

REPORT

Toxicological investigations of *Aloe ferox* Mill. extracts using Brine shrimp (*Artemia salina* L.) assay

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Abstract: Cytotoxicity of the extracts of *Aloe ferox* using brine shrimp was evaluated. Effects of the extracts on hatchability and lethality of brine shrimps were reported in terms of minimum inhibitory concentration and LD₅₀ respectively. The hatching success was in the order: aqueous root extract (39.8%) >aqueous leaf (26.5%) >acetone leaf (13.7%) > ethanol leaf extracts (2.5%). Hatchability in incubations of ethanol and acetone extracts was found to be dose - dependent, with hatching success decreasing as the concentration of the extracts increased. The lethality of extracts was in the order: aqueous leaf extract (4.7%) >aqueous root extract (4.2%) >acetone leaf extract (3.5%) >ethanol root extract (2.6%) > ethanol leaf extract (1.8%) >acetone root extract (0.7%). Mean mortality of nauplii in the control was higher (6.7%) than that of all the extracts. Based on Meyer's index of toxicity, the acetone leaf extract with LD₅₀ >1.0mg/mL could be considered as non toxic, while the ethanol root extract (LD₅₀ <1.0mg/mL was significantly toxic to the brine shrimp. Since the extracts of *A. ferox* have consistently exhibited significant pharmaceutical properties in-vitro, the non-toxic extracts could further be exploited for the development of plant-based pharmaceuticals.

Keywords: *Aloe ferox*, cytotoxicity, brine shrimp, hatchability, lethality.

INTRODUCTION

The genus *Aloe* contains about 500 species that are native to Africa, Madagascar and the Arabian Peninsula (Viljoen, 2008). Members of this genus are widely used in folk medicine. *Aloe ferox* Mill. is associated with hot dry climates but can grow in grasslands, coastal or mountain locations. It is distributed in the Southern, Western and the Eastern Cape Provinces of South Africa (Watt and Brayer-Brandwijk, 1962; Van Wyk *et al.*, 2002; Steenkamp and Stewart, 2007; Grace *et al.*, 2009).

A. ferox (cape aloe) popularly called *Ikhala* in Xhosa has been widely used as food, for medicinal purposes and as a component of many cosmetic preparations (Shackleton and Gambiza, 2007; Viljoen, 2008). It is also used extensively in herbal medicine as a laxative (Watt and Breyer-Brandwijk, 1962; Wintola and Afolayan 2010), anti-inflammatory (Steenkamp and Stewart, 2007), immune-stimulant, antitumour and antidiabetic agent (Watt and Breyer-Brandwijk, 1962) and also for the control of gastro-intestinal parasites (Mwale and Masika, 2008), sexually transmitted diseases (Kambizi *et al.*, 2004) and for the treatment of wounds (Van Wyk *et al.*, 2002).

In some other instances *in vitro* studies, Botes *et al.* (2008) and Afolayan *et al.* (2002) have also found the growth of tumour to be suppressed by the *A. ferox* leaf extract, while Jia *et al.*, (2008) reported the presence of

various types of metabolites including alkaloids, flavonoids, tannins, anthraquinone glycosides, flavonols and phenols. Several antibacterial and antiviral compounds have been isolated from the plant (Afolayan *et al.*, 2002; Kambizi *et al.*, 2004; Loots *et al.*, 2007; Botes *et al.*, 2008; Viljoen, 2008; Grace *et al.*, 2009). Viljoen *et al.* (2001) reported that *A. ferox* leaf exudates contained aloeresin, aloesin, aloin B, aloin A, aloinoside B and aloinoside A as major compounds in the leaf exudates. In an earlier investigation of medicinal plants used as laxatives in Nkonkobe district Municipality of the Eastern Cape Province three species; *A. ferox*, *A. tenuior* and *A. arborescence* and seven other plants were mentioned by the rural dwellers and the traditional healers. Based on the frequency of usage, perceived efficacy and availability, *A. ferox* had the highest importance value index (IVI) with respect to the treatment of constipation in the study area (Wintola and Afolayan, 2010).

