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Toxicity evaluation of white spike head (*Kyllinga nemoralis*) using brine shrimp lethality test and anthelminthic assay

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Abstract

Kyllinga nemoralis has been utilized for the treatment of various illnesses in developing countries because it is inexpensive and readily available but toxicity evaluation of this plant is still unreported in scientific publications. This study investigated the toxicity level of combined roots and leaves of *K. nemoralis* (40g) using Brine Shrimp Lethality Assay and Earthworm Assay. The study employed Complete Randomized Design in triplicates (n= 210 *A. salina* nauplii and n=126 *E. euginiae*). For BSLA, four *K. nemoralis* ethanolic root+leaf extracts [KNERLE] (T1=1000ppm; T2=100ppm; T3=10ppm; and T4=1ppm) were tested for toxicity while in earthworm assay, four *K. nemoralis* aqueous root+leaf extracts [KNARLE] (T1=25g/ml; T2=20g/ml; T3=15g/ml; and T4=10g/ml) were tested for time paralysis and mortality. After 24h experimental period, mortality data were analyzed using ANOVA ($p \leq 0.01$) and post hoc analysis (Tukey's test) while Probit analysis was employed to determine toxicity level of KNERLE at LC_{50}. Result showed that there is a linear dose-dependent trends of KNERLE and KNARLE vs. nauplii (LC50 = 18.97ppm) and earthworm mortality (100% mortality comparable to positive control, Albendazole), respectively, suggesting that higher treatment concentration exhibit toxicity and anthelminthic properties. The study suggests that *K. nemoralis* root+leaf extracts are strongly toxic, and further investigation of toxicity using higher vertebrate models' tissue and organ level is recommended.

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Introduction

In the countryside and rural communities of Cebu Island, Philippines, one of the commonly used, easy to find monocot (i.e. sedge) with extensive medicinal benefit is *busikad* or White Spike Head (*Kyllingane moralis*) (Gruyal *et al.*, 2014; Stuart, 2016). Anecdotal reports from upland communities in Central Cebu reported that *K. nemoralis* has antidiabetic, antiviral, antibacterial, antibleeding and anti-poison properties (Lucresio Son, *personal communication,* 2018). In Alcoy and Naga, Southern Cebu, *K. nemoralis* is used to relieve muscle pain, headache and fever through boiling of either its leaves or stem by soaking it overnight in water and orally administered in the morning (Miano *et al.*, 2011). At the country-level surveys, Quisumbing (1978) had already reported that it is traditionally used for the treatment of skin diseases such as pruritus, relief for malarial fever, and diabetes and in Surigao del Sur, the infusion of stem and leaves are widely used for headaches, muscle pains and fever (Gruyal, 2014).

Current researches in some parts of the world noted remarkable medicinal properties of *K. nemoralis*, as follows: treatment of snake bites due to its anti-venom property (Raju *et al.,* 2011); analgesic activity(Amor *et al.*, 2009); anti-diabetic activity (Sudipta*et al.,* 2012); nematocidal activity (Majumder, 2013); diuretic agent (Karthikeyan *et al.,* 2009); hepatoprotective activity (Somasundaram *et al.,* 2009) andantioxidant and antibacterial activity (Sindhu *et al.,* 2014). Medicinal property of this plant is attributed to its presence of very important chemical constituents such as alkaloids, glycosides, phenol, and flavonoid (Devendra *et al.,* 2013) as well as limited phytoconstituents like carbohydrates and volatile oils in ethanolic tuber extract (Majumder, 2013). Despite all of these claimed medicinal properties, there has been no reported toxicity evaluation of *K. nemoralis* relative to its oral mode of administration in treating various illnesses as well as to completely establish its safety.

This study aims to determine the toxicity levels of *K. nemoralis* root and leaf ethanolic extracts *in vitro* (i.e. in terms of LC_{50} of *Artemia salina*) and *K. nemoralis* root and leaf aqueous extracts (in terms of its anthelminthic effect of *Eudrilus euginiae*). This study evaluated the toxicity level of this herbal plant through invertebrate animal models (*A. salina* and *E. euginiae*) that may demonstrate physiological similarities to other vertebrate models.

