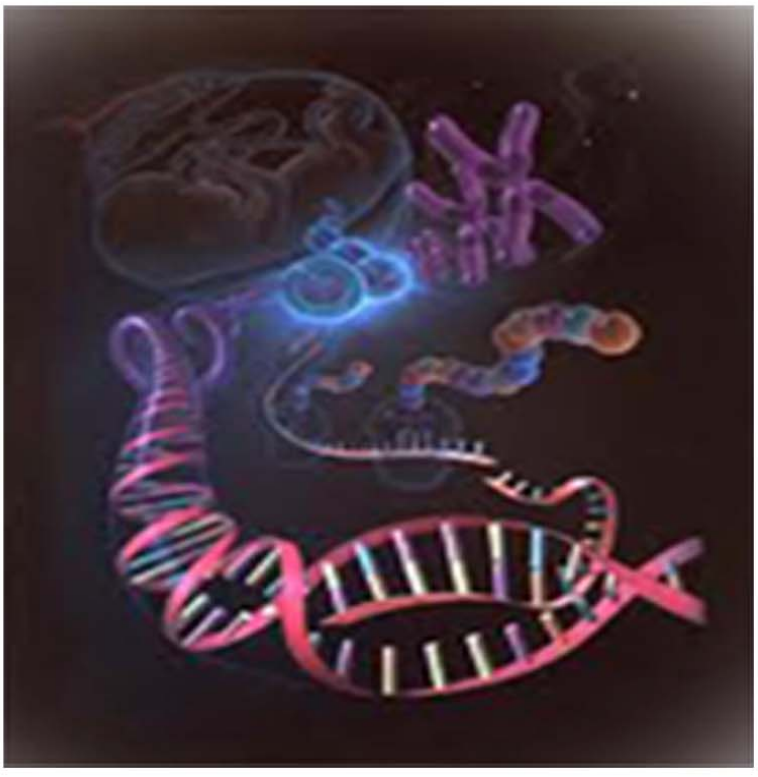
IJLBPR

ISSN 2250 - 3137
Vo1. 2, No. 4, October 2013

International Journal of

Life Sciences Biotechnology and Pharma Research



International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA

www.ijlbpr.com

Email : editorijlbpr@gmail.com or editor@ijlbpr.com

International Journal of Life Sciences Biotechnology and Pharma Research

ISSN 2250-3137 www.ijlbpr.com
Vol. 2, No. 4, October 2013

Research Paper

ACUTE AND SUB ACUTE TOXICITY OF *MORINGA OLEIFERA* STEM BARK EXTRACT IN SWISS ALBINO MICE

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Different parts of *Moringa oleifera* have been shown to exhibit wide pharmacological activities. Testing the toxicity and safety of plant extracts is a key step before further efficacy tests could be performed. In the current study we determined the safety of methanolic extract of *M. oleifera* bark by performing acute and sub acute (28 days) oral toxicity studies in Swiss albino mice. In the acute study oral administration of bark extract up to 2000 mg/kg body weight (b.wt) did not show any toxicity or mortality in mice during the 14 days. In the sub acute study the bark extract was administered orally at 500, 1000 and 2000 mg/kg b.wt for 28 days. Toxicity was assessed through biochemical, haematological, relative organ weights and histopathology tests apart from weekly measurements of body weight and food consumption. In all these tests no significant difference was observed between treated (extract administered) and control group mice. Histopathological examination of organs from the mice treated with extract at 2000 mg/kg b.wt for 28 days did not show any toxic effects compared to control group. Current study determined the non toxic nature of oral administration of *M. oleifera* bark extract.

Keywords: *Moringa oleifera,* Acute toxicity, Sub acute toxicity, OECD, AST, ALT

INTRODUCTION

Moringa oleifera (Family: *Moringaceae*) is a multipurpose tree with significant medicinal and nutritional value. Different parts of *moringa* (leaves, pods, seeds, bark and flowers) have been shown to exhibit wide pharmacological activities and used in Ayurvedic medicine. The leaves have been shown to exhibit antihypertensive (Dangi *et al*., 2002), hypocholesterolaemic (Ghasi *et al*., 2000), antiulcer (Pal *et al.*, 2006) and wound healing properties (Rathi *et al*., 2006). Leaf, fruit and seed extracts of *M. oleifera* protect against oxidative DNA damage by scavenging free radicals (Bharali *et al.,* 2003). The roots are mildly diuretic, contain antifertility activity (Shukla *et al*., 1988) and are used as a stimulant in paralytic, epilepsy and hysteria conditions. The bark is regarded as an antiscorbic, antifungal,