There is information in literature on toxicological study of *Aloe* species. In a recent study by Mwale and Masika (2012) aqueous extract of *A. ferox* was found to exert no toxic effect on rat at all grade levels tested during the acute, sub acute and chronic toxicity test. In the study conducted by Wintola *et al.* (2010), *A. ferox* aqueous leaf extract did not cause necrosis and inflammation on the liver and kidney of the Wistar rats at all dosage investigated when compared to senokot (the standard laxative for constipation). The toxicity of *Aloe* gel against HeLa cell and rabbit kidney cell was reported (Bransher

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et al 1969). In their conclusion, it was obvious that the *Aloe* gel solution was completely toxic to both cell lines at 5×10^{-1} (Bransher *et al.*, 1969).

Chandu *et al.* (2012) showed that the extract from *A. vera* had a cytoprotective effect on normal cells but demonstrated some level of toxicity against the cancerous cells at concentration of 300 μ g/mL. Ihsan-ul-Haq *et al.* (2012) reported the use of brine shrimp (*Artemia franciscana*) for the determination of the acute toxicity of *A. vera* gel. He concluded that both the extract and the isolated fraction (RP-HPLC) of the extract were toxic to the larva within 24 h of exposure in relation to the control. Despite the wide use of this plant, further work is needed on its toxicity. Thus, cytotoxicity screening will contribute immensely to the clarification of the plant safety, which is crucial to the users. In view of the lack of appraisal on the cytotoxicity effect of *A. ferox* using brine shrimp assay, this study was designed to evaluate the cytotoxicity of *A. ferox* using brine shrimp (*Artemia salina* L).

MATERIALS AND METHODS

Plant collection and identification

Fresh leaves and roots of *A. ferox* were harvested from their natural population in 2011 in the University of Fort Hare, Alice campus, Eastern Cape Province, South Africa with GPS coordinate of 32° 47' 0'' to the South and 26° 50' 0'' to the East (Erasto *et al.*, 2005). An authenticated voucher specimen (Wintola Med 2009/01) was deposited at the Griffen's Herbarium of the University of Fort Hare.

Preparation of extracts

The harvested samples were oven dried to constant weight at 40°C and milled to a homogenous powder. The powder plant materials (25g) were extracted with 200ml of distilled water/acetone/ethanol on a Start Scientific Orbital Shaker, Essex, UK for 48h and then filtered using a Buckner funnel and Whatman No 1 filter paper. The filtrate obtained from the water extract was quickly frozen at -40°C and others dried for 48h using a freeze drier (Savent Trap RV T41404 Refrigerated Vapour, USA) This gives a yield of 3.9 and 2.8g respectively for the leaf and root, while extracts from the other solvents were reduced by concentrating under reduced pressure at 40°C to give the following yields acetone (1.9, 1.2g) ethanol (2.6, 1.8g) for the leaf and root in that order. The various extract were later reconstituted in respective solvent to give a series of concentration as follows; (2.0, 1.0, 0.5, 0.25, 0.125mg/mL). All solvent were of analytical grades (Merck, Gauteng, South Africa).

Brine Shrimp hatchability Assay

The brine shrimp (*Artemia salina*) eggs (Sera, Heidelberg, Germany) were obtained from aquaculture shop in East London, 5200, South Africa. The brine shrimp eggs were hatched in the different concentrations of plant extracts

dissolved in seawater (38g/L NaCl) and pH 8.5, obtained from the East London sea (32° 59 S 27°52 E) for 24h at 26°C under constant illumination and aeration. Twenty milligrams of the extracts were dissolved in 1 ml of the respective solvents to give a crude extract concentration of 20mg/mL. A two-fold serial dilution was carried out with seawater (38g/L NaCl) to obtain a series of concentrations (2.0, 1.0, 0.5, 0.25 and 0.125mg/mL). Each concentration was tested in triplicate. Petri dishes containing 5mL seawater were used as blank controls. A second set of test tube containing senokot dissolved in seawater with the same concentration served as the positive control. Ten brine shrimp eggs were introduced into the petri dishes bearing 2.0, 1.0, 0.5, 0.25 and 0.125mg/mL plant extracts and controls. The effect of the test solutions on the hatching success was examined at 12h intervals and the set up was allowed to stand for 72h. The hatching success was computed as the ratio of the hatched nauplii to the number of cysts at present at the beginning of the experiment and expressed in percentage.

