

**TOXICITY STUDY OF *Warbugia ugandensis* USED BY TRADITIONAL HEALERS TO TREAT HERPES ZOSTER IN HIV PATIENTS USING *Drosophila melanogaster* AS A MODEL****Ahmad Halilu Ringim<sup>1</sup>, Marta Vicente Crespo<sup>1</sup>, Barkat Ali Khan<sup>2\*</sup>**<sup>1</sup>Institute of Biomedical Research, Kampala International University Western-Campus Uganda<sup>2</sup>Faculty of Pharmacy, Gomal University D.I Khan, PakistanReceived 9<sup>th</sup> November 2016; revised accepted 16<sup>th</sup> January 2017**ABSTRACT**

**Objectives:** To carryout toxicity study of *Warbugia ugandensis*, also to participate in the compilation of ethnopharmacological knowledge of Rukararwe and makes it available for future research, and also to avail the healers with the information on scientific literature.

**Methods:** Different concentrations of aqueous and ethanol extracts of *Warbugia ugandensis* with or without food were fed on *Drosophila melanogaster* and acute toxicity, locomotion assay, longevity study of the flies was done in 3 replicates, together with preliminary phytochemical screening of different extracts.

**Results:** Traditional healers mostly used oral formulation (80%), herbs for treating herpes zoster (40%); Steroids, Terpenoids and Glycosides were present in all extracts of *W. ugandensis*. Extracts of *W. ugandensis* were not toxic at acute exposure, ethanol extract of *W. ugandensis* decreased negative geotaxis ( $P < 0.01$ ) and aqueous extract also ( $P < 0.05$ ). Finally longevity study has shown toxicity of ethanolic extract of *W. ugandensis* at concentration of 1% and 2%.

**Conclusion:** The herb appears to be non toxic at acute exposure and toxic (ethanolic extract) at higher dose on chronic exposure.

**Key words:** *Warbugia ugandensis*, Toxicity, *Drosophila melanogaster*, Herpes zoster

\*Corresponding Author. E-mail: barki.gold@gmail.com; Mobile No. ++2348067964580; Phone/Fax : +92-966-750284

**INTRODUCTION**

The World Health Organization (WHO) estimated that 4 billion people (80% of the World's population) use herbal medicines for some phase of primary healthcare [1]. Herbal medicine has a long history in African traditional medicine, and its use is still inevitable especially in rural settings. Traditional herbal medicine still remains the main source of health care in the rural communities and the majority of the sub-Sahara African population depends on it as the most preferred form of treatment of HIV-related symptoms [2].

The human immunodeficiency virus (HIV) is a lentivirus which is subgroup of retrovirus that causes HIV infection and if left without treatment leads to acquired immunodeficiency syndrome (AIDS).

Herpes zoster (shingles or zona) is viral disease caused by varicella zoster virus usually manifested by a painful skin rash with blisters and single dermatomal involvement in a limited area on one side

of the body, often in a stripe. Although the rash usually doesn't exceed two to four weeks, some patients experience outstanding nerve pain for months or years, a condition known as postherpetic neuralgia [3]. Varicella zoster virus (VZV) a member of the subfamily Alphaherpesvirinae and it is known to infect humans, causing chicken pox in children and herpes zoster in adults and rarely in children [4]. The same article stated that VZV can remain dormant in the nervous system and later in life reactivates to cause shingles in 10-20% of cases. The exact mechanisms of how VZV remains latent and reactivates itself it's not clear [5]. Incidence of Herpes zoster in the general population was estimated to be between 1.5 and 3 per 1000, and up to 10 to 15 times higher in HIV-positive patients than in the general population [6, 7]. Other studies, though, give numbers 15 to 25 higher [8].

*Drosophila melanogaster* is widely employed in many researches because of its advantages which

includes; short lifecycle of about 10 days, easy to handle, ability to reproduce many genetically identical flies in a shorter time and their cost-effectiveness [9, 10].

Researchers have proved that about 75% of disease-related genes in human have a homologue in *D. melanogaster*, making it a suitable candidate model from which findings can easily be extrapolated to human beings [11]. The adult fly has structures that perform functions corresponding to those of the heart, gastro-intestinal tract, kidneys and the reproductive tract [10] making it possible to study the effect of drugs and medicinal remedies in the context of a whole organism. The fly also has other advantages over many models in use since it can be used at any of the four developmental stages of life (embryo, larva, pupa, and adults) depending on the nature of research questions and objectives of the study [10].