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antitubercular (Bhatnagar *et al*., 1961) and antiurolithiatic activity (Jameel *et al*., 2010). Bark exudes reddish gum used in the treatment of diarrhoea and known to have filaricidal activity. Bark has been shown to decrease the cardiotoxicity and lipid peroxidation by stimulating endogenous antioxidant enzymes (Mahendra *et al*., 2010). All the biological activities are attributed to the presence of many bioactive compounds in different parts which include; glucosinolates, isothiocyanates, pterygospermin, gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin (Brahma *et al*., 2009; Jed W Fahey and ScD, 2005).

The World Health Organization (WHO) estimates that 80% of the world's population still depends on herbal medicine for primary health care. Modern system of medicine has many examples of plant products that are used successfully such as; reserpine (*Rauwolfia serpentina*) for hypertension, digoxin and digitoxin (*Digitalis purpurea*) in congestive heart failure and aspirin (*Salix alba*). Modern medicine has the advantage of extensive research to evaluate safety but herbal medicines do not undergo such screening process. Most of the drugs including the natural compounds are known to have side effects. It is therefore important that all herbal medicines are subjected to safety tests by the methods that are used for synthetic drugs. As the search to discover new therapeutic agents and dietary supplements derived from plants offers many advantages, determining the toxicity of the plant extracts is crucial to assure the safety. Therapeutic benefits of herbal drugs can be delivered only with strong scientific evidence that proves efficacy and toxicity. Interestingly a toxic substance might elicit pharmacological effects at a lower non-toxic dose. Hence toxicity tests in animals play key role in determining the safety of medicinal plants that are found to contain bioactive compounds. The objective of the current study is to determine the safety of *moringa* bark extract by performing, acute and sub acute (28 days repeated dose) oral toxicity studies in Swiss albino mice.

MATERIALS AND METHODS

Collection of Plant Material and Preparation of Extracts

M. oleifera stem bark was collected in the month of March 2012 from the trees available locally and authenticated by Dr. K. Madhava Chetty, Department of Botany, S V University, Tirupati, India. A voucher specimen was deposited in the herbarium with register number 921. Bark was shade dried for one week. Dried material was pulverized to coarse powder and soaked (in 1:3 ratio) in aqueous methanol (water: methanol, 20:80 v/v) for two days with intermittent mixing. The mixture was filtered and evaporated at 40 ^{o}C in rotary evaporator and stored in sterile glass container in a refrigerator (4 ^{o}C). The yield of the extract was found to be 10.2% w/w of dried bark powder.

Animals

The experiments were performed using male and female Swiss albino mice (8 weeks old) obtained from Sri Venkateswara Enterprises (Bangalore, India). Female mice were nulliparous and non pregnant. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC). The mice were housed in separate polypropylene cages in experimental room. All the animals were maintained at 24 ± 2°C and the relative humidity set at 30-70% with a 12:12-h light-dark cycle. They were fed with

standard pellet feed purchased from Provimi Animal Nutrition India Pvt. Ltd., Bangalore, India and UV sterilized water *ad libitum*. Mice were acclimatized in the experimental room for five days before beginning the experiment.

EXPERIMENTAL PROCEDURE

Acute Oral Toxicity

Swiss albino mice (n=12) were divided into three groups, viz., Group I, Group II and Group III. Each group comprises of three female mice (n=3). Group I mice after overnight fasting were administered orally with single dose of *M. oleifera* bark extract in 1% Carboxy Methyl Cellulose (CMC) at 300 mg/kg body weight (b.wt). The food was withheld for further 4 h. Mice were observed individually after dosing during the first 30 min, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter for a period of 14 days. There was no mortality at 300 mg/kg b.wt, hence Group II mice were administered at the dose of 2000 mg/kg b.wt and observations were recorded as above. As the extract did not show mortality at 2000 mg/kg b.wt, the same dose was administered to Group III mice for confirmation. Weekly body weight and food consumption were recorded. At the end of observation period mice were sacrificed by CO_2 asphyxiation and necropsy was performed to examine gross pathology of visceral organs (OECD- 423, 2001).

