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Original Article

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Abstract

The safety of ethanolic extract from the seeds of *Moringa oleifera* was evaluated for its oral toxicity by acute toxicity test and subchronic toxicity study in experimental animals. The acute toxicity test in mice showed that the extract at doses ranging from 5.1 to 10.0 g/kg caused toxic signs and dose dependent mortality. Oral administration of the extract in rats at doses of 100, 500 and 1000 mg/kg/day for 90 consecutive days revealed that male rats treated with the extract at 1000 mg/kg had a significant decrease in RBC but this alteration was within rats reference range. A significant decrease in eosinophil cells in the female rats receiving the extract at the doses of 500 and 1000 mg/kg and in the male rats receiving 1000 mg/kg were within normal range. A significant increase in glucose level in the female rats receiving the extract at 1000 mg/kg and a significant decrease in total protein in the male rats receiving 1000 mg/kg extract were within normal ranges. Stomachs of the male and female rats receiving the extract at the doses of 500 and 1000 mg/kg showed significantly higher relative weights than those of their corresponding water and tragacanth controls. Histopathology of visceral organs indicated that the extract did not produce any dose related lesions. The data from this experiment may be a guideline for establishing the appropriate dose range of *M. oleifera* seed extract on further development of safety herbal products.

Keywords: acute toxicity, mice, *M. oleifera* seed extract, rat, subchronic toxicity

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บทคัดย่อ

การประเมินความปลอดภัยของสารสกัดเมล็ดมะรุมด้วยเอทานอลในสัตว์ทดลอง

ทรงพล ชีวะพัฒน์ [1] พรชัย สินเจริญโภไคย [1] พราว ศุภจริยาวัตร [1] อนุเทพ รังสีพิพัฒน์ [2] สุพรรณ ภัทรพรชัยวัฒน์ [1] วนิดา จันทรเทพเทวัญ [3]*

ทำการประเมินความปลอดภัยของสารสกัดเมล็ดมะรุมด้วยเอทานอล โดยการทดสอบพิษเฉียบพลันและศึกษาความเป็นพิษระยะกึ่งเรื้อรังในสัตว์ทดลองที่ได้รับสารสกัดเม็ดมะรุมทางปาก การทดสอบพิษเฉียบพลันต่อหนูเม้าส์แสดงให้เห็นว่า สารสกัดเมล็ดมะรุมขนาดตั้งแต่ 5.1 ถึง 10.0 ก./กก. ทำให้เกิดอาการพิษเฉียบพลัน และมีจำนวนหนูเสียชีวิตสัมพันธ์กับขนาดที่ได้รับ จากการให้สารสกัดเมล็ดมะรุมแก่หนูแรทขนาด 100, 500 และ 1000 มก./กก./วัน ติดต่อกันนาน 90 วัน พบว่า หนูเพศผู้กลุ่มที่ได้รับสารสกัดขนาด 1000 มก./กก./วัน มีปริมาณเม็ดเลือดแดงต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญแต่อยู่ในค่าอ้างอิง ส่วนหนูเพศเมียกลุ่มที่ได้รับสารสกัดขนาด 500 และ 1000 มก./กก./วัน และเพศผู้กลุ่มที่ได้รับสารสกัดขนาด 1000 มก./กก./วัน มีเซลล์อิโอสิโนฟิลต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญแต่อยู่ในช่วงค่าปกติเช่นกัน การเพิ่มขึ้นของระดับกลูโคสอย่างมีนัยสำคัญในหนูเพศเมียที่ได้รับสารสกัดขนาด 1000 มก./กก./วันและค่าโปรตีนรวมที่ลดลงอย่างมีนัยสำคัญในหนูเพศผู้ที่ได้รับสารสกัดขนาด 1000 มก./กก. ยังคงอยู่ในช่วงค่าปกติของหนูแรท กระเพาะอาหารของหนูเพศผู้และเพศเมียที่ได้รับสารสกัดเมล็ดมะรุมขนาด 500 และ 1000 มก./กก. มีน้ำหนักสัมพัทธ์สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ผลการตรวจอวัยวะภายในทางจุลพยาธิวิทยาแสดงให้เห็นว่า สารสกัดเมล็ดมะรุมไม่ก่อให้เกิดรอยโรคที่สัมพันธ์กับขนาดที่ได้รับ ข้อมูลที่ได้จากการทดลองนี้จะเป็นแนวทางเพื่อปรับขนาดของสารสกัดเมล็ดมะรุมให้เหมาะสมต่อการนำมาพัฒนาเป็นผลิตภัณฑ์สุขภาพที่มีความปลอดภัยต่อไป

คำสำคัญ: พิษเฉียบพลัน หนูเม้าส์ สารสกัดเมล็ดมะรุม หนูแรท พิษกึ่งเรื้อรัง

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Introduction

Moringa oleifera Lam. is a small sized tree, native to South Asia, Africa and Arabia. The plant is known as drumstick or horse radish tree in some parts of the world (Goyal et al., 2007; Anwer et al., 2007). In Thailand, it is locally known as 'Marum'. Seed of this plant is used as human food, medicine, in oil production (Anwer et al., 2007) and also for efficient treatment of hard water (Muyubi and Evison, 1995). Extracts from the seeds were reported to possess many interesting biological activities such as anti-inflammatory, antiasthmatic, antioxidant, anticancer, antimicrobial activities, hepatoprotective and hypotensive effects (Caceres et al., 1992; Faizi et al., 1998; Guevara et al., 1999; Lalas and Tsaknis, 2002; Mahajan et al., 2007; Mehta and Agrawal., 2008; Hamza, 2010). Phytochemicals analysis revealed that the seed contained various compounds such as O-ethy1-4-(alpha-L-rhamnosyloxy)benzyl carbamate, 4(alpha-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin, niasirin, beta-sitosterol, glycerol-1-(9-octadecanoate), 3-O-(6'-O-oleoyl-beta-D-glucopyranosyl)-beta-sitosterol and beta-sitosterol-3-O-beta-D-glucopyranoside (Guevara et al., 1999).

