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In vivo toxicological investigations of standardized ethanolic extract of *Curcuma xanthorrhiza* Roxb. rhizome.

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ABSTRACT

Curcuma xanthorrhiza Roxb. known as 'Temu Lawak' in Malaysia, is widely used in South East Asian countries in the traditional treatment of many ailments including migraines, constipation, liver complaints and inflammatory conditions. The present study was designed to investigate the toxicity effects of the standardized ethanolic extract of *Curcuma xanthorrhiza* in experimental animals. Brine shrimp lethality test and acute oral toxicity were conducted to evaluate the toxicity effects of this plant. The ethanolic extract of *Curcuma xanthorrhiza* screened for toxicity against brine shrimps showed lethal concentration (LC₅₀) values of more than 1.0 mg/ml confirming that the extract was not toxic and bioactive. Oral administration of standardized ethanolic extract of *Curcuma xanthorrhiza* at the doses 300, 2,000 and 5,000 mg/kg resulted in no mortalities or evidence of adverse effects, indicating that *Curcuma xanthorrhiza* is non-toxic. This is further proven with the normal behavioral pattern, clinical signs of animals and histopathology analysis of the vital organs. The experimental results suggest that the standardized *Curcuma xanthorrhiza* ethanolic extract is non-toxic with a high margin of safety.

Keywords: *Curcuma xanthorrhiza*, standardized ethanolic extract, acute oral toxicity, Brine shrimp lethality assay,

INTRODUCTION

Medicinal plants constitute a source of raw materials for both traditional systems of medicine (e.g. Ayurvedic, Chinese, Unani, Homeopathy, and Siddha) and modern medicine. Nowadays, plant materials are employed throughout the industrialized and developing world as home remedies, over-the-counter drugs, and ingredients for the pharmaceutical industry. As such, they represent a substantial proportion of the global drug market [1].

Curcuma xanthorrhiza Roxb. is a member of the ginger family (Zingiberaceae) and a native Indonesian plant. It is grown in Thailand, Philippines, Sri Lanka and Malaysia. It is commonly known as 'Temu Lawak' in Malaysia. *Curcuma xanthorrhiza* is low growing plant with a root (rhizome) which is similar to ginger with aromatic, pungent odor and bitter taste. *Curcuma xanthorrhiza* is reported to be useful for hepatitis, liver complaints, diabetes, rheumatism, cancer, hypertension and heart disorders. *Curcuma xanthorrhiza* has also shown diuretic, anti-cancer, anti-inflammatory, anti-oxidant, anti-hypertensive, anti-rheumatic, anti-hepatotoxic, anti-dysmenorrheal, anti-spasmodic, anti-leucorrhoea, anti-bacterial and antifungal effects [2]. It reduces cholesterol, treats constipation, migraines and increases flow of milk during breast feeding. The traditional benefits of *Curcuma xanthorrhiza* are further supported by the isolation and identification of several active chemical constituents including xanthorrhizol, curcumin and few volatile substances. Xanthorrhizol, the major component of the essential oil of *Curcuma*

xanthorrhiza, is a bisabolane-type sesquiterpenoid. This compound makes up nearly 46.3% of the total component of essential oil through hydrodistillation technique. Investigations on functional plants provide evidence on the presence of substances that are potential for human health benefits. However, there should be a vital requirement to determine the toxicity effects of some substances contained in the plants [3].

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells [4]. Traditionally, herbs and herbal products have been considered to be nontoxic and have been used by the general public and traditional medicinal doctors worldwide to treat a range of ailments. The fact that something is natural does not necessarily make it safe or effective. The active ingredients of plant extracts are chemicals that are similar to those in purified medications, and they have the same potential to cause serious adverse effects [5]. Hence, evaluation of toxic properties of a substance is crucial when considering for public health protection. In practice, the evaluation typically includes acute, sub-chronic, chronic, carcinogenic and reproductive effects [4].

In the present study of the standardized *Curcuma xanthorrhiza* ethanolic extract rhizome, both the acute oral toxicity test in animal model and brine shrimp test were applied to determine its toxic properties.

MATERIALS AND METHODS

Plant material identification

Curcuma xanthorrhiza plants were obtained from Johor Plantation, Malaysia. A voucher specimen (11022) was authenticated and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia by a botanist, Mr Shanmugam.

Preparation of the extracts

The rhizome portion of *Curcuma xanthorrhiza* was purchased in powder form from Chemical Engineering Pilot Plant (CEPP), UTM, Skudai, Johor, Malaysia. The coarsely powdered material (800g) was macerated with 8L of ethanol (99.5%) for 72 hours with occasional shaking. The maceration was repeated thrice. The extract was filtered and concentrated at reduced pressure on rotary evaporator resulting in dark yellow colored semisolid mass (yield 5.2%).

