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EVALUATION OF ANTI-NOCICEPTIVE ACTIVITY AND BRINE SHRIMP LETHALITY BIOASSAY OF ROOTS OF *LEEA MACROPHYLLA* ROXB.

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ABSTRACT

The ethanolic extracts and its different partitioning fractions of roots of *Leea macrophylla* (Leeaceae) were evaluated for their analgesic and preliminary cytotoxic activities. The analgesic activity was evaluated using the acetic acid-induced writhing test in mice while the screening of cytotoxic activity was done using brine shrimp lethality bioassay. In acetic- acid induced writhing test, the ethanolic extract at the dose of 200 mg/kg body weight significantly (p<0.001) reduced the number of writhes with 62.37% of inhibition when compared to the control group which was comparable to that of the standard drug diclofenac sodium (61.85% inhibition, p<0.001). The petroleum ether, carbon tetrachloride and ethyl acetate soluble fractions of the ethanolic extract at the same dose exhibited moderate anti-nociceptive activities with 36.59, 30.92 and 27.83 % inhibition of writhing compared to control, respectively. In Brine shrimp lethality bioassay, the LC_{50} values of the ethanolic extract and carbon tetrachloride, chloroform and ethyl acetate soluble fractions were found to be 2.39, 0.049, 4.53 and 0.09 μg/ml, respectively which were comparable to the standard vincristine sulphate, (LC_{50}: 0.34μg/ml). The results of the study demonstrated both the anti-nociceptive and preliminary cytotoxic activity of the roots of *Leea macrophylla* Roxb.

INTRODUCTION: Medicinal plants represent a rich source of new molecules with pharmacological properties, which are lead compounds for the development of new drugs. Since the chemical constituents of medicinal plants, particularly the secondary metabolites (alkaloids, sterols, terpenes, flavonoids, saponins, glycosides, cyanogenics, tannins, resins, lactones, quinines, volatile oils, etc.) have pronounced pharmacological action on animal systems and organs; they are capable of mitigating sufferings and curing ailments. Natural products and related drugs are used to treat 87% of all categorized human diseases including bacterial infections, cancer and immunological disorders [1]. About 25% of prescribed drugs in the world originate from plants [2]. About 80% of the population in developing countries relies on traditional plant based medicines for their primary health care needs [3]. Plants have played an important role as a source of effective anti-cancer agents, and it is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms [1].

Drugs which are in use presently for the management of pain and inflammatory conditions are either

narcotics e.g. opioids or non-narcotics e.g. salicylates and corticosteroids e.g. hydrocortisone. All of these drugs present well known side and toxic effects [4]. Moreover synthetic drugs are very expensive to develop. On the contrary, many medicines of plant origin had been used since long time without any adverse effects. It is therefore essential that efforts should be made to introduce new medicinal plants to develop cheaper drugs.

Leea macrophylla Roxburgh (Family: Leeaceae; Bengali name: Dholsamudra, Dinda) is a robust herb or shrub, stem erect, 30-90 cm high, root tuberous, perennial, red. Leaves simple, broadly ovate, nearly as broad as long, the lower leaves up to 60 cm long, the upper 15-23 cm long, base cordate, apex acute or acuminate, coarsely serrate or sublobed, dark green and glabrous above, pubescent beneath, main nerves opposite, 8-10 pairs, very prominent; petioles 5-12 cm long, deeply striate, glabrous. Inflorescence terminal, much branched, puberculous, corymbose cymes, up to 30 cm long, flower white. Berry globose, 6-8 cm in diameter, black, 3-6 celled, depressed-globose, usually 3-6 lobed [5].

The plant is occurring in Dinajpur, Shavar and Chittagong Hill Tracts of Bangladesh, Yunnan of China, Cambodia, India, Laos, Myanmar, Nepal and Thailand. The plant has various ethnopharmacological uses and almost all parts of the plants possess potential curative properties. *Leea macrophylla* is claimed to be anti-cancerous. Powder of leaves mixed with honey is given to patient of cancer [6, 7]. Leaf juice is recognized as local anti-inflammatory agent and used in boils, arthritis, gout and rheumatism [8]. A paste of the leaf is applied to cuts and wounds. Roots are used in fracture and healing cut injury. Roots are used externally to allay pain, used for ringworm and guinea worm; it has astringent, styptic and antiseptic activity. The plant parts are also used by tribal people in cold, cough, headache, body pain, rheumatic pain etc. It has also Ethnobotanical uses in goiter, gastric tumor, lipoma and tetanus [5].

