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## PHYTOCHEMICAL ANALYSIS AND TOXICITY EVALUATION OF ACETONE, AQUEOUS AND METHANOLIC LEAF EXTRACTS OF *AGAPANTHUS PRAECOX* WILLD

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**ABSTRACT:** *Agapanthus praecox* Willd of the family Amaryllidaceae is widely used in South African traditional medicine to treat heart disease, paralysis, coughs, colds, chest pains and diarrhoea. The decoction is used to prevent pregnancy complications and argument labour. The study aims at determining the phytochemical constituents and investigating the potential toxicity of the solvent extracts of *A. praecox*. Quantitative phytochemical analysis was done using standard procedures and spectro-photometric techniques while the toxicity assessment was carried out with brine shrimp toxicity bioassay. The data were subjected to two way analysis of variance (ANOVA) followed by Tukey's test. Methanol had the highest percentage yield of extract (16.2 %) while water (9.3 %) was the least. The quantities of tannins (335.23 mg g<sup>-1</sup>), phenols (91.11 mg g<sup>-1</sup>), proanthocyanidins (139.47 mg g<sup>-1</sup>), flavonoids (46.53 mg g<sup>-1</sup>) and flavanols (74.02 mg g<sup>-1</sup>) in acetone extract were significantly higher ( $p > 0.05$ ) than in aqueous and methanolic extracts. Highest hatching success was recorded in aqueous extract (50.33%) and the lowest hatching success ( $p < 0.05$ ) was recorded in the positive control (amoxicillin). The LC<sub>50</sub> of all the solvent extracts was greater than 1mg/mL (LC<sub>50</sub> > 1 mg/mL) while the positive control (amoxicillin) had a lesser LC<sub>50</sub> (0.76 mg/mL). Organic solvents of fresh leaves of *A. praecox* contained significant phytochemicals that were not toxic according to Meyer's index of toxicity, which states that any substance with LC<sub>50</sub> > 1mg/mL were considered non-toxic. Further toxicological evaluation assays are recommended to confirm the toxicity of *A. praecox*.

**INTRODUCTION:** The ever-increasing interest in the use of medicinal plants to treat different ailments and diseases necessitates the investigation of their potential toxicities. Most people in developing countries rely on medicinal plants for their primary health care.

Diversity, flexibility, easy accessibility, relative low cost, low levels of technological input and relative low side effects are some of the positive features of traditional medicine<sup>1</sup>. All medicinal plants contain active principles such as saponins, tannins, alkaloids, phenols, flavonoids, anthocyanins, glycosides etc. which are responsible for their biological activities<sup>2</sup>. These active principles have been reported to possess therapeutic as well as toxicological properties<sup>3</sup>. The toxicity of the medicinal plants has been attributed to the quantities and strength of their active principles<sup>4</sup>. As the global interest in the use of medicinal plants is increasing, public health issues and concerns

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surrounding their safety are also increasingly recognized. The ethno pharmacological significance of *Agapanthus praecox* in South African folkloric medicine made it suitable for the current study.

*Agapanthus praecox* locally called "Isicakathi" (Isixhosa) is a rhizomatous herb of the family Amaryllidaceae. This species occurs in Eastern Cape Province of South Africa. It usually grows to between 0.8 and 1m tall and has 10-11 leathery leaves. The blue to purple or white flowers are clustered in a large globular flower head. The fruit is a greenish capsule. It possesses anti-inflammatory, anti-oedema, antitussive, immunoregulatory, antibacterial, antifungal, and antitumour properties<sup>5</sup>. It is used in folkloric medicine to treat chest pains, coughs and colds<sup>6</sup>. Duncan *et al.*,<sup>7</sup> reported its inhibition of angiotensin converting enzyme *in vitro* hence, used to treat high blood pressure and related disorders. The leaves with *Dianthus* sp. are used to treat gastrointestinal tract trouble and abdominal pains<sup>8</sup>. The leaves are used to hold dressings in place and wound around wrists to bring down fevers<sup>9</sup>. Koduru *et al.*,<sup>10</sup> also reported the anticancer potential of the root infusion.