Materials and methods

K. nemoralis as source of phytochemicals

Whole mature plant (erect stem with leaves and single flower, ~40 to 50cm height, 2kgs) of *Kyllingane moralis* was collected from shaded meadows of Sudlon I, Cebu City. Since it is a weed of Least Concerned status under IUCN (2018), permit for collection was no longer secured from the Department of Environment and Natural Resources (DENR). Plant sample authentication was performed by resident plant taxonomist of the Research Institute of Tropical Biology and Pharmacological Biotechnology [RITBPB], Cebu Normal University, Cebu City. After collection, plant samples were washed three times under running tap water to completely remove soil debris and was finally rinsed with distilled water. Roots and leaves section of the plant were then separated, and air-dried using a nylon net in an open space for 3 days. Leaf samples were separated from individual leaf sheaths, cut into smaller pieces (~2-3 cm) prior to homogenizing using electric blender. Meanwhile, root parts were also cut into smaller pieces (~0.5-1cm), and homogenized using an electric blender. Powdered root and leaf components were mixed, and were stored in a zipper bag (Ziploc™) with silica gel, stored in a dry container (~25°C) until use.

Extraction of K. nemoralis bioactive compounds and preparation of test solution

Brine Shrimp Lethality Assay (BSLA)

Finely powdered leaves (20 grams) were soaked in 95% ethanol (250mL) in a 500ml beaker. The mixture was shaken for 1 minute for every after 30 minutes interval throughout the 6 hours soaking period and left for 48 hours in room temperature thereafter. The beaker was shaken once more (around 1 minute) and

the solution was promptly separated using Whatmann No. 1 filter paper. The filtrate was subjected to rotary evaporator (<55°C) to separate the crude extract from the solvent (Olowa and Nuneza, 2013).

The researchers used DMSO (dimethyl sulfoxide) with 1.25% (v/v) (1.25mL of DMSO per 100mL of double distilled water) as the vehicle for the crude extract (Geethaa *et al.*, 2013). In preparing different dilutions, the researchers adopted McLaughlin *et al.*, (1998) procedure. Twenty (20) mg of the crude extract was dissolved in 2 mL of solvent (DMSO) and this served as the stock solution. Range finding test was first performed before arriving at a concentration level that satisfies LC50. Serial dilution procedure yielded four concentrations of the final test extract: 1000, 100, 10, and 1 ppm.

Anthelminthic assay

Twenty-five grams (25g) of dried and pulverized plant sample (i.e. root+leaf powder) was boiled in 250mL of double distilled water with constant stirring for 45 minutes. The solution was allowed to cool, and subsequently filtered using Whatmann No. 1 filter paper. Range finding test was first performed to determine the concentration that would be low enough to paralyze and kill earthworm. The same procedure was done consecutively using 18.75g, 12.5g, and 6.25g of mixed root+leaf powder in 250mL of triple distilled water to yield the concentrations 100mg/mL, 75mg/mL, 50mg/mL, and 25mg/mL, respectively.

Experimental organisms and design of the experiment

Brine Shrimp Lethality Assay (BSLA)

A pack of brine shrimp eggs (approx. 20 grams) were procured from a local aquarium supplies dealer in Manalili Street, Cebu City. Prior to incubation, eggs were kept in a sealed zipper bag and stored in a refrigerator (around 10°C-12°C) to avoid premature hatching (Brine Shrimp Direct, Inc. 2018). The BSLA experiment was laid under Completely Randomized Design (CRD) with equal replications, producing four treatments of test solutions (Treatment1: 1000 ppm, T2: 100 ppm, T3: 10 ppm, and T4: 1 ppm), one negative control (natural seawater), positive control (100ppm $K_2Cr_2O_7$), and a vehicle control (DMSO). In each of the treatments and control groups, there were three replicates. Ten (10) active nauplii (larvae of *A. salina*) were placed in each of the three replicate test tubes. Each replicate contains 10 nauplii as sample summing up to 30 *A. salina* for every treatment. Therefore, for the entire assay, there were 240 A. salina nauplii larvae subjected for observation (Table 1).