Standardization of *Curcuma xanthorrhiza* ethanolic extract

The standardization of *Curcuma xanthorrhiza* ethanolic extract was performed based on the validated method described by Devaraj *et al* [6].

Brine Shrimp Toxicity Assay

Brine shrimp (*Artemia salina*) lethality bioassay technique referring to the modified method of Meyer *et al* [8] was conducted for the determination of general toxicity of standardized ethanolic extract of *Curcuma xanthorrhiza*. In brief, brine shrimp eggs were hatched in a glass beaker filled with artificial seawater on the day prior to the experiment. Stock solution of *Curcuma xanthorrhiza* ethanolic extract at 10 mg/ml was prepared by dissolving the extract in 5% DMSO solution. From the stock solution, different concentrations of *Curcuma xanthorrhiza* ethanolic extract (0.5-10 mg/ml) were added into separate test tubes containing artificial seawater. Subsequently, 10 newly hatched shrimps were added to each test tube and incubated at room temperature for 24 h. The final volume of each test tube was 5 ml. The percentage of mortality was calculated after 24 h. Lethality concentration of extract that kills 50 % of the shrimps (LC₅₀) was calculated. The same procedure using potassium dichromate served as positive control was conducted. All the experiments were performed in triplicate.

Acute Oral Toxicity Study

Experimental animals

Female Swiss mice (20-25g) were obtained from the Animal House, Universiti Sains Malaysia. The animals were acclimatized to laboratory conditions for seven days prior to the experiments. Five rats were housed per polycarbonate cage, with free access to food (normal laboratory chow, Gold Coin) and tap water *ad libitum*. The animals were maintained at room temperature under a light/dark cycle of 12 h. All experiments were performed between 9.00 a.m. to 2.00 p.m. in order to prevent confrontation with circadian rhythm. Experimental protocols and procedures employed in this study were approved by the Animal Ethics Committee of the Universiti Sains Malaysia with the reference number USM/PPSF/50(054) Jld 2.

Acute toxicity evaluation

An acute toxicity study was carried out by using OECD (2001) Guidelines 423 [9]. Standardized ethanolic extract of *Curcuma xanthorrhiza* was administered orally to Swiss albino mice (n=6) at doses of 300, 2000 and 5000 mg/kg. Co solvent was used as control. The behavioral changes (abdominal constriction, hyperactivity, sedation, grooming), mortality and body weight 14 were observed for 14 days. On the day 14, necropsy was carried out on the animals under diethyl ether anesthesia and organs as liver, kidney, spleen, heart and lung were withdrawn for the histological procedures. The organ body index and relative organ weight of tested groups was calculated and compared with control group as follows:

$$ROW = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of mice on sacrifice day (g)}} \times 100$$

Histopathological studies

After the animals were sacrificed, postmortem examination was performed according to Tsung *et al.* [20]. All the organs were sliced into small pieces and preserved in 4% formalin before further treatment. Then the organs were dehydrated using solvents followed by waxing and clearing process. Thereafter, the tissues were embedded in paraffin and processed into 4-5 μm thick sections and fixed in slides. Finally, samples were stained using hematoxylin-eosin (H&E) and assessed for any tissue damage under photomicroscope.

Statistical analysis

Data were expressed as mean \pm SEM for brine shrimp lethality evaluation and acute oral toxicity studies. Data were analyzed in Probit analysis (SPSS version 12.0.1) software programme for brine shrimp lethality evaluation. One way ANNOVA followed by Tukey's test using Sigma Stat® version 3.5 Software were performed for acute toxicity studies. Data were expressed as mean \pm SEM for both toxicity studies. A difference was considered significant at $p < 0.05$. LC50 values were calculated from regression line obtained from Probit analysis.

RESULTS**Standardization of *Curcuma xanthorrhiza* ethanolic extract**

Xanthorrhizol was identified and quantified at 9.58 minute retention time. 0.1238 mg of xanthorrhizol was quantified in 1 mg of *Curcuma xanthorrhiza* ethanolic extract [6].

Brine Shrimp Lethality Test

Brine shrimp lethality assay is a preliminary toxicity screening that enables determination of medium lethal concentration (LC₅₀) values for the extract [7]. The LC₅₀ values of the standardized *Curcuma xanthorrhiza* ethanolic extract were recorded in Table 1. Based on the results obtained, the respective extract showed non-toxic, indicating that the samples are biologically active [8]. Crude extracts resulting in LC₅₀ values of less than 1 mg/ml are considered significantly active which, 4.17 mg/ml and 2.16 mg/ml at 6 and 24 hours respectively, have a low toxicity and considered safe for consumption in comparison of potassium dichromate standard. Potassium dichromate served as the positive control for this brine shrimp lethality assay and exhibit toxic expressions (LC₅₀ less than 1.0 mg/ml) against brine shrimp.