*Agapanthus praecox* has been considered a plant of fertility and pregnancy; it has aphrodisiac property and is used as a medicine for impotency and barrenness<sup>5</sup>. It has been reported that the decoction can induce labour and ensure smooth expulsion of the placenta<sup>11,12</sup>. Pooley<sup>13</sup> stated that "bathing the new born baby with the decoction of *A. praecox* makes them strong, keep them free from bowel problems and prevents crusts on the head". The anti-depressant property was reported by Nielsen *et al.*,<sup>14</sup>. It was listed among the plant species traditionally used in Southern Africa for the treatment of various central nervous system-related ailments<sup>15</sup>. Despite, the wide usage of *A. praecox* in indigenous medicine system, its phytochemical constituents and potential toxicity remain unknown. Though, Notten<sup>6</sup> suspected its haemolytic poisoning in humans but this has not been proven. To furnish the general public including the traditional medicine practitioners with adequate information to facilitate better understanding of the risks associated with the use of this plant; this study aims at estimating the phytochemical constituents

as well as evaluating the potential toxicity of *Agapanthus praecox* using brine shrimp toxicity testing as a preliminary test to ascertain its level of toxicity.

## MATERIALS AND METHODS:

**Plant Collection:** Fresh leaves of *Agapanthus praecox* were collected from the University of Fort Hare, Alice Campus, South Africa in June, 2016. The plant was identified by Dr. O. J. Sharaibi and a voucher specimen (SHAMed 2016/07) was deposited in the Giffen's herbarium of the University.

**Sample Preparation and Extraction:** The fresh leaves were rinsed, air dried and pulverized before extraction. One hundred gram each of the powdered materials was soaked in 1000mL of distilled water, acetone and methanol respectively. The aqueous extract was filtered through Whatman no.1 paper and the filtrate was freeze-dried for 48 hr using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY). The acetone and methanolic extracts were concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator (Strike 202 Steroglass, Italy). The resulting extracts were reconstituted in their respective solvents to give the desired concentrations used in the study.

## Phytochemical Analysis:

**Determination of Total Phenol Contents:** A volume of 0.5mL of the extract (1mg/mL) was mixed with 5mL Folin-Ciocalteu reagent (previously diluted with water 1:9 v/v) and 4mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15 sec and allowed to stand for 30 min at 40 °C Absorbance was measured at 765 nm using AJI-C03 UV-VIS spectrophotometer. The results were expressed as mg/g tannic acid equivalent using the equation based on the calibration curve:

$$Y = 0.1216x, R=0.9365$$

Where  $x$  is the absorbance and  $Y$  was the tannic acid equivalent.

**Determination of Flavonoids Contents:** A volume of 0.5mL of 2 % AlCl<sub>3</sub> ethanol solution was added to 0.5mL of the sample solution. After 1 hr at room temperature, the absorbance was measured at 420 nm. Extract samples were evaluated at a final concentration of 0.1mg/mL.

The results were calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve:

$$Y = 0.0255x, R^2 = 0.981$$

Where  $x$  was the absorbance and  $Y$  was the quercetin equivalent.

**Determination of Total Flavanols Contents:** The reacting mixture of 2mL each of the extracts with 2 mL of  $AlCl_3$  in ethanol solution and 3mL of 50g/L sodium acetate solution was allowed to stay for 2.5 hr at 20 °C in a water bath. The absorbance was measured at 440 nm. The flavanols contents were calculated thus:

$$Y = 0.0255x, R^2 = 0.9812$$

Where  $x$  was the absorbance and  $Y$  is the quercetin equivalent in mg/g.

**Determination of Proanthocyanidins Contents:** A volume of 0.5mL each of the plant extracts was added to 3 mL of vanillin- methanol (4 % v/v). 1.5 mL of hydrochloric acid was added to the reacting mixture and vortexed. The mixture was allowed to stand for 15 min at room temperature. Absorbance was measured at 500nm. Proanthocyanidins content was expressed as:

$$Y = 0.5825x, R^2 = 0.9277$$

Where  $x$  was the absorbance and  $Y$  was the catechin equivalent in mg/g.

**Determination of Tannin Contents:** The extracts weighing 0.20g was added to 20mL of 50 % methanol, vortexed vigorously and later incubated at 80 °C in a water bath for 1 hr. The filtrate was mixed with 20mL of distilled water, 2.5mL of Folin-Dennis reagent and 10mL of 17% aqueous  $Na_2CO_3$ . The mixture was made up to 100mL with distilled water, mixed and allowed to stand for 20 min. The absorbance of the tannic acid standard solutions and the sample was measured at 706 nm. Results were expressed as mg/g of tannic acid equivalent using the calibration curve:

$$Y = 0.0763x, R^2 = 0.9644$$

Where  $x$  was the absorbance and  $Y$  was the tannic acid equivalent.

