Original Article

Evaluation of dermal toxicity study of ethanolic extract of *Morinda citrifolia fruit* in Spraque Dawley rats

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Abstract

Plant-based preparations are widely known to complement dermatological therapy, however toxic profiles of these plants are lacking and limited. To investigate the toxicity of *Morinda citrifolia* fruit ethanol extract applied to the skin of rats daily for 28 days, thirty female Sprague Dawley rats were grouped into five groups (n=5 rats for each group) for each study. The experimental period was set at 14 days and 28 days for acute and subacute dermal toxicity, respectively. Rats in the treatment groups were topically applied with the plant extract at three different doses; 2.5%, 5% and 10% on a shaved area of dorsal skin. For the acute toxicity study, rats received a single application of the extract on the first day of study, whereas rats in the subacute toxicity study were applied with the extract every single day for 28 days. All rats were observed for changes in appearance and behaviour and were assessed for changes of body weight, organ weight, haematology, serum biochemistry and histopathological analyses as a result of the interaction between the extract and the skin. The study indicated no mortality and sudden changes in physical appearances and behaviour of the rats. The results depicted no significant changes ($p > 0.05$) in the body weight, relative organ weight, haematological and biochemical values. No microscopic changes were detected in the assessment of the liver, kidneys and skin. It is demonstrated that *M. citrifolia* fruit extract is non-dermal toxic and these results may be useful in determining dosages for further pre-clinical evaluation and product development.

Keywords: dermal toxicity, haematology, histology, liver enzymes, kidney parameters, *Morinda citrifolia*

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Introduction

The clinical use of *M. citrifolia* for dermatological purposes has been reported, however data on its dermal toxicity is still lacking. Previous study has documented irritant contact dermatitis, phytophotodermatitis and delayed hypersensitivity contact reactions developing upon application of certain herbal plants (Mantle *et al*., 2001). In addition, some of the herbal plants can be absorbed through the skin and cause systemic toxicity including nephron-, hepato-, neuro- and cardiovascular-related toxicity. This study was conducted to assess the toxicity effects of *M. citrifolia* fruit extract upon 14 and 28-day repeated topical application on Sprague Dawley rats' skin. The experiments were carried out following the OECD guidelines No. 402 (acute dermal toxicity) and No. 410 (subacute dermal toxicity).

Materials and Methods

Plant collection and extraction: *Morinda citrifolia* ripe fruit was obtained from the Malaysian Agricultural and Research Development Institute (MARDI), Muadzam Shah, Pahang (Figure 1). The ripe fruit was sliced, dried and ground into powder form before being mixed with ethanol at a ratio of 0.20 g sample to 400 mL of 100% ethanol for one day. Ethanol was removed from the mixture by filtration and evaporation using a rotor paper evaporator (R-215, Buchi rotavaporator, Switzerland). The percentage yield was found at 10% and the ethanolic extract which contained phenolic compounds mainly anthraquinones (damnacanthal, morindone and morindin, etc) was kept in the refrigerator at 4°C for later use.

Figure 1 *M. citrifolia* ripe fruits.

Ointment preparation: Ten percent (w/w) ointment of *M. citrifolia* fruit extract was formulated by incorporating 10g of the extract in 100g of white paraffin. Similar preparations were also done for 2.5% and 5.0% (w/w) ointments of the extract, accordingly.
Animal husbandry: Thirty female Sprague Dawley rats were used in each of the toxicity studies (n=60). Animals were housed in polypropylene plastic cages in a ventilated room at a room temperature of 22±2°C with 12 hours light/dark cycle at the Animal Metabolism, Toxicity and Reproductive Centre (AMTREC), MARDI, Serdang, Selangor. Animals were acclimatised for 7 days to the laboratory conditions prior to experimentation and were fed with commercial rat pellets and water *ad libitum*. The experimental procedure was authorised by the Animal Care and Use Committee (ACUC), MARDI.

Skin preparation for dermal toxicity study: Skin preparation was conducted under general anaesthesia and the rats received an intramuscular injection of ketamine at 50 mg/kg and xylazine at 5 mg/kg. Fur at the dorsal thoracic region of the rats was shaved using a razor blade. The shaved area (2 cm width x 5 cm length) was applied with herbal extract, vehicle (white paraffin) or no application (Figure 2).

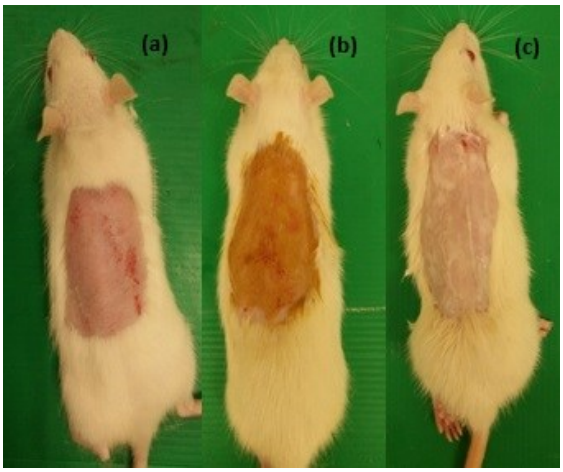

Figure 2 Shaved area of skin in negative control (a), applied with herbal extract (b) and white paraffin (c) rats. Note: Minimal haemorrhages on the skin due to shaving procedure prior to the experiment were wiped off with 70% alcohol.

