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Safety of *Prosopis juliflora* (Sw.) DC. (*Fabaceae*) and *Entada leptostachya* Harms (*Leguminosae*) Extract Mixtures Using Wistar Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author DK designed the study, wrote the protocol, performed the statistical analysis and wrote the first draft of the manuscript. Author PGK supervised and managed all aspects of the study and review of the draft. Authors HLK and FKN managed the animal welfare aspect including pathology. Author GCN managed the analytical and biochemical analyses. Authors PG and GM managed all information on natural products chemistry. Authors JMK and KM helped in the practical aspect of the study and also contributed in review of the manuscript from time to time. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Many medicinal plants have been used traditionally in treating ailments in humans and animals. However, for most of herbal remedies, no scientific toxicity profiles exist in literature. In this study, the safety profile of an herbal extract mixture containing *Entada leptostachya* (EL) and *Prosopis juliflora* (PJ) was determined using acute oral toxicity tests using adult female Wistar albino rats.
Place and Duration of Study: Laboratories in the departments of Chemistry, Zoology, Botany and Biochemistry of Jomo Kenyatta University of Agriculture and Technology (J.K.U.A.T.) between March 2012 and April 2012.
Methodology: The OECD 425 guidelines (Up-and-Down procedure) were followed. Different dosages (control, 175, 550, 1750 and 5000 mg/kg body weight) were used in the experiment. Selective observations and analysis were made and recorded on mortality, signs of pain or distress and moribund animals, biochemical and macroscopic (pathological, organ and live body weights) analyses.
Results: During the entire period of the study, no signs of pain or enduring distress were observed neither was there any mortality. Alanine aminotransferase (ALT) values were within range (for experimental rats) apart from the rat in control while Aspartate aminotransferase (AST) values were within range (for experimental rat) apart from two rats in the upper limit. Macroscopic organ observations did not show colour or texture consistent with drug-induced inflammation or lesions. The toxicity studies of the extract mixture showed that the median lethal dose (LD_{50}) was above the upper limit of 5000mg/kg body weight.
Conclusion: In conclusion, the LD_{50} of the extract mixture was found to be greater than 5000 mg/kg body weight and was, therefore, considered safe and has potential as a novel herbal preparation.

Keywords: Toxicity; Prosopis juliflora; alanine aminotransferase; LD_{50}; upper limit.

1. INTRODUCTION

Herbs and herbal formulations as medicinal alternatives have continued to receive increased attention because of strong belief that they are safe. It is this assumption, to a larger extent, that has influenced the indiscriminate use of herbal formulations leading to incidences of adverse effects and sometimes life-threatening conditions [1,2]. The traditional uses of any plant for medicinal purposes do not necessarily guarantee their safety, especially with regard to mutagenicity, carcinogenicity, embryotoxicity, nephrotoxicity and hepatotoxicity, especially if such effects are complex and not easily recognized by the local populations [3].

Although phytochemicals or plant secondary metabolites (PSMs) with pharmacological properties continue to play a vital role in the health management of people and animals, in most cases, not much information is known about their possible toxic effects. For this reason, alongside confirming their activities, it becomes important to investigate and confirm their toxicity profiles in order to appraise adequately their suitability for use by humans or target host animals [4]. These experiments fall into four categories: a) microorganism systems, b) mammalian cell culture systems, c) tissue preparations, and d) organ cultures [5]. The animal tests investigate the effect of a test substance on the various body systems such as respiratory, nervous, and cardiovascular systems [6]. In the context of this study, OECD guideline Up-and-Down procedure (UDP) provides a more humanely acceptable

animal use protocol. OECD's UDP uses a maximum of six animals in dose administration of test substances in a sequential manner after survival of previously dosed animal has been assured. Animal found in moribund state or showing signs of severe pain or enduring signs of severe distress are used as end-points rather than mortality [7].

