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Pathological Evaluation of Subchronic Administration of *Moringa Oleifera* Ethanolic Leaf Extract on Mice

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Objectives: This research evaluated the consequences of repeated administration of *M. oleifera* ethanolic leaf extract (MOEL) orally on kidneys, liver and the blood of female Institute of Cancer Research (ICR)-mice.

Methods: Fifty (50) 8-week-old female mice were assigned into 5 groups of 10 mice each: groups 1 (control), 2 (125 mg/kg), 3 (250 mg/kg), 4 (500 mg/kg) and 5 (1000 mg/kg) for the sub-chronic toxicity studies of the extract. A 90-day repeated daily oral doses of MOEL extracts were administered to each mouse in the treatment groups through oral gavage. However, distilled water was administered to the control group (group 1). The mice were euthenised at the end of the experiments to collect and analysed samples.

Results: An obvious ($p < 0.05$) elevation in the levels of alanine aminotransferase (ALT) was observed in group 5 (437.50 $\pm$ 28.63 U/L) compared to 1 (239.10 $\pm$ 22.50 U/L), and then a significant ($p < 0.05$) elevation in aspartate aminotransferase (AST) concentration in group 5 (355.90 $\pm$ 26.45 U/L) compared to 1 (207.90 $\pm$ 19.67 U/L). Histopathological evaluation of the liver revealed a moderate liver degeneration indicated by moderate vacuolation of the cytoplasm in group 5 (1.70 $\pm$ 0.24) compared to 1 (0.35 $\pm$ 0.18), as well as mild hepatic necrosis characterised by mild eosinophilic cytoplasm (1.10 $\pm$ 0.3) of the hepatocytes in group 5 compared to 1 (0.00 $\pm$ 0.00). There was also a moderate renal cytoplasmic vacuolation in group 5 (2.20 $\pm$ 0.08) compared to 1 (0.00 $\pm$ 0.00). Moreover, a moderate to severe kidney necrosis indicated by significant ($p < 0.05$) eosinophilic cytoplasm was observed in groups 4 (1.95 $\pm$ 0.09) and 5 (2.45 $\pm$ 0.05) compared 1 (0.00 $\pm$ 0.00), pyknosis (0.90 $\pm$ 0.27) and karyolysis (0.60 $\pm$ 0.26) were significantly ($p < 0.05$) higher in groups 4 (1.15 $\pm$ 0.34) and 5 (1.75 $\pm$ 0.24) compared to 1 (0.00 $\pm$ 0.00).

Conclusions: It is concluded from this study that MOEL extract at high dose of 1000 mg/kg is associated with hepatic and renal toxicity in ICR-mice.

Keywords: sub-chronic toxicity; *Moringa oleifera*; hepatotoxicity; nephrotoxicity

Introduction

Moringa oleifera Lam of the Moringaceae family, is commonly called Horseradish, Drumstick or Ben oil tree in English and Pokok Kelor in Malay. It is a versatile pharmaceutical plant that is generally used in traditional Medicine [1]. The plant is naturally found in the Indian subcontinent, and Pakistan. Additionally, the plant is broadly grown in the African countries, Malaysia, India, Sri-Lanka, Mexico, America and the Philippines. The plant is likewise used in industries, as food (leaf and pods), and as a wholesome herb. In addition, the leaves, seeds, bark, sap, roots, oil and flowers parts of the plant have been reported to have important pharmacological activities [2]. Furthermore, the plant is important as food additive and phyto medicine for the cure of numerous illnesses, including starvation microbial, hyperlipidemic, cancer, hepatitis, arthritis, prostrate problems, rheumatism, etc., [2]. The pharmacological effects connected with *M. oleifera* could be due to the various phytocompounds present in various parts of the plant re-

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ported earlier; including phenolic compounds, carotenoids, flavonoids, polyphenol and vitamins [3,4].

Nevertheless, there is dearth of information on the toxic effect of long-term repeated administration of the ethanolic leaf extract of the plant on some vital organs, including kidneys and liver on laboratory animals. This is particularly imperative, because treatment of several chronic illnesses such as cancers involved repeated administration of the treatment for a longer period of 3 months or more [3]. A previous study from our research group [3] has shown that repeated daily administration of *M. oleifera* ethanolic leaf extract for as long as 28 days can have a toxic effects on the liver and kidney of mice [3]. It is therefore very necessary to assess the safety levels of this plant when administered repeatedly for 3 months, so that appropriate and safer doses for future treatment of chronic diseases in both humans or animals subjects could be suggested.

This research therefore was designed to investigate the effect of 3 months repeated daily administration of different doses of *M. oleifera* ethanolic leaf extract (MOEL) on the blood, liver and kidney of Institute of Cancer Research (ICR)-mice. The findings from this research would be valuable in the assessment of the plant's safety and the importance of plant natural products in healthcare management and medicine.

Materials and Methods

Plant Materials

The Institute of Bioscience, UPM provided the fresh leaves for this research. The leaves were processed as reported earlier by Aliyu *et al*. [3].

Botanical Identification

Botanical identification of the plant was conducted by Dr. Mohd Firdaus Ismail, at the Institute of Bioscience (IBS), UPM withthe voucher number: (SK3168/17) been deposited at the herbarium, UPM.

Extraction Procedure

The plant was extracted according to the method explained by [2,3,5,6] with some adjustments.

Sub-Chronic Toxicity Evaluation of Moringa Oleifera Ethanolic Leaf Extract

The research were carried out according to the guidelines described by the Organisation for Economic Co-operation and Development (OECD) 408 [7–10] with slight modifications [11]. The mice for this research were purchased from a market vendor from Selangor, Malaysia. The animals were supsequently kept at the Animal Metabolism, Toxicology and Reproductive Centre (AMTREC), Malaysian Agricultural Research Development Institute (MARDI), Serdang. An optimum housing condition with temperature of 22–25 ºC, relative humidity (40%–70%) and a cycle of 12 h light/12 h dark was used to acclimatize the mice as described earlier [3,11–13].

