, 1993). With the use of Brine Shrimp Bioassay method, a factorial completely randomized design with a total of 27 mangrove extracts which includes; crude extract, ethanolic extract and aqueous extract.

Table 2. Main effect of different concentrations

Treatment	Mean
2 (10 Î¼g/ml)	7.4815 a
3 (100 Î¼g/ml)	6.8148 ba
1 (1 Î¼g/ml)	6.6667 ba
4 (1000 Î¼g/ml)	6.296 ba
5 (Positive control: Potassium dichromate)	6.0370 b
6 (Negative control: DMSO)	0.0000 c

Note: Means with the same letter are not significantly different. N=54

Table 2 shows the analysis of variance on the effects of concentrations. Treatment 2= 10 µg/ml is significantly different compared to Treatment 3= 100 µg/ml, Treatment 1=1 µg/ml and Treatment 4= 1000µg/ ml are not significant different. Since C is highly significant among all dosage/ concentrations in terms of lowest mortality mean with 0.0000.

The mortality rate of nauplii exposed in different concentration of leaf aqueous extract of *Rhizophora apiculata* through BSLA was used to estimate the LD_{50} (Fig. 7). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Rhizophora apiculata* leaf extract were estimated to be 446497230.938ppm at lower and upper limits of 599771.279ppm and 332393003926.ppm respectively. This further means that the value 446497230.938ppm is 95 percent confidence that it can kill 50 percent of population of the sample, and any value that is lower than 599771.279ppm and any value that is higher than 332393003926.045ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the aqueous leaf extract of *Rhizophora apiculata* are classified non-toxic.

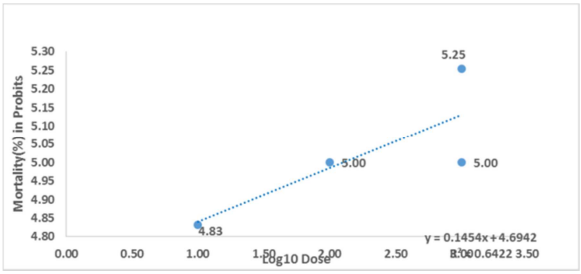

Fig. 7. Log dose of mortality: The graph showing the mortality of the aqueous leaf extract of bakawan lalake (*Rhizophora apiculata*) using probit analysis

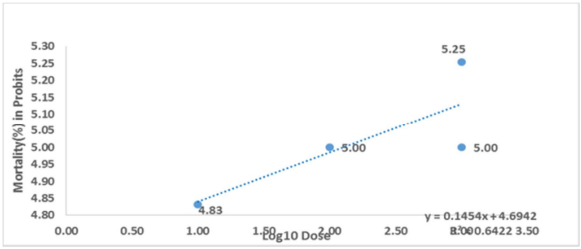

Fig. 8. Log dose of mortality: The graph showing the mortality of bakawan babae (*Rhizophora mucronata*) using probit analysis

The mortality rate of nauplii exposed in different concentrations of leaf aqueous extract of *Rhizophora mucronata* through BSLA was used to estimate the LD_{50} (Fig. 8). Based on the result of probit analysis at 95% Fiducial CI, The LD50 of *Rhizophora mucronata* leaf extract were estimated to be 30575780.454ppm at lower and upper limits of 3172.470ppm and 294684737909.678ppm respectively. This further means that the value 30575780.454ppm is 95 percent confidence that it can kill 50 percent of the population of the sample, and any value that is lower than 3172.470ppm and any value that is higher than 294684737909ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition,

according to Meyer's toxicity index, the aqueous leaf extract of *Rhizophora mucronata* are classified as non-toxic.

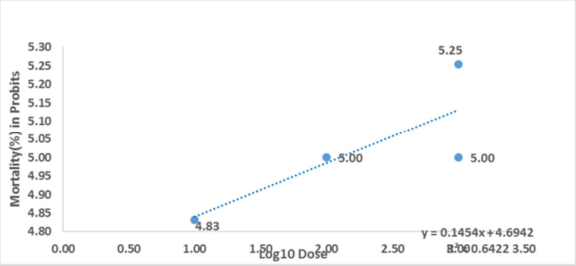

Fig. 9. Log dose of mortality: The graph showing the mortality of the aqueous leaf extract of pototan (*Bruguiera sexangula*) using probit analysis

The mortality rate of nauplii exposed in different concentrations of leaf aqueous extract of *Bruguiera sexangula* through BSLA was used to estimate the LD_{50} (Fig. 9). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Bruguiera sexangula* leaf extract were estimated to be 57189.517ppm at lower and upper limits of 10.063 and 325032319.601ppm respectively. This further means that the value 57189.517ppm is 95 percent confidence that it can kill 50 percent of population of the sample, and any value that is lower than 10.063ppm and any value that is higher than 325032319.ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the aqueous leaf extract of *Bruguiera sexangula* are classified non-toxic.