**Determination of Alkaloids Contents:** A volume of 200mL of 10% acetic acid prepared in ethanol

was added to 5g each of the extracts. This was covered and allowed to stand for 4 hr. The mixture was filtered and the filtrate was concentrated to one-fourth of the original volume in a water bath. Concentrated ammonium hydroxide was added drop-wisely to the extract, pending the completion of the precipitation. The solution was allowed to settle and re-filtered after washing with dilute ammonium hydroxide. The residue obtained was dried, weighed and the percentage composition was determined using the formula:

$$\% \text{ Alkaloid} = \text{Final weight of the sample} / \text{Initial weight of the extract} \times 100$$

**Determination of Saponins Content:** The extracts weighing 20g was added to 100mL of 20% aqueous ethanol and kept in a shaker for 30 min. The mixture was heated over the water bath for 4 hr at 55 °C and then filtered to collect the residue which was later re-extracted with 200mL of 2 % aqueous ethanol. The filtrate was concentrated over the water bath at 90 °C to approximately 40mL. The concentrate was transferred into a 250mL separatory funnel and extracted twice with 20mL diethyl ether. The ether layer was discarded while the aqueous layer was retained and to which 60ml *n*- butanol was added. The mixture was washed twice with 10mL of 5% aqueous sodium chloride. After evaporation, the samples were dried in the oven at 40 °C to a constant weight. The saponins content was calculated using the formula:

$$\% \text{ Saponin} = \text{Final weight of residue} / \text{Initial weight of the sample} \times 100$$

### Brine Shrimp Toxicity Bioassay:

**Preparation of the Assay System:** The assay system was prepared according to Ohikhena *et al.*,<sup>16</sup>. Five Petri dishes containing 30ml of the plant extracts dissolved in filtered seawater were prepared in a two-fold dilution to yield a series of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg/mL) of the plant extracts. Amoxicillin dissolved in seawater (30µl/ml) served as a positive control while Petri dishes containing sea water served as the negative controls. The experimental setup was allowed to stand in open air for 30 min for the solvents to evaporate.

**Brine Shrimp Hatchability Assay:** *Artemia salina* (Brine shrimp) cysts were stocked at a density of 10 individuals per Petri dish. Each Petri dish

contained 30ml of the incubation medium at varying concentrations (1, 0.5, 0.25, 0.125 and 0.0625mg/mL). They were partly covered, incubated at 28 °C and allowed to stand for 72 hr under constant illumination. The number of free nauplii in each Petri dish was counted after every 12 hr. The percentage of hatchability was assessed by comparing the number of hatched nauplii with the total number of cysts stocked. The minimum inhibitory concentration (MIC) was determined as the minimum concentration of the plant extracts (or control drug) that inhibited hatching of the cysts.

**Brine Shrimp Lethality Assay:** The lethality of *A. praecox* extracts against brine shrimp nauplii was evaluated by pipetting an aliquot (0.1ml) containing 10 nauplii into each Petri dish of extract solutions and controls. The setup was allowed to remain for 72 hr under constant illumination; dead larvae in each Petri dish were counted after every 12 hr.

The percentage mortality (M %) was calculated as:

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

**Statistical Analysis:** Statistical analysis was performed using Graph Pad Prism 5 statistical package (Graph Pad Software, San Diego MA, USA). The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey test. All the results were expressed as mean  $\pm$  SEM (n = 3) and were considered statistically significant when p < 0.05.

## RESULTS AND DISCUSSION:

**Dry Extract Yield:** Each 100 gm of powdered leaves of *A. praecox* extracted in water, acetone and methanol yielded 9.3g, 11.6g and 16.2g of dry extract respectively. Methanol extracted more phytochemicals (16.2g) than water and acetone. The high dry extract yield recorded in methanol extract (16.2%) may be due to high polarity of methanol which can draw high variety of plant constituents than the other solvents. This result agreed with some reports in the literatures. Anokwuru *et al.*,<sup>17</sup> reported higher yields of *Acalypha wilkesiana*, *Azadirachta indica* and *Solanum scabrum* in methanol extraction than in acetone. Also, Dhawan and Gupta<sup>18</sup> observed the

highest extract yields in methanol solvent for all the plants studied.

