EFFECTS OF EUPATORIUM ADENOPHORUM LEAF EXTRACT ON CELL-MEDIATED IMMUNE RESPONSE IN MICE

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ABSTRACT

Twenty five apparently healthy male albino mice (25-30 g) were randomly divided into 5 groups of 5 animals each. Group-I and II served as vehicle (5% tween 80) and positive control (cyclophosphamide @ 25 mg/kg orally, daily till the termination of the experiment) respectively. The remaining groups (III-V) received methanolic extract of E. adenophorum @ 1/20\textsuperscript{th}, 1/10\textsuperscript{th} and 1/5\textsuperscript{th} of ALD\textsubscript{50} orally daily for 14 days before sensitization with the contactant, dinitrochlorobenzene (DNCB) and continued till the termination of the experiment. Daily oral administration of MEA @ 700 mg/kg for 19 days resulted in marked reduction in the ear thickness in DNCB-induced delayed type hypersensitivity test (DTH) in mice, while MEA doses of 175 and 350 mg/kg produced a marginal increase and reduction in the ear thickness; respectively. The dose levels of MEA @ 350 and 700 mg/kg significantly lowered the total leukocyte and absolute lymphocyte counts. However, MEA @ 175 mg/kg caused a marginal increase in total leukocyte count, while marginal decrease in absolute lymphocyte counts. Daily oral administration of MEA @ 350 and 700 mg/kg for 19 days resulted in significant reduction in per cent lymphocyte, while increase in both per cent neutrophils and monocytes in mice. The present study suggests that the highest dose of MEA (700 mg/kg) might be having some immunosuppressant effects on cell-mediated immunity (CMI) of mice.

KEYWORDS: Eupatorium adenophorum; cell-mediated immune response; mice.
INTRODUCTION

Millions of people in various traditional systems have resorted to the use of medicinal plants to treat their ailments; this could be as a result of the high cost of orthodox health care, or lack of faith in it, or maybe as a result of the global shift towards the use of natural, rather than synthetic products. While the craze for natural products has its merits, care must be taken not to consume plants or plant extracts that could have deleterious effects on the body, either on the short term or on the long term.\(^1\)

*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.\(^2\) *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.\(^3\)

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.\(^7\) Exposure of mice to feed containing *E. adenophorum* freeze-dried leaf powder caused hepatotoxicity.\(^8\) *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.\(^9,10\) Methanolic extract of *E. adenophorum* leaf samples collected from Mizoram (India) has also been reported to induce hepatotoxicity in albino mice.\(^11\) However, no reports were found about the detailed studies of the plant *E. adenophorum* on cell-mediated immune responses of any species of animal.
Keeping the above information in view, the present study was undertaken to study the humoral immune responses in mice subjected to oral administration of methanolic extract of *E. adenophorum* (MEA).

**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).

**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.

![Fig.1. Leaves and flowers of plant *Eupatorium adenophorum*](image)

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 3\textsuperscript{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\textdegree 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\textdegree 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, 55 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, Kolkata (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 (LD₅₀). 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 LD₅₀ value calculated.[¹⁴] All animals that died during the observation period and euthanatized mice were subjected to necropsy.

**Assessment of Cell-mediated immune (CMI) response**
For this study, 25 apparently healthy male albino mice (25-30 g) were randomly divided into 5 groups of 5 animals each. Group-I and II served as vehicle (5% tween 80) and positive control (cyclophosphamide @ 25 mg/kg orally, daily till the termination of the experiment) respectively. The remaining groups (III-V) received methanolic extract of *E. adenophorum* @ 1/20th, 1/10th and 1/5th of ALD₅₀ orally daily for 14 days before sensitization with the
contactant, dinitrochlorobenzene (DNCB) and continued till the termination of the experiment.

(i) **Delayed type of hypersensitivity test (Dinitrochlorobenzene-induced DHT):** Contact sensitivity in mice was accomplished by sensitization of the abdominal skin with dilute solution of contactant (Dinitrochlorobenzene; DNCB). The animals were challenged 4 days later by applying a more dilute challenge solution of contactant to the ear. The response (increase in ear swelling) was measured after 24 hours.\(^{[15]}\)

The stock solution of DNCB (5% w/v) was prepared in 4:1 acetone: olive oil. For sensitization, the 5% stock DNCB solution was diluted 1:10 in 4:1 acetone: olive oil, yielding 0.5% solution. The solution (5% DNCB) was further diluted to 1:25 in 4:1 acetone: olive oil yielding 0.2% working challenge solution.\(^{[16]}\)

On Day-0, i.e. Day 15 of the experiment, the mice were sensitized by applying 20 µl of 0.5% of DNCB in area of 2 cm\(^2\) on the shaved abdominal skin evenly with a glass rod. The procedure was repeated on Day-1. On Day-0, the basal ear thickness was measured with a Vernier caliber in outer 2/3rd of the ear to avoid skin fold. Ninety six (96) hours after sensitization, the mice were again applied with challenge solution @ 10 µl of 0.2% DNCB to the dorsal surface of the ear with a pipette dispenser and evenly distributed with a glass rod. The response (increase in ear swelling) was again measured after 24 hours of secondary sensitization.\(^{[17]}\) The difference in the ear thickness between pre-challenge and post-challenge was calculated and results expressed in mm.

