Subramanien D and Ravikumar S. / ***International Journal of Pharmacy & Therapeutics*****, 7(4), 2016, 174-183.**
e- ISSN 0976-0342
Print ISSN 2229-7456

International Journal of Pharmacy & Therapeutics

Journal homepage: www.ijptjournal.com

IJPT

ACUTE AND CHRONIC TOXICITY STUDIES OF THE EXTRACTS OF STEM BARK OF *NEISOSPERMA OPPOSITIFOLIUM (LAM.) FOSB. & SACHET*

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ABSTRACT

The present study confirms the safety of stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet* by subjected to acute and chronic toxicological studies. Acute oral toxicity study was performed as per OECD 423 guidelines, albino Wistar rats of either sex selected by random sampling technique were used for acute toxicity study. Test group animals received test extracts (500mg/kg, oral) once daily for 90days. Toxicological indications and symptoms were recorded daily. All groups of animal feed consumption, and animal's weight were monitored at the end of the day 0, day 30, day 60 & day 90. Similarly, hematological and biochemical parameters were estimated on day 0, day 30, day 60 & day 90. In conclusion, at the oral dose 500mg/kg, of Ethyl acetate extract of stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet* (EAE*No*), Ethanol extract of stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet* (EAE*No*), can be considered as safe and these extracts did not cause any lethality and adverse effects or changes in general behaviour, haematological and biochemical parameters in the chronic toxicity study in rats.

Key Words:- *Neisosperma oppositifolium*, Acute toxicity, Chronic toxicity.

INTRODUCTION

Toxicology can be described as that category of pharmacology, it deals with harmful substances and poisons when received either by planned or accident, to a living organism in mammalian. In screening of plant extracts, determination of the LD50 (The dose which has proved to be lethal to 50% of the tested group of animals is usually an initial step in the estimation of the toxic characteristics of a substance. It is an initial assessment of toxic manifestations and provides information on health hazards likely to arise from short term exposure to drugs. The acute toxicity studies give the initial information on the biological mechanism & systemic toxic effect of chemical substances and find the dose determination in animal studies. Its help to determine LD_{50} values that provide many indices of potential types of drug activity (Rhodes *et al*., 1993).

For confirming the safety of stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet*, this plant has subjected to acute and chronic toxicological studies.

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MATERIALS AND METHODS

Collection and Authentication of the plant material

Neisosperma oppositifolium (Lam.) Fosb. & Sachet Stem bark (Family: Apocynaceae) was collected from Tirupati (Andhra Pradesh, India) during September 2015, and identified and authenticated in Dr.

Madhavachetty, Assistant Professor, Department of Botany, Sri Venakateswara university, Tirupati 517 502, A.P., India.

Procedure for extraction

Neisosperma oppositifolium (Lam.) Fosb. & Sachet Stem bark (Family: Apocynaceae) was shade dried at room temperature individually. The dried of each plant were subjected to size reduced to a coarse powder (2000 grams) by using a dry grinder and passed through sieve. The powder was packed into soxhlet apparatus and extracted successively with ethyl acetate and ethanol. The extraction was carried out until the extract becomes colorless. The solvent is removed by distillation under reduced pressure and stored in desiccators for further experiment.

Experimental Animals

Male and female Wistar albino strain rats were supplied by Srinivasa Enterprises, Bengaluru, India. Nonpregnant and nulliparous female animals were selected. On receipt the animals were randomly allocated to cages. While the starting of the study the animals weighed between 200 to 250 g, and approximately eight weeks of age. The animals were housed in groups of three (single sex) in solid-floor polypropylene cages with well-ventilated room. The rate of air exchange was approximately 15 changes/hour and the lighting was controlled by a time switch to give 12 h continuous light (light period 6.00 a.m. to 6.00 p.m.) and 12 h darkness. The experimental animals were kept for overnight fast immediately before dosing and for approximately three to four hours after dosing, free access to mains drinking water and food (commercial diet from Hindustan Lever Limited, Bangalore, India) was allowed throughout the study. The animals were maintained under standard housing conditions (room temperature 21±2 ˚C and relative humidity 55-70%.

