Phytochemistry, Brine shrimp lethality and mice acute oral toxicity studies on seed extracts of *Vernonia anthelmintica*

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Abstract: Despite the widespread use of *Vernonia anthelmintica* seeds in traditional medicine, the need to establish the safety of the *Vernonia anthelmintica* is required to ascertain the safe use of this herbal medicine. The aim of the present study is to establish the acute toxicity profile of different extracts of *Vernonia anthelmintica*. Hexane and ethanol extract of *Vernonia Anthelmintica* has been studied for its brine shrimp lethality potential. Water decoction (WDVA), Hexane (HEVA) and Ethanol (EEVA) extracts of *Vernonia anthelmintica* has also been evaluated for their *in-vivo* acute oral toxicity in mice by Lorke's method. Phytochemistry of all three extracts was also evaluated for the presence of their secondary metabolites. All three extracts showed the presence of flavonoids and terpenoids, while alkaloids, tannins and fixed oils were present in HEVA and EEVA. Furthermore EEVA also showed presence of carbohydrates and HEVA also showed the presence of cardiac glycosides. Ethanol and hexane extracts of *Vernonia anthelmintica* showed a positive cytotoxicity in brine shrimp lethality test at 24 hours with LC50 104.16 (224.0-48.05)µg/ml and 216.11µg/ml (378.2-128.7) respectively as compared to standard drug etoposide LC50 7.46µg/ml. The oral LD50 for EEVA, HEVA and WDVA in mice by Lorke's method was greater than 5000mg/kg. The result of brine shrimp lethality test clearly exhibited the presence of bioactive compounds with cytotoxic potential; however seems to be safe for oral use since LD50 was higher than 5000mg/kg and thus safety of acute dosing *in vivo* practices is justified.

Keywords: Acute oral toxicity, brine Shrimp assay, Lorke's method, Phytochemistry, *Vernonia anthelmintica* seed.

INTRODUCTION

Traditional medicine with respect to medicinal plants still holds an immense role in cure of various ailments and a large proportion of the population has firm faith on traditional healing practices and herbal plants of medicinal importance. However it is worth mentioning that most of the knowledge regarding their medicinal role is usually not evident through reliable scientific literature thus ongoing research is required to establish the safety and risks associated with the non-prescription use of these remedies.

Vernonia anthelmintica is a medicinal plant, which belongs to the family Asteraceae, it is an annual terrestrial erect plant, Vernonia is the biggest genus in the tribe of Vernoniae constituting about 1000 species. Genus Vernonia is named after English Botanist William Vernon (Toyang and Verpoorte, 2013). Most frequently used synonym is *Centratherum anthelminticum* (Bewley *et al.,* 2006).

Vernonia anthelminticum commonly known as kalijiri is an important plant of the sub-continent. It has a valid role in alternative medicine for the treatment of differing illnesses. The seeds have a prominent role as anthelmintic agent(Kirtikar and Basu, 1999). It has been used in asthma, kidney troubles, inflammatory and arthritic swellings and convulsions. Various review studies give the clue of its role against inflammation, diabetes, cancers and much more (Manvar and Desai, 2012). According to traditional claims the fatty oils yielded from the seeds are commonly used as anthelmintic and alexipharmic. In folk medicine it is also used in cough preparations, flatulence, intestinal colic, dysuria and chronic skin problems. Literature also exhibited its role in pediculosis (Misra *et al.*, 1984).

Because of the diverse pharmacological potential this plant has provided a wide area of continuous research and has been stamped as a medicinal plant. Present study is designed to evaluate the phytochemistry, cytotoxicity and acute oral toxicity of different extracts of *Vernonia Anthelmintica.*

As a general rule bioactive compounds are usually toxic to shrimp larvae Artemia salina. This is a quick, cheap and generalized developed method for the screening of bioactive natural products (Krishnaraju *et al.,* 2005). This method used for assessing cytotoxic potential of a drug has a clear advantage of not requiring higher animals for preliminary screening of bioactive compounds (McLaughlin *et al.,* 1998).

The aim of the present study is to establish the acute toxicity profile of different extracts of *Vernonia anthelmintica*.