Sub Acute Oral Toxicity

Mice (n=40) were divided into four groups, viz., Group 1 (Vehicle control), Group 2, Group 3 and Group 4 (*M. oleifera* bark extract). Each group (n=10) comprises of five male and five female mice. Group 1 mice were administered orally with 1% CMC at 10 ml/kg b.wt daily once for a period of 28 days; Group 2, Group 3 and Group 4 mice were administered orally with *M. oleifera* extract (suspended in 1% CMC) at the dose of 500, 1000 and 2000 mg/kg b.wt ,respectively. The volume of bark extract for oral administration was 10 ml/kg b.wt daily once for a period of 28 days. Mice were observed daily for clinical signs of toxicity. Body weight measurements and food consumption were recorded weekly.

Blood Analysis

At the end of the study (day 29) blood samples (approximately 0.5 ml) were collected in 1% ethylene diamine tetra acetic acid (EDTA) from retro orbital sinus plexus to perform hematological tests in an auto hematology analyzer (Mindray BC-2800Vet, China). Similarly blood samples were collected and serum was obtained by centrifuging at 425 g for 10 min to perform biochemical tests in semi auto biochemistry analyzer (Merck microlab 300, Germany).

Haematological Parameters

Erythrocyte count (RBC), Leucocyte count (WBC), Hemoglobin (Hb), Haematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Platelet (PLT) count were evaluated in the control and bark extract treated groups.

Biochemical Parameters

Glucose, Alanine amino transferase (ALT), Aspartate amino transferase (AST), Alkaline Phosphatase (ALP), Total protein, Albumin, Creatinine, Urea, Cholesterol and Triglycerides were determined in all the groups.

Pathology

All the mice were sacrificed by CO_2 asphyxiation and necropsy was performed to examine gross pathology of visceral organs. Histopathological

examination of the organs was performed for high dose (2000 mg/kg b.wt) and control group animals. Tissues were fixed in 10% buffered formalin and processed by automatic tissue processor (Thermo Scientific- Microm STP-120 and EC-350, Germany). Paraffin sections (4-5 μm thickness) were prepared (Thermo Scientific-HM-315, Germany), stained with hematoxylin and eosin (H & E) and examined by light microscopy (Olympus CX21, Japan). Organ weights were measured and relative organ weights were calculated for organs such as; brain, heart, liver, kidneys, adrenals, spleen, thymus, testes, epididymides, uterus and ovaries (OHAUS Scout *pro* SPG 202F, USA). Paired organs were weighed together (OECD- 407, 1995).

STATISTICAL ANALYSIS

Data were analyzed using SPSS 16.0 to obtain group means and standard deviations (SD) for comparison between the control and test groups. All the parameters characterized as continuous data such as, body weight, food consumption, haematological, biochemical and organ weight data were subjected to Bartlett's test to meet the homogeneity of variance before conducting Analysis of Variance (ANOVA) and Dunnett's t-test. Where the data did not meet the homogeneity of variance, Kruskal-Wallis test was performed to calculate the significance $p < 0.05$ is considered as significant.

