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Research Article

Biochemical Changes in Mice Treated with *Eupatorium adenophorum* (Sticky snakeroot) for 60 Days

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ABSTRACT

The leaves of *Eupatorium adenophorum* Spreng were powdered and extracted with methanol. An acute oral toxicity study was conducted in male Swiss albino mice and a LD_{50} of 3501 mg/kg was obtained during 14 days observation period. Twenty Swiss albino mice (male) randomly divided into four groups were administered orally with vehicle (5% tween 80), $1/20^{th}$ (i.e. 175 mg/kg), $1/10^{th}$ (i.e. 350 mg/kg) and $1/5^{th}$ (i.e. 750 mg/kg) LD_{50} doses of methanolic leaf extract of *E. adenophorum* Spreng; respectively for a period of 60 days. Treatment of the mice with methanolic extract of *E. adenophorum* at the dose level of 750 mg/kg (i.e. $1/5^{th}$ LD_{50}) elicited hepatotoxicity and the animals had marked enlargement of liver without yellowish discoloration of liver, subcutaneous tissue and musculature. The sera samples revealed marked increase in bilirubin levels and activities of alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH), while reductions in glucose, cholesterol, triglycerides, total protein and albumin levels. Histopathological examination of the livers of the group IV animals had dilated bile ducts and focal areas of necrosis with mononuclear cells infiltration. Elevation of plasma bilirubin concomitant with alterations in enzyme profile and histopathological lesions are consistent with liver injury and cholestasis.

Keywords: *Eupatorium adenophorum*; Hepatotoxicity; Biochemical; Mice

1. INTRODUCTION

Eupatorium adenophorum (syn. *Ageratina adenophora*, common name: Crofton weed; Sticky snakeroot), a native of Central America has appeared as a major weed in several areas in different parts of the world and has infested the grazing areas in the lower and mid hills in the Himalayan region of India[1]. *E. adenophorum* is an important weedy colonizer in early succession communities developing after slash and *jhum* (shifting cultivation) at high elevations of North Eastern Hill Region of India[2,3].

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There are many reports of using the whole plant, leaves and shoots of *E. adenophorum* as folklore medicines in different parts of the world. Traditional practitioners in Darjeeling Himalaya give the young leaves and shoots of *Eupatorium adenophorum* Linn (Asteraceae) orally against dysentery[4]. A decoction of the plant has been recommended to treat jaundice and ulcers[5] and that of the leaves is given to cure stomachache among the tribal people of Meghalaya and Nagaland[6].

Although, *E. adenophorum* is having many medicinal values, the plant has been reported by some workers to possess pneumotoxic as well as hepatotoxic effects in different species of animals. Regular ingestion of *E. adenophorum* caused chronic pulmonary disease mainly in Australia, New Zealand, and the Himalayas[7]. *E. adenophorum* leaf samples collected from Kangra Valley (India) and partially purified extracts from leaf samples

mixed in the diet caused hepatotoxicity and cholestasis in rats[8,9]. Exposure of mice to feed containing *E. adenophorum* freeze-dried leaf powder caused hepatotoxicity[10]. Methanolic extract of *E. adenophorum* leaf samples collected from Mizoram (India) has also been reported to induce hepatotoxicity in albino mice[11].

Liver is the vital organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders. Certain medicinal agents when taken in overdoses and sometimes even when introduced within therapeutic ranges may injure the liver.

Keeping the above information in view, the present study was undertaken to investigate the biochemical changes in mice treated with *Eupatorium adenophorum* (*Sticky snakeroot*) for 60 days.

2. MATERIALS AND METHODS

2.1 Chemicals

All the chemicals and solvents were of analytical grade and were procured from E. Merck (India) Ltd, Mumbai and Sigma (St. Louis, MO, USA). The standard kits for AST, ALT, ALP, LDH and bilirubin were obtained from Crest Biosystems (Goa), India.

2.2 Plant material and preparation of extract

The fresh leaves of the plant of *Eupatorium adenophorum* was collected at the flowering stage from bushes in the vicinity of the College of Veterinary Sciences and A.H., Central Agricultural University, Selesih, Aizawl, Mizoram (India) (Fig-1). The plant was authenticated by Botanical Survey India, Shillong (Ref. No.BSI/ERC/Tech/2010/052 dated 27.04.2010) and a voucher specimen was deposited as herbarium to the Regional Office, BSI, Shillong.

