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Acute and Subchronic Toxicity of *Momordica Charantia* L Fruits Ethanolic Extract in Liver and Kidney

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ABSTRACT

Background: *Momordica charantia* L (MC) fruit is used as anti-malaria in Indonesia, and long-term application requires testing of acute and subchronic toxicity.

Aim of the study: The aim of this study, therefore, is to investigate the histopathological effect of MC ethanolic extract on the liver and kidney of mice.

Materials and methods: For acute toxicity study, the mice were randomly divided into four groups (n=6), where the control received Na-CMC 0.5%, and the experimental units were administered a single dose of 175, 1250, 2000 and 5000 mg/Kg ethanolic extract of MC fruit per oral. Moreover, sub chronic toxicity study involved the random division of mice into three experimental groups (n=6), that received 40, 80, and 320 mg/Kg ethanolic extract of MC per orally for 28 days.

Results: The acute toxicity study demonstrated no detrimental effects, hence LD_{50} is greater than 5000 mg/Kg. In addition, the subchronic evaluation led to elevation in hepatic and kidney biomarker, including ALT, AST, BUN, and creatinine, although none attained twice the normal level. The increase was observed to be significantly different ($p>0.05$) at 80 mg/Kg and 320mg/Kg doses, compared to the control. Conversely, the liver histology sections showed congestion at the sinusoids with higher doses (320mg/Kg), while the sections of the kidney verified the presence of mild distortion and congestion at 320 mg/Kg.

Conclusion: Despite within the safe limits, it is possible for the 28 days administration of *Momordica charantia* L ethanol extract to affect liver and kidney function.

Keywords: Acute and subchronic toxicity, *Momordica charantia* L fruits, ethanolic extract, liver, kidney

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INTRODUCTION

Momordica charantia L (MC) or bitter melon is a plant, which is widely used as a source of antioxidants, antidiabetic, antihepatoma, supplements, and also as an antimalarial [1–7]. Meanwhile, malaria is known to be a major health problem, with an estimate of 228 million cases worldwide, leading to about 405,000 deaths, based on data from the World Health Organization (WHO) on the World Malaria Report 2019 [8]. Recently, there has been some resistance from plasmodium to some modern antimalarial drugs, which subsequently triggered the development of new antimalarial drugs [9–12].

MC has been used in the Sei Kepayang area, Asahan, North Sumatra, Indonesia as an ethnopharmacological malaria drug [13,14], due to the presence of confirmed active compounds, including momordisin, momordin, charantin, hydroxitryptamine, resin [15,16]. Prior studies have shown effectiveness with the alkaloid fraction of the MC fruit both in vitro against P. falciparum and in vivo towards mice infected with P. berghei.

An evaluation on the toxicity towards zebrafish showed abnormal embryonic growth [17,18], while the consumption for pregnant women is limited due to an experiment in mice show the juice ability to initiate a miscarriage [19]. There is a need to prove the safety of traditional drugs through a series of tests, including toxicity evaluation, with the aim of determining the short-term, long-term effects, as well as the appropriate dosage of ingredients containing the active compound. This is, therefore, performed on animals to identify the potential toxic effect of plants and drugs, subsequently helping in determining the limit of provision, as not all plant extracts possess the drug characteristics. Furthermore, some of the observed parameters include changes in the liver and kidneys, being important organs for the excretion of toxic substances from the body

The MC fruit possesses a bitter taste, due to the presence of momordisin and triterpen glycosides [2]. Therefore, reception, practicality and stability, is possibly improved through the formulation of granular MC extract, which are further developed into other dosage forms, including capsules. In addition, there is also a need to prove the safety of granules created, as humans are accountable for the efficacy and possible toxic effects during clinical practice.

The purpose of this study, therefore, was to evaluate the acute and subchronic toxicity of the ethanolic extract of M. charantia fruit, as well as the histopathological changes in the liver and kidney following daily oral administration for 28 days.

