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Original Article

Acute and Sub-acute Toxicity Study of Aqueous Extracts of *Enicostemma axillare* (Lam.) Raynal in Animal models

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ABSTRACT

Background: *Enicostemma axillare* (Lam.) Raynal is used in traditional practice for the treatment of diabetes, malaria and liver disorders. No systematic toxicity study was described for this plant and hence the present was undertaken to evaluate acute and sub-acute toxicity of aqueous extract of *Enicostemma axillare* (AEEA). **Objective:** The acute oral toxicity study of AEEA was carried out as per the OECD guidelines 423 in mice and the sub-acute toxicity was carried out as per the guidelines set by OECD 407 in male and female rats. **Materials and Methods:** Body weight, food and water consumption, hematological parameters, biochemical parameters, organ weight and histopathological analysis were carried out. **Results**: No gross toxicity and mortality was observed upto a dose of 2000 mg/kg. For sub-acute toxicity test, 200 mg/kg and 400 mg/kg daily dose of AEEA administered orally for 28 days in male and female group of rats not exhibited any signs of toxicity and mortality. **Conclusion**: In acute oral toxicity study, the oral administration of AEEA in mice was found to be safe up to a dose of 2000 mg/kg. Both male and female treated rats showed no change in hematological, biochemical and histological investigations and no signs of toxicity were observed upto the dose of 400 mg/kg in rats.

Key words: Acute toxicity, *Enicostemma axillare,* Histology, Sub-acute toxicity, OECD guidelines.

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INTRODUCTION

Enicostemma axillare (Lam.) Raynal (EA) also called as Vellarugu in Tamil and Chotachirayata in Hindi is an important group of medicinal plant belonging to the Gentianaceae family. EA (Figure 1) is a glabrous perennial rainy season herb, growing on moist, damp and shady ridges and slopes of the borders of cultivated fields, chiefly near the sea and often on black cotton soil.[1] Whole plant of EA was reported for the presence of phenols, tannins, flavonoids, glycosides, anthraquinones and sterols.[2] The decoctions of the leaves of EA are used in rheumatism, abdominal ulcers, diabetes, cold swelling, antipyretic, and cough and for stomachache.[3] Anti-diabetic effect of the aqueous extract of EA on streptozotocin induced diabetes mellitus in rats was reported.[4-6] Antidiabetic efficacy on alloxan induced diabetes mellitus in rats for the extracts of EA was reported.[7-10] Swertiamarin was isolated from EA[11] and screened for antioxidant and hepatoprotective effect of compound against D-galactosamine induced acute liver damage in rats. Hepatoprotective effect of EA was reported on carbon tetrachloride induced hepatic damage in rats.[12-13] Hypolipidaemic and antioxidant effect of aqueous extract of EA was reported in cholesterol fed rats.[14] Aerial parts of EA was reported to possess hypolipidaemic effect in *p*-dimethyl aminobenzene (PDAB) induced hepatotoxic animals.[15]

In India, *Enicostemma axillare* is used by the traditional healers for the treatment of liver disorders, which we have scientifically validated in our earlier study in rats.[16] To ascertain and establish the safety for its use in traditional practice, acute and sub-acute toxicity studies of the aqueous extract of *Enicostemma axillare* were carried out as per OCED guidelines.

MATERIALS AND METHODS

Plant materials

Enicostemma axillare was collected during August 2014 and the plants were authenticated (BSI/SRC/5/23/2014-15/Tech/873) by Dr. M. Palanisamy, Scientist C, Botanical survey of India, Coimbatore, India. Voucher specimens of the plants were deposited at the Department of Pharmacognosy, KMCH College of Pharmacy, and Coimbatore for future reference.

Preparation of extracts

Whole plant of *Enicostemma axillare was* cleaned with distilled water to remove earthy matter and residual materials. It was then shade dried at room temperature (32 ± 2°C) for 10 days, pulverized to coarse powder, passed through a #40 mesh sieve. Then d*ecoction was prepared* by boiling 100 g of the ground *plant* material in 500 mL of distilled *water* for about 30 min. Filtered and concentrated separately under reduced pressure IKA Rotary evaporator (Model No RN 10 digital V, ILMAC Germany) at 40°C. Percentage yield of AEEA was 16.2 % w/w.

Animals

Female Albino mice (7-8 weeks old, 20-30 g) and Wistar strains of either sex rats (10-12 weeks old, 150-200 g) were obtained from the animal house of Kovai medical center research and educational trust, Coimbatore, India. The animals were kept in polypropylene cages at a temperature of 25 ± 2°C, RH (50 ± 5%), 12 h light and dark cycles. They were fed with standard laboratory animal diet and water *ad libitum*. Animals were acclimatized to laboratory conditions before the test. Experiments were designed and conducted in accordance with ethical norms approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Institutional Animal Ethical Committee (IAEC)(KMCRET/AICTE/01/2014-15, dt.31/01/2015).

Acute toxicity study

Acute toxicity test was performed according to OECD[17] guideline 423 for testing of chemicals (2001). Healthy young adult albino nulliparous, non-pregnant female mice weighing about 20-30 g were administered as a single dose (1 ml) orally using oral feeding needle with 5, 50, 300 and 2000 mg/kg of AEEA in distilled water. Animals were observed individually for first 30 min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days to observe toxicity signs like changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems and behavioural pattern. On 15th day animals were anaesthetized, blood was

Figure 1: Entire plant of *Enicostemma axillare*

collected from the animals for haematological and biochemical analysis. Mice were then sacrificed, dissected and the organs lungs, liver, spleen and kidney were carefully collected, weighed, processed and observed under photomicroscope for histopathological examination.