Brine shrimp lethality assay

In the present study, the brine shrimp lethality of the plant extracts was investigated using Meyer *et al.* (1982) procedure. Using a micropipette, 10 freshly hatched nauplii were pipetted into each petri dish of the respective treatment. Lethality of larvae for each concentration was determined every 12h by counting the number dead when compared to the amount stocked and left up to 72h under constant illumination. The index of lethality was the absolute difference between the mean number of larvae in the test and control petri dish and the resultant percentage lethality obtained was regressed with the concentration of the plant extracts to compute the LD₅₀ values.

STATISTICAL ANALYSIS

Linear regression analysis on MINITAB version 12 was used to determine the LD₅₀. This was taken as the concentration required producing a reduction of 50% in mortality of controlled experiments.

RESULTS

Brine shrimp hatchability assay

Highest hatching success was recorded in aqueous root extract (39.8%), followed by aqueous leaf (26.5%) and acetone leaf (13.7%) extracts (fig. 1). However, senokot used as control exhibited higher hatchability than the aqueous root and leaf extracts of *A. ferox*. Increasing concentrations of the plant extracts and in the senokot led to a dose-dependent decrease in brine shrimp hatchability observed in incubations containing ethanol and acetone extracts. In the ethanol extracts of the leaf, hatching was completely inhibited at higher concentrations (>0.25).

Lethality test on the brine shrimp

The lethality result of the extract of the leaf and root extract of the plant of *A. ferox* and the standard drug (senokot) (fig. 3 and 4) and the LD₅₀ shown in table 1. The mean value for the lethality of the aqueous leaf extract (4.7%), was the highest followed closely by the aqueous root extract (4.2%), acetone leaf extract (3.5%), ethanol root extract (2.6%), ethanol leaf extract (1.8%) but significantly lower in the acetone root extract (0.7%). However, there is no significant difference in the % mortality of the acetone and the ethanolic extracts when compared with the % mortality of the control (sea water). Furthermore, senokot showed the most prominent lethality activity (6.7%) compared to the control. Increased concentration of the plant extract led to a dose dependent increase in the brine shrimps lethality observed in incubations containing all the test solutions and control (fig. 4).

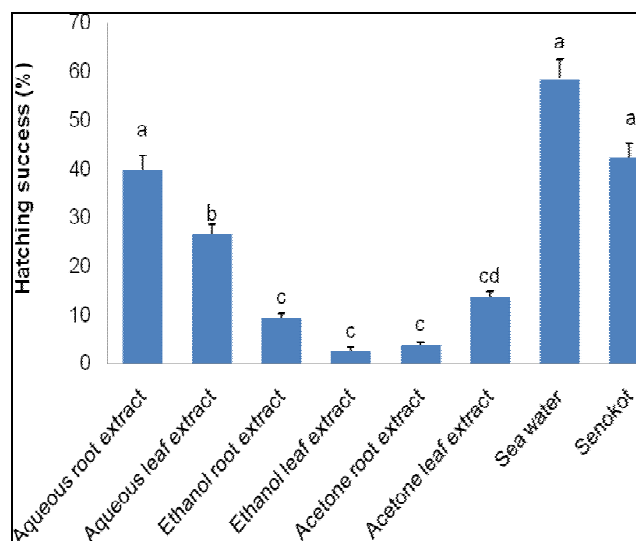


Fig. 1: Effect of the leaf/root extracts of *A. ferox* on the hatchability of brine shrimps. Bar represent hatchability success for 3 replicates \pm SD. Values having the same letter are not significantly different ($P>0.05$).

DISCUSSION

Brine shrimp assay is a very useful method of assessing the bioactivity of plant extracts and activities of several plant species that are used in traditional medicine (McLaughlin *et al.*, 1993; Ramachandran *et al.*, 2010). The hatching potency of the aqueous extract was lower than all other solvent used. This showed that the organic solvents have higher hatchability strength than the aqueous extract. This could be as a result of a reduced toxic effect of the aqueous extracts to the brine shrimps. Water is used traditionally to prepare extracts of medicinal plants. Most remedies are prepared as simple aqueous extracts, thus avoiding potent toxic effects.

