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Assessment of potential toxicity of three South African medicinal plants using the brine shrimp (*Artemia salina*) assay

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As the use of medicinal plants increases, screening of their toxicity is crucial to guarantee the safety of the users. Hexane and acetone extracts of three South African plants, traditionally used for the management of opportunistic fungal infections in human immune deficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients were assayed for toxicity to hatching and larval mortality of *Artemia salina.* Lowest percentage values of hatching success were observed in cysts incubated with hexane and acetone extracts of *Arctotis arctotoides,* respectively and were significantly lower ($P < 0.05$) than that of the positive control (amphotericin B). Based on Meyer's toxicity index, both extracts of *Pittosporum viridiflorum* and the acetone extract of *A. arctotoides* with LC_{50} values > 1 mg/ml were considered as non-toxic and may be further explored for development of plant-based pharmaceuticals. The hexane and acetone extracts of *A. arctotoides* and the hexane extract of *Gasteria bicolor* with LC_{50} values < 1 mg/ml have shown significant biological activity, indicative of the presence of potent cytotoxic components which warrant further investigation. The *in vivo* lethality of *A. salina* has been used as a convenient toxicological screening system, while the resistance to harmful effects by *Artemia* cysts made the hatchability assay less desirable than the lethality test.

Key words: Medicinal plants, *Artemia salina*, hatchability assay, lethality test.

INTRODUCTION

Arctotis arctotoides (L.f.) O. Hoffm. (Asteraceae) is a decumbent herb commonly found as a roadside weed in most coastal districts of South Africa (Afolayan, 2003). *Gasteria bicolor* Haw. belongs to the succulent genus *Gasteria* (Aloaceae) which comprises 16 species and is endemic to South Africa, with its main centre of distribution in the savanna region of the Eastern Cape (Dagne et al., 1996). *Pittosporum viridiflorum* Sims, (Pittosporaceae) is a shrub used in traditional medicine to treat fever, malaria, inflammation and stomach ache and as an antidote for insect bites (Seo et al., 2002). A number of investigations have been performed on *P. viridiflorum* indicating that its leaves possess antimicrobial properties and contains compounds such as volatiles monoterpenes, sesquiterpenes, and cytotoxic saponins (Seo et al., 2002). Based on our previous ethnobotanical survey (Otang et al., 2012), the above medicinal plants were commonly cited by human immune deficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients and traditional healers for their usage in the management of symptoms of opportunistic fungal infections. The *in vitro* antifungal activity of these plants has also been investigated (Seo et al., 2002; Sultana et al., 2008).

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As the use of these medicinal plants increases, experimental screening of their toxicity is crucial to guarantee the safety of the users (Asgarpanah and Ramezanloo, 2012; Nazri et al., 2012). Toxicity is an expression of being poisonous, indicating the state of adverse effects caused by the interaction between toxicants and cells (Alam et al., 2012; Syahmi et al., 2010). Recent years have seen the development of a number of toxicity tests in which the response has been measured in invertebrates. These tests have the virtue of being inexpensive, reproducible, easy to carry out, and environmentally relevant (Favilla et al., 2006). Invertebrates are already used in tests that are required by some regulatory authorities for the environmental risk assessment of pesticides, chemicals and pollutants (Commission of the European Communities (CEC), 1991; United States Environment Protection Agency (US EPA), 2002).

The crustacean *Artemia salina* Leach (brine shrimp) is an invertebrate that has been widely used for studies of ecotoxicology, as well as of general toxicology of chemicals (Cleuvers, 2003) and natural compounds (Caldwell et al., 2003). *A. salina* cysts are easily available commercially and inexpensive and hence this assay may be useful in situations where rapidity and low cost make it practical to test large number of samples for preliminary toxicity screening. The present study was therefore designed to evaluate the potential toxicity (using *A. salina*) of three South African medicinal plants, traditionally used for the management of opportunistic fungal infections in HIV/AIDS patients.

While most researchers (Alluri et al., 2006; Favilla et al., 2006; Manilal et al., 2009; Syahmi et al., 2010) have made use of the hatched nauplii for toxicity analysis of natural products, few assays (Caldwell et al., 2003) based on the inhibition of hatching of the cysts have been used. In this study, toxicity assessment was based on both the percentage of hatching of cysts and lethality of hatched nauplii in different concentrations of plant extracts and controls. Although brine shrimp assay was (and still is) routinely used for estimation of cytotoxicity, in fact it does not correlate with cytotoxicity determined on human cell lines (Hisem et al., 2011). The present study therefore provides toxicological data that are of importance to assess the ethnopharmacological relevance of the medicinal plants investigated and also points out the possible application of invertebrate bioassays for the preliminary evaluation of the general toxicity of herbal remedies.