Fifty (50) 8-week-old female mice were allocated at random to five groups (1–5) of ten mice each using RCBD (randomized complete block design). Group 1 was given distilled water and used as control. The remaining four groups including groups 2, 3, 4 and 5 were respectively treated with 125, 250, 500 and 1000 mg/kg MOEL extract [suspended in 5% dimethyl sulfoxide (DMSO)]. The extract was given at the ratio of 1 mL/100g body weight daily once for 90 days via oral gavage using a stainless steel needle [3,11,13]. The mice were carefully monitored for any symptoms of toxicity and were then euthenised on day 91 of the research by the use of CO_2 chamber [3,11–13].

Analysis of Weekly Body Weight Gain

The bodyweight of each group of mice was monitored weekly by an electric weighing scale and documented as reported hitherto [3,9,11,13].

Samples Collection

The mice were euthenised on days 91 of the research, using CO_2 chamber (Labquip Sdn. Bhd, Malaysia) [3,11–14]. Blood samples were obtained through cardiac venipuncture by as reported previously [11], and transferred to clean anticoagulated (EDTA) sample bottles. The samples were then used to analyse haematological and biochemical parameters of the mice [15,16].

Post-mortem was done on each of the mice and tissue specimens from the kidneys, liver, heart, spleen, brain, uterus and lungs, were collected and analysed accordingly [3,12–14].

Haematological Analyses

The method described earlier [3,11–14] was adopted. The anticoagulated blood samples obtained earlier were transported to the Universiti Putra Malaysia, Faculty of Veterinary Medicine, Haematology and Clinical Biochemistry Laboratory, ice packs and evaluated for full blood count (FBC) using automated haematology analyser (ABC Vet®, ABX Diagnostics, Port-Saint-Louis-du-Rhône, France). The factors evaluated included total white blood cell count, total red blood cells count, haemoglobin concentration, platelet count, mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) [12, 17]. Thin blood smears were made and examined under a light microscope after been stained with Wright stain. Packed cell volume (PCV), concentration of plasma protein, differential white blood cell (WBC) count and the absolute values for each WBC type were determined according to the standard methods earlier [8,9,12,13].

Analysis of Plasma Biochemical Parameters

The anticoagulated blood samples taken earlier were centrifuged with the use of a bench centrifuge (Centrifuge

Table 1. Relative organ weights in % (mean ± SEM) of female ICR-mice treated with different doses of MOEL continuously for 90 days.

Organs (%)	1	2	3	4	5
Liver	6.49 ± 0.24	6.97 ± 0.36	5.85 ± 0.29	6.26 ± 0.72	5.92 ± 0.35
Right Kidney	0.88 ± 0.07	0.78 ± 0.04	0.75 ± 0.03	0.72 ± 0.04	0.72 ± 0.02
Left Kidney	0.81 ± 0.05	1.06 ± 0.30	0.72 ± 0.02	0.73 ± 0.06	0.66 ± 0.02
Spleen	0.63 ± 0.07	0.61 ± 0.06	0.70 ± 0.06	0.72 ± 0.07	0.46 ± 0.04*
Heart	0.68 ± 0.05	0.76 ± 0.03^a	0.80 ± 0.06	0.75 ± 0.05	0.63 ± 0.05
Lungs	1.41 ± 0.06	1.36 ± 0.08^a	1.28 ± 0.14	1.32 ± 0.13	1.57 ± 0.18
Brain	2.34 ± 0.09	1.88 ± 0.12*	1.58 ± 0.08*	1.78 ± 0.15^b	1.55 ± 0.10*
Uterus	1.22 ± 0.18	1.42 ± 0.20	1.31 ± 0.16	1.24 ± 0.15	1.22 ± 0.18

MOEL, *Moringa oleifera* ethanolic leaf extract; 1, control; 2, 125 mg/kg MOEL; 3, 250 mg/kg MOEL; 4, 500 mg/kg MOEL; 5, 1000 mg/kg MOEL. Values in rows with different superscripts or with asterisk are significantly ($p < 0.05$) different.

S417R, Eppendorf, Murrieta, CA, USA) at 3000 rpm for 15 minutes to separate the plasma from whole blood. The separated plasma sample was then evaluated using an automated clinical chemistry analyser (BioLis 24i Chemistry Analyzer, Tokyo Boeki Japan Ltd, Tokyo, Japan) for the presence of creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine kinase (CK), total protein (TP), globulins and albumin (ALB) [12,13,18].

Histopathological Evaluation of Liver and Kidneys

At the end of the experiments, liver and kidneys samples were obtained from individual mouse and prepared at the Histopathology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia according to the standard methods [3,8,11–13,19]. The processed tissue samples were stained using haematoxylin and eosin (H&E) stains, then evaluated accordingly [3,11–13].

Lesion Scoring

Lesions on the liver and kidneys including pyknosis, karyolysis, karyorrhexis, acidophilic cytoplasm, sinusoidal dilatation, activated Kupffer cells, regeneration, degeneration (cytoplasmic vacuolation), inflammatory changes, cellular degenerative change (hydropic degeneration), formation of epithelial and hyaline casts were scored for the respective stained section as reported earlier [3,8,9,11,13,20]. The scoring methods and severity of each lesion in liver and kidney are shown on Table 1.

Statistical Analysis

The findings from the experiments were reported as mean ± standard error of the mean (SEM). It was then evaluated by one-way analysis of variance (ANOVA) statistical tool via *IBM *SPSS statistics 23 (IBM Corp., Armonk, NY, USA) [12]. Statistical differences among the experimental groups (between three or more groups) were established by tukey post hoc test [12]. Conversely, the findings from the histopathological observation from the liver and kidneys were subjected to Kruskal Wallis H (non-parametric) test [12]. Significant was set at $p < 0.05$.

