PyrexJournals

Pyrex Journal of Medicinal Plant Research
Vol 1 (3) pp. 010-018 December, 2015
http://www.pyrexjournals.org/pjmpr

Full Length Research Paper

Brine Shrimp Lethality and Acute Oral Toxicity of Commiphora swynertonii (Burrt) Exudate

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Accepted 24th November, 2015

Commiphora swynertonii **exudate is used by people in Central and Northern Tanzania for treatment of various ailments, including upper respiratory infection and urinary tract infections, as well as anthelminthic and insecticide. Its antimicrobial and acaricidal activities have been demonstrated, but its toxicity has not been studied. The aim of the study was to determine its toxicity using brine shrimp test and oral administration of various doses of the exudates. Brine shrimp nauplii were subjected to various concentrations of the exudates in simulated sea water and by noting the mortalities against each concentration, the median lethal concentration (LC50) was determined using Fig P computer program. As for acute toxicity test, mice were administered with various doses of the exudates by gastric intubation and observed for signs of physical and behavioral changes as compared to mice which were given a placebo. After a 14-day observation period, the mice were sacrificed, and their internal vital organs (heart, liver, lungs, kidneys and spleen) checked/examined for weights / histological changes as compared to the mice which were given placebo. The Brine Shrimp test exhibited moderate toxicity of LC50=15.30 µg/mL. In Acute Toxicity test, no changes in mice were observed at doses below 2000 mg/kg body weight. Some behavioral and physical changes were observed at 3000 mg/kg and some mice died at 4000 mg/kg. The mouse which was dosed 5000 mg/kg died after 3 hours. The LD50 was 3400mg/kg. Histological analysis revealed no significant changes below 2000mg/kg doses. But at 3000 mg/kg and beyond, major changes, including organ degeneration, vasocongestion and behavioral changes began to manifest. Liver and lungs were severely affected at 4000 mg/kg dose and pneumonitis was advanced as the cause of death for the mice that died. We conclude that at doses below 2000 mg /kg, CS exudate is relatively safe. We also recommend further studies to be carried out on CS exudates for anticancer activity.**

Keywords: *Commiphora swynetonii*, Brine shrimp lethality, acute toxicity test, Histological analysis.

INTRODUCTION

Use of plants for medicinal purposes is indubitable and ubiquitous. The World Health Organization (WHO) estimates that 80% of the population in developing countries continues to use primarily traditional medicines based on plants (WHO, 1991). Plants can be used directly or as a source of modern medicines. At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants (Askew, 2002). Examples of medicines directly extracted from plants include quinine from Cinchona, Morphine from poppy and dioxin from foxglove (Meskin, 2002). Heroine, acetyl salicylic acid, aminophyline and ephedrine are chemicals extracted from plants that have been modified chemically to improve potency or reduce adverse reactions. *Commiphora swynertonii* (CS) exudate tapped from the stem and branch barks is used traditionally for treatment of a multitude of diseases by many tribes in Tanzania and Kenya (Kalala *et al.,* 2014). Some bioactivities of this plant has been studied and proved. These include Tick repellency (Bakari *et al.,* 2012), Antibacterial and antifungal (Mkangara *et al.,* 2012) as well as acaricidal activity (Kalala *et al.,* 2014).

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Although people who utilize traditional plant based medicines have faith in their pharmacology, little is thought of regarding toxicity. The general belief is that plant based medicines are safe and free from toxicity when used in the "usual" dose (Atsamo *et al.,* 2011). However, information on the toxicity of any chemical or material intended for medicinal use is paramount and serves as a baseline for further exploration to establish it as a new herbal medicine (Saha *et al.,* 2011). In the same context, toxicity of CS exudate has hitherto not been explored. The objective of this study was to test for the acute toxicity of CS exudate so as to validate its use in an effective and safe way. Brine shrimp test (BST) and Oral acute toxicity tests were performed to achieve this objective. BST, developed by Meyer *et al.,*1982, is a simple tool to guide screening active plant extracts, where one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. BST reflects a good correlation with cytotoxicity (McLaughlin (1991) and bioactivity of a given compound or material (Sorgeloos, 1978).

Acute toxicity has been defined by the organization for economic corporation and development (OECD) panel of experts as "the adverse effect occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hrs". The purpose of toxicity testing is to provide evidence concerning the toxicological properties of plant extracts, products, chemicals and commercial products, so as to decide whether they are safe for use and to establish the safe limits in conditions of use. This process is called hazard evaluation. When information on the cumulative toxicity of a substance in the body is needed, sub-acute toxicity is tested. This is the advance effects occurring as a result of the repeated daily oral dosing of a chemical to experimental animal for part of their life span usually not exceeding 10% (Lee and Dixon, 1978) which can range from 6 days to 6 months depending on the lifespan of the animal in question.