In vitro Earthworm Assay

A total of 126 healthy African night crawlers (*Eudrilus eugeniae)* were procured from the City Agricultural Office, Maguikay, Mandaue City. A selection criterion was utilized in the selection of earthworm that will be used in the experiment include approximate length of 3-5cm and 0.1- 0.2 cm width. Prior to testing, all earthworms were acclimatized for 24 hours then washed with distilled water to remove soil and dirt.

This Earthworm Assay also utilized a Completely Randomized Design (CRD) with four treatments of test extracts (T1: 100mg/mL, T2: 75mg/mL, T3: 50mg/mL, and T4: 25mg/mL) and three control groups, namely: negative control – represented by double distilled water; positive control – represented byAlbendazole (Majumder, 2013) and dimethyl sulfoxide (DMSO) as the vehicle control (i.e. the vehicle for the albendazole). In each treatment and control group, there were six (6) earthworms used in triplicates, summing up to 18 earthworms for every treatment and controlgroup. Therefore, for the entire assay, there were 126 earthworms subjected for observation (Table 2).

Experimental protocol

Brine Shrimp Lethality Assay (BSLA)

This research used an integrated protocol adopted, with slight modification, from different brine shrimp lethality assay studies (Meyer *et al.*, 1982; McLaughlin *et al.*, 1998; Hamidi *et al.*, 2014; Sarah *et al.*, 2017).

Hatching brine shrimp

Natural seawater was prepared. The tip of an airline tube connected from an aerator was placed in the bottom of the aquarium maintaining proper aeration and enough oxygen levels. Equal distribution of the oxygen is achieved by constant aeration from the aerator. Hatching brine shrimp eggs needs at least 90% oxygen-saturated ASW (Hamidi *et al.*, 2014). Temperature was optimized for hatching *A. salina* at about 30 ± 0.5°C. Brine shrimp eggs (20g) were gently sprinkled at the top section of the water and the water was mixed shortly after. A light bulb (60 to 100 Watts) located few inches away from the aquarium was switched on (i.e. light is an important stimulus for hatched nauplii). After 20-24 hours, *A. salina* eggs hatched. Twenty-four (24) more hours later, several nauplii from the specialized aquarium were collected using a Pasteur pipette and were immediately transferred to a prepared container with seawater and aerator. After 12 more hours, the 1 ½-day old nauplii were then incubated for another 12h to reach 2nd - 3rdlarval stage (2-day old nauplii) which will be used for toxicity testing. Similarly, 6 mg of dry yeast was added to the container where nauplii were newly transferred. Dry yeast is an ideal substitute food for brine shrimps (Carolina Biological Supply Company, 2012).

Toxicity testing (Two-day old nauplii)

Nauplii larvae were exposed to different treatments of *K. nemoralis* ethanolic extracts as well as to control groups. Each test tube with its respective treatment (0.50mL) was filled with about 4mL of seawater. At the same time, 10 nauplii were transferred to each test tube using Pasteur pipette (i.e. 30 brine shrimps per treatment). Afterwards, the volume of the seawater was adjusted to 5mL per test tube. Test tubes were then left uncovered for the whole duration of toxicity testing (24hours) yet safe under a light. Finally, the number of surviving nauplii was counted and recorded using a magnifying glass and flashlight; and the percentage of death after 24 hours was calculated. (A dead nauplius larva was recognized by the absence of controlled forward motion at least within 30 seconds of observation even if light stimulus is present.)