Although the seed extracts of *M. oleifera* were shown to possess a variety of health benefits especially its efficiently hepatoprotective effects, the results of its safety or toxicity study have been contradictory. A study of Faizi et al.(1998) stated that the seed extract up to 3 g/kg caused neither behavioral change nor lethal effect in mice. Observations of renal hemorrhages, hydropic degeneration of hepatocytes, decrease in platelets, monocytes and total white blood cells in rats treated with seed extract were reported by Ajibade et al. (2012). Moreover, the liver of albino rats receiving the extract for 21 days revealed presence of marked aggregation of bile canaliculi around the portal vein (Oluduro et al., 2009). Therefore, this study aimed to evaluate the safety of *M. oleifera* seed extract in experimental animals after acute and subchronic oral exposure in order to be guidelines for establishing suitable dose range on further health product development.

Materials and Methods

Moringa oleifera seed extract (MOS): **S**eeds of *Moringa oleifera* were collected from a plantation of villagers of Amphur Thavung, Lopburi Province, Thailand**.** The plant specimens were identified by Office of the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation. A voucher specimen of *M. oleifera* (BKF No.174540) was deposited at the Forest Herbarium, Thailand. The dried seeds of *M. oleifera* were finely pulverized into powder and then macerated in 70% ethanol at 4^0C for 48 hours. The extract solution was filtered and evaporated using rotary evaporator under reduced pressure and then the concentrated extract was dried using lyophilizer. The average yield of dried *M. oleifera* seed extract (MOS) from the dried seed was 15.95% (w/w). Total phenolic content in MOS was determined using Folin-Ciocalteu phenol test (Singleton et al., 1999). It was found that the total phenolic content was equal to 15.08 mg gallic acid equivalents/100 g MOS. MOS was suspended in 0.5% tragacanth solution and adjusted to the desired concentration for further toxicological investigation.

Animals: Sixty ICR mice (30 males and 30 females) weighing 20-22 g and 120 Wistar rats (60 males and 60 females) weighing approximately 180-200 and 170-190 g, respectively, were purchased from The National Laboratory Animal Center, Mahidol University. The animals were housed in a hygienic conventional animal room of the Laboratory Animal Center, Department of Medical Sciences where the environment of the room was maintained at 25 ± 1^0C with 60% humidity and 12 hour-light-dark cycle. They were raised with commercial pellet diet (082 CP® feed, Perfect Companion Group, Thailand) and clean water *ad libitum*. The mice were fasted for two hours before acute toxicity test. For subchronic toxicity study, the rats were acclimatized with the environment for two weeks. The protocol study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Department of Medical Sciences (Code no. 53-018).

Acute toxicity test: Sixty ICR mice were randomly divided into six groups, each of ten animals (five males and five females). Four experimental groups were orally given with MOS suspension at the doses of 5.1, 6.4, 8.0 and 10.0 g/kg while two control groups received distilled water and 0.5%tragacanth at the volume of 10 ml/kg. Following administration, they were closely observed for abnormal signs and mortality for 14 days. At the end of the observation period, the mice were sacrificed with CO_2 inhalation and necropsy was performed to examine gross lesions of their vital visceral organs. The number of dead animals in each treatment group was calculated for LD_{50} using probit analysis.

Subchronic toxicity study: Wistar rats were randomly allocated to five groups of twelve animals of each sex. Group1 and 2 were control groups receiving distilled water and 0.5% tragacanth, respectively at the volume of 10 ml/kg. Group 3 to 5 were experimental groups that were orally administered with MOS suspension at the doses of 100, 500 and 1000 mg/kg/day, respectively, for 90 days. During the experimental period, body weight and food intake were recorded weekly and the animals were observed for general appearance, behavior and signs of abnormalities. At the end of the treatment period, the animals were fasted overnight, and anesthesized with diethyl ether inhalation. Blood samples were collected from posterior vena cava for determining hematological and serum biochemical values.

Hematological analysis was performed using automatic hematological analyzer Cell Dyn®3500 (Abbot Laboratories Ltd, USA). Parameters examined were hematocrit (Hct), hemoglobin, erythrocyte (RBC), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell (WBC), neutrophil, eosinophil, lymphocyte, monocyte, basophil and platelet. Biochemical values were measured by using automatic chemistry analyzer Cobas®Integra 400 plus (Roche Diagnostics Ltd., Switzerland) and parameter assays were alkaline phosphatase (ALP), alanine transminase (ALT), aspartate transminase (AST), total protein, albumin, bilirubin, blood urea nitrogen (BUN), creatinine, glucose, uric acid, triglyceride, cholesterol, sodium, potassium and chloride ions. A complete necropsy was performed to determine gross lesions of various visceral organs. Brain, heart, lung, liver, kidney, stomach, spleen, testis, uterus, urinary bladder and adrenal glands were weighed by using Mettler Toledo®PB 153 balance (Metler Toledo International Inc, Switzerland). Organ weights were calculated into relative organ weight (g /1000 g bw). The visceral organs were fixed in 10% buffered formalin, and then subjected to conventional histological process. Histopathological examination was performed on the organs mentioned above including trachea, lymph node, esophagus, pancreas, intestines, thyroid gland, lacrimal and salivary glands, prostate gland, seminal vesicle, ovary, uterus, and mammary glands.

Statistical analysis: The data were statistically evaluated by one way ANOVA. Comparison between treatment and control groups were made by Bonferroni test. For histopathological results, Fisher's exact was applied. Differences between groups were considered significant at $p< 0.05$.

