EFFECTS OF *EUPATORIUM ADENOPHORUM* LEAF EXTRACT ON HUMORAL IMMUNE RESPONSE IN MICE

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**ABSTRACT**

Fifty apparently healthy male albino mice (25-30 g) were randomly divided into 5 groups of 10 animals each. Groups I and II served as vehicle (5% tween 80) and positive control (cyclophosphamide @ 25 mg/kg, orally) respectively. The remaining groups (III-V) received methanolic extract of *E. adenophorum* (MEA) @ 1/20th, 1/10th and 1/5th of ALD$_{50}$ orally daily for 14 days before immunization and continued till the experiment was over. The positive control as well as the MEA-treated mice @ 175, 350 and 700 mg/kg did not produce any significant changes in the globulin and A/G ratios in primary immune response, whereas a marked reduction of A/G ratio was observed in the cyclophosphamide and MEA-treated mice @ 350 and 700 mg/kg in secondary immune response. Both the cyclophosphamide and MEA-treated mice @ 700 mg/kg showed significant reductions in total proteins and albumin in both primary and secondary immune responses, while animals receiving MEA @ 350 mg/kg had marked decrease in total proteins and albumin in secondary immune response. Cyclophosphamide-treated animals exhibited significant reduction in absolute and relative spleen weights after 20 and 30 days exposure for primary and secondary immune responses; respectively, while MEA at dose levels of 175 and 350 mg/kg did not cause any significant change in relative spleen weight. However, the dose level of MEA @ 700 mg/kg showed marginal reduction in relative spleen weight following 20 and 30 days of exposure. The higher doses of MEA (350 and 700 mg/kg) did not produce any significant change of the antibody (IgM) titer in the primary humoral immune response, while the lowest dose (175 mg/kg) showed a marginal increase of IgM titer. The highest dose of MEA (700 mg/kg) caused a significant reduction of antibody (IgG) titer in the secondary
humoral immune response, while the lower doses (175 and 350 mg/kg) showed marginal increase and decrease of IgG; respectively. The present study suggests that the highest dose of MEA (700 mg/kg) might probably be having some immune-suppressant effects on the primary (IgM) and secondary (IgG) immune responses of mice.

**KEYWORDS:** *Eupatorium adenophorum;* humoral 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 snakeroott), 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.\textsuperscript{[9,10]} Methanolic extract of \textit{E. adenophorum} leaf samples collected from Mizoram (India) has also been reported to induce hepatotoxicity in albino mice.\textsuperscript{[11]} However, no reports were found about the detailed studies of the plant \textit{E. adenophorum} on humoral 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 \textit{E. adenophorum} (MEA).

\textbf{MATERIALS AND METHODS}

\textbf{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).

\textbf{Plant material and preparation of extract}

The fresh leaves of the plant of \textit{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.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{flowers.png}
\caption{Leaves and flowers of plant \textit{Eupatorium adenophorum}}
\end{figure}

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 \textit{E. adenophorum} was subjected to cold maceration technique\textsuperscript{[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, 80 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 22-24\textdegree C 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 (LD\textsubscript{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.

**Assessment of humoral immune (HI) response**

(a) *Animal and treatment:* Fifty apparently healthy male albino mice (25-30 g) were randomly divided into 5 groups of 10 animals each. Groups I and II served as vehicle (5% tween 80) and positive control (cyclophosphamide @ 25 mg/kg, orally) respectively. The remaining groups (III-V) received methanolic extract of *E. adenophorum* @ 1/20$^{th}$, 1/10$^{th}$ and 1/5$^{th}$ of ALD$_{50}$ orally daily for 14 days before immunization and continued till the experiment was over.

(b) *Antigen:* Bovine serum albumin (Sigma, USA) was used as antigen which was dissolved in PBS and diluted to 2% solution.

(c) *Immunization:* All the mice were immunized intraperitoneal (i.p.) with 0.1 ml of 2% BSA at day 14 after administration and the day of immunization was taken as Day-0. On Day-6 of post-immunization, half of the animals (5 mice from each group) were used for measurement for primary immune response (IgM). For evaluating secondary immune response (IgG), inoculation was repeated in the remaining animals with the same antigen on Day-6.

(d) *Collection of blood:* On Day-0 (i.e. before commencement of the experiment), the mice were bled from the tail vein into a glass centrifuge tube and the blood was allowed to clot. The clotted blood was rimmed and the serum was separated after centrifugation at 2000 rpm (Remi-8C, India) and frozen stored at -20°C, until use as negative control for the determination of IgM and IgG antibody responses by ELISA. On Day-6 of post-immunization, half of the animals (5 mice from each group) were bled from the orbital plexus under ether anaesthesia and blood samples were collected for determination of IgM and the remaining animals on Day-11 for IgG antibody titer.$^{[15]}$ Sera were separated by centrifugation at 2000 rpm for the determination of BSA specific IgM and IgG antibody responses by ELISA.

(e) *Estimation of Total proteins, albumin and albumin/globulin ratios:* Total plasma proteins and albumin were estimated on Day-6 and at the termination of the experiment, i.e. Day-11 post-immunization. The total proteins and albumins were estimated using commercially available reagent kits (Crest Biosystems, Goa, India) according to manufacturer’s protocol. By subtracting albumin from total proteins, globulin content and albumin/globulin (A/G) ratios were determined.
(f) **Measurement of antibody titre:** BSA specific primary (IgM) and secondary antibody (IgG) responses in the sera of control and test groups were determined by an ELISA test as described by.\[16\]

**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≤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 ≤ 3501 ≥ 5682 mg/kg with 95% confidence limit).