Sub-acute toxicity study

The sub-acute toxicity assessment of AEEA was performed as per OECD Guideline 407.[18] Wistar strain of rats (150-200 g) was divided into six groups, each consisting of six male and six female rats. Male and female Control groups received only distilled water. Two other groups of each male and female were administered with AEEA in distilled water orally using rat oral feeding needles, daily for 28 days at a dose of 200 mg/kg and 400 mg/kg respectively. During the treatment period the body weight of animals were monitored on 0, 7th, 14th, 21stand 28th day. Food consumption and water intake for all the groups were observed from day 1 to 28 days.

On 29th day, the overnight fasted rats were anaesthetized with diethyl ether inhalation in a jar containing cotton soaked with diethyl ether. Then blood samples were withdrawn from retro-orbital sinus and the collected blood samples were evaluated for hematological parameters viz. Red blood cells (RBC), White blood cells (WBC), Hemoglobin (Hb), Platelet count, Packed cell volume (PCV), Differential count, Mean Platelet Volume (MPV), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC) and Red blood cell distribution width (RDW).

A portion of the blood samples were centrifuged at 10000 rpm for 10 min and the separated serum was analyzed for biochemical parameters viz. Cholesterol, triglycerides, VLDL levels SGOT, SGPT, ALP, total bilirubin, total protein, albumin, globulin, urea, uric acid and creatinine. Biochemical investigations were carried out in an auto analyser (Photometer 5010 V5+, Robert Riely, Berlin) using Piramal healthcare limited reagent kit. The animals were sacrificed by cervical dislocation and the organs, heart, liver, spleen, kidneys, stomach, testes and ovary were isolated and weighed processed and observed under photomicroscope for histopathological examination.

Statistical analysis

The results are expressed as the mean ± SEM. The significance of the difference was evaluated by one-way ANOVA followed by Dunnett's test. Data were considered statistically significant if $p<0.05$.

RESULTS

Acute Toxicity Study

Single oral administration of AEEA at a dose of 5, 50, 300 and 2000 mg/kg as per OECD guideline 423 for 14 days did not produce any mortality in tested animals (Table 1). No observable sign of toxicity was detected during the experimental period.

Sub-acute toxicity study

Daily oral administration of AEEA at a dose of 200 mg/kg and 400 mg/kg elicited no change in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems and behaviour pattern of the animals observed. Absence of tremors, convulsions, salivation, diarrhoea, and sleep also noted (Table 2). Thus in sub-acute toxicity study,

Table 1: Observations of Acute toxicity study of AEEA

Group	Dose (mg/kg)	Number of animals	Number of survival	Number of deaths	Percentage of mortality
1	Control	3	3	0	0
2	5	3	3	0	0
3	50	3	3	0	0
4	300	3	3	0	0
5	2000	3	3	0	0

Table 2: Behavioural Studies of AEEA at a dose 200 and 400 mg/kg on rats in sub-acute toxicity study

Gross activity	30 Min	1h	2 h	3 h	4 h	24h	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15	Day 17	Day 19	Day 21	Day 23	Day 25	Day 28
Respiration	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Writhing	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tremor	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Convulsion	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sense of touch/sound	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salivation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urination	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Defecation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diarrhoea	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sedation	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Locomotor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
edema	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Mortality	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

+ Indicates normal; – Indicates no effect.

no sign of toxicity viz. jerk, convulsion and mortality was observed for both the male and female rats treated with 200 and 400 mg/kg dose of AEEA.

Parameters observed during acute and sub-acute toxicity studies

Body weight

Gain in body weight of mice and rats was observed in acute and sub-acute toxicity study respectively for control group and AEEA administered animals and the results are recorded in Table 3 and 4.

Table 3: Assessment of body weights of mice in acute toxicity study

Treatment	Body weight (g)			
	0 day	7th day	14th day	Weight gain on 14th day
Control	19.98 ± 0.52	21.75 ± 0.35	23.91 ± 0.49	3.93 ± 0.36
AEEA 2000 mg/kg	19.56 ± 0.54	23.58 ± 0.75[a]	26.95 ± 1.44[b]	7.38 ± 1.39[c]

Data provided as mean ± SEM (n=6); [a]p<0.05 treated groups vs control [b]p<0.01 treated groups Vs control; [c]p<0.001 treated groups vs control.

Table 4: Assessment of body weights of rats in sub-acute toxicity study

Groups	Day 1	Day 7	Day 14	Day 21	Day 28	Weight gain (g) on day 28 th day
Male Control	153.6 ± 1.49	182.16 ± 2.61	212.66 ± 2.94	231.66 ± 4.58	250.5 ±1.62	96.83 ± 2.72
Male AEEA 200 mg/kg	127.66 ± 1.35	144.5 ± 2.89	163.66 ± 2.99	184.5 ± 3.25	200.16 ± 4.06	71.33 ± 3.06b
Male AEEA 400 mg/kg	167.33 ± 1.94	190.33 ±5.55	199.16 ± 4.74	229.0 ±2.46	247.5 ± 2.54	80.16 ± 2.79 b
Female Control	124.66 ± 0.98	146.3 ± 0.71	164.33 ± 1.66	182.0 ± 2.35	199.5 ± 2.37	74.83 ± 2.45
Female AEEA 200 mg/kg	132.5 ± 1.76	151.0 ± 4.179	168.66 ± 5.53	187.66 ± 6.23	205.66 ± 3.52	73.16 ± 4.30 c
Female AEEA 400 mg/kg	154.16 ± 1.49	182.5 ± 1.05	199.16 ± 2.72	217.66 ± 3.49	233.66 ± 4.01	81.16 ± 5.54c

Data provided as mean ± SEM (n=6); ap<0.05 treated groups vs control bp<0.01 treated groups Vs control;cp<0.001 treated groups vs control.