Results

Acute toxicity test

The mice receiving MOS at the doses of 5.1, 6.4, 8.0 and 10.0 g/kg manifested abnormal signs including increased motor activity, bradypnea, cyanosis (purple mucous membrane of tail and nail) and piloerection. The number of dead animals in each group within 24 hours showed the tendency to be dose dependent (Table 1). The survival animals in each dose group became normal after 24 hours of administration. Therefore, LD_{50} value of MOS according to probit analysis was 6.68 g/kg. Gross pathology of the survival revealed no remarkable lesions as compared to the both control groups.

Table 1 Acute toxicity study in mice after 24 hours of treatment

Groups	Treatment (g/kg)	D/T*	Sign of toxicity observed
1	DW	0/10	-
2	TG	0/10	-
3	MOS 5.1	1/10	Decrease in motor activities and curiosity in the first 24 hours of extract administration. Bradypnea was observed after 3 hours of administration but after 6 hours past the sign disappeared; cyanosis and piroerection occurred after 4 hours of extract administration. The survival became normal after 24 hours of extract administration.
4	MOS 6.4	5/10	Decrease in motor activities and curiosity in the first 24 hours of extract administration. Bradypnea was observed after 15 minutes of administration; cyanosis and piloerection occurred after 4 hour of extract administration. The survival became normal after 24 hours of extract administration.
5	MOS 8.0	8/10	Decrease in motor activities and curiosity in the first 24 hours Bradypnea was observed after 15 minutes of administration but after 24 hours they became normal; cyanosis and piroerection occurred after 4 hours of administration. The survival became normal after 24 hours of administration
6	MOS 10.0	9/10	Decrease in motor activities and curiosity in the first 24 hours Bradypnea was observed after 15 minutes of administration but after 24 hours they became normal; cyanosis and piroerection occurred after 4 hours of administration. The survival became normal after 24 hours of administration.

MOS: *M. oleifera* seed extract, DW: distilled water, TG: tragacanth, D/T: number of deaths /number of mice treated

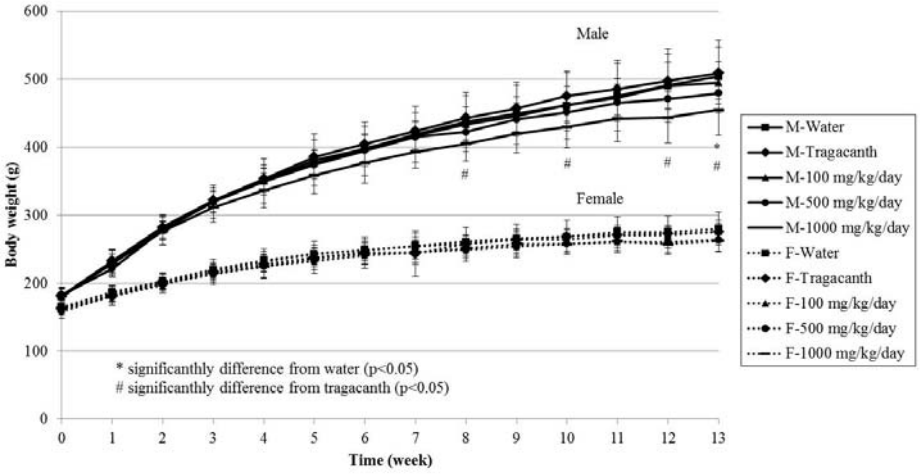

Figure 1 Growth curves of male and female rats receiving MOS for 3 months.

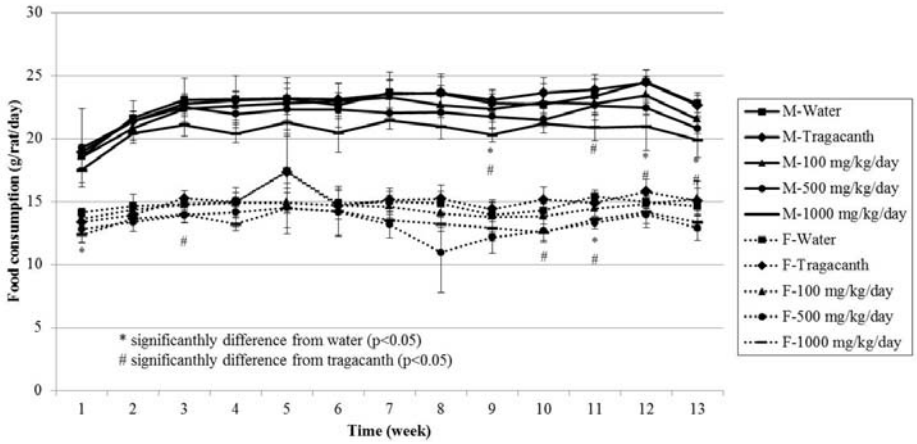

Figure 2 Food consumption of male and female rats receiving MOS for 3 months.

