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# Pathology of *Eupatorium adenophorum* (Sticky snakeroot) toxicity in mice

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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 30 days. The mice were sacrificed on day-31 and the liver dissected out freed from adherent tissue weighed to nearest milligram. The liver histology, estimations of biochemical contents and enzyme activities were carried out. 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 yellow discoloration of liver, subcutaneous tissue and musculature indicating jaundice. Study on liver enzymes revealed marked increase in the activities of alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH), while significant increase in serum bilirubin level. Histopathological examination of the livers of the group IV animals had focal areas of necrosis and bile duct proliferation. Elevation in plasma bilirubin concomitant with alterations in enzyme profile and histopathological lesions are consistent with liver injury and cholestasis

**Keywords:** *Eupatorium adenophorum;* hepatotoxicity; bilirubin; liver enzymes; mice

## 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 widely growing shrub in most parts of NE region particularly in Mizoram. *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]. It is a perennial herb, nearly 1 meter high, and erect [4]. Grazing animals get accidentally exposed to the plant under scarcity conditions. A considerable variation between the animal species exists in terms of susceptibility to toxicity due to *E. adenophorum.*

There is a lot of variation in the susceptibility of different animal species to the noxiousness of *E. adenophorum*. The content of natural products in the plants is known to vary with geographical region, soil and other environmental factors [5]. Consumption of *E. adenophorum* by horses results in pulmonary toxicity [6, 7]. Moreover, regular ingestion of *Eupatorium adenophorum* [*Ageratina adenophora (Spreng.)*] or Crofton weed causes chronic pulmonary disease in horses mainly in Australia, New Zealand, and the Himalayas [8].

Toxicity due to consumption of this plant by other grazing animals is not clear. It has been reported that no toxic effects were seen in goats when *E. adenophorum* collected from Nepal comprising up to 67% of their intake, was administered [9]. However, experimental studies reported that feeding of *E. adenophorum* plant growing in north-eastern India to cattle caused anorexia, suspension of rumination and photosensitization [10]. In studies with laboratory animals, mice were shown to be suitable test animals, but in this species lesions occur in the liver rather than the lungs. Exposure of mice to feed containing *E. adenophorum* freeze-dried leaf powder caused hepatotoxicity. In mice, hepatotoxicity involved multiple areas of focal necrosis of the parenchyma associated with degeneration and loss of epithelial cells lining of the bile duct [11].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 [12, 13]. We report here the $LD_{50}$ of *E. adenophorum* (methanolic extract) and its toxic effects on albino mice in Mizoram.

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The present study was undertaken to evaluate effects of methanolic leaf extract of *Eupatorium adenophorum* on liver histology and activity of liver enzymes such AST, ALT, ALP and LDH in albino mice.

## MATERIALS AND METHODS

### 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.

Fig.1. Leaves and flowers of plant *Eupatorium adenophorum*

### 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 & A.H., Central Agricultural University, Selesih, Aizawl, Mizoram (India). 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 as described by Manjunatha *et al.* (2005) [14] and Harborne (1998) [15] 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 3$^{rd}$ 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.

### 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.

### 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 & 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 & Fishery Sciences (Reg. No. 763/03/a/CPCSEA dated. 05.06.03) vide Ref. No. E.C./93 dated 24.06.2011.

### 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 [16] (Miller and Tainter, 1944). All animals that died during the observation period and euthanazied mice were subjected to necropsy.

### Sub-acute toxicity study

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 30 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. Gross lesions present in the liver, lungs, kidneys, heart and spleen were recorded. 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.

### Biochemical assays

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).

The liver enzymes, ALT, AST, ALP and LDH 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 [17] (Bergmeyer, 1974).

**Histopathological examination**

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] (Bancroft and Stevens, 1980).

**Statistical analysis**

The data generated during the present investigation were subjected to statistical analysis. 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 12.0 version software).

**RESULTS**

**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 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).

**Sub-acute toxicity**

**Clinical signs**

The vehicle-control mice remained normal throughout the experimental period, while the animals treated with MEA @ 175 mg/kg showed a partial loss of appetite, dullness and slight depression. MEA-treated mice at 350 mg/kg became dull, depressed and had rough hair coat after 10 days of treatment. However, the animals in dose level of 700 mg/kg showed dullness and depression 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.

**Changes in the biochemical parameters**

The total bilirubin levels as well the conjugated bilirubin were much higher ($P \leq 0.01$) in the MEA-treated mice @ 700 mg/kg, when compared with those in the control and MEA dose levels of 175 and 350 mg/kg (data not shown). There was marked increase ($P \leq 0.05$ or $P \leq 0.01$) in the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in the animals of MEA @ 350 and 700 mg/kg, as compared to those in the control and lowest dose of MEA at 175 mg/kg. However, a significant increase of ALT ($P \leq 0.05$) activity could be observed in group-II mice when compared to the control (Table-1).