MATERIAL AND METHODS

Material

Chemical and reagents

The reagent kits used to determine creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and blood urea nitrogen (BUN), as well as 70% ethanol, sodium carboxymethyl cellulose (Na-CMC), polyvinylpyrrolidone (PVP), Avicel PH 102, Aerosil, aqua demineralization, physiological NaCl, and other chemicals were of good analytical quality.

Plant material

The fresh fruit of *Momordica charantia* L (MC) was obtained from the BALITRO experimental garden,

Cimanggu, Bogor, Indonesia. Therefore, the determination of MC was conducted at the Biology Research Center Laboratory, Indonesian Research Institute, Cibinong.

Animals

A total of 24 female white mice of DDY strain, aged 2-3 months, with a weight of 20-30 grams [20] were obtained from the Non-Ruminant and Animal Hope section of the Faculty of Animal Husbandry, Bogor Agricultural University, Indonesia. These healthy specimens were allowed to acclimatize for 1 week, with a feed dose of 5 grams per head per day, and then weighed a day prior to the commencement of treatment, in order to ensure group homogeneity in body weight. Furthermore, the room temperature was set within the range of 27 ± 2oC, with humidity of 50-70%, and the lighting was adjusted at 12 hours of light and darkness, respectively, following the Organization for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals [21].

Methods

Extraction

The Fresh fruit of *Momordica charantia* L (MC) was mashed and macerated with 70% ethanol (1:10 w / v) at room temperature, using a maserator for 6 hours, and the product was then allowed to stand for 18 hours, filtered, and collected in a container. Therefore, a remaseration process was conducted with up to 2 repetitions, and the maserate was subsequently concentrated with a rotary evaporator, in order to obtain a thick extract, which is then dried with the freeze-drying method.

Making granules and test preparation

Granulation of the dried *Momordica charantia* L (MC) extract was performed by wet granulation method, using Avicel PH 102 fillers and PVP binder. Furthermore, the test preparations were produced by dispersing MC extract granules at 0.5% Na-CMC.

Acute toxicity (LD_{50} evaluation)

The provision of test solutions was conducted with the administration of a single oral dose, according to the Organization for Economic Co-operation and Development (OECD) test no 425 [21,22]. Furthermore, all mice were weighed a day prior to the experiment, and reserved for 4 hours, followed by a random selection for testing. Therefore, the control group was only provided with CMC 0.5%, while the experimental group for acute toxicity was given a dose in stages, up to a maximum of 5000 mg / Kg.

The first observation was carried out individually at least 30 minutes to 3 hours after the administration, and then the observations were repeated on the 24th hour, up to 7 days, depending on the life status of mouse. Therefore, clinical and toxicity symptoms was evaluated for 14 days, through observation of tremors, convulsions, salivation, diarrhea, allergies, coma, reactions to the skin, eyes, righting reflexes and activity. In addition, the LD50 values were statistically determined following the principle of maximum likehood method, and then all doses and responses of test animals were entered into the AOT425StatPgm software.

Subchronic (28-days) toxicity

The administration of test preparations was performed through a method, referring to the Organization for Economic Co-operation and Development (OECD) test no. 408: Subchronic Oral Toxicity - Repeated Dose 90-day Oral Toxicity Study in Rodents [23]. The DDY strain mice were divided into one control (given 0.5% CMC-Na) and three experimental groups, where 40 mg / Kg, 80 mg / Kg and 320 mg / Kg doses were provided orally to each unit consisting of 6 female mice, daily for 28 days. Therefore, observation for reversibility was performed in the satellite group for 14 days post-administration. The experiment was initiated by acclimatizing the animals in the test environment for about 7 days, which were then grouped randomly to attain an even distribution in body weight to all groups, with variation of <20% from the average.