Table 1. Brine shrimp toxicity expressed as the LC₅₀ values of the standardized *Curcuma xanthorrhiza* ethanolic extract.

Sample	LC ₅₀ (mg/ml)
<i>Curcuma xanthorrhiza</i> (6h)	4.17
<i>Curcuma xanthorrhiza</i> (24h)	2.16
Potassium dichromate (24h)	0.014

Acute Oral Toxicity**Lethality and Behavioral analysis**

Oral administration of standardized *Curcuma xanthorrhiza* ethanolic extract showed no mortality at doses up to 5 g/kg in mice as shown in Table 2. There were no toxicity signs observed for skin, fur or eyes of the animals. No noticeable behavioral changes in salivation, sleeping pattern, diarrhea or lethargy were spotted in the treated animals as shown in Table 3. This result indicates the *Curcuma xanthorrhiza* ethanolic extract is non-toxic, safe at 300 mg/kg, 2000 mg/kg and 5000 mg/kg. This explains the rational usage of this extract in folk medicine practices.

Table 2. Potential toxic effects of the standardized *Curcuma xanthorrhiza* ethanolic extract in mice.

Female mice	
Control ^a	Crude extract ^b
0/5 ^c	0/5

^a Control group (treatment without crude extract); ^b Test group (treatment with 5000 mg/kg crude extract) ; ^c Number of dead mice/number of mice used.

Table 3: General appearance and behavioural observations for control and treated groups.

Observations	Control group		Test group	
	6 h	14 days	6 h	14 days
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Behavioral patterns	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Diarrhea	Normal	Normal	Normal	Normal

Organ and body weight statistical analysis

The body weight and relative organ weight of the vital organs of the animals were recorded in Table 4 and Table 5 respectively. Based on the results obtained, there were no significant changes observed in the body as well as the organ weight compared to the control indicating that standardized *Curcuma xanthorrhiza* ethanolic extract is non-toxic.

Table 4. Effect of standardized *Curcuma xanthorrhiza* ethanolic extract on body weight of mice.

Groups	Day 0	Day 14	Weight gained
Control	28.6 ± 0.60	30.6 ± 0.43	2.2 ± 0.68
300 mg/kg	25.7 ± 0.44	29.1 ± 1.23	3.4 ± 0.94
2000 mg/kg	24.7 ± 0.44	28.3 ± 0.94	3.6 ± 1.02
5000 mg/kg	23.2 ± 0.34	28.0 ± 1.64	4.8 ± 1.32