MATERIALS AND METHODS

Plant

A. arctotoides (leaf) was collected at the Agricultural Research Farm of the University of Fort Hare, South Africa while *G. bicolor* (leaf) and *P. viridiflorum* (bark) were supplied by an herbalist in Alice. The plants were authenticated at the Giffin herbarium of the Department of Botany, University of Fort Hare where voucher specimens were deposited (*A. Arctotoides:* W28, *G. bicolor*: W31 and *P. viridiflorum:* W9).

Extraction procedure

The plant materials were chopped and dried in an oven at 40°C and ground into fine powder. The ground samples were put into separate conical flasks containing 250 ml of acetone or hexane and shaken for 24 h on an orbital shaker. After filtering with a Buchner funnel and Whatman No. 1 filter paper, the hexane and acetone filtrates were concentrated to dryness under reduced pressure at a maximum of 40°C using a rotavapor (Sultana et al., 2008). Each extract was re-suspended in the respective solvent to yield a 20 mg/ml stock solution.

Preparation of the assay system

The assay system was conducted by preparing 5 petri dishes containing 10 ml of filtered seawater each and a two-fold dilution was set up to yield a series of concentrations (2, 1, 0.5, 0.25, and 0.125 mg/ml) of the plant extract. A second set of test tubes containing amphotericin B dissolved in seawater (30 µl/ml) served as a positive control, while petri dishes containing sea water only served as the blank controls. Due to their low solubility in water, the extracts were initially dissolved in 0.5 ml of their corresponding solvent before being transferred to the filtered seawater. The setup was allowed to stand in open air for 30 min to allow the solvents to evaporate.

A. salina hatching assay

Brine shrimp hatchability assay was evaluated by assessing the hatching success of *A. salina* cysts in different concentrations of the plant extracts and positive control (Manilal et al., 2009). *A. salina* cysts were stocked at a density of 10 individuals per petri dish containing 10 ml of the incubation medium at varying concentrations. The Petri dishes were partly covered and incubated at 28°C. The number of free nauplii in each petri dish was counted after every 12 h and the setup was allowed to remain for 72 h under constant illumination. The percentage of hatchability was assessed by comparing the number of hatched nauplii with the total number of cysts stocked (Carballo et al., 2003). The minimum inhibitory concentration (MIC) was determined as the minimum concentration of the plant extracts (or control drug) that inhibited hatching of the cysts.

A. salina lethality assay

An aliquot (0.1 ml) containing 10 nauplii was pipetted into each petri dish for each extract solution and controls. The dead larvae in each petri dish were counted after every 12 h and the setup was allowed to remain for 72 h under constant illumination. Larvae were considered dead if they did not exhibit any external movement during several seconds of observation (Carballo et al., 2003). The percentage of mortality (M%) was calculated as:

M% = percentage of survival in the blank control - percentage of survival in the treatment or positive control

Data analysis

The Petri dishes were stocked according to a completely

randomized design with 2 replicate incubations per treatment. Mortality data obtained from 5 different concentrations of each extract and control experiments were used to construct the dose /response curves and to determine their corresponding LC_{50} values. The LC_{50} was taken as the concentration required for producing 50% mortality (Syahmi et al., 2010). LC_{50} values were determined from the best-fit line obtained by linear regression analysis of the percentage lethality versus the concentration. The analysis was done on MINITAB version 12 for windows. One-way analysis of variance followed by Fisher's least significant difference post-hoc analysis was used to test for the effect of concentration and time of exposure of the plant extracts (Caldwell et al., 2003).

RESULTS AND DISCUSSION

Brine shrimp hatchability assay

The hatching success of *A. salina* cysts incubated with different plant extracts and controls are shown in Figure 1. The lowest percentage values of hatching success of 5.10 and 5.20 were observed in cysts incubated with the hexane and acetone extracts of *A. arctotoides,* respectively and were significantly lower ($P < 0.05$) than that of the controls. On the other hand, the hatching success of brine shrimps in the hexane and acetone extracts of *G. bicolor* and *P. viridiflorum,* respectively was not significantly different from that of the positive control (amphotericin B). It is worth noting that the hatching success of the blank control was significantly higher ($P < 0.05$) than those of all the extracts tested, and that of the positive control. The inhibitory effects of the extracts and controls on hatching were expressed as MIC values (Table 1) and depicts the potential of the extracts to completely inhibit hatching of brine shrimps (0% hatching success).