Table 2 Relative organ weight (g/1000 g of body weight) and body weight (g) of male rats receiving MOS for 90 days

Organs	Control		Dose of MOS administered (mg/kg/day)		
	DW	0.5% TG	100	500	1000
	n =12	n =12	n =12	n =12	n =12
Brain	4.35 ± 0.38	4.26 ± 0.36	4.42 ± 0.30	4.65 ± 0.30	4.83 ± 0.37*,#
Heart	3.03 ± 0.26	3.08 ± 0.42	2.90 ± 0.20	3.14 ± 0.24	3.11 ± 0.19
Lung	3.46 ± 0.21	3.56 ± 0.34	3.53 ± 0.29	3.61 ± 0.26	3.70 ± 0.36
Liver	27.61 ± 2.37	29.07 ± 3.12	30.40 ± 1.86	30.59 ± 2.19*	32.82 ± 2.02*,#
Stomach	4.22 ± 0.41	4.36 ± 0.53	4.77 ± 0.53	5.13 ± 0.43*,#	5.72 ± 0.53*,#
Spleen	1.75 ± 0.14	1.69 ± 0.22	1.78 ± 0.19	2.01 ± 0.24*,#	2.04 ± 0.16*,#
Right kidney	2.72 ± 0.21	2.81 ± 0.30	2.90 ± 0.54	2.97 ± 0.22	3.08 ± 0.29
Left kidney	2.67 ± 0.21	2.63 ± 0.31	2.52 ± 0.60	2.85 ± 0.17	3.03 ± 0.33
Right testis	6.02 ± 0.57	5.96 ± 0.69	6.15 ± 0.52	6.21 ± 0.32	6.44 ± 0.55
Left testis	6.00 ± 0.57	6.00 ± 0.69	6.13 ± 0.56	6.26 ± 0.28	6.50 ± 0.42
Right adrenal	0.090 ± 0.01	0.076 ± 0.01	0.081 ± 0.02	0.085 ± 0.02	0.084 ± 0.01
Left adrenal	0.085 ± 0.01	0.084 ± 0.01	0.088 ± 0.02	0.093 ± 0.02	0.093 ± 0.01
Bladder	0.287 ± 0.04	0.273 ± 0.04	0.289 ± 0.05	0.314 ± 0.04	0.308 ± 0.04
Initial body weight	157.37 ± 9.27	158.44 ± 10.85	158.57 ± 11.40	159.53 ± 6.80	158.93 ± 11.06
Final body weight	485.94 ± 52.35	490.04 ± 39.58	474.54 ± 30.75	458.44 ± 26.11	436.44 ± 37.69*,#

The values are expressed as mean±SD, DW: Distilled water, TG: Tragacanth; * significantly different from water control group($p< 0.05$); # significantly different from tragacanth control group ($p< 0.05$)

Table 3 Relative organ weight (g/1000g body weight) and body weight (g) of female rats receiving MOS for 90 days

Organs	Control		Dose of MOS administered (mg/kg/day)		
	DW	0.5% TG	100	500	1000
	n =12	n =12	n =12	n =12	n =10
Brain	7.41 ± 0.58	7.40 ± 0.40	7.62 ± 0.38	7.68 ± 0.43	7.58 ± 0.42
Heart	3.63 ± 0.35	3.56 ± 0.35	3.89 ± 0.46	3.70 ± 0.27	3.71 ± 0.18
Lung	4.67 ± 0.40	4.60 ± 0.21	4.60 ± 0.34	4.70 ± 0.34	4.71 ± 0.27
Liver	28.70 ± 2.74	29.44 ± 3.42	30.57 ± 4.59	31.98 ± 4.66	32.20 ± 3.26
Stomach	5.80 ± 0.43	5.74 ± 0.44	6.02 ± 0.77	7.09 ± 0.83*,#	7.06 ± 0.72*,#
Spleen	2.30 ± 0.19	2.40 ± 0.27	2.56 ± 0.25	2.53 ± 0.33	2.42 ± 0.26
Right kidney	3.18 ± 0.25	3.37 ± 0.37	3.18 ± 0.24	3.25 ± 0.27	3.77 ± 1.23
Left kidney	2.97 ± 0.28	3.17 ± 0.18	3.02 ± 0.20	3.18 ± 0.28	3.26 ± 0.26
Right adrenal	0.157 ± 0.03	0.187 ± 0.03	0.175 ± 0.03	0.178 ± 0.03	0.181 ± 0.04
Left adrenal	0.188 ± 0.04	0.196 ± 0.03	0.191 ± 0.02	0.183 ± 0.03	0.193 ± 0.03
Bladder	0.346 ± 0.07	0.350 ± 0.06	0.356 ± 0.07	0.332 ± 0.05	0.532 ± 0.55
Uterus	2.33 ± 0.45	2.24 ± 0.37	2.48 ± 0.83	2.43 ± 0.80	2.72 ± 0.81
Right ovary	0.336 ± 0.06	0.341 ± 0.07	0.340 ± 0.05	0.361 ± 0.06	0.328 ± 0.07
Left ovary	0.324 ± 0.10	0.352 ± 0.08	0.340 ± 0.07	0.367 ± 0.06	0.342 ± 0.08
Initial body weight	152.26 ± 9.64	152.72 ± 5.69	151.22 ± 9.00	151.28 ± 9.11	152.21 ± 8.09
Final body weight	263.98 ± 22.67	260.73 ± 10.28	260.08 ± 15.91	248.86 ± 16.46	247.64 ± 16.03

The values are expressed as mean±SD, DW: Distilled water, TG: Tragacanth; * significantly different from water control group($p< 0.05$); # significantly different from tragacanth control group ($p< 0.05$)

Subchronic toxicity study

***Effect of MOS on body weight, food consumption and health status*:** The average body weight of the male rats receiving MOS at the dose of 1000 mg/kg/day was significantly lower than that of the tragacanth control group at week 8, 10 and 12, and also than that of both water and tragacanth control groups at the last week of the experiment. Furthermore, the body weight gain of this group was significantly lower when compared with that of both control groups. However, the females receiving different doses of MOS showed no difference in their body weights as compared to both controls (Fig 1). Measurement of the weekly food intake showed that the highest dose-treated male rats had significantly lower food intake than both control groups at week 9, 12 and 13 and only the tragacanth control group at week 11. The female rats receiving MOS at 500 and 1000 mg/kg showed significantly less food intake in only week 10 and 11, and thereafter no difference was determined. The detail of weekly food consumption was illustrated in Fig 2.