Results

Body Weight Gain

The effects of 90 days repeated administrations of various doses of MOEL on the mean body weight gain of the mice are demonstrated on Fig. 1. There were obvious ($p < 0.05$) changes in the body weight gain of the treated mice within the 13 weeks period of the study as shown by repeated measures ANOVA with a Greenhouse-Geisser correction and Bonferroni post hoc test (Fig. 1).

Analysis of Relative Organs Weight

The results of 90 days continuous oral administrations of varying dosages of MOEL on relative organs weights of female ICR-mice are described on Table 1. Significant ($p < 0.05$) difference was found among the groups using one-way ANOVA. Tukey post hoc test revealed that the mice administered with MOEL had 27% and 33.8% significant ($p < 0.05$) decreases respectively in the relative organ weight of spleen (0.46 ± 0.04) and brain (1.55 ± 0.10) in group 5 compared to 1 (0.63 ± 0.07 and 2.34 ± 0.09) (Table 1). Moreover, the relative organ weights of liver were 9.9% and 8.8% lower ($p > 0.05$) in groups 3 (5.85 ± 0.29) and 5 (5.92 ± 0.35) respectively, compared to 1 (6.49 ± 0.24). Likewise, the relative organ weights of right kidney were 11.4%, 14.8%, 18.2% and 18.2% lower ($p > 0.05$) in groups 2 (0.78 ± 0.04), 3 (0.75 ± 0.03), 4 (0.72 ± 0.04) and 5 (0.72 ± 0.02) respectively, compared to 1 (0.88 ± 0.07).

Evaluation of Haematological Parameters

Table 2 (Ref. [17]) presents the results of continuous oral gavage of varying doses of MOEL daily for 90 days on the blood parameters of female ICR-mice. Significant ($p > 0.05$) changes were not found among the groups, as shown by one-way ANOVA (Table 2). However, the haemoglobin concentrations were 4.6% greater ($p < 0.05$) in group 5 (165.67 ± 4.22 g/L) compared to 1 (158.40 ±

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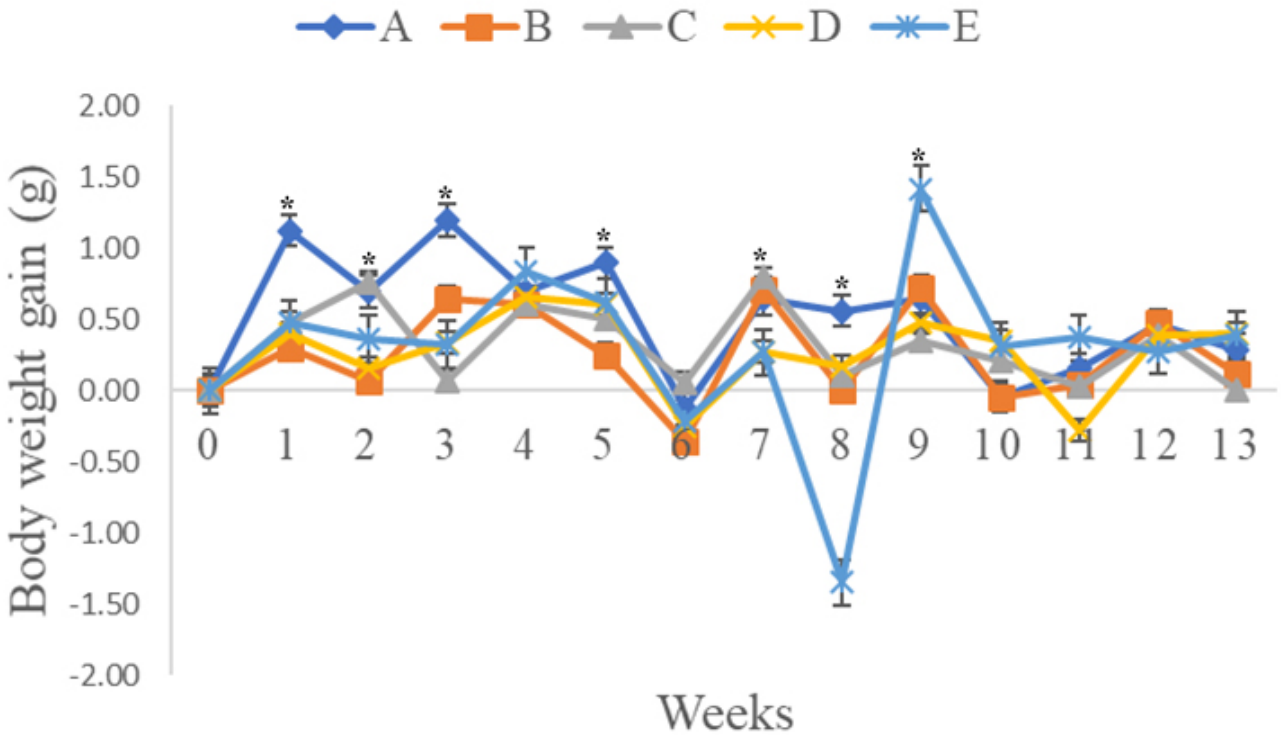

Fig. 1. Average weekly body weight gain (g) of female ICR-mice treated with different doses of MOEL continuously for 90 days. A, control; B, 125 mg/kg MOEL; C, 250 mg/kg MOEL; D, 500 mg/kg MOEL; E, 1000 mg/kg MOEL; *significantly different at $p < 0.05$; SEM, standard error of mean; MOEL, *Moringa oleifera* ethanolic leaf extract; ICR, Institute of Cancer Research.