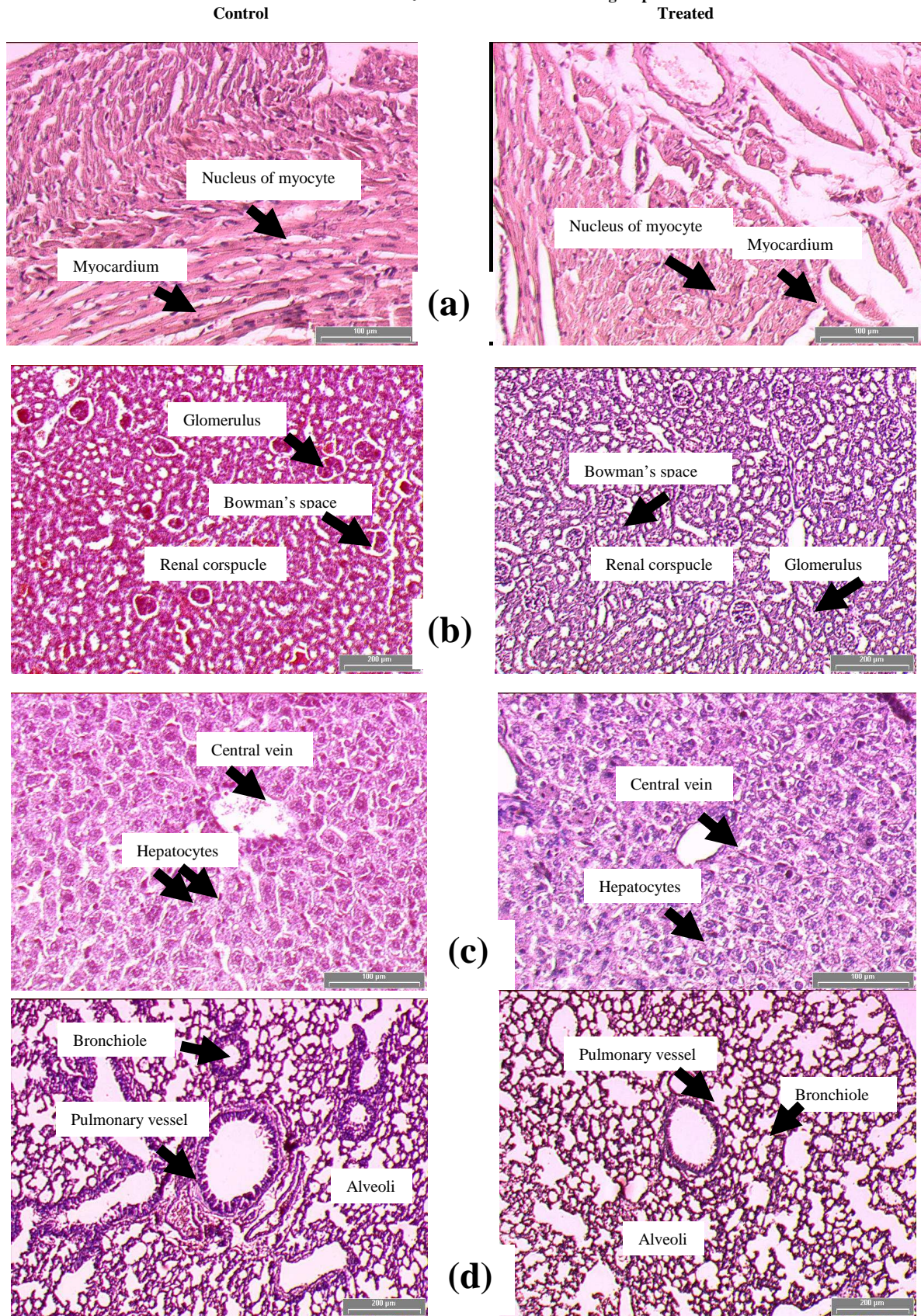
Table 5. Effect of standardized *Curcuma xanthorrhiza* ethanolic extract on relative organ weight of mice.

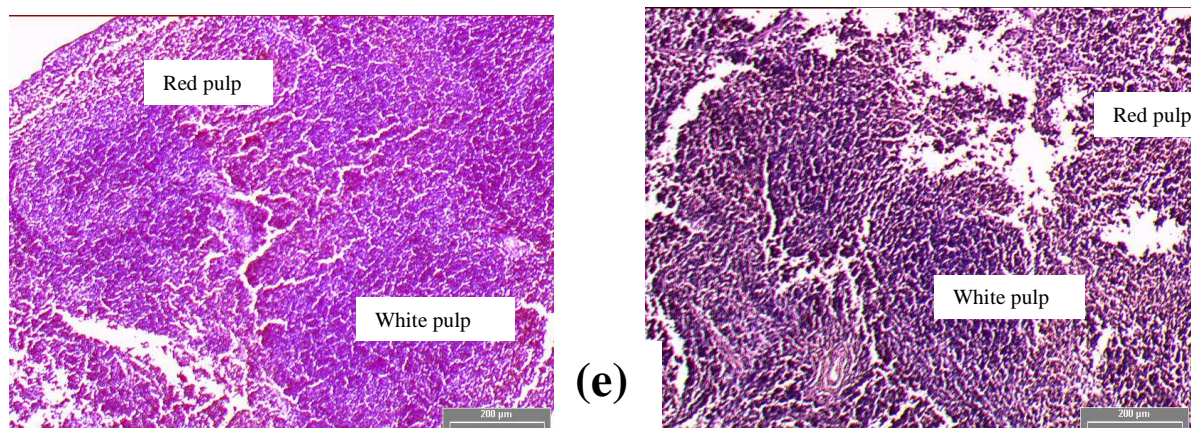
Groups	Organ				
	Liver	Kidney	Spleen	Lung	Heart
Control	6.89 ± 0.40	1.20 ± 0.07	0.69 ± 0.03	1.06 ± 0.05	0.47 ± 0.02
300 mg/kg	6.80 ± 0.36	1.26 ± 0.08	0.73 ± 0.08	1.08 ± 0.05	0.54 ± 0.02
2000 mg/kg	7.09 ± 0.45	1.44 ± 0.07	0.70 ± 0.11	1.06 ± 0.09	0.61 ± 0.04
5000 mg/kg	6.84 ± 0.33	1.41 ± 0.03	0.97 ± 0.13	1.06 ± 0.10	0.56 ± 0.02

Histopathology analysis of Heart, Liver, Kidneys, Lung and Spleen

The microscopic examination of internal organs through Figure 1 shows unnoticeable differences between the control and test groups. There were no abnormalities, cell degradation or any unfavorable defects were observed in the organs under the light microscope. Microscopic assessment in the lower dose was not performed in agreement with the OECD guidelines that consider the microscopic evaluation of organs in low dose group as unnecessary when no histopathological abnormalities are found in the high dose group [9].