Entada leptostachya is found in several parts of Kenya (Machakos, Meru, Embu and Mbeere districts) and other parts of Africa such as Somalia, Ethiopia and Tanzania. The communities in Embu and Mbeere districts of Eastern Province, Kenya use the root bark decoction to treat worms in humans and animals [8]. *Prosopis juliflora* (locally known as 'Mathenge') was introduced from Latin America into Kenya in the early 1970s [9,10] and is generally considered a noxious weed locally. This study, therefore, sought to determine the toxicity of a formulated herbal extract mixture containing the two plants in order to determine its safety as well as confirm possible application as an anthelmintic for ruminants as a way of mitigating the negative attributes of *P. Juliflora* while providing a cheaper alternative for controlling worm-infestations, especially among flocks of small holder farmers and pastoralists. For animal welfare reasons, OECD 425 guideline was used as it uses fewer animals and considers moribund conditions or severe signs of pain and distress as end-points.

2. MATERIALS AND METHODS

Standard procedures using OECD 425 guidelines (Up-and-Down procedure) were followed [7] during the toxicity studies with minor, selective additional data on biochemical analysis and relative organ weights. The main test was performed at a starting dose of 175 mg/kg b.w. as no toxicity data for the herbal mixture existed in literature or was documented. Approval letter from the ethics sub-committee (ref: JKU/2/4/IPC/IP/013) was obtained from Jomo Kenyatta University of Agriculture and Technology. The herbal preparation is at the pre-patent stage hence, the ratio of the mixture could not be divulged at this stage.

2.1 Collection and Preparation of Medicinal Plant Material

Entada leptostachya root barks were collected from Embu and Mbeere areas of Kenya. *Prosopis juliflora* leaves were collected from Marigat, Baringo County, Kenya. The plant specimen were identified in the field and authenticated by a plant taxonomist from the Botany department at Jomo Kenyatta University of Technology (J.K.U.A.T.) where voucher specimens were also deposited (voucher numbers: **En-jkuat/092010** and **Pro-jkuat/092010** for *E. Leptostachya* and *P. Juliflora* respectively). The *E. Leptostachya* root and *P. Juliflora* leaf samples were sorted, cleaned using tap water to remove adhering soil and other foreign matter and air dried at room temperature with intermittent turning for about three weeks on the laboratory benches away from direct sunlight before being ground into fine powder using an electric grinder (manufactured by J.K.U.A.T. mechanical engineering department) and separately stored in air-tight plastic bags at room temperature for further use to avoid contact with moisture.

2.2 Care and Preparation of Experimental Animals

Seven (1 control) adult female Wistar albino rats, 10-12 weeks old and weighing 165-196g, were used in the study. The animals were procured from Zoology Department animal house of Kenyatta University at the age of between 7-9 weeks. The animals were left to acclimatize in their cages (bedded with wood shavings) for three weeks with *ad libitum* access to food

(standard commercial rat pellets from Unga Feeds Limited, Kenya) and tap water. Twelve hours artificial lighting and 12 hours darkness sequence was followed. The rats were uniquely marked on their tails for easy individual identification using permanent ink.

2.3 Preparation and Administration of Doses

The plant powders were weighed and separately soaked for one hour in hot, distilled water and filtered through cotton swabs using a funnel. Separate aqueous stock solutions for the two plants at various concentrations were prepared using the equation below:

$$Concentration\ (mg/ml) = \frac{Dose\ rate\ (mg/kg\ b.w.) \times Body\ weight\ (kg)}{Volume\ (ml)}$$

The rats were fasted overnight with *ad libitum* access to water and weighed prior to dosing with the extract mixture. A constant volume of 2 ml per rat based on a single dose was given by gavage using a stomach tube. A dose progression factor of half log (equivalent to 3.2) was followed with a starting dose of 175 mg/ml. Food, but not water, was withheld for a further 3 hours after dosing and any toxicity signs noted during the first 30 minutes and then hourly for the next six hours. Individual observations were made daily for the next 48 hours for any signs of pain or enduring distress, being moribund or mortality before dosing the next animal. Each animal was then observed on a daily basis for 14 days and each rat weighed once weekly and at the end of the study. The dose progression sequence and LD_{50} estimate in toxicity testing was done using AOT425statpgm (version 1) program software.

2.4 Biochemical and Macroscopic Analyses

Each animal was humanely sacrificed at the end of the study by euthanizing it using carbon dioxide gas in a closed dessicator. Fresh blood samples were obtained by cardiac puncture using a needle and syringe into blood sample tubes containing EDTA (anticoagulant) and the biochemical parameters recorded using an auto analyzer using assay kits from Roche diagnostics, GmbH, Mannheim, Germany. The lungs, spleen, kidneys and liver were immediately weighed on an electronic balance and their weights recorded.