ACUTE ORAL TOXICITY STUDY

Experimental Procedure

Acute oral toxicity study was performed as per OECD 423 guidelines, albino Wistar rats (n=6) of either sex selected by random sampling technique were used for acute toxicity study. The animal was kept fasting for overnight providing only water, after which the extracts were administered orally at the starting maximum dose level 2000 mg/kg body weight by oral needle and observed for deaths of overt signs of toxicity ½, 1, 2 and 4 h after dosing and subsequently once daily for fourteen days. The signs of changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, motor activity and behavior pattern were noted. The toxicity signs of fits, excessive salivation, tremors, diarrhea, lethargy, sleep and coma, as well as the onset of toxicity and signs of toxicity were also noted (OECD, 2002).

CHRONIC ORAL TOXICITY STUDY

Experimental Group Design

Animals were separated into five groups (each group contains 6 rats).

Group I – animals received 1% tween 80 (5ml/kg, p.o) once daily for 90days and Considered as a vehicle control.

Group II – animals received an Ethyl acetate extract of the stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet* (EAE*No*), 5*0*0mg/kg, p.o.

Group III - animals received Ethanol extract of stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet* (EE*No*), 500mg/kg, p.o. once daily for 90days respectively.

Toxicological indications and symptoms were recorded daily. All groups of animal feed consumption, and animal's weight were monitored at the end of the Day 0, Day 30, Day 60 & Day 90. Similarly, hematological and biochemical parameters were estimated on Day 0, Day 30, Day 60 & Day 90 (Mukinda & Syce, 2007). After the last dose, all groups of animals were sacrificed by cervical dislocation at the end of the study. All animal organs (heart, spleen, liver and kidneys) were dissected out, observed necropsy and weighed. Histopathological studies have done on stomach, liver, heart and kidney.

Collection of blood samples in heparinised and non-heparinised bottles

Blood samples were obtained through a capillary tube by retro orbital puncture during mild diethyl ether anesthesia. At the end of the each 30day period, blood samples were collected. The blood samples were collected in heparinized bottles for hematological estimation and non-heparinised bottles for biochemical estimation. The collected blood samples in non-heparinised bottles and allowed to clot and for separation of serum for biochemical analysis. Biochemical analyses were performed in serum obtained after centrifugation of total blood without anticoagulants, at 2500 rpm for 15min (Sanaa Lahlou *et al*., 2008).

Assessment of hematological parameters

Assessment of Red Blood Cells (RBC), White Blood Cells (WBC), hemoglobin (Hb), Hematocrit, Lymphocyte and Neutrophils were determined using Sysmex automated hematology analyzer (Sysmex K4500) (Dacie & Lewis, 1958; Wintrobe *et al*., 1994).

Estimation of biochemical parameters

Blood Glucose determination was done with Accu-Chek One Touch Glucometer (Accu-Chek, Roche Diagnostics, USA). Total Cholesterol, Triglycerides, Total protein, Alanine Amino transferase (ALT) also known as Serum Glutamate Pyruvate Transaminase (SGPT), Aspartate Amino transferase (AST) also known as Serum Gluconate Oxaloacetate Transaminase (SGOT), bilirubin, Urea (Urease- UV Kinetic-GLDH), Creatinine (Jaffe's Method) were estimated using semi-automatic analyzer (Clinical Chemistry Photometer Analyzer 90) followed by International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standard procedures (Bergmeyer *et al*., 1977; Young, 2001; Vasiliades, 1976).

Histopathological Assessment

After sacrificed all group animals, they are dissected and collected each organ such as stomach, liver, heart and kidney. The organs were anchored in 10% formaldehyde solution and histologically prepared. The organ tissues were embedded in paraffin, solid sections of 5 µm thickness were procured from rotary microtome and stained with haematoxylin-eosin (HE) (Luna, 1968). The microscopical analysed were examined for histopathological alteration such as necrosis, oedema, congestion and haemorrhage.