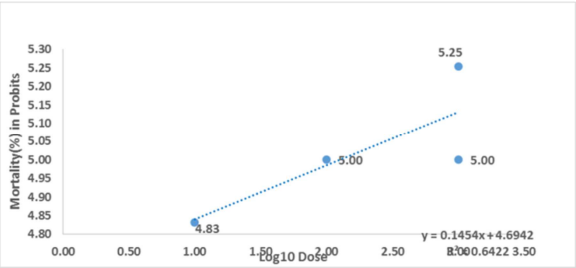

Fig. 10. Log dose of mortality: The graph showing the mortality of the ethanol leaf extract of *Rhizophora apiculata* using probit analysis

The mortality rate of nauplii exposed in different concentration of leaf ethanol extract of *Rhizophora apiculata* through BSLA was used to estimate the LD_{50} (Fig. 10). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Rhizophora apiculata* leaf extract were estimated to be 3134295.584ppm at lower and upper limits of 21089.273ppm and 465820175.091ppm respectively. This further means that the value 3134295.584ppm is 95 percent confidence that it can kill 50 percent of population of the sample, and any value that is lower than 21089.273ppm and any value that is higher than 465820175.091ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the ethanol leaf extract of *Rhizophora apiculata* are classified non-toxic.

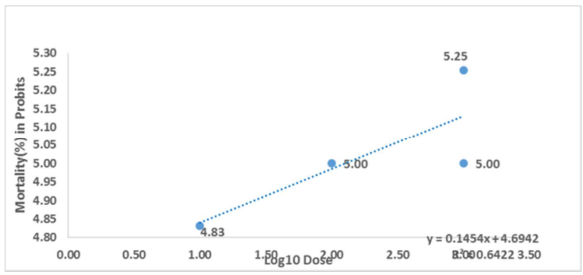

Fig. 11. Log dose of mortality: The graph showing the mortality of the ethanol leaf extract of *Rhizophora mucronata* using probit analysis

The mortality rate of nauplii exposed in different concentration of leaf ethanol extract of *Rhizophora mucronata* through BSLA was used to estimate the LD_{50} (Fig. 11). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Rhizophora mucronata* leaf extract were estimated to be 55683429.015ppm at lower and upper limits of 15057.875ppm and 205915129567.225ppm respectively. This further means that the value 55683429.015ppm is 95 percent confidence that it can kill 50 percent of population of the sample, and any value that is lower than 15057.875 and any value that is higher than 205915129567.225ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the ethanol leaf extract of *Rhizophora mucronata* are classified as non-toxic.

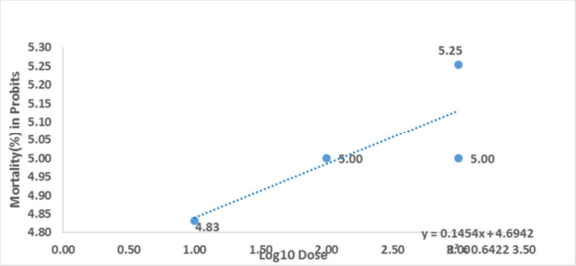

Fig. 12. Log dose of mortality: The graph showing the mortality of the ethanol leaf extract of *Bruguiera sexangula* using probit analysis

The mortality rate of nauplii exposed in different concentrations of leaf ethanol extract of *Bruguiera sexangula* through BSLA was used to estimate the LD_{50} (Fig. 12). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Bruguiera sexangula* leaf extract were estimated to be 292540697.413ppm at lower and upper limits of 994.268ppm and 86073447341681.900ppmppm respectively. This further means that the value 292540697.413ppm is 95 percent confidence that it can kill 50 percent of the population of the sample, and any value that is lower than 994.268ppm and any value that is higher than 86073447341681.900ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the ethanol leaf extract of *Bruguiera sexangula* are classified as non-toxic.