Though the plant has traditionally been used in the treatment of various types of pain and neoplastic diseases in Bangladesh, to the best of our knowledge, there have been no scientific reports on the analgesic and cytotoxic effects of this plant in animals. Therefore, the present study was designed to evaluate the analgesic activity of the ethanolic extracts and its different partitioning fractions of roots of *Leea macrophylla* using the acetic acid-induced writhing inhibition method in albino mice and to screen preliminary cytotoxic activities by brine shrimp lethality bioassay.

MATERIALS AND METHODS:

Chemicals and Reagents: The chemicals used in this study were: ethanol, petroleum ether, carbon tetrachloride, chloroform, ethyl acetate, dimethyl-sulfoxide and acetic acid (Merck, Germany), diclofenac sodium (Square Pharmaceuticals Ltd; Dhaka, Bangladesh), vincristine sulphate (Sigma-Aldrich), tween-80 (BDH Chemicals Ltd) and normal saline solution or 0.9% NaCl (Beximco Infusion Ltd., Bangladesh).

Plant Material: The roots of *Leea macrophylla* were collected from Savar, Bangladesh in September, 2010 at the flowering stage. The plant was identified and authenticated by Bangladesh National Herbarium and a voucher specimen (accession No: DACB 35293) was deposited there for future reference. The roots parts were cut into small pieces and sun dried for several days. The plant materials were then oven dried for 24 hours at considerably low temperature for better grinding. The dried cutting pieces were pulverized by a mechanical grinder and stored into an air-tight container.

Extraction of the Plant Material: The dried and ground root powder (1.2 kg) was extracted with absolute ethanol (5.0 liters) in an air-tight, clean, round bottomed flask for 15 days at room temperature with occasional stirring and shaking. The whole mixture was then filtered through cotton plug followed by Whatmann No. 1 filter paper and the filtrate thus obtained was concentrated at 39°C under reduced pressure with a Heidolph rotary evaporator. The concentrated extract was then air dried to solid residue. The weight of the crude extract obtained from the roots of the *Leea macrophylla* was 50.3 gm. Solvent–solvent partitioning was done using the protocol designed by Kupchan [9] and modified version of Wagenen *et al*., [10]. The crude extract (5 gm) was dissolved in 10% aqueous methanol which was

subsequently extracted first with petroleum ether, then carbon tetrachloride, chloroform and finally with ethyl acetate. All the four fractions were evaporated to dryness by using rotary evaporator and kept in air tight containers for further analysis.

Experimental Animals: Swiss albino mice of either sex, weighing 25-30 gm were purchased from the animal resource branch of International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR,B). The animals were kept in standard environmental conditions (temperature: 23±2^{o}C; relative humidity: 55±10 % and 12 hours light/ dark cycle). The animals were fed with standard rat food (ICDDR, B formulated) and water *ad libitum* and acclimatized to laboratory conditions for 7 days before the experimentation. The design and performance of research study involving mice have been approved by the Ethical Review Committee, Faculty of Biological Science, University of Dhaka, through the submission of a research protocol before the study.

Acetic acid-induced Writhing Response in mice: The ethanolic extract of *L. macrophylla* roots and its different solvent soluble fractions were evaluated for their analgesic activity by acetic-acid induced writhing inhibition method [11]. Thirty Swiss albino mice were divided into six groups of five mice each. The control group (Group I) received the vehicles (1% Tween-80 in normal saline) at a dose of 10 ml/kg p.o. The reference group (Group II) received diclofenac sodium 50 mg/kg body weight p.o. whereas the test groups (Group III, IV, V and VI) were administered with ethanolic extract of roots, the petroleum ether, CCl_4, and ethyl acetate fractions, respectively at a dose of 200 mg/kg p.o.

After 40 minutes acetic acid (0.7% v/v in saline) was administered intraperitoneally to each of the animals of all the groups at a dose of 0.1 ml/10 g to create pain sensation. The forty minutes interval between the oral administration of test materials and intra-peritoneal administration of acetic acid was given to assure proper absorption of the administered samples. Five minutes after the administration of acetic acid, the numbers of squirms or writhes characterized by contraction of the abdominal musculature together with turning of trunk and extension of hind limbs was counted for each mouse over a period of 15 min. The response of the extract and diclofenac sodium treated groups was compared with those of the animals in the control group. Percentage inhibition of writhing compared to control group was taken as an index of analgesia and was calculated using the following formula:

$$\text{Inhibition (\%)} = [(Wc-Wt) \times 100]/Wc$$

Where, Wc is the average number of writhing reflex in the control group and Wt is the average number of writhing in the test groups.

