

Article

Cytotoxic Activity of *Vernonia mespilifolia* Less Used in the Folk Medicine in the Eastern Cape Province, South Africa

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Abstract: *Vernonia mespilifolia* is widely used in folk medicine in the Eastern Cape Province, South Africa. The aim of this study was to evaluate the biological activity of the acetone, aqueous and ethanol extracts of *Vernonia mespilifolia* using brine shrimp hatchability and lethality assay. The result showed hatching success in this order: aqueous extract (48.6%) > acetone extract (38.2%) > ethanol extract (26.8%). The LC₅₀ of the lethality assay were in this order: acetone extract (67.8 µg/mL) > aqueous extract (132 µg/mL) > ethanol extract (383 µg/mL). According to Meyer's toxicity index (using brine shrimps), LC₅₀ < 1000 µg/ mL is toxic. Therefore, the results of the three solvent extracts could be said to be toxic as do have LC₅₀ < 1000 µg/ mL. However, the toxicity of the crude extracts could suggest or confer some antitumor properties, hence further *in vitro*, *in vivo* and antitumour assays are recommended to further substantiate these claims.

Keyword: *Vernonia mespilifolia*; *Artemia salina*; toxicity; biological activity; hatchability; lethality

1. Introduction

The pivotal role medicinal plants and traditional health systems play in solving the health care problems of the world is gaining increasing attention. As a result of this rebirth of interest, research on medicinal plants is rising impressively at the international level, particularly developing countries where traditional medical practice is imbibed as an essential part of their culture [1]. Bioactive compounds present in medicinal plants are responsible for their efficacy [2]. These compounds are mainly secondary metabolites and they include alkaloids, essential oils, tannins and resins to mention a few, which function either in their original form or in semi-synthetic form [3]. In spite of these bioactive compounds exhibiting therapeutic potential, there is insufficient knowledge about their toxicogenic effects when consumed in large amount [4]. Many research studies at present focus on both pharmacology and toxicity of medicinal plants used by humans to promote safety with the use of plant products for the treatment of various ailments [5].

To this end, it is of great importance to verify the pharmacological qualities of herbal-derived remedies and also their level of toxicity contrary to the putative view of the innocuity/inocuousness of natural products [6].

Various assays are being employed for the research of potential toxicity of herbal extracts based on different biological models, such as *in vivo* assays on laboratory animals. Brine Shrimp Lethality Assay (BSLA) has gained recognition as an alternative bioassay technique to screen the toxicity of algae [7], of dental materials [8], toxicity of heavy metals [9] and metal ions [10], toxicity of nanoparticles [11], as well as screening of marine natural products

[12], the toxicity plant extracts [13, 14, 15, 16]. It also indicates cytotoxicity of a myriad of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor of compounds [13, 14].

Vernonia mespilifolia Less. popularly known as Uhlunguhlungu (Xhosa) among the indigenous people of the Nkonkobe Municipality of the Eastern Cape of South Africa is one of the five Southern African species of the *Vernonia* genus that is endemic or near-endemic to this subcontinent [17]. It is a climbing shrub that is 0.6–9.0 m tall, with pinnately-veined leaves, epaleate receptacle with obtuse involucre bracts, and white to violet florets [17]. *Vernonia mespilifolia* is commonly found in the Eastern Cape, Kwazulu-Natal, Limpopo, Mpumalanga and Western Cape provinces of South Africa [18]. It is used in the Eastern Cape of South Africa for ethno-medicinal management of weight loss and hypertension [19] and also for the treatment of heart water disease in goats [20].

Although *V. mespilifolia* is used for ethno-medicinal purposes there is limited knowledge about its toxicity level. This study aims to investigate the cytotoxic activity of the crude extracts of *Vernonia mespilifolia* using a brine shrimp model.

2. Materials and methods

2.1 Materials

The whole plant parts used for this study were collected in June 2015 from its natural habitat in the wild at Zihlahleni Village Maipase, Nkonkobe Rural Eastern Cape, South Africa which lies at Latitude 32° 51' 41.846" S and Longitude 27° 10' 59.318" E. The plant was authenticated by Mr. Tony Dold of Selmar Schonland Herbarium, Rhodes University, South Africa, and a voucher specimen (Unuofin Med, 2015/1) was prepared and deposited at the Giffen Herbarium, University of Fort Hare.

