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Research Article
Investigation of Hepatotoxic Effects of Zerumbone in Female Balb/c Mice

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Abstract

Background and Objective: It was reported recently that zerumbone (ZER), isolated from the rhizomes of a local plant *Zingiber zerumbet*, possesses intriguing bioactivities. The present studies investigate the effects of ZER on the liver of female Balb/c mice. **Materials and Methods:** In the acute and subacute dosing studies, twenty-four female Balb/c mice (14-19 g) were randomly divided into 4 groups (n = 6/group) and were given intraperitoneal (IP) doses of 0, 20, 100 and 250 mg/kg of ZER dissolved in 0.05 mL 5% ethanol (v/v). The animals were sacrificed 24 hrs post-treatment for hepato-histological and biochemical analysis. Acute study results showed no indication of liver toxicity or high plasma enzyme levels [Aspartate Aminotransferase (AST)] have been seen in all treated groups of mice. In the sub-acute study, dosing was once daily for 14 days consecutively. Changes in body weight and liver weight were recorded once in 2 days for each mouse. **Results:** The significant increase in body weights of all treated and control groups ($p<0.05$). In contrast, no significant difference in AST and relative liver weight has been observed in treatment compared to control groups ($p>0.05$). Histological analyses of the liver control group showed degenerative cell changes, moderate portal infiltration and mild lobular inflammation, while in treatment groups, livers showed few degenerative cell changes, moderate portal infiltration and mild lobular inflammation. Treatment groups showed few hepatocyte non-significant changes when compared to the control group ($p<0.05$). **Conclusion:** This investigation supports that the compound, ZER at 20 to 250 mg/kg dose, given daily for up to 14 days, had no ill effect on the livers of the female Balb/c mice.

Key words: ZER, hepatotoxicity, Balb/c mice, acute toxicity, AST

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer[1-6] and remedy for several diseases[7-9]. Several naturally derived agents have been entered into clinical trials and terminated due to lack of efficacy, unacceptable toxicity or fail with respect to safety[10,11]. This toxic effect could be studied through histopathology and biochemistry of the liver. The liver plays an essential role in the metabolism of most of the nutrients since it is the major metabolic organ carrying out a series of physiological and metabolic processes related to drug metabolism[12].

Traditional plants have been used since ancient times for healing several diseases and to improve health status[13,14]. Zerumbone (ZER), a monocyclic sesquiterpene from rhizomes of the edible plant *Zingiber zerumbet* (L.) Smith. The ZER has gained popularity for its pharmacological activity against several pathological conditions[15]. It has been found to suppress tumor promoter 12-O-tetradecanoylphorbol-13 acetate (TPA)-induced Epstein-Barr virus activation in a patent manner[16]. Additionally, ZER has been found to induce apoptosis in cervical intraepithelial neoplasia in cervical tissues from Balb/c mice[17]. The ZER is known to be a potent suppressant of cyclooxygenase (COX)-2 and inducible nitric oxide synthase expression[16]. The ZER is a food phytochemical that has a distinct potential for use as an effective anti-leukemic as well as anticancer agent[18-21], markedly suppresses free radical generation, proinflammatory protein production[16], possibly by its apoptosis-inducing, antiproliferative influences[22] and activates phase II drug metabolizing enzymes[23].

With all the promising properties of ZER as a drug candidate for different kinds of tumours, a preclinical evaluation of this compound should be carried out to introduce it for further clinical application. Therefore, the current study aims to evaluate the hepatotoxicity of zerumbone on female Balb/c mice.

MATERIALS AND METHODS

Isolation of ZER: *Zingiber zerumbet* rhizomes were obtained from the Laboratory of Natural Products, IBS, University Putra Malaysia, Serdang and the experimental work of this study was carried out in UPM-MAKNA-Cancer Research Lab, IBS, University Putra Malaysia during 2010-2013. The rhizomes were obtained from the wet market of Kuala Lumpur, cleaned under running tap water, cut into thin slices and soaked in absolute acetone for 3 days at room temperature. The soaking step was repeated thrice, for efficient extraction. The crude extract was obtained by evaporating acetone under vacuum pressure. The crude extract was dissolved in 10% methanol and fractionated using hexane in the ratio of 1:1. Non-polar hexane fractions were obtained, dried using rotary-evaporation and mixed with silica and hexane. The mixture was subjected to rotary-evaporation again to allow the non-polar compound to adhere to the silica. After that, it was separated using column chromatography (33 cm height and 3 cm diameter) and the ZER, which is the second fraction was collected with a flow rate of approximately 50 mL/hr and subjected to thin-layer chromatography to evaluate its purity. The pure ZER compound was stored at stored crystalized at -4°C for future use.