In vitro Earthworm Assay

Earthworm assay was conducted using the integrated protocols from Ajaiyeoba *et al.*, (2001) and Panda *et al.,* (2011). All the solutions used in the assay were freshly prepared. The treatment and control solutions were poured in its respective petri dishes with 20mL volume for each. Six earthworms were introduced for each of the three replicate Petri dishes. Changes in earthworms such as reduced movement, rapid involuntary movement, localized tissue damage and lesions, and fading of color were monitored. Time until paralysis and mortality rate after 24 hours under each treatment (when there is no movement within the observation period, unless the dish is shaken vigorously) was taken cautiously. Death of the earthworm was characterized as the loss of their motility even after being immersed in warm water (approximately 50°C) and the eventual fading of the body color.

Data analysis

Brine Shrimp Lethality Assay (BSLA)

Mean number of dead nauplii for the three replicates was utilized in evaluating % lethality for all experimental and control groups. The formula was utilized: % mortality = no. of dead nauplii / total no. of tested nauplii x 100%. Linear regression analysis using Microsoft Excel 2010 was employed to evaluate relationship between independent variable (varying concentrations of *K. nemoralis* ethanolic extracts) and dependent variable (mortality rate) (Biswas *et al.,* 2011). In calculating median lethality concentration (LC_{50}) value, Probit regression analysis was applied wherein percent mortality was transformed into probits or probability units based on Finney (1952). Probit was graphed against $\log_{10}$ concentrations thus yielded a slope-intercept equation. From this equation (y = ax + b), the slope (x) was then calculated. Subsequently, LC_{50} was inferred as the antilog of x.

The toxicity level of herbal extracts expressed in lethal concentration 50 or LC_{50} was validated using the

toxicity index as follows. Hamidi *et al.*, (2014) modified the toxicity index from Clarkson *et al.*, (2004), in which plant extracts with an LC_{50}>1000 ug/ml will be considered as non-toxic while the plant extracts in a following order with a LC_{50} of 500-1000 ug/ml, 100-500 ug/ml and 0-100 ug/ml will be considered as low toxic, medium toxic and highly toxic, respectively.

In vitro Earthworm Assay

All data were expressed as mean time (in minutes) of worm paralysis and death per treatment [mean±S.E.M]. One-way Analysis of Variance (ANOVA) was used in comparing the mean values of treatment and control groups (p<0.01). Statistically significant results were followed with Tukey Post Hoc Test to determine which among the groups' means differ. All these analyses were conducted using IBM SPSS version 20 software.

Results and discussion

Brine Shrimp Lethality Assay

The toxicity of the combined leaf and root ethanolic extracts was evaluated in *A. salina*. Results show that the highest mortality was reported in the highest KNELRE concentration (T1), and decreases correspondingly as the concentration of the ethanolic extract decreases (Table 3). This pattern shows a linear dose-response trend. Expectedly, positive control (100ppm $K_2Cr_2O_7$) and solvent control (70% ethanol) registered 100% mortality, and are statistically comparable to T1 (p<0.000) in terms of mortality. This result suggests that T1 concentration is extremely toxic to *A. salina* with no reported survivors after 24h exposure to KNELRE.

Table 1. Complete Randomized Design (CRD) with equal replications per trial for Brine Shrimp Lethality Assay**.

Treatments	Replicates	Sample*per replicate	Total number of *A. salina* per treatment
Control Groups			
Negative (sea water)	3	10	30
Solvent (ethanol)	3	10	30
Vehicle (DMSO)	3	10	30
Positive ($K_2Cr_2O_7$)	3	10	30
Experimental Groups			
T1 (1000 ppm)	3	10	30
T2 (100 ppm)	3	10	30
T3 (10 ppm)	3	10	30
T4 (1 ppm)	3	10	30
Total			240 nauplii for 8 treatments

* Each active nauplii of *A. salina* represents 1 sample

** For the experiment, 240 nauplii of *A. salina* were used.

Meanwhile, survival of *A. salina* was highest in negative control (100% survival), followed by vehicle control and T4 (1ppm) with 97% survival. Remarkably, the experimental groups were statistically incomparable to each other in terms of mean survival rates.