Aloe ferox extracts showed inhibitory activity against hatchability with MIC ranging from 0.5mg/mL to

>2.0 mg/mL in all the extracts. This suggests that the cyst has more resistant to hatching in all the acetone and ethanol extracts than in the aqueous root and leaf extracts. Such resistant could be due to the permeability barrier provided by the cysts or to the membrane barriers provided by the cysts or to the membrane accumulation mechanism of the cysts (Adwan and Abu-Hassan, 1998; Abu-Shanab *et al.*, 2004).

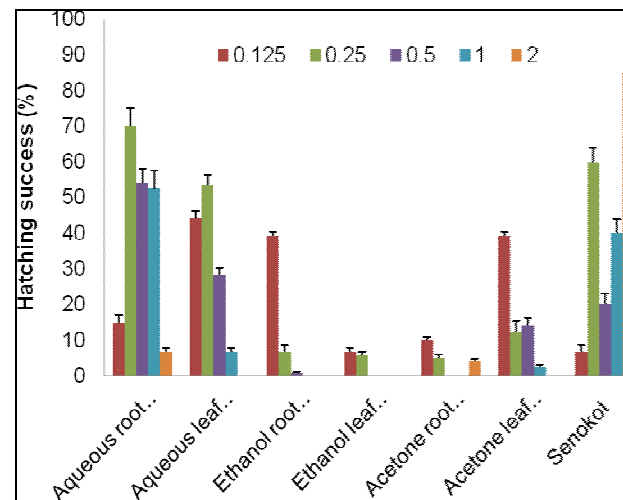


Fig. 2: Effects of the different concentration of the leaf/root extracts of *A. ferox* on the hatchability success of brine shrimp. Bar represent hatchability success for 3 replicates \pm SD.

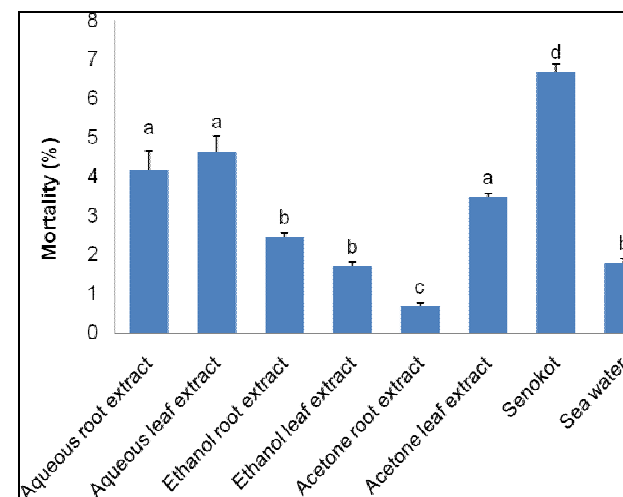


Fig. 3: Effect of the leaf/root extract of *A. ferox* on the mortality of brine shrimp. Bar represent mortality for 3 replicates \pm SD. Values having the same letter are not significantly different ($P>0.05$).

Artemia salina is vulnerable to toxins at early developmental stage (Sorgeloos *et al.*, 1978; Lewan *et al.*, 1992), which explains why the degree of inhibition observed in the hatchability assay was directly related to the concentration of the extracts and control. The dose dependent decrease in brine shrimp hatchability as concentration increases, suggests the toxic chemical

Table 1: LD₅₀ and MIC values of the mortality and hatchability assays of *A. salina* incubated in different concentration of the extracts

Plant extract/control	MIC (mg/ml)	LD ₅₀ (mg/ml)	R ² (%)	Regression equation	P value
Aqueous root	> 2.0	0.90	95.50	Y= 3.28x + 1.22	0.00
Aqueous leaf	> 2.0	0.90	95.10	Y= 3.80x +1.15	0.01
Ethanol leaf	0.50	0.86	90.20	Y=1.75x +0.37	0.01
Ethanol root	0.50	0.77	83.20	Y= 1.58x +1.24	0.03
Acetone leaf	0.50	1.05	96.30	Y= 5.28x +0.59	0.00
Acetone root	1.00	0.84	92.10	Y= -1.54x +0.32	0.01
Senokot (control)	0.13	0.61	62.90	Y= 2.37x-0.275	0.11

principles which revealed that at a very low concentration of the extracts, toxicity to the cysts may not occur as a result of the arrest in the hatchability of the cysts (Subhadra *et al.*, 2011). The fact that the potency of all the *A. ferox* extracts to hatching was lower than that of senokot, suggests that *A. ferox* could be used for the development of plant based pharmaceutical with lesser toxicity threats to users. Dose-dependent experiments are indicative of the sensitivity of *A. salina* to *A. ferox* extracts.