3.27 g/L). Moreover, there were 10.6%, 36.7% and 16.8% elevation ($p > 0.05$) in the number of platelets in groups 3 ($963.60 \pm 154 \times 10^9$/L), 4 ($1191.90 \pm 149 \times 10^9$/L) and 5 ($1018.00 \pm 145 \times 10^9$/L) respectively, compared to 1 ($871.60 \pm 124 \times 10^9$/L). Correspondingly, the plasma proteins were 4.4%, 7.3% and 10% higher ($p > 0.05$) in groups 3 (81.00 ± 1.94 g/L), 4 (83.30 ± 2.87) and 5 (85.33 ± 2.51 g/L) respectively, compared to 1 (77.60 ± 3.34 g/L). Interestingly, the values for red blood cells, haemoglobin, and platelets in all the groups were within the reference limits (Table 2). Furthermore, the values for total white blood cell count (TWBC) were 26.7% higher ($p > 0.05$) in group 4 ($9.29 \pm 0.77 \times 10^{12}$/L) compared to 1 ($7.33 \pm 0.45 \times 10^{12}$/L). The lymphocytes in group 4 ($5.89 \pm 0.45 \times 10^9$/L) were 28.3% higher ($p > 0.05$) than group 1 ($4.59 \pm 0.31 \times 10^9$/L). Similarly, the monocytes were 8.5% higher ($p > 0.05$) in group 4 ($0.51 \pm 0.05 \times 10^9$/L) compared to 1 ($0.47 \pm 0.03 \times 10^9$/L).

Evaluation of Plasma Biochemical Parameters

The changes on the biochemical parameters of female ICR-mice administered with varying doses of MOEL repeatedly daily for 90 days are described on Table 3 (Ref. [17]). Significant ($p < 0.05$) changes in the plasma biochemical markers of the mice were observed among the treatment groups, as shown by one-way ANOVA (Table 3). Tukey post hoc test indicated that there were 35.4% and 34.6% significant ($p < 0.05$) elevation in the blood concentration of urea in groups 2 (9.52 ± 0.79 mmol/L) and 4 (9.46 ± 0.80 mmol/L) respectively, compared to 1 (7.03 ± 0.25 mmol/L). Moreso, an 83% significant ($p < 0.05$) elevation in the plasma concentration of ALT was noticed in group 5 (437.50 ± 28.63 U/L) compared to 1 (239.10 ± 22.50 U/L), in addition to a 71.2% significant ($p < 0.05$) elevation in AST level in group 5 (355.90 ± 26.45 U/L) compared to 1 (207.90 ± 19.67 U/L). The plasma concentration of CK were also 51.2% and 54.2% significantly ($p < 0.05$) elevated in groups 4 (962.60 ± 78.88U/L) and 5 (981.90 ± 132.37 U/L) respectively, compared to 1 (636.80 ± 45.81 U/L). Furthermore, the levels of plasma total proteins were 8.56%, 5.67% and 6.63% higher ($p > 0.05$) in groups 3 (62.51 ± 3.95 g/L), 4 (60.84 ± 3.87 g/L) and 5 (61.40 ± 2.01 g/L) correspondingly, compared to group 1 (57.58 ± 4.20 g/L).

Histological Evaluation of Lesions in the Liver

The results of the histopathological analysis of liver of female ICR-mice following administration of different dosages of MOEL repeatedly for 90 days are presented on Table 4. Significant ($p < 0.05$) changes in the lesion score were observed among the groups treated with the extract, as shown by Kruskal Wallis H test (Table 4). Pairwise comparisons test demonstrated a moderate hepatic degeneration (Table 4) characterised by moderate cytoplas-

Table 2. Haematological parameters (mean ± SEM) of female ICR-mice treated with different doses of MOEL continuously for 90 days.

Parameters	1	2	3	4	5	Reference[a]
Red blood cells ($\times 10^{12}$/L)	9.50 ± 0.25	9.49 ± 0.71	9.84 ± 0.32	9.31 ± 0.32	9.96 ± 0.24	9.12–10.74
Haemoglobin (g/L)	158.40 ± 3.27	153.70 ± 11.24	157.50 ± 4.21	155.80 ± 4.50	165.67 ± 4.22	145–179
PCV (L/L)	0.33 ± 0.02	0.34 ± 0.03	0.34 ± 0.02	0.35 ± 0.02	0.34 ± 0.02	0.433–0.485
Platelets ($\times 10^9$/L)	871.60 ± 124	825.60 ± 102	963.60 ± 154	1191.90 ± 149	1018.00 ± 145	926–1539
MCV (fl)	65.80 ± 0.79	66.70 ± 0.84	65.20 ± 1.10	66.00 ± 1.07	65.67 ± 0.97	41.8–47.9
MCH (pg)	16.71 ± 0.33	16.23 ± 0.36	16.11 ± 0.38	16.79 ± 0.36	16.67 ± 0.40	15.1–17.8
MCHC (g/L)	253.50 ± 3.51	243.70 ± 3.78	250.20 ± 5.20	254.60 ± 3.88	253.56 ± 3.05	351–385
Plasma proteins (g/L)	77.60 ± 3.34	79.20 ± 0.61	81.00 ± 1.94	83.30 ± 2.87	85.33 ± 2.51	-
White blood cells ($\times 10^9$/L)	7.33 ± 0.45	7.01 ± 0.49	7.03 ± 0.76	9.29 ± 0.77	7.59 ± 0.79	7.8–16.85
Neutrophils ($\times 10^9$/L)	2.02 ± 0.16	1.83 ± 0.17	1.79 ± 0.30	2.61 ± 0.31	2.40 ± 0.32	0.82–2.36
Lymphocytes ($\times 10^9$/L)	4.59 ± 0.31	4.62 ± 0.31	4.72 ± 0.44	5.89 ± 0.45	4.53 ± 0.50	5.5–14.32
Monocytes ($\times 10^9$/L)	0.47 ± 0.03	0.42 ± 0.03	0.38 ± 0.04	0.51 ± 0.05	0.47 ± 0.06	0.09–0.27
Eosinophils ($\times 10^9$/L)	0.24 ± 0.04	0.14 ± 0.03	0.14 ± 0.04	0.28 ± 0.05	0.19 ± 0.06	0–0.79
Basophils ($\times 10^9$/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0–0.08

MOEL, *Moringa oleifera* ethanolic leaf extract; 1, control; 2, 125 mg/kg MOEL; 3, 250 mg/kg MOEL; 4, 500 mg/kg MOEL; 5, 1000 mg/kg MOEL; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; a, Serfilippi *et al.* [17]. Readings in rows with no asterisk did not differ significantly ($p > 0.05$).

