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Research Article

In-vivo Antioxidant Enzyme Activities of *Eupatorium Adenophorum* (Sticky Snakeroot) Leaf Extract

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ABSTRACT

Twenty Swiss albino mice (male) randomly divided into four groups were administered orally with vehicle (5% Tween 80), 1/20th (i.e. 175 mg/kg), 1/10th (i.e. 350 mg/kg) and 1/5th (i.e. 750 mg/kg) LD_{50} doses of methanolic leaf extract of *Eupatorium adenophorum* respectively; for a period of 90 days and the levels of various antioxidant enzymes such as lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were studied. Treatment of the mice with methanolic extract of *E. adenophorum* at the dose levels of 350 and 750 mg/kg revealed marked increase in lipid peroxidation (LPO) levels and decreased activities of superoxide dismutase (SOD) as compared to control and mice treated with 175 mg/kg, while all the treated animals showed significant reductions in catalase (CAT) and glutathione (GSH) activities as compared to control. The present findings suggest that methanolic leaf extract of *E. adenophorum* may be having dose dependant hepatotoxic effect on mice as it markedly induced lipid peroxidation and reversed the activities of the antioxidant enzymes.

Keywords: *Eupatorium adenophorum; H*epatotoxicity; Antioxidant activity; Mice

1. 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].

Liver is the vital organ responsible for drug metabolism and appears to be sensitive target site for substances modulating biotransformation[2]. 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. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative stress in liver[3].

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[4]. *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[5].

There are many reports of using the whole plant, leaves and shoots of *E. adenophorum* as folklore medicines in different

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parts of the world. Traditional practitioners in Darjeeling Himalaya give the young leaves and shoots of *Eupatorium adenophorum* Linn (Asteraceae) orally against dysentery[6]. A decoction of the plant has been recommended to treat jaundice and ulcers[7] and that of the leaves is given to cure stomachache among the tribal people of Meghalaya and Nagaland[8].

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[9]. Exposure of mice to feed containing *E. adenophorum* freeze-dried leaf powder caused hepatotoxicity[10]. *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 [11,12]. Methanolic extract of *E. adenophorum* leaf samples collected from Mizoram (India) has also been reported to induce hepatotoxicity in albino mice[13].

The present study was undertaken to investigate the status of antioxidant enzymes in mice subjected to oral *E. adenophorum* extract administration and also to find out whether consumption of the plant is safe or not.

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).

2.2 Collection, identification and extraction of plant material

The fresh leaves of the plant of *Eupatorium adenophorum* were 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). 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. The dried leaf powder of *E. adenophorum* (100g) 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 was 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, Kolkata (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 $1/20^{th}$, $1/10^{th}$ and $1/5^{th}$ LD_{50} respectively for 90 days. Food and water were freely available during the experiment. On termination of the experiment, all the animals were euthanatized using ether anesthesia. Livers were removed immediately, weighed, rinsed in ice-cold saline, blotted, and used for estimation of antioxidant enzyme activities.

2.7 Estimation of antioxidant enzyme activities

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 estimating the activities of antioxidant enzymes such as lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH).

Lipid peroxidation (LPO) was estimated spectrophotometrically by measuring malondialdehyde (MDA) formation[15]. In brief, 0.1 ml of 10% liver homogenate was treated with 2 ml of a 1:1:1 ratio of TBA-TCA-HCl (TBA 0.37%, TCA 15%, HCl 0.25 N) and placed in a water bath at 65°C for 15 min, cooled, and centrifuged at 5,000 rpm for 10 min at room temperature. The optical density of the clear supernatant was read at 535 nm wavelength in UV-VIS Spectrophotometer (Spectrascan UV 2600, Chemito) against a reference blank. The concentration of malondialdehyde (MDA) was then calculated from the standard curve and expressed as micromole (µM) of malondialdehyde mg^{-1}wet tissue.

Estimation of superoxide dismutase (SOD) activity in liver was measured in UV-VIS Spectrophotometer[16]. 0.2 ml of the 10% tissue supernatant samples was placed to each of the reference and test cuvettes. Reference cuvette contained 1.5 ml of sodium carbonate buffer (100mM, pH 10.2) and 1.3 ml of water, while the test cuvette contained 1.5 ml of sodium carbonate buffer (100mM, pH 10.2) and 1.1 ml of water. The reaction was initiated by adding 0.2 ml of epinephrine (10 mM) in the test cuvette, so that the final volume of reaction mixture would be 3.0 ml. Both the reference and test cuvettes were read simultaneously at 480 nm wave length. The gradual increase of OD till attainment of maximum increase of test cuvette against reference cuvette was recorded. One unit of SOD activity is the amount of SOD giving a 50% inhibition.

Catalase (CAT) activity in the liver was spectrophotometrically determined[17]. The reaction mixture (2 ml), which consisted of 10 µl of liver homogenate, 90 µl of 1% Triton X-100 and 1.9 ml of 20 mM of phosphate buffer, was added to a photometric quartz cuvette containing 1 ml of H_2O_2 (0.03 M). The decrease in optical density was measured at 240 nm for 1-min time interval. The catalase activity expressed as unit of wet tissue with standard deviations (S.D.) were calculated with 43.6 as the molar absorption index for H_2O_2 at 240 nm in a 1 cm cuvette.

Reduced glutathione (GSH) level of liver was measured in UV-VIS spectrophotometer[18]. Total aliquot was 3 ml, containing 2 ml of 0.6 mM DTNB in 0.2 M Tris-hydrochloric acid buffer (pH 8.0), 0.1 ml of tissue supernatant and 0.9 ml of 0.2 M Tris-hydrochloric acid buffer (pH 8.0). The reference cuvette contained 0.1 ml of 5% Trichloroacetic acid (TCA) instead of tissue sample and after 5 minute, absorbance was read at 412 nm wavelength. Reduced glutathione content of tissue homogenate was expressed as µmole/gm wet tissue.