## **MATERIALS AND METHODS**

### **Compilation of Knowledge**

Questionnaires of 'Designing a Holistic Approach for the Management of HIV/AIDS for the Institute of Biomedical research; Kampala International University, was used for this purpose.

### **Plant Collection and Pre-Extraction Preparation**

*Warbugia ugandensis* Sprague Plants specimens was collected from Rukararwe Nyakabiri Sub-county, Bushenyi District, Western Uganda. Specimens were taken to Mbarara University of Science and Technology, Department of Botany for authentication and issue of collection number. The leaves were dried under shade for about two to three weeks, and then they were pounded using wooden mortar and pestle and sieved to obtain fine powder.

### **Plant Extraction and Preparation**

The powdered leaves of *Warbugia ugandensis* Sprague were macerated in ethanol and water. Two hundred and fifty grams of powdered plant leaves was soaked in 2.5 liters of ethanol and three hundred grams of powdered plant leaves was soaked in 3 liters of distilled water, which was divided into different aliquots using conical flasks. The above was taken to a shaker for 24 hours [12]. The macerated fluid obtained was filtered with Whatman No. 1 filter paper and the filtrate, ethanol was concentrated in a round bottomed flask using a rotary evaporator and water extract filtrate was poured onto petridishes and dried in oven under low temperature [12]. The process was repeated to obtain higher yield of crude extract.

Another four hundred and eighty seven grams of powdered plant leaves was soaked in 4.87 liters of distilled water, which was divided into different aliquots using conical flasks. This was taken to a shaker for 24 hours [12]. The macerated fluid

obtained was filtered with Whatman No. 1 filter paper and the filtrate; water was partitioned with chloroform to obtain two different layers (water and chloroform layer). The water extract filtrate was poured onto petridishes and dried in oven under low temperature and the chloroform extract was concentrated in a round bottomed flask using a rotary evaporator to obtain crude extract [13].

### **Percentage Yield Determination**

Percentage yield was calculated using the formula  $W_2 - W_1 / W_0 \times 100\%$ . Where  $W_0$  is the weight of the initial dried sample,  $W_1$  is the weight of container alone and  $W_2$  is the weight of both extract and container [14].

### **Preliminary Phytochemical Screening Tests**

#### ***Test for Alkaloids***

10mls of the extract was added to 5-10 drops of Wagner's reagent (2g of iodine + 6g of KI in 100mls of distilled water). Formation of opalescent or yellow white precipitate will confirm the presence of alkaloids [15].

#### ***Test for Anthraquinones (Borntrager's Test)***

3ml of aqueous extract was shaken with 3 ml of benzene, filtered and 5 ml of 10% ammonia solution was added onto it. The mixture was shaken and the presence of a pink, red or violet colour in the ammonical (lower) phase indicates the presence of free anthraquinones [16].

#### ***Test for Flavonoids***

0.5g of extract was dissolved in sodium hydroxide (NaOH) solution. The appearance of yellow solution that disappears on addition of HCl indicates the presence of flavonoids [15].

#### ***Test for Glycosides***

##### ***Liebermann's Test***

1ml of the organic extract was dissolved in 2 ml of chloroform and 2 ml of acetic acid was added carefully. A color change from violet to blue to green indicates the presence of a steroidal nucleus (i.e. aglycone portion of glycoside) [16].

##### ***Keller Killiani's Test***

Test solution was treated with few drops of glacial acetic acid and ferric chloride solution and mixed. Concentrated sulphuric acid was added which forms two-layers. Lower reddish brown layer and upper acetic acid layer which turns bluish green would indicate a positive result for presence of glycoside [17].

##### ***Test for Saponins (Froth test)***

5mls of distilled water was added to 0.2g of the extract and shaken vigorously. Froth (foam) that persisted for more than 10 minutes will indicate the presence of saponins [18].

##### ***Test for Steroids***

2 ml of the extract was added in 2 ml of chloroform and 2 ml of concentrated sulphuric acid. A red colour

produced in the lower chloroform layer indicates the presence of steroids [16].