2.2 Preparation of extracts

The whole plant was rinsed with deionised water and gently blotted with paper towel to remove the water and subsequently oven-dried (LABOTEC, South Africa) at 55°C for 72 hours until constant weight was achieved. The dried sample was then ground into powder (Polymix® PX-MFC 90D Switzerland) and stored at 4°C until needed for analyses. The ground sample (200g) was weighed into 3 separate conical flasks containing (2 L) acetone, ethanol, and water respectively and shaken in an orbital shaker (Orbital Incubator Shaker, Gallenkamp) for 48 hours. The crude extracts were filtered under pressure using a Buchner funnel and Whatman No. 1 filter paper. The acetone and ethanol extracts were further concentrated to dryness to remove the solvents under reduced pressure using a rotary evaporator (Strike 202 Steroglass, Italy), while the aqueous filtrate obtained was concentrated using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY).

2.3 *A. salina* hatching assay

The method described by [21] was employed with little modifications. Five petri dishes containing 30 mL of the extracts were prepared in filtered sea water by dissolving them in minute amounts of the parent solvents to yield a two-fold dilution of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg/mL). A positive control was also prepared by dissolving potassium dichromate in seawater in the same concentrations as the plant extracts. Sea water alone was used as the negative control. The setup was allowed to stand for 30 minutes to allow the solvents to evaporate.

Ten (10) *A. salina* cysts were stocked in each of the petri dishes containing 30 mL of the prepared two-fold concentrations (1 to 0.0625 mg/mL) of the plant fractions and positive

control. The petri dishes were partly covered, incubated at 30°C and under constant illumination for 72 hours. The number of free nauplii in each petri dish was counted after every 24 hours till the end of 72 hours. The percentage of hatchability was calculated by comparing the number of hatched nauplii with the total number of cysts stocked.

2.4 *A. salina* lethality assay

A. salina cysts were hatched in sea water and 10 nauplii were pipetted into each petri dish containing the two-fold concentrations of the extracts and control as in the hatchability assay described above. The petri dishes were then examined and the number of living nauplii (that exhibited movement during several seconds of observation) was counted after every 24 hours and the set up was allowed to stand for 72 hours under constant illumination. The percentage of mortality (M %) was calculated as: $\text{Mortality (\%)} = (\text{Total nauplii} - \text{Live nauplii}) \times 100 / \text{Total nauplii}$.

2.5 Data analysis

The percentage hatchability success and mortality data obtained from the 5 different concentrations of each fraction and control experiments were used to construct the dose-response curves. These were used to determine their corresponding LC₅₀ values. The LC₅₀ was taken as the concentration required for producing 50% mortality of the nauplii. LC₅₀ values were determined from the best-fit line obtained by regression analysis of the percentage hatchability and lethality versus the concentration. The statistical analysis was done on MINITAB version 17 for Windows. One-way analysis of variance (ANOVA) followed by Fischer's Least Significant Difference (for means separation) was used to test the effect of concentration and time of exposure of the plant extracts on the hatchability success of the cysts and mortality of larvae respectively.

3.0 Results and Discussion

3.1 Brine shrimp hatchability

Brine shrimp hatchability test was used to determine the biological activity of *Vernonia mespilifolia*. The hatching success of *A. salina* incubated with different plant extracts and control is as shown in Figure. The sea water exhibited a significantly higher ($P < 0.05$) hatching success (71%) than the solvent extracts and the positive control (potassium dichromate) (5.4%). The hatching success of the cysts in the acetone, aqueous and ethanol extracts were 38.2%, 48.6% and 26.8% respectively and were significantly different ($P < 0.05$) from each other. The hatching success of *A. Salina* cysts incubated with aqueous extracts had the highest hatching success and presumably least toxic of the solvent extracts used (Figure 1). This could explain why most traditional herbal medicines are prepared using water as a solvent because it is not or less toxic. This could also suggest why the cyst showed more resistant to hatching in the acetonic and ethanol extracts than in the aqueous extracts. This resistant could be attributed to the permeability barrier provided by the cysts [22, 23].