Experimental design for acute and subacute dermal toxicity study: All rats were randomly divided into 5 groups (n= 6 rats) in each study, according to OECD 402 (acute dermal toxicity study) and 410 (subacute dermal toxicity study) guidelines as follows:

Group 1: Treatment group; animals received 2.5 % of the extract, topically.
Group 2: Treatment group; animals received 5.0 % of the extract, topically.
Group 3: Treatment group; animals received 10.5 % of the extract, topically.
Group 4: Vehicle control group; animals received paraffin.
Group 5: Negative control group; animals received no treatment.

Rats in the acute dermal toxicity study were applied with extract on the skin once on the first day of the experiment and were monitored until day 14 of the study. Meanwhile, rats in the sub-acute dermal toxicity study received a daily application of the extract for 28 days. For each treatment group, approximately 3 g of herbal extract was applied topically on the skin of the rats.

General observation of signs and behaviour of rats: Each rat was monitored constantly during the first 4 hours, regularly for the first 24 hrs and twice daily for 14 or 28 days. All the rats were monitored for changes in their behaviour, for example, abnormal body posture, breathing and sleep pattern and any signs of tremor, convulsions, lethargy, hypoxia and salivation that might develop due to toxicity. Impairment in the physical appearance of the fur, skin, eye and mucous membranes was also monitored. Alterations in body weight and feed and water intake were recorded.

Euthanasia and necropsy: All rats were humanely euthanized using the carbon dioxide inhalation method. Gross pathological examination of the major internal organs (the liver and kidneys) and skin was conducted. Blood samples were obtained from the posterior vena cava for haematological and biochemical analyses in both treated and control groups. The kidneys and livers of all rats were preserved in 10% buffered formalin after determining the relative organ weight for histopathological evaluation.

Haematological and serum biochemical analysis: An automated haematology analyser (Cell Dyn, 3700, Abbot, USA) was used to analyse the blood samples for the total red blood cell (RBC), white blood cell (WBC), platelet count and haemoglobin concentration. For the determination of packed cell volume (PCV), icteric index and plasma protein concentration, a microhematocrit tube was filled with the EDTA blood. Blood smears were stained with Wright's staining for determination of a manual WBC differential count. An automated biochemistry analyser (TRX 7070, Biorex, Germany) was used to determine the serum concentrations of alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate transaminase (AST), creatine kinase (CK), creatinine, urea, total protein and albumin. Estimated globulin values were calculated by subtracting the albumin from the total protein.

Histopathological evaluation: Skin, liver and kidney samples were fixed in 10% neutral buffered formalin for 48 hours and trimmed to 0.5 cm thickness and placed in plastic cassettes before being processed automatically in an automated processor (Leica ASP300, Germany). The tissue samples were later embedded with paraffin, followed by trimming and sectioning at 4 µm thickness using a rotary microtome (Leica RM 2155, Germany). The tissue sections were mounted on glass slides, placed on a hot plate (Leica H11220, Germany) and allowed to dry overnight. Afterwards, tissue section deparaffinisation using xylene took place for 5 minutes, followed by rehydrating with two changes of different ethanol dilutions (100% and 80%) for 5 minutes each, respectively. The tissue sections were then rinsed in tap water and stained with Haematoxylin and Eosin (H&E) staining. Tissue sections were examined and scored under a light microscope and photomicrographs were taken at ×10 and ×40. Hepatic architectural changes were scored based on the presence of activated Kupffer cells, necrosis, inflammation, degeneration and regeneration. For the kidneys, the architectural changes were assessed for the presence of granular, cellular and protein casts, necrosis, inflammation and degeneration. Meanwhile, skin tissues were examined for evidence of degeneration, necrosis, inflammation, hyperkeratosis and epidermal hyperplasia. All the lesions were scored as 0: no lesion observed, 1 (mild = 1-30%), 2 (moderate = 31-69%) and 3 (severe = 70-100%) (Nurul *et al*., 2018).

Statistical analysis: Bodyweight, organ weight, haematology and serum biochemistry data was statistically analysed using Statistical Package for Social Science (SPSS) software version 23. The values were reported as mean ± standard errors of the mean (SEM). Analysis of variance (ANOVA) tests was done to compare the differences of data between and within groups. Differences were considered significant at $p < 0.05$ using the Duncan test. Histopathological scoring results were expressed as mean ± SEM. Kruskal-Wallis test for global comparison of organ lesions in all groups was performed and comparison between the two groups was analysed using Mann-Whitney-U test.

Results

General signs and behaviour of rats: During the 24-hour observation period, no group of rats in either toxicity study displayed any significant changes or impairment in their behaviour, body appearance (skin, fur, eyes, body posture), breathing and respiratory patterns and feed and water consumption. During the 14 and 28 days of the experimental periods, there were no signs of toxicity or mortality noticed.