Laboratory animal: Inbred female Balb/c mice (14-19 g) were kept under 12 hrs light/12 hrs dark at 23-25°C and given a commercial diet and tap water *ad libitum*. Mice were acclimatized at laboratory conditions in plastic cages for 1 week before the study.

Acute toxicity test: The 24 female Balb/c mice were randomly selected into 4 groups with 6 mice in each group. The animals were given intraperitoneal (IP) injections of 20, 100 and 250 mg/kg of ZER dissolved in 5%(v/v) ethanol in distilled water at 50 µL volume. Control animals were treated with the dose vehicle similarly. The mice were given a single dose and later sacrificed using 10% chloroform after 24 hrs of the injected doses. Before sacrifice, 0.5 mL of blood was taken via cardiac puncture from each animal and kept in EDTA tubes. Livers were quickly removed, cleaned and fixed in 10% formalin.

Sub-acute toxicity test: Mice were randomly distributed (CRD) into 4 groups with 6 mice in each group. Animals were given intraperitoneal (IP) injections of 20, 100 and 250 mg/kg of ZER dissolved in 5% (v/v) ethanol in distilled water at 50 µL volume. Control animals were treated with the dose vehicle similarly. Dosage was given once daily for 14 days and the body weight of each animal was recorded once in 2 days until day 14. On day 14, 0.5 mL of blood was taken via cardiac puncture and kept in EDTA tubes.

Biochemical analysis Aspartate Aminotransferase (AST): Blood samples were centrifuged at 3000 rpm for 15 min at -4°C. This step was repeated twice to obtain the maximum volume of plasma which was stored at -80°C for future

Table 1: Staging and grading of liver injury

Score	Portal	Lobular	Fibrosis
0	None	None	None
1	Mild portal inflammation, mild degeneration	Inflammation, no necrosis, mild hyperemia	Enlarged portal tracts
2	Moderate portal infiltration	Focal necrotic cells, moderate hyperemia	Fibrosis of periportal area
3	Severe portal infiltration	Severe focal cell damage, severe hyperemia	Fibrosis in septa
4	Severe necrosis of periportal hepatocytes	Necrosis of liver cells bridges between portal tracts	Fibrosis with regenerative nodules

analysis. Analysis of Aspartate Aminotransferase (AST) was carried out using Roche Diagnostics AST test kit. As 250 µL of plasma were transferred into labeled sample cups and inserted into the Roche Cobas Miras machine. The photometric tests were run automatically.

Histological study: Fresh liver sections from Balb/c mice were processed for light microscopy after fixation in 10% formalin. Fixed tissues were dehydrated in ascending ethanol concentrations (70, 80, 90 and 95%) and finally 3 times in 100% ethanol. Processed tissues were embedded later in paraffin wax. Thin sections of 5 µm were sectioned using a microtome and placed on microscope slides. These slides are then stained using Hematoxylin and Eosin (H&E) staining method. Pathological changes were assessed under a light microscope (Leica, Wetzlar, Germany), after Hematoxylin and Eosin (H&E) staining. For each slide, scoring of liver injury was done at magnification (X200) based on the following Table 1.

Ethical consideration: The experimental protocol and procedures were performed in accordance with the regulations and recommendations of the Animal Care and Use Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). Mice were obtained from the Institute for Medical Research (IMR), Ministry of Health, Kuala Lumpur, Malaysia.

Statistical analysis: All data were analyzed using SPSS 26.0. The data were presented as Means±SEM. The mean values of the various treatment groups were compared using One-way Analysis of Variance (ANOVA). For non-parametric analysis, Kruskal-Wallis was used. For both statistical tests, the significance level was set at $p<0.05$. Linear regression statistical analysis was performed to test the effect of day on the body weights of mice in each group.