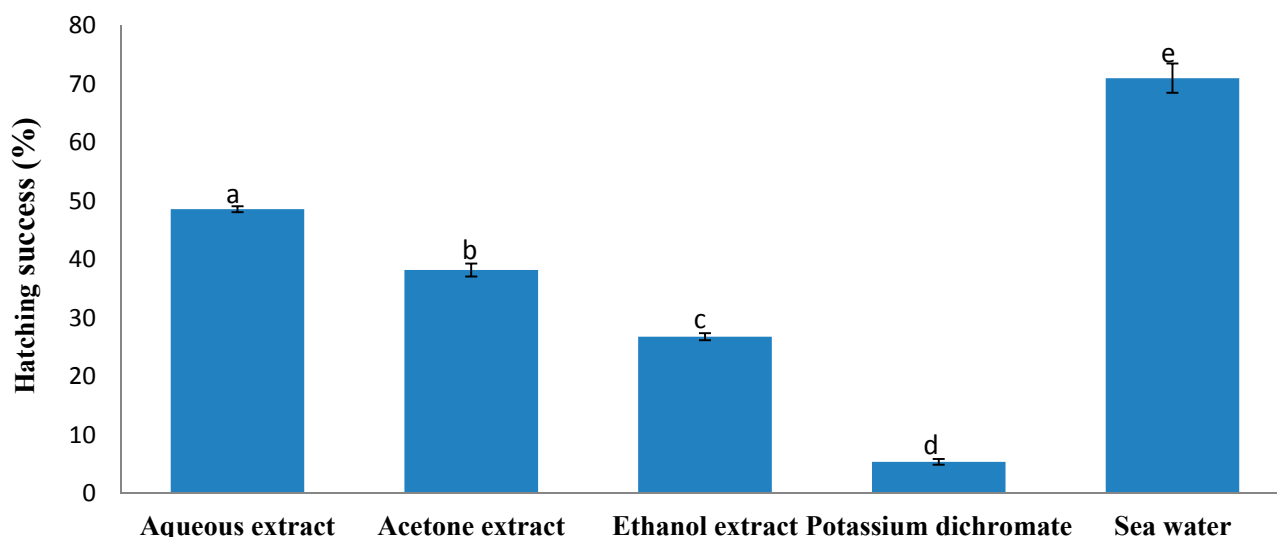


Figure 1: Percentage hatching success of *A. salina* cysts incubated in different solvent extracts and controls.

*The values are means of five concentrations for each plant extract/control \pm SD of three replicates. Bars with different letters are significantly different ($P < 0.05$).

The *A. salina* embryos are highly defenseless to toxins at early developmental stages [24, 25, and 26]. In the brine shrimp hatchability assay, the hatching success significantly decreased with increasing concentrations of the crude extracts in a dose dependent manner. The hatching success of the cyst was also evaluated at different concentrations and the result is depicted in Figure 2. The aqueous extract exhibited its maximum hatching success at 0.5 mg/mL (76%). The acetone and ethanol extracts had similar hatching pattern, with the highest hatching success observed at 0.125 mg/mL (Figure 2). The overall best percentage hatching success potential of cysts was observed with cyst incubated at 0.5 mg/mL in the aqueous extract which was significantly ($P > 0.05$) greater when compared with other solvent used. Potassium dichromate showed the lowest hatching success. At 0.0625 mg/mL, there was no significant difference in the hatching success of aqueous and potassium dichromate. There was also no significant difference in the hatching success of cysts incubated at 0.125 mg/mL in both the aqueous and acetone extracts. The acetone and ethanol extracts had no significant difference in their hatching success at 0.25 mg/mL. At concentrations of 0.25 mg/mL -1 mg/mL for potassium dichromate, no hatching was observed. Also, cysts incubated in 1 mg/mL of acetone and ethanol extracts did not hatch. The inhibitory effect of *Vernonia mespilifolia* crude extracts could have attributed to the toxic compounds present in the extracts that have ovicidal property. Also reports have shown that secondary metabolites in plants could have effect on the embryonic development [27].