Body and relative organ weight: Body weights (Figures 3 and 4) and relative organ weight (Table 1) measurements in both toxicity studies showed no significant ($p>0.05$) changes compared to the controls.

Haematology and serum biochemistry analyses: There were no significant changes ($p>0.05$) observed in the erythron, thrombon and leukon parameters in any groups in both studies compared to the control groups (Tables 2 and 3). Results of serum biochemistry for kidney parameters, liver enzymes, muscle enzymes and total serum protein concentration were within normal ranges and demonstrated no significant changes ($p>0.05$) compared to the control rats (Tables 4 and 5).

Gross and histopathological findings: The gross morphology of the liver, kidneys and skin of rats in both studies showed no abnormal findings. No histopathological changes were noted in any group (Figures 5, 6 and 7).

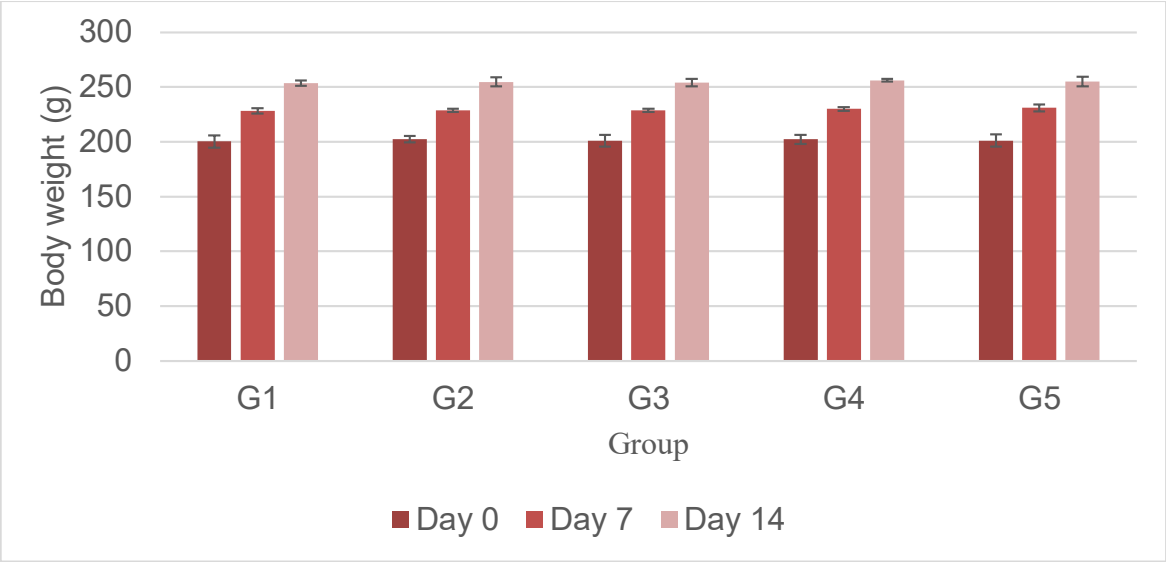

Figure 3 Mean body weights (g) of rats in acute dermal toxicity study of *M. citrifolia* fruit ethanolic extract. Note: G1: 2.5%, G2: 5% *M.*, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups. All values (mean±SE) are insignificantly different (p>0.05) between groups.

