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COMPARATIVE ANTIANXIETY POTENTIAL OF *EUPHORBIA NERIIFOLIA* LINN. LEAVES AND *EUPHORBIA HIRTA* LINN. AERIAL PARTS

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Keywords:

Euphorbia neriifolia, *Euphorbia hirta*, Antianxiety, Elevated plus maze, Diazepam, TLC densitometry

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ABSTRACT: *Euphorbia neriifolia* and *Euphorbia hirta* have been traditionally used for relieving anxiety and other nervous disorders. The present study was designed to evaluate and compare the antianxiety activity of *E. nerrifolia* leaves and *E. hirta* aerial parts using elevated plus maze model. Petroleum ether (60-80°C), chloroform, ethanol and aqueous extracts of both the plants were prepared and evaluated for antianxiety activity. Chloroform extract of *E. neriifolia* and ethanol extract of *E. hirta* showed significant antianxiety activity at the dose of 200 and 400 mg/kg, respectively, comparable to that of diazepam (2 mg/kg). The study was extended to develop the TLC fingerprint profile of the two plants using CAMAG HPTLC system, and the relative percentage of the separated constituents was calculated from the area of peaks. Further, phytochemical screening of the bioactive chloroform extract of *E. neriifolia* showed the presence of flavonoids, steroids/terpenoids, phenols, and tannins while the bioactive ethanol extract of *E. hirta* tested positive for flavonoids and steroids/terpenoids.

INTRODUCTION: Anxiety disorders are one of the most prevalent and impairing psychiatric problems and ranked as the sixth largest contributor to non-fatal health loss globally [1]. Evolutionarily, anxiety is considered to be the normal behavioral repertoire and adaptive response to stress to deal with potential risks. However, when anxiety increases beyond a certain level, interfering with everyday functioning, it is considered to be pathological [2]. Pharmacological treatment of anxiety includes different drugs like barbiturates, benzodiazepines, and serotonin reuptake inhibitors but their use is limited because of unwanted side effects such as psychological and physical dependence, impaired cognition and coordination, withdrawal reactions and suicidal ideation [3].

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Plants have been used traditionally for their ameliorative effect in anxiety since the ancient times as they are equally efficacious as conventional drugs with the added advantage of having fewer side effects [4]. *E. neriifolia* (Euphorbiaceae), commonly known as Thohar (Hindi) and Common milk hedge (English), is found throughout the Deccan peninsula of India and around North and Central India. It is a large succulent shrub with stipular thorns, growing up to 4.5 m. Leaves are succulent, deciduous, 6-12 inches long and ovular in shape with reticulate venation [5, 6, 7].

The plant has been extensively used in Ayurveda as an aphrodisiac, rubefacient, anticancer agent and to heal anal fistula. Besides this, it is also used in Unani medicine as a respiratory stimulant, local anesthetic, antibacterial, antiviral, and paronychia. *E. neriifolia* leaves have been claimed to possess aphrodisiac, expectorant, wound healing, diuretic, immunomodulatory, antianxiety and CNS depressant properties [8, 9]. *E. hirta* (Euphorbiaceae), commonly called Dhudhi (Hindi) or Asthma weed

(English) is a small annual herb, frequently seen along roadsides, grasslands and pathways. It is usually erect, grows up to a height of 40 cm and can also be seen lying down. The plant has been used widely in the traditional system for treating bowel complaints, worm infestations, and kidney stones. The whole plant has also been reported to possess anti-bacterial, antiamoebic, antifungal, antiviral, spasmolytic, antidiarrheal, sedative, anxiolytic, analgesic, antipyretic, anti-inflammatory, anti-malarial and antihypertensive properties [10, 11]. Despite the widespread use of *E. neriifolia* and *E. hirta* as a traditional medicine for treating various ailments, including anxiety, no exhaustive reports to substantiate their therapeutic claims are available. Thus, the objective of the present study was to evaluate and compare the antianxiety activity of various extracts *viz*. petroleum ether, chloroform, ethanol and water extracts of *E. neriifolia* leaves and *E. hirta* aerial parts

MATERIAL AND METHODS:

Plant Material: The leaves of *E. neriifolia* and aerial parts of *E. hirta* were collected from the Medicinal Plant Garden of University Institute of Pharmaceutical Sciences (UIPS), Panjab University, Chandigarh. These were air-dried in the shade. Morphologic and microscopic characters of the collected samples were found to match completely with those reported in the literature [7, 12]. Voucher specimens of *E. neriifolia* leaves (Lf/1502) and *E. hirta* aerial parts (Wp/1501) have been deposited in Herbarium-cum-Museum of UIPS, Panjab University, Chandigarh.

Chemicals and Reagents: Solvents used include petroleum ether 60-80 °C (Merck India Ltd., Mumbai), chloroform (Thermo Fisher Scientific India Pvt. Ltd., Mumbai), ethanol (Panipat Sugar Mill, D-Unit, Panipat) and distilled water prepared in our laboratory. Diazepam (Java Pharmaceuticals, Gurugram) was used as the standard antianxiety agent.