Table 1. Effect of oral administration of MEA on liver enzyme activities of mice (30 days exposure)

| Group/Treatment (mg/kg/day) | AST (IU/l) | ALT (IU/l) | ALP (IU/l) | LDH (IU/l) |
|---|---|---|---|---|
| I (Vehicle control) | $71.06^{c}\pm2.44$ | $64.94^{d}\pm1.53$ | $32.58^{c}\pm0.75$ | $107.99^{c}\pm4.55$ |
| II (MEA-175) | $75.12^{c}\pm1.32$ | $81.29^{c}\pm1.66$ | $37.79^{c}\pm0.73$ | $117.32^{c}\pm5.10$ |
| III (MEA-350) | $122.08^{b}\pm2.78$ | $166.75^{b}\pm4.34$ | $50.87^{b}\pm1.19$ | $177.98^{b}\pm5.33$ |
| IV (MEA-700) | $201.87^{a}\pm3.75$ | $302.33^{a}\pm4.49$ | $85.38^{a}\pm3.43$ | $476.62^{a}\pm11.78$ |
| F-value | 501.55** | 1070.36 ** | 158.29 ** | 565.25** |

Values are Mean ± SEM (n=5). ** Significance at $p \leq 0.01$; * $p \leq 0.05$.

**Gross and histopathological changes**

Post-mortem examination of the animals in the groups I (vehicle control) and II (MEA @ 175 mg/kg) revealed no appreciable gross changes of the liver and other visceral organs, while the animals receiving MEA @ 350 mg/kg showed enlargement of the liver and spleen as compared to control (Figs. 2a, 2b and 2c). However, the group IV mice which received the highest dose of MEA (i.e. 700 mg/kg) had yellowish discoloration of liver, subcutaneous tissue and musculature with marked enlargement of liver and spleen (Figs. 2d and 2e).

Histopathological studies also provided supportive evidence for the biochemical analysis depicted by the following photomicrographs. Figs. 3a and 3b showed the normal architecture

and mild degenerative changes of liver in groups I (control) and II animals respectively. Liver sections of group III animals revealed mild to moderate bile duct proliferation and focal areas of necrosis (Fig. 3c). In the animals of Gr-IV (MEA @ 700 mg/kg), the bile ducts were dilated and showed proliferative changes with mononuclear cells infiltration. The hepatocytes around the bile ducts showed necrotic changes as well as some focal areas of necrosis (Fig. 3d). No significant microscopic lesions were observed in other tissues collected.

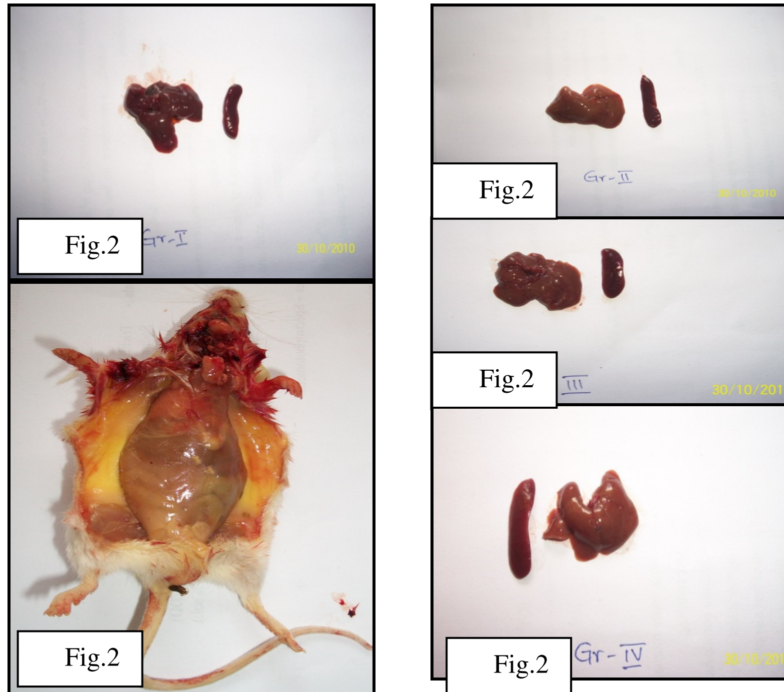


Fig. 2 (a, b, c, e): Photographs showing the gross changes of mice from different groups (I-IV) for 30 days exposure to MEA.
Fig.2d: Post-mortem examination a mouse from Gr-IV showing yellowish discoloration of liver, subcutaneous tissue and musculature indicating jaundice.