MATERIALS AND METHODS

Study design

This was a matched case-control study where cases included mice exposed to a plant exudate and controls given a placebo (0.01% Tween 80 in distilled water) and these were observed for clinical and morphological evidence of toxicity. These mice were matched for age, sex and body weight.

Study site

The Institute of Traditional Medicine (ITM), The Laboratory Animal House as well as the Departments of Pharmacognosy and Pathology at the Muhimbili University of Health and Allied Sciences (MUHAS), Dar es Salaam, Tanzania.

Collection of *C.swynertoniibark* exudate

The exudate of CS bark was collected in June 2015 from Kitwai village in Simanjiro District, Arusha region in Tanzania (4°00′ 00″S and 36°30 ′ 00″ E; 1,455 m above sea level). This was a dry season in which local residents believe the abundant availability of the exudate. The plant was positively identified as *Commiphora swynertonii* by John Elia, a botanist from the National herbarium of Tanzania and its voucher specimen no. WK01 is deposited in the National herbarium in Arusha City. The exudate was collected by incising stem and branch and barks with a sharp knife and collecting the oozing exudate in a clean bottle. Each tree can generate about 100 mL of the exudate.

Preparation of the exudate

CS exudate was used in its undiluted form and in a 10% emulsion. The exudate is practically insoluble in water.In order to dilute it; a water-in-oil emulsion was made using 0.01% Tween 80 as an emulsifying agent. The most stable emulsion was 10% v/v in water. To make an emulsion 0.01 g of Tween 80 was dissolved in 90 mLs of water. Then 10 mL of the exudate was added and the mixture was blended at high speed using Kenwood blender model SB 054. A milky white emulsion resulted and was kept in a well stoppered glass bottle.

Brine Shrimp Lethality Test (BST)

BST was done as a monitor for bioactivity and as a predictor for the presence of cytotoxic activity in the exudate. The medium in which the test was conducted was artificial sea water, made by dissolving 38 g of crude salt in 1 L of distilled water. The crude salt was obtained by evaporating actual sea water collected from a reasonably clean sea shore of Dar es Salaam. Artificial sea water was introduced in a Thin-Layer Chromatography (TLC) tank (size 10cm x 20 cm) which was covered by a thin manila paper on one side while the other side was not covered. A lamp was placed above the open side of the tank to attract the shrimps that will hatch close to the tank wall. Brine shrimp eggs, *Artemia salina* were introduced into the vessel and left for 48 h at room temperature to allow hatching of eggs into nauplii. The nauplii were collected on the bright side of the vessel (near the light source) by using micropipette and placed in a Petri dish containing artificial sea water for ease of counting them for further procedure.

BST assay was conducted by preparing 10 bijoux bottles filled with2 mL of artificial seawater each and a twofold dilution were set up to yield a series of concentrations of the CS exudate. From the 10 mg/mL of the prepared CS exudate emulsion, a 10-fold dilution was made to obtain 1mg/ml emulsion. Then from this, 30, 15, 10, 5, and 3 µL were drawn and filled in the bijoux bottles above in duplicate and each bottle was filled with more artificial sea water to 5 mL. By calculation the bijoux bottles now contained 60, 30, 20, 10 and 6 µg/mL of the exudate in pairs. Potassium dichromate was dissolved in artificial seawater and functioned as a positive control with concentrations ranging from 0.1 to 0.9 mg/mL. 10 nauplii were withdrawn from the Petri dish and introduced in each bijoux bottle and incubated at room temperature for 24 hours. The bottles were observed, and the dead larvae from each bottle were counted after 24 h. Based on the percentage of the mortality, the concentration that led 50% lethality (LC50) to the nauplii was determined by using the graph of mean percentage mortality versus the log of concentration (WHO, 2007).