2.8 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).

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 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).

3.2 Changes in the antioxidant enzyme activities

There was significant increase in LPO levels ($P \leq 0.05$ or $P \leq 0.01$), while activities of superoxide dismutase (SOD) were significantly decreased ($P \leq 0.05$ or $P \leq 0.01$) in Groups III and IV animals when compared with the control and Group-II mice. All the MEA-treated animals of Group-II, III and IV showed significant reductions in catalase (CAT) and reduced glutathione (GSH) activities ($P \leq 0.05$ or $P \leq 0.01$) as compared to the control group. However, there was no significant alteration in both LPO and SOD activities in Group-I (control) and Group-II animals. The effects of daily oral administration of MEA (175, 350 and 700 mg/kg) in mice for 90 days on antioxidant enzyme activities are shown in Figures 2-4.

Lipid peroxidation has been postulated to be the destructive process in liver injury due to CCl_4/paracetamol administration[19]. The increase in LPO levels of liver of animals treated with MEA @ 350 and 700 mg/kg in the present study suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals.

Serum activities of superoxide dismutase (SOD) and catalase (CAT) are the most sensitive enzymatic index in liver injury caused by ROS and oxidative stress. SOD is one of the most abundant intracellular antioxidant enzymes present in all aerobic cells and it has an antitoxic effect against ROS[20]. CAT is a haemoprotein; it protects the cells from the accumulation of H_2O_2 by dismutating it to form H_2O and O_2 [21]. Therefore reduction in the activities of these enzymes may indicate the toxic effects of ROS produced by toxicants. The decrease in SOD and CAT activities in the present study suggests that high dose levels of MEA can increase ROS that may enhance the oxidative damage to the hepatocytes and decrease the activities of the liver antioxidant enzymes.

Glutathione (GSH) is of the most abundant naturally occurring tripeptide, non-enzymatic biological antioxidant present in the liver[22]. Its functions are concerned with the removal of free radicals such as H_2O_2 and superoxide radicals, maintenance of membrane protein, detoxification of foreign chemicals and biotransformation of drugs[23]. The decrease level of GSH of the present study has been associated with an enhanced level of lipid peroxidation (LPO) in MEA intoxicated groups of mice in a dose dependant manner. Thus MEA may act by reducing the detoxifying enzymes and these enzymes may not detoxify the ROS following administration of toxicants.

Findings of the present study are in the line with the earlier reports of significantly increased LPO levels while decreased SOD, CAT and GSH activities in both paracetamol and acetaminophen induced hepatotoxicity in rats[24,25].

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

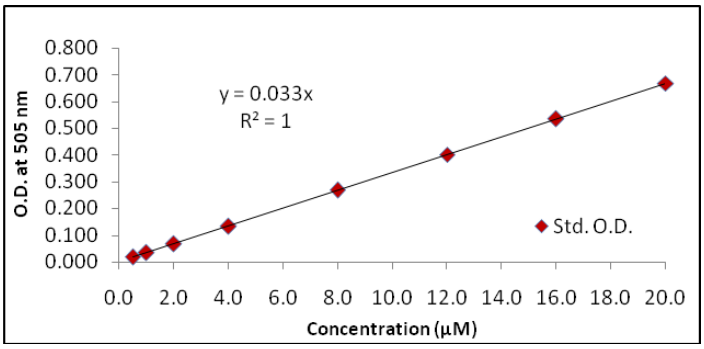

Fig 2: Graphical representation of standard curve of MDA

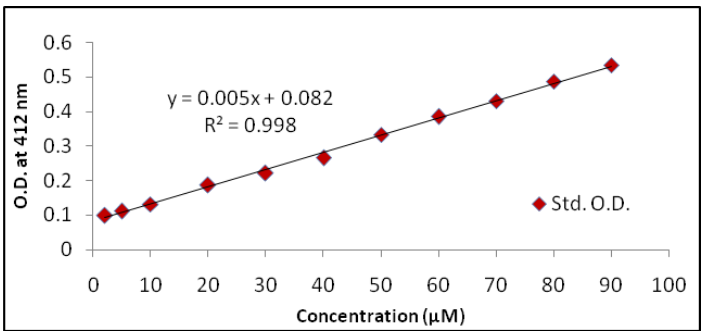

Fig-3: Graphical representation of standard curve of GSH

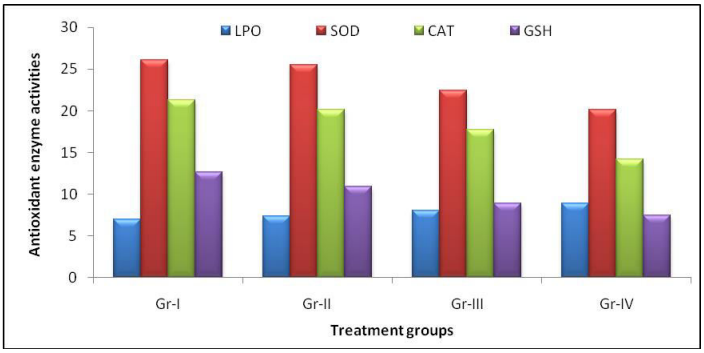

Fig 4: Multiple bar diagram showing changes in antioxidant enzyme activities of mice (90 days exposure)

4. CONCLUSION

From the present study, it can be concluded that *Eupatorium adenophorum* causes oxidative stress by reducing the activities and consequently the effectiveness of the antioxidant enzyme defense mechanism indicating hepatotoxicity to mice. Therefore, the oral administration 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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