#### **Test for Tannins**

10mls of distilled water was added to 0.5mls of each extract and mixed with few drops of ferric Chloride (FeCl<sub>3</sub>) solution. An immediate visible green precipitate indicates positive test for presence of tannins [19].

#### **Test for Terpenoids**

About 0.5g of the extract was dissolved in 1ml of chloroform and 1ml of concentrated H<sub>2</sub>SO<sub>4</sub> to form a layer. A reddish brown layer formed at the interface indicates positive result for presence of terpenoids [15].

#### **Fly Stock Maintenance**

The wild-type flies strain w<sup>1118</sup> were maintained in cornmeal standard media (containing agar 1.05% w/v, corn meal 7.0% w/v, glucose 7.5% w/v, distilled water 100ml, nipagin 2.33% v/v, propionic acid 0.37% v/v and dried yeast 1.5% w/v) at 25°C under 12hours light-dark cycle. The emerging flies were collected and separated by sex [20]. All experiments were conducted at 25°C under 12hours light/dark.

#### **Acute Toxicity Test**

Eight (8) different concentrations of aqueous and ethanol extracts of *Warbugiaugandensis Sprague* were prepared as 0.0952µg/100µl (equivalent to human dose), 9.52µg/100µl, 20µg/100µl (equivalent to 15g/kg), 1000µg/100µl, 10,000µg/100µl, 75,000µg/100µl, 90,000µg/100µl and 0µg/100µl (control) each with 5% glucose consistently on filter paper (2.5cm by 1cm). Having it in mind that 1 fly weighs approximately 1mg [21] and a fly eats on average a 75µg (5µg to 150µg; [22]).

For each experiment a total of 240 flies (30 flies per groups) were starved for 14 to 16 hours in empty vials, and then fed on the different concentrations of the extracts for 30 minutes. The number of deaths was counted after 1 hour of feeding [23], [10]. Three independent experiments were conducted each with three replicas; the last experiment was conducted with 120 flies (15 flies per groups) but 1fly per vial to ensure quality of the results obtained.

#### **Climbing Assay**

Aqueous and ethanol extracts of *Warbugia ugandensis Sprague* was separately prepared at dose of 0.0952µg/100µl (equivalent to human dose), 20µg/100µl (equivalent to 15g/kg), 1000µg/100µl, 10,000µg/100µl and 0µg/100µl (control) each formulated in corn meal medium as 0.0000952%, 0.02%, 1%, 10% and 0% respectively. Wild type flies were treated in 5 groups for 13 days prior to the test. Ten (10) flies from each group were transferred into empty vials, around which a horizontal line 8 cm above the bottom of the vial was drawn as a marker.

The flies were allowed to acclimatize for 10 min at room temperature. The procedure entails gently tapping the vials of the flies down to the bottom trice; then after 10 sec the number of flies that crossed the 8cm mark was recorded. Three (3) replicates was used for each group, and average values was calculated to get the mean [24]. All flies used for this assay were one day old.

#### **Longevity Assay**

Aqueous and ethanol extracts of *Warbugia ugandensis Sprague* was prepared at dose of 0.0952µg/100µl (equivalent to human dose), 20µg/100µl (equivalent to 15g/kg), 1000µg/100µl, 10,000µg/100µl and 0µg/100µl (control) each formulated in corn meal medium as 0.0000952%, 0.02%, 1%, 10% and 0% respectively, and then 10 wild type virgin flies were raised in a vials containing corn meal medium with one of the aforementioned percentage of *W. ugandensis* for 40 days. Food vial was consistently changed after every 4 days, and dead flies were counted after every 3 days [20]. Three replicates were used (1 for females and 2 for males) each containing 10 flies and all flies used were 1 to 2 days old.

## **RESULTS**

### **Compilation of Knowledge**

Results from interviewing traditional healers (compilation of knowledge) showed a high use of *Zanthoxylum Gillette* by different traditional healers to treat different ailments (Table 1). The same table showed that herpes zoster (shingles) appears to be the ailment for which there are more herbs available (40% of remedies) followed by diarrhea (20%). Leaves appear to be the most common part of plant used.

### **Percentage Yield**

The results showed slightly higher yield with ethanol extract compare to water extract of *W. ugandensis*, with loss of some crude extracts after partitioning aqueous extract with chloroform (Table 2).