**Phytochemical Constituents:** The quantities of tannins (335.2mg g<sup>-1</sup>), phenols (91.1mg g<sup>-1</sup>), proanthocyanidins (139.5 mg g<sup>-1</sup>), flavonoids (46.5 mg g<sup>-1</sup>) and flavanols (74.0 mg g<sup>-1</sup>) in acetone extract were significantly higher (p > 0.05) than in aqueous and methanolic extracts. The amount of saponins in all the extracts was considerably while all the extracts contained little amounts of alkaloids (Table 1).

**TABLE 1: PHYTOCHEMICALS OF AQUEOUS, ACETONE AND METHANOLIC LEAF EXTRACTS OF A. PRAECOX**

Phytochemicals	Aqueous	Acetone	Methanol
Proanthocyanidins	22.368	139.47	137.0428
Phenols	48.3725	91.1149	74.0422
Flavonoids	14.56	46.5299	10.4805
Flavanols	20.6627	74.0148	35.5419
Tannins	302.2396	335.4071	321.2306
Saponins	123.0764	142.0865	132.0743
Alkaloids	0.2961	0.4532	0.3562

Values are the mean of the three replicates (n = 3).

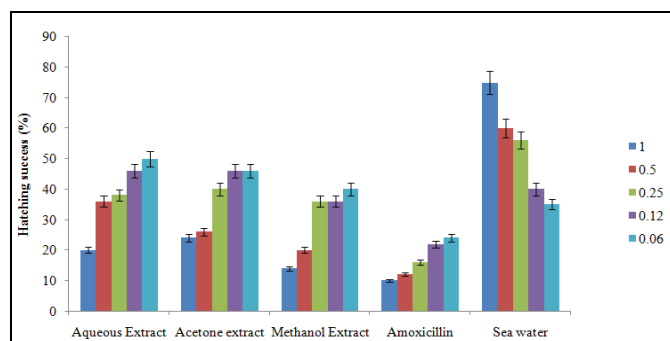
The extracts of *A. praecox* contained a considerable amount of phytochemicals that have significant therapeutic and exhibit biological activities in humans. The presence of these phytochemicals may be responsible for the diverse uses of this plant in traditional medicine. The amount of tannins, phenols and proanthocyanidins in all the extracts were considerably high. According to Ben Mohamed *et al.*,<sup>19</sup> polyphenols levels are considerably affected by genetic factors like plant species, plant organ, phenological stage, and environmental factors like climatic conditions, biotic and abiotic stresses occurring during plant growth. Basma *et al.*,<sup>20</sup> and Chew *et al.*,<sup>21</sup> also identified higher amounts of polyphenols in leaves than the remaining plant organs.

The phenolics have been reported to have antimicrobial, anti-inflammatory and antioxidant activities<sup>22</sup>. Proanthocyanidins have a putative role as antioxidants, they affect the inflammatory process and protect against H<sub>2</sub>O<sub>2</sub> - induced lipid peroxidation<sup>23</sup>. Yamakoshi *et al.*,<sup>24</sup> investigated the toxicity of proanthocyanidins from grape seed extracts and reported lack of toxicity in both acute and sub-acute toxicity studies and supported the use of proanthocyanidins as food supplements.

