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Research Article
Toxicity Evaluation of *Vernonia mespilifolia* Less (A South Africa Medicinal Plant) Using Brine Shrimp

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Abstract

Background and Objective: *Vernonia mespilifolia* is widely used in folk medicine in the Eastern Cape province, South Africa. This study evaluated the toxicity of acetone, aqueous and ethanol extracts of *Vernonia mespilifolia* using brine shrimp model. **Materials and Methods:** Different concentrations (0.0625-1 mg mL^{-1}) of the extracts were used to incubate the cysts and nauplii (hatched cysts) of brine shrimp (*Artemia salina*) to evaluate their effects on the hatching success of the cyst and lethality of the nauplii respectively. The percentage hatching success of cyst and Lethal Concentration (LC_{50}) to kill 50% of the nauplii were recorded. **Results:** The hatching success was in order: Aqueous extract (48.6%)>acetone extract (38.2%)>ethanol extract (26.8%). The hatching of nauplii was in a concentration dependent fashion, with hatching success decreasing with increase in concentration of extracts. Lethality of extract was based on Meyer's index of toxicity. **Conclusion:** All the three extracts showed high levels of toxicity with LC_{50} <1 mg mL^{-1} which signify toxicity in a brine shrimp model. In this respect, *V. mespilifolia* possesses cytotoxic behavior suggesting the presence of potential bioactive chemical component in the plant extract. Further *in vivo* and cell lines cytotoxicity test is recommended to substantiate these findings.

Key words: *Vernonia mespilifolia*, toxicity, brine shrimp, hatchability, lethality, nauplii, cyst, extracts

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

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, study on medicinal plants is rising impressively at the international level, particularly in 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 forms[3]. In spite of these bioactive compounds exhibiting therapeutic potential, there is insufficient knowledge about their toxicogenic effects when consumed in large amounts[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/innocuousness of natural products[6].

Various assays are being employed for the study 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], dental materials[8], heavy metals[9] and metal ions[10], toxicity of nanoparticles[11], as well as screening of marine natural products[12] and the toxicity plant extracts[13-16]. It also indicated 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, 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 involucral 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 ethnomedicinal 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 ethnomedicinal purposes, there is limited knowledge about it toxicity level. This study aim to investigate the potential toxicity of the crude extracts of *Vernonia mespilifolia* using brine shrimp model.

MATERIALS AND METHODS

The whole plant parts used for this study was collected in June, 2015 from its natural habitat in the wild at Zihlahleni village Maipase, Nkonkobe Municipality of the Eastern Cape, South Africa; which lies at latitude 32°51′ 41.846″ S and longitude 7° 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.

Preparation of extracts: The whole plant was rinsed with deionised water and gently blotted dry with paper towel and subsequently oven-dried (LABOTEC, South Africa) at 55°C for 72 h until constant weight was achieved. The dried sample was then ground into powder (Polymix® PX-MFC 90D Switzerland) and stored at 4°C till needed for analyses. The ground sample (200 g) 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 h. 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).

***Artemia salina* hatching assay:** The method described by Otang *et al.*[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 amount (1 mL) of the parent solvents to yield a two-fold dilution of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg mL^{-1}). 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 min to allow the solvents to evaporate.

Ten *A. salina* cysts were stocked in each of the petri dishes containing 30 mL of the prepared two-fold

concentrations (1-0.0625 mg mL^{-1}) of the plant fractions and positive control. The petri dishes were partly covered, incubated at 30°C and under constant illumination for 72 h. The number of free nauplii in each petri dish was counted after every 24 h till the end of 72 h. The percentage of hatchability was calculated by comparing the number of hatched nauplii with the total number of cysts stocked.

***Artemia salina* lethality assay:** *Artemia 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 h and the set up was allowed to stand for 72 h under constant illumination. The percentage of mortality (M%) was calculated as following in Eq. 1:

$$\text{Mortality (\%)} = \frac{\text{Total nauplii} - \text{Live nauplii}}{\text{Total nauplii}} \times 100 \quad (1)$$

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 LC_{50} values. The LC_{50} was taken as the concentration required for producing 50% mortality of the nauplii. The LC_{50} 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.

RESULTS AND DISCUSSION

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 Fig. 1. 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 (Fig. 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].