### **Preliminary Phytochemical Screening Tests**

Phytochemical screening showed a high level of terpenoids and steroids in both aqueous and ethanolic extracts of *W. ugandensis*, which persist even after partitioning aqueous extract with chloroform (Table 3). Saponins were lost after partitioning aqueous extract with chloroform. And test for glycoside was positive with Keller killiani's test and negative with Lieberman's test.

### **Acute Toxicity**

Acute toxicity tests for both aqueous and ethanolic extracts of *W. ugandensis* showed that the plant is not toxic at acute exposure (Figure 1).

### **Climbing Assay**

Climbing assay for aqueous and ethanolic extracts of *W. ugandensis* showed a significant difference

between control and flies treated with 100mg/ml (10%) of both aqueous and ethanolic extracts of *W.*

*ugandensis* with  $P < 0.05$  and  $P < 0.01$  (Figure 2) respectively, calculated using T-test

**Table 1:** Medicinal plants used by the traditional healers of Rukarwe in the management of opportunistic infections in HIV-positive patients

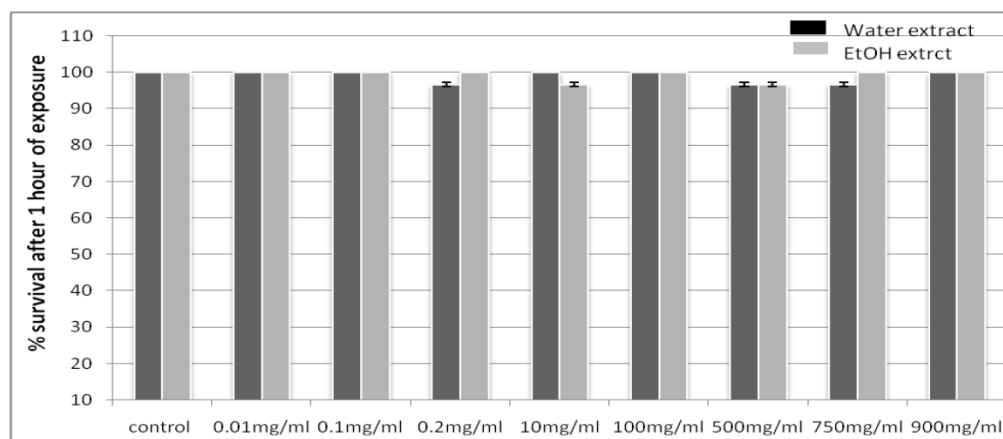
Sr.	Scientific name	Local name (rnyankole)	Part(s) used	Indication	Route
1	<i>Zanthoxylum Gillette</i>	Omutatembwa	Bark	Anorexia	Oral
2	<i>Aloe vera</i>	Rukaka	Leaves	Malaria	Oral
3	<i>Phytolaccadodecandra</i>	Omuhoko	Leaves	Herpes zoster	Topical
4	<i>Sesbania sesban</i>	Omungyeganyegye	Leaves	Herpes zoster	Oral
5	<i>Zanthoxylum Gillette</i>	Omutatembwa	Bark	Oral thrush	Oral
6	<i>Warbugia ugandensis</i>	Omwiha	Leaves	Herpes zoster	Oral
7	<i>Mangifera indica</i>	Omuyemba	Leaves /Bark	Cough	Oral
8	<i>Psidium guajava</i>	Ampera	Leaves	Diarrhea	Oral
9	<i>Phytolaccadodecandra</i>	Omuhoko	Seeds /Leaves	Herpes zoster	Topical