3. RESULTS AND DISCUSSION

3.1 Clinical Toxicological Signs

Table 1 shows the effects of the drug mixture on mortality after single drug administration.

Table 1. Toxicity test data for the extract mixture

Test sequence	Animal ID	Dose (mg/kg b.w.)	Short-term result (48hrs)	Long-term result (14 days)
1	A	175	O	O
2	B	550	O	O
3	C	1750	O	O
4	D	5000	O	O
5	E	5000	O	O
6	F	5000	O	O

Key: (X = Died, O = Survived)

During the entire study, no signs of toxicity were observed after administration of a single oral dose of aqueous extract mixture. No signs of pain or enduring distress were evident during the 14 days (long-term) of observation and no mortalities were observed as seen in Table 1. The study had to be stopped when no deaths were observed after three consecutive rats were dosed at the upper bound (5000 mg/kg b.w.). The median lethal dose (LD_{50}) was, therefore, estimated at >5000 mg/kg b.w. following the stopping rule procedure. No delayed behavioural toxicity signs were observed.

3.2 Biochemical Data

Table 2 shows the drug effect on the various blood chemistry parameters. From Table 2, the creatinine values obtained from all the animals treated and the control were similar at <44.2 µmol/L which was within the normal range of between 15-61 µmol/L [6] for experimental rats. This implies that none of the rats suffered from impaired renal functioning. Creatinine blood levels rise if the kidney filtering process is deficient and this is a reliable indicator of kidney malfunction/impaired function. The alanine aminotransferase (ALT) values for all the animals apart from the control were within range of 35-80 U/L [11] for experimental rats. Low ALT levels in the control may indicate a normal healthy liver or a low/non-functioning liver which may fail to release a lot of ALT into the blood. A high level of ALT in the blood usually signifies liver cell damage [1]. ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles and pancreas [12]. Aspartate aminotransferase (AST) values were within range of 65-203 U/L for experimental rats [13] for all the rats apart from two rats in the 5000mg/kg b.w. category. AST is also a hepatic health/function indicator but may also be used to assess damage in the heart and cell necrosis of many tissues [1]. A mild elevation of AST level has been associated with liver injury or myocardial infarction. The amount of AST in the blood is directly related to the extent of the tissue damage. An AST/ALT ratio is sometimes useful in differentiating causes of tissue damage. A typical myocardial infarction gives an AST/ALT ratio greater than 1 while an AST/ALT ratio less than 1 is due to liver injury and AST/ALT of more than 2 indicates alcoholic hepatitis or cirrhosis [14]. The AST/ALT ratio (>2) for the two rats in upper-bound doses indicate possible plant extract-induced liver cirrhosis as the cause of elevated AST levels. However, macroscopic observation of the organs from the two animals did not show any form of inflammation or colour consistent with cirrhosis or abnormal texture. The AST levels were consistently higher than the ALT levels which was expected since the body cells contain more AST than ALT. Since ALT is localized mainly in cytosol of hepatocytes, this enzyme is considered a more sensitive marker of hepatocellular damage than AST [12,14,15]. Toxin-induced hepatocellular damage is caused by leakage of cytosolic enzymes out of the cells due to increase in cell permeability, membrane damage and cell necrosis [16]. Cell permeability, and hence the cellular leakage, could be caused by saponins which combine with membrane-associated sterols reducing the membrane integrity [17].

Table 2. Effect of treatment of rats with the aqueous extract mixture on biochemical parameters (n=1)

		Dose (mg/kg b.w.)					
Parameter	**Control**	**175**	**550**	**1750**	**5000**	**5000**	**5000**
Creatinine (µmol/L)	<44	<44	<44	<44	<44	<44	<44
ALT (U/L)	18.5	66.6	38.1	63.3	44.4	73.5	36.6
AST (U/L)	110	67	152	82.1	423	336	148

3.3 Changes in Organ and Live Body Weights

Figs. 1 to 2 show the drug effects on the organ weights and live body weights, respectively, of the rats during and after the study.