RESULTS

No change in caging activity was observed after a single injection dose of ZER. There were no changes to fur colour, food consumption behaviour, nor any physical signs of illness observed within the 14 days of sub-acute study.

The mean body weight (gm) of each group of mice in the sub-acute study (Mean±SEM) was shown in Fig. 1. A graph of the mean body weight of each mice group during the 14 day's treatment was plotted (Fig. 1). Linear regression statistical analysis was performed to test the effect of day on the body weights of mice in each group. Curve estimations of linear regression in control and treated groups were also plotted. Linear regression statistical analysis revealed that there was a positive significant effect of day on body weight for the control, 20 and 100 mg/kg treated group ($p<0.05$), while the 250 mg/kg treated group showed insignificant effect of day on body weight ($p>0.05$).

Table 2 shows the activities of the liver enzyme, AST in plasma from the control and ZER treated mice. No significant changes were observed in the AST liver enzyme in both acute and sub-acute studies ($p<0.05$). Average liver weight per unit body weight (liver enlargement) (Table 2) was also not significantly changed for all treated groups when compared to the controls, which received dose vehicle only during sub-acute and acute study ($p>0.05$).

Histological examinations of liver sections from a single dose of ZER-treated mice showed no significant changes observed in both control and treated groups (20, 100 and 250 mg/kg), either in the centrilobular, liver parenchyma or the periportal regions (microscopic photos not shown). No statistical analyses were done.

For the 14 day repeated dose sub-acute study, histological examination of liver sections showed minor liver injuries (Fig. 2a). At 20 mg/kg doses, the liver section showed few hepatocytes undergoing degenerative changes (Fig. 2b). These hepatocytes showed characteristics of small, dense pycnotic nuclei and eosinophilic cytoplasm. The other mice of a similar group also showed some focal inflammation in liver parenchyma. At 100 mg/kg doses (Fig. 2c), the liver section showed few degenerative changes in the periportal area and focal inflammation in the centrilobular region. The liver section from the 250 mg/kg doses treated mice also showed degenerative changes and focal inflammation in the periportal area (Fig. 2d). The control mice, however, showed some

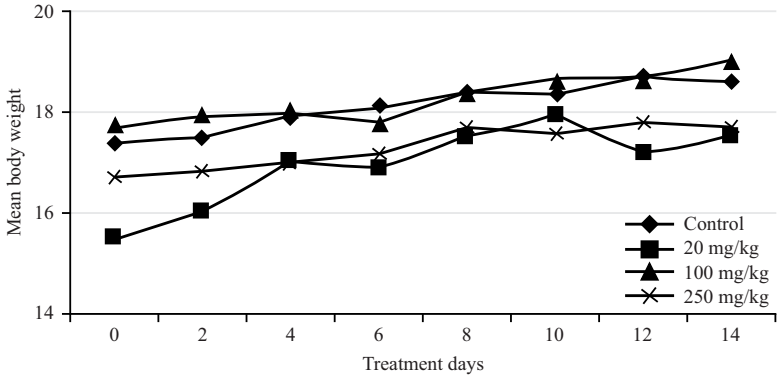

Fig. 1: Graph showing the mean body weight (g) for each treatment group in sub-acute toxicity study