Majority of researcher have used brine shrimp assay to determine potential toxicity of substances in plant material (Meyer *et al.*, 1982; Solis *et al.*, 1993). The extracts could contain cytotoxic agents since the LD₅₀ obtained in this study was lower than the standard set by Meyer's index on cytotoxicity, for bioactive compounds to be toxic, it must have an LD₅₀ <1.0mg/mL which shows the extracts may contain some cytotoxic compounds which may be toxic at a higher dose and may not be toxic when low. All the solvent extracts of *A. ferox* used in this study showed LD₅₀ <1.0mg/mL with the exception of the acetone leaf extract with LD₅₀ of 1.05 mg/mL. The mild toxic effects observed in the extracts of *A. ferox* may suggest that the potent components of the extracts be subjected to further investigation before being recommended for drug development. This was however less toxic than the positive control with LD₅₀ of 0.61 mg/mL. For example, paracetamol could be hepatotoxic at higher concentration while it is analgesics at lower dosage.

With respect to the lethality assay, the same dose dependent relationship just like in the hatchability assay was observed wherein the percentage mortality increases as the concentration of the extracts increased. Similar observation was shown by Mojica and Micor (2007), while studying the effects of the aqueous extract bioactivity of *Barringtonia asiatica* (Linneaus) Kurz. Seed on *Artemia salina* L, the result shows high biological activity of the extract in both assays. Toxicity evaluation of herbal materials is necessary in order to guarantee the safety of the users. (Parra *et al.*, 2001). Studies have been carried out on the inherent poisonous activities of the constituents of *A. ferox* (Williams *et al.*, 2010). They reported a positive stimulation of a gene mutation assay in

their investigation on aqueous plant extract of *A. ferox* and *Bacillus subtilis* and a negative effect of *A. ferox* plant extract on the strains of *Salmonella typhimurium* (Williams *et al.*, 2010). Therefore, an LD₅₀ >1.0mg/mL of aqueous root and leaf extracts is an indication that the extracts does not exhibit any apparent toxicity and could be used conventionally because of it's affordability to the people in the rural areas (Sahgal *et al.*, 2010).

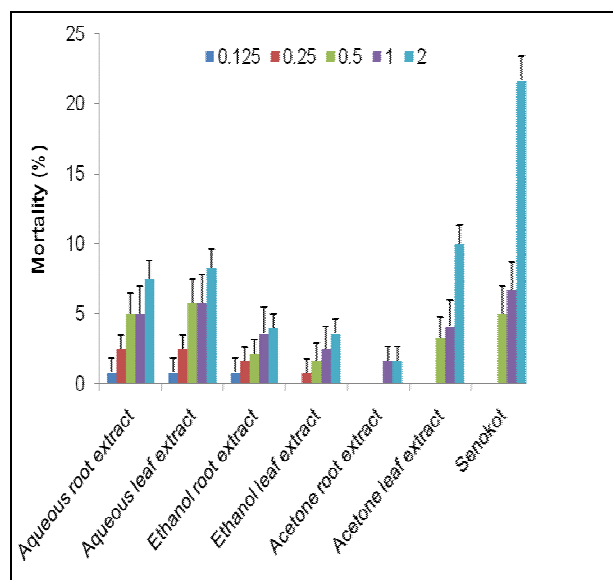


Fig. 4: Effects of the different concentration of the leaf/ root extracts of *A. ferox* on the mortality of brine shrimp. Bar represent mortality for 3 replicates ± SD.