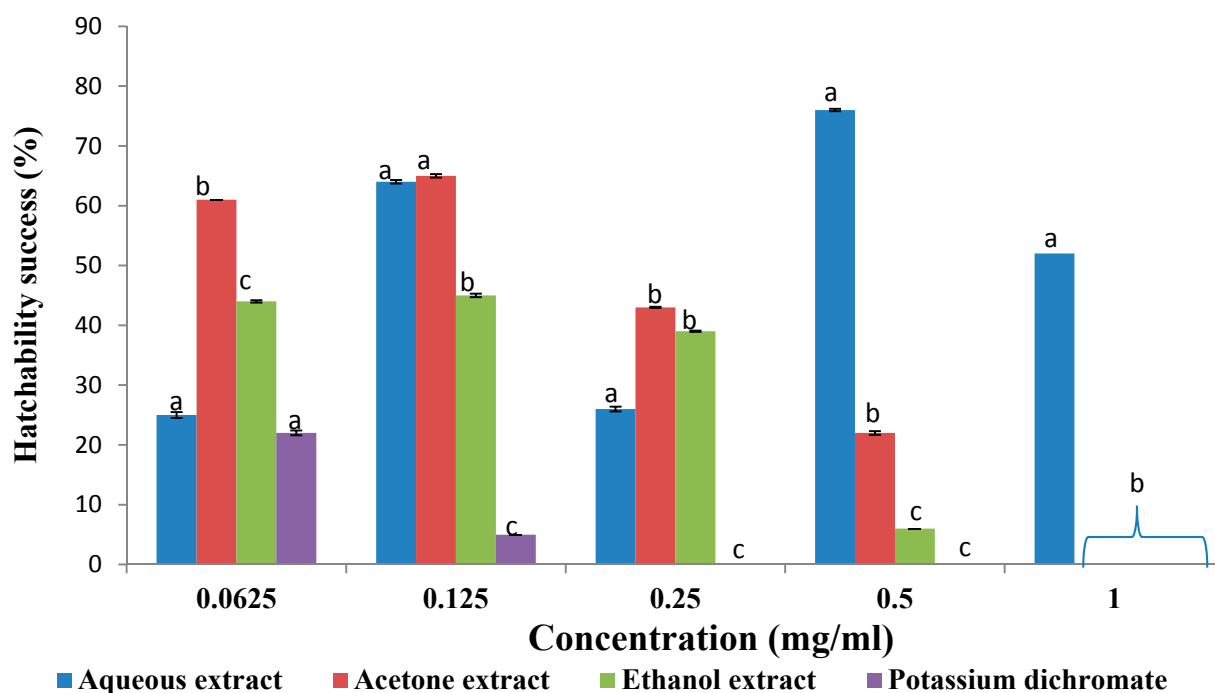


Figure 2: Effect of varying concentrations on hatching success (%) of *A. salina* cysts.

*Values are means \pm SD of three replicates of the concentrations for each plant extract/control. Set of bars with different letters are significantly different ($P > 0.05$).

The results of the effect of exposure time on hatchability showed that the sensitivity of *A. salina* cyst to the plant extracts was strongly dependent upon exposure period (Figure 3). A similar trend was observed with the aqueous and acetone extracts as there was significant differences in hatching success from 24 h to 48 h ($P > 0.05$). The lowest hatching success was observed after 24 h treatment in all the extracts. This result is in line with reports from [26, 27] which stated that *A. salina* is extremely susceptible to toxin during its early stage of development. The acetone and ethanol extracts experienced maximum hatching at 48 h with 45% and 32% hatching success respectively, after which death set in for the already hatched nauphlii. The aqueous extract had the highest hatching success at 60 hours. Also in Figure 3 there was a 1.3 and 1.1 fold decrease respectively in hatched cyst from 48 to 72 h in both acetone and ethanol extracts while aqueous extract decreased significantly by 1.1 fold after 60 h ($P < 0.05$). Sea water exhibited optimum hatching at 36 h and remained the same throughout the experiment. Cysts incubated in potassium dichromate decreased significantly by 3.5-fold after 36 hours ($P < 0.05$), followed by no hatching of cyst after 48 hours.