The lethal concentration (Table 4) were calculated and estimated using Probit Analysis. Under laboratory conditions, the lethal concentration that can cause 50% nauplii mortality in leaf+root ethanolic extract of *K. nemoralis* is 20.049 ppm. The LC_{50} value was estimated using a Probit regression analysis, and this concentration value is considered to possess high toxic effects based on Clarkson toxicity criterion (Hamidi *et al.*, 2014). Nguta *et al.* (2011) also suggests that plant extracts that has an LC_{50}ranging from 0-500 ppm is known to possess

high level of toxicity.

Fundamentally, the graph below depicts a clear linear dose-dependent toxicity trend indicating that the two variables are directly proportional to one another (Fig. 1). It also shows that the R^2 =0.958, suggesting that the linear model fits with the data almost completely. The slope in the graph, y = 0.5393x + 4.5444 for the leaf+root extract represents the steepness of the line, it defines linear relationship between the variables (dose and response), and this can be used in estimating the average rate of change where x is the concentration and y is the mortality rate (response of the nauplii).

Table 2. Complete Randomized Design (CRD) with equal replications per trial for Anthelminthic Assay**.

Treatments	Replicates	Sample*per replicate	Total No. of earthworms per treatment
Control Groups			
Negative (distilled water)	3	6	18
Positive (Albendazole)	3	6	18
Vehicle control (DMSO)	3	6	18
Experimental Groups			
T1 (25g/ml)	3	6	18
T2 (20g/ml)	3	6	18
T3 (15 g/ml)	3	6	18
T4 (10 g/ml)	3	6	18
Total			126 earthworms for 7 treatments

* Each active *Eudrilus euginiae* represents 1 sample

** For the experiment, 108 earthworms were used.

The observed lethality of the different concentration of KNELRE may suggest the presence of potent toxic phytochemicals. Raju *et al.*, (2013); Amor *et al.*, (2009) and Sudipta *et al.*, (2012) reported the presence of different phytochemicals such as humulene, β-caryophyllene, α-amyrin, β-amyrin and other secondary metabolites in the leaves of *K. nemoralis*. Some of these phytochemicals are toxic in elevated dosages such as phenolic substances. In a research article of Devendra *et al.,* (2013), the study found that the higher concentration of phenolics and flavonoids in the plant, the greater is the toxicity.

Table 3. Surviving nauplii after 24h exposure to *Kyllinga nemoralis* ethanolic leaf and root Extract (KNELRE) treatments and control groups.

Group	Content	Surviving nauplii after 24 hrs. exposure to KNELRE			Mean±S.D.*	Ave. no. of dead A. salina	% mortality
		R1	R2	R3			
		n=10	n=10	n=10			
NegCon	35-36 ppt sea water	10	10	10	10.0 ± 0.0^{a}	0	0
SolvCon	70% ethanol	0	0	0	0 ± 0.0^{d}	30	100
VehCon	2% DMSO	10	10	9	9.67 ± 0.58^{a}	1	4
PosCon	100ppm $K_2Cr_2O_7$	0	0	0	0 ± 0.0^{d}	30	100
T1-1000 ppm	1 µg/mL	0	0	0	0 ± 0.0^{d}	30	100
T2-100 ppm	0.1µg/mL	3	3	2	2.67 ± 0.58^{c}	22	73
T3-10 ppm	0.01 µg/mL	4	5	5	4.67 ± 0.58^{b}	16	53
T4-1 ppm	0.001 µg/mL	10	10	9	9.67 ± 0.58^{a}	1	3
ANOVA (p<0.01)			0.000**				

*Similar letter superscript under column S.D. indicates significant difference of the mean at p<0.01 based on Tukey's HSD; ** significant at p<0.01.

The study further argue that these phytochemicals have the potential to prevent the metabolism of carbohydrates that can cause a condition where there will be an abnormal deposition of harmful chemicals such as glycogen in the different organs in the organisms. These different studies support that the leaves of *K. nemoralis* possessed toxic phytochemicals. Since this research cannot identify which potent toxic phytochemical are present in the crude extract, this research warrants for further investigation.