Food and distilled water consumption

Food and distilled water consumption of mice (acute toxicity) and rats (sub-acute toxicity) were continuously monitored, where there is no change in consumption was observed for treated groups and the control group animals (Figure 2 to Figure 5).

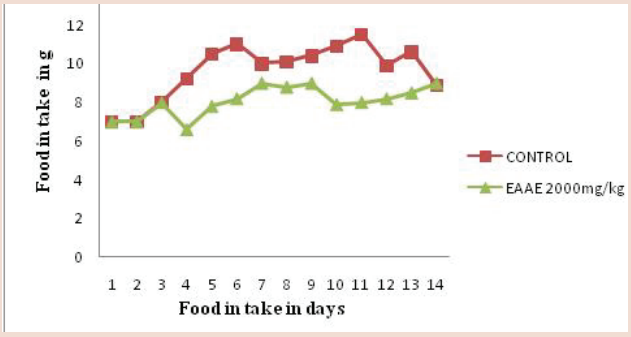

Figure 2: Food intake in g during acute toxicity study for 14 days

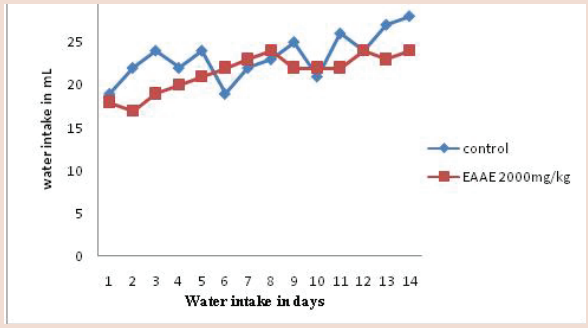

Figure 3: Water intake in ml during acute toxicity study for 14 days

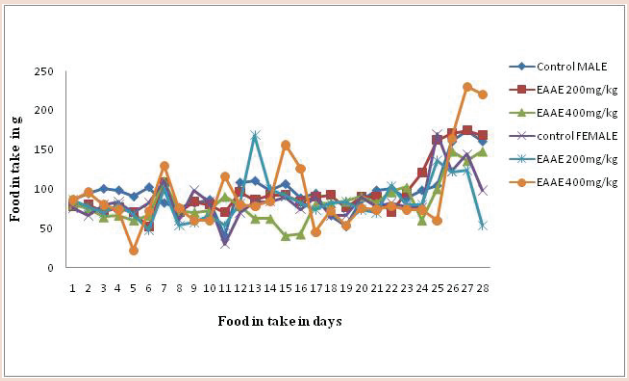

Figure 4: Food intake in g during acute toxicity study for 28 days

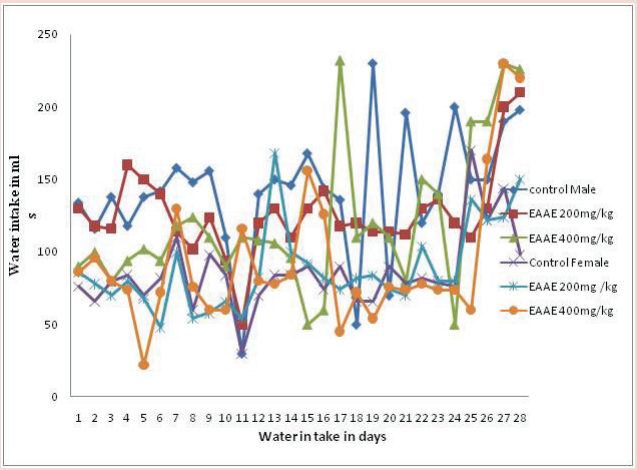

Figure 5: Water intake in mL during acute toxicity study for 28 days

Hematological analysis

The results of hematological investigations (Table 5 and 6) conducted on day 14th day for acute toxicity study and on 29th day for sub-acute toxicity study revealed no significant changes in the values of RBC, WBC, Hb, platelet count, PCV, differential count, MPV, MCV, MCH, MCHC and RDW of treated groups when compared with the respective control mice and rats respectively.

Biochemical Investigations

Biochemical investigations were performed in order to review any toxic effects produced after administration of AEEA on liver and kidney. There was no significant alteration in cholesterol triglycerides and VLDL levels in treated groups of sub-acute toxicity study when compared with control group of rats (Table 7). No significant change observed in (serum glutamic oxaloacetic transaminase) SGOT, (Serum glutamic pyruvic transaminase) SGPT, (alkaline phosphatase) ALP and total bilirubin content of treated group animals when compared with control group animals (Table 8 and 9). There was no significant alteration observed in creatinine, urea and uric acid levels of treated group animals when compared with control group animals (Table 10 and 11).

Table 5: Evaluation of hematological parameters of mice in acute toxicity study

Groups	Total RBC ($X10^6$cells/mm^3)	Total Hb (g/dl)	Total WBC ($X10^6$cells/mm^3)	Platelet Count ($X10^6$cells/mm^3)	PCV (%)	Differential Count				MPV (fL)	MCV fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
						Polymorphs (%)	Lymphocytes (%)	Monocytes (%)	Eosnophils (%)					
Control	14.40 ± 0.69	14.48 ± 0.42	13.98 ± 0.75	789.5 ± 13.67	45.58 ± 1.49	5.16 ± 0.30	80.50 ± 2.23	3.66 ± 0.42	2.83 ± 0.30	8.05 ± 0.31	60.3 ± 0.43	19.15 ± 0.19	32.31 ± 0.54	18.9 ± 0.48
AEEA 2000 mg/kg	16.25 ± 0.73[a]	13.82 ± 0.73[a]	13.55 ± 0.57[a]	757.16 ± 32.89[a]	42.33 ± 0.63	4.83 ± 0.60[a]	85.33 ± 2.41[a]	3.0 ± 0.36[a]	3.66 ± 0.55[a]	8.01 ± 0.27[a]	60.01 ± 1.02[a]	19.15 ± 0.18[a]	32.06 ± 0.55[a]	17.66 ± 0.79[a]

Data provided as mean ± SEM (n=6); ap>0.05 treated groups vs control.