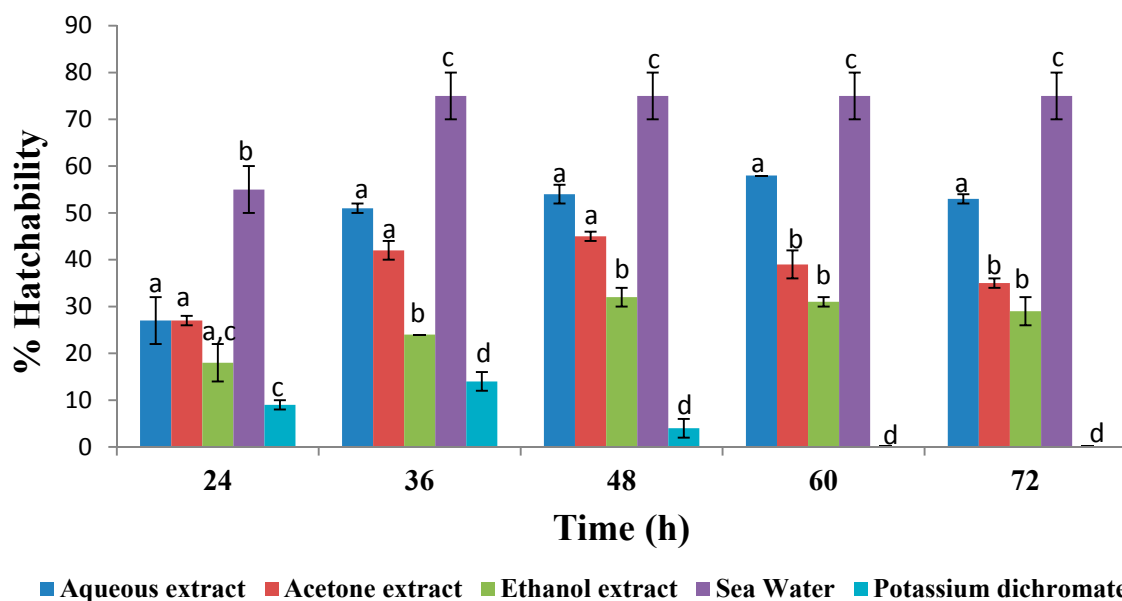


Figure 3: Effect of time (h) on the hatching success of *A. salina* cysts.

*Values are means \pm SD of three replicates for each plant extract/control. Set of bars with different letters are significantly different ($P < 0.05$).

3.2 Brine shrimp lethality

Brine shrimp cytotoxicity test is considered as a preliminary assessment of toxicity. This assay determines lethal concentration of active compounds such as heavy metals, pesticides, and medicines in brine medium [28, 29, 30] and has been employed to determine toxicity of various active compounds because of it is reliable, rapid and very convenient to carry out [13, 31, 21]. The percentage mortality of *A. salina* larvae (nauplii) incubated in different solvent extracts of *Vernonia mespilifolia* and controls are shown in Figure 4. There was high mortality of nauplii incubated in both the aqueous and acetic extracts although there was no significant difference in their percentage mortality, whereas the nauplii incubated with potassium dichromate had a significantly greater mortality when compared to all the extracts and sea water ($P < 0.05$). The ethanolic extract and sea water showed a mortality of 49.60% and 0% respectively.