Table 4. Calculated lethal concentration (LC_{50}, in ppm) after the 24h exposure of *Artemia salina* nauplii to the varying concentrations of KNELRE.

LC_{50} of ethanolic extract of *K. nemoralis* (ppm)	95% confidence Limit*	R^2	Slope Value	Intercept	Fitting
20.049 ppm	Upper limit: 52.7 Lower limit: 7.6	0.958	1.198	3.44	Good fit

*Fiducial value.

Earthworm Assay

Table 5 shows the average mortality rate of *E. eugeniae* exposed to the two control and various concentrations of the *K. nemoralis* aqueous leaf and root extract (KNALRE). Across the different treatment concentrations, the statistically comparable treatment to that of the positive control (Albendazole, 78% mortality) isT1 (100mg/ml), T2 (75mg/ml) and T3 (50mg/ml) with 100%, 94% and 78% mortality, respectively. Both 2%DMSO (used as the vehicle for Albendazole) and distilled water (used as the solvent of the plant extract) did not induce paralysis and death towards earthworms during the experiment. This comparison may further suggest that physiological damages was more likely manifested in T1 and T2 with 100% and 94% mortality, respectively and were very detrimental to the health and life of the earthworm.

Table 5. Mortality rate of *E. eugeniae* under various treatments of *Kyllingane moralis* aqueous Leaf and Root Extracts [KNALRE] and control groups after 24h exposure.

Treatment	Content	Dead earthworm after 24 hrs. exposure to KNALRE			Mean±S.D.	% mortality
		R1	R2	R3		
		n=6	n=6	n=6	N=18	100%
NegCon	dH_2O	0	0	0	$0.0+0.00^{d}$	0
PosCon	Albendazole	4	5	5	$14.0+0.58^{b}$	78
VehCon	2% DMSO	0	0	0	$0.0+0.00^{d}$	0
Treatment 1	100mg/ml	6	6	6	$18.0+0.00^{b}$	100
Treatment 2	75mg/ml	6	5	6	$17.0+0.58^{b}$	94
Treatment 3	50mg/ml	4	5	5	$14.0+0.58^{b}$	78
Treatment 4	25mg/ml	2	2	3	$7.00+0.58^{c}$	39
ANOVA ($p<0.01$)		0.0000*				

*Similar letter superscript under column S.D. indicates significant difference of the mean at $p<0.01$ based on Tukey's HSD; ** significant at $p<0.01$.

These treatment registered a higher number of deaths than the positive control Albendazole, where twisting motion, sudden hyperactively, and crawling to the cover of the petri dish were manifested, as if avoiding the solution. As observed by the researchers, exposure to T1 and T2 showed rapid and remarkable manifestation of changes in the behavior and physiology of the earthworms under study. While the lowest number of death was observed in the lowest concentration (T4, 25mg/ml) with only 39%

mortality, no deaths were reported in both negative control (distilled water) and vehicle control (DMSO). Further analysis of the trend and patterns from the mortality data showed that mortality followed a linear dose – response curve, where the number of mortality increases as the concentration of KNALRE increases. Similar patterns of mean mortality was observed in the study of Bilbao *et al.*, (2019) where anthelminthic activity of *Cinnamomum cebuense* leaf extract showed a concentration-dependent response trend. In this study, they also noticed that 100mg/ml, 75 mg/ml and 50mg/ml concentrations were more potent than the standard drug Albendazole™.