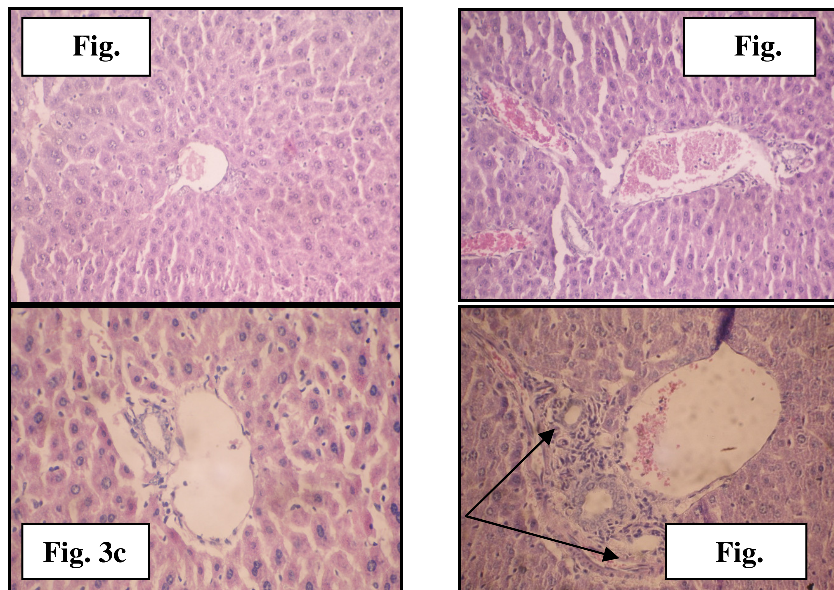


Fig. (3a) Control liver showing normal architecture (H&E x 200); (3b) Group-II liver showing mild degenerative changes (H&E x 200); (3c) Group-III liver showing mild to moderate bile duct proliferation and focal areas of necrosis (H&E x 400) and (3d) Group-IV liver showing dilated bile ducts with proliferative changes (arrows), necrotic changes of hepatocytes around the bile ducts with mononuclear cells infiltration (H&E x 200).

## DISCUSSION

The present $LD_{50}$ was lower than 3761 mg/kg reported by Singh et al. [19] in mice with methanolic extract of the plant leaves using refined vegetable oil as vehicle. 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. This $LD_{50}$ value was also lower than that of Gao et al. [20] (2005) who obtained an acute $LD_{50}$ of 5000 mg/kg in mice with alcoholic extract of *E. adenophorum Sprengel.* However, the methanolic extract of *E. adenophorum* at 2000 mg/kg did not produce any signs of overt toxicity in rats [21]. The difference in the $LD_{50}$ values might be due to variation of species/strain, geographical region, soil and other environmental factors and also suggests that the *E. adenophorum* plant growing in the region is apparently more toxic.

The clinical picture of the animals in the test groups after oral administration of MEA (175, 350 and 700 mg/kg) was almost similar to the earlier observations reported by several workers. Mice intoxicated with the toxin, 9-oxo-10,11-dehydroageraphorone @ 350 mg/kg and methanolic leaf extract of *E. adenophorum* @ 752 mg/kg became icteric within 2-10 days of dosing and the jaundice persisted for at least 3 weeks [8, 19]. The rats fed with freeze dried leaf powder, 9-oxo-10,11-dehydroageraphorone, methanolic extract of leaf powder as well as oven-dried leaves of *E. adenophorum* were icteric, dull, and hair coat appeared rough and erect. The ear pinnae and paws were yellow. The urine was also yellow [1, 12, 13, 22].

The elevation of serum enzymatic activity (AST, ALT, ALP and LDH) in the present study is attributed to *E. adenophorum*-induced hepatic damage/or necrosis as confirmed from histopathological observations. The increase in the activity of transaminases is known to be the indicator of degenerative changes in organs or tissues like liver and myocardium [23, 24]. Moreover, 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 [25]. 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, 12, 13, 22]. Similar observations of the biochemical alterations were also reported by Singh et al. [19] (2011), in a short-term toxicity study of *E. adenophorum* in Swiss albino mice conducted.

The gross and microscopic changes of present study were in the line of earlier observations during development of toxicity due to the whole leaf powder, methanolic extract and partially purified fraction of *E. adenophorum* in rats [1, 12, 13, 22] and also in mice fed with methanolic leaf extract of the plant by Singh et al. [19]. Sani et al. [11] (1989) also observed that administration of *E. adenophorum* leaf powder to mice caused degeneration of intrahepatic bile ducts and hepatocellular necrosis.

The present study shows that the toxicity of methanolic leaf extract of E. adenophorum is dose dependent and the dose level of 1/5th LD 50 (i.e. 700 mg/kg) is highly hepatotoxic in mice. Therefore, feeding of *E. adenophorum* to livestock should be avoided as it could potentially toxic to higher animals too. Further long term toxicity studies and other toxicological aspects on *E. adenophorum* are advocated

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