**Table 2:** Results of percentage yield of four different extracts of *W. ugandensis*

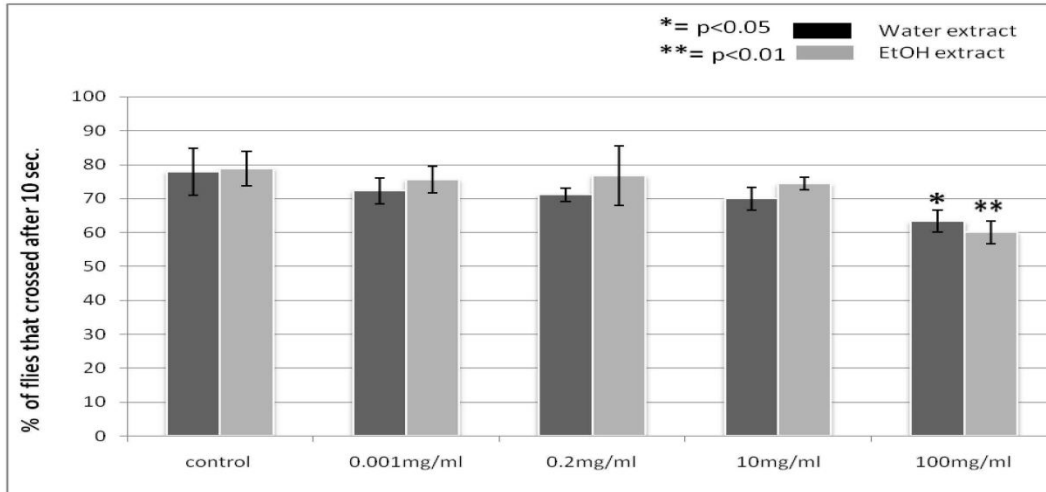
Medicinal herb and solvent used	Initial powder used (g)	Percentage yield (%)
<i>W. ugandensis</i> , water extract	300g	7.3%
<i>W. ugandensis</i> , ethanol extract	250g	7.6%
<i>W. ugandensis</i> , water-chloroform partitioning (water part)	487g	5.0%
<i>W. ugandensis</i> , water-chloroform partitioning (chloroform part)	487g	1.5%

**Table 3:** Preliminary phytochemical screening tests of 4 extracts of *W. ugandensis*, (+) showing positive test and (–) showing negative test

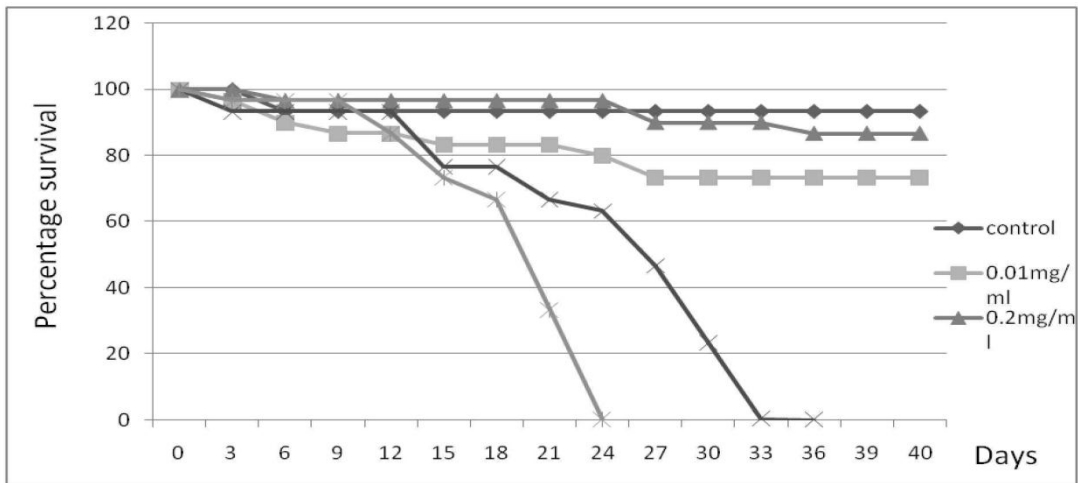
Phytochemical tests	Aqueous extract	Ethanol extract	Aqueous—Chloroform partitioning (H <sub>2</sub> O extract)	Aqueous—Chloroform partitioning (CHCl <sub>3</sub> extract)
Alkaloid	–	+	–	–
Antraquinones	–	–	–	–
Flavonoids	+	–	+	–
Glycosides				
Keller killiani's test	+	+	+	+
Lieberman's test	–	–	–	–
Saponins	+	+	–	–
Steroids	+	+	+	+
Tannins	+	+	+	–
Terpenoids	+	+	+	+