**Humoral Immune Response**

*Total proteins, albumin, globulin and albumin/globulin (A/G) ratios*

During 20 days treatment to study the primary immune response, all animals in the positive control (cyclophosphamide @ 25 mg/kg) as well as the MEA-treated mice (175, 350 and 700 mg/kg; respectively) did not produce any significant changes in the globulin and A/G ratios. The positive-control mice showed a significant reduction in total proteins (P ≤ 0.05) and albumin (P ≤ 0.01) as compared to Group-I (vehicle control) and Group-III. Mice treated with MEA @ 350 mg/kg also showed significant decrease in total proteins (P ≤ 0.05), but not the albumin, from the animals in the Group-I (control) and Group-III (MEA, 175 mg/kg). However, the highest dose of MEA (700 mg/kg) treated mice exhibited a marked reduction in
both total proteins and albumin (P ≤ 0.01) when compared to Group-I (control) and Group-III mice (MEA, 175 mg/kg) (Fig.2).

Fig.2: Multiple bar diagram showing effect of MEA on total proteins, albumin, globulin and A/G ratio in mice (Primary immune response).

During 30 days study for the secondary immune response, at all dose levels of MEA (175, 350 and 700 mg/kg) and cyclophosphamide did not produce any significant change in the globulin concentration. The positive-control mice showed a marked reduction in total proteins (P ≤ 0.05), albumin (P ≤ 0.01) and A/G ratio (P ≤ 0.05 or P ≤ 0.01) as compared to vehicle-control, Groups II and III. Mice treated with MEA @ 350 mg/kg also caused significant decrease in total proteins, albumin and A/G ratio (P ≤ 0.05) compared to Group-I (control) and Group-III animals. However, the highest dose of MEA (700 mg/kg) treated mice showed a marked reduction in total proteins, albumin and A/G ratio (P ≤ 0.01) when compared to Groups I and III. The reduction of A/G ratio was significantly higher (P ≤ 0.05) in the cyclophosphamide-treated mice than the MEA-treated group @ 700 mg/kg (Fig.3).

Fig.3: Multiple bar diagram showing effect of MEA on total proteins, albumin, globulin and A/G ratio in mice (Secondary immune response).
In both the primary and secondary immune responses, the highest dose of MEA (700 mg/kg) showed significant reduction in total proteins (P ≤ 0.05) even lower than that of cyclophosphamide-treated mice, but no significant difference was observed in the albumin concentrations. These results, thus, suggested that the highest dose of MEA (700 mg/kg) has more suppressing effects on protein synthesis than the cyclophosphamide which might be due to severe liver damage/necrosis caused by this dose level as evidenced by histological findings of liver.

**Absolute and relative spleen weights**

Cyclophosphamide-treated animals showed significant reduction in absolute and relative spleen weights (18.97 to 22.90 % of the vehicle-control values) after 20 and 30 days exposure for primary and secondary immune responses; respectively. The MEA did not cause any significant change in relative spleen weight at dose levels of 175 and 350 mg/kg. However, at the dose level of 700 mg/kg, it caused marginal reduction (1.06 to 1.21 % of the vehicle-control values) in mean relative spleen weight following 20 and 30 days exposure (Figs. 4 and 5) which indicates the possibility of having mild immunosuppressant effect at this dose.

![Fig.4: Multiple bar diagram showing effect of MEA on absolute and relative spleen weights in mice (Primary immune response).](image)

![Fig.5: Multiple bar diagram showing effect of MEA on absolute and relative spleen weights in mice (Secondary immune response).](image)
**Humoral antibody titers**

Injection of 2% bovine serum albumin (BSA) solution i.p. in mice gave rise to primary antibody (IgM) with a serum titer of 2.54±0.06 (log₂) in vehicle-treated controls. At dose levels of MEA @ 350 and 700 mg/kg caused marginal decrease in IgM titer, while significantly increased (P ≤ 0.05) in dose level of MEA @ 175 mg/kg. As compared to the vehicle-treated control groups, cyclophosphamide produced marked reduction (P≤0.01) in IgM titer by 24.13 per cent.

There was no significant difference of IgG titers at the dose levels of 175 and 350 mg/kg in the secondary immune response, but the highest dose of MEA @ 700 mg/kg caused a significant reduction (P ≤ 0.05) in IgG titer (7.87% of the normal control value). Cyclophosphamide-treated mice showed marked reduction (P ≤ 0.01) in IgG titer (50.14% of control values) when compared to vehicle-treated animals (Figs. 6, 7 and 8).

**Fig.6: Graphical representation of IgM standard curve.**

**Fig.7: Graphical representation of IgG standard curve.**
Fig. 8: Multiple bar diagram showing effect of MEA on Primary (IgM) and secondary (IgG) antibody levels in mice.

The present findings thus suggest that the lowest dose of MEA (175 mg/kg) shows mild immunomodulatory effect on both primary and secondary responses, while the highest dose of MEA (700 mg/kg) has mild as well as marked immunosuppressant effect on the primary and secondary immune responses respectively, but these effects are comparatively much lower than that cyclophosphamide.

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 the primary (IgM) and secondary (IgG) immune responses of mice.

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