Effect of MOS on relative organ weight: the male rats receiving the highest dose of MOS had significantly higher relative brain weight than the water and tragacanth control groups. The relative weights of stomach and spleen in the male rats receiving MOS at the doses of 500 and 1000 mg/kg/day were significantly higher than those of both control groups.

The relative liver weight was significantly higher than the water control group in the former and than the both controls in the latter group (Table 2). In the females, there was no significant differences in relative weights of almost all organs between the treatment and both control groups, except the significant increases of the stomach relative weight in the groups treated with 500 and 1000 mg/kg/day (Table 3).

Effects of MOS on hematological values: RBC and eosinophil cells in the highest dose-treated male group significantly decreased as compared to the water control group, and to both control groups, respectively (Table 4). The female rats receiving MOS

at the dose of 100 mg/kg had significantly lesser WBC than the tragacanth control group. Eosinophil cells in the female rats treated with 500 and 1000 mg/kg were significantly lower than those of the water control (Table 5).

Table 4 Hematological values of male rats receiving MOS for 90 days

Parameters	Control		Dose of MOS administered (mg/kg/day)		
	DW	0.5% TG	100	500	1000
	n =12	n =12	n =12	n =12	n =12
Hematocrit (%)	31.27 ± 1.91	29.63 ± 2.49	30.78 ± 1.59	30.06 ± 1.80	29.87 ± 1.39
RBC (x10^{6}cells/mm^3)	9.50 ± 0.60	9.19 ± 0.36	9.20 ± 0.60	8.96 ± 0.55	8.85 ± 0.43*
Hemoglobin (g/dl)	16.33 ± 0.91	15.95 ± 0.58	16.15 ± 0.93	15.72 ± 1.01	15.72 ± 0.73
MCV (µm^3/red cell)	32.95 ± 1.34	32.91 ± 0.93	33.42 ± 1.17	33.58 ± 0.68	33.80 ± 0.72
MCH (pg/red cell)	17.20 ± 0.64	17.38 ± 0.44	17.55 ± 0.49	17.56 ± 0.46	17.73 ± 0.50
MCHC (g/dl RBC)	52.21 ± 0.92	52.83 ± 0.68	52.39 ± 0.76	52.27 ± 0.58	52.60 ± 0.71
WBC (x10^{3}cells/mm^3)	4.35 ± 1.18	3.87 ± 0.59	4.07 ± 0.40	3.83 ± 0.82	4.06 ± 1.22
Neutrophil (%)	21.86 ± 4.28	21.96 ± 5.70	22.51 ± 3.86	21.62 ± 3.42	22.83 ± 4.38
Eosinophil (%)	1.20 ± 0.26	1.28 ± 0.40	1.57 ± 0.77	1.04 ± 0.26	0.80 ± 0.19*,#
Lymphocyte (%)	75.09 ± 5.68	75.46 ± 5.51	74.77 ± 3.80	74.54 ± 4.66	72.05 ± 2.76
Monocyte (%)	0.944 ± 0.90	0.527 ± 0.14	0.559 ± 0.14	1.300 ± 2.07	1.528 ± 2.08
Basophil (%)	1.10 ± 1.14	0.76 ± 0.48	0.59 ± 0.29	1.49 ± 0.81	1.72 ± 1.20
Platelet (x10^{3}cells/mm^3)	1,006.08 ± 142.44	989.75 ± 60.95	988.83 ± 101.24	948.79 ± 98.83	982.00 ± 95.01

The values are expressed as mean±SD, DW: Distilled water, TG: Tragacanth; * significantly different from water control group (p< 0.05); # significantly different from tragacanth control group (p< 0.05)

Table 5 Hematological values of female rats receiving MOS for 90 days

Parameters	Control		Dose of MOS administered (mg/kg/day)		
	DW	0.5% TG	100	500	1000
	n =12	n =12	n =12	n =12	n =10
Hematocrit (%)	29.89 ± 1.75	29.72 ± 1.86	30.05 ± 1.00	29.93 ± 1.61	29.63 ± 0.88
RBC (x10^{6}cells/mm^3)	8.65 ± 0.43	8.49 ± 0.60	8.66 ± 0.47	8.59 ± 0.47	8.56 ± 0.43
Hemoglobin (g/dl)	15.75 ± 0.93	15.64 ± 1.02	15.90 ± 0.52	15.82 ± 0.76	15.69 ± 0.46
MCV (µm^3/red cell)	34.53 ± 1.02	34.98 ± 0.86	34.74 ± 1.24	34.83 ± 0.77	34.71 ± 1.12
MCH (pg/red cell)	18.25 ± 0.58	18.42 ± 0.47	18.39 ± 0.73	18.42 ± 0.49	18.34 ± 0.72
MCHC (g/dl RBC)	52.78 ± 0.68	52.74 ± 0.63	52.91 ± 0.56	52.86 ± 0.50	52.85 ± 0.91
WBC (x10^{3}cells/mm^3)	2.04 ± 0.49	2.70 ± 0.42	1.96 ± 0.43#	2.47 ± 0.64	2.88 ± 1.21
Neutrophil (%)	24.50 ± 10.45	22.64 ± 8.87	21.41 ± 3.86	18.75 ± 7.43	18.14 ± 6.45
Eosinophil (%)	2.00 ± 0.73	1.54 ± 0.68	1.38 ± 0.53	1.02 ± 0.57*	0.79 ± 0.48*
Lymphocyte (%)	70.42 ± 10.68	73.33 ± 9.08	75.39 ± 3.98	78.04 ± 7.42	78.95 ± 6.90
Monocyte (%)	2.18 ± 2.38	1.32 ± 1.48	0.99 ± 0.50	0.95 ± 0.41	1.00 ± 0.32
Basophil (%)	0.92 ± 0.97	1.29 ± 1.17	0.82 ± 0.45	1.22 ± 0.44	1.12 ± 0.75
Platelet (x10^{3}cells/mm^3)	1,040.25 ± 112.81	986.88 ± 80.79	976.21 ± 74.53	1,009.75 ± 98.02	1,101.15 ± 146.12