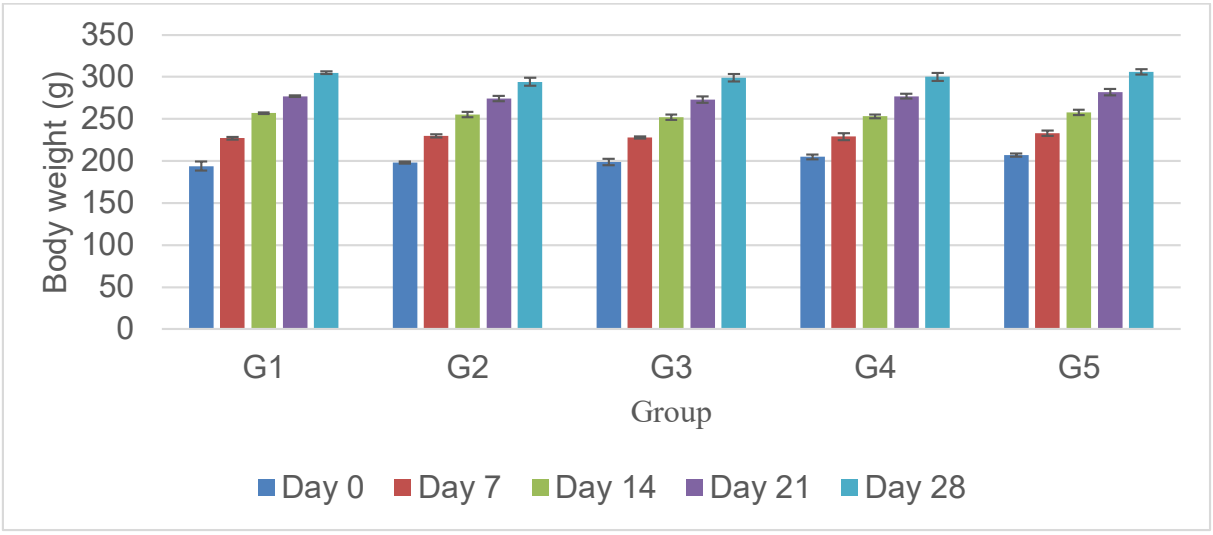

Figure 4 Mean body weights (g) of rats in all groups in sub-acute dermal toxicity study of *M. citrifolia* fruit ethanolic extract. Note: G1: 2.5%, G2: 5%, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups. All values (mean±SE) are insignificantly different (p>0.05) between groups.

Table 1 Relative organ weights of all rats in acute and subacute dermal toxicity study of *M. citrifolia* fruit ethanolic extract

Group	Liver	Kidneys	Spleen
Acute dermal toxicity study			
G1	3.34 ± 0.05[a]	0.84 ± 0.01[a]	0.26 ± 0.01[a]
G2	3.37 ± 0.06[a]	0.83 ± 0.01[a]	0.25 ± 0.01[a]
G3	3.39 ± 0.04[a]	0.86 ± 0.01[a]	0.27 ± 0.02[a]
G4	3.47 ± 0.09[a]	0.85 ± 0.02[a]	0.25 ± 0.02[a]
G5	3.44 ± 0.06[a]	0.88 ± 0.02[a]	0.27 ± 0.01[a]
Subacute dermal toxicity study			
G1	3.68 ± 0.02[a]	0.81 ± 0.01[a]	0.29 ± 0.01[a]
G2	3.67 ± 0.02[a]	0.82 ± 0.01[a]	0.24 ± 0.01[a]
G3	3.70 ± 0.02[a]	0.79 ± 0.01[a]	0.25 ± 0.01[a]
G4	3.73 ± 0.07[a]	0.82 ± 0.03[a]	0.24 ± 0.02[a]
G5	3.73 ± 0.07[a]	0.80 ± 0.02[a]	0.23 ± 0.01[a]

Values in the same row with similar superscripts are not significantly different at p>0.05. Note: G1: 2.5% *M. citrifolia*, G2: 5% *M. citrifolia*, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups.

Table 2 The values of erythron, thrombon and plasma protein values (mean ± SEM) of rats in acute and subacute dermal toxicity study of *M. citrifolia* fruit ethanolic extract

Parameter	G1	G2	G3	G4	G5
Acute dermal toxicity study					
RBC ($x10^{12}$/l)	7.61±0.22[a]	7.88 0±0.28[a]	7.59±0.24[a]	7.76±0.40[a]	7.64±0.41[a]
Hb (g/l)	155.97±0.56[a]	154.93±2.67[a]	155.93±0.87[a]	154.1±3.20[a]	156.36±2.06[a]
PCV (l/l)	39.97±1.15[a]	41.88±0.10[a]	40.98±1.84[a]	39.0±1.40[a]	42.16±1.38[a]
PP (g/l)	71.77±1.12[a]	70.86±0.78[a]	70.58±0.83[a]	72.80±1.62[a]	74.73±0.81[a]
MCV (fl)	63.95±0.39[a]	63.67±0.86[a]	64.98±0.49[a]	62.87±1.05[a]	63.54±0.61[a]
MCHC (g/l)	304.82±3.89[a]	304.15±7.23[a]	311.58±11.94[a]	341.23±4.34[a]	308.2±5.77[a]
Icterus Index	2.00±0.00[a]	2.00±0.00[a]	2.00±0.00[a]	2.00±0.00[a]	2.00±0.00[a]
Platelets (10^9/l)	1432.60±22.23[a]	1464.92± 28.80[a]	1422.49± 41.69[a]	1414.44± 45.07[a]	1450.98± 17.03[a]
Subacute dermal toxicity study					
RBC ($x10^{12}$/l)	7.34±0.23[a]	7.20±0.11[a]	7.28±0.11[a]	7.23±0.15[a]	7.45±0.08[a]
Hb (g/l)	151.93±2.30[a]	144.26±2.72[a]	147.91±5.27[a]	145.29±1.97[a]	146.45±2.31[a]
PCV (/l)	42.17±0.72[a]	41.98±0.43[a]	42.68±0.73[a]	43.0±1.47[a]	44.11±0.92[a]
PP (g/l)	77.34±0.84[a]	76.08±1.24[a]	75.41±1.10[a]	60.60±15.21[a]	76.29±2.85[a]
MCV (fl)	60.83±0.41[a]	61.03±0.79[a]	60.20±0.86[a]	60.76±1.22[a]	59.87±0.79[a]
MCHC (g/l)	332.60±9.39[a]	334.49±9.25[a]	346.71±4.00[a]	326.80±4.63[a]	325.80±1.53[a]
Icterus Index	2.00±0.00[a]	2.00±0.00[a]	2.00±0.00[a]	2.00±0.00[a]	2.00±0.00[a]
Platelets (10^9/l)	1430.12± 65.69[a]	1465.35± 104.21 [a]	1337.84± 67.46[a]	1319.17± 134.46[a]	1478.91± 67.76[a]

Values in the same row with similar superscripts are not significantly different at p>0.05. Note: G1: G1: 2.5% *M. citrifolia*, G2: 5% *M. citrifolia*, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups.