Statistical analysis

The present research observations were signified as Mean ± Standard Error Mean. The statistical significance of dissimilarities amid the groups was evaluated by one way and multiple way analysis of variance (ANOVA) followed by Dunnett's test. P values less than 0.05 were deliberated as significance.

RESULTS

Individual morbidity, mortality data and necroscopy were observed. Acute oral toxicity studies confirmed that plant extracts [Ethyl acetate extract of the stem bark of *Neisosperma oppositifolium (Lam.) Fosb. & Sachet* (EAE*No*), Ethanol Extract of stem bark of *Neisosperma oppositifolium (Lam.) Fosb.* (EE*No*)] were non-toxic nature. After the administration of all plant extracts, the all group rats were observed immediately observed for 4hrs for autonomic and central nervous system, motor activity and behavior pattern for any changes or lethality for the next 14 days. There was no lethality or toxic reactions such as tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma found at the dose of 2000mg/kg until the end of the study period.

EFFECT OF DAILY ORAL ADMINISTRATION OF EAE*No* & EE*No* FOR UP TO 90 DAYS

General Behavior and Mortality Observation

During 90 days of the study, daily at the dose of 500mg/kg of EAE*No* & EE*No* did not produce any significant changes in behaviour, alteration in skin, no abnormalities in breathing, postural and defecation when compared to vehicle (Control Group) treated rats. Throughout the chronic toxicity study, no mortality was observed in plant extracts and vehicle treated animals.

Body weight Measurement

The mean of rat's body weight was measured for every 30 days in 90 days of the chronic toxicity study and presented in Table 1. During 90 days of the study all group animals were gaining weight as expected. But no significant difference in weight gain between vehicle (1% tween 80, 5ml/kg, p.o) and EAE*No* & EE*No* (500mg/kg, p.o) treated rats.

Observation of Feed intake

The amount food consumption was noted initial day and every 30 days of the study of the quantity food given and the amount remaining after 24 hrs. The food consumption of group II & III animals showed similar to that of vehicle treated animals. The feed intake data were represented in Table 2. There was no treatment related significant changes in feed intake in extracts treated group rats include vehicle treated rats.

Assessment of hematological parameters

The hematological parameters, Red Blood Cells (RBC), White Blood Cells (WBC), hemoglobin (Hb), Hematocrit, Lymphocyte and Neutrophils were estimated and expressed in Table 3 to 8. Chronic oral administration (90days of treatment) of EAE*No* & EE*No* did not showed significant changes in Hematocrit and Neutrophils when compared to vehicle treated group animals. But other hematological parameters, Red Blood Cells (RBC), White Blood Cells (WBC), Haemoglobin (Hb), and Lymphocytes were significantly elevated in EAE*No* & EE*No* when compared to vehicle treated group animals. But it is not abnormal than normal values.

Estimation of biochemical parameters

Biochemical parameter profiles of the extracts treated and control group animals shown in Table 9 to 17. A 90 day oral administration of EAE*No* & EE*No* did not cause any significant changes in total protein, bilirubin, urea and creatinine when compared to vehicle treated group animals. As for biochemical estimation, in the groups that received EAE*No* & EE*No* at the dose of 400mg/kg showed a significant difference in following

parameters such as blood glucose, total cholesterol, triglycerides, ALT and AST. Hepatic enzymes like ALT and AST was used for biological markers to identify the any early hepatic injury or hepatic toxicity. These hepatic enzymes were significantly decreased in extracts treated groups (within physiological range) when compared to vehicle treated group animals. Hence it was understood the protective and safety of the all plants extract. Simultaneously, the blood glucose, total cholesterol and triglycerides were decreased (within physiological range) significantly in extracts treated rats.

Observation of necropsy and measurement of organ weight

The stomach, heart, spleen, liver and kidneys were examined macroscopically. In plant extracts treated rats, no macroscopic lesions observed in the stomach and no pathological changes in heart, spleen, liver and kidneys. The oral ingestion of EAE*No* & EE*No* at the dose of 400mg/kg over 90 days caused mild significant changes (increased) in weight of the organs such as heart, spleen, liver and kidneys in the treated as compared to the vehicle treated group animals. This result indicates that all extracts not affect the growth of organs (Table 18).