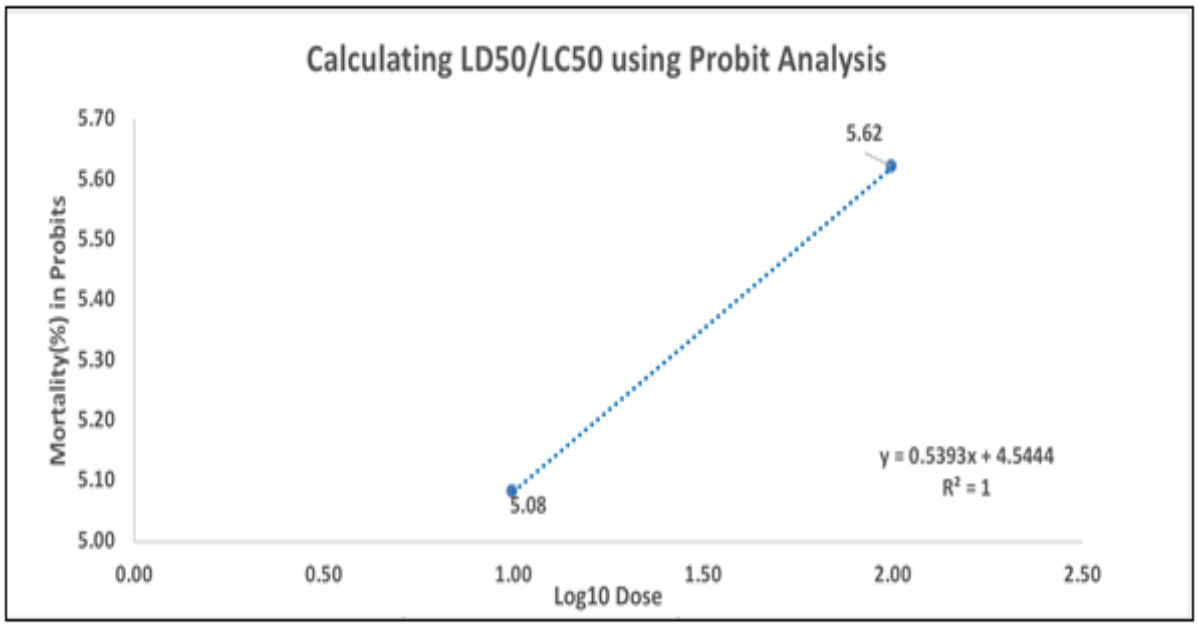

Fig. 1. Percent mortality of *A. salina* exposed to leaf+root ethanolic extracts of *K. nemoralis*plotted against the log10 of the sample concentration. X-axis shows the independent variable (log concentration) while y-axis shows the dependent variable (mortality rate) to illustrate the relationship of the two variables (log concentration and mortality rate).

Toxic effects of *K. nemoralis* aqueous extract evaluated in terms of mean time until paralysis and death (Fig. 2) showed an inversely proportional trend (T1 to T4) where the time taken until complete paralysis and death are increasing as the concentration of the KNALRE decreases. Meanwhile, the highest time taken until both complete paralysis and death was observed in the positive control (Albendazole). ANOVA results (not shown in the figure) for both time until paralysis and death are both highly significant (p=0.000 for both parameters). Post-hoc test (Tukey's HSD) indicated significant differences of the mean time for paralysis across treatments indicating that the mean difference among the 4 experimental group and 1 positive control are not statistically similar to each other. On the hand, post hoc result for mean time until death revealed that T2 (75mg/ml) and T3(50mg/ml) are comparable, similar to T1 (100mg/ml) vs T2 (75mg/ml). These results may indicate that the compared treatments T1 and T2, as well as T2 and T3 are comparable in terms of its potential to kill the earthworm completely. Treatment 4 (25mg/ml) and PosCon (Albendazole) registered a statistical significance against each other, as well as against all treatments.

The present study believed that the differences in the time taken until paralysis or death due to standard drug and *Kyllingane moralis* derived extracts could be attributed to the different target mechanisms at the cellular level. While Albendazole inhibits tubulin polymerization, reducing metabolic and cellular transport in worms and thus killing it eventually

(Vercruysse and Claerebout, 2016), the plant extracts' phytochemicals may have resulted to muscle spasms and fast paralysis, suggesting *K. nemoralis* extract's target mechanism towards muscle and nerves paralysis.