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MATERIAL AND METHODS

Collection and extraction of seed

The dried seeds of *Vernonia Anthelmintica* were obtained from commercial market of Karachi, Pakistan. The identification of the seed was carried out by the Centre of plant conservation, Karachi University Herbarium and Botanic Garden, University of Karachi through Herbarium no. G.H.No. 92629. The seeds were cleaned manually to remove any impurities and crushed to coarse powder using a mechanical grinder. The coarse seeds were thereafter extracted with n-hexane using maceration method for 10 days with occasional shaking. On day 10 the extract was sequentially filtered with muslin clothes and filter papers. The filtrate was poured and concentrated in a rotary evaporator at reduced pressure at 50ºC. The concentrated extract was dried in an oven at 40ºC and stored at room temperature until further use. The resulted extract is referred to as hexane extract of *Vernonia anthelmintica* (HEVA). The residue remained after filtration of hexane was used for preparation of ethanol extract by the same procedure and referred to as ethanol extract of *Vernonia anthelmintica* (EEVA). Water decoction of *Vernonia anthelmintica* (WDVA) of the seeds was prepared freshly each time simply by boiling the seeds in distilled water prior to use.

Phytochemistry

Various tests were employed to analyze the phytochemical constituents of HEVA, EEVA and WDVA.

Wagner test for alkaloids

Small quantity of HEVA, EEVA and WDVA was dissolved in Methanol (2ml) and few drops of HCl (1%) were added. The mixture was then heated and cooled and few drops of Wagner reagent were added. Formation of precipitate or turbidity confirms the presence of alkaloids in the sample (Rajesh, *et al.,* 2013)

Ferric chloride test for cardiac glycosides

Small quantities of HEVA, EEVA and WDVA were dissolved in 1% glacial acetic acid. Few drops of ferric chloride solution were added to this mixture and 0.5 ml of concentrated sulphuric acid was added along the walls of test tubes. Appearance of brown ring at interface is indicative of presence of cardenolide (Rajesh, *et al.,* 2013).

Benedict test for carbohydrates

Small quantities of HEVA, EEVA and WDVA were allowed to mix with few drops Benedict solution, which contains cupric citrate complex in alkaline solution. The mixture was observed for reddish brown precipitate after boiling it in a water bath. Presence of precipitate confirms the presence of carbohydrate in the sample(Yadav and Agarwala, 2011)

Xanthoprotein test for proteins

Small amount of each extract was taken in separate test tubes. 1 ml sulphuric acid concentrated was added sliding from the sides of test tubes. Presence of yellow precipitate is the indication of presence of Protein in the sample (Raju *et al.,* 2011)

Ferric chloride test for flavonoids

Sequential addition of 0.5ml, 1% ammonia solution and concentrated sulphuric acid to small amount of each extract already dissolved in distilled water may resulted in the appearance of yellow color indicative of flavonoids. On standing this color would disappear(Rajesh *et al.,* 2013)

Foam test for saponins

Small amount of each extract was dissolved in distilled water and this mixture was shaken briskly to observe persistent froth. The froth was observed for emulsion on shaking with 3 drops of olive oil. (Rajesh *et al.,*2013)

Lead test for tannins

Small amount of each extract was dissolved in distilled water in the separate test tubes and 2 drops of 0.1%ferric chloride were added to the solution. The mixture may turn blue or green if tannins are present(Rajesh *et al.,* 2013).

Salkowaski's test for terpenoids

Small quantity of each extract was dissolved in 1 ml of chloroform and 1ml of concentrated sulphuric acid. Terpenoids if present may appear as reddish brown discoloration at the interface (Rajesh *et al.,* 2013).

Spot test for fixed oil and fatty acid

Place a spot of different extracts on separate filter papers. Oil staining on the filter paper is the indication of presence of fixed oil and fats (Rajesh *et al.,* 2013).

Brine Shrimp lethality assay

The brine shrimp lethality bioassay was carried out on HEVA and EEVA using the standard procedure(Meyer *et al.,* 1982)**.**A rectangular dish was used as a hatching tray and it is half filled with already filtered brine shrimp solution containing artificial sea water (38g/L of distilled water, pH 7.4). The eggs of Artemia salina (50mg) was then sprinkled on it and left for hatching under incubation at 37ºC.Hatching period of eggs of Artemia Salina take 48 hrs. when placed in sea water artificially prepared for this purpose. EEVA and HEVA (20mg each) were dissolved in 2ml of ethanol and hexane respectively. From this solution 5, 50 and 500µl was transferred to 3 different vials, this process is carried out in triplicate (3 test/Concentration). The final concentration was 10,100 and 1000µg/ml respectively. The solvent used is allowed to evaporate overnight. After hatching, active nauplii that were free from their eggshells were collected from illuminated part of the hatching tray and used for the test. 10 mature Nauplii were then placed in each vial using a Pasteur pipette. Sea water was used to make up the