Table 3 The values of leukon parameters (mean ± SEM) of rats in acute and subacute dermal toxicity study of *M. citrifolia* fruit ethanolic extract

Acute dermal toxicity study					
Parameter	**G1**	**G2**	**G3**	**G4**	**G5**
WBC($x10^9$/l)	6.51 ± 0.55[a]	6.88 ± 0.31[a]	6.36 ± 0.33[a]	6.94 ± 0.51[a]	6.84 ± 0.45[a]
Neu ($x10^9$/l)	1.18 ± 0.12[a]	1.30 ± 0.08[a]	1.19 ± 0.12[a]	1.24 ± 0.12[a]	1.27 ± 0.16[a]
Lym ($x10^9$/)	4.79 ± 0.43[a]	4.38 ± 0.23[a]	4.49 ± 0.25[a]	4.93 ± 0.31[a]	5.02 ± 0.38[a]
Mo ($x10^9$/l)	0.47 ± 0.06[a]	0.44 ± 0.04[a]	0.43 ± 0.04[a]	0.42 ± 0.05[a]	0.47 ± 0.07[a]
Eos ($x10^9$/l)	0.19 ± 0.02[a]	0.18 ± 0.04[a]	0.16 ± 0.03[a]	0.14 ± 0.01[a]	0.15 ± 0.02[a]
Baso ($x10^9$/l)	0.09 ± 0.02[a]	0.10 ± 0.02[a]	0.08 ± 0.01[a]	0.06 ± 0.02[a]	0.07 ± 0.02[a]
Subacute dermal toxicity study					
WBC($X10^9$/l)	6.58 ± 0.24[a]	6.29 ± 0.34[a]	6.27 ± 0.25[a]	6.70 ± 0.92[a]	6.87 ± 0.33[a]
Neu ($x10^9$/l)	1.22 ± 0.10[a]	1.21 ± 0.14[a]	1.23 ± 0.09[a]	1.17 ± 0.09[a]	1.09 ± 0.07[a]
Lym ($x10^9$/l)	4.67 ± 0.18[a]	4.59 ± 0.24[a]	4.39 ± 0.22[a]	4.70 ± 0.61[a]	4.95 ± 0.11[a]
Mo ($x10^9$/l)	0.55 ± 0.14[a]	0.66 ± 0.10[a]	0.57 ± 0.07[a]	0.43 ± 0.04[a]	0.43 ± 0.05[a]
Eos ($x10^9$/l)	0.15 ± 0.02[a]	0.16 ± 0.03[a]	0.16 ± 0.03[a]	0.14 ± 0.02[a]	0.15 ± 0.01[a]
Baso ($x10^9$/l)	0.12 ± 0.01[a]	0.08 ± 0.01[a]	0.09 ± 0.02[a]	0.07 ± 0.01[a]	0.07 ± 0.00[a]

Values in the same row with similar superscripts are not significantly different at p > 0.05. Note: G1: G1: 2.5% *M. citrifolia*, G2: 5% *M. citrifolia*, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups.

Table 4 Renal and hepatic serum biochemical parameters (mean ± SEM) of rats in acute and subacute dermal toxicity study of *M. citrifolia* fruit ethanolic extract

Acute dermal toxicity study					
Parameter	**G1**	**G2**	**G3**	**G4**	**G5**
Urea(mmol/l)	6.01 ± 0.11[a]	6.02 ± 0.19[a]	6.20 ± 0.09[a]	6.02 ± 0.25[a]	5.90 ± 0.26[a]
Crea (µmol/l)	63.20 ± 1.15[a]	52.51 ± 1.30[a]	62.66 ± 0.33[a]	63.0 ± 1.92[a]	63.0 ± 2.78[a]
ALT (U/l)	71.33±4.24[a]	68.93±1.88[a]	70.61±1.23[a]	72.00±7.76[a]	73.48±4.79[a]
ALP (U/l)	207.12±10.96[a]	204.68±10.31[a]	188.45±3.53[a]	214.0±22.26[a]	226.40±13.32[a]
Subacute dermal toxicity study					
Urea(mmol/l)	7.10 ± 0.09[a]	6.96 ± 0.15[a]	7.14 ± 0.18[a]	7.62 ± 0.60[a]	6.23 ± 0.21[a]
Crea (µmol/l)	70.53 ± 0.64[a]	71.52 ± 0.72[a]	69.93 ± 2.55[a]	68.60 ± 1.60[a]	71.50 ± 2.60[a]
ALT (U/l)	59.98 ± 2.02[a]	59.32 ± 1.93[a]	59.54 ± 2.54[a]	62.91 ± 4.59[a]	56.53 ± 3.39[a]
ALP (U/l)	153.73±6.41[a]	152.70±6.06[a]	151.72±5.84[a]	152.89±8.59[a]	154.09±14.87[a]

Values in the same row with similar superscripts are not significantly different at $p > 0.05$. Note: G1: G1: 2.5% *M. citrifolia*, G2: 5% *M. citrifolia*, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups.