Statistical analysis: The results obtained were expressed as mean ± standard error of means (SEM). The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *t* test to determine the level of significance. A value of $P<0.05$ was considered to be significant. The statistical analysis was carried out using the SPSS program (version 17.0).

Brine Shrimp Lethality Bioassay: Preliminary cytotoxic activities of the crude ethanolic extract and its different solvent soluble fractions were screened by brine shrimp lethality bioassay [12]. Four mg of each of the test samples (crude extract, petroleum ether, carbon tetrachloride, chloroform, and ethyl acetate soluble fraction of ethanolic extract) was taken and dissolved in 200 μl of pure dimethyl sulfoxide (DMSO) in vials to get stock solutions. Solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.123, 1.563, 0.781 μg/ml) of each sample were prepared from the stock solution by serial dilution technique.

The solutions were then added to the pre-marked vials containing ten live brine shrimp nauplii in 5 ml simulated sea water. In the present study vincristine sulphate is used as the positive control. Measured amount of the vincristine sulphate is dissolved in DMSO to get an initial concentration of 20 μg/ml from which serial dilutions are made using DMSO to get solutions of varying concentrations (10 μg/ml to 0.0390 μg/ml). Then the positive control solutions are added to the premarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent of lethality of the brine

shrimp nauplii was calculated for each concentration. The median lethal concentration (LC_{50}) of the test samples were obtained by plotting percentage of the shrimp killed against the logarithm of the sample concentration.

RESULTS:

Acetic acid-induced Writhing Response in mice: The crude ethanolic extract of roots of *L. macrophylla* at the dose of 200 mg/kg body weight significantly (p<0.001) reduced the number of writhes with 62.37% of inhibition when compared to the control group (**Table 1**) which is almost similar to that of the standard drug diclofenac sodium (61.85% inhibition, p<0.001). The partitioning fractions such as pet ether, carbon tetrachloride and ethyl acetate soluble fractions at the same dose exhibited moderate anti-nociceptive activities with 36.59, 30.92 and 27.83 % inhibition of writhing, respectively.

TABLE 1: EFFECTS OF ETHANOLIC EXTRACT AND ITS DIFFERENT FRACTIONS OF ROOTS OF *LEEA MACROPHYLLA* ON ACETIC ACID-INDUCED WRITHING RESPONSE IN MICE

Treatment	Dose (mg/kg)	Writhing [a]	% inhibition
Control (Vehicle)	10 mL/kg	38.8±2.07	-
Diclofenac sodium	50	14.8±1.16**	61.85
EELMR	200	14.6±1.80**	62.37
PEF	200	24.6±1.72**	36.59
CCF	200	26.8±1.35**	30.92
EAF	200	28.0±2.98*	27.83

[a] values represent mean ± SEM (n=5). One -way ANOVA followed by Dunnett's *t* test, **p<0.001 compared to control. *p<0.05 compared to control. EELMR: ethanolic extract of roots of *L. macrophylla*; PEF: pet ether fraction of ethanolic extract; CCF: carbontetrachloride fraction; EAF: ethyl acetate fraction of ethanolic extract of roots of the plant.

Brine Shrimp Lethality Bioassay: In Brine shrimp lethality bioassay, the LC_{50} values of the crude ethanolic extract, carbon tetrachloride, chloroform and ethyl acetate soluble fractions of the ethanolic extract were found to be 2.39, 0.049, 4.53 and 0.09 µg/ml, respectively (**Table 2 and Fig. 1-2**). However, varying degree of lethality to *Artesia salina* was observed with exposure to different dose levels of the test samples. In other words, % mortality increased gradually with the increase in concentration of the test samples. The CCl_4 and ethyl acetate fractions were more toxic to brine shrimp than the reference standard, vincristine sulphate, (LC_{50}: 0.34 µg/ml) whereas the toxicity shown by ethanolic extract and chloroform fraction were comparable to that of the vincristine sulphate.