Table 3. Biochemical parameters (mean ± SEM) of female ICR- treated with varying doses of MOEL continuously for 90 days.

Parameters	1	2	3	4	5	Reference[a]
Urea (mmol/L)	7.03 ± 0.25*	9.52 ± 0.79	8.30 ± 0.58	9.46 ± 0.80	9.24 ± 0.29	4.6–7.5
Creatinine (µmol/L)	30.50 ± 1.31	27.90 ± 1.73	35.10 ± 3.14*	25.30 ± 0.86	29.50 ± 1.67	26.52–35.36
ALT (U/L)	239.10 ± 22.50	258.90 ± 19.80	269.20 ± 25.90	274.00 ± 24.03	437.50 ± 28.63*	19–42
AST (U/L)	207.90 ± 19.67	227.30 ± 19.53	264.60 ± 31.59	178.10 ± 17.68	355.90 ± 26.45*	46–79
CK (U/L)	636.80 ± 45.81	603.30 ± 53.82	806.50 ± 77.94	962.60 ± 78.88*	981.90 ± 132.37*	35–246
Total Protein (g/L)	57.58 ± 4.20	58.12 ± 2.07	62.51 ± 3.95	60.84 ± 3.87	61.40 ± 2.01	53–60
Albumin (g/L)	32.05 ± 2.01	33.91 ± 1.26	34.62 ± 1.80	35.89 ± 3.78	35.53 ± 1.57	36–43
Globulins (g/L)	25.53 ± 2.66	24.21 ± 2.00	27.89 ± 2.46	24.95 ± 2.71	25.87 ± 2.54	15–21

MOEL, *Moringa oleifera* ethanolic leaf extract; 1, control; 2, 125 mg/kg MOEL; 3, 250 mg/kg MOEL; 4, 500 mg/kg MOEL; 5, 1000 mg/kg MOEL; CK, creatinine kinase; a, Serfilippi *et al.* [17]; * indicates statistical significance at $p < 0.05$.

mic vacuolation (Fig. 2B,D) in group 5 (1.70 ± 0.24) compared to 1 (0.35 ± 0.18; Fig. 2A). Moreover, there were mild (1.40 ± 0.19) activated Kupffer cells (Fig. 2B,D), as well as mild necrosis of the hepatocytes indicated by mild eosinophilic cytoplasm (1.10 ± 0.3) of the hepatocytes in group 5 (Fig. 2B,C) compared to 1 (0.00 ± 0.00) (Table 4).

Histological Evaluation of Lesions in the Kidney

The findings from the histopathological evaluation of kidneys of female ICR-mice administered with different dosages of MOEL repeatedly for 90 days are presented on Table 5. Significant ($p < 0.05$) changes in the lesion score were observed among the groups treated with the extract, as shown by Kruskal Wallis H test. Pairwise comparisons test demonstrated a moderate cytoplasmic vacuolation in the kidneys (Fig. 3C,F) in group 5 (2.20 ± 0.08) compared to 1 (0.00 ± 0.00; Fig. 3A,B). In addition, a moderate to severe kidney necrosis indicated by significant ($p < 0.05$) eosinophilia of the cytoplasm (Fig. 3C–F) in groups 4 (1.95 ± 0.09) and 5 (2.45 ± 0.05) compared 1 (0.00 ± 0.00; Fig. 3A,B). Furthermore, pyknosis (0.90 ± 0.27) and karyolysis (0.60 ± 0.26) were significantly ($p < 0.05$) elevated (Fig. 3C,D) in groups 4 (1.15 ± 0.34) and 5 (1.75 ± 0.24) compared to 1 (0.00 ± 0.00). The protein casts (Fig. 3E) were significantly ($p < 0.05$) elevated in group 5 (1.20 ± 0.20) compared to 1 (0.00 ± 0.00) (Table 5).

Discussion

The evaluation of toxic potentials of plants is necessary for use in both human and animal. Toxicological invstigations are generally carried out in animals to determine how a substance or chemical can affect the body of the animals, so as to forecast the potential consequences and/or dosages of the substance in humans [21]. Liver and kidney are the major organs for to be evaluated in evaluating oral toxicity. This is important due to the fact that compounds taken via the oral route are first being processed by the liver and later excreted from the body via the kidneys [22]. Acute toxicity study may perhaps offer important information for

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Table 4. Lesion scores (mean ± SEM) of the liver of female ICR-mice treated with different doses of MOEL continuously for 90 days.

Lesion	1	2	3	4	5
Hydropic degeneration	0.35 ± 0.18	1.45 ± 0.26	0.35 ± 0.24	1.90 ± 0.12*	1.70 ± 0.24*
Eosinophilic cytoplasm	0.00 ± 0.00	0.20 ± 0.20	0.45 ± 0.24	0.00 ± 0.00	1.10 ± 0.3*
Pyknosis	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.15
Karyolysis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.20
Sinusoidal dilatation	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00	0.10 ± 0.10	0.60 ± 0.28*
Activated Kupffer cells	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.18	1.40 ± 0.19*
Inflammation	0.10 ± 0.10	0.45 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	0.40 ± 0.16*
Regeneration	0.55 ± 0.24	0.40 ± 0.27	1.50 ± 0.33	1.05 ± 0.25	0.80 ± 0.23

MOEL, *Moringa oleifera* ethanolic leaf extract; 1, control; 2, 125 mg/kg MOEL; 3, 250 mg/kg MOEL; 4, 500 mg/kg MOEL; 5, 1000 mg/kg MOEL; * indicates statistical significance at $p < 0.05$.