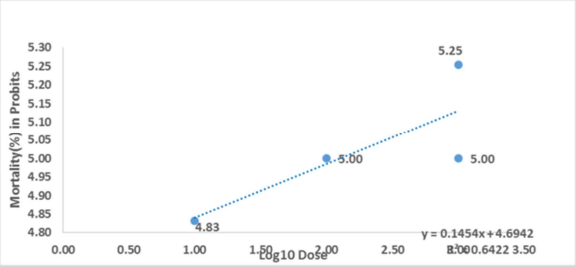

Fig. 13. Log dose of mortality: The graph showing the mortality of the crude leaf extract of *Rhizophora apiculata* using probit analysis

The mortality rate of nauplii exposed in different concentrations of leaf crude extract of *Rhizophora apiculata* through BSLA was used to estimate the LD_{50} (Fig. 13). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Rhizophora apiculata* leaf extract were estimated to be 128130.472ppm at lower and upper limits of 1197.291ppm and 13712133.ppm respectively. This further means that the value 128130.472ppm is 95 percent confidence that it can kill 50 percent of the population of the sample, and any value that is lower than 1197.291ppm and any value that is higher than 13712133ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the ethanol leaf extract of *Rhizophora apiculata* are classified non-toxic.

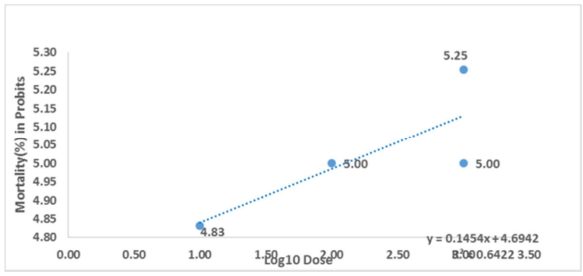

Fig. 14. Log dose of mortality: The graph showing the mortality of the crude leaf extract of *Rhizophora mucronata* using probit analysis

The mortality rate of nauplii exposed in different concentrations of leaf crude extract of *Rhizophora mucronata* through BSLA was used to estimate the LD_{50} (Fig. 14). Based on the result of probit analysis at 95% Fiducial CI, The LD_{50} of *Rhizophora mucronata* leaf extract were estimated to be 3017766.544ppm at lower and upper limits of 0.057ppm and 159983959711361.000ppm respectively. This further means that the value 3017766.544ppm is 95 percent confidence that it can kill 50 percent of the population of the sample, and any value that is lower than 0.057 and any value that is higher than 159983959711361.000ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index the ethanol leaf extract of *Rhizophora mucronata* are classified as non-toxic.

The mortality rate of nauplii exposed in different concentrations of leaf crude extract of *Bruguiera sexangula* through BSLA was used to estimate the LD_{50} (Fig. 15). Based on the result of probit analysis at

95% Fiducial CI, The LD_{50} of *Bruguiera sexangula* leaf extract were estimated to be 561.704ppm at lower and upper limits of 0.001ppm and 351826188.813ppm respectively. This further means that the value 561.704ppm is 95 percent confidence that it can kill 50 percent of the population of the sample, and any value that is lower than 0.001ppm and any value that is higher than 351826188.813ppm is 2.5 percent confidence that can kill 50 percent of the total population. In addition, according to Meyer's toxicity index, the ethanol leaf extract of *Bruguiera sexangula* is classified as toxic.

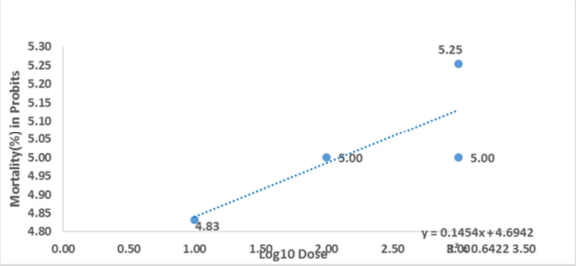

Fig. 15. Log dose of mortality: The graph showing the mortality of the crude leaf extract of *Bruguiera sexangula* using probit analysis

Conclusion

The conclusion highlights significant implications regarding the toxicity of *Bruguiera sexangula* crude extract. The findings reveal that this extract exhibited a remarkably high level of toxicity, with a mortality rate of 50% at a concentration of 1mg/ml, which is on par with the positive control treatment using potassium dichromate at the same concentration.

This indicates that the extract contains potent toxic compounds, which raises concerns about its environmental impact if released into natural habitats. These results underscore the need for further research to identify and characterize the specific toxic compounds within the extract, helping us better understand its potential risks to ecosystems. Additionally, this information could have consequences for the conservation and management of mangrove species, as the high toxicity of *Bruguiera sexangula* suggests the necessity of considering its ecological implications and the potential impact responsible for both its toxicity and its biopesticide properties.

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