RESULTS

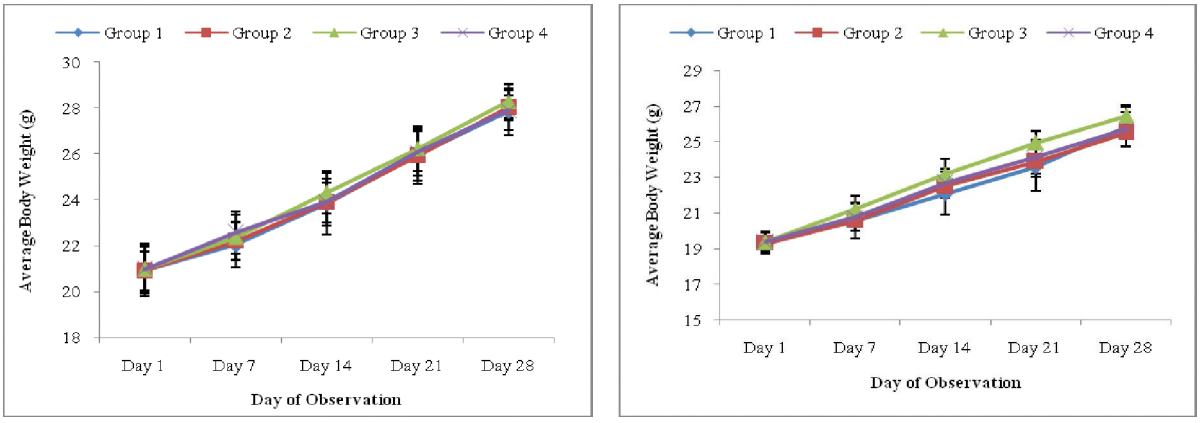
Acute Oral Toxicity

No mortality and clinical signs were observed in mice treated with *moringa* bark extract at 2000 mg/kg b.wt during the 14 days observation period. Gross pathological examination of organs did not show any lesions or abnormal changes.

Sub Acute Oral Toxicity

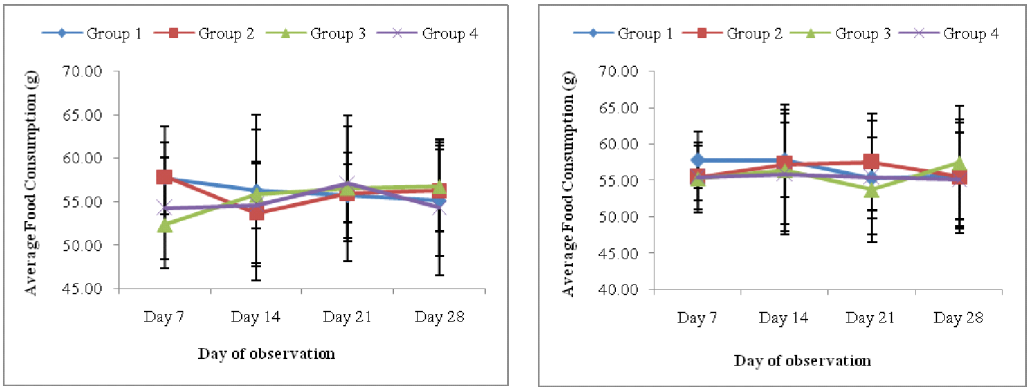
Cage side observations: No mortality or clinical signs were observed in mice administered orally with bark extract at 500, 1000 and 2000 mg/kg b.wt for a period of 28 days. General behavior of the mice was found to be normal throughout the study period. Results of mean weekly body weight and food consumption are shown in Figures 1 and 2, respectively. No significant variation was observed in the mean body weight and food

Figure 1: Effect of 28 days Repeated Oral Administration Of *M. Oleifera* Bark Extract On Mean Weekly Body Weight (G) In Male (A) And Female (B) Mice



Note: Group1-Vehicle control; Group2- *M.oleifera* extract at 500 mg/kg b.wt; Group3- *M.oleifera* extract at 1000 mg/kg b.wt; Group4- *M.oleifera* extract at 2000 mg/kg b.wt; g- grams. Differences were not significant between test (Group 2, 3, 4) and control group (Group 1) in both sexes. However an increase in body weight was observed in both sexes compared to their initial weights on day 1.

Figure 2: Effect of 28 Days Repeated Oral Administration of *M. Oleifera* Bark Extract On Mean Weekly Food Consumption (G) In Male (A) And Female (B) Mice



Note: Group1-Vehicle control; Group2- *M.oleifera* extract at 500 mg/kg b.wt; Group3- *M.oleifera* extract at 1000 mg/kg b.wt; Group4- *M.oleifera* extract at 2000 mg/kg b.wt; g- grams. Differences were not significant between test (Group 2, 3, 4) and control group (Group 1) in both sexes.

consumption of mice treated with bark extract at different doses compared with control. However gain in the body weight was observed in all the groups compared to their initial weights on day 1.