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

Acute Oral Toxicity Test

The experiment was conducted on 30 healthy Swiss albino mice weighing 20 to 25 g and aged 8 to 10 weeks, acquired from the Animal House at the Muhimbili University of Health and Allied Sciences (MUHAS). The mice were selected at random and brought into the laboratory from the animal house

72 hours prior to the start of the experiment to allow them to acclimatize to the laboratory environment. Drinking water and food were provided *ad libitum* throughout the experiment, except for the short fasting period where the drinking water was still in free access but no food supply was provided 6 hours prior to treatment. Following a 6 hour fasting period, at random again six mice for each dose were chosen, weighed and given a special mark on their tails for individual identification and kept in their own cage. Each cage measured 7 × 27 × 14 cm. All of the cages were located in a room at a temperature approximately 23 °C with constant humidity and cycles of 12 h of light and 12 h of darkness per day. The acute oral toxicity of CS exudate was evaluated in mice, according to the procedures outlined by the OECD, 2001 and Wallum *et al.,* 1995. The dose for each mouse was calculated in reference to the body weight. The volume administered to a mouse should not exceed 10mL/kg. Thus, where the calculated dose exceeded this mark, a crude exudate was administered in place of the emulsion. A single high dose of 2000 mg/kg of the exudate emulsion and 3000, 3500, 4000 and 4500 mg/kg of crude exudate was administered to mice through the oral route. Other six male mice were allotted 0.01% Tween 80 in distilled water and were regarded as the control groups receiving a placebo. Food was provided to the mice approximately an hour after treatment. The mice were observed in detail for any indications of toxicity effect within the first six hours after the treatment period, and daily further for a period of 14 days. Visual observations of mortality, signs of injury and or pain, weakness or aggressiveness, food refusal, loss of weight, diarrhea, discharge from eyes and ears, noisy breathing if any were observed. Change in behavioral pattern (Compulsive behavior, excitability, apathy or depression), changes in physical appearance (reflexes, pruritus, circling, locomotor behavior) and signs of illness (moribund, ataxia and apathy) were conducted daily during the period. On the 15th day all mice were weighed again and sacrificed for histological analysis. Their vital internal organs including heart, liver, kidney, spleen and lungs were weighed in order to determine Organ-to-body weight index which was calculated as follows:

$$\text{OBWI} = \text{Organ-to-body weight Index} = \frac{\text{organ weight}}{\text{bodyweight}} \times 100$$

Biopsies collection and evaluation

After sacrificing the mice, vital organs such as heart, kidneys, liver, lung and spleen were isolated, weighed and examined for macroscopic and microscopic changes and/or development of any lesions as previously described (Dammeyer *et al.,* 2009). All of the individual organs were weighed and their features were compared between both treated and control groups and these were histologically evaluated by the Department of Pathology at MUHAS.

Tissue processing, histological staining, microscopic evaluation and photomicrography

After macroscopic evaluation (grossing), tissues from the mice were fixed for 24 hours in neutral well-buffered (40%) formalin, embedded in paraffin and sections (5 µm) mounted on SuperFrost slides (Menzel GmbH & CoKG, Braunschweig, Germany) as previously described (Dammeyer *et al.,* 2009, Mwakigonja *et al.,* 2007). These were then deparaffinized, rehydrated and stained with haematoxylin and eosin (H&E) as previously described (Mwakigonja *et al.,* 2007). Histological evaluation and photomicrography was performed by the Histopathologist (Amos Mwakigonja) using an Olympus (CX31RBSF Model) light microscope equipped with a digital camera (Olympus Corporation, Tokyo, Japan). Tissue toxicity (damage) was evaluated under the microscope on 7 low-power fields (x10 magnification) as well as on their high-power fields (x40 magnification) while taking pictures. Picture processing and printing was performed using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA) and Microsoft-Power Point 2003 (Microsoft Corporation, Redmond, WA, USA) as previously described (Dammeyer *et al.,* 2009, Mwakigonja *et al.,* 2007).

Statistical analysis

The LC_{50} in BST was found from the regression equations obtained from a plot of the Logarithm of concentration of the exudates against brine shrimp lethality generated using Fig P computer program (Biosoft Inc, USA).Confidence intervals (95% CI) were calculated according to a previously reported method (Litchfield and Wilcoxon, 1949). In acute toxicity tests, statistical analysis to assess the significant difference between groups in weights and organ to body indices was conducted by running the student's t- test using SPSS (SPSS Inc., Chicago Ill) as well as Microsoft Excel spreadsheet application. The level of significance used in this analysis was 5%. The Fisher exact test was used were numbers were small.

Ethical Considerations

The protocol of the study was approved by the Ethics Committee of MUHAS and the use of mice in acute toxicity study followed guidelines for use of animals in experiments as adopted from internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC).