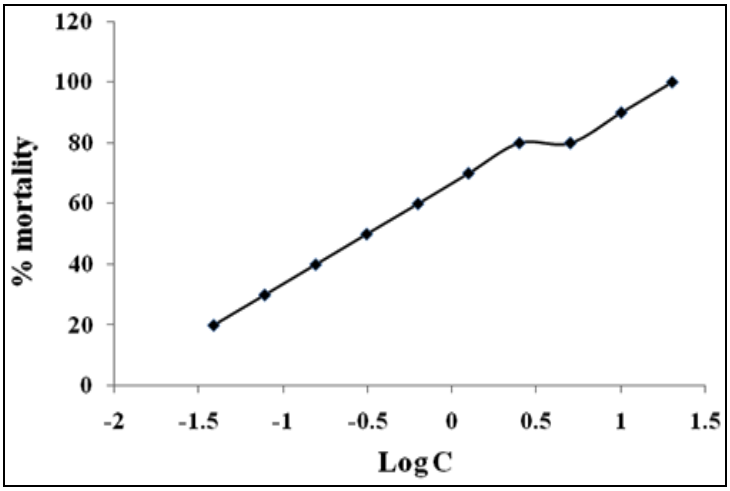

FIG. 1: PLOT OF % MORTALITY OF BRINE SHRIMP NAUPLII VS. CONCENTRATION OF VINCRISTINE SULPHATE

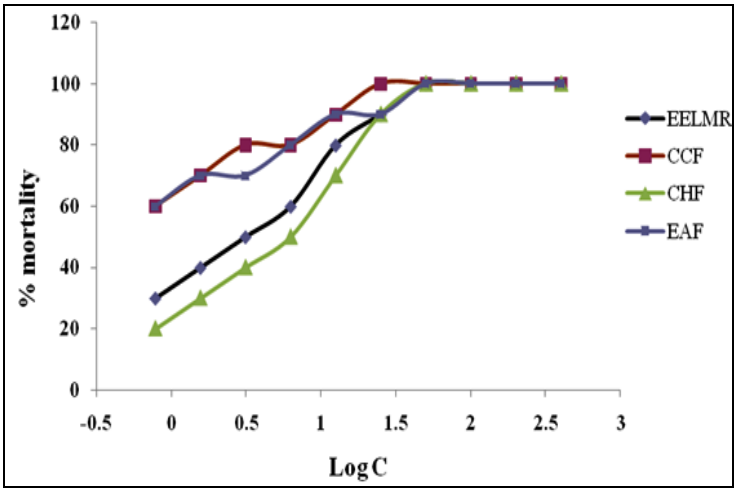

FIG. 2: PLOT OF % MORTALITY OF BRINE SHRIMP VS. CONCENTRATION OF ETHANOLIC EXTRACT AND ITS DIFFERENT PARTITIONATES OF ROOTS OF *LEEA MACROPHYLLA*

EELMR: ethanolic extract of roots of *L. macrophylla*; CCF: carbontetrachloride fraction; CHF: chloroform fraction; EAF: ethyl acetate fraction of ethanolic extract of roots of the plant

TABLE 2: LC_{50} VALUES OF THE DIFFERENT TEST SAMPLES OF *LEEA MACROPHYLLA* ROOTS AND VINCRISTINE SULPHATE

Test samples	Regression line	R^2	LC_{50} values (µg/ml)
Vincristine sulphate	y= 28.991x + 63.548	0.988	0.34
EELMR	y= 28.793x + 39.081	0.9046	2.39
CCF	y= 14.9x + 69.412	0.8466	0.049
CHF	y= 33.827x + 27.801	0.91	4.53
EAF	y= 15.705x + 66.408	0.9037	0.09

EELMR: ethanolic extract of roots of *L. macrophylla*; CCF: carbontetrachloride fraction; CHF: chloroform fraction; EAF: ethyl acetate fraction of ethanolic extract of roots of *Leea macrophylla*

DISCUSSION: The acetic acid-induced writhing test in mice is widely used to screen and study compounds for

peripherally mediated analgesic activity. Peritoneal administration of acetic acid (0.7%) induces endogenous pain mediators, such as prostaglandins, histamine, serotonin (5-HT), bradykinin and substance P that sensitize pain nerve endings [13, 14]. The released prostaglandins, mainly prostacyclin (PGI_2) and to lesser extent PGE_2 and $PGF_{2\alpha}$ have been held responsible for pain sensation [15, 16]. The significant inhibition of writhing induced by acetic acid in this study by ethanolic extract of roots of *L. macrophylla* suggest a peripherally mediated analgesic activity and the extract exert the activity probably by inhibiting the synthesis or action of prostaglandins.