The *A. salina* embryos are highly defenseless to toxins at early developmental stages[24-26]. In the brine shrimp hatchability assay, the hatching success significantly

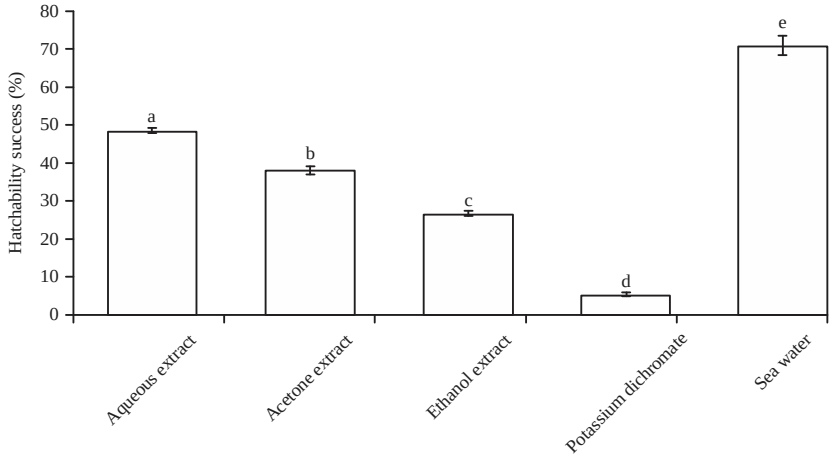

Fig. 1: Percentage hatching success of *A. salina* cysts incubated in different solvent extracts and controls

Values are means of 5 concentrations for each plant extract/control ±SD of three replicates. Bars with different letters are significantly different at $p<0.05$

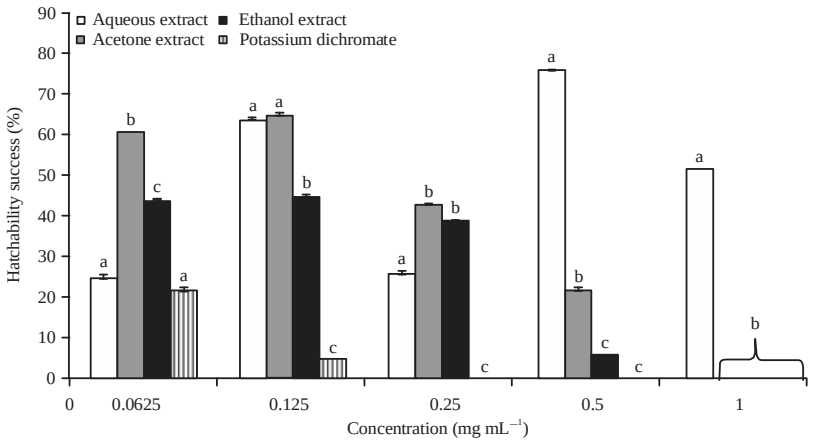

Fig. 2: Effect of varying concentrations on hatching success (%) of *A. salina* cysts

Values are Means±SD of three replicates of the concentrations for each plant extract/control. Set of bars with different letters are significantly different at p>0.05

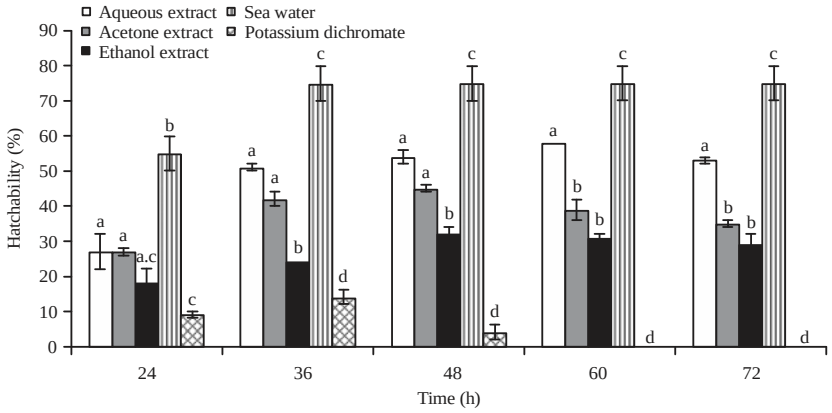

Fig. 3: Effect of time (h) on the hatching success of *A. salina* cysts

Values are Means±SD of three replicates for each plant extract/control. Set of bars with different letters are significantly different at p<0.05

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 Fig. 2. The aqueous extract exhibited its maximum hatching success at 0.5 mg mL^{-1} (76%). The acetone and ethanol extracts had similar hatching pattern, with the highest hatching success observed at 0.125 mg mL^{-1} (Fig. 2). The overall best hatching success potential was observed with cysts incubated at 0.5 mg mL^{-1} in the aqueous extract which was significantly (p>0.05) greater when compared with other solvents used. Potassium dichromate showed the lowest hatching success. At 0.0625 mg mL^{-1}, there was no significant difference in the hatching success of aqueous extract and potassium dichromate. There was also no significant difference in the hatching success of cysts incubated at 0.125 mg mL^{-1} 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^{-1}. At concentrations of 0.25-1 mg mL^{-1} for potassium dichromate, no hatching was observed. Also, cysts incubated in 1 mg mL^{-1} of acetone and ethanol extracts did not hatch. The inability of cysts to hatch in *V. mespilifolia* crude extracts could be 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].