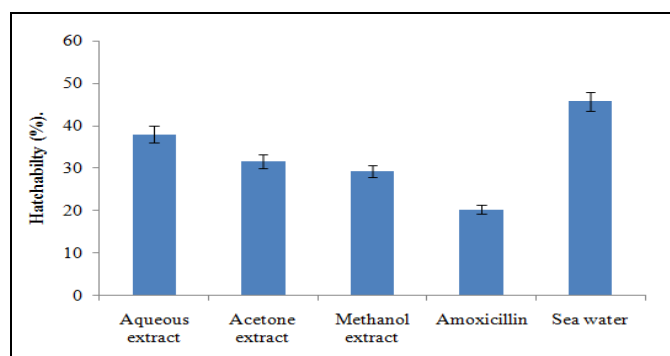


The quantities of saponins in *A. praecox* leaves in all the extracts were very high; acetone extract (142%), methanolic extract (132%) and aqueous extract (123%). Ezeabara *et al.*,<sup>25</sup> also reported higher levels of saponins in the leaves of *Citrus sinensis* and peel of *Citrus aurantifolia*. Liu and Henkel<sup>26</sup> considered saponins and polyphenols as key ingredients in traditional Chinese medicines which are responsible for most of the observed biological activities. The saponins in *A. praecox* leaves may also possess anti-inflammatory and antibacterial activity which may justify its usage in the traditional medicine for the treatment of wounds. The amount of alkaloids in the leaves of *A. praecox* was very small (0.296%). The alkaloids present in the leaves of *A. praecox* may be responsible for its antimicrobial activity reported in the literatures; since it plays role in the defence of systems against pathogens<sup>27</sup>.

**Brine Shrimp Hatchability Assay:** The percentage hatching success of *A. salina* in different solvent extracts and the controls were represented in **Fig. 1a** and **1b**.



**FIG. 1A: PERCENTAGE HATCHABILITY OF A. SALINA CYSTS IN DIFFERENT CONCENTRATIONS OF EXTRACTS OF AGAPANTHUS PRAECOX, AMOXICILLIN AND SEA WATER**



**FIG. 1B: PERCENTAGE HATCHABILITY OF A. SALINA CYSTS IN DIFFERENT EXTRACTS OF AGAPANTHUS PRAECOX, AMOXICILLIN AND SEA WATER**

Highest hatching success was recorded in the aqueous extract (50.33%) compared with methanol extract (40.45%) and acetone extract (40.35%). The positive control, amoxicillin produced the least hatching success (24%). The order of percentage hatchability of *A. salina* cysts was aqueous extract > methanol extract > acetone extract > amoxicillin. This suggested that aqueous extract had low inhibitory activity against the *A. salina* cysts and this may justify the use of water in herbal preparations. Similar hatching success recorded in acetone extract (40.35%) and methanolic extract (40.45%) may be due to the strength and the quantity of the phytochemicals present in these extracts. According to Tulay<sup>3</sup>, the toxicity of the medicinal plants can be attributed to the strength and quantity of their secondary metabolites.

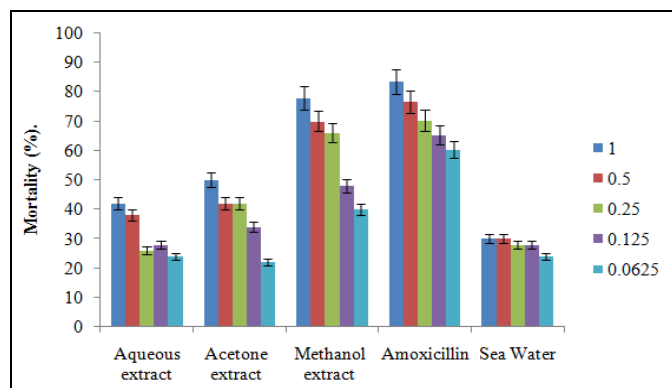
The hatchability increased with decrease in concentrations of all the solvent extracts with highest hatching success observed in 0.0625 mg/mL. This may be due to tolerance of *A. salina* being at resistant cyst stage to a wide range of salinity and until the dormancy is broken, hatching will not occur. It may also mean that all the extracts at 1mg/mL had inhibitory effects and therefore prevented the cysts from hatching.

The increase in the rate of hatchability with time suggests the nutritive potential of the extract that the cysts thrived on as incubation progresses. The highest hatching rate of the cysts for all the extracts was observed after 72 hr of incubation. This contradicted Ohikhena *et al.*,<sup>16</sup> who reported highest hatching success after 36 hr of incubation in various solvent extracts of *Phragmanthera capitata*. The difference in significant hatching time may be due to difference in plant species as reported by Tulay<sup>3</sup>.