Results of haematological and biochemical parameters are shown in Tables 1 and 2, respectively. No significant difference was observed in all the haematological and biochemical parameters of the test groups treated with bark extract at 500, 1000 and 2000 mg/kg b.wt compared to control. All the parameters were found to be within the normal range. Results of organ weights are shown in Table 3. Differences were not significant in the relative organ weights

Table 1: Effect of 28 days repeated oral administration of *M. oleifera* bark extract on haematological parameters

Parameter	Group1		Group2		Group3		Group4		Normal
	M	F	M	F	M	F	M	F	Range
RBC ($1x10^6/\mu l$)	7.37±0.36	7.12±0.98	7.39±0.55	7.13±0.61	7.37±0.44	7.14±0.53	7.39±0.51	7.14±0.87	5.0-9.5
WBC ($1x10^3/\mu l$)	10.07±1.75	8.31±1.27	10.15±2.03	8.51±1.36	10.31±2.92	8.39±0.88	10.1±2.04	8.4±0.82	3.0-14.2
Hb (g/dl)	14.7±0.94	13.89±1.05	14.87±1.36	13.89±1.97	14.69±0.83	13.89±1.04	14.84±1.37	13.94±0.99	10.9-16.3
HCT (%)	50±5.09	49.55±3.74	49.97±3.80	49.72±3.99	49.97±4.04	49.8±4.47	49.95±2.78	49.55±3.11	38.5-45.1
MCV (fL)	58.75±3.28	60.13±3.25	58.9±3.45	60.22±3.64	58.9±2.84	60.27±2.42	58.92±3.74	60.28±2.90	48.0-56.0
MCH (pg)	19.18±1.97	19.32±1.05	19.32±0.91	19.42±0.83	19.3±1.06	19.43±1.35	19.32±1.18	19.43±0.87	11.9-19.0
MCHC (g/dl)	30.82±0.92	31.63±1.99	30.93±1.39	31.62±1.75	30.78±0.98	31.45±1.65	30.87±0.39	31.57±1.02	25.9-35.1
Plt ($1x10^3/\mu l$)	826.5±46.72	785.5±36.68	848.5±38.37	783.5±52.32	806.5±28.38	833.5±64.69	896.5±47.23	753.5±40.47	1084-1992

Note: Results are expressed as mean±SD. n=5. In all the haematological parameters differences were not significant between test groups (Group 2, 3, 4) and control group (Group1). M - Male; F- Female; RBC -Red blood cells; WBC - White blood cells; Hb- Hemoglobin; HCT - Haematocrit; MCV - Mean Corpuscular Volume; MCH - Mean Corpuscular Hemoglobin; MCHC- Mean Corpuscular Hemoglobin Concentration; PLT - Platelet; fl - Femtolitre; pg - Picogram.

Table 2: Effect of 28 Days Repeated Oral Administration of *M. Oleifera* Bark Extract on Biochemical Parameters

Parameter	Group1		Group2		Group3		Group4	
	M	F	M	F	M	F	M	F
Glucose(mg/dl)	108.25±10.78	105.17±15.35	104.17±13.44	97.12±9.53	101.50±11.55	95.83±10.59	96.33±10.32	101.67±9.91
ALT (IU/l)	41.82±4.37	38.90±2.32	42.70±3.38	39.27±4.22	42.48±3.41	40.50±4.23	42.40±4.03	39.20±3.95
AST (IU/l)	193.50±56.62	154.83±35.24	189.17±63.70	159.50±42.67	180.50±52.89	146.33±25.19	166.50±43.51	165.67±42.01
ALP (IU/l)	167.67±24.18	77.17±14.30	156.67±36.05	86.17±14.32	180.33±47.56	84.00±12.93	147.00±47.44	77.17±12.80
Total Protein(g/dl)	6.75±0.92	6.98±1.51	7.52±1.24	6.95±0.96	7.35±1.45	7.11±1.52	6.97±1.32	7.53±1.70
Albumin (g/dl)	4.37±0.42	4.87±0.53	5.25±0.40	4.50±0.57	4.27±0.42	4.63±0.47	5.37±0.53	4.67±0.41
Creatinine(mg/dl)	0.66±0.05	0.74±0.08	0.70±0.06	0.62±0.06	0.56±0.08	0.69±0.07	0.68±0.08	0.71±0.06
Urea (mg/dl)	16.50±2.74	15.67±2.87	15.67±2.71	16.83±3.19	14.93±3.60	15.17±2.86	15.67±2.16	16.50±3.08
Cholesterol (mg/dl)	98.67±8.44	106.83±10.64	88.00±7.64	102.67±9.50	87.67±7.20	105.33±8.44	92.50±7.62	105.33±8.94
Triglyceride(mg/dl)	58.23±8.08	29.98±5.83	62.67±7.27	39.83±6.14	71.20±9.90	38.63±6.94	65.17±7.79	30.20±7.58

Note: Results are expressed as mean±SD, n=5. Differences were not significant between test (Group2, 3, 4) and control group (Group1) in both sexes. ALT- alanine amino transferase; AST- aspartate amino transferase; ALP- alkaline phosphatase; TP- total protein; IU- international units.