The results of the effect of exposure time on hatchability showed that the sensitivity of *A. salina* cysts to the plant extracts was strongly dependent upon exposure period (Fig. 3). A similar trend was observed with the aqueous and acetone extracts as there was significant differences in hatching success from 24-48 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 Vasconcelos *et al.*[26] and

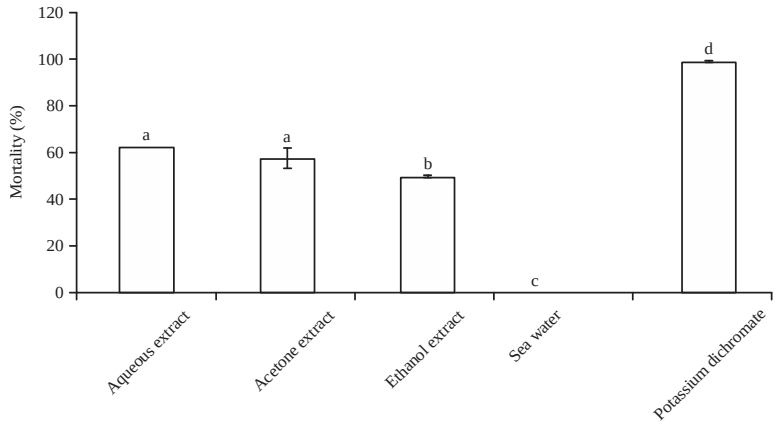

Fig. 4: Percentage mortality of *A. salina* nauplii incubated in different plant extracts and controls
Values are means of 5 concentrations for each plant extract/control ±SD of three replicates. Bars with different letters are significantly different at p<0.05

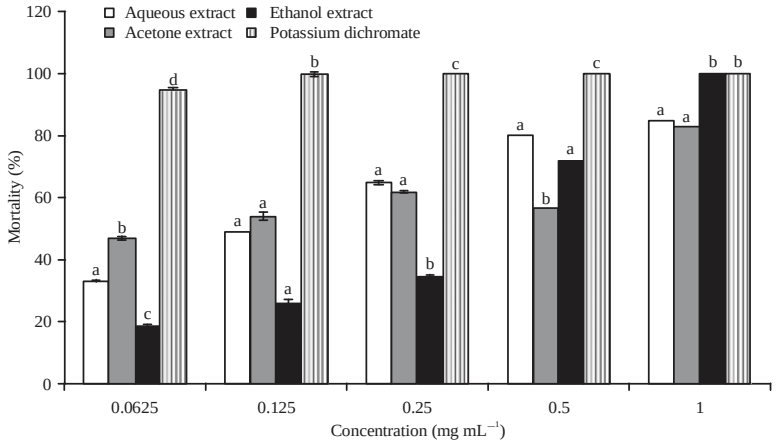

Fig. 5: Percentage mortality of *A. salina* cysts incubated at different concentrations of the plant extracts and control
Values are means of three replicates ±SD (at different hours). Set of bars with different letters are significantly different at p<0.05

Subhadra *et al.*[27], which stated that *A. salina* is extremely susceptible to toxin during its early stage of development. The cysts in 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 nauplii. The aqueous extract had the highest hatching success at 60 h. Also, in Fig. 3 there was a 1.3 and 1.1 fold decrease respectively in hatched cyst from 48-72 h in both acetone and ethanol extracts, while aqueous extract decreased significantly ($p<0.05$) by 1.1 fold after 60 h. Sea water exhibited optimum hatching at 36 h and remained the same throughout the experiment. Hatching of cysts incubated in potassium dichromate decreased significantly by 3.5-folds after 36 h ($p<0.05$), followed by no hatching of cysts after 48 h.

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[28-30] and has been employed to determine toxicity of various active compounds because 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 Fig. 4. There was high mortality of nauplii incubated in both the aqueous and acetone extracts, although there was no significant difference in the 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.