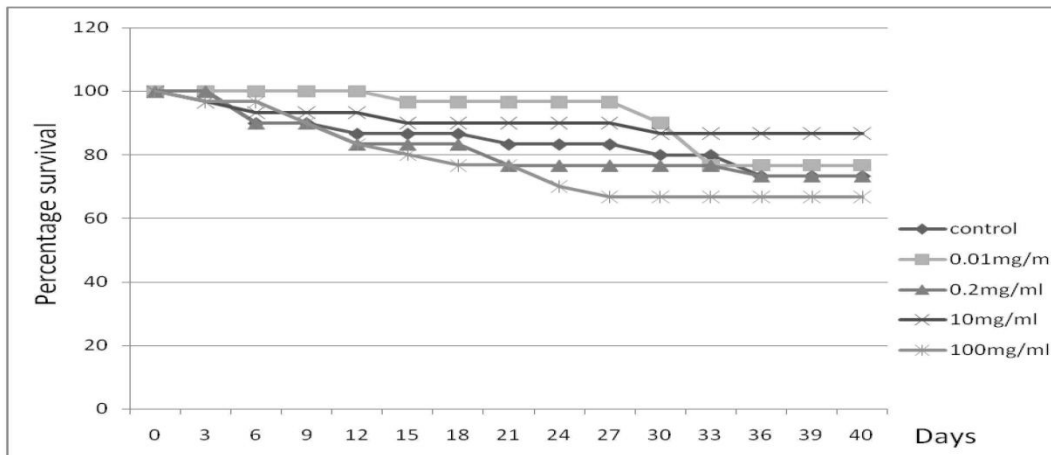
**Figure 1:** Percentage survival of *D. melanogaster* after one (1) hour of exposure to different concentrations of aqueous and ethanolic extracts of *W. ugandensis* (Standard deviation of 0 to 0.57)



**Figure 2:** Percentage of flies that crossed 8cm after 13 days treatment with different concentrations of aqueous extract of *W. ugandensis*



**Figure 3:** Percentage survival after 40 days of exposure to different concentrations of ethanolic extract of *W. ugandensis*



**Figure 4:** Percentage survival after 40 days of exposure to different concentrations of aqueous extract of *W. ugandensis*

## LONGEVITY ASSAY

Longevity study showed toxicity of ethanolic extract of *W. ugandensis* at high dose (**Figure 3**) and relatively safety of its aqueous counterpart at equipotent doses (**Figure 4**).

## DISCUSSION

After compilation of healers' knowledge the results showed that remedies to treat herpes zoster (shingles) appear to be the commonest and oral dosage is usually prepared and prescribed in 80% of cases, followed by topical formulations with 20%. The routine use of oral and topical formulations partly explained the lack of technical know-how by the traditional healers to produce parenterals and other advance formulations, also give an insight onto their attempt to reduce production and product costs, since it is the only main source of health care in rural settings [2].

Percentage yield results (**Table 2**), shows loss of some crude extracts after partitioning aqueous extract with chloroform due to formation of three phased system (aqueous, chloroform and residues) after partitioning, middle layer being residue of clotting materials.

Preliminary phytochemical screening (**Table 3**), showed a high level of terpenoids and steroids in both extracts. The presence of the two metabolites together is due to similar metabolic pathway of their synthesis from Acetyl CoA+AcetoacetylCoA to Squalene (C<sub>30</sub>), although their quantities can differ [15]. Saponins were lost after partitioning aqueous extract with chloroform due to their inherent nature to form froth in mostly in water but not in chloroform

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and other organic solvents. Test for glycoside was positive with Keller killiani's test and negative with Lieberman's test probably due to the absence of the specific class of glycosides that react to Lieberman's test.

Acute toxicity is the adverse effects of a substance seen after an exposure or multiple exposures within a short term interval [25], which showed to be non-toxic after one hour of exposure with standard deviation of 0 to 0.57. This shows that the local irritation caused by this plant does not really reflect same systemically. The results create a room for another question, which is whether the extracts are toxic on chronic exposure or not. Which was answered by climbing assay and longevity study.

Climbing assay provides behavioral measure to determine motor activity, neurological damage and aging in *Drosophila* [26]. Climbing assay showed a significant difference between control and flies treated with 100mg/ml (10%) of both aqueous and ethanolic extracts of *W. ugandensis* with P<0.05 and P<0.01 respectively (Figure 2). The cause for significant decreased in negative geotaxis activity is not established in this study.

## CONCLUSION

My study provides some basic information on toxicity (acute and chronic) of commonly prescribed herb by Rukararwe traditional healers to treat herpes zoster. It appears to be non toxic at acute exposure and toxic at higher dose on chronic exposure.

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