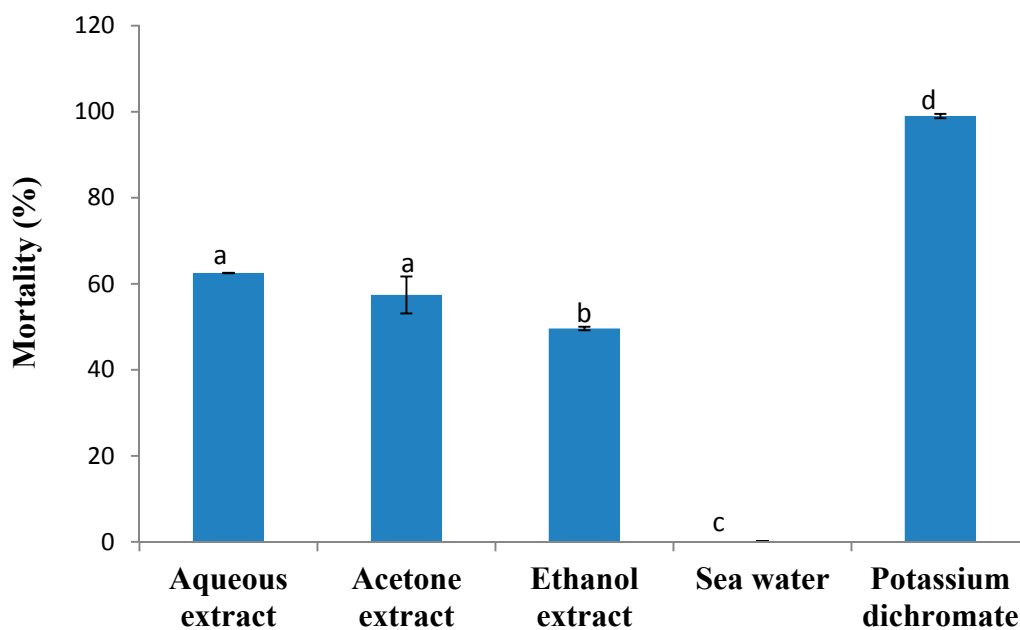


Figure 4: Percentage mortality of *A. salina* nauplii incubated in different plant extracts and controls.

*The values are means of five concentrations for each plant extract/control \pm SD of three replicates. Bars with different letters are significantly different ($P < 0.05$).

The effect of different concentrations of the plant extracts on the mortality of larvae is shown in Figures 5. The degree of mortality of nauplii was in a concentration-dependent fashion. The highest mortality was observed in all the extracts at 1 mg/mL compared to potassium dichromate which showed maximum mortality (100%) at 0.125 mg/mL. There was no significant difference ($P < 0.05$) in percentage mortality of the nauplii between the aqueous extract and acetone extract at concentrations of 0.125, 0.25 and 1 mg/mL. At 0.125 and 0.5 mg/mL, aqueous and ethanol extracts also exhibited no significance difference in percentage mortality ($P < 0.05$). At 1 mg/mL, there was no significance difference in percentage mortality between aqueous and acetone extracts and also between the ethanolic extract and potassium dichromate ($P < 0.05$) in Figure 5. The results revealed that the effect of varying concentrations of all the plant extracts on the mortality of larvae was in a concentration dependent fashion, therefore it can be postulated that though these are toxicological data, this plant possesses pharmacological activity based on the dosage administered [21, 32].