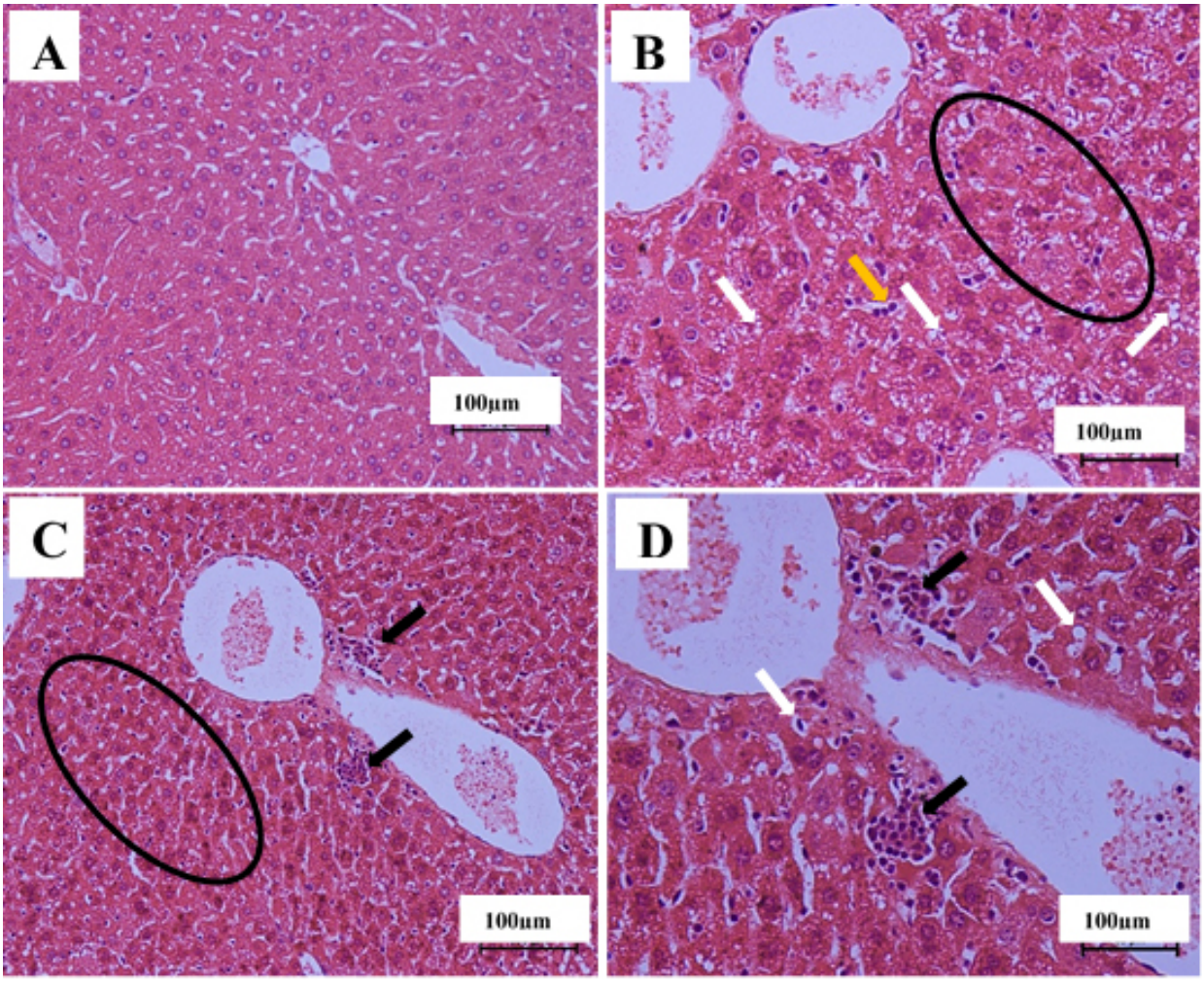

Fig. 2. Effects of continuous oral administration of MOEL for 90 days on histology of liver of female ICR-mice. (A) Micrograph of a liver histology (H&E stain ×200 stain) in a mouse in group 1 (control) presentating typical structute of liver, (B) Micrograph of a liver histology (H&E stain ×200) in a mouse in group 5 (1000 mg/kg MOEL) presentating eosinophilic cytoplasm (encircled), activated Kupffer cells (yellow arrow), (C) Micrograph of a liver histology (H&E stain ×100) in a mouse in group 5 presentating cellular infiltrates (black arrows) and eosinophilic cytoplasm (encircled), (D) Higher magnification of E (H&E stain ×200). H&E, haematoxylin and eosin.

the detection of the targeted organs by the substances after acute administration [22]. On the other hand, the subacute and sub-chronic toxicity studies evaluate the toxic effects of recurrent gavage of a substance or chemical on specific markers of the animals under investigation. This is to produce proofs on the target-organ toxicity and possibili-