Figure 1. Histological examination of heart (a), kidneys (b), liver (c), lung (d) and spleen (e) of control and 5000 mg/kg standardized *Curcuma xanthorrhiza* ethanolic extract treated groups.





DISCUSSION

Herbal medicines are gaining popularity in developing countries. Such remedies are often believed to be harmless, since these treatments are 'natural' and commonly used for self-medication without supervision. Although medicinal plants may cause several biological activities in humans, very little is known regarding the potential toxicity for many of these bioactive substances, such as *Curcuma xanthorrhiza* [10]. The present extraction method offers several advantages. Firstly, this method closely resembles the traditional bioactive dosage form [10]. Previous studies indicated that the important bioactive ingredients are present in ethanolic extract [11]. Second, the extract can be freeze-dried to produce a lyophilized form that can be standardized in terms of constituents (marker compounds), which it easily converted into other high quality pharmaceutical dosage forms [12].

Brine shrimp lethality assay is an *in vivo* lethality test using simple zoologic organism, *Artemia salina* as to screen and monitor the toxicity level of bioactive natural products [13]. *Artemia salina* is an invertebrate component of the fauna of saline aquatic and marine ecosystems. It can be used in laboratory bioassay in order to determine the toxicity by estimation of the medium lethality concentration (LC_{50}) [14], which have been reported for a series of toxins and plant extracts [15]. This brine shrimp bioassay is an efficient, rapid and inexpensive test that requires only a relatively small amount of sample. This bioassay has a good correlation with cytotoxicity and genotoxicity, and has led to the discovery of the new classes of natural pesticides and active antitumor agents [16]. According to Meyer *et al.* [8], extracts derived from natural products which have $LC_{50} \geq 1.0$ mg/ml are known to be no toxic and bioactive. In this study, *Curcuma xanthorrhiza* ethanolic extract is not toxic since the LC_{50} values are more than 1.0 mg/ml indicating this plant is bioactive and can be further explored for the biological screening tests to be developed as pharmaceutical products.

The acute oral toxicity study indicated that standardized *Curcuma xanthorrhiza* ethanolic extract at a dose of 5,000 mg/kg caused neither visible signs of toxicity nor mortality. Generally, the reduction in body weight gain and internal organ weight is a simple and sensitive index of toxicity after exposure to potentially toxic substances [17]. In the present study, standardized *Curcuma xanthorrhiza* ethanolic extract (300 mg/kg, 2000 mg/kg and 5000 mg/kg) showed no significant changes in the body or organ weight as compared to the control group, which suggests the extract, did not hinder mice growth as well as no major impact on the general metabolic status of animals [18]. This analysis is based on the toxicity classification where substances with an LD_{50} between 500-5,000 and 5,000 – 15,000 mg/kg body weight are categorized as slightly toxic and practically non-toxic respectively [19]. Therefore, according to the chemical labeling and classification of acute systemic toxicity recommended by OECD, standardized *Curcuma xanthorrhiza* ethanolic extract is assigned in class 5 status ($LD_{50} > 5,000$ mg/kg) which considered to be non-toxic.

Based on histopathology analysis, standardized *Curcuma xanthorrhiza* ethanolic extract did not adversely affect the morphology of mice organs. The tissues presented good morphological structures with no cell degradation or distortion being observed as compared to the control group. This suggests that this extract did not interact with the target cells or change the biological systems of the animals [20]. Extrapolation of these results to human suggests that *Curcuma xanthorrhiza* ethanolic extract should be relatively safe for usage at the dose of 5,000 mg/kg and suitable for the development of pharmacological agent to treat various types of ailment.

CONCLUSION

The experimental evidences acquired in this study imply that standardized ethanolic extract of *Curcuma xanthorrhiza* is non-toxic and suggest no potential health risk for human consumption. It can be concluded that this extract may has high level of safety margin, thus justifying its wide application in various communities coupled with lack of any reported serious side effects with the traditional use of this plant. However, further toxicity studies are required in order to justify the safety margin of this respective extract.

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