Table 6: Evaluation of hematological parameters of rats in sub-acute toxicity study

Groups	Total RBC ($X10^6$ cells/mm^3)	Total Hb (g/dl)	Total WBC ($X10^6$ cells/mm^3)	Platelet Count ($X10^6$ cells/m^3)	PCV (%)	Differential Count				MPV (fL)	MCV fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
						Polymorphs (%)	Lymphocytes (%)	Monocytes (%)	Eosnophils (%)					
Control Male	4.98 ± 0.13	14.95 ± 0.590	11.55 ± 0.82	740.16 ± 40.67	46.48 ± 1.66	5.0 ± 1.06	84.83 ± 2.05	3.33 ± 0.21	5.66 ± 0.55	7.33 ± 0.20	63.95 ± 0.73	22.25 ± 0.68	31.88 ± 0.35	17.84 ± 0.41
Male AEEA 200 mg/kg	4.94 ± 0.46[a]	15.31 ± 1.19[a]	11.7 ± 1.25[a]	808.33 ± 23.93[a]	45.8 ± 3.39[a]	6.83 ± 1.60[a]	84.16 ± 2.24[a]	3.5 ± 0.71[a]	3.83 ± 0.40[a]	7.96 ± 0.22[a]	66.86 ± 1.57[a]	22.58 ± 0.61[a]	28.33 ± 2.37[a]	17.11 ± 0.45[a]
Male AEEA 400 mg/kg	4.58 ± 0.18[a]	14.66 ± 0.73[a]	12.13 ± 0.90[a]	735.66 ± 38.92[a]	42.86 ± 1.83[a]	4.83 ± 1.07[a]	86.0 ± 2.23[a]	3.66 ± 0.33[a]	5.0 ± 0.77[a]	7.431 ± 0.23[a]	65.36 ± 0.50[a]	21.26 ± 0.37[a]	31.85 ± 0.77[a]	17.42 ± 0.32[a]
Control Female	5.0 ± 0.10	15.36 ± 0.36	11.65 ± 0.39	777.83 ± 30.75	52.78 ± 0.96	5.00 ± 0.81	86.16 ± 1.60	3.76 ± 0.33	5.66 ± 0.33	8.1 ± 0.06	71.5 ± 1.54	32.88 ± 0.36	32.80 ± 0.36	18.45 ± 0.14
Female AEEA 200 mg/kg	5.66 ± 0.40[a]	16.61 ± 0.20[a]	12.28 ± 0.91[a]	823.83 ± 12.65[a]	54.36 ± 1.31[a]	6.83 ± 0.48[a]	90.33 ± 2.49[a]	4.33 ± 0.42[a]	5.0 ± 0.25[a]	8.2 ± 0.15[a]	73.66 ± 1.22[a]	32.93 ± 0.36[a]	32.40 ± 0.35[a]	17.83 ± 0.31[a]
Female AEEA 400 mg/kg	4.83 ± 0.31[a]	14.81 ± 0.31[a]	12.54 ± 0.69	836.16 ± 10.87[a]	50.95 ± 1.17[a]	5.83 ± 0.30[a]	89.00 ± 1.15[a]	4.50 ± 0.34[a]	5.66 ± 0.49[a]	8.41 ± 0.13[a]	70.91 ± 0.58[a]	33.5 ± 0.46[a]	34.28 ± 0.35[a]	18.48 ± 0.14[a]

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Table 7: Effect of sub-acute dose of AEEA on lipid profile of rats

Groups	Dose mg/kg (p.o.)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	VLDL (mg/dl)
Control Male	Distilled water (5 ml/kg)	95.05 ± 5.76	262.43 ± 34.98	52.58 ± 7.03
Male AEEA	200 mg/kg	93.51 ± 3.13[a]	217.36 ± 39.86[a]	43.47 ± 7.97[a]
Male	400 mg/kg	110.95 ± 3.47[a]	290.03 ± 7.66[a]	58.00 ± 1.53[a]
Control Female	Distilled water (5 ml/kg)	89.03 ± 4.50	295.76 ± 14.55	59.15 ± 2.91
Female AEEA	200 mg/kg	93.51 ± 3.09[a]	290.66 ± 17.61[a]	58.13 ± 3.52[a]
Female AEEA	400 mg/kg	96.0 ± 1.61[a]	288.66 ± 7.07[a]	57.73 ± 1.4[a]

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Table 8: Assessment of liver function test of mice in acute toxicity study

Groups	Dose mg/kg *(p.o.)*	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total Bilirubin (mg/dl)
Control	Distilled water (5 ml/kg)	33.01 ± 1.01	29.08 ± 2.03	249.08 ± 6.83	0.8 ± 0.07
AEEA	2000 mg/kg	37.08 ± 0.98[a]	33.61 ± 1.88[a]	251.23 ± 9.88[a]	0.83 ± 0.09[a]

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Organ weight

No abnormal change in the relative weight of internal organs of mice and rats was observed when compared to control group as shown in Table 12 and 13.