The acetone and hexane extracts of *A. arctotoides* and *P. viridiflorum,* respectively exhibited more potent inhibitory effects, with MICs of 0.50 mg/ml each. In the present study, the evaluation of the *in vitro* toxicity of the medicinal plant extracts was done quantitatively by determining the MIC and LC_{50} values using the brine shrimp hatchability and lethality assays, respectively. The experimental screening of the toxicity of medicinal plants is crucial to assure their safety and effectiveness (Syahmi et al., 2010).

Effect of extract concentration on hatching success

In the brine shrimp hatchability assay, the hatching success of *A. salina* cysts decreased with increasing concentrations of the plant extracts (Figure 2). The hatching success of cysts in incubations with extracts of *G. bicolor* and *P. viridiflorum* (acetone) showed a significant dose-dependent response. In *G. bicolor*, hatching success dropped from 29 to 15%, with increase in concentration from 0.125 to 2 mg/ml. In treatments incubated with the acetone extracts of *A. arctotoides,* lower hatching percentages of 15.9 and 15.8 were observed at minimum extract concentrations of 0.125 and 0.25 mg/ml, respectively, while hatching was completely inhibited at higher concentrations (> 0.25 to 2 mg/ml). Similarly, the hexane extracts of *P. viridiflorum* elicited 100% hatching inhibition within the concentration range of 0.5 to 2 mg/ml (Figure 2).

Artemia rely on the resistant cyst stage which makes it tolerant to a wide range of salinities ranging from almost freshwater to almost saturated saline (Caldwell et al., 2003). Hence, the fact that hatching continued even at a high test concentration of 1 mg/ml albeit a reduced rate was expected. This resistance to harmful effects by *Artemia* cysts makes the hatchability assay less desirable than the lethality test for the preliminary screening of herbal toxicity. The use of freshly hatched nauplii has the benefit of circumventing the toxin tolerant cyst stage, and this increases the sensitivity of the lethality assay (Caldwell et al., 2003).

Effect of exposure time on hatching success

Results of the effect of exposure time on hatchability indicated that the sensitivity of *A. salina* to the medicinal plant extracts was strongly dependent upon exposure period (Figure 3). For example, hatching success in incubations of *G. bicolor* and *A. arctotoides* extracts at 36 h of exposure ranged from 3 to 35% and 0 to 35%, respectively and was significantly lower ($P < 0.05$) when compared to the positive and blank controls. On the other hand, incubations in *P. viridiflorum* extracts exhibited higher hatching success which ranged from 30 to 75% at 36 h of exposure. Incubations at longer exposure periods (>48 h) resulted in higher hatching success, which ranged from 45 to 80% and was not significantly different from the controls. The improved hatchability detected when cysts were subjected for longer exposure periods was observed in all the extracts, with the exception of the acetone extract of *G. bicolor*. The very low hatching rate of *Artemia* cysts detected at 24 h of exposure to the plant extracts was probably due to an alteration in the development of *Artemia* embryos. It has been shown that *Artemia* is highly vulnerable to toxins at the early developmental stages (Alluri et al., 2006).

Brine shrimp lethality assay

Brine shrimp lethality results and LC_{50} values obtained are shown in Figure 4 and Table 1, respectively. Crude extracts resulting in LC_{50} values of less than 1 mg/ml are considered as significantly active (Meyer et al., 1982).