The observations included measurements of relative weights of the liver and kidneys, followed by clinical biochemical examination of the following parameters: AST, ALT, BUN, and Creatinine. On day 29, the blood samples of live mice were obtained through the orbital sinus, followed by the the killing of specimen (euthanasia) and subsequent dissection. Therefore, the organs of kidney and liver were taken, cleaned and weighed, and the relative weight was calculated based on the similarity between the value obtained for organ and body (relative weight = organ weight of mice / body weight of mice). In addition, the samples were cleaned again and weighed for histopathological examination.

Biochemical analysis

Clinical biochemical tests, including blood urea nitrogen (BUN), creatinine (CRE), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using Microlab 300 & 300 LX EliTech Clinical Systems.

Histopathological examination

The liver and kidneys are then soaked in 10% formalin solution for 48 hours, and then routinely processed, embedded in paraffin wax, cut into 2-3µm sections and stained. Subsequently, the organs were submitted to the Primate Animal Study Center, Bogor Institute of Agriculture, for further histopathological examination for possible organ damage, using a light microscope.

Statistical analysis

Data obtained is presented as mean ± SD, while the statistical analysis using the ANOVA one-way method, which was previously tested for normality (Kolmogorov-smirnov test) and homogeneity (Levene test). In addition, the inability to fulfil one of the ANOVA requirements, necessitated the conduction of a non-parametric Kruskal Wallis analysis, while least significance difference (LSD) method was used to further differentiate between the test groups. Moreover, all statistical analyzes were performed using the IBM Statistical Package for the Social Sciences (SPSS) V.22 program at a 95% confidence level ($p = 0.05$).

RESULTS

Acute toxicity (LD 50 evaluation)

The LD_{50} result for *Momordica charantia* L. (MC) extract granule greater than 5000 mg / Kg, hence included in the practically non-toxic category, as neither the smallest nor the largest dose showed any mortality within 24 hours to 14 days. The dose of 1750 mg / kg showed no symptoms of tremor, salivation, convulsions, coma, or allergies, alongside normal motor, eye, and skin activity, accompanied by the presence of righting reflexes in test animals up to day 14. Meanwhile, at 5000 mg / Kg, there was a marked decline in activity, characterized by the standing of mice fur, staying away and hiding. These symptoms are only visible for up to 30 minutes after administration, followed by the reversion of normal behaviors. However, tremor, salivation, coma, and

convulsions were not observed at the beginning up to the 14th day, and also no reactions were observed on the eye and skin. The results of body weight testing from the day before treatment up to the day 14 showed the absence of any conditions influencing the decline in consumption of food and beverage by test animals.

3.2. Subchronic toxicity

The test for weight from the day prior to treatment, up to the 28th day, showed the absence of any significant difference between each test group, based on the normal and homogeneous data distribution ($p > 0.05$). In addition, the relative weight of liver and kidney were not significantly different, although there was substantial variation between the average measures for hearts between each experimental group and the control. These weights serve as indicators for the evaluation of toxic effects [24], and the data shows a proportional increase in the relative value for kidney, in contrast with the administered dose, which was not significantly different between treatments (Table 1).

Table 1. Data on average relative weight of the liver and kidneys in a 28-day subchronic toxicity test.

Groups	The relative weight of the heart	The relative weight of the kidney
Control	0,0538 ± 0,0059	0,0049 ± 0,0004
40 mg/Kg	0,0546 ± 0,0053	0,0050 ± 0,0004
80 mg/Kg	0,0559 ± 0,0026	0,0051± 0,0003
320 mg/Kg	0,0628 ± 0,0127	0,0054 ± 0,0004

Biochemical analysis

Blood urea nitrogen (BUN) and creatinine (CRE) levels are indicators of kidney function, which the level of 22 mg / dL in control mice. The ethanol extracts of the fruit *Momordica charantia* L. (MC) led to an increase in BUN levels, following the elevation of doses (24.17, 29.5, and 32.5 mg / dL). Furthermore, the administration for 28 days in each dose group did not significantly increase the creatinine level. However, the decline in kidney function by about 50-70% is required to demonstrate elevated creatinine levels. The statistical tests showed substantial variation between the control and the experimental group, which contains higher values (Figure 1). The results showed the absence of any subsequent kidney damage, at levels less than twice the normal value. Following the administration of MC fruit extracts, a marked increase was observed in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which were proportional to the dose (Figure 1). Therefore, the provision of 40 mg / Kg, 80 mg / Kg, and 320 mg / Kg dosages showed no damages to the liver function, as the elevation in ALT and AST enzymes were less than twice the normal values [25,26].