Table 3: Effect of 28 Days Repeated Oral Administration of *M. oleifera* Bark Extract on Relative Organ Weights (g)

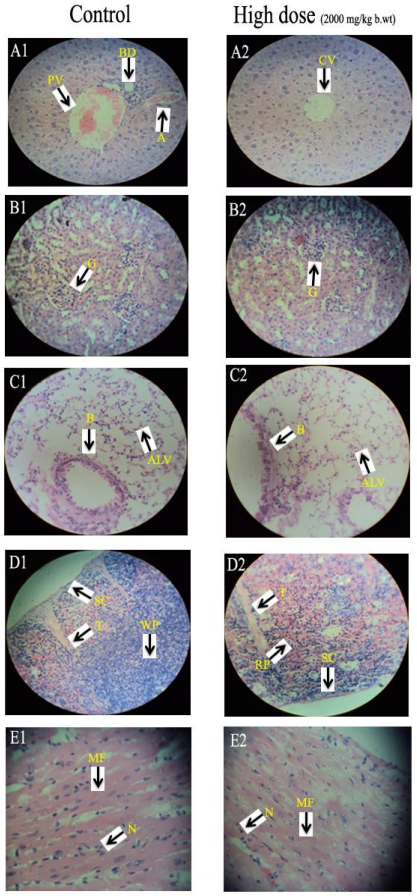
Parameter	Group1		Group2		Group3		Group4	
	M	F	M	F	M	F	M	F
Brain	1.64±0.02	1.72±0.08	1.51±0.11	1.83±0.07	1.54±0.11	1.71±0.06	1.61±0.06	1.75±0.08
Heart	0.79±0.09	0.74±0.05	0.79±0.06	0.73±0.05	0.76±0.07	0.71±0.06	0.81±0.07	0.74±0.03
Liver	7.19±0.57	5.83±0.41	7.24±0.99	6.18±0.21	7.42±0.86	5.71±0.29	7.37±1.54	5.96±0.14
Kidney	2.19±0.12	1.66±0.19	2.17±0.08	1.69±0.14	2.19±0.09	1.66±0.07	2.23±0.09	1.66±0.09
Adrenals	0.06±0.02	0.07±0.02	0.06±0.02	0.07±0.02	0.06±0.02	0.07±0.02	0.06±0.02	0.07±0.02
Spleen	0.77±0.05	0.72±0.07	0.76±0.08	0.78±0.07	0.76±0.06	0.72±0.07	0.77±0.04	0.72±0.09
Thymus	0.31±0.04	0.23±0.03	0.29±0.03	0.26±0.05	0.28±0.03	0.24±0.03	0.30±0.03	0.26±0.04
Testis/ Ovaries	0.75±0.06	0.21±0.03	0.78±0.07	0.21±0.04	0.76±0.05	0.21±0.03	0.79±0.08	0.20±0.03
Epididymis/Uterus	0.30±0.03	0.27±0.04	0.30±0.04	0.30±0.06	0.30±0.03	0.30±0.04	0.30±0.03	0.29±0.06

Note: Results are expressed as mean±SD, n=5. Differences between test (Group2, 3, 4) and control group (Group1) were not significant in both sex.

of mice treated with the extract at different concentrations compared to control group. Differences were also not significant between male and female mice in the parameters monitored.

Pathology: Toxicity changes or effects were not identified in the gross pathological examination of visceral organs from mice treated orally with bark extract for 28 consecutive days. Histopathological examination of organs was performed in Group 4 (2000 mg/kg b.wt) and Group 1 (control) mice. Microscopic images of histological sections are shown in Figure 3. No adverse changes were detected in the microscopic architecture of liver, kidney, lungs, spleen and heart and cellular organization was found to be normal in Group 4 mice compared to control group.