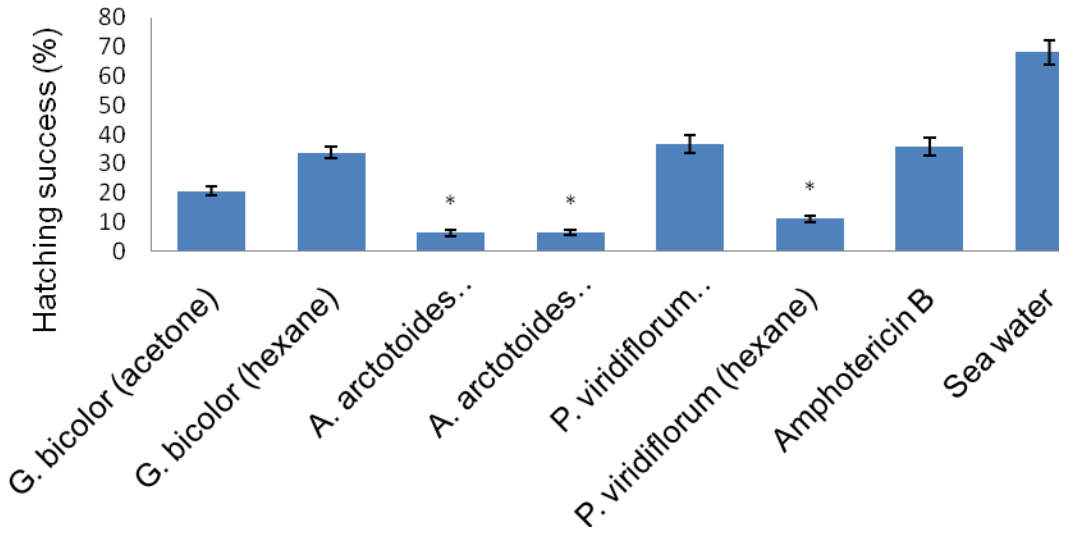

Figure 1. Percentage hatching success of *A. salina* cysts incubated in different plant extracts. Mean are values of 5 concentrations for each extract ± standard error (SE). Hatching success of extracts marked with an asterisk are significantly different (P < 0.05) from the positive control (amphotericin B).

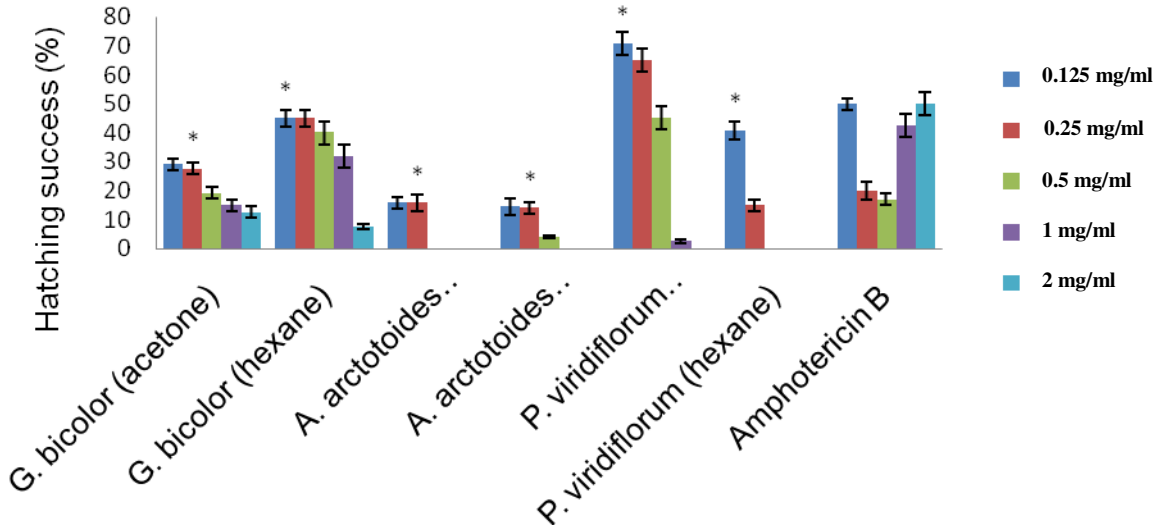

Figure 2. Percentage hatching success of *A. salina* cysts incubated in different extract concentrations of plant extracts.

This suggests that the hexane extract of *P. viridiflorum,* the acetone extracts of *G. bicolor* and *P. viridiflorum,* with LC_{50} values of 1.13, 1.10 and 1.10 mg/ml, respectively exhibited low brine shrimp toxicity. On the other hand, the acetone extract of *A. arctotoides* and the hexane extracts of *A. arctotoides* and *G. bicolor* exhibited significant brine shrimp lethality with LC_{50} values of 0.87, 0.89 and 0.82 mg/ml, respectively. Hence, based on Meyer's threshold of toxicity, both the hexane and acetone extracts of *P. viridiflorum*, and the acetone extract of *A. arctotoides* with LD_{50} values of > 1 mg/ml could be considered as non-toxic and may be further explored for the development of plant-based pharmaceutical products. On the other hand, both the hexane and acetone extracts of *A. arctotoides*, and the hexane extract of *G. bicolor* with LD_{50} values of < 1 mg/ml could be considered to have shown significant biological activity, indicative of the presence of potent toxic components which warrants further investigation (Alluri et al., 2006).