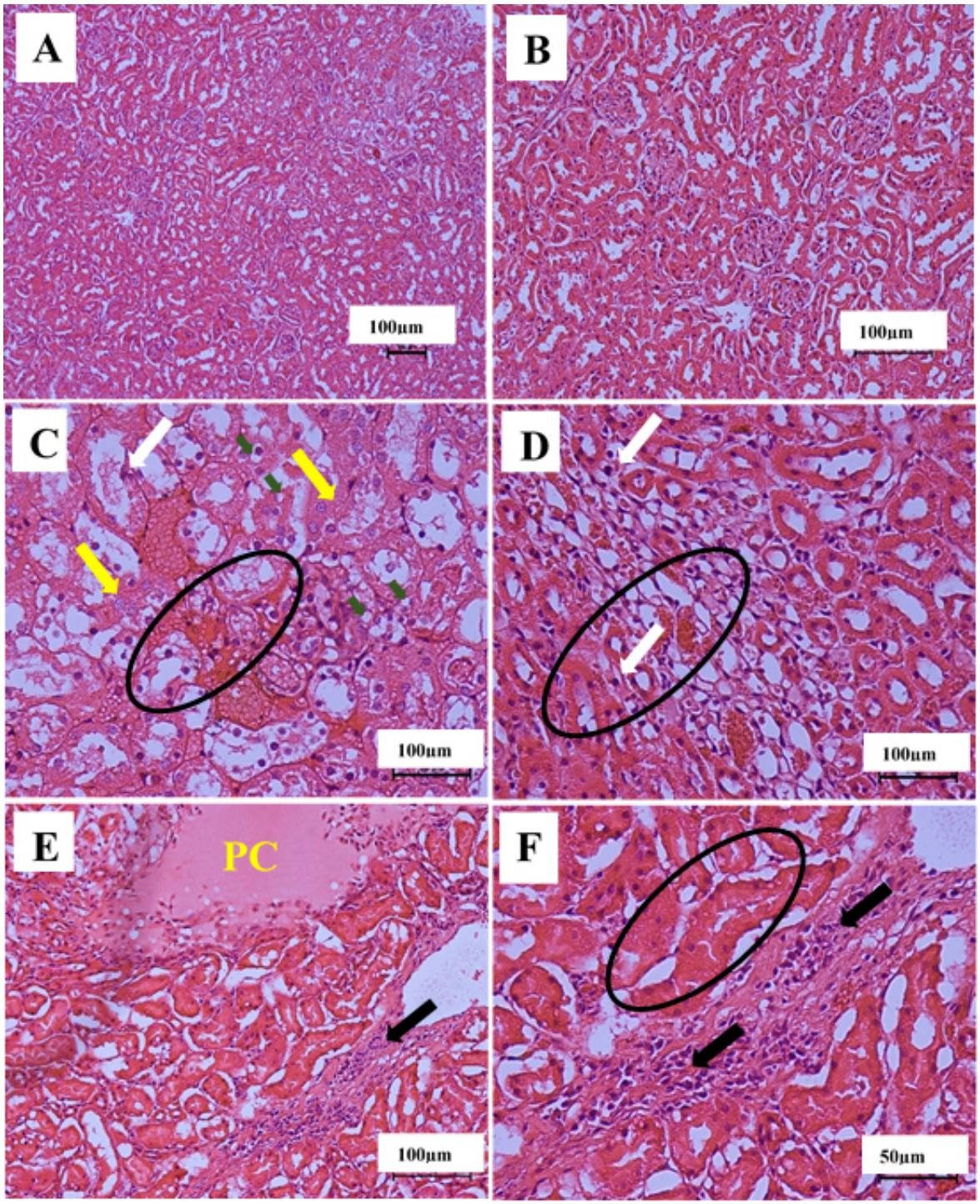

Fig. 3. Effects of continuous oral administration of MOEL for 90 days on the histology of kidney of female ICR-mice. (A) Micrograph of a kidney histology (H&E stain ×100) in a mouse in group 1 presentating typical renal structute, (B) Higher magnification of A (H&E ×200), (C) Micrograph of a kidney histology (H&E stain ×400) in a mouse in group 4 (500 mg/kg MOEL) presentating vacuolations (green arrows), eosinophilic cytoplasm (black encircled), pyknosis of the renal tubular cells (white arrow), as well as karyolysis of the renal tubular cells (yellow arrows), (D) Micrograph of a kidney histology (H&E stain ×400) in a mouse in group 5 (1000 mg/kg MOEL), presentating eosinophilic cytoplasm (black encircled) and pyknosis of the renal tubular cells (white arrows), (E) Micrograph of a kidney histology (H&E stain ×200) in a mouse in group 5 (1000 mg/kg MOEL), presentating protein cast (PC) and cellular infiltrations (black arrows), (F) Higher magnification of E presentating eosinophilic cytoplasm of the renal tubules (black encircled) and cellular infiltrations (black arrows) (H&E stain ×400).

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Table 5. Lesion scores (mean ± SEM) of the kidney of female ICR-mice in sub-chronic toxicity study of MOEL.

Lesion	1	2	3	4	5
Hydropic degeneration	0.00 ± 0.00	0.20 ± 0.20	0.00 ± 0.00	0.65 ± 0.33	2.20 ± 0.08*
Eosinophilic cytoplasm	0.10 ± 0.10	0.00 ± 0.00	0.90 ± 0.21	1.95 ± 0.09*	2.45 ± 0.05*
Pyknosis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.15 ± 0.34*	1.75 ± 0.24*
Karyolysis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.80 ± 0.34	1.65 ± 0.20*
Nephritis	0.30 ± 0.15	0.25 ± 0.17	0.50 ± 0.21	0.20 ± 0.20	0.85 ± 0.30
Protein casts	0.65 ± 0.18	0.10 ± 0.10	0.50 ± 0.17	0.45 ± 0.23	0.70 ± 0.25
Cellular casts	0.00 ± 0.00	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Granular casts	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.75 ± 0.21	1.20 ± 0.20*

MOEL, *Moringa oleifera* ethanolic leaf extract; 1, control; 2, 125 mg/kg MOEL; 3, 250 mg/kg MOEL; 4, 500 mg/kg MOEL; 5, 1000 mg/kg MOEL; * indicates statistical significance different at $p < 0.05$.

ties of bioaccumulation of the test substance, that are necessary to determine the no-observed-adverse-effect levels (NOAELs), so that, the doses for human exposure can be established [10].