CONCLUSIONS

The toxicity testing using brine shrimp is a faster and cheaper means of determining toxicity of herbal material. Out of three *A. ferox* whole leaf extracts screened for toxicity, the aqueous extracts showed a lesser inhibitory activity on the hatchability of cysts as compared to the acetone leaf and ethanol extracts. The standard laxative drug (senokot) showed a higher inhibition on hatchability than all the *A. ferox* extracts tested. The present study observed reasonable cytotoxicity activity of the extract which is an indication that the plant could be used for subsequent *in vivo* assay as reported in literature, there are reports on the correlation between cytotoxic effect and

activity on brine shrimp. The active ingredients from the root and the whole leaf extracts could provide leads to interesting pharmaceuticals of plant origin.

REFERENCES

- Abu-Shanab B, Adwan G, Abu-Safiya D, Jarrar N and Adwan K (2004). Anti-bacterial activities of some plant extracts utilized in popular medicine in Palestine. *Turk. J. Bio.*, **28**: 99-102.
- Adwan K and Abu-Hassan N (1998). Gentamicin resistance in clinical strains of enterobacteraceae associated with reduced gentamicin uptake. *Fol. Microbiol.*, **43**: 438-440.
- Afolayan AJ, Grierson DS, Kambizi L, Madamombe I and Masika P (2002). *In vitro* Anti-fungal activity of some South African medicinal plants. *S. Afr. J. Bot.*, **68**: 72-76.
- Botes L, Van der Westhuizen FH and Loots DT (2008). Phytochemical contents and anti-oxidant Capacities of two *Aloe* greatheadii var. davyana extracts. *Molecules.*, **13**: 2169-2180.
- Brasher WJ, Zimmermann ER and Collins CK (1969). The effect of prednisolone, indomethacin and *A. vera* gel on tissue culture cells. *Oral. Surg. Oral. Med. oral Pathol.*, **27**: 122-128.
- Chandu AN, Kumar SC, Bhattacharjee C and Debnath S (2012). Cytotoxicity study of plant aloe vera (Linn). *Chronicles of Young Scientist*, **3**(3): 233-235.
- Erasto P, Adebola PO, Grierson DS and Afolayan AJ (2005). An ethnobotanical study of plants used for the treatment of diabetes in Eastern Cape Province, South Africa. *Afr. J. Biotechnol.*, **4**: 1458-1460.
- Grace OM, Simmonds MSJ, Smith GF and Van Wyk AE (2009). Documented utility and biocultural value of *Aloe* L. (Aspodelaceae): A review. *Econ. Bot.*, **64**: 167-178.
- Ihsan-ul-Haq, Mannan A, Ahmed I, Hussain I, Jamil M, Mirza B (2012). Antibacterial activity and brine shrimp toxicity of *Artemisia dubia* extract. *Pak. J. Bot.*, **44**(4): 1487-1490.
- Jia S, Lang M and Guo LH (2008). Photoelectrochemical detection of oxidative DNA damage induced by Fenton reaction with low concentration and DNA associated Fe^{++} . *J. Phy. Chem. Bull.*, **112**: 4461-4464.
- Kambizi L, Sultana N and Afolayan AJ (2004). Bioactive compounds isolated from *Aloe ferox*: A Plant traditionally used for the treatment of sexually transmitted infections in the Eastern Cape. *S.A. Pharm. Biol.*, **42**: 636-639.
- Lewan L, Anderson M and Morales PG (1992). The use of *Artemia salina* in toxicity. Testing alternatives. *Laboratory Animal*, **20**: 297-301.
- Loots DT, Van Der Westhuizen FH and Botes L (2007). *Aloe ferox* leaf gel phytochemical content, antioxidant capacity and possible health benefit. *J. Agric. Chem.*, **55**: 6891-6896.
- McLaughlin JL, Chang CJ and Smith DL (1993). Simple bench-top bioassays (brine shrimp and potato discs) for the discovery of plant antitumor compounds. *In: Human Medicinal Agents from Plants*. Kinghorn AD and Balandrin MF. (Eds.), ACS Symposium 534, American Chemical Society, Washington, D.C, pp.112-137.
- Meyer BN, Ferrigni NR, Putnam JE, Jacobson LB, Nicholas DE and Mc Laughlin JL (1982). Brine shrimp: A convenient general bioassay for active plant constituents. *Plant. Med.*, **45**: 31-34.
- Mojica ERE and Micor JRL (2007). Bioactivity study of Bioactivity of *Barringtonia asiatica* (Linnaeus) Kurz. Seed aqueous extract in *Artemia salina*. *Intern. J. Bot.*, **3**: 325-328.
- Mwale M and Masika PJ (2008). Medicinal plants used in ethno-veterinary control of gastrointestinal parasites in village chickens in the communal areas of the Eastern Cape. The Annual Indigenous Plant Use Forum Conference; Value Adding. Graaff Reinet South Africa, **51**: 7- 10.
- Mwale M and Masika PJ (2012). Toxicological studies on the leaf extract of *Aloe ferox* Mill. (Aloaceae). *Sci. Res. Essay.*, **7**(15): 1605 -1613,
- Parra AL, Yhebra RS, Sardinias IG and Buela I (2001). Comparative study of the assay lethal dose (LD 50 value) in mice, to determined oral acute toxicity of plant extracts. *Phytomed.*, **8**(5):395-400.
- Ramachandran S, Vamsikrishna M, Gowthami KV, Heera B and Dhanaraju MD (2010). Assessment of cytotoxicity activity of *Agave cantula* using brine shrimp (*Artemia Salina* L) lethality bioassay. *Asian. J. Sci. Res.*, **4**: 90-94.
- Sahgal G, Ramanathan S, Sasidharan S, Mordi MN, Sabariah I and Mansor SM (2010). Brine shrimp lethality and acute oral toxicity studies on *Swietenia mahagoni* (Linn.) Jacq. Seed methanolic extract. *Pharmacog. Res.*, **4**: 215-220.
- Shackleton CN and Gambiza J (2007). Growth of *Aloe ferox* Mill. at selected sites in the Makana region of the Eastern Cape. *S. Afr. J. Bot.*, **73**: 266-269.
- Solis PN, Wright CW, Anderson MM, Gupta MP and Phillipson JD (1993). A microwell cytotoxicity assay using *Artemia salina*. *Planta Med.*, **59**: 250-252.
- Sorgeloos P, Remiche-Van Der Wielen C and Persoone G (1978). The use of *Artemia nauplii* for toxicity tests: A critical analysis. *Ecotoxicol. Environ. Safety*, **2**: 247-255.
- Steenkamp V and Stewart MJ (2007). Medicinal Applications and Toxicological Activities of *Aloe*. *Prod. Pharm. Bio.*, **45**: 411-420.
- Subhadra S, Kanacharalapalli VR, Rivindran VK, Parre, SK, Chintala S and Thatipally R (2011). Comparative toxicity assessment of three *Tephrosia* species on *Artemia salina* and animal cell lines. *J. Nat. Pharm.*, **3**: 143-148.