The pharmacological properties of these plants have been demonstrated *in vitro*, including the antibacterial and antifungal properties of *A. arctotoides* (Sultana et al., 2008; Afolayan, 2003). Hence, the extracts with LD_{50} values of < 1 mg/ml which have been labeled as "toxic",

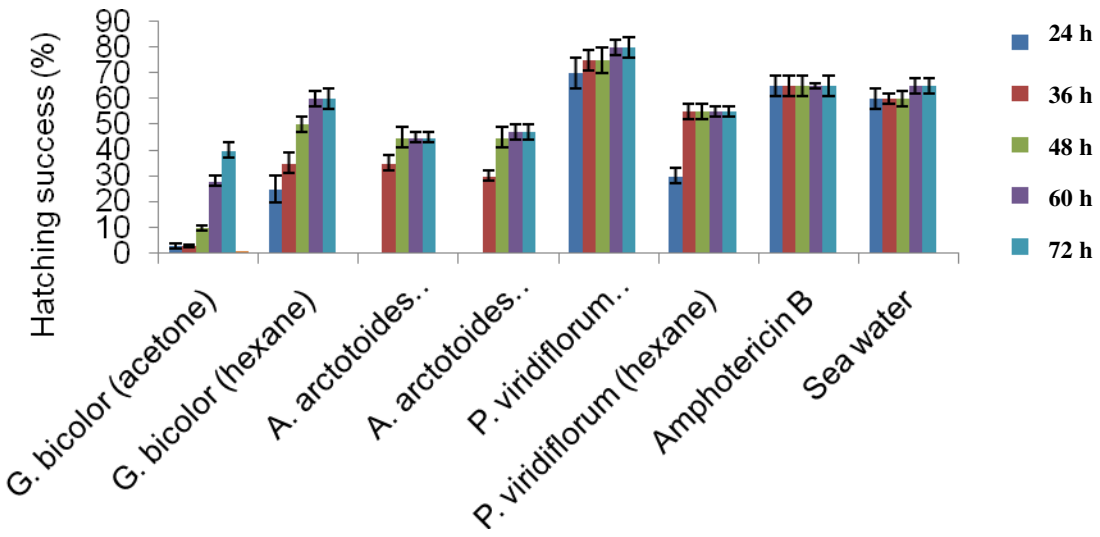

Figure 3. Percentage hatching success of *A. salina* cysts incubated at different durations in plant extracts. Mean are values of two replicate experiments for each extract ± SE. Exposure periods that are not significantly different (P > 0.05) from the positive control (amphotericin B) are marked with an asterisk.

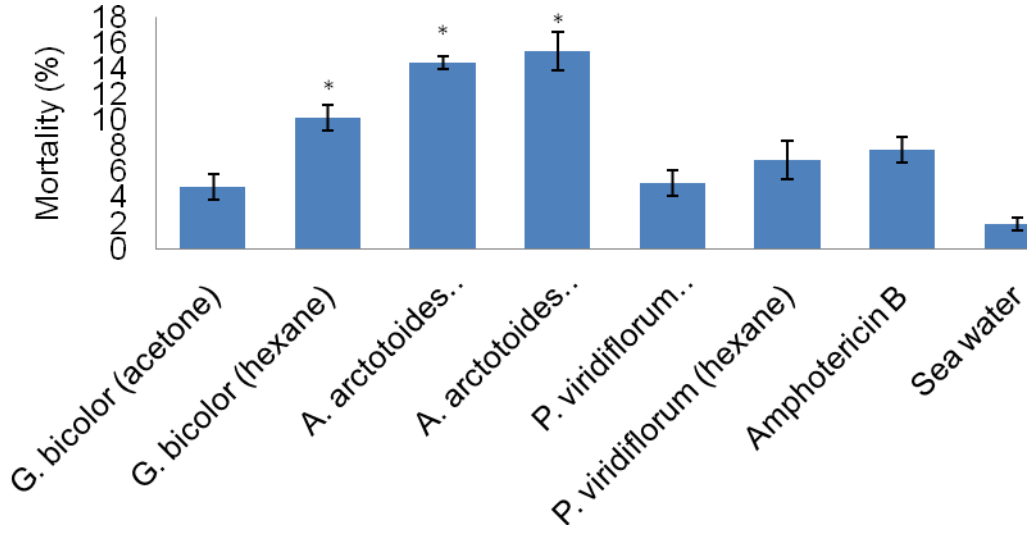

Figure 4. Percentage mortality of *A. salina* cysts incubated in different plant extracts. Mean value of two replicate experiments for each extract ± S.E. Percentage mortality values that are significantly different (P < 0.05) from the positive control (amphotericin B) are marked with an asterisk.

according to Meyer's threshold should not preclude their inclusion in future investigations and development of plant-based pharmaceuticals.