Table 1: Qualitative analysis of HEVA, EEVA and WDVA

Secondary Metabolites	Test Employed	HEVA	EEVA	WDVA
Alkaloids	Wagner test	+ ve	+ ve	- ve
Carbohydrates	Benedict test	-ve	+ ve	- ve
Cardiac glycosides	Ferric chloride test	+ ve	- ve	+ ve
Flavonoids	Ferric chloride test	+ ve	+ ve	+ ve
Proteins	Xanthoproteins test	- ve	- ve	- ve
Saponins	Foam test	- ve	- ve	- ve
Tannins	Lead test	+ ve green	+ ve blue	- ve
Terpenoids	Salkowaski's test	+ ve	+ ve	+ ve
Fixed oils and fatty acids	Spot test	+ve	-ve	-ve

+ve sign indicates the presence of secondary metabolite
- ve sign shows the absence of secondary metabolite

Table 1: *In vivo* oral acute toxicity of HEVA, EEVA and WDVA in mice

Groups	Doses (mg/Kg) No. of Mortality / total no. of mice		
PHASE 1:	10	100	1000
HEVA	0/3	0/3	0/3
EEVA	0/3	0/3	0/3
WDVA	0/3	0/3	0/3
PHASE 2:	1600	2900	5000
HEVA	0/3	0/3	0/3
EEVA	0/3	0/3	0/3
WDVA	0/3	0/3	0/3

Table 3: Median Lethal dose (LD_{50}) of Hexane extract of *Vernonia anthelmintica* (HEVA) against brine shrimp lethality bioassay

Dose (µg/ml)	No. of shrimps	No. of survivors	LD_{50} (µg/ml)
10	30	30	216.1
100	30	20	
1000	30	5	

Table 4: Median Lethal dose (LD_{50}) of Ethanol extract of *Vernonia anthelmintica* (EEVA) against brine shrimp lethality bioassay

Dose (µg/ml)	No. of shrimps	No. of survivors	LD_{50} (µg/ml)
10	30	24	104.1
100	30	20	
1000	30	5	

Standard drug: etoposide (LD_{50}: 7.4µg/ml)

volume to 5ml and placed under illumination at 25-27ºC for 24hrs. Same method was repeated to prepare other vials containing solvent and cytotoxic drug Etoposide as negative and positive controls respectively. The vials were examined and the number of dead larvae in each bottle was counted after the incubation period of 24 hrs. The death Percentage was calculated using simple mathematical equation (1) and LD_{50} was analyzed using Finney program with 95% confidence Interval(Finney, 1971).

Percentage of Death%=(Total nauplii-alive nauplii)×100 (1)

In vivo acute oral toxicity in mice

Locally bred albino mice were kept in cages and acclimatized in the standard environment of 12h/12 h light/dark cycles and standard temperature. Animals had free access to standard pellet diet and tap water. The studies are in accordance with the standard ethics of animal use and Lorke's method was adopted to carry out Acute toxicity studies (Lorke, 1983). This method is divided into 2 phases. In the first phase 9 mice were selected and divided into 3 groups constituting 3 mice each. The first group received HEVA orally at a dose of 1000mg/kg; second group received the same extract at the dose of 100mg/kg; whereas the third group received the extract at the dose of 10mg/kg body weight. Animals were observed for 24 hours periodically to assess general signs and symptoms of gross toxicity including mortality. In the second phase 3 mice were selected and assigned to different groups containing one animal each. HEVA is administered orally to these groups in the doses of1600,

2900 and 5000mg/kg. Same procedure was repeated for oral acute toxicity of EEVA and WDVA on fresh animals. Based on the results of both the phases LD_{50} was calculated as follow:

$LD_{50} = \sqrt{(D_0 \times D_{100})}$

Where D_0 = Highest dose producing mortality

RESULTS

Table 1 shows the results of qualitative phytochemistry of HEVA, EEVA and WDVA. The results of median lethal dose of *in vivo* acute oral toxicity (LD_{50}) for water decoction, hexane and ethanol seed extracts of *Vernonia Anthelmintica* are given in table 2. The results clearly revealed that both extract and water decoction of *Vernonia anthelmintica* has median lethal dose above 5000mg/kg on acute dosing. No mortality or toxicity signs with respect to behavior and general appearance of animal were observed after oral administration of water decoction, hexane and ethanol extract of *Vernonia anthelmintica* seeds at doses 10, 100, 1000, 1600, 2900 and 5000mg/kg bodyweight. Furthermore no alteration in color of stool, urine or eye was noted in both control and treated groups.