The values are expressed as mean±SD, DW: Distilled water, TG: Tragacanth; * significantly different from water control group (p< 0.05); # significantly different from tragacanth control group (p< 0.05)

Effects of MOS on biochemical values: The male rats receiving MOS at 100 mg/kg/day had significantly lower potassium level than the water control. Every rat receiving MOS at 500 mg/kg/day had significantly lower glucose level than the water control group. Total protein level of the male rats receiving the highest dose of MOS was significantly lower than that of both control groups (Table 6). The female rats receiving MOS at 100 mg/kg/day had significantly higher albumin level than the water control group. The glucose level of the highest dose group was significantly higher than that of the both control groups (Table 7).

Effects of MOS on histopathological alterations: At necropsy, there was no remarkable macroscopic lesions in any visceral organs of all MOS-treated and the both control groups**.** Histopathological study revealed that the incidence of dilated sinusoid and congestion in the liver of the male rats receiving MOS at 500 and 1000 mg/kg and the female rats receiving MOS at 1000 mg/kg significantly decreased when compared with those of the both control group. Furthermore, the female rats receiving MOS at 100 and 1000 mg/kg had significantly lower incidence of such lesions than the tragacanth control and both control groups, respectively. The female rats receiving MOS at 500 and 1000 mg/kg/day had significantly higher incidence of bronchiolar associated lymphoid tissue proliferation than the both control group. However, the incidence of kidney congestion in these groups were significantly lower when compared with the tragacanth control group. The incidence of histopathological lesions in the intestines, adrenal glands and mammary glands in all treatment groups were not significantly different from the water and tragacanth control group in both sexes. In addition, no remarkable lesions were found in other organs. Histopathological lesions of the visceral organs were summarized in Table 8.

Table 6 Biochemical values of male rats receiving MOS for 90 days

Parameters	Control		Dose of MOS administered (mg/kg/day)		
	DW	0.5% TG	100	500	1000
	n =12	n =12	n =12	n =12	n =12
ALP (U/L)	56.58 ± 9.38	54.92 ± 13.37	46.00 ± 6.19	50.42 ± 6.82	57.42 ± 12.43
ALT (U/L)	31.92 ± 6.84	33.58 ± 11.77	30.17 ± 9.73	39.50 ± 23.40	37.82 ± 7.08
AST (U/L)	83.25 ± 10.07	85.25 ± 17.87	80.67 ± 6.92	95.50 ± 15.89	89.18 ±17.29
Total protein (g/dl)	6.33 ± 0.19	6.39 ± 0.22	6.36 ± 0.27	6.16 ± 0.14	6.04 ± 0.26*,#
Albumin (g/dl)	4.40 ± 0.13	4.42 ± 0.18	4.45 ± 0.26	4.39 ± 0.10	4.44 ± 0.13
Total bilirubin(mg/dl)	0.061 ± 0.02	0.044 ± 0.02	0.058 ± 0.02	0.062 ± 0.01	0.064 ± 0.02
BUN (mg/dl)	18.15 ± 2.88	19.13 ± 3.38	27.94 ± 28.09	18.38 ± 2.76	19.79 ± 4.01
Creatinine (mg/dl)	0.440 ± 0.05	0.446 ± 0.09	0.454 ± 0.07	0.446 ± 0.06	0.462 ± 0.07
Glucose (mg/dl)	256.73 ± 44.43	251.42 ± 39.80	227.71 ± 45.43	208.56 ± 30.02*	264.09 ± 27.44
Uric acid (mg/dl)	4.96 ± 1.30	4.54 ± 0.97	4.33 ± 1.52	3.78 ± 1.19	5.03 ± 1.36
Triglyceride(mg/dl)	90.80 ± 61.59	65.47 ± 32.04	80.13 ± 44.96	58.24 ± 33.35	44.88 ± 11.49
Cholesterol (mg/dl)	65.75 ± 9.58	63.20 ± 9.16	68.22 ± 21.11	61.21 ± 12.84	55.88 ± 11.76
Sodium	142.25 ± 0.87	141.92 ± 0.90	142.33 ± 1.15	142.25 ± 0.97	142.92 ± 1.00
Potassium	7.34 ± 0.53	6.71 ± 0.33	6.67 ± 0.48*	6.71 ± 0.73	6.78 ± 0.82
Chloride	103.33 ± 1.15	102.42 ± 1.51	102.75 ± 1.42	96.00 ± 25.84	103.25 ± 1.22

The values are expressed as mean±SD, DW: Distilled water, TG: Tragacanth; * significantly different from water control group (p< 0.05); # significantly different from tragacanth control group (p< 0.05)