Effect of varying concentrations on brine shrimp mortality

The effect of varying concentrations on the mortality of brine shrimps is shown in Figure 5. A direct proportional relationship was observed between the concentration of the extracts and the degree of lethality. This is shown by the fact that maximum mortalities of 15.8, 33.3, and 35% occurred at the highest extract concentrations of 2 mg/ml in incubations of *P. viridiflorum* (hexane), *A. arctotoides* (acetone) and *A. arctotoides* (hexane), respectively. Similarly, least mortalities of 0% were observed at the lowest extract concentration of 0.125 mg/ml (Figure 5). The percentage mortality of cysts in all the extract concentrations was not significantly different (P > 0.05)

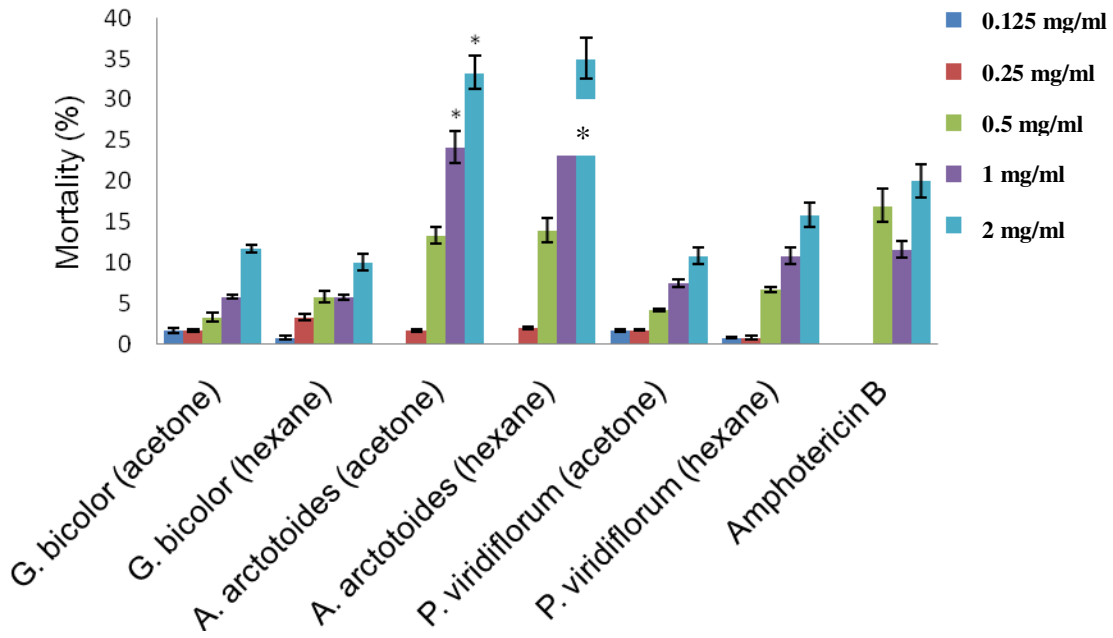

Figure 5. Percentage mortality of *A. salina* cysts incubated in different concentrations of plant extracts. Mean are values of two replicate experiments for each concentration ± SE. Concentrations marked with an asterisk are significantly different (P < 0.05) from that of the positive control (amphotericin B).

from that of the positive control (amphotericin B), except for *A. arctotoides* incubations where the percentage mortality was significantly higher (P < 0.05) than that of the positive control at highest concentrations of 1 to 2 mg/ml (Figure 5).

Both the toxicological and pharmacological effect of a drug could be highly versatile and interchangeable, based on the dosage administered. Thus, the *in vivo* lethality of a simple zoologic organism has be used as a convenient toxicological screening system for the plant extracts and a baseline for the definition of their intrinsic toxicity in order to prevent the effects of acute overdose in future *in vivo* trials.

Effect of exposure time on brine shrimp mortality

In the brine shrimp lethality test, mortality of nauplii in incubations of plant extracts was first observed after 48 h of exposure (Figure 6). Exposure to extracts for longer periods (>60 h) did not induce any significant increase in mortality when compared to the controls. In contrast, in the brine shrimp lethality test, mortality of nauplii was first reached at 48 h of exposure. In a related study, the bioactivity of the isopropanolic (2-PrOH) extracts of 14 species of marine invertebrates and 6 species of macroalgae was evaluated with the shrimp lethality assay (Carballo et al., 2003). Maximum sensitivity was reached after 48 h of exposure. At this stage in their life cycle, the nauplii have reached their second and third instar and exhibit their greatest sensitivity to test compounds (Lewis, 1995). Delayed toxic effects of the plant extracts which resulted in delayed mortality observed only after 48 h of exposure suggests that long exposure times are advisable for the evaluation of the toxicological risks of plant extracts with the brine shrimp lethality assay (Favilla et al., 2006). Notwithstanding, it is important to mention here that data from brine shrimp assays are not so easily transferable to potential toxicity to humans, and this study is the first attempt to select appropriate plants for further selection of candidate plant, and more tests (for example, *in vitro* cytotoxicity tests with human cells) are needed to validate the toxicity results of the plant extracts.