(ii) **Total leukocyte, differential leukocyte and absolute lymphocyte counts:** On termination of the experiment, i.e. after 19 days exposure to *E. adenophorum* extract, the blood samples were collected for the study of the parameters like total leukocytes count (TLC), differential leukocyte count (DLC) and absolute lymphocyte count (ALC).

The total leukocytes count (TLC) and differential leukocyte counts (DLC) were analyzed by using automatic blood analyzer (Vet Scan HM5). Absolute lymphocyte count (ALC) was obtained by using the following formula:

\[
ALC = \frac{TLC \times \% \text{ lymphocytes}}{100}
\]
Statistical analysis
One way analysis of variance (ANOVA) was employed to find the significant differences among 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).

RESULTS AND DISCUSSION
Acute toxicity
Mice administered with methanolic leaf extract of *E. adenophorum* (MEA) at the dose level of 1350 mg/kg body weight showed no mortality, while those at dose levels of both 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 ($2157 \leq 3501 \geq 5682$ mg/kg with 95% confidence limit).

Cell-mediated Immune Response
*Delayed type hypersensitivity test (DTH)*
Dinitrochlorobenzene (DNCB) – induced DTH was used to assess the effect of MEA treatment on cell-mediated immunity (CMI). Application of challenge dose of DNCB caused erythema and oedema of the ear (Fig. 2). These changes were more pronounced in the vehicle-control group than in the MEA treated mice. The mean increase in the ear thickness after challenge is presented in Fig. 3. In the control mice, the mean increase in the ear thickness was 0.180±0.01 mm. MEA treatment (350 mg/kg) resulted in slight reduction (3.33 %), while 700 mg/kg caused a significant reduction ($P \leq 0.05$) in ear thickness (14.44% of the normal control values) which is suggestive for suppression of CMI response.. However, cyclophosphamide-treated (25 mg/kg) mice revealed a significant decrease ($P \leq 0.01$) in the ear thickness (28.89% of the normal control values).
Fig. 2: Photograph showing erythema and oedema of the ear of a mouse after application of challenge dose of DNCB for delayed type hypersensitivity test (DTH).

![Fig. 2: Photograph showing erythema and oedema of the ear of a mouse after application of challenge dose of DNCB for delayed type hypersensitivity test (DTH).](image)

Fig. 3: Multiple bar diagram showing effect of MEA on ear thickness of mice in dinitrochlorobenzene (DNCB) -induced DTH test.

![Fig. 3: Multiple bar diagram showing effect of MEA on ear thickness of mice in dinitrochlorobenzene (DNCB) -induced DTH test.](image)

**Total leukocyte and absolute lymphocyte counts**

The effect of MEA (175, 350 and 700 mg/kg, orally for 19 days) treatment on total leukocyte and absolute leukocyte counts is presented in Fig.4. The MEA at dose levels of 350 and 700 mg/kg; significantly reduced (P ≤ 0.05 or P ≤ 0.01) both the total leukocyte and absolute leukocyte counts by 14.95 to 27.17% and 24.40 to 52.14%; respectively on day 20 and 30 as compared to vehicle-treated control. Cyclophosphamide also caused marked decrease (P ≤ 0.01) in the total leukocyte and absolute lymphocyte counts by 50.31% and 76.63%; respectively on day 20 and 30 when compared with the vehicle control. However, MEA @ 175 mg/kg showed slight increase in total leukocyte counts, while decrease in absolute lymphocyte counts when compared with the vehicle control values. The present results indicate that the higher doses of MEA are having a dose-dependent antilymphocytic effect which is comparatively lower than that of cyclophosphamide.
Differential leukocyte counts

The result of daily oral administration of MEA (175, 350 and 700 mg/kg) in mice on differential leukocyte count is shown in Fig. 5. MEA at 350 and 700 mg/kg produced significant reduction (P ≤ 0.05 or P ≤ 0.01) in % lymphocyte, while increase (P ≤ 0.05 or P ≤ 0.01) in both % neutrophil and % monocyte as compared to normal vehicle-control values. Similarly, cyclophosphamide also caused significant reduction in % lymphocyte with significant increase in both % neutrophil and % monocyte (P ≤ 0.05 or P ≤ 0.01) as compared to control values. However, MEA at 175 mg/kg did not show any significant difference in differential leukocyte count compared to normal control values.
The present findings thus suggest that the highest dose of MEA (700 mg/kg) might be having some immunosuppressant effects on cell-mediated immunity (CMI) of mice.

CONCLUSION
From this present study, it may be concluded that the methanolic leaf extract of Eupatorium adenophorum (MEA) possibly contain a moderately toxic principle and the highest dose of MEA (700 mg/kg) might probably be having some immune-suppressant effects on cell-mediated immunity (CMI) of mice.

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REFERENCES