- Van Wyk B-E, van Oudtshoorn B and Gericke N (2002). Medicinal Plants of South Africa, 2nd Ed. Briza Publications, Pretoria, pp.40-41.
- Viljoen A (2008). Indigenous South African medicinal plants part 7: *Aloe ferox* (Cape aloe). *S. Afr. Pharm. Journ.*, 1: 47.
- Viljoen AM, Van Wyk BV and Newton LE (2001). The occurrence and taxonomic distribution of the anthrones aloin, aloinoside and microdotin in aloe. *Biochem. Syst. Ecol.*, **29**: 53-67.
- Watt J and Breyer-Brandwijk MG (1962). The Medicinal and poisonous plants of Southern and Eastern Africa. An account of their medicinal and other uses, chemical composition, pharmacological effects and toxicology in man and animal. E and S Livingstone (Ltd). Edinburg, pp.996-1000.
- William LD, Burdock GA, Shin E, Kim S, Jo, TH, Jones KN and Matulka RA (2010). Safety studies conducted on proprietary high- purity *A. vera* inner leaf fillet preparation, Qmatrix. *Regulatory Toxicol. Pharmacol.*, doi:10.1016/j.yrtph.2010.01.002 (in press).
- Wintola OA and Afolayan AJ (2010). Ethnobotanical survey of plants used for the treatment of constipation within Nkonkobe Municipality of South Africa. *Afr. Journ. Biotechnol.*, **9**(45): 7767-7770.
- Wintola OA, Sunmonu TO and Afolayan AJ (2010). Toxicological evaluation of aqueous extract of *Aloe ferox* Mill. In Loperamide-induced constipated rats. *Hum. Exptl. Toxicol.*, **30**(5): 425-431.