Histopathological Assessment

No histological changes were observed in plant extracts treated rats similar to that of vehicle treated rats. No lesions and epithelial damages were observed in the stomach with extracts and vehicle treated rats (Figure 1). Histopathological abnormalities of liver such as vascular congestion, hepatocyte damage, vacuole formation and oedema were not observed in extract treated rats (Figure 2). In heart & kidney, there are no morphological changes observed in plant extract treated rats when compared to vehicle treated rats (Figure 3 & 4). Therefore, the increased weight of heart, spleen, liver and kidneys found in rats was considered unrelated to treatment.

Table 1. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on body weight

Treatment	Body Weight (g)			
	D_0	D_{30}	D_{60}	D_{90}
Group I -1% tween 80 (5ml/kg, p.o)	155.17±1.14	184.17±1.167	208.33±0.88	233.67±1.12
Group II EAE*No* 500mg/kg, p.o	153.50±1.18	186.50±0.99[a]	207.83±1.14[a]	220.67±1.02[a]
Group III EE*No* 500mg/kg, p.o	153.67±0.56[a]	189.83±0.75[a]	210.67±0.80[a]	227.67±0.76[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 2. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on feed intake

Treatment	Feed Intake (g/day/rat)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	23.52±1.22	24.21±1.42	24.66±1.54	24.41±1.54
Group II EAE*No* 500mg/kg, p.o	22.17±1.41[a]	23.27±1.14[a]	24.21±1.42[a]	23.19±1.33[a]
Group III EE*No* 500mg/kg, p.o	23.36±1.24[a]	24.14±1.22[a]	24.28±1.61[a]	24.27±1.64[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 3. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Red Blood Cells

Treatment	Red Blood Cells ($X10^6$ / mm^3)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	8.26±0.04	8.23±0.06	8.33±0.04	8.33±0.06
Group II EAE*No* 500mg/kg, p.o	8.23±0.04[a]	8.42±0.03[a]	8.57±0.03[a]	8.70±0.05[a]
Group III EE*No* 500mg/kg, p.o	8.18±0.03[a]	8.43±0.05[a**]	8.77±0.04[a**]	9.03±0.07[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 4. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on White Blood Cells

Treatment	White Blood Cells ($X10^3/mm^3$)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	9.59±0.04	9.58±0.02	9.61±0.05	9.67±0.06
Group II EAE*No* 500mg/kg, p.o	9.55±0.02[a]	9.74±0.03[a*]	9.86±0.02[a**]	9.89±0.01[a*]
Group III EE*No* 500mg/kg, p.o	9.56±0.05[a]	9.80±0.04[a*]	9.88±0.03[a**]	10.32±0.04[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 5. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Haemoglobin

Treatment	Haemoglobin (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	10.55±0.04	10.55±0.04	10.78±0.07	10.82±0.04
Group II EAE*No* 500mg/kg, p.o	10.58±0.02[a]	10.58±0.02[a]	11.31±0.02[a]	11.77±0.03[a]
Group III EE*No* 500mg/kg, p.o	10.62±0.02[a]	10.62±0.02[a**]	11.84±0.03[a**]	12.59±0.08[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 6. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Hematocrit

Treatment	Hematocrit (%)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	38.24±1.17	38.41±1.58	38.59±1.47	39.22±1.14
Group II EAE*No* 500mg/kg, p.o	37.94±1.37[a]	38.16±1.33[a]	39.39±1.42[a]	39.66±1.28[a]
Group III EE*No* 500mg/kg, p.o	38.32±1.52[a]	39.62±1.29[a]	40.19±1.33[a]	41.17±1.32[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 7. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Lymphocyte