The collected leaves of the plant were washed; mopped by blotting paper and then dried under shade. On complete drying, whole of the leaves were ground to powder with Willey grinder and sifted through sieve number 22. The dried leaf powder of *E. adenophorum* was subjected to cold maceration technique[12, 13] with slight modification. One hundred (100g) grams of powder was soaked in 500 ml of methanol (1: 5 w/v) in a conical flask and stirred for a period of 3 days with intermittent stirring and at the end of 3rd day the content was filtered with muslin cloth followed by Whatman filter paper No-1. For complete extraction of the active principles, this process is repeated three times using fresh solvent on each occasion or until the color of the methanol becomes light. The filtrate obtained was pooled and further subjected to vacuum evaporation at 30^{0}C in a rotary evaporator and lyophilized for successive 24 hours. Lyophilization was stopped when the extract appeared sufficiently dry. Further the material was stored at -40^{o}C in deep freezer in air tight containers until use.

2.3 Preparation of oral suspension

The methanolic extract was found insoluble in water; therefore, for different dose levels, a stock suspension was prepared in tween 80 and diluted with the vehicle (5% tween 80) immediately before use for oral administration.

2.4 Experimental animals

In the present study, 50 male Swiss albino mice (*Mus musculus*) of 25-30 g were obtained from the colony stock of Laboratory Animal House, College of Veterinary Sciences and A.H., Central Agricultural University, Selesih, Aizawl, Mizoram. They were given a standard pelleted diet and water *ad libitum* throughout the experimental period. A twelve-hour day and night cycle was maintained in the animal house. The ambient temperature and relative humidity during the experimental period were 22-24ºC and 65-70%, respectively. The experimental protocol met regulatory guidelines on the proper care and use of animals in laboratory research and was approved by the Institutional Animal Ethics Committee (IAEC) of West Bengal University of Animal and Fishery Sciences (Reg. No. 763/03/a/CPCSEA dated. 05.06.03) vide Ref. No. E.C./93 dated 24.06.2011.

2.5 Acute toxicity study

Thirty (30) male mice were randomly selected and divided into six groups of five animals each. The animals were fasted overnight. Group-I animals were orally administered the vehicle (5% tween 80), while the animals of Groups II-VI were given

single doses of methanolic leaf extract of *E. adenophorum* (MEA) in progressively increased manner (1350, 2025, 3050, 4575 and 6900 mg/Kg respectively) for determination of the acute lethal dose (LD_{50}). However, food and water were provided throughout the experiment. Immediately after dosing, the animals were observed continuously for the first 72 hours for mortality and any signs of overt toxicity. The surviving animals were also observed up to 14 days for signs of toxicity. The number of mice that died within the period of study was noted for each group, and subsequently the LD_{50} value calculated[14]. All animals that died during the observation period and euthanatized mice were subjected to necropsy.

2.6 Experimental design

Twenty (20) male mice were randomly divided into four groups of five animals each. Animals of Group-I served as vehicle (5% tween 80) treated controls, while animals of Groups II, III and IV were administered orally with the methanolic leaf extract of *E. adenophorum* (MEA) at daily doses of 175 mg/kg ($1/20^{th}$ LD_{50}), 350 mg/kg ($1/10^{th}$ LD_{50}) and 700 mg/kg ($1/5^{th}$ LD_{50}) respectively for 60 days. Food and water were freely available during the experiment. The animals in treated groups were observed daily for physical and behavioral changes as signs of toxicity. On termination of the experiment, all the animals were weighed and then euthanatized using ether anesthesia. Livers were removed immediately, weighed, rinsed in ice-cold saline, blotted, and used for various biochemical assays and histological studies. Half of each liver was processed for biochemical analysis and the other half preserved in 10% formalin for histological examination.

2.7 Biochemical assays

Biochemical analysis was performed on serum obtained after centrifugation of total blood (without anticoagulant) at 4000 rpm for 10 minutes. Standardized diagnostic kits (Crest Biosystems, Goa, India) were used for spectrophotometric (Spectrascan UV 2600, Chemito) determination of the following biochemical parameters: glucose, cholesterol, triglycerides, total proteins, albumin, total bilirubin, direct (conjugated) bilirubin, urea and creatinine in different experimental groups of the animals, according to manufacturer's protocol[15].

Liver was minced and homogenized (10% w/v) in ice-cold 0.1 M sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 rpm for 15-20 min at 4°C twice to get the enzyme fraction. The supernatant was used for estimation of liver enzymes like alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) which were estimated spectrophotometrically (Spectrascan UV 2600, Chemito) in different experimental groups of the animals using standard commercial kits (Crest Biosystems, Goa, India) according to manufacturer's protocol[16,17].