Table 7 Biochemical values of female rats receiving MOS for 90 days

Parameters	Control		Dose of MOS administered (mg/kg/day)		
	DW	0.5% TG	100	500	1000
	n =12	n =12	n =12	n =12	n =10
ALP (U/L)	25.50 ± 4.03	26.42 ± 4.25	25.67 ± 4.81	28.17 ± 7.35	28.10 ± 11.18
ALT (U/L)	24.00 ± 3.38	24.00 ± 2.41	21.67 ± 3.73	28.00 ± 10.78	26.90 ± 5.24
AST (U/L)	86.08 ± 8.71	84.33 ± 10.48	82.75 ± 9.19	85.50 ± 12.67	79.70 ± 11.39
Total protein (g/dl)	6.30 ± 0.25	6.33 ± 0.26	6.48 ± 0.24	6.33 ± 0.32	6.10 ± 0.21
Albumin (g/dl)	4.54 ± 0.15	4.62 ± 0.22	4.82 ± 0.16*	4.73 ± 0.20	4.54 ± 0.25
Total bilirubin(mg/dl)	0.076 ± 0.02	0.083 ± 0.02	0.088 ± 0.02	0.085 ± 0.03	0.082 ± 0.03
BUN (mg/dl)	22.11 ± 4.42	23.03 ± 4.71	21.02 ± 3.35	24.28 ± 5.60	26.89 ± 9.74
Creatinine (mg/dl)	0.440 ± 0.05	0.449 ± 0.08	0.423 ± 0.03	0.458 ± 0.05	0.519 ± 0.12
Glucose (mg/dl)	120.93 ± 23.30	121.44 ± 16.58	117.14 ± 16.46	139.69 ± 36.26	166.76 ± 51.35*,#
Uric acid (mg/dl)	2.51 ± 0.69	2.28 ± 0.83	2.50 ± 0.91	2.81 ± 1.07	3.68 ± 1.78
Triglyceride(mg/dl)	37.34 ± 6.13	32.89 ± 4.12	30.73 ± 7.96	37.75 ± 10.42	37.23 ± 19.47
Cholesterol (mg/dl)	66.38 ± 13.75	63.86 ± 12.44	62.19 ± 15.53	67.93 ± 16.51	65.32 ± 20.01
Sodium (mmol/l)	141.33 ± 1.23	141.08 ± 1.16	141.58 ± 1.08	141.58 ± 1.16	142.10 ± 2.08
Potassium (mmol/l)	6.82 ± 0.60	6.42 ± 1.35	6.38 ± 1.41	5.99 ± 1.02	6.12 ± 1.05
Chloride (mmol/l)	105.25 ± 1.42	104.83 ± 1.47	105.17 ± 1.34	103.92 ± 2.35	104.40 ± 1.07

The values are expressed as mean±SD, DW: Distilled water, TG: Tragacanth; * significantly different from water control group (p< 0.05); # significantly different from tragacanth control group (p< 0.05)

Table 8 Histopathological results of male and female rats receiving MOS for 90 days

Organs	Microscopic findings	Male					Female				
		Control		Dose of MOS (mg/kg/day)			Control		Dose of MOS (mg/kg/day)		
		DW	0.5%TG	100	500	1000	DW	0.5%TG	100	500	1000
Lung	BALT proliferation	4/12	8/12	7/12	3/12	4/12	0/12	1/12	2/12	6/12*, #	5/12*,#
Heart	NRL										
Liver	Centrilobular fatty degeneration	0/12	1/12	1/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
	Dilated sinusoid and congestion	7/12	9/12	3/12	2/12*,#	2/12*,#	6/12	7/12	2/12#	4/12	0/12*, #
Spleen	NRL										
Kidney	Congestion	2/12	4/12	4/12	4/12	0/12	4/12	9/12	3/12	2/12#	2/12#
Small intestine	GALT proliferation in submucosa	1/12	2/12	1/12	4/12	2/12	2/12	1/12	1/12	NRL	1/12
Large intestine	GALT proliferation in submucosa	NRL	1/12	NRL	NRL	1/12	1/12	NRL	NRL	NRL	1/12
Adrenal gland	Cortical fatty infiltration	3/12	7/12	3/12	4/12	3/12	NRL	0/12	0/12	0/12	0/12
	Medullary congestion	0/12	1/12	1/12	1/12	1/12	NRL	3/12	2/12	2/12	2/12
Mammary gland	Glandular hyperplasia						4/12	3/12	6/12	2/12	3/12

Data shown were number of rats with histopathological lesions / total number of rats in each group
*significantly different from water control group (p< 0.05), # significantly different from tragacanth control group
(DW: Distilled water, TG: tragacanth, NRL: No remarkable lesions, BALT: Bronchiole-associated lymphoid tissue, GALT: Gut-associated lymphoid tissue)

Discussion

The results of acute toxicity test in mice by gavaging MOS at the doses ranging from 5.1 to 10.0 g/kg indicated that MOS cause toxic signs, e.g. decreased motor activities, bradypnea and cyanosis. Such toxic signs reflect that MOS may act as central nervous system depressant and may affect the blood circulation system of the animal (Chan and Hayes, 1994). According to its oral LD_{50} value, MOS may be categorized as slightly toxic (Hodge and Sterner, 1999); whereas Faizi et al. (1998) reported that when subcutaneously given up to 3 g/kg, the ethanolic seed extract of *M. oleifera* was relatively safe. This discrepancy may possibly be due to the effects of some toxic metabolites that may occur after the oral ingestion. In addition, the variation in phytochemical constituents from different plant sources and temperatures of extraction may be concerned. The ethanolic extract of *M. oleifera* seeds was reported to possess some potent hypotensive principles as found in the leaf extract (Faizi et al., 1998). In addition, it was demonstrated that the seeds of *M. oleifera* contained hydrogen cyanide (0.58 mg/100g) (Anhwange et al., 2004). Since cyanide inhibits cytochrome oxidase thus halting electron transport, oxidative phosphorylation and aerobic glucose metabolism. Inhibition of glucose metabolism resulted in the buildup of lactate and the increase in the concentration of oxygenated hemoglobin. Increased oxyhemoglobin in the venous circulation reflects that oxygen is not being utilized in the peripheral tissue. The most serious consequences of oxidative phosphorylation inhibition are related to neurological and cardiovascular problem including adverse neurological sequelae, respiratory arrest, arrhythmia and cardiac failure (Robert and Budinsky, 2000). Therefore, the possible causes of acute toxic signs and mortality in this study may contribute to the blood circulatory failure induced by acute sudden hypotension and also be due to tissue anoxia.