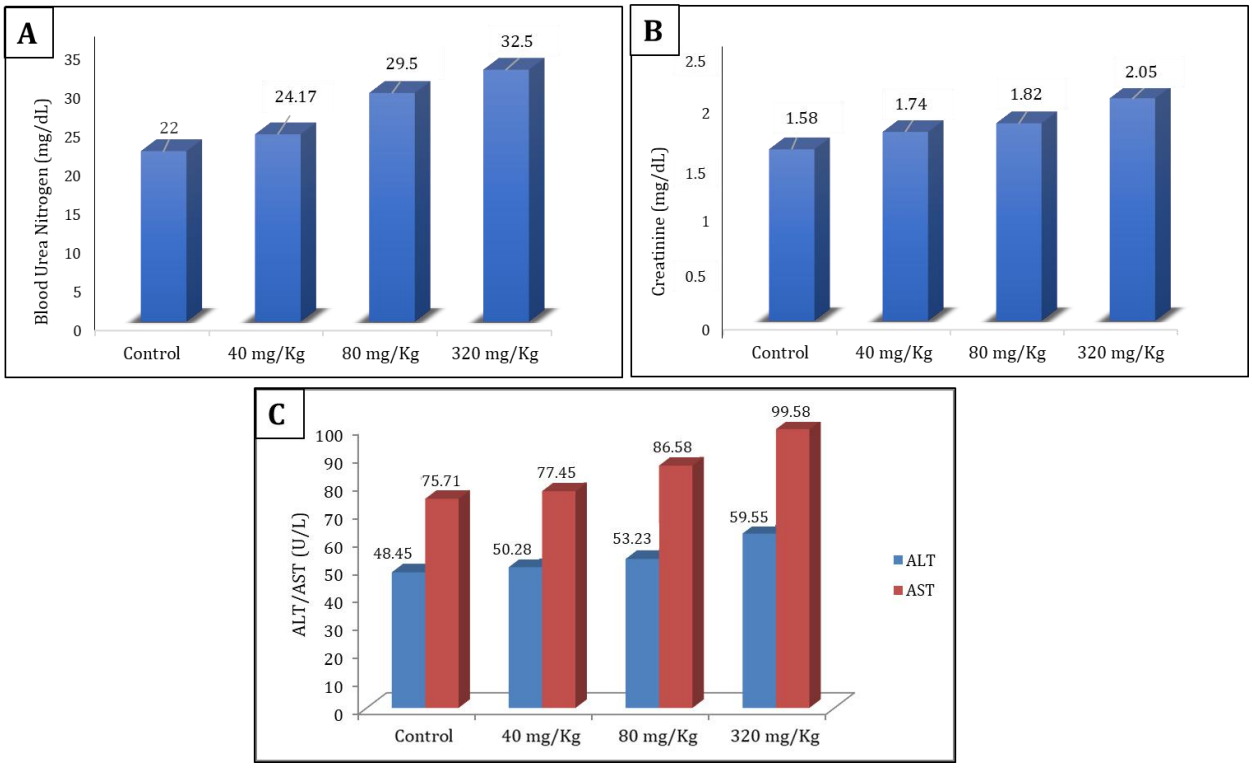
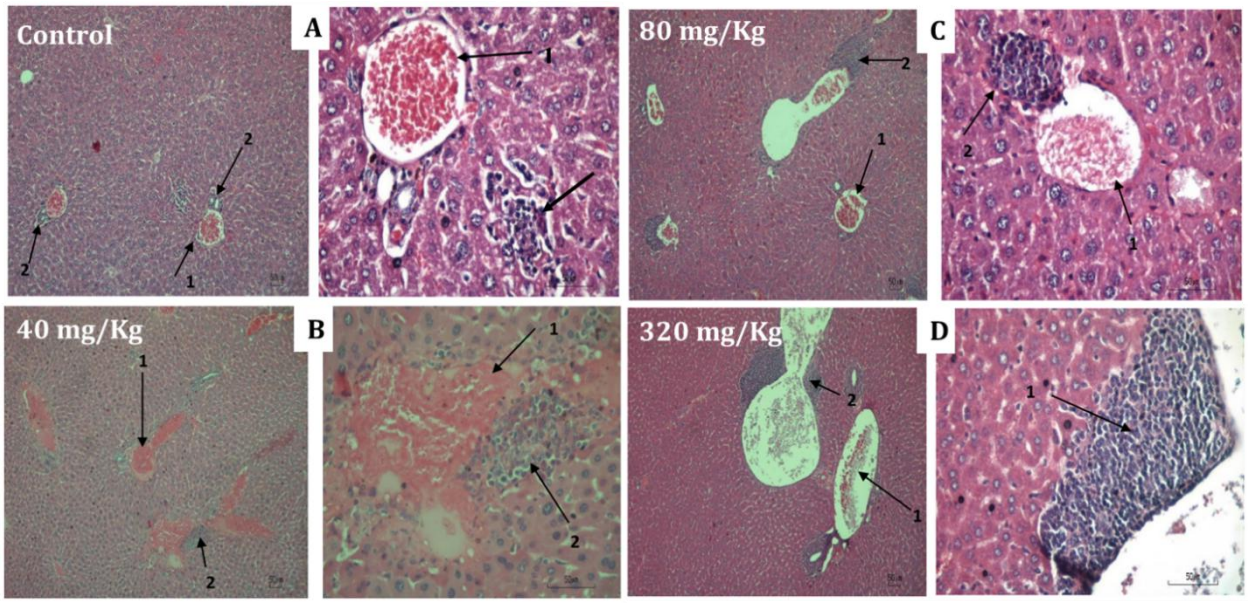