2.8 Gross and histopathological examination

After collection of blood on termination of the study (i.e. on 61), the mice were humanely sacrificed for macroscopic examinations. The external parts as well as the subcutaneous tissue and musculature were properly observed to record the visible gross changes. The vital organs such as liver, heart, lungs, spleen and kidneys were carefully isolated and examined for any lesions. All these vital organs were fixed in 10% formaldehyde solution for histopathological examinations.

Formalin fixed liver tissues (2-3 mm thick) were taken, washed overnight in running tap water and then dehydrated in ascending grades of alcohol starting from 50%, 70%, 90% and absolute alcohol I, alcohol II, alcohol III and finally cleared in cederwood oil or xylene. These dehydrated tissue pieces were then embedded in molten paraffin. Sections were cut at 3-5 µm thick and stained with Mayer's hematoxylin and eosin method of staining for histopathological examinations[18].

2.9 Statistical analysis

The data generated during the present investigation were analysed using suitable statistical formulae[19]. One way analysis of variance (ANOVA) was employed to find the significant differences between the groups. For any significant value of F, least significant difference (lsd) test was used to determine the significant differences between any two groups. A significant difference at $P \leq 0.05$ was considered statistically significant. All

the statistical analyses were done using a computer programme (SYSTAT 6.0.1 version software).

3. RESULTS AND DISCUSSION

3.1 Acute toxicity

Mice administered with methanolic leaf extract of *E. adenophorum* (MEA) at the dose level of 1350 mg/kg body weight showed no signs of toxicity and mortality, while those at dose levels of 2025 and 3050 mg/kg body weight showed partial loss of appetite, pilo-erection and hypoactivity with 20% mortality in 48 hours. The dose level of 4575 mg/kg body weight produced hypoactivity, disorientation, hyperventilation, convulsion and 60% mortality. However, the dose level of 6900 mg/kg body weight had severe clinical signs and all animals died within 4-6 hours.

The doses of LD_{50} study thus obtained were then plotted on semi-logarithmic paper against the probit and a best fitted linear scale was drawn. In the present study, the Log LD_{50} was 3.544 and the acute oral LD_{50} of methanolic leaf extract of *E. adenophorum* (MEA) was found to be 3501 mg/kg body weight (2157≤ 3501 ≥ 5682 mg/kg with 95% confidence).

This LD_{50} value was lower than 5000 mg/kg reported by other workers in mice with alcoholic extract of *E. adenophorum Sprengel*[20]. This LD_{50} value was also lower than 3761 mg/kg in mice with methanolic leaf extract of the plant using refined vegetable oil as vehicle[11]. However, methanolic extract of *E. adenophorum* at 2000 mg/kg did not produce any signs overt toxicity in rats[21]. The difference in the LD_{50} values might be due to using of different vehicles and also due to variation of geographical region, soil and other environmental factors. This suggests that the vehicle, 5% tween 80, used in the present study might have increased the ready absorption of the plant extract in mice and also the *E. adenophorum* plant growing in the region is apparently more toxic.

3.2 Clinical signs

The vehicle controlled mice (Gr-I) remained normal throughout the experimental period, while the animals in group-II (treated with $1/20^{th}$ LD_{50} i.e. 175 mg/kg) showed a partial loss of appetite, dullness and slight depression. The group-III animals (treated with $1/10^{th}$ LD_{50} i.e. 350 mg/kg) became dull, depressed and had rough hair coat after 10 days of treatment. However, the animals in group-IV (treated with $1/5^{th}$ LD_{50} i.e. 700 mg/kg) became dull and depressed within 10 days and had very less appetite leading to body weight loss (data not shown). They had rough hair coat and appeared jaundiced when observed after 7 days of treatment. The ear pinnae and paws became yellowish. However, signs of jaundice had disappeared during the later part of the study.

3.3 Changes in the biochemical parameters

Groups III and IV animals exhibited a marked decrease in the serum levels of glucose ($P \leq 0.05$), cholesterol and triglycerides ($P \leq 0.01$) as compared to group-I (control). The animals of groups II, III and IV showed significant reduction in total proteins and albumin levels ($P \leq 0.05$ or $P \leq 0.01$) as compared to those in group-I (control). The total bilirubin as well as the conjugated bilirubin levels were significantly higher ($P \leq 0.05$ or $P \leq 0.01$) in the animals of groups III and IV when compared with that of control and group-II. No significant changes were noted in the levels of urea and creatinine in all experimental groups of animals (Table-1).