The results of cytotoxicity of HEVA and EEVA by brine shrimp lethality assay are depicted in tables 3 and 4. It shows that HEVA is moderately cytotoxic to brine shrimp with LC_{50} value 216.1µg/ml with upper toxic concentration 378.2 and lower toxic concentration 128.7µg/ml whereas EEVA showed positive cytotoxicity in the range of upper toxic concentration 224.0 and lower toxic concentration 48.05 with the LC_{50} value 104.1 µg/ml at 24 hours.

DISCUSSION

Herbal medicines have a prominent role in treatment and prophylaxis of varying ailments and usually contain potent pharmacological compounds Apart from their known pharmacological activities in conventional medical systems it is worth mentioning that these herbs are usually therapeutic at certain dose but show toxicity on increasing the dose (Saad *et al.,* 2006). Previous reviews on safety of herbal medicine reported that most of the present herbs do not correspond to the plants described in the earlier literature as most of the herbs has undergone various primitive changes due to cultivating and environmental factors (Azaizeh *et al.,* 2006). It is therefore recommended that every time any herb to be undertaken for pharmacological screening must go through acute toxicity and cytotoxicity assays and phytochemical assays to validate the exact data for the specific herb. To address the similar concerns the present study is undertaken to evaluate the safety of different extracts of *Vernonia anthelmintica* seeds.

The phytochemical screening of HEVA revealed the presence of alkaloids, cardiac glycosides, flavonoids, tannins, terpenoids and fixed oils. EEVA found to be similar in phyto-composition with HEVA except for presence of carbohydrates and absence of cardiac glycosides in the former. WDVA differs drastically in comparison with ethanol and hexane extract as it contains only cardiac glycoside, flavonoids and terpenoids.

In present study *in vivo* acute oral toxicity test in mice clearly revealed the safety of water decoction, hexane and ethanol extract of *Vernonia anthelmintica* seed and LD_{50} estimated by Lorke's method is found to be greater than 5000mg/kg body weight. All extracts are nontoxic and comes under the category 5 of global harmonization system that encircle all drugs with LD_{50} value >2000-5000mg/kg (Pratt, 2002)

The present study suggested that both hexane and ethanol extracts contains bioactive compounds that holds cytotoxic effects. The cytotoxicity potential of drugs on the basis of brine shrimp lethality test was classified in various previous studies such that LC_{50} value above 1000µg/ml is considered as nontoxic, LC_{50} value between 500-1000µg/ml is stratified as weakly cytotoxic, 100-500µg/ml reveals moderate toxicity and 0-100µg/ml is a predictor of strong cytotoxicity (Meyer *et al.,* 1982; Santos Pimenta *et al.,* 2003). In the present study hexane and ethanol extracts of *Vernonia anthelmintica* seeds show to have moderate toxicity with the LC_{50} value of 216.1 and 104.1µg/ml at 24 hours.

Apart from the individual results of LD50 of acute oral toxicity and LC50 of brine shrimp assay this study also strengthen the correlation between LC50 of brine shrimp and LD50 of acute oral toxicity assay in accordance with Parra (Parra *et al.,* 2001). According to Parra correlation data the brine shrimp LC_{50} <10µg/ml holds LD_{50} in the range of 100-1000 mg/kg; LC_{50} <20µg/ml possesses LD_{50} in the range of 1000-2500 mg/kg and LC_{50} >25 µg/ml indicates LD_{50} in the range of 2500-8000 mg/kg with good correlation ($r=0.85$; $P<0.05$) (Parra *et al.,* 2001). Thus in accordance with this correlation study, the LC_{50} of both extracts of *Vernonia anthelmintica* correlates with LD_{50} of acute oral toxicity in mice.

CONCLUSION

Present study provides valuable information regarding phytochemistry, acute oral toxicity and cytotoxic potential of *Vernonia anthelmintica*. This study is unique in the way as it presents the comparative effects of both hexane and ethanol extracts in contrast to previous studies that worked on single extract. This study provides valuable data for various *in vivo* animal studies in order to elucidate its chronic toxicity and pharmacological activities on scientific grounds.

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