Table 9: Assessment of liver function test of rats in sub-acute toxicity study

Groups	Dose mg/kg *(p.o.)*	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total Bilirubin (mg/dl)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/L)
Control male	Distilled water (5 ml)	54.29 ± 4.38	48.25 ± 2.07	237.35 ± 16.92	0.76 ± 0.09	7.23 ± 0.28	3.8 ± 0.18	3.08 ± 0.29
Male AEEA	200	38.58 ± 7.47 [a]	36.56 ± 1.39 [a]	238.53 ± 18.29 [a]	0.82 ± 0.01 [a]	8.03 ± 0.22 [a]	3.38 ± 0.26 [a]	4.41 ± 0.31 [a]
Male AEEA	400	57.36 ± 5.20 [a]	48.95 ± 6.23 [a]	231.21 ± 31.22 [a]	0.95 ± 0.05 [a]	7.75 ± 0.31 [a]	3.8 ± 0.18 [a]	3.76 ± 0.35 [a]
Control Female	Distilled water (5 ml)	51.33 ± 1.85	44.61 ± 1.25	272.75 ± 5.36	0.86 ± 0.04	7.16 ± 0.26	4.26 ± 0.11	2.86 ± 0.16
Female AEEA	200	45.08 ± 4.09 [a]	38.50 ± 1.40 [a]	268.66 ± 4.96 [a]	0.84 ± 0.02 [a]	7.78 ± 0.22 [a]	4.03 ± 0.11 [a]	3.40 ± 0.08 [a]
Female AEEA	400	48.35 ± 2.11 [a]	43.50 ± 3.03 [a]	286.66 ± 6.16 [a]	0.97 ± 0.03 [a]	7.36 ± 0.15 [a]	4.28 ± 0.08 [a]	3.31 ± 0.18 [a]

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Table 10: Assessment of kidney function test of mice in acute toxicity study

Groups	Dose mg/kg (P.O.)	Urea (mg/dl)	Uric acid (mg/dl)
Control	Distilled water (5 ml/	26.90 ± 2.81	4.810 ± 0.09
AEEA	2000	28.38 ± 1.33 [a]	4.616 ± 0.07 [a]

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Table 11: Assessment of kidney function test of rats in sub-acute toxicity study

Groups	Dose mg/ kg *(p.o.)*	Creatinine	Urea (mg/dl)	Uric acid (mg/dl)
Control male	Distilled water (5 ml/kg)	0.7 ± 0.05	18.63 ± 1.29	2.3 ± 0.15
Male AEEA	200 mg/kg	0.7 ± 0.03 a	22.18 ± 2.01 a	1.75 ± 0.11 a
Male AEEA	400 mg/kg	0.73 ± 0.05 a	20.35 ± 1.88 a	2.2 ± 0.12 a
Control Female	Distilled water (5 ml/kg)	0.8 ± 0.02	19.06 ± 0.42a	2.34 ± 0.08
Female AEEA	200 mg/kg	0.75 ± 0.03 a	20.83 ± 0.65 a	2.23 ± 0.09 a
Female AEEA	400 mg/kg	0.8 ± 0.02 a	20.73 ± 1.29 a.	2.38 ± 0.14 a

Data provided as mean ± SEM (n=6); ap>0.05 treated groups vs control.

Table 12: Assessment of relative organ weight (g per 100 g body wt) in acute toxicity study

Groups	Dose |mg/kg *(p.o.)*	Heart (g)	Lungs (g)	Liver (g)	Spleen (g)	Left kidney (g)	Right kidney (g)
Control	Distilled water (5 ml/kg)	0.40 ± 0.02	1.01 ± 0.06	4.85 ± 0.17	0.50 ± 0.02	0.72 ± 0.03	0.65 ± 0.02
AEEA	2000 mg/kg	0.74 ± 0.03 a	1.05 ± 0.10 a	5.76 ± 0.69 a	0.49 ± 0.04 a	0.77 ± 0.08 a	0.70 ± 0.07 a

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Table 13: Assessment of relative organ weight (g per 100 g body wt) in sub-acute toxicity study

Groups	Heart (g)	Lungs (g)	Liver (g)	Spleen (g)	Left kidney (g)	Right kidney (g)	Thymus (g)	TESTES/ OVARIES (g)
Control Male	0.352 ± 0.006	0.698 ± 0.021	2.658 ± 0.041	0.726 ± 0.018	0.473 ± 0.013	0.387 ± 0.013	0.293 ± 0.015	1.07 ± 0.024
Male AEEA 200 mg/kg	0.361 ± 0.007 [a]	0.762 ± 0.044 [a]	2.515 ± 0.059 [a]	0.758 ± 0.013 [a]	0.459 ± 0.020 [a]	0.375 ± 0.012 [a]	0.257 ± 0.018 [a]	1.07 ± 0.050 [a]
Male AEEA 400 mg/kg	0.362 ± 0.009 [a]	0.755 ± 0.040 [a]	2.726 ± 0.069 [a]	0.692 ± 0.014 [a]	0.461 ± 0.017 [a]	0.359 ± 0.007 [a]	0.258 ± 0.01 [a]	1.16 ± 0.015 [a]
Control Female	0.373 ± 0.012	0.756 ± 0.023	2.34 ± 0.036	0.628 ± 0.035	0.291 ± 0.007	0.367 ± 0.12	0.187 ± 0.013	0.499 ± 0.044
Female AEEA 200 mg/kg	0.391 ± 0.015 [a]	0.742 ± 0.037 [a]	2.463 ± 0.074 [a]	0.762 ± 0.075 [a]	0.295 ± 0.019 [a]	0.342 ± 0.005 [a]	0.197 ± 0.008 [a]	0.521 ± 0.013 [a]
Female AEEA 400 mg/kg	0.389 ± 0.011 [a]	0.749 ± 0.023 [a]	2.242 ± 0.054 [a]	0.691 ± 0.018 [a]	0.276 ± 0.010 [a]	0.336 ± 0.010 [a]	0.204 ± 0.007 [a]	0.513 ± 0.021 [a]

Data provided as mean ± SEM (n=6); [a]p>0.05 treated groups vs control.

Histopathological investigation

Acute toxicity study in mice

Lungs

Normal alveoli with blood vessels and normal bronchioles were observed in histopathological investigation of lungs of control group of animals in both 10 x and 40 x magnifications. Normal alveoli with bronchioles was observed in treated group of animals in both the magnifications (Figure 6).