There were marked increases ($P \leq 0.05$ or $P \leq 0.01$) in the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) in the animals of groups II, III and IV as compared to those in group-I (control), while the alkaline phosphatase (ALP) activity was significantly increased ($P \leq 0.05$ or $P \leq 0.01$) only in groups III and IV animals when compared with the control and group-II (Table-2).

The increase in the activity of transaminases is known to be the indicator of degenerative changes in organs or tissues like liver and myocardium[15,22,23]. Increased levels of transaminases and ALP activities are known to occur in a wide range of diseases of liver like cholestasis, biliary obstruction and hepatic necrosis[24]. The elevation of serum enzymatic activity in the present study is attributed to *E. adenophorum* - induced hepatic damage/ or necrosis as confirmed from histopathological observations.

Similar biochemical changes have been observed in the plasma of rats exposed to leaf powder, methanolic extract and partially purified fraction of *E. adenophorum*[1,9,10,25]. In a short-term toxicity study of *E. adenophorum* in Swiss albino mice conducted[11], similar observations of the biochemical alterations were reported.

3.4 Gross and histopathological changes

Postmortem examination of the animals in the groups I and II revealed no appreciable gross changes of the liver and other visceral organs, while the group-III mice which received MEA @ 350 mg/kg showed enlargement of the liver when compared with the control. However, a marked enlargement of liver was observed in the group IV mice which received the highest dose of MEA (700 mg/kg) without yellowish discoloration of liver, subcutaneous tissue and musculature.

Histopathological studies also provided supportive evidence for the biochemical analysis depicted by the photomicrographs of the liver (Figs. 2a, 2b, 2c and 2d). The mice of group-I (control) showed no changes in the normal architecture of liver (Fig. 2a), while mild degenerative changes were observed in group-II animals (Fig. 2b). Liver sections of group-III animals revealed mild to moderate bile duct proliferation and focal areas of necrosis with mononuclear cells infiltration (Fig. 2c). However, group-IV mice showed dilated bile ducts and marked focal areas of necrosis with mononuclear cells infiltration (Fig. 2d). Similar changes have been observed during development of toxicity due to the whole leaf powder, methanolic extract and partially purified fraction of *E. adenophorum* in rats[1,9,10,25] and also in mice fed with methanolic leaf extract of the plant[11]. It was also observed that administration of *E. adenophorum* leaf powder to mice caused degeneration of intrahepatic bile ducts and hepatocellular necrosis[26].

Table 1: Changes in the biochemical parameters of mice treated with methanolic leaf extract of *E. adenophorum* (MEA) (60 days exposure, n = 5)

Parameters	Vehicle Control	Treatment with MEA			F-value
		175 mg/kg	350 mg/kg	700 mg/kg	
Glucose (mg/dl)	88.11^{a}±1.82	83.78^{ab}±2.64	80.43^{b}±1.46	78.70^{b}±1.26	4.91*
Cholesterol (mg/dl)	127.13^{a}±4.95	118.39^{ab}±3.78	107.82^{b}±3.61	106.66^{b}±3.97	5.52**
Triglycerides (mg/dl)	102.10^{a}±3.78	100.98^{ab}±3.32	$91.19b^{c}$±3.17	86.99^{c}±3.32	4.72**
Total protein (g/dl)	5.17^{a}±0.21	4.52^{b}±0.07	3.92^{c}±0.12	3.53^{c}±0.14	24.57**
Albumin (g/dl)	3.33^{a}±0.09	2.61^{b}±0.13	2.52^{b}±0.09	2.09^{c}±0.12	21.10**
Total bilirubin (mg/dl)	0.673^{c}±0.03	0.749^{c}±0.06	0.970^{b}±0.03	1.230^{a}±0.04	34.03**
Conj. Bilirubin (mg/dl)	0.484^{d}±0.04	0.658^{c}±0.06	0.845^{b}±0.03	1.102^{a}±0.04	38.09**
Urea (mg/dl)	41.30±0.91	39.74±0.72	38.89±0.66	39.52±1.04	1.47^{NS}
Creatinine (mg %)	0.446±0.03	0.456±0.02	0.495±0.01	0.491±0.02	1.24^{NS}

Values are Mean ± SEM (n=5). ** Significance at $p \leq 0.01$; * $p \leq 0.05$ and NS = Not significant

Table 2: Changes in the liver enzyme activities of mice intoxicated with methanolic leaf extract of *E. adenophorum* (MEA) (60 days exposure, n = 5).