Treatment	Lymphocyte (%)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	71.25±1.24	71.28±1.29	72.33±1.17	73.28±1.41
Group II EAE*No* 500mg/kg, p.o	72.17±1.32[a]	73.54±1.32[a]	74.56±1.42[a]	75.22±1.32[a]
Group III EE*No* 500mg/kg, p.o	74.22±1.61[a]	75.69±1.62[a]	76.23±1.67[a**]	77.62±1.63[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 8. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Neutrophils

Treatment	Neutrophils (%)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	13.21±0.42	13.33±0.34	13.42±0.31	14.17±0.42
Group II EAE*No* 500mg/kg, p.o	13.67±0.22[a]	13.19±0.61[a]	13.22±0.48[a]	13.32±0.60[a]
Group III EE*No* 500mg/kg, p.o	13.33±0.17[a]	13.52±0.27[a]	13.46±0.43[a]	14.32±0.42[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 9. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Blood Glucose

Treatment	Blood Glucose (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	92.00±2.02	94.17±0.75	95.67±0.95	100.83±1.08
Group II EAE*No* 500mg/kg, p.o	95.67±1.05[a]	95.17±0.83[a*]	95.50±1.12[a*]	95.50±1.09[a**]
Group III EE*No* 500mg/kg, p.o	100.00±1.41[a]	95.67±1.36[a*]	91.00±1.00[a**]	86.50±0.89[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01; [a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 10. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Total Cholesterol

Treatment	Total Cholesterol (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	115.29±1.33	112.32±1.54	114.24±1.28	116.39±1.49
Group II EAE*No* 500mg/kg, p.o	113.33±1.42[a]	115.21±1.84[a**]	112.52±1.37[a**]	110.24±1.36[a**]
Group III EE*No* 500mg/kg, p.o	110.69±1.19[a]	113.36±1.27[a**]	114.17±1.64[a**]	112.31±1.52[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 11. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Triglycerides

Treatment	Triglycerides (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	33.29±1.33	36.22±1.19	36.14±1.32	37.66±1.27
Group II EAE*No* 500mg/kg, p.o	34.19±1.42[a]	35.62±1.41[a]	35.17±1.24[a]	34.21±1.42[a]
Group III EE*No* 500mg/kg, p.o	33.64±1.24[a]	34.17±1.39[a]	31.94±1.21[a*]	30.37±1.46[a**]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 12. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Total protein

Treatment	Total protein (g/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	5.27±0.05	5.64±0.03	6.42±0.04	6.41±0.04
Group II EAE*No* 500mg/kg, p.o	5.33±0.04[a]	5.49±0.04[a]	5.54±0.03[a]	6.37±0.05[a]
Group III EE*No* 500mg/kg, p.o	5.45±0.03[a]	5.47±0.03[a]	6.69±0.05[a*]	6.88±0.03[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 13. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on ALT

Treatment	ALT (1Unit/L)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	30.21±0.52	31.27±0.46	32.52±0.68	33.52±0.52
Group II EAE*No* 500mg/kg, p.o	31.22±0.41[a]	31.17±0.57[a*]	30.33±0.56[a*]	29.60±0.33[a**]
Group III EE*No* 500mg/kg, p.o	29.46±0.56[a]	29.33±0.34[a*]	29.12±0.67[a*]	28.14±0.41[a**]

ALT- Alanine Aminotransferase; D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01; [a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 14. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on AST

Treatment	AST (1Unit/L)			
	D_0	D_{30}	D_{60}	D_{90}
Group I1% tween 80 (5ml/kg, p.o)	102.67±2.33	104.52±1.82	107.41±2.61	110.67±2.14
Group II EAE*No* 500mg/kg, p.o	103.22±2.41[a]	101.36±1.67[a*]	99.54±2.17[**]	98.33±2.27[a**]
Group III EE*No* 500mg/kg, p.o	105.41±2.86[a]	103.12±1.46[a*]	101.33±2.14[a**]	99.52±2.36[a**]

AST- Aspartate Aminotransferase; D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01; [a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 15. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Bilirubin