Figure 1: Mortality rate % of *Artemisia salina* nauplii at 24 hour after exposure to various concentrations of CS exudate

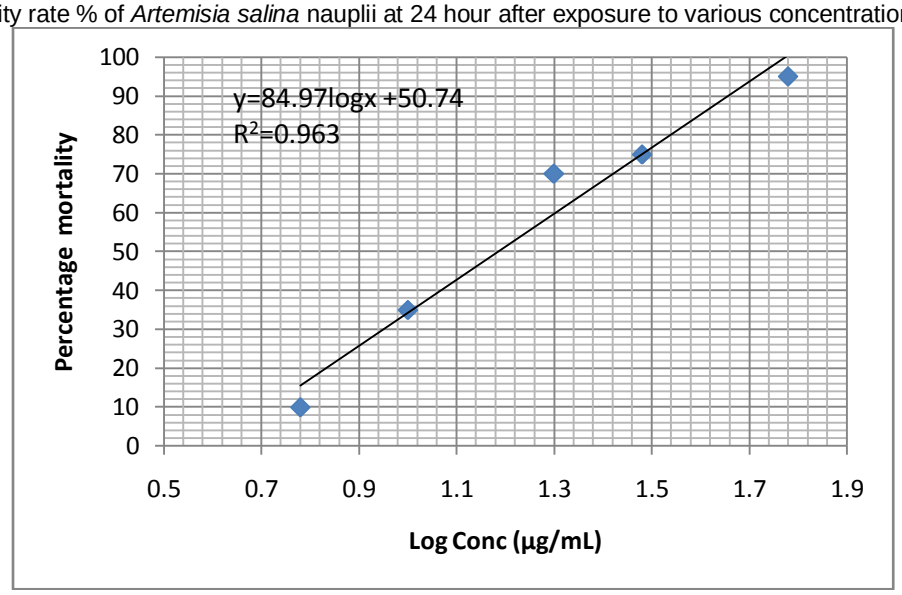

Figure 2: Dose-dependent mortality rate of mice after administration of CS exudate

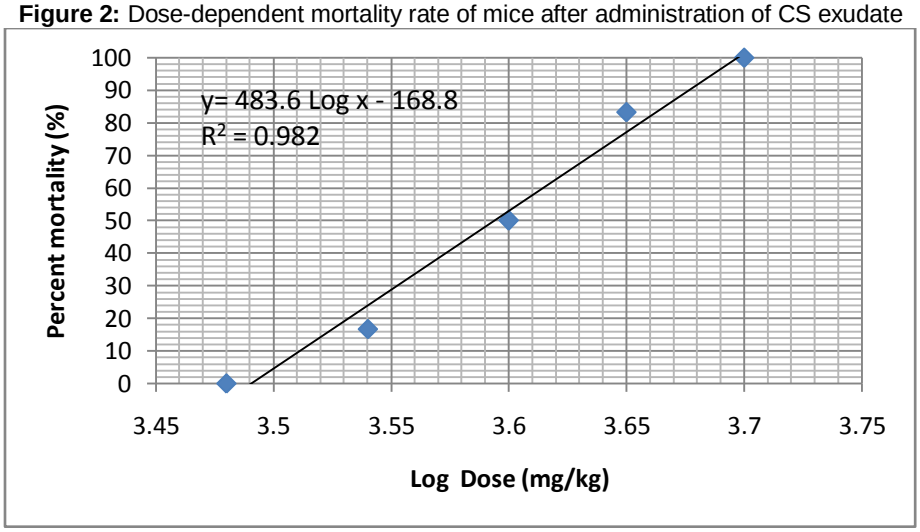

Figure 3: Variation of Organ-to-body weight Index of Mice in The Acute Toxicity Study of *C swwynertonii* exudate