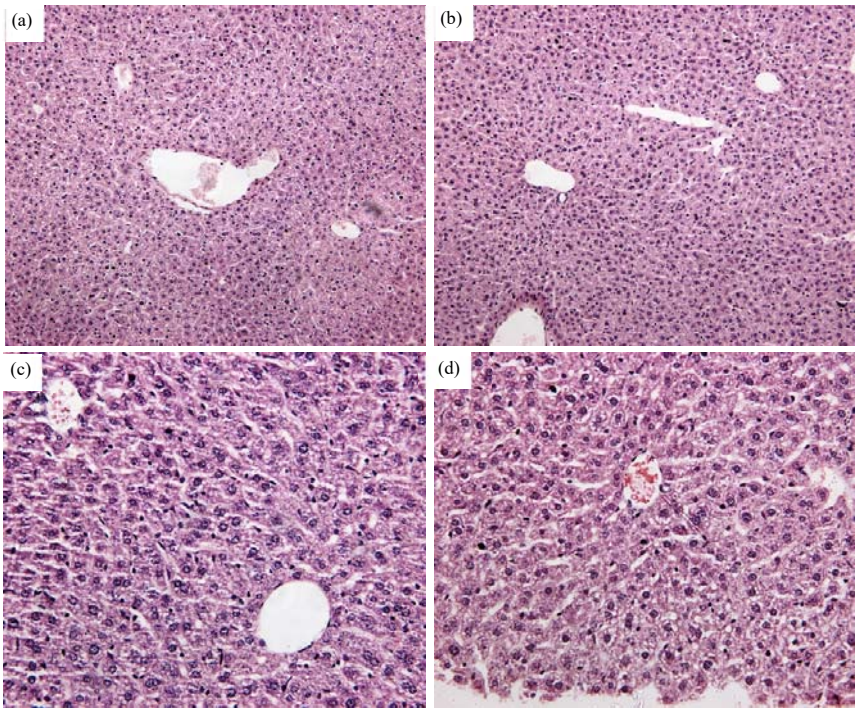

Fig. 2(a-d): Liver histology of treated mice with ZER for 14 days (a) Liver section from control mice (received 5%(v/v) ethanol only), showing normal histology in the centrilobular region of liver parenchyma, (b) Liver section from 20 mg/kg ZER treated mice, showing normal histology in the liver parenchyma, (c) Liver section from 100 mg/kg ZER treated mice, showing normal histology in the periportal region and (d) Liver from 250 mg/kg ZER treated mice, showing normal histology in the portal region
Magnification 200X

degenerative hepatocyte and focal inflammation. Liver lesions were scored according to the scoring method modified and developed by Feist *et al.*[24]. Lesion score data were given as mean rank using Kruskal-Wallis analyses. Although the liver sections of the treated group showed some liver histological changes, these changes however were insignificant when compared to the controls ($p>0.05$) (Table 3).

Liver sections were stained using Hematoxylin and Eosin (H&E) method. The Liver sections were then viewed and lesions were scored under X200 magnification. Lesion score data are given as mean rank using Kruskal-Wallis analysis. The significance value that $p>0.05$ revealed the lesion scores were not significantly different between all treatment groups including the control.

Table 2: Liver function test in both acute and sub-acute study

Group	AST (U/L)	Liver weight (g/kg b.wt.)	n
Acute study			
Control (5% ethanol)	79.9±7.5	NA	3
20 mg/kg	79.7±14.8	NA	6
100 mg/kg	98.4±7.7	NA	5
250 mg/kg	90.7±9.3	NA	5
Sub-acute study			
Control (5% ethanol)	77.9±18.5	50.12±2.86	5
20 mg/kg	53.1±9.4	53.32±1.48	4
100 mg/kg	59.6±4.5	51.83±1.20	4
250 mg/kg	60.4±9.7	53.22±3.72	6

Significant value (p>0.05) revealed plasma AST levels were not significantly different in all treatment groups including control

Table 3: Liver histology of treated mice with ZER for 14 days

Group	Portal	Lobular	Sum (portal+lobular)
Control	16	13.5	15.25
20 mg/kg	14	15.5	15.67
100 mg/kg	10	9.5	9.00
250 mg/kg	10	11.5	10.08

DISCUSSION

The ZER is a natural compound that possesses versatile biological activities[15,25]. The ZER is known to have antioxidant, anti-inflammatory, anti-cancer properties[26,27], activates phase II drug metabolizing enzymes[23] and promotes cytotoxicity in human malignant glioblastoma cells through generation of ROS[28]. Intensive research is being conducted in our laboratory to verify the mechanistic and toxicological effects of this compound[29-32].

This acute toxicity study (24 hrs) was carried out to evaluate the hepatotoxicity effect of ZER on Balb/c female mice. The mice were given single intra peritoneal (IP) doses of ZER at 3 different concentrations, 20, 100 and 250 mg/kg. Healthy mitochondria are essential for the integrity and function of hepatocytes. However, excessive stress can damage these vital organelles[33]. Aspartate Aminotransferase (AST), a key enzyme located within mitochondria, is a marker of liver cell injury. An increase in serum AST levels may indicate mitochondrial dysfunction, including potentially excessive proliferation, in response to stress[34]. During liver cell damage, enzymes such as AST, ALT and GGT leak out, causing the blood levels of these enzymes to rise. Increased level of these enzymes in the blood is considered good markers and indicators for the detection of liver cell damage. Current results showed no significant difference in plasma AST in all groups of treated mice as compared to the control group (p>0.05). This finding indicated absence of the hepatocellular injury. This was confirmed by histological light microscope examinations of liver sections stained with H&E staining. Results of this study suggest that a single dose of ZER at 20 to 250 mg/kg had no ill effect on the livers of Balb/c mice.