Treatment	Bilirubin (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	0.32±0.02	0.33±0.01	0.32±0.01	0.34±0.03
Group II EAE*No* 500mg/kg, p.o	0.33±0.01[a]	0.32±0.02[a]	0.34±0.03[a]	0.33±0.02[a]
Group III EE*No* 500mg/kg, p.o	0.34±0.02[a]	0.35±0.01[a]	0.32±0.01[a]	0.34±0.02[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 16. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Urea

Treatment	Urea (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	39.24±1.52	38.21±1.22	39.14±1.63	39.29±1.36
Group II EAE*No* 500mg/kg, p.o	40.67±1.42[a]	39.72±1.52[a]	39.33±1.28[a]	40.62±1.56[a]
Group III EE*No* 500mg/kg, p.o	39.52±1.22[a]	40.19±1.28[a]	41.32±1.14[a]	40.28±1.29[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 17. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on Creatinine

Treatment	Creatinine (mg/dl)			
	D_0	D_{30}	D_{60}	D_{90}
Group I 1% tween 80 (5ml/kg, p.o)	0.54±0.02	0.53±0.02	0.55±0.02	0.54±0.03
Group II EAE*No* 500mg/kg, p.o	0.52±0.01[a]	0.51±0.02[a]	0.53±0.02[a]	0.52±0.03[a]
Group III EE*No* 500mg/kg, p.o	0.53±0.01[a]	0.54±0.01[a]	0.52±0.03[a]	0.53±0.02[a]

D- Days; Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Table 18. Effect of daily oral administration of EAE*No* & EE*No* for up to 90 days on organ weight in rats

Treatment	Heart (g)	Spleen (g)	Liver (g)	Kidney	
				Right (g)	Left (g)
Group I 1% tween 80 (5ml/kg, p.o)	1.12±0.01	0.89±0.02	7.32±0.03	0.60±0.01	0.63±0.02
Group II EAE*No* 500mg/kg, p.o	1.17±0.02[a**]	0.91±0.02[a**]	7.72±0.02[a**]	0.62±0.02[a]	0.66±0.02[a*]
Group III EE*No* 500mg/kg, p.o	1.19±0.02[a**]	0.96±0.01[a**]	7.96±0.02[a**]	0.64±0.02[a*]	0.67±0.01[a**]

Data are expressed as Mean ± SEM; n=6 rats each group; *p< 0.05; **p< 0.01;
[a] statistically significant difference compared to the vehicle control: [a] Group I *Vs* Group II & III.

Figure 1. Histopathological studies on stomach (10X)

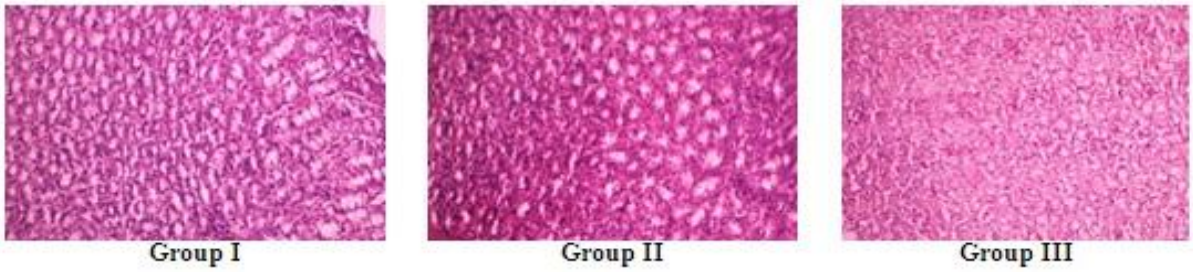

Figure 2: Histopathological studies on liver (10X)

Figure 3: Histopathological studies on Heart (10X)

Figure 4: Histopathological studies on Kidney (10X)

DISCUSSION AND CONCLUSION

In developed and developing countries, herbal medicine frequently playing a role in therapeutic system to which people are referred for their basic health care (WHO, 2007). Many times, phytotherapeutic drugs are falsely regarded as safe on account of "natural". Nevertheless, these herbal drugs contain bioactive constituents which produce potential adverse effects due to their poor Pharmacovigilance service because of difficult to estimate the adverse effects of them. Therefore, to confirm the safety use of plant based medicine, this medicinal herb must be subjected to the toxicological evaluation (Saad *et al*., 2006; Olson *et al*., 2000).