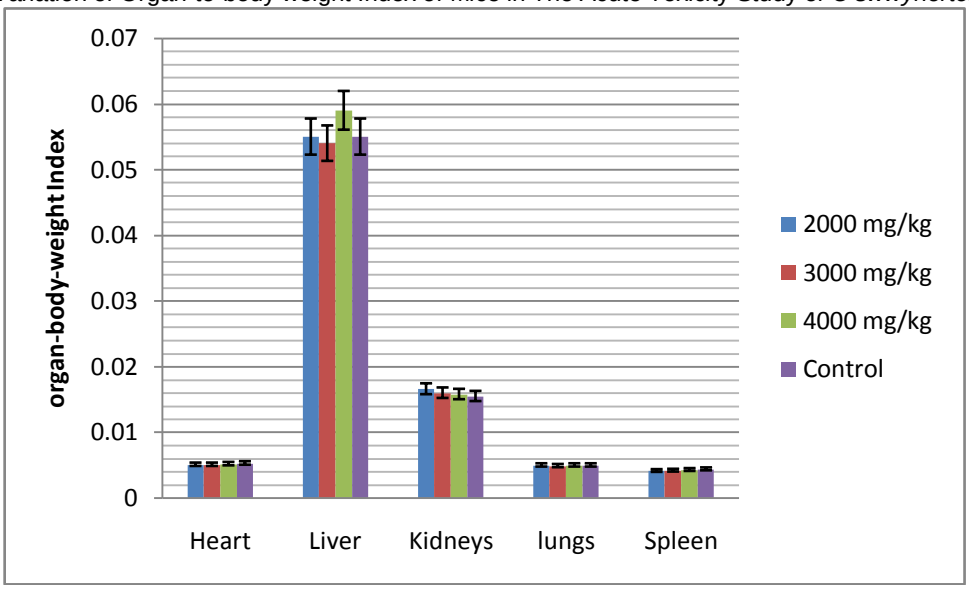

Table 1: General clinical appearance and behavioral observations for control and treated groups

General macroscopic and behavioral changes	2000mg/kg	3000- 3500 mg/kg	≥4000mg/kg	**Control**
Skin and fur Eyes Behavioral patterns Salivation Lethargy Sleep Diarrhea Coma Tremors	Mice showed discomfort soon after administration of the exudate, but immediately recovered and within 30 minutes they did not show anything abnormal in terms of behavior, breathing, sensory nervous system or gastrointestinal products (salivation and defecation). No other abnormalities were observed and all survived 14 day testing.	At 3000 mg/kg Mice exhibited lethargy, restricted their movements for a few moments (10-15 minutes) and exhibited hyperventilation. Soon after they recovered and started feeding and drinking normally. No other sensory, motor, behavioral or gastrointestinal effects were observed. No death was observed within 14 days. At 3500mg /kg dose mice were hyperexcited for about half an hour, then went into depression. One mouse died on the 7th day. All other mice survived the 14 days observation period.	At 4000 mg/kg the mice immediately became hyper-excited and jumpy and their fur appeared wet as if they were sweating. 3 mice developed pilo-erection within one hour. 1 mouse died within 12 hours, two mice died after 72 hours. 3 mice survived 14 days. At 4500 mg kg only one mouse survived after 14 days. Only one mouse was administered dose of 5000mg/kg body wt. This mouse was immediately hyperactive, jumpy, showing some tremors and died within three hours after administration. There was no further indication to dose another mouse	Mice did not show any change in behavior and they survived the 14 days of testing

Table 2: Histological analysis of internal organs after administration of test drug at different doses, and control (no-drug)

Organ	**Control (No Drug)**	**Test Drug**	**Test Drug**	**Test Drug**
	Tween 80	**Dose = 2000 mg/kg**	**Dose =3000 mg/kg**	**Dose = 4000 mg/Kg**
Heart (Figure 4a)	Normal Histology	Slight haemorrhage and mild myocardial degeneration. No inflammation.	Mild-moderate myocarditis and myocardial degeneration	More severe myocarditis and myocardial degeneration
Kidney (Figure 4b)	Normal Histology	Vasocongestion and thrombosis without glomerular injury and without tubulo-interstitial inflammation	Increased dilated tubules, cylinders, vasocongestion and thrombosis as well as glomerular thrombosis and focal tubulo-interstitial inflammation	Increased dilated tubules, cylinders, more severe vasocongestion and thrombosis as well as glomerular thrombosis and focal tubulo-interstitial inflammation
Liver (Figure 4c)	Normal Histology	No significant changes from normal	Sinusoidally dilated veins including vasocongestion and minimal inflammation	More prominent and widespread sinusoidally dilated veins including vasocongestion and minimal inflammation
Spleen (Figure 4d)	Normal Histology	Mild expansion of red pulp, mild shrinking of the white pulp, follicularization of the white pulp	Moderate expansion of red pulp, disorganization of the white pulp, sinus histiocytosis	Severe expansion of red pulp, disorganization and dispersal of the white pulp, Increased sinus histiocytosis
Lungs (Figure 4e)	Normal Histology	Mild to moderate pneumonitis/ consolidation	Severe pneumonitis (severe consolidation) including vasocongestion, thrombosis and haemorrhage	Severe pneumonitis (severe consolidation) including more prominent vasocongestion, thrombosis and haemorrhage