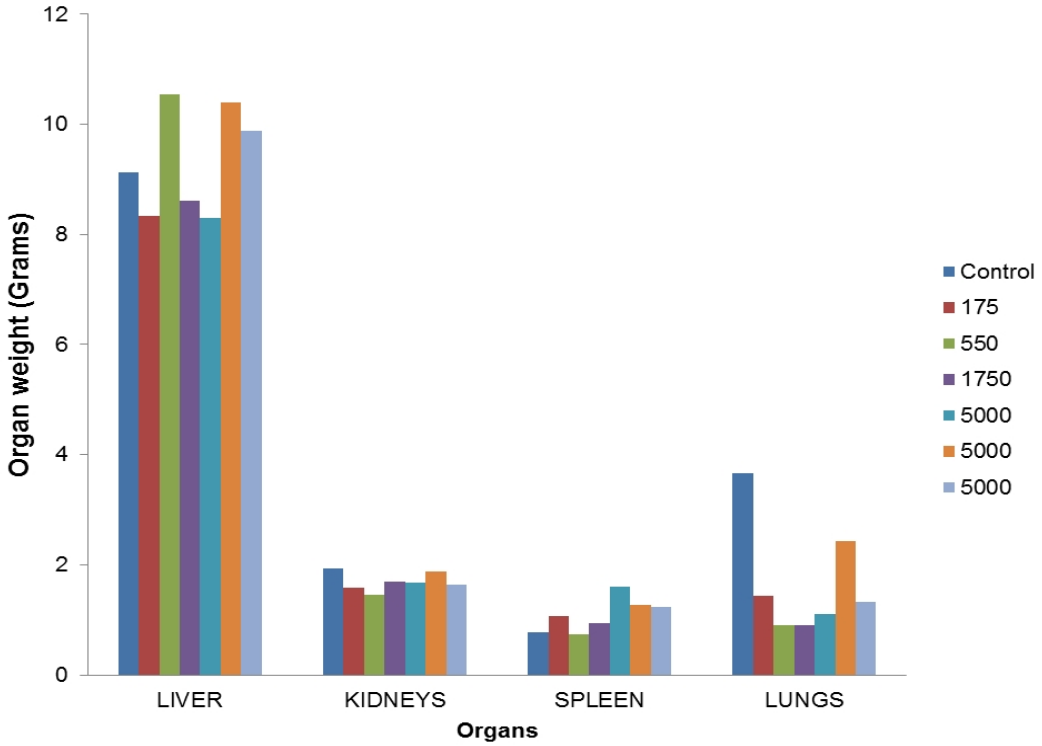

Fig. 1. Internal organ weights of rats after acute oral administration of the aqueous extract mixture, n=1

There were no major differences in the gross pathological parameters in the study. In Fig. 1, the lung weights of the rat in control and one rat in upper bound were the only conspicuously visible outliers. There were no major deviations in the other organ weights compared to control. The slight differences in liver and spleen weights could not be directly attributed to any internal pathological processes. Macroscopic examination of the organs showed no changes in colour and texture or any visible inflammation compared to the control.

Generally, the animals had progressive weight gains (Fig. 2). The progressive weight gains indicate positive growth response.

Similar studies using similar and other guidelines to establish toxicity of plant extracts in rat or mice models are documented. Acute oral toxicity testing of *Hunteria umbellate* was done using the Up-and–Down procedure (OECD guideline 425). The study showed that the plant extract had an LD_{50} of 1020mg/kg and was therefore slightly toxic [18]. Akanmu *et al.* [4] investigated acute and sub-acute oral toxicity of methanolic extract of *Bauhinia monandra* (used for treatment of diabetes). Acute administration of the extract up to a dose of 8000 mg/kg b.w. did not cause any deaths nor any toxicity signs. The study concluded that *B.*

Monandra may possess relatively low toxicity. Acute toxicity study of the leaves of *Sphenocentrum jollyanum* showed no toxicity when administered up to 11000 mg/kg b.w. orally while intra-peritoneal (IP) administration produced dose dependent mortality with an LD_{50} of 1445.4 mg/kg b.w. The results suggested that the leaves extract was potentially safe for oral consumption [2]. Acute and sub-acute toxicity of 95% ethanolic extract of aerial parts of *Cansjera rheedii* J. Gmelin (*Opiliaceae*) was evaluated in Swiss mice and Wistar albino rats. The acute toxicity study was conducted following the OECD 420 guideline where a limit test dose of 2000 mg/kg b.w. was used. No significant changes in the organ weights between the control and treatment groups were observed nor were there any gross pathological and histopathological changes observed after 28 days. There were no mortalities during the entire treatment period. In conclusion, the study presented strong evidence of non-toxic effects of the ethanol extract of *C. rheedii* and the extract was considered safe and could be extensively used [14].