Brine shrimp lethality bioassay [12] is a rapid and comprehensive bioassay for the bioactive compound of the natural and synthetic origin. By this method, natural product extarcts, fractions as well as the pure compounds can be tested for their bioactivity. In this method, *in vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products. This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal and anti-tumor etc. of the compounds [12].

From the results of the brine shrimp lethality bioassay, it can be well predicted that the crude ethanolic extract and its different partitioning fractions possess cytotoxic principles and have considerable cytotoxic potency. Further bioactivity guided investigation can be done to find out potent antitumor and pesticidal compounds.

CONCLUSION: In conclusion, the overall results of the present study indicate the anti-nociceptive and preliminary antitumor activities of the roots of *L. macrophylla* and further investigations are required to isolate the active constituents responsible for these activities and to establish the mechanisms of action. The results of this study provided a scientific support for the use of the roots of the plant in the treatment of pain and tumor in traditional system of medicine.

REFERENCES:

1. Newman DJ and Cragg GM: Natural products as sources of new drugs over the last 25 years. Journal of Natural Products 2007; 70:461-477.
2. Rates SMK: Plants as source of drugs. Toxicon 2001; 39:603-613.
3. FAO: Trade in medicinal plants. In: Economic and social Department, Food and Agriculture Organization of the United Nations. Rome, 2-3.
4. Ahmad F, Khan RA and Rasheed S: Study of analgesic and anti-inflammatory activity from plant extracts of *Lactuca scariola* and *Artemisia absinthium*. Journal of Islamic Academy of Sciences 1992; 5:111-114.
5. Uddin SN: Traditional uses of Ethnomedicinal plants of the Chittagong Hill Tracts. Bangladesh National Herbarium, First Edition 2006.
6. Swarnkar S and Katewa SS: Ethnobotanical observation on tuberous plants from tribal area of Rajasthan (India). Ethnobotanical Leaflets 2008; 12.
7. Pracihi G, Haruyo I, Nikita M, Gautam S, Bharat B and Aggarwal: From Ancient Medicine to Modern Medicine: Ayurvedic Concepts of Health and Their Role in Inflammation and Cancer. Journal of the Society for integrative Oncology 2007; 5:10-15.
8. Uddin MZ, Hassan MA and Sultana M: Ethnobotanical survey of Medicinal plants in Phulbari Upozila of Dinajpur District, Bangladesh. Bangladesh Journal of Plant Taxonomy 2006; 13:63-68.
9. Kupchan SM and Tsou G: Tumor inhibitors. LXXXI, structure and partial synthesis of fabacein. The Journal of Organic Chemistry 1973; 38:178.
10. Van Wagenen BC, Larsen R, Cardellina JH, Ran Dazzo D, Lidert ZC and Swithenbank C: Ulosantoin, a potent insecticide from the Sponge Ulosa ruetzleri. The Journal of Organic Chemistry 1993; 58:335-337.
11. Koster R, Anderson M and De Beer EJ: Acetic acid for analgesic screening. Federation Proceedings 1959; 18:412.
12. Meyer BN, Ferringni NR, Puam JE, Lacobsen LB, Nichols DE and McLaughlin JL: Brine shrimp: a convenient general bioassay for active constituents. Planta Medica 1982; 45:31-32.
13. Collier HO, Dinneen LC, Johnson CA and Schneider C: The abdominal constriction response and its suppression by analgesic drugs in the mouse. British Journal of Pharmacology 1968; 32:295-310.
14. Ronaldo AR, Mariana LV, Sara MT, Adriana BP, Steve P, Ferreira SH and Fernando QC: Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. European Journal of Pharmacology 2000; 387:111-118.
15. Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S and Narumiya S: Altered pain perception and inflammatory response in mice lacking prostacycline receptor. Nature 1997; 388:678-682.
16. Deraedt R, Jougney S, Delevalcee F and Falhout M: Release of prostaglandin E and F in an algogenic reaction and its inhibition. European Journal of Pharmacology 1980; 61:17-24.

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