RESULTS

The results of BST are presented in the plot which was generated using Fig P computer program (BiosoftInc, USA) as depicted in Figure 1. The median lethal concentration (LC_{50}) in Brine shrimps was 15.30 µg/mL (11.86 -19.74 µg/ml at 95% confidence) at 24 hour exposure. The acute toxicity test showed that, the exudate was very well tolerated by mice after gastric intubation. At 2000 mg per kg and below this dose, the mice did not show any significant change in behavior, sensory, gastrointestinal or breathing activities. However, when the dose was increased to 3000 mg /Kg and 3500 mg/kg some gross changes were observed for a few moments but recovered afterwards. No deaths were recorded at 3000 mg/kg but one mouse died seventh day at 3500 mg/kg dose. At 4000 mg/Kg and beyond, major behavioral manifestations were observed and 3 mice died within four days. At 4500 mg /kg a total of 5 mice died. Table 1 gives a summary of observed behavior and appearance in control (placebo group) and at different doses. The mouse that was administered 5000 mg/kg died immediately and for this reason there was no indication to administer such a dose to other mice. A plot of percent mortality against the logarithm of concentration of the exudates (figure 2), generated using Fig P program, and subsequent calculations revealed that, the median lethal dose (LD_{50}) was 3614.01 mg/kg.

After the 14thday post-dose administration period, the animals were weighed and thereafter sacrificed, their vital organs carefully removed and weighed. For each dose, the Organ-to-body weight Index (OBWI) which is the relative weight of each organ relative to the body weight of the mouse was found according to the methodology above. The OBWI of each organ is illustrated in Figure 3. There was no significant difference in the changes of the respective weights in all organs at all doses except the liver, who's OBWI was higher at 4000 mg/kg dose. After measuring OBWI, the organs were analyzed histologically, Table 2 summarizes the observation of histological analysis of the organs at various doses. Below 2000 mg/kg there were no observable changes between test animals and controls. At 2000 mg/kg and above changes began to be observed as narrated in Table 2. Diagrammatically, the histology of vital organs after administration of respective doses in comparison with the placebo is presented in Figure 4 (a-e). In the figures low power microscopy means x100 objective and high power means x400.

DISCUSSION

LC50 of CS exudates in BST was 15.30 µg/ml. It has been interpreted that when LC50<1.0 µg/ml, the substance is highly toxic; LC_{50} 1.0-10.0 µg/ml the substance is classified as toxic; LC_{50} between 10.0-30.0 µg/ml it is moderately toxic; LC50>30 <100 µg/ml – mildly toxic, and > 100µg/ml a substance is non-toxic (Moshi et al. 2010). Thus, CS exudate is moderately toxic according to this classification. It has also been demonstrated that BST correlates well with several biological activites including pesticidal and cytotoxic activities (mcLaughlin 1991), screening for natural toxins (Harwig, 1971), and anticancer activity (McLaughlin and Rodgers (1998). Indeed CS exudates have been found to have a very good acaricidal activity against common ticks in Tanzania (Kalala et al 2014). This fact also makes the exudate a potential candidate for anticancer activity study. In the acute oral toxicity test, the LD_{50} was 3614 mg /kg body weight. The chemical labeling and classification of acute systemic toxicity based on oral LD_{50} values recommended by the Organization for Economic Co-operation and Development (OECD, Paris, France) are as follows: very toxic ≤ 5 mg/kg; 5 > toxic ≤ 50 mg/kg; 50 > harmful ≤ 500 mg/kg; and 500 > no label ≤ 2000 mg/kg, [OECD Guideline, 2001)].The oral LD_{50} value in this study suggests that the CS bark exudates are relatively nontoxic. These results concur with the use of this plant by traditional healers as traditional medicine.