In the subchronic toxicity study, MOS at the doses of 100, 500 and 1000 mg/kg did not cause any behavioral changes, overt toxic signs or mortality in the rats. The findings of the lesser food consumption in the males receiving MOS at 1000 mg/kg in the last month of experiment and also in the female rats receiving MOS at 500 and 1000 mg/kg at some time-points suggested that MOS may affect some regulation signals of food intake and metabolism of the animals (Bernadier, 2004). The poorer body weight in the highest dose male rats may be the result of the growth depression effect caused by the extract. It was shown that the kernels of *M. oleifera* contained some antinutritional factors such as glucosinolates and phytate which may cause reduced growth and decrease in the digestibility of starch and protein, respectively (Makkar and Becker, 1999). In the present study, the absolute weight of stomach in the male and female rats receiving 500 and 1000 mg/kg was found to significantly increase, whereas that of other organs including liver and spleen showed no differences. Therefore, the increased relative stomach weight may result from the increase in its absolute weight while the increase in liver and spleen relative weights in the male treated with 500 and 1000 mg/kg MOS may be partly due to their poorer body weight induced by MOS. However, histopathology of the organs mentioned above showed no correlated morphological changes. Organ weight can be the most sensitive indicator of an effect of an experimental compound, as significant differences in organ weight between treated and control animals may occur in the absence of any morphological changes (Bailey et al., 2004). Therefore, MOS might possibly induce some gastric subcellular changes, which needs to be further investigated. The observation of a significant decrease in RBC in the highest dose male group might possibly contribute to MOS, as the kernel and extracted- kernel of *M. oleifera* were demonstrated to produce hemolytic activity (Makkar and Becker, 1999). However, this finding was within the normal range of Wistar rat (Gad, 1992). In addition, hematocrit, hemoglobin, MCV and MCHC values in this group showed no significant differences. Therefore, it may be concluded that subchronic exposure of MOS produced no anemic status in Wistar rats. The decrease in eosinophil cells in the highest dose-treated male and the female rats receiving MOS at 500 and 1000 mg/kg were within normal range (Gad, 1992; Pimainog et al., 2003). Taken together, MOS did not cause any abnormalities in hematological values.

Measurement of biochemical values indicated that MOS at the tested doses did not affect the hepatic serum enzymes. However, our results were different from the study of Ajibade et al. (2012), which states that the oral administration of methanol extract of *M. oleifera* in Wistar rats for 21 days induced a significant increase in ALT and AST levels. The plausible causes for these discrepancies may be due to the variation of chemical constituents in herbal materials from different sources, the differently given doses and the duration of administration. The increase in glucose in the female rats receiving the highest dose of MOS tended to be dose-dependent and therefore this phenomenon may be produced by MOS. This finding was in contrast with a few reports demonstrating the antidiabetic action of the *M. oleifera* fruit and stem bark (Kar et al., 2003) and the insulin secretagogues activities from the four compounds isolated from the methanol extract of *M. oleifera* fruits (Francis et al., 2004). The reasons for this discrepancy may also contribute to the difference in chemical constituents between these extracts as mentioned earlier and, therefore, the mechanism of this phenomenon require further study. The significant decrease in glucose in the male receiving MOS at 500 mg/kg and in potassium in the male receiving MOS at 100 mg/kg did not show any dose dependency; therefore, it may not contribute to MOS. Similar reason may account for the increase in albumin level in the low dose female group. The decrease in serum total protein level in the highest dose-treated male rats was still within the reference range (Gad, 1992) and it may probably be due to the less food intake of this group.

The increased incidence of mild degree BALT proliferation at the peribronchiolar area in the lung of the female receiving MOS at 500 and 1000

mg/kg/day may not contribute to MOS. The lesionsare common in association with inflammation such as chronic alveolitis, bronchitis and pneumonia in the lung of control laboratory rats (Peckham, 1995). The possible cause of this lesion may be concerned to the immune response to some aspirated antigens which irritate the breathing airways such as ammonia or dusts from the bedding materials and also from gavage. The decrease in dilated sinusoid and congestion in the liver of the male rats receiving MOS at 500 and 1000 mg/kg and that of the female rats receiving MOS at the doses of 100 and 1000 mg/kg/day may be, in part, due to MOS. The underlying causes of these findings might, in part, be due to its hypotension effects (Faizi et al., 1998). In addition, niazimicin, a compound isolated from the ethanolic extract of *M. oleifera* leaves and seeds was demonstrated to produce hypotension and bradycardia effects in anesthesized rats and also cause negative inotropic and chronotropic in isolated guinea-pig atria (Gilani et al., 1994; Anwar et al., 2007). Therefore, these cardiovascular effects of MOS might possibly cause the decrease in arterial blood supply resulting in the reduction of congestion. Similar reasons may account for the decrease in kidney congestion in the medium and highest dose-treated female rats. The incidence of histopathological lesions in some organs were not significantly different between the treatment and control groups. Furthermore, no remarkable lesions were found in other organs. Therefore, the 90-day oral exposure of MOS at differently tested doses did not induce any pathological changes in the visceral organs of the experimental animals.

In conclusion, the acute toxicity test of MOS at the doses ranging from 5.1-10.0 g/kg produced acute toxic signs and lethality in mice. The 90-day oral administration of MOS in Wistar rats at the doses of 100, 500 and 1000 mg/kg/day revealed that MOS did not produce any overt pharmacotoxic signs and abnormalities on hematological and biochemical values. In addition, it did not cause any dose-related pathological lesions in the visceral organs. Thus, it could be said that MOS produce no serious subchronic toxicity in the rats. Further studies on many aspects concerning the *M. oleifera* seed extract such as phytochemical profiles, antinutritional factors, standardization and quality control, and including chronic toxicity should be investigated.

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