The continuous oral administration of MOEL daily for 90 days showed certain changes in the physiological and histological structures of the mice. The results demonstrated an obvious decline in body weight gain throughout the investigation period. This corroborate the results reported by Aliyu *et al*. [3], which observed a remarkable reduction in the body weight gain in mice gavaged with *Moringa oleifera* continuously for four weeks. This can indicate further that the repeated oral administration of the extract might have influenced the appetite of the mice or might have influenced the normal fat metabolism of the mice tested [11,23]. Moreover, the relative internal organ weights give information on the possibility of the test substance to affect certain organs of the body [3]. These results contradict the results of other previous investigators including Chivapat *et al*. [24] and Kwaghe *et al*. [25], perhaps of the variations in the solvents for extraction and the species of animals used [2]. The researchers found that daily administration of *M. oleifera* aqueous extract in Wistar rats at different doses, including 10, 100 and 1000 mg/kg daily for six months did not present any significant alterations in the average body weight of the rats during the study period [24]. Correspondingly, oral gavage of ethyl acetate soluble fraction of aqueous extract of *M. oleifera* leaves in cockerels at different doses, including 100, 200, 300 and 400 mg/kg daily for 28 days did not indicate any remarkable changes in body weight of the treated mice in comparism with the control mice [25]. The variations in the findings of this research with of the ones highlighted by Chivapat *et al*. [24] and Kwaghe *et al*. [25] might be because of the variation in the animal species and extraction solvents used in the respective studies [2].

The remarkable elevation in the plasma concentrations of ALT and AST in the mice administered with 1000 mg/kg dose of MOEL daily for 90 days suggests the extract at this high dose has remarkable adverse effect on the liver [26,27] of the experimental mice, as reported elsewhere [3] following daily oral gavage of *M. oleifera* extract at 1000 mg/kg for 28 days. Hepatic necrosis has been associated with increased permeability of cell membrane, leading to leakage of cytosolic ALT and subsequent elevation of the enzyme in the blood [28]. Furthermore, ALT has been established to be elevated in hepatic injuries particularly liver necrosis, in different animals including mice [3,26]. The elevation of ALT and AST in this study supports the previous findings by Adedapo *et al*. [29], who reported a significant elevation in serum concentration of ALT and AST in rats administered with 400 and 1600 mg/kg *M. oleifera* leaves extracts for three weeks [29]. Moreover, a significant reduction in liver enzyme serum glutamic oxalo-actetic transaminase (SGOT), also known as AST has been demonstrated by Awodele *et al.* [15] in rats administered with *M. oleifera* extract at 250, 500 and 1500 orally, indicating liver injury in the treated animals. In another study be Olayemi *et al*. [30] the activities of liver enzymes; AST, ALP and ALT were found to be significantly lower following daily administration of 100, 200, 400 and 1000 mg/kg of the seed and leaf methanolic extracts of *M. oleifera* in rats for 28 days. The significant elevstion in the plasma levels of CK (creatinine kinase) enzyme at 500 and 1000 mg/kg doses could be as a result of muscle injury in the treated mice and/or an increase in the activity of the mice [26,27]. This is true because increased serum activities of AST, and CK, have been shown to be associated with myocardial infarction, injury in muscle or increased muscle activity [31]. The obvious rise in plasma concenyration of urea throughout the treatment groups might be associated with dehydration as indicated by the decrease bodyweight of the animals. Dehydration has been reported to decrease the glomerular filtration rate, thereby decreasing the tubular reabsorption of urea by renal tubules and subsequent reduction in the concentration of urea in the blood [26]. However, the elevation in the plasma levels of creatinine in the group of mice administered with 250 mg/kg of the extract may indicate kidney pathology in the mice because creatinine has been considered as an important marker for kidney function [32,33].

Evaluation of the histology of liver in this research showed that the extract only induced a very mild histologi-

cal lesions at the highest (1000 mg/kg) dose of the extract. The mild eosinophilic cytoplasm and cytoplasmic vacuolation and of the hepatocytes may indicate that the affected mice might have been recovering from the effects of early administration of the extract and the activated Kupffer cells signified that phagocytic process is ongoing probably to remove out some of the earlier affected hepatocytes [8,9,34]. This may further explain the significant changes observed in the plasma hepatic injury markers earlier in the research. Conversely, the result of the renal histological evaluation of the mice revealed moderate to severe renal lesions. The moderate cytoplasmic vacuolation noted in the group administered with 1000 mg/kg of the extracts suggested that the extract elicited tubular degenerative changes [25,35] in the kidney of the mice, likewise the moderate and moderate to severe eosinophilic cytoplasm respectively noted in the mice administered with 500 and 1000 mg/kg suggested that the extract at these dosages could have induced renal necrosis in the treated mice [9,30,35].

Conclusions

It is concluded that, 90 days daily continuous oral administrations of MOEL for in female ICR mice at 500 mg/kg and 1000 mg/kg caused moderate hepatic degeneration and necrosis respectively, as well as mild renal necrosis at 1000 mg/kg. Hence, the plant extract at 1000 mg/kg dose, is deemed toxic and unsafe for long term continuous intake as food additive and/or as complementary medicine; but, smaller doses can be used safely for medical reasons. However, the current research did not include molecular mechanisms and signaling pathways for organ toxicity. The authors encourage future researchers to consider this aspect in designing a similar study.

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting the corresponding authors if needed.

Author Contributions

AA, MRS, NSAS, MFHR, SS, SNNA, MMN, KS, QAR, AAA and HH made the design of the experimental protocol. AA, AAA and QAR drafted this manuscript. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The present study followed institutional guides for humane animal treatment and complied with the relevant legislation, according to the guide for the care and use of laboratory animals of Malaysian Agricultural Research Development Institute (MARDI). The study was approved by the Animal Ethic Committee (approval reference number: 20170717/R/MAEC00023).

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Conflict of Interest

The authors declare no conflict of interest.

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