In this study, the earthworm assay of combined leaf and root extracts of *K. nemoralis* is in consonance to the study of Majumder (2013) where the pharmacological properties of this plant species are attributed to the phytochemical constituents present in both leaves and roots of the plant. *K. nemoralis* leaves possess many biologically active chemicals like essential oils (terpenes, α-cyperone, β-selinene, and α-humulene), terpenoids, saponins and phenolic compounds. Meanwhile, the root part possess also flavonoids, triterpenoids and glycosides (Amor *et al.*, 2008) and the most representative compounds include α-muurolol, α-humulene, and α-atlantone (Majumder, 2013).

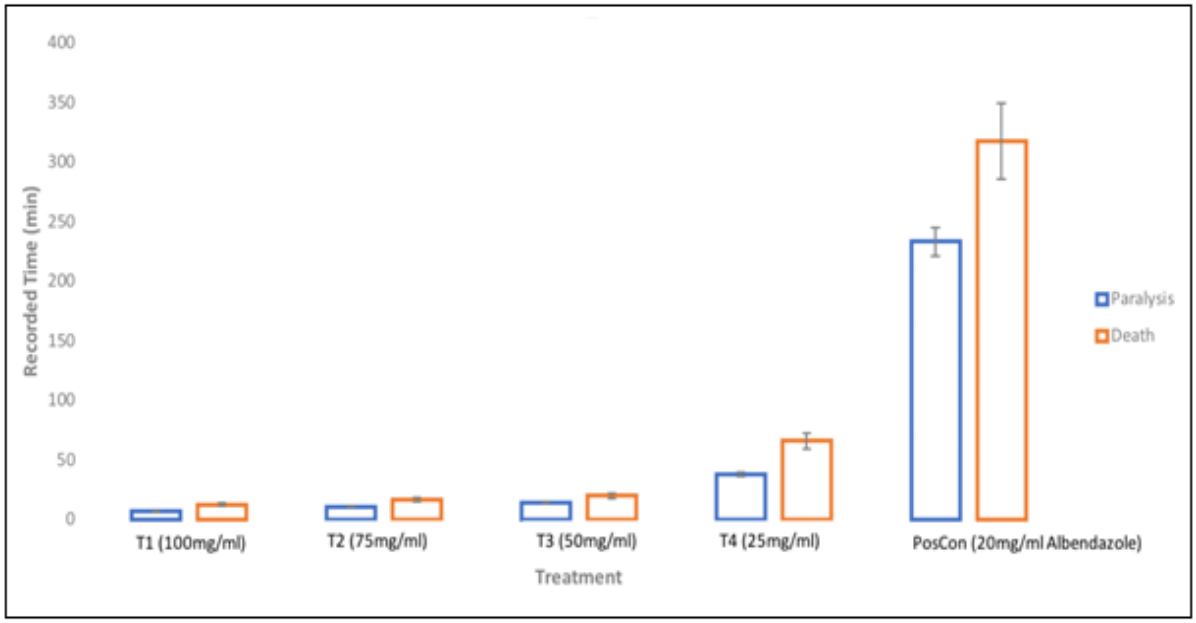

Fig. 2. Toxicity of KNALRE against earthworms in terms of time (in min.) until paralysis (blue bars) and death (orange bars). Pattern shows that time taken until paralysis or death are inversely proportional to the level of concentration of KNALRE in the solution.

Conclusion

K. Nemoralis manifested high acute toxicity as evidenced by high mortality of *A. salina* and *E. euginiae* in a dose-dependent manner.

To pursue further pharmacological investigation, lower vertebrate models (e.g. zebrafish, mice, guinea pig, rabbits) are suggested to extrapolate results to higher mammals. However, non-polar solvents must also be used to explore the potential effect of non-polar compounds in the synergism or antagonism of toxicity of *K. nemoralis*. Quantitative analysis of bioactive compounds is also recommended to increase accuracy if chronic toxicity testing will be pursued in the future. For further research on toxicity testing of *Kyllinga nemoralis*, the researchers recommend the use human cultured cell line to investigate the cytotoxic effects of *K. nemoralis*; and employed chronic toxicity test to determine the long-term toxicity effect of *K. nemoralis*.

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