Since a single dose of ZER did not show any toxic effect on the liver of female Balb/c mice, repeated doses of ZER study (14 days) were carried out to examine whether there was any cumulative toxic effect. In this sub-acute study, the mice were given similar doses (20, 100 and 250 mg/kg) of ZER per day. Sub-acute treatment of the mice with ZER did not cause any significant changes in plasma AST as compared to the control group (p>0.05). The liver weight per body weight of each mouse was also not significantly different in all groups (p>0.05), both treated and control groups. This strongly suggests that ZER did not cause any liver enlargement. Body weights in all groups were recorded every other day. There was however significant increase of body weights in all 4 groups (p<0.05), but no association could be found in the body weights of the mice that related to liver toxicity caused by ZER. These changes in body weights may be explained in terms of appetite or the normal growing pattern of the mice.

Histological examination of the liver section using H&E showed some hepatocytes degenerative changes and focal inflammation in all treatment groups and control group. Inflammation is predominantly located in the portal area and consists of a mixture of mononuclear cells and lymphocytes. The liver lesions were scored under 200X magnification using the scoring method modified from Feist *et al.*[24]. The results revealed that there were no significant differences in the lesion scoring in all 4 groups (p>0.05), indicating that ZER did not cause any toxic effect on the liver. The lesions observed in the liver may be due to the vehicle (5% (v/v) ethanol, that was used to dissolve ZER, initially.

The results of this repeated doses study showed no ill effect on the livers of female Balb/c mice. These results supported that the natural compound, ZER at 20 to 250 mg/kg

dose, given daily (IP) for up to 14 days will not have any cumulative toxic effect on the liver of female Balb/c mice. These findings were in the same line with the investigations of Hamid *et al.*[35], who speculated that ZER has hepatoprotective effects against paracetamol-induced hepatotoxicity in rats, in addition to the observation of Kim *et al.*[36] reporting the hepatoprotective effects of ZER in mice acute liver injury. Meanwhile, the previous results obtained by Rahman *et al.*[37], showed the absence of toxicological changes after doses of ZER-loaded nanostructure lipid carrier in the liver tissue. Similarly, ZER has been observed to have no genotoxic effects when evaluated against cultured human peripheral blood lymphocytes[31].

CONCLUSION

The ZER, a natural compound with diverse biological activities, was investigated for its potential liver toxicity in mice. Single doses (20-250 mg/kg) showed no significant effect on liver enzymes, weight or histology, suggesting no acute hepatotoxicity and repeated doses (daily for 14 days) also revealed no significant changes in liver enzymes, weight or histology, indicating no cumulative toxicity. The observed minor liver lesions were likely due to the vehicle used to dissolve ZER, not the compound itself. These findings support the safety of ZER at tested doses and align with previous reports of its potential hepatoprotective effects.

SIGNIFICANCE STATEMENT

While nature has presented us with powerful cancer-fighting tools in the form of plant-derived medicines, the road to effective treatments isn't always smooth. While some natural agents show promising results, others failed in clinical trials due to inefficacy, harmful side effects or safety concerns. Studying the effects of these drugs on the liver, a key player in drug metabolism, through histopathology and biochemistry, is crucial to understanding and mitigating potential toxicity. Zerumbone (ZER), isolated from the rhizomes of a local plant *Zingiber zerumbet*, was reported recently to possess intriguing bioactivities. The present studies investigate the effects of ZER on the liver of female Balb/c mice. The results of the present study investigate the toxicity of zerumbone in female Balb/c mice using histopathological and biochemical parameters. With all the promising properties of ZER as a drug candidate for different kinds of tumors, a preclinical evaluation of this compound should be carried out to introduce it for further clinical application. Therefore, the current study aims to evaluate the hepatotoxicity of zerumbone on female Balb/c mice.

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