The effect of different concentrations of the plant extracts on the mortality of larvae is shown in Fig. 5. The degree of mortality of nauplii was in a concentration-dependent

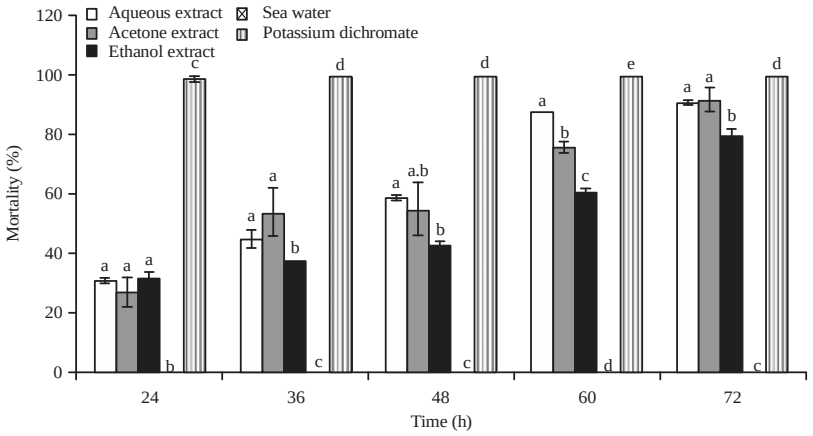

Fig. 6: Percentage mortality of *A. salina* cysts incubated at different time durations in the plant extracts and controls

Values are Means±SD of 3 replicates (of all the concentrations) for each plant extract/control ±SD. Set of bars with different letters are significantly different at p<0.05

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

Sample	Regression equation	LC_{50} (µg mL^{-1})	Toxicity status	R^2 (%)
Aqueous extract	Y = 19.476ln(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 = 1.4427247ln(x)+101	<0.100	Toxic	50

LC_{50} is the concentration (µg mL^{-1}) 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

fashion. The highest mortality was observed in all the extracts at 1 mg mL^{-1} compared to potassium dichromate which showed maximum mortality (100%) at 0.125 mg mL^{-1}. 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^{-1}. At 0.125 and 0.5 mg mL^{-1}, aqueous and ethanol extracts also exhibited no significance difference in percentage mortality ($p<0.05$). At 1 mg mL^{-1}, 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 Fig. 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].

All extracts were screened at 5 different concentrations viz., 62.5, 125, 250, 500 and 1000 µg mL^{-1} and observed for their toxic effect on *A. salina* rom 24-72 h. Potassium dichromate was used as a standard[33].

The percentage mortality due to exposure time is as shown in Fig. 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 were 2.94, 3.41 and 2.5-folds 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 ($p<0.05$) (Fig. 6). The mortality of nauplii incubated in these plant extracts increased exponentially with time with the highest mortality observed at 72 h with all the extracts. The nauplii attain the second and third instars of their life cycle within 48 h, hence their greatest sensitivity to toxins at this time[34,35]. However, the findings of this study indicated that the maximum sensitivity was reached after 72 h of exposure.

According to Meyer *et al.*[17] and Bastos *et al.*[36] the brine shrimp lethality were interpreted in accordance to the criterion by Meyer toxicity index that LC_{50} values >1000 µg mL^{-1} (1 mg mL^{-1}) are considered non-toxic, LC_{50} values $\geq$500 µg mL^{-1} (0.5 mg mL^{-1}) but not >1000 µg mL^{-1} are considered to have weak toxicity, while those having LC_{50} values <500 µg mL^{-1} are considered toxic. The LC_{50} values were calculated as 132, 6.78 and 383 µg mL^{-1} for aqueous extract, acetone extract and the ethanol extracts, respectively (Table 1).

The BSLA result of all the crude extracts of *V. mespilifolia* showed that the extracts were toxic with LC_{50} <1 mg mL^{-1} (Table 1); hence these extracts may not be considered safe for consumption as herbal medicine. These toxic results from this study could be employed as promising alternative in the treatment and management of tumors as brine shrimp lethality test now serves as an indicator for the preliminary screening of bioactivity including for anticancer[37] properties. The combination of larval mortality and increased exposure period further improved the bioassay sensitivity, providing values which are typically lower than embryotoxicity assays. The use of *Artemia salina* 72 h larval mortality assay may be used as an alternative bioassay to screen for toxic effects of novel bioactive compounds, particularly in situations where the availability of mature invertebrates such as sea urchins is problematic.

CONCLUSION

The brine shrimp lethality bioassay now serves as a useful tool for the preliminary assessment of toxicity from plant extracts. It can be deduced that the acetone, aqueous and ethanol extracts of *Vernonia mespilifolia* contains certain useful bioactive compounds which though toxic can be harnessed and purified into useful therapeutic drugs. Although the brine shrimp lethality cytotoxicity bioassay is rather insufficient as to the elucidation of the mechanism of action.

SIGNIFICANT STATEMENT

This study sought to screen the potential toxicity of *Vernonia mespilifolia* Less., a medicinal plant widely used for the management of weight loss and hypertension in South Africa using the brine shrimp toxicity assay. The findings revealed that all the solvent extracts screened were toxic. Therefore, these results indicate that *V. mespilifolia* may not be safe for human consumption. However, further studies are on-going to establish the toxicity and its alternative use in cancer cells.

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