Table 5 Muscle enzymes and protein concentrations (mean ± SEM) in serum of rats in acute and subacute dermal toxicity study *M. citrifolia* fruit ethanolic extract

Acute dermal toxicity study					
Parameter	**G1**	**G2**	**G3**	**G4**	**G5**
AST (U/l)	173.32 ± 2.79[a]	170.90 ± 3.02[a]	170.91 ± 4.88[a]	179.82 ± 19.88[a]	176.17 ± 9.75[a]
CK (U/l)	1364.85 ± 125.67[a]	1480.49 ± 133.23[a]	1371.77 ± 63.46[a]	1298.69 ± 183.41[a]	1389.56 ± 342.11[a]
TP (g/l)	72.70 ± 0.93[a]	73.38 ± 1.16[a]	73.44 ± 0.94[a]	74.6 ± 2.17[a]	71.64 ± 1.13[a]
Alb (g/l)	43.39 ± 1.29[a]	42.65 ± 1.22[a]	41.42 ± 0.83[a]	44.16 ± 1.37[a]	42.80 ± 2.58[a]
Glob(g/l)	29.41 ± 1.58[a]	30.73 ± 1.88[a]	32.02 ± 1.22[a]	30.84 ± 1.33[a]	29.26 ± 3.04[a]
Subacute dermal toxicity study					
AST (U/l)	203.32 ± 5.53[a]	202.18 ± 5.31[a]	201.25 ± 3.85[a]	213.88 ± 12.23[a]	214.96 ± 11.80[a]
CK (U/l)	1467.64 ± 36.96 [a]	1427.12 ± 46.64[a]	1455.32 ± 61.84[a]	1499.31 ± 96.72[a]	1567.58 ± 178.11[a]
TP (g/l)	61.02 ± 0.41 [a]	61.71 ± 0.83 [a]	61.57 ±1.30[a]	62.20 ±1.81[a]	60.87 ±1.56[a]
Alb (g/l)	48.93 ± 0.47 [a]	48.69 ± 0.89 [a]	47.97 ± 1.31[a]	47.90 ± 2.30[a]	49.44 ± 0.42[a]
Glob (g/l)	12.09 ± 0.70 [a]	13.03 ±1.71 [a]	13.60 ± 1.78[a]	11.32 ±1.95[a]	12.98 ± 0.99[a]

Values in the same row with similar superscripts ae not significantly different at $p > 0.05$. Note: G1: G1: 2.5% *M. citrifolia*, G2: 5% *M. citrifolia*, G3: 10% *M. citrifolia*, G4: negative control, G5: paraffin groups.

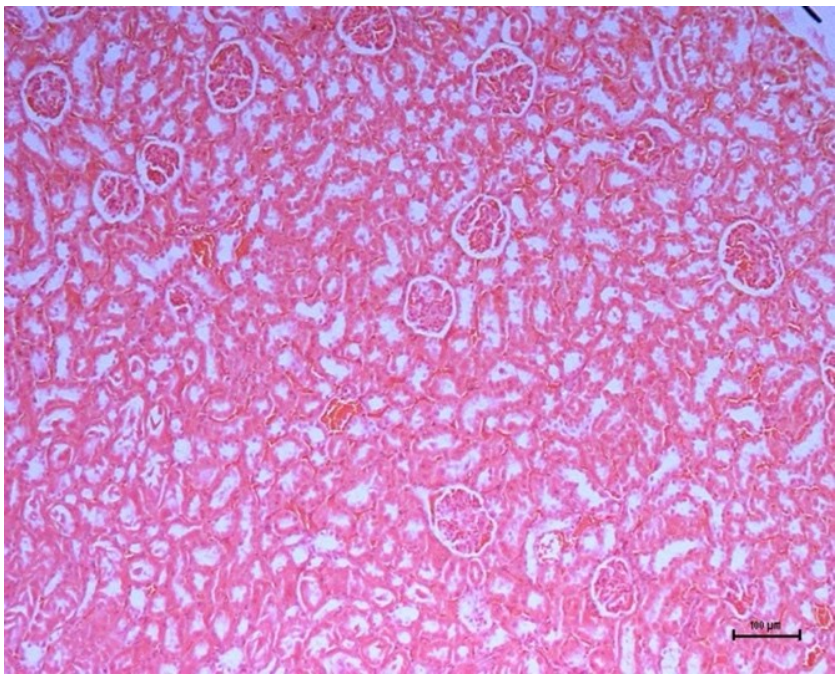

Figure 5 Histopathological section of kidney. There are no significant pathological lesions observed in all groups in acute and subacute dermal toxicity study of *M. citrifolia* fruit ethanolic extract. H&E staining x10 (scale bar: 100 µm).