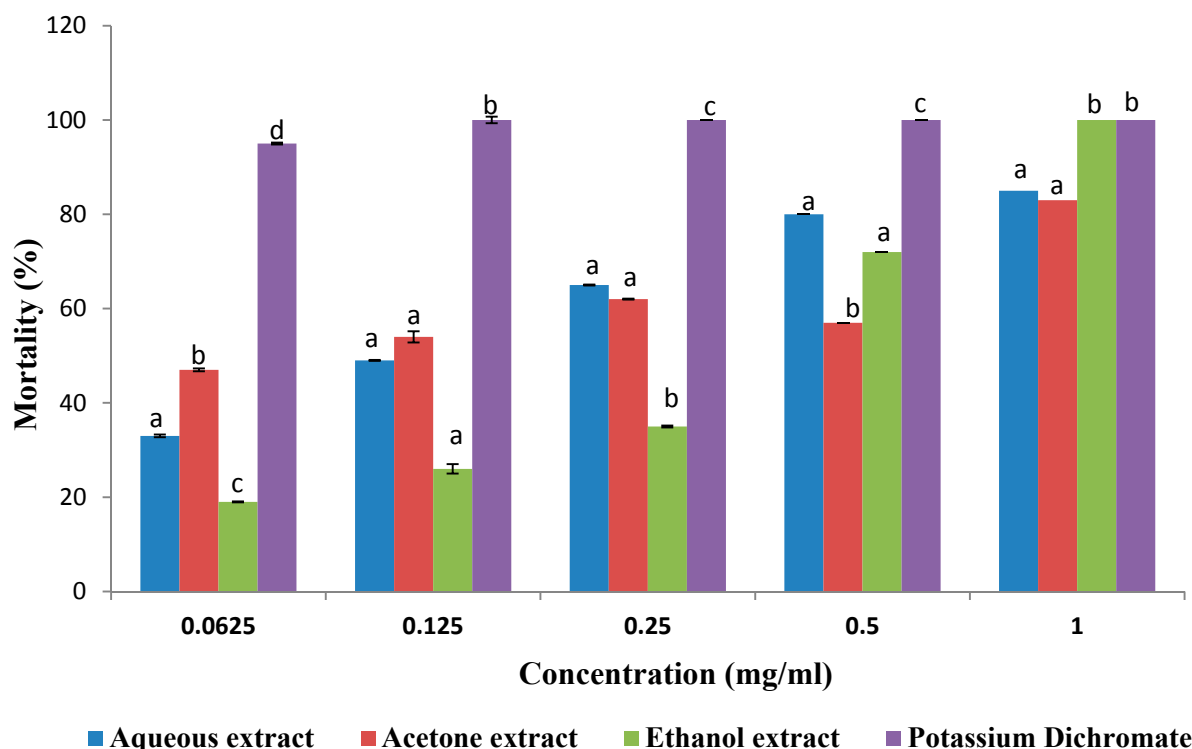


Figure 5: Percentage mortality of *A. salina* cysts incubated at different concentrations of the plant extracts and control.

*The values are means of three replicates \pm SD (at different hours). Set of bars with different letters are significantly different ($P < 0.05$).

All extracts were screened at five different concentrations viz. 62.5, 125, 250, 500 and 1000 $\mu\text{g/mL}$ and observed for their toxic effect on *A. salina* from 24 - 72 h. Potassium dichromate was used as a standard [33].

The percentage mortality due to exposure time is as shown in Figure 6. The result revealed that the percentage mortality was time dependent; the longer the exposure of nauplii to the plant extracts, the greater the mortality. It was observed that between 24-72 h of exposure of the nauplii to aqueous, acetone and ethanol extracts, there was 2.94, 3.41 and 2.5-fold increase in the mortality of nauplii respectively. The nauplii incubated in sea water did not die throughout the duration of the experiment. Generally, the mortality of nauplii was significantly similar when incubated with the ethanol, acetone, and the aqueous extracts at 24 h and was significantly higher than sea water ($P < 0.05$) (Figure 6). The mortality of nauplii incubated in these plant extracts increased exponentially with time with the highest mortality observed at 72 hours with all the extracts. The nauplii attain the second and third instars of their life cycle within 48 hours hence reveal their greatest sensitivity to toxins at this time [34, 35]. However, the findings of this study indicate that the maximum sensitivity was reached after 72 hours of exposure.