Figure 3: Histopathological examination of liver (A), kidney (B), lungs (C), spleen (D) and heart (E) after 28 days repeated oral Administration of *M. oleifera* bark extract



Note: Microscopic images of H & E stained sections of organs from control and high dose group (2000 mg/kg b.wt) mice. Magnification: 40X. (PV- Portal vein; BD- Bile duct; A- Artery= portal triad); CV- Central vein; G- Glomerulus; B- Bronchiole; ALV- Alveoli; SC- Splenic capsule; T- Trabeculae; WP- White pulp; RP- Red pulp; MF- Myocardial fibers; N- Nucleus. No adverse changes were detected in the microscopic structure of the organs from mice treated with 2000 mg/kg b.wt of *moringa* bark extract compared to control group.

DISCUSSION

Plant extracts are good source of biologically active substances but knowing the side effects before therapeutic application is essential to know the safety of the extract. Many Ayurvedic formulations are in use without valid scientific data on safety and efficacy. This is due to lack of stringent regulations for use and approval of natural products/extracts. Taking into account the basic premise that pharmacology is simply toxicology at a lower dose (Sasidharan *et al.*, 2008), in the current study the safety of methanolic extract of *M. oleifera* bark was evaluated by performing acute and sub acute oral toxicity tests in mice. In acute oral toxicity study the median lethal dose (LD_{50})of *moringa* bark extract was found to be >2000-5000 mg/kg b.wt and it is being classified as "Category 5" according to Globally Harmonized Classification System (GHS) (OECD 423, 2001).

In sub acute oral toxicity study non toxic nature of bark extract is indicated by lack of significant changes in the hematologic parameters because it is one of the most sensitive targets of toxic compounds and serves as an important index of physiological and pathological status of man and animals (Adeneye *et al.,* 2006; Diallo *et al.,* 2008). Haematopoietic components are initially exposed

to significant concentrations of toxic compounds as blood forms the medium for xenobiotics transport. Hence haematological tests are relevant in risk evaluation because of their high predictive value for human toxicity when assays involving rodents (Olson *et al.,* 2000).

Disease or response to toxic substances is indicated by alterations in the key biochemical parameters which are the sensitive indicators of organ function or metabolic defects. Liver plays a major role in the metabolism and detoxification of compounds that reach the liver and hence the prime target organ for drugs and toxic substances. Liver function tests such as; AST, ALT and ALPare useful in determining the extent of damage (Shah *et al.,* 2011). Similarly creatinine and urea tests are critical and sensitive indicators of kidney function (Obidah *et al.*, 2009). Hepato-renal toxicity is particularly liable to occur because these are the organs involved in drug metabolism and elimination. Non toxic nature of the bark extract is also indicated by these biochemical parameters which did not alter significantly compared to control mice. Biochemical findings further corroborated with the histopathological findings.

The heart, liver, kidney, spleen and lungs are more sensitive organs affected by toxic substances (Dybing *et al.,* 2002). Hence the relative organ weight is also an important index to determine whether the organ was exposed to the toxic manifestations of the compounds. Differences in relative organ weights were not significant in mice administered with the bark extract. In addition safety nature of bark extract is also indicated by histopathological examinations of the organs which did not show any adverse changes in microscopic structure as histopathological examinations is also the standard index for evaluating treatment related pathological changes in tissues and organs. In general, the histopathology analysis is in agreement with the results of body weight and organ weights. In conclusion the current study determined the safety of oral administration of *M. oleifera* bark extract in Swiss albino mice.

ACKNOWLEDGMENT

The authors thank Sugen Life Sciences Pvt. Ltd., #4/86, S V Nagar, Perumallapalli, Tirupati, 517505 for financial support.

CONFLICTS OF INTEREST

Authors declare no conflict of interest.

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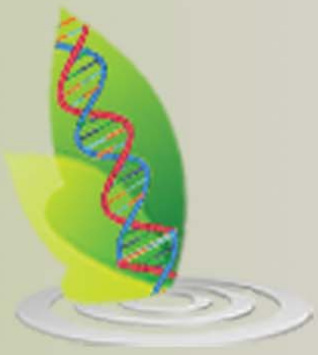

International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA. Ph: +91-09441351700, 09059645577
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ISSN 2250-3137
9 772250 313001 04