Group/Treatment (mg/kg/day)	AST (IU/l)	ALT (IU/l)	ALP (IU/l)	LDH (IU/l)
I (Vehicle control)	72.48^{d}±1.77	64.08^{d}±2.34	31.68^{c}±2.45	109.32^{d}±4.76
II (MEA-175)	88.61^{c}±2.96	95.33^{c}±4.23	33.93^{c}±1.97	133.99^{c}±5.72
III (MEA-350)	132.68^{b}±3.49	178.13^{b}±4.00	51.02^{b}±1.76	205.31^{b}±7.20
IV (MEA-700)	226.97^{a}±5.26	325.94^{a}±5.32	93.93^{a}±3.07	533.95^{a}±12.71
F-value	371.88**	810.97 **	148.59**	574.72**

Values are Mean ± SEM. ** Significance at $p \leq 0.01$; * $p \leq 0.05$.

Fig-1: Leaves and flowers of plant *Eupatorium adenophorum*

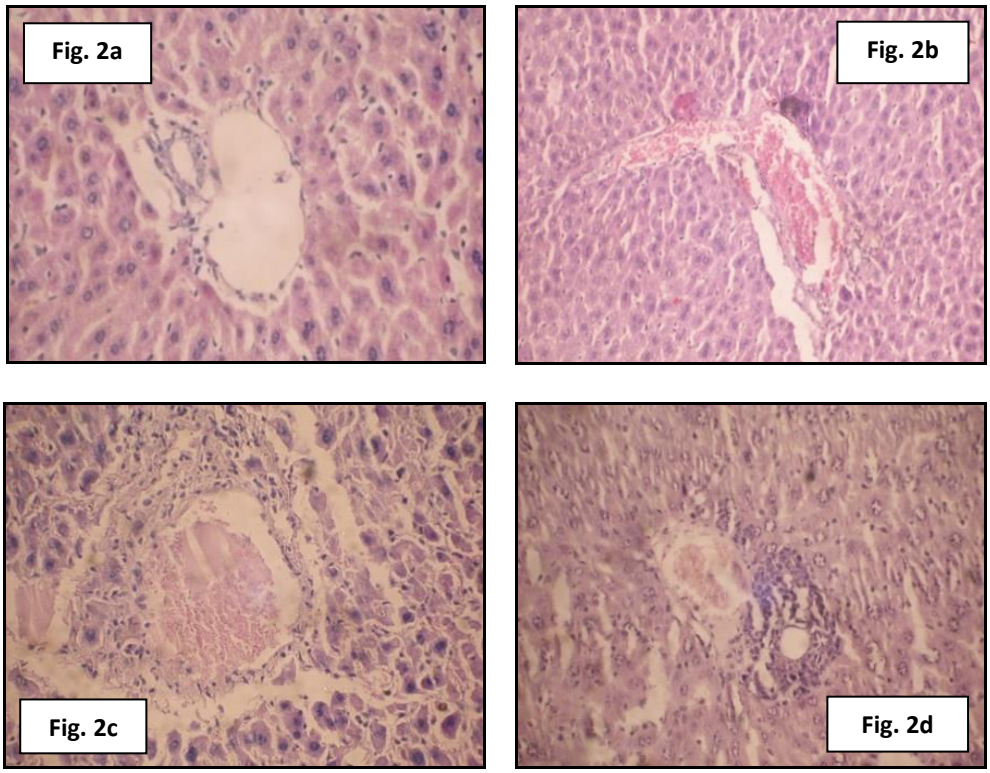

Fig. (2a): Control liver showing normal architecture (HandE x 400); **(2b)** Group-II liver showing mild degenerative changes (HandE x 200); **(2c)** Group-III liver showing mild to moderate bile duct proliferation and focal areas of necrosis with mononuclear cells infiltration (HandE x 400) and **(2d)** Group-IV liver showing dilated bile ducts and focal areas of necrosis with mononuclear cells infiltration (HandE x 200).

4. CONCLUSIONS

The present study shows that the toxicity of methanolic leaf extract of *Eupatorium adenophorum* is responsible for alterations in biochemical parameters which is dose dependant and the dose level of $1/5^{th}$ LD_{50} (i.e. 700 mg/kg body wt.) is highly hepatotoxic to mice. Therefore, the oral consumption of *E. adenophorum* for medicinal purposes without proper dosing should be avoided as it could potentially toxic to higher animals too.

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