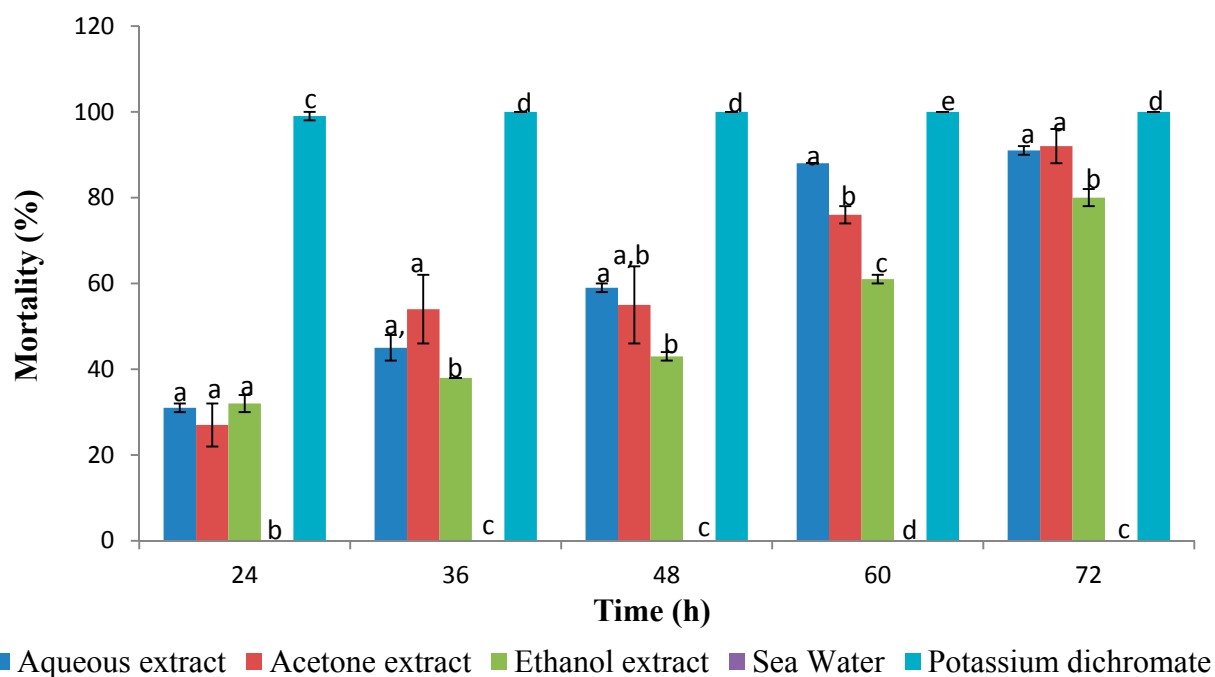


Figure 6: Percentage mortality of *A. salina* cysts incubated at different time durations in the plant extracts and controls.

*The values are means \pm SD of 3 replicates (of all the concentrations) for each plant extract/control \pm SD. Set of bars with different letters are significantly different ($P < 0.05$).

According to Meyer et al [13] and Bastos et al [36] the brine shrimp lethality were interpreted in accordance to the criterion by Meyer toxicity index that LC_{50} values greater than 1000 $\mu\text{g/mL}$ (1 mg/mL) are considered non-toxic, LC_{50} values equal/greater than 500 $\mu\text{g/mL}$ (0.5 mg/mL) but not greater than 1000 $\mu\text{g/mL}$ are considered to have weak toxicity while those having LC_{50} values less than 500 $\mu\text{g/mL}$ are considered toxic. The LC_{50} values were calculated as 132 $\mu\text{g/mL}$ for aqueous extract, 67.8 $\mu\text{g/mL}$ for acetone extract and 383 $\mu\text{g/mL}$ for the ethanol extracts, respectively (Table 1).

All of the crude extracts of *V. mespilifolia* were found to be lethal with $LC_{50} < 1$ mg/mL (Table 1) and hence it could be employed as promising alternative in the treatment and management of tumor, as brine shrimp lethality test now serves as an indicator for the preliminary screening of bioactivity including for anticancer [37].

Table 1: Lethal dose concentration (LC_{50}) of acetone, ethanol and aqueous extracts of *Vernonia mespilifolia* against Brine Shrimp

Sample	Regression equation	LC_{50} ($\mu\text{g/mL}$)	Toxicity status	R^2 (%)
Aqueous extract	$Y = 19.476\ln(x) + 89.4$	132	Toxic	97.4
Acetone extract	$Y = 33.161x + 47.75$	67.8	Toxic	85.8
Ethanol extract	$Y = 88.667x + 16.042$	383	Toxic	96.3
Potassium dichromate	$Y = 1.4427247\ln(x) + 101$	< 0.100	Toxic	50

* LC_{50} is the concentration ($\mu\text{g/mL}$) of the plant extracts and positive control (Potassium dichromate) sufficient to obtain 50% of inhibition of nauplii mortality of *A. salina*, respectively. R^2 is the coefficient of determination of the regression equation.

4.0 Conclusion

This study showed that the different crude extracts of *Vernonia mespilifolia* were lethal ($LC_{50} < 1$ mg/mL) in the brine shrimp lethality assay. Further *in vivo* and *in vitro* studies with different human cell lines is required to ascertain the antitumor activity of this plant and also the isolation of the lead compound responsible for this activity, might be utilized for the development of novel anticancer drug.

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Author Contributions: Jeremiah Oshiomame Unuofin, Gloria Aderonke Otunola and Anthony Jide Afolayan conceived and designed the experiments; Jeremiah Oshiomame Unuofin performed the experiments; Jeremiah Oshiomame Unuofin and Gloria Aderonke Otunola analyzed the data; Anthony Jide Afolayan contributed reagents/materials/analysis tools; Jeremiah Oshiomame Unuofin wrote the paper.” Authorship must be limited to those who have contributed substantially to the work reported.

Conflicts of Interest: The authors declare no conflict of interest.

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