Liver

Histopathological section of liver in control group and AEEA treated group (2000 mg/kg) showed normal lobular architecture. The central

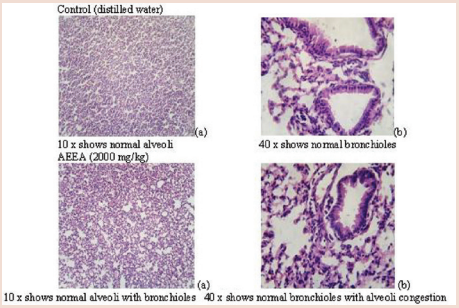

Figure 6: Histology of lungs from control and treated mice (10x (a) and 40x (b) magnifications)

veins, sinusoids are found to be normal in the AEEA treated groups. Toxic signs like inflammation, fatty change or fibrosis were not found (Figure 7).

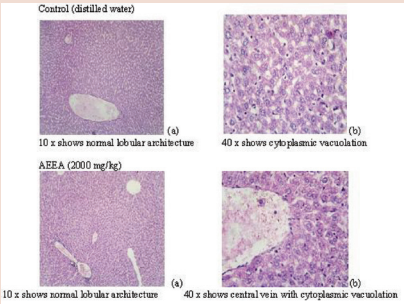

Figure 7: Histology of liver from control and treated mice groups (10x (a) and 40x (b)magnifications)

Spleen

Section from the spleen of control group and EAAE treated group (2000 mg/kg) showed red pulp congestion. No change was observed in white pulp, pencillar artery and the red pulp (Figure 8).

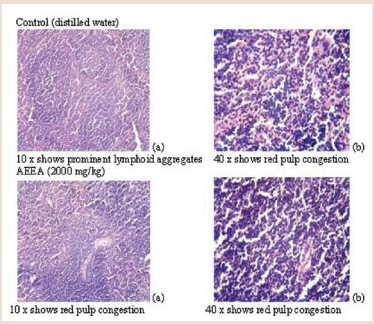

Figure 8: Histology of spleen from control and treated mice (10x (a) and 40x(b) magnifications)

Kidney

Sections from kidney of control group and AEEA treated group (2000 mg/kg) showed normal cortex, medulla and normal glomeruli. The interstitium was found to be normal and no inflammation or necrosis was observed (Figure 9).

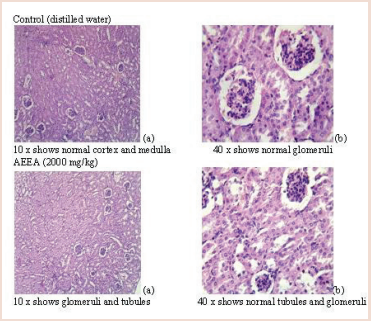

Figure 9: Histology of kidney from control and treated mice (10x (a) and 40x(b) magnifications)

Sub-acute toxicity study in rats

Heart

Section from the heart of control group and AEEA treated to male and female groups at a dose of 400 mg/kg showed normal myocytes (Figure 10 and 11).

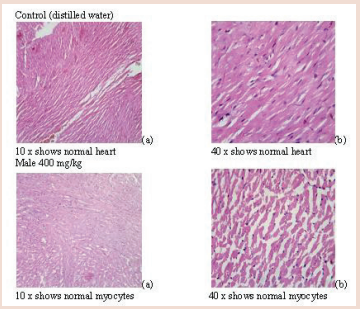

Figure 10: Histology of heart from control& treated rat (male) (10x (a) and 40x (b) magnifications)

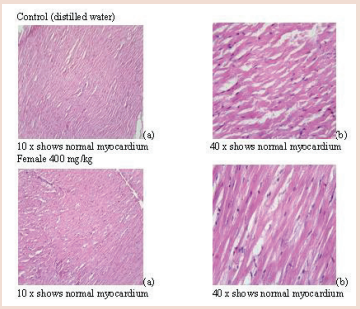

Figure 11: Histology of heart from control& treated rats (female) (10x (a) & 40 x (b) magnifications)

Lungs

Normal alveoli and normal bronchioles were observed in control group and AEEA treated to male and female groups at a dose of 400 mg/kg (Figure 12 and 13).

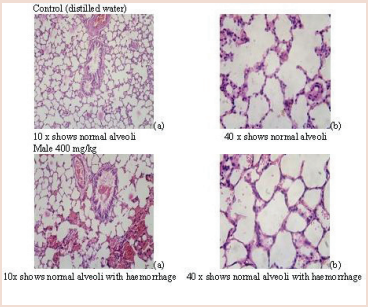

Figure 12: Histology of lungs from control& treated rat (male) groups(10x (a)&40x(b)magnifications)

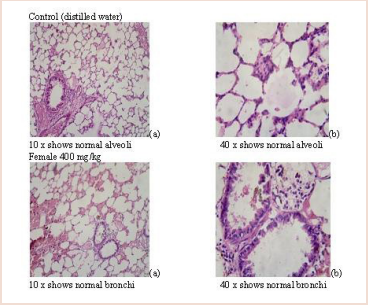

Figure 13: Histology of lungs from control& treated (female) groups (10x(a)&40x(b)magnifications)

Liver

Histopathological section of liver in control group and AEEA treated to male and female groups at a dose of 400 mg/kg showed normal lobular architecture. The portal tracts, hepatocytes, central veins, sinusoids are found to be normal. No evidence of toxic signs observed as there is no inflammation, fatty change or fibrosis (Figure 14 and 15).