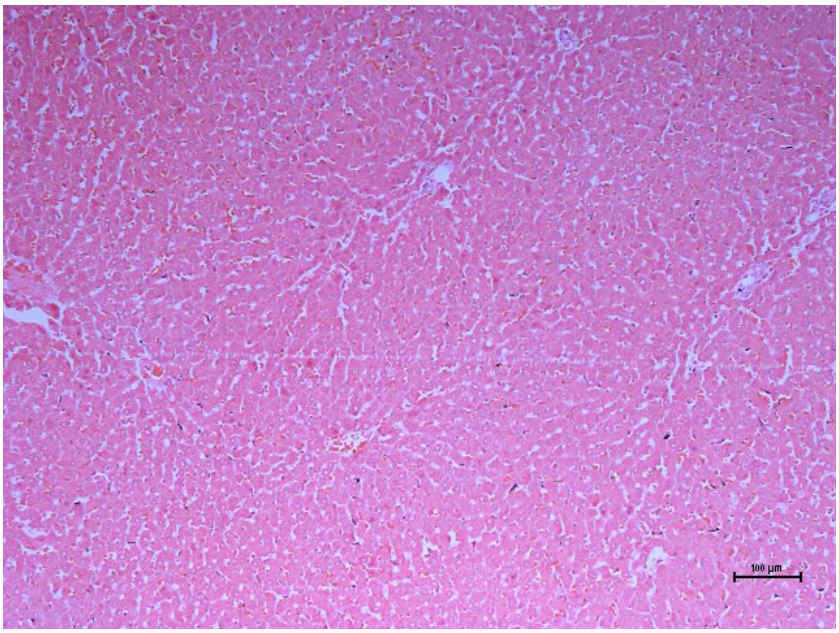

Figure 6 Histopathological section of liver. There are no significant pathological lesions observed in all groups in acute and subacute dermal toxicity study of *M. citrifolia* fruit ethanolic extract. H & E staining x10 (scale bar: 100 µm).

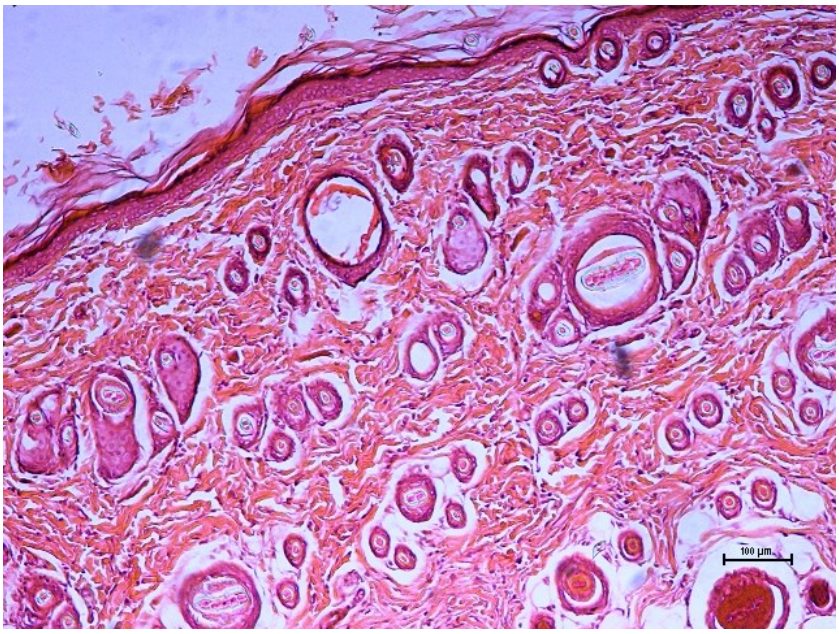

Figure 7 Histopathological sections of skin. Normal skin layers were observed in acute and subacute of dermal toxicity study of *M. citrifolia* fruit ethanolicextract. H&E staining x10 (scale bar: 100 µm).

Discussion

Several research articles have documented therapeutic uses of *M. citrifolia* as an important alternative medicinal product in various diseases (Matsuda *et al*., 2013; West, 2018; Almeida *et al*., 2019). The investigation of the plant's safety profile is vital to guide its dermal application management. Toxicity studies on incompatible animal models are widely used in evaluating possible human health risks. This can be achieved by examining blood samples and organ tissues. In this study, acute and subacute dermal application of female rats with *M. citrifolia* fruit ethanolic extract at doses of 2.5 %, 5.0% and 10.0% had no effect on mortality, toxic signs and the general behaviour of the rats. Therefore, the approximate median lethal dermal dose was determined to be higher than 10.0 % (w/w). Referring to the Hodge and Sterner toxicity scale, *M. citrifolia* is classified as a practically non-toxic herbal medicine (Hodge & Sterner, 2005).