It has been previously demonstrated that there is a good correlation between the LC_{50} of the brine shrimp lethality assay and the LD_{50} of the acute oral toxicity assay in mice (Logarto *et al.,* (2001). Brine shrimp LC_{50}< 10 µg/ml possesses LD_{50} between 100 and 1000 mg/kg; LC_{50}< 20 µg/ml possesses LD_{50} between 1000 and 2500 mg/kg, and LC_{50}> 25 µg/ml possesses LD_{50} between 2500 and 8000 mg/kg. Using BST data only we could therefore have assumed that the LD_{50} of oral acute toxicity for CS exudates is around 2500 mg/kg and that it is moderately toxic. The LD 50 extracted from a plot of Percent Mortality against Log Concentration, was actually 3614.01 (3592.4 – 3634.3 mg/kg at 95% confidence) LD_{50}. (figure 2). Histopathological analysis supported macroscopic observation. At doses below 2000 mg/kg there was no significant difference between drug and placebo treated animals. At 3000 mg/kg and above severe tissue injury began to be noticed. Furthermore, histopathological analysis revealed that the test drug caused a dose dependent tissue injury in all the vital organs; although this was minimal if at all, for the liver. The control cases did not show any tissue injury and this was also expected. This tissue injury was generally minimal (if at all) at the dose of 2000mg/kg. The severe tissue injury was seen at very high test doses of 3000mg/kg, 3500 mg/kg and 4000mg/kg. This tissue injury corresponds well to the observed clinical and behavioral changes listed in table 2 above. Of all organs studied, lungs were the most severely affected organs while liver was the least affected organ implying that the drug tends to be pneumotoxic at high doses, and it does not seem to be hepatotoxic at all doses. Therefore, these clinical, behavioral as well as histomorphological changes at the high test doses seem to be minimal (if any) at "normal therapeutic levels." implying that consequently, the test drug might be well tolerated.

It is important to note that the animals may appear well physically, but changes could be realized histologically. These results should caution us that although physical or macroscopical observation reveals no abnormality, close microscopical analysis histologically exhibits looming toxicity and clearly caution against the use of high dose albeit not killing the animals.

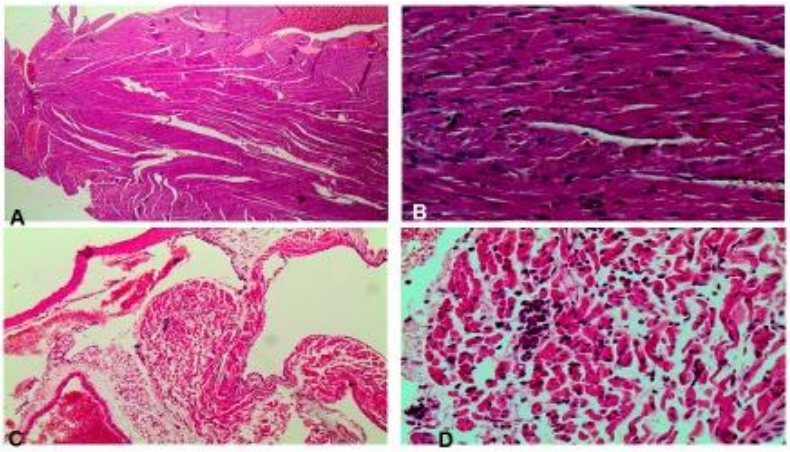

Figure 4a: Histology of the Heart (A)Control: Normal, low power;(B)Control, Normal high power; (C) Dose 4000 mg/kg Myocarditis and myocardial degeneration, low power, (D) Myocarditis and myocardial degenaration, high power

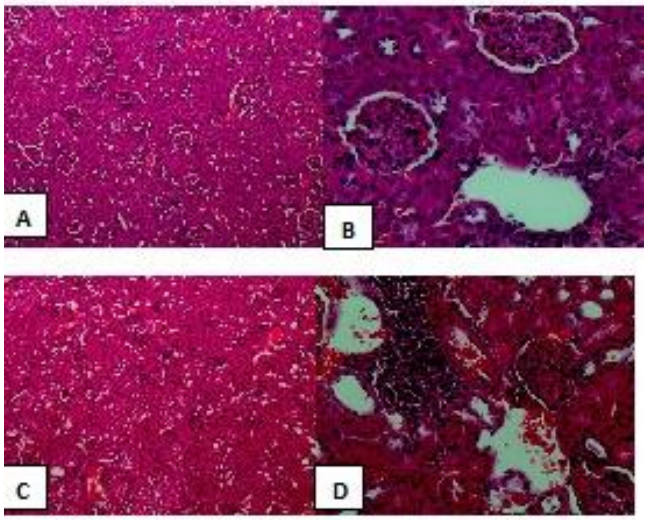

Figure 4b: Histology of the Kidneys (A) Control Normal, low power, (B) Control Normal, high power, (C) 4000mg/kg dose, increased number of dilated tubules, vasocongestion, (D) 4000 mg /kg vasocongestion, glomerular thrombosis, tubulo-interstitial inflammation