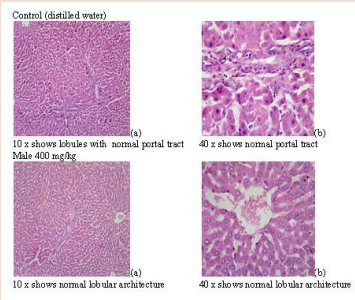

Figure 14: Histology of liver from control& treated rat (male) groups(10x (a)&40x(b) magnifications)

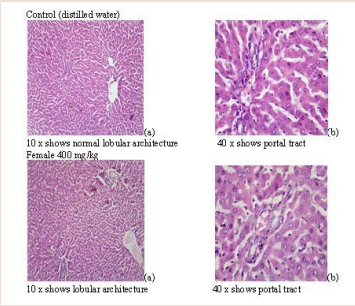

Figure 15: Histology of liver from control& treated rats (female) (10x(a)&40x(b) magnifications)

Stomach

Section from the stomach of control group and AEEA treated to male and female groups at a dose of 400 mg/kg showed normal mucosa, muscle layer and stomach glands (Figure 16 and 17).

Spleen

Section from the spleen of control group and AEEA treated to male and female groups at a dose of 400 mg/kg showed normal spleen with red

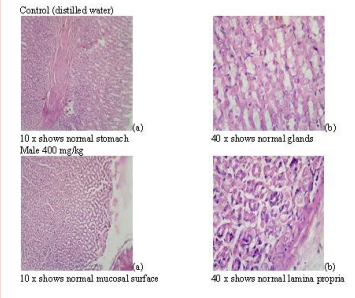

Figure 16: Histology of stomach from control& treated rat (male) (10x(a) and 40x (b) magnifications)

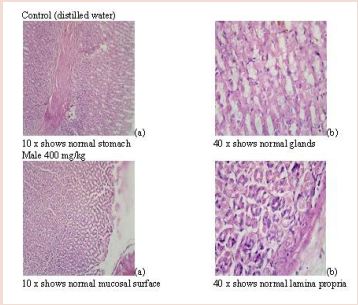

Figure 17: Histology of stomach from control& treated rats (female) (10x(a)&40x(b)magnifications)

pulp and germinal center formation. No abnormality was observed in histological sections of spleen (Figure 18 and 19).

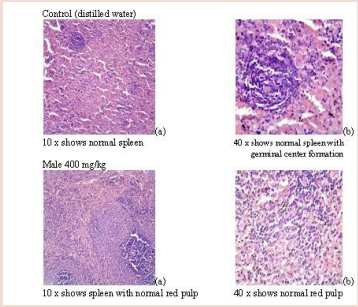

Figure 18: Histology of spleen from control& treated rat (male) (10x (a) and 40x (b) magnifications)

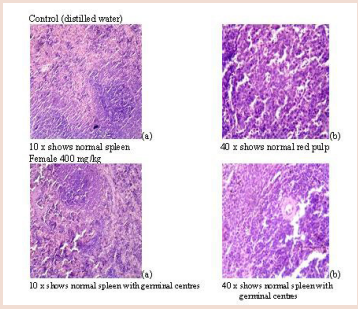

Figure 19: Histology of spleen from control& treated rats (female) (10x (a)& 40 x(b) magnifications)

Kidney

Sections from kidney of control group and AEEA treated to male and female groups at a dose of 400 mg/kg showed normal cortex and medulla. The cortex showed normal glomeruli. The interstitium and distal convoluted tubules are found to be normal. No inflammation or tubular necrosis was observed (Figure 20 and 21).

Testes

Sections from kidney of control group and AEEA treated to male groups at a dose of 400 mg/kg showed normal testes and spermatozoa (Figure 22).

Ovary

Sections from kidney of control group and AEEA treated to female groups at a dose of 400 mg/kg showed normal ovarian follicles (Figure 23).

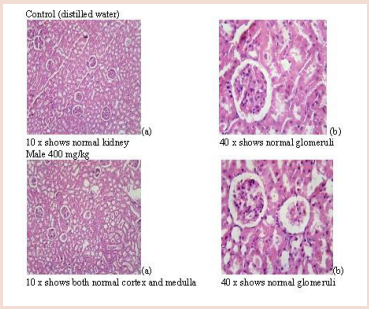

Figure 20: Histology of kidney from control& treated rat (male) (10x (a) and 40x (b) magnifications)

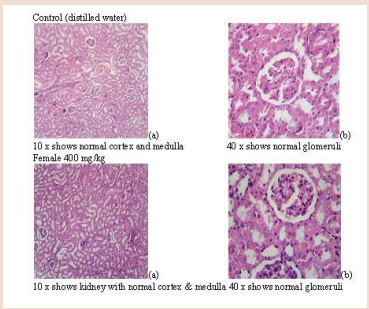

Figure 21: Histology of kidney from control& treated rats (female) (10x (a)& 40 x(b)magnifications)

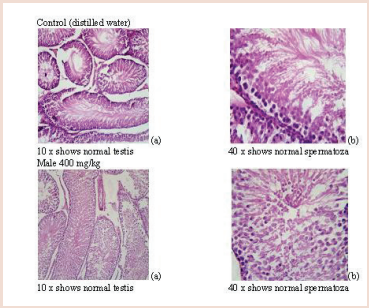

Figure 22: Histology of testes from control& treated rat (male) (10x (a) and 40x (b) magnifications)

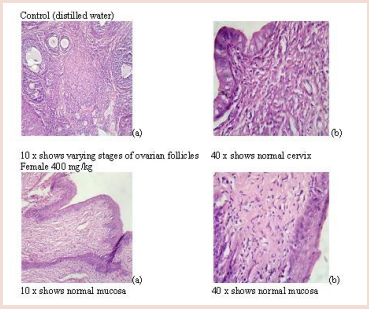

Figure 23: Histology of ovary from control& treated rats (female) (10x (a) & 40 x(b) magnifications)

DISCUSSION

Acute and sub-acute studies in mice and rats were performed as per OECD guidelines 423 and 407 respectively to assess the safety profile of AEEA, as there were no earlier reports on the safety assessment of aqueous extract of *Enicostemma axillare*. In acute toxicity test, mortality was not observed in mice with the maximum dose of 2000 mg/kg throughout the 14 days of study. In sub-acute toxicity test also no mortality was observed in male and female rats with the maximum dose of 400 mg/kg throughout 28 days of study. Significant changes were not found in breathing, sense of touch/sound, central nervous systems, behaviour pattern and locomotor activity. Convulsion, tremor, excessive salivation, diarrhea, sedation and edema were not observed.