Acute dermal toxicity is characterized by an adverse effect after dermal application within a short period of time of one single dose of a test substance (OECD guideline No. 402). Meanwhile, subacute dermal toxicity warns of possible health hazards resulting from repeated dermal exposures over a short period of time, such as 21 or 28 days (OECD guideline No. 410). All rats applied topically with the ethanolic extract of *M. citrifolia* fruit in both studies showed no signs of toxicity or mortality at the doses of 2.5%, 5% and 10% w/w. A similar finding was reported in the dermal toxicity analysis of *Melastoma malabathricum* where no toxic signs were observed at doses of 2.5%,

5.0% and 10% w/w after application of the plant extract (Reduan *et al*., 2020).

Changes in body weight are a sensitive and predictive toxicity marker caused by the drug tested. In the present study, there were no significant (p>0.05) changes in body weight observed in the treated groups compared with the controls after 14 and 28-days application of *M. citrifolia*. The results of this study are consistent with our previous studies on *M. citrifolia* fruit at concentrations of 2000 and 5000 mg/kg (acute dermal toxicity) and 500, 1000 and 2000 mg/kg (subacute dermal toxicity) (Ali *et al*., 2015). As optimal intake of nutrients is essential for the physiological health of the animal, it is also important to monitor water and feed intake during toxicological studies to determine the safety of herbal products. In the toxicity study, neither feed intake nor water consumption of the rats in all groups were affected by the topical application of *M. citrifolia* fruit. These results suggest that the extract is stable and has no direct effect on carbohydrate, protein and fat metabolisms. Similar findings have been reported by Maharan *et al*., (2019) who demonstrated the topical application of herbal cream containing *Zingiber officinale Roscoe* and *Phyllanthus amarus* extracts on the skin of mice once daily for 28 days showed no changes in feed intake and water consumption. In contrast to the present study, a significant decrease in the feed and water intake (p<0.001) and body weight (p<0.05) were reported in rats administered with *Caralluma dalzielii* (Ugwah-Oguejiofor *et al*., 2019).

The gross appearance of organs and their weight on necropsy are also important tools in assessing the safety of medicinal plants in a toxicity study. In this study, no gross abnormalities such as atrophy or hypertrophy were found. The relative organ weights in both the control and treated groups also showed no significant differences (p>0.05). In contrast, rats administered orally with *Citrullus colocynthis* methanol extract reported to have reductions in body weight and relative organ weight. Increases in the serum levels of liver enzymes and kidney parameters, total protein, uric acid and organ lesions have been shown to be associated with these changes (Soufane *et al*., 2017).

Blood serves as transporter and carrier for many drugs and xenobiotics in the body and plays an important role in the assessment of the toxic effects of medicinal plants. The toxicants may directly damage the mature cells in the circulation of the blood or indirectly suppress the cell precursors in the bone marrow, leading to reduced cell numbers in the circulatory system (Reduan *et al*., 2020). Haematological parameters of the rats in both studies revealed no significant (p>0.05) differences between the treated and control groups. The results are similar to our previous analysis of the acute and sub-acute dermal toxicity study of *M. citrifolia* fruit (Zahi *et al*., 2015). In contrast to this study, Ugwah-Oguejiofor *et al*., (2019) reported lymphocytes and mean platelet volume counts were significantly (p<0.05) increased in rats receiving oral administration of *Caralluma* dalzielii, which suggests the toxic potential of the plant.

Results seen from biochemical analyses suggest that *M. citrifolia* fruit ethanolic extract did not produce toxic effects in either study which indicates the absence of any harmful effects on the organs. Serum biochemicals parameters such as ALP, ALT and AST are among liver enzymes use for liver injury assessment (Fazliana *et al*., 2008; Tamsir *et al*., 2019; Arsad *et al*., 2014; Aliyu *et al*., 2020). Measurements of the levels of urea and creatinine in the blood are usually done to assess renal function (Dina *et al*., 2011; Pariyani *et al*., 2017). These two variables are frequently raised markedly to reflect renal toxicity. Previous studies showed an increase in ALT (Ugwah-Oguejiofor *et al*., 2019) and blood urea nitrogen and potassium concentration (Teshome *et al*., 2008) which suggests the toxic potential of the plant extracts.

Histological examination is the gold standard for evaluating the toxic effects of medicinal plants (Sajjaratul *et al*., 2016; Nurul *et al*., 2018; Aliyu et al., 2020). The lack of any histopathological changes in the kidneys and liver in both toxicity tests have been shown to be compatible with haematological and biochemical analyses indicating the safety of *M. citrifolia* ethanolic extract. In addition, the microscopic examination of the skins of rats treated with the extract in both studies showed no changes in the skin layers compared to the control rats. In contrast to the findings reported in this study, skin lesions of irritant contact dermatitis and granuloma were observed with the dermal application of *Pistacia lentiscus* fruit fatty oil (Djerrou *et al*., 2013). Besides, mice treated with 2000 mg/kg sesame oilseed showed degeneration and necrosis of the liver and focal degeneration and tubular dilation of the kidneys (Khan *et al*., 2016).

The results of these studies demonstrate *M. citrifolia* fruit ethanolic extract at concentrations of 2.5%, 5.0% and 10% is non-dermal toxic. These results may be useful in determining dosages for further pre-clinical evaluation and product development.

Conflict of interest statement: We declare that there is no conflict of interest.

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