Now days, evaluation of safety of plant based medicine is mostly done in rats due to their good correlation between toxicological insults in humans and rats. It is weaker between human and mice (Rhiouani *et al*., 2008). Therefore, numerous chronic studies investigated that the chronic effects evaluated with lower

doses in rats, including the potential doses which used in humans (Lahlou *et al*., 2008; Teo *et al*., 2002).

In the 90 days chronic toxicity study, EAE*No* & EE*No* at the dose of 500mg/kg, did not show any toxicity, not affect the body weight or general behaviour of rats, and no significant changes in feed intake. Because decreasing the body weight of animals would be an indicator of adverse effects. It was confirmed that these plant extracts did not affect the normal metabolism and retard the growth of rats (Teo *et al*., 2002).The haemopoietic system is the most sensitive targets for toxic

agents and a substantial index of pathophysiological status in human and animal (Mukinda & Syce, 2007). In hematological parameters, there were no significant difference between the chronic oral administration (90days of treatment) of EAE*No* & EE*No* and vehicle treated groups in hematocrit and neutrophils. But EE*No* were significantly increased the Red Blood Cells (RBC) and hemoglobin (Hb) and it suggested that methanol extracts can be used to fight against anemia (Mukinda & Syce, 2007). At the same time, White Blood Cells (WBC) and Lymphocytes were increased significantly results in strengthening the immune system (Odou, 2005).

Estimation of Biochemical parameters, the EAE*No* & EE*No* at the dose of 500mg/kg showed a significant decrease in the level of ALT and AST when compared to the control group. Serum transaminase enzymes (ALT and AST) are used as biomarkers of liver function and act as indicators of liver toxicity. Hence, the results confirmed that oral administration of these extracts were safe and protected from liver damage in rats represented by significant depression of serum transaminase enzymes (Upur *et al*., 2009). Additionally, the above effect was confirmed by no significant changes in the level of bilirubin with extracts and vehicle treated group animals. Similarly, there were no significant changes in urea and creatinine in extracts and vehicle treated groups. These two parameters are effective indicator of proper kidney function (Lameire *et al*., 2005). This effect was also confirmed by histological examination of the kidney. Hence, the results recorded that these extracts did not affect the renal function.

These extracts significantly decreased the blood glucose, total cholesterol and triglyceride level when compared to the vehicle control during 90 days study. These hypoglycemic and hypolipidemic effects can be attributed to the plant extract. But these parameters exhibit within physiological range. At the same time, these extracts did not cause any significant changes in total protein.

According to Teo *et al* (2002), There will be a mild reduction in body weight and internal organ weights after exposure to potential toxic agents. The chronic (90 days) oral administration of EAE*No* & EE*No* at the dose of 500mg/kg showed slightly increased the weight of the organs (heart, spleen, liver and kidneys) as compared to the vehicle treated group animals. This result indicates that these extracts did not affect the growth of organs and organ to body weight ratio. In addition, these extracts did not change to their macroscopic characteristics of appearance, color, and size. Histopathological analysis of selected organs (stomach, liver, heart and kidney) was fatherly confirmed the non-toxic effect of these plant extracts. These extracts did not produce any detrimental effect and morphological changes in stomach, liver, heart and kidney similar to that of the vehicle control group (Luna, 1968).

The present study provides valuable information on the chronic toxicity profiles of EAE*No* & EE*No* that should be very helpful for further *in vivo* experimental studies of the pharmacological potentiality with the mode of administration and clinical study of these plants. In conclusion, at the oral dose 500mg/kg, of EAE*No* & EE*No* can be considered as safe and these extracts did not cause any lethality and adverse effects or changes in general behavior, hematological and biochemical parameters in the chronic toxicity study in rats.

ACKNOWLEDGEMENT: None

CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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