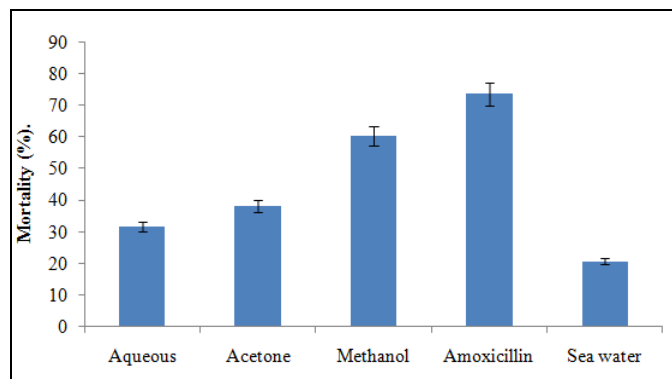
**Brine Shrimp Lethality Assay:** The results of the percentage lethality of *A. salina* cysts in different solvent extracts of *Agapanthus praecox*, amoxicillin and sea water were shown in **Fig. 2a** and **2b**.

Among the extracts studied, the highest percentage lethality was observed in methanol extract (78.53%) compared with acetone extract (50.22 %) and aqueous extract (42.35 %). Ogugu *et al.*,<sup>28</sup> stated that the type of solvent for the extraction

process is vital for the toxicity testing, because different solvents show different extracting potential. The highest mortality rate observed in methanol extract can be due to the quantity and the type of phytochemicals present in the extract; since, methanol had the highest yield of extract during the extraction process of this study.



**FIG. 2A: PERCENTAGE LETHALITY OF *A. SALINA* IN DIFFERENT CONCENTRATIONS OF EXTRACTS OF *A. PRAECOX*, AMOXICILLIN AND SEA WATER**



**FIG. 2B: PERCENTAGE LETHALITY OF *A. SALINA* IN DIFFERENT CONCENTRATIONS OF EXTRACTS OF *A. PRAECOX*, AMOXICILLIN AND SEA WATER**

The highest mortality rate of *A. salina* cysts was observed in 1mg/mL of all the solvent extracts tested. This means increase in concentration increased the mortality rate and that the LC<sub>50</sub> of all the extracts studied were greater than one (>1), hence the extracts were not toxic. According to Meyer's toxicity index, extracts with LC<sub>50</sub> < 1000µg/ml are considered as toxic, while extracts with LC<sub>50</sub> > 1000µg/ml are considered as non-toxic<sup>29</sup>. The rate of mortality increased with increase in time hence, the highest mortality rate was observed after 72 hr of incubation of *A. salina* cysts in all the extracts. The longer the nauplii were exposed to the plant extracts, the higher the mortality. Significant mortality observed in all the plant extracts after 72

hr of incubation suggests that the plant extracts have some nutrients on which the nauplii fed on and that the toxic effects of the plant extracts on the nauplii were delayed but later manifested after a long time of exposure. This agreed with the reports of Ohikhena *et al.*,<sup>16</sup> and Otang *et al.*,<sup>30</sup>.

According to Ahmed *et al.*,<sup>31</sup> the toxicity of plant samples was determined by comparing their LC<sub>50</sub> values with highly toxic substances suitable to be used as positive controls for this test. In **Fig. 2b**, amoxicillin had the highest percentage lethality (78.05%) in 1mg/mL than all the plant extracts and this showed that amoxicillin used as positive control in this study was more toxic than all the plant extracts.

**TABLE 2: HATCHABILITY AND LETHALITY OF *ARTEMIA SALINA* CYSTS IN DIFFERENT SOLVENT EXTRACTS OF *AGAPANTHUS PRAECOX* AND POSITIVE CONTROL AS SHOWN BY THEIR MIC<sub>50</sub> AND LC<sub>50</sub> VALUES**

Extract	MIC <sub>50</sub> (mg/ml)	LC <sub>50</sub> (mg/ml)
Acetone extract	0.16	>1
Aqueous extract	0.62	>1
Methanol extract	0.12	>1
Amoxicillin	< 0.0625	0.76

**CONCLUSION:** The different phytochemicals present in the leaf extracts of *A. praecox* are of significant biological activities; their presence may justify the diverse uses of *A. praecox* in South African traditional medicine. It was discovered from this study that, all the solvent extracts of *Agapanthus praecox* were not toxic (LC<sub>50</sub>>1 mg/mL) in brine shrimp toxicity bioassay. It was confirmed from this study that water is the best solvent for preparation of *A. praecox* as it poses less risk to the users. Caution should be taken when using this plant and continuous use should be discouraged. Further investigation is necessary to determine the toxicity of the methanol extract of this plant as methanol extract exhibited the highest toxicity when compared with aqueous and acetone extracts.

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**CONFLICT OF INTEREST:** The authors declare no conflict of interest.

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