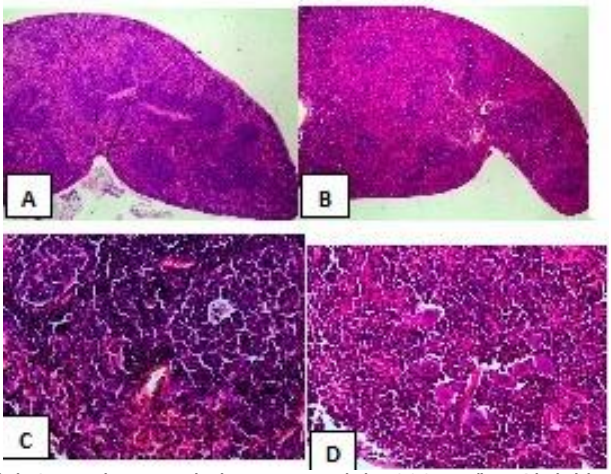

Figure 4c: Histology of the Spleen. (A) Control: Normal , low power. (B) 4000 mg/kg: Shrinking white pulp, expanding red pulp; (C) 2000 mg/kg Follicularization of white pulp, high power, (D) 4000 mg/kg: Expanded white pulp, histiocytosi

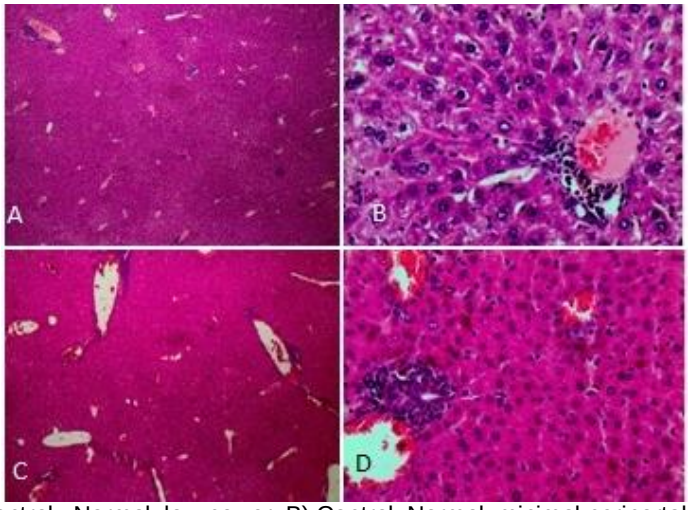

Figure 4d: Histology of Liver. A) Control: Normal, low power; B) Control: Normal, minimal periportal inflammation, high power; C) 4000 mg/kg Sinusoidally dilated portal veins, congestion; D) 4000 mg/kg mild inflammation

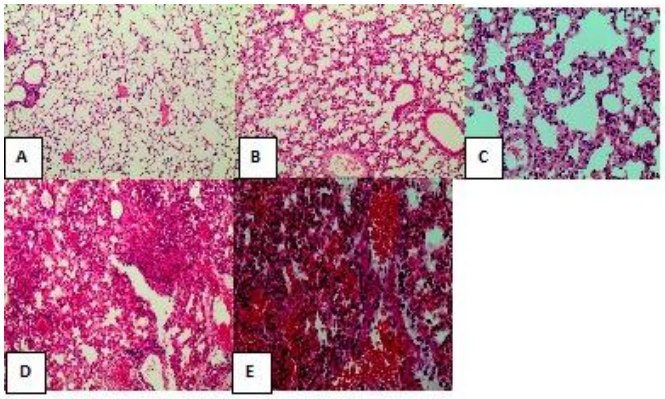

Figure 4e: Histology of Lungs: (A) Control, Low power,(B) 2000 mg/kg: Mild-moderate pneumonitis, low power, (C) Mild-moderate pneumonitis, high power, (D) 4000 mg/kg: Severe consolidation, low power, (E) 4000mg/kg: Severe consolidation, high power.

CONCLUSION

Our results from BST and Acute toxicity studies show that, the CS exudate is well tolerated at ≤ 2000 mg/kg doses since there were no notable behavioral and physical changes except for the minimal histomorphological changes observed in a few mice. Below 3000 mg /kg there were no death observed up to 14 days of the study period. The LC_{50} for BST and the LD50 for acute toxicity studies were 15.30 µg /ml and 3614.01 mg/kg respectively. Severe tissue injury and deaths were observed at high doses of ≥ 3000 mg/kg which are therapeutically not applicable. From the BST results, the CS exudate is a good candidate for further studies aiming at anti-cancer activity.

ACKNOWLEDGEMENT

We are thankful to the Muhimbili University of Health and Allied Sciences (MUHAS) and the staff for the greatly needed assistance during the study. More importantly, we thank the Swedish International Development Cooperation Agency (SIDA) for funding the study.

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