Figure 1. The effect of administering *Momordica charantia* L. (MC) ethanol extract on the average values of BUN (A), CRE (B), ALT, and AST (C), during subchronic toxicity test results (n = 6).

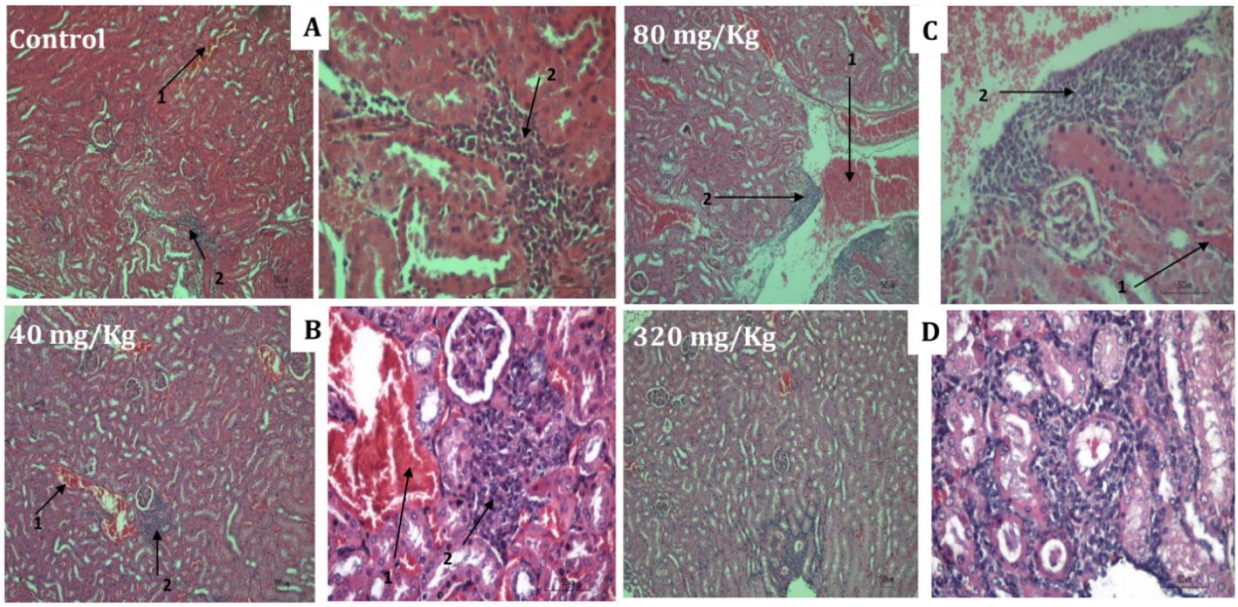
Histopathological examination

Therefore, changes in the histopathological pictures observed in the liver and kidneys include mononuclear inflammatory cell infiltration and vascular congestion, which was identified in all treatment groups. Furthermore, the liver examination showed changes in the infiltration of inflammatory cells, which were worse in relation with elevated doses. Therefore, the administration of higher *Momordica charantia* L. (MC) ethanol extract increases the propensity for liver toxicity (Figure 2). The histopathological examination results on the kidney of mice provided with the treatment did not demonstrate any necrosis or fat degeneration in either the control or test group, at 40 mg / Kg, 80 mg / Kg, and 320 mg / Kg doses (Figure 3).



Description: (1) vascular congestion (2) infiltration of mononuclear inflammatory cells

Figure 2. Histopathology of the liver in control mice (A), and treatment with *Momordica charantia* L. (MC) fruit ethanol extract at 40 mg / Kg (B), 80 mg / Kg (C), and 320 mg / Kg (D) doses



Description: (1) vascular congestion (2) infiltration of mononuclear inflammatory cells

Figure 3. Histopathology of the kidneys in control mice (A), and the treatment with *Momordica charantia* L. (MC) fruit ethanol extract at doses of 40 mg / Kg (B), 80 mg / Kg (C), and 320 mg / Kg (D)

DISCUSSION

The long history shows numerous experience-based traditional medical practices, passed on through the generations, which provides good effectiveness, although not proven scientifically. According to the WHO, the world's population relies on traditional medicine, hence the the need to thoroughly evaluate the safety and toxic effects of herbal ingredients [27].