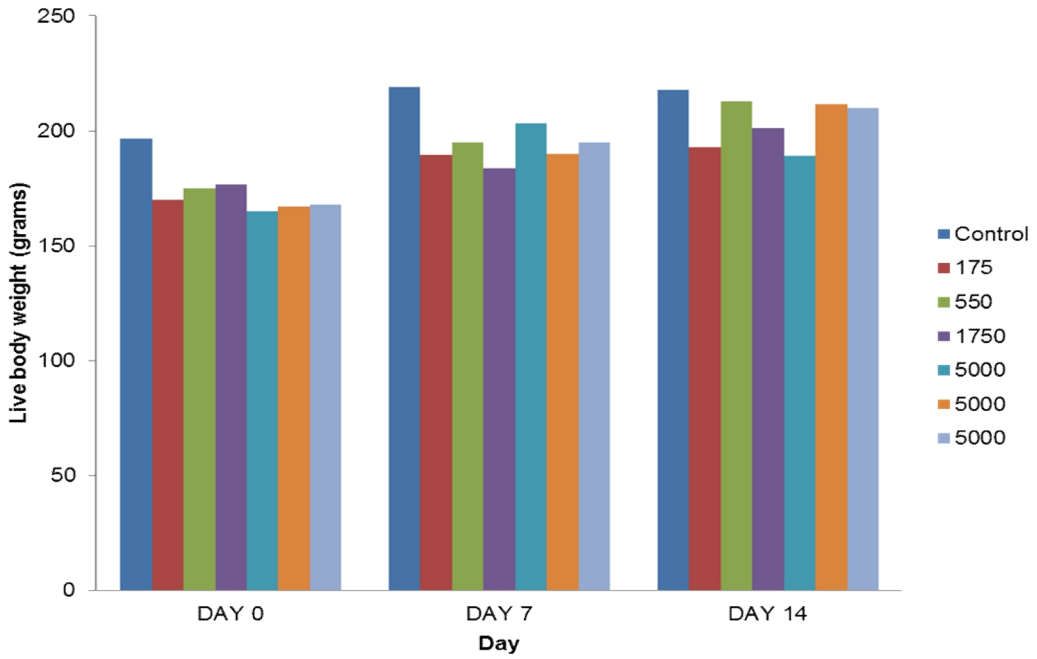

Fig. 2. Effect of the aqueous extract mixture on live body weights of rats (n=1)

In this study, the constituent phytochemicals in the extract mixture may not have had any or may have had very minimal negative effects on the internal vital organs to produce any noticeable physical toxicity signs. This could have been due to poor absorption and bioavailability of the herbal extract mixture from the gastrointestinal tract [19]. According to the American Society for Testing and Materials (ASTM) [20], any chemical substance with an LD_{50} estimate less than 2000 mg/kg/oral route but greater than 1000 mg/kg/oral route could be considered to be slightly toxic although Clarke and Clarke [21] consider any compound with an estimated LD_{50} greater than 1000 mg/kg/oral route to be safe. A scale proposed by Lorke [22], roughly classifies substances according to their LD_{50} as follows: very toxic (LD_{50}<1.0 mg/kg), toxic (LD_{50} up to 10.0 mg/kg), less toxic (LD_{50} up to 100.0 mg/kg) and only slightly toxic (up to 1000.0 mg/kg). Substances with LD_{50} values greater than 5000

mg/kg are practically non-toxic [23]. The extract mixture formula qualified as a safe substance (non-toxic) using the proposed toxicological scales [7,20,21,22].

4. CONCLUSION

The study confirmed that *Entada leptostachya* (EL) and *Prosopis juliflora* (PJ) extract mixture is safe at the prescribed acute exposure, hence it is recommended for use by farmers and pastoralists.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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