Conclusion

The *in vivo* lethality of *A. salina* has be used as a convenient toxicological screening system for the plant extracts, while the resistance to harmful effects by *Artemia* cysts made the hatchability assay less desirable than the lethality test. The hexane extract of *P. viridiflorum,* the acetone extracts of *G. bicolor* and *P. viridiflorum* with LD_{50} values > 1 mg/ml which exhibited low brine shrimp toxicity could be further explored for the development of plant-based pharmaceuticals. On the other hand, the acetone extract of *A. arctotoides* and the hexane extracts of *A. arctotoides* and *G. bicolor* exhibited significant brine shrimp lethality with LD_{50} values < 1 mg/ml, indicative of the presence of potent toxic components which warrants further investigation. Delayed mortality observed only after 48 h of exposure suggests

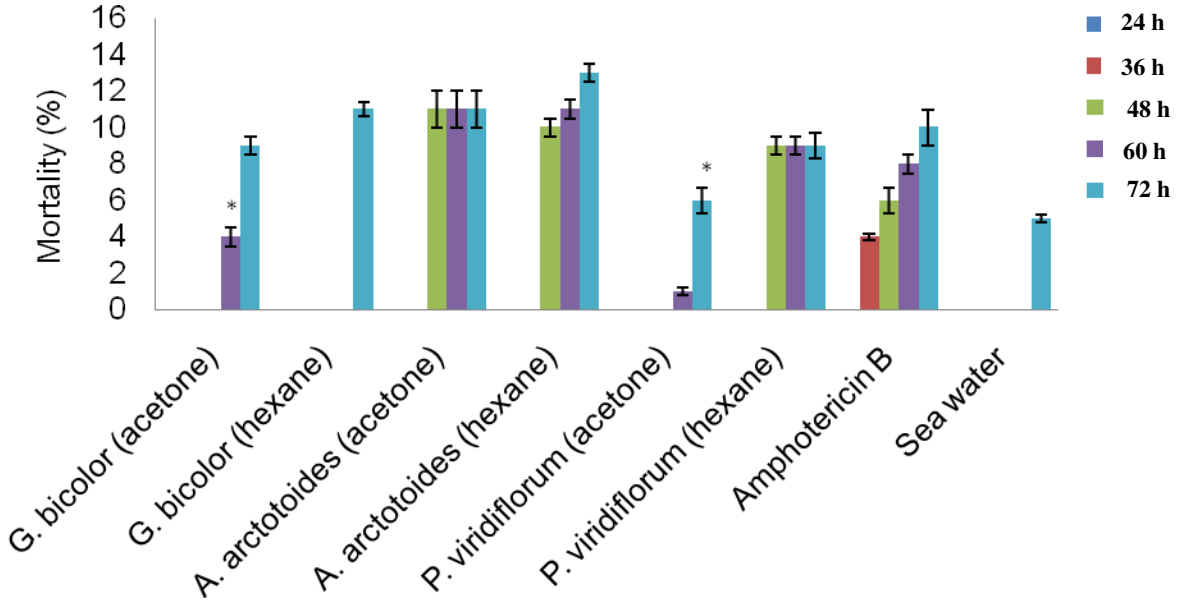

Figure 6. Percentage mortality of *A. salina* cysts incubated at different durations in plant extracts. Mean are values of two replicate experiments for each extract ± SE. Exposure periods that are significantly different ($P < 0.05$) from the positive control (amphotericin B) are marked with an asterisk.

Table 1. Hatchability and lethality of *A. salina* in different plant extracts, as shown by their MIC and LC_{50} values respectively.