Body weight and internal organ weight changes is sensitive and indicative marker for the first sign of toxicity when exposed to toxic substances.[19] All animals were found to be active with increase in body weight both in acute and sub-acute toxicity study. Normal food and water consumption of animals clearly support the gain in body of animals throughout the study period.

Hematopoietic system is the most sensitive target for toxic substances and it is the important index of physiological and pathological status[20-21] and hence hematological investigation was carried out. No abnormality was observed in hematopoietic function indices for AEEA treated groups compared with control groups indicating the extract is safe.

Lipid profile assessment was performed for the animals treated with AEEA to verify any changes observed in serum levels of triglycerides, cholesterol and VLDL.[22] No significant variation in cholesterol, triglycerides and VLDL levels in AEEA treated groups when compared with control groups.

SGOT, SGPT, ALP and bilirubin levels of the experimental animals were monitored as they are the specific markers for liver damage or injury.[23] No significant changes in SGOT, SGPT, ALP and bilirubin level was observed in all the treated group animals. Thus animals treated with AEEA were found to be non-hepatotoxic.

To assess whether treatment of AEEA to rats causes any damage to kidney, kidney function test was performed. GLDH-UV Kinetic method, Jaffe method and Uricase POD method was adopted to evaluate the important markers of renal dysfunction viz. urea, uric acid and creatinine levels respectively.[24] AEEA was found to be safe and nontoxic to kidney as there is no significant change observed in creatinine, urea and uric acid levels of AEEA treated group of animals both in acute and sub-acute toxicity when compared with control group animals. When compared with control groups, no variation in relative organ weight was observed for AEEA treated groups both in acute and sub-acute toxicity study.

The above mentioned biochemical investigations were in correlation with the histopathological studies.

Section of the heart of the control and AEEA treated animals showed no abnormality as shown in Figures 10 and 11. Alveoli and bronchioles were found to be normal both in control and treated group confirming that AEEA did not cause any toxicity to lungs of the animals. Inflammation or cirrhosis or necrosis was not observed in the histological study of liver in mice and rats tested with AEEA at a dose of 400 mg/kg. Normal cortex, medulla and normal glomeruli were observed indicating that AEEA did not cause any damage to kidney. Cross section of stomach under low and high power magnification showed normal mucosal surface, normal stomach glands and normal lamina propria indicated that AEEA not caused any toxicity to rats. Histological sections of testes clearly implicated that no testicular toxicity was observed to the treated male group of rats for 28 days in sub-acute study. Observation of normal cervix, mucosa and follicles from histological sections of ovary confirmed that AEEA was non toxic to ovary of female rats.

CONCLUSIONS

Based on our results, we conclude that AEEA were found to be safe up to a dose of 2000 mg/kg/day. Hematological, biochemical and histopathological investigations clearly demonstrates that single oral administration upto 2000 mg/kg in acute toxicity study and daily oral administration of the AEEA for 28 days upto 400 mg/kg in sub-acute toxicity study caused no damage to the organs like heart, lungs, liver, spleen and kidney. Abnormalities were also not observed in the organs stomach, testes and ovary of the animals tested at a dose of 200 and 400 mg/kg respectively. The study provided significant data on the sub-acute toxicity profile of AEEA which may be valuable in the clinical study of medicinal herb *Enicostemma axillare*. The aqueous extract of *Enicostemma axillare* thus may be used for manufacturing pharmaceutical formulations.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors have declared that there is no Conflict of interest.

ABBREVIATION USED

EA: *Enicostemma axillare* (Lam.) Raynal; AEEA: Aqueous extract of *Enicostemma axillare*; OECD: Organisation for Economic Co-operation and Development PDAB: p-dimethyl aminobenzene; RH: Relative Humidity; CPCSEA: Committee for the Purpose of Control and Supervision on Experiments on Animals; IAEC: Institutional Animal Ethical Committee; RBC : Red blood cells; WBC: White blood cells; Hb: Hemoglobin; PCV: Packed cell volume; MPV: Mean Platelet Volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW: Red Blood Cell Distribution Width; VLDL: Very low density lipoprotein; SGOT : Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic-pyruvic transaminase; ALP: Alkaline phosphatase; GLDH: Glutamate dehydrogenase; POD: Peroxidase.

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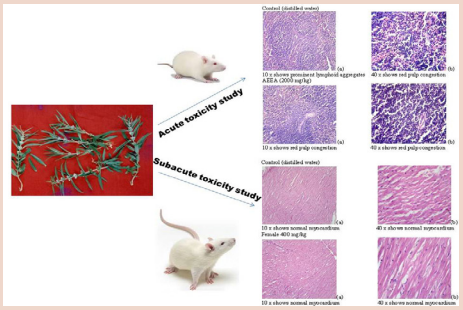
PICTORIAL ABSTRACT



SUMMARY

- In acute oral toxicity study, the oral administration of AEEA in mice was found to be safe up to a dose of 2000 mg/kg.
- Both male and female treated rats showed no change in hematological, biochemical and histological investigations.
- No signs of toxicity or mortality were observed in sub-acute toxicity study upto the dose of 400 mg/kg in rats.

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