Momordica charantia L. (MC) fruit is known to contain saponins, flavonoids, polyphenols, alkaloids,

triterpenoids, momordicin, cucurbitacin glycosides, charantin, butyric acid, palmitic acid, linoleic acid, and stearic acid [28,29]. In addition, the isolated momordicin components have been proven to possess antimalarial effects, both in vitro and in vivo, although there are limited scientific articles related to toxicity on the liver and kidneys.

This study involved a 28-day observation of acute and subchronic toxicity evaluation, particularly for the effects on the liver and kidneys. Furthermore, the acute toxicity, and LD50 assessment of MC fruit extract granules was over 5000 mg / Kg, as the treatment group remained alive without any symptoms of tremor, salivation, coma, convulsions, or reactions to the eyes and skin. However, a decline in motor activity, which is a symptom of toxicity, was only experienced for less than 30 minutes post-administration of large dose.

Subchronic toxicity evaluations were conducted within 28 days by administering the following doses: 40, 80, and 320 mg / Kg, followed by observations for effects on kidney and liver. The bioclinical parameters measured to determine kidney health include BUN and creatinine, while AST and ALT were used for the liver assessment [30]. Furthermore, urea nitrogen was identified in the blood and is subsequently excreted through the kidney tubules, and the value obtained describes the excretion function. Normally, urea is only reabsorbed in the distal tubules and also in the proximal tubules, where about 40% of the filtered urea is reabsorbed in the proximal tubules [31]. In addition, a significant increase was observed at high doses (320 mg / kg) although approaching the higher limit but still within the normal range (10-33 mg / dL).