Extract	MIC (mg/ml)	LC_{50} (mg/ml)	Regression equation	R^2 (%)	P-value
A. arctotoides (acetone)	0.50	0.87	Y = 5.47x+0.59	87.80	0.01
A. arctotoides (hexane)	1.00	0.89	Y = 18.8x+0.79	87.00	0.01
G. bicolor (acetone)	>2.00	1.10	Y = 5.47x+0.59	99.40	0.00
G. bicolor (hexane)	>2.00	0.82	Y = 4.16x+1.94	82.20	0.02
P. viridiflorum (acetone)	2.00	1.10	Y = 5.10x+1.22	94.50	0.00
P. viridiflorum (hexane)	0.50	1.13	Y = 8.18x+0.66	89.50	0.01
Amphotericin B (positive control)	>2.00	1.06	Y = 11.5x–2.22	95.40	0.00

R^2: Coefficient of determination of the regression equation. P values indicate the level of significance of the regression equation; values less than 0.05 are significant and those greater than 0.05 are not significant at 5% level of probability.

that long exposure times are advisable for the evaluation of the toxicological risks of plant extracts with the brine shrimp lethality assay.

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REFERENCES

Afolayan AJ (2003). Extracts from the shoots of *Arctotis arctotoides* inhibit the growth of bacteria and fungi. Pharm. Biol. 41:22–25.

Alam M, Ghiasuddin, Sadat A, Muhammd N, Khan AA, Siddiqui BS (2012). Evaluation of *Viburnum grandiflorum* for its in-vitro pharmacological screening. Afr. J. Pharm. Pharmacol. 6(22):1606-1610.

Alluri VK, Tayi V, Rao N, Sundararaju D, Mulabagal V, Hsin-Sheng T, Gottumukkaka SV (2006). Biological screening of medicinal plants collected from Eastern Ghats of India using *Artemia salina* (Brine Shrimp Test). Int. J. Appl. Sci. Eng. 4:115-125.

Asgarpanah J, Ramezanloo F. (2012). Chemistry, pharmacology and medicinal properties of *Peganum harmala* L. Afr. J. Pharm. Pharmacol. 6(22):1573-1580.

Caldwell GS, Bentley MG, Olive PJW (2003). The use of a brine shrimp (*Artemia salina*) bioassay to assess the toxicity of diatom extracts and short chain aldehydes. Toxicon. 42:301–306.

Carballo JL, Hernández-Inda ZL, Pérez P, García-Grávalos MD (2003). A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. BMC Biotechnol. 2–17.

Cleuvers M (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142:185–194.

Commission of the European Communities (1991). Council Directive of 15 July 1991, concerning the placing of plant protection products on the market, consolidated text. Office for official publications of the European communities, Luxembourg, LU.

Dagne E, van Wyk, BE, Mueller M, Steglish W (1996). Three dihydroanthracenones from *Gasteria bicolor.* Phytochemistry 41:795-799.

Favilla M, Macchia L, Gallo A, Altomare C (2006). Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) invertebrate bioassays. Food Chem. Toxicol. 44:1922–1931.

Hisem D, Hrouzek P, Tomek P, Tomšick ová J, Zap omelová E, Skácelová K, Lukešová A, Kopeck ý J, (2011). Cyanobacterial cytotoxicity versus toxicity to brine shrimp *Artemia salina*. Toxicon 57:76–83.

Lewis GE (1995). Testing the toxicity of extracts of Southern African plants using brine shrimp (*Artemia salina*). S. Afr. J. Sci. 91:382.

Manilal A, Sujith S, Kiran GS, Selvin J, Shakir C (2009). Cytotoxic potentials of red alga, *Laurencia brandenii* collected from the Indian coast. Global J. Pharm. 3:90-94.

Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL (1982). Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med. 45:31–34.

Nazri S, Anoush M, Khatami N (2012). Evaluation of analgesic and anti-inflammatory effects of fresh onion juice in experimental animals. Afr. J. Pharm. Pharmacol. 6(23):1679-1684.

Otang WM, Grierson DS, Ndip RN (2012). Antifungal activity of *Arctotis arctotoides* (L.f.) O. Hoffm. and *Gasteria bicolor* Haw. against opportunistic fungi associated with HIV/AIDS. Pharmacogn. Mag. 80:135-140

Seo Y, Berger JM, Hoch J, Neddermann KM, Bursuker I, Mamber SW, Kingston DG (2002). A new triterpene saponin from *Pittosporum viridiflorum* from Madagascar rainforest. J. Nat. Prod. 65:65–68.

Sultana N, Afolayan AJ, Bhuiyan RF (2008). Antimicrobial compounds from the shoots of *Arctotis arctotoides.* Bangladesh J. Sci. Ind. Res. 43:89-96.

Syahmi ARM, Vijayarathna S, Sasidharan S, Latha, LY, Kwan YP, Lau YL, Shin LN, Chen Y (2010). Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* jacq., (oil palm leaf) methanol extract. Molecules 15:8111–8121.

United States Environment Protection Agency. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms**.** 2002, EPA/821/R-02-012.