Creatinine levels were higher in the treatment groups, compared to the control ($p < 0.05$), although not an indicator of kidney damage. This is because according to Spitalnik (2015) kidney damage is characterized by an increase in blood creatinine levels, reaching twice the normal range, and persistent for over two weeks [32]. Furthermore, this manifestation occurs because creatinine is a waste product of muscle metabolism within the body and is mainly filtered out through the kidneys [33].

The results of kidney histopathology show the presence of congestion in blood vessels as well as infiltration of inflammatory cells in treatment groups, administered doses of 40, 80, and 320 mg / Kg. These possibly influence the rate of creatinine excretion and are also indicative of more severe changes in line with increasing doses, as seen in the outcome of biochemical analysis.

The process of kidney filtration is not possible, and the chronic persistence of this situation is assumed to influence other healthy tubules, subsequently causing a common blockage that leads to kidney failure [34]. In addition, a research conducted by Mardani *et al*. (2014) showed the propensity for the single dose administration of 4000 mg / kg MC to not cause damage to kidney tissue, although a week of exposure led to some pathological changes. The histological study of Nazrul-Hakim *et al*, showed the inability for the administration of up to 1000 mg / Kg MC for 72 hours to cause changes in the kidney structure of mice [35,36].

In this study, there was an increase in the level of AST and ALT enzyme, which serve as liver health biomarkers in the treatment group, compared to the control. This effect was dose dependent, as higher amounts elevates the work of the liver in terms of metabolizing the drug, which is subsequently excreted through urine or bile. This process allows for a conversion into more toxic compounds with the propensity to cause inflammation and impairments. According to Price and Wilson (2003), liver damage is only demonstrated by at least a two-time increase, up to 20-100 times in the activity of ALT and AST enzymes from the normal levels, which lasts for two to six weeks [26]. This event often results from injury to the hepatocyte cell membrane, leading to the escape of some enzymes. Therefore, the provision of MC fruit extracts in doses of 40, 80, and 320 mg / Kg is not implicated in any form of destruction to liver function due to the inability to increase ALT and AST enzymes above twice the normal value.

The histological picture of liver cells demonstrated mild to severe inflammation of the phagocytic cells, including the monocyte and liver polymorphonuclear of mice. Moreover, at a dose of 320 mg / kg, inflammatory cell infiltration occurred with sinusoid dilatation. This is in line with the study by Kietzmann's (2006), where the area of necrosis was also observed to have enlarged sinusoids, a conscientious liver with distorted structure [37].

Prior studies conducted by Narendra S. Deshmukh (2016) show the inability for MC fruit extracts administered at dose levels of 250, 500, and 1000 mg / kg per day for 28 days to alter hematological and blood chemistry parameters, and also urinalysis. In addition, there were no absolute and relative changes in organ weights, as well as pathological, and histopathological modification in all organs. Meanwhile, a study conducted by Ume Kalsoom (2014) reported on the presence of differences in the parameters of hemoglobin and platelets, which increase in the value at high and low doses, respectively [38,39].

The variation in results possibly results from numerous factors, including the instrument used, conditions at the time of measurement, the method of collecting samples and the number taken. Furthermore, the preparation techniques, as well as the number of anticoagulants used, were also identified as possible influences on the measurement outcome. Conversely, the handling of blood samples, including medium, pH, temperature, tonicity, mechanical treatment, and others have been associated with the results of hematological examination.

CONCLUSION

Based on the results and discussion, the LD_{50} of MC fruit extract granules was estimated to be over 5000 mg / Kg. Also, the administration of 40, 80, and 320 mg / Kg doses for 28 days had no detrimental effect on liver and kidney function. This was due to the high levels of ALT and AST enzymes, which were less than twice the normal value, as well as the poor elevation in creatinine, although a significant increase only occurs on instances where about 50-70% decline in kidney function is reported.

Declaration of Competing Interest

The authors declare no conflflict of interest.

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