Preparation of Extracts: Coarsely powdered leaves of *E. neriifolia* and aerial parts of *E. hirta* (1kg each) were exhaustively soxhlet extracted successively with pet ether, chloroform, and ethanol. The marc was finally boiled (1 h) with distilled water to prepare the water extract. The extracts were dried using Eyela N 1100 rotary vacuum evaporator and were preserved in a vacuum desiccator containing anhydrous silica gel blue.

Experimental Animals: Laca mice (either sex), housed at the Central Animal House, Panjab University, were allowed standard pellet diet (Ashirwad, Chandigarh) and water ad libitum. Groups of 6 mice (20-30 g) were used in all sets of experiments. The animals fasted for 18 h before use. Approval (PU/IAEC/S/16/112) from the Institutional Animal Ethical Committee of Panjab University, Chandigarh was taken before carrying out biological studies.

Preparation of Doses: Polysorbate 80 (5%) in aqueous carboxymethylcellulose (CMC 0.5% w/w) was used as a vehicle for preparing the suspension of extracts/standard drug. Doses of various test substances were prepared by suspending appropriate quantities in the vehicle for administering these to mice in volumes ranging between 0.20-0.30 ml per oral route.

Acute Toxicity Studies of the Extracts: Acute toxicity studies of pet ether, chloroform, ethanol and water extracts of *E. neriifolia* and *E. hirta* were carried out on mice as per OECD 423 guidelines [13]. After 12 h of fasting, different groups of mice were administered single oral dose (500, 1000 or 2000 mg/kg) of the four extracts. Immediately after dosing, animals were observed for signs of toxicity during the first 0.5, 1, 2, 4, 8 and 12 h, and at every 24 h for 14 days. Behavioral parameters, tremors, lethargy, death, amount of water and feed taken were observed.

Anti-anxiety Activity: Anti-anxiety activity was evaluated using elevated plus maze (EPM) model [14, 15]. The apparatus consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) having an open roof. It was kept elevated (25 cm) from the floor for evaluating the anxiolytic behavior. Doses were administered orally using tuberculin syringe fitted with an oral cannula. The dose administration schedule was so adjusted that each mouse was having its turn on the EPM 60 min after the administration of the vehicle, diazepam (2mg/kg) or the test extracts (100, 200 or 400 mg/kg). Each mouse was placed at the center of

EPM with its head facing towards the open arm. During 5 min duration of the experiment, behavior of the mouse was recorded as (a) the number of entries into the open arms and (b) mean time spent by the mouse in open arms.

TLC Fingerprint Profile:

a) Preparation of Extracts: *E. neriifolia* leaves, and *E. hirta* aerial parts (2 g each in filter paper sachets) were separately macerated (30 min) and refluxed (30 min) with methanol. The extracts were concentrated, transferred to 5 ml volumetric flasks, and the volume was made up to the mark. These were used to prepare the fingerprint profiles.

b) TLC Densitometry: A CAMAG HPTLC system (Switzerland) comprising of CAMAG Linomat 5 applicator, CAMAG TLC Scanner 3, CAMAG win CATS software, version 1.3.3, Hamilton syringe (100 µl), CAMAG Reprostar 3, were used for the study. The chromatography was carried out using precoated silica gel 60 aluminum sheets (Merck, Germany; 10 × 5 cm) as the stationary phase. A band (6 mm length) of 4 µl for both extracts was applied, and the plates were developed using toluene: ethyl acetate: formic acid (9.5:0.5:0.1) and toluene: ethyl acetate: formic acid (8:2:1) as mobile phase for *E. neriifolia* and *E. hirta*, respectively.

The developed plates were visualized after spraying with an anisaldehyde-sulphuric acid reagent. The chromatograms obtained were scanned at 540 nm. The relative percentage of the separated constituents was calculated from the area of peaks.

Phytochemical Screening: The bioactive chloroform extract of *E. neriifolia* and ethanol extract of *E. hirta* were screened for different classes of phytoconstituents using the standard procedures [16].

RESULTS:

Yield of the Extracts: Percentage yield of extracts is listed in **Table 1**.

TABLE 1: PERCENTAGE YIELD (w/w) OF VARIOUS EXTRACTS OF *E. NERIIFOLIA* AND *E. HIRTA*

Extract	*E. neriifolia*	*E. hirta*
Pet ether	3.91	4.31
Chloroform	2.23	3.65
Ethanol	1.88	4.11
Water	1.01	2.67

Acute Toxicity Studies: All the extracts neither exhibited signs of acute toxicity nor mortality up to the dose of 2000 mg/kg, p.o.

Antianxiety Activity of Extracts: Administration of diazepam (2 mg/kg) significantly increased the number of entries and the time spent in the open arms compared to the control group. Among all the extracts chloroform extract (200 mg/kg) of *E. nerrifolia* leaves **Fig. 1** and ethanol extract 400 mg/kg) of *E. hirta* aerial parts **Fig. 2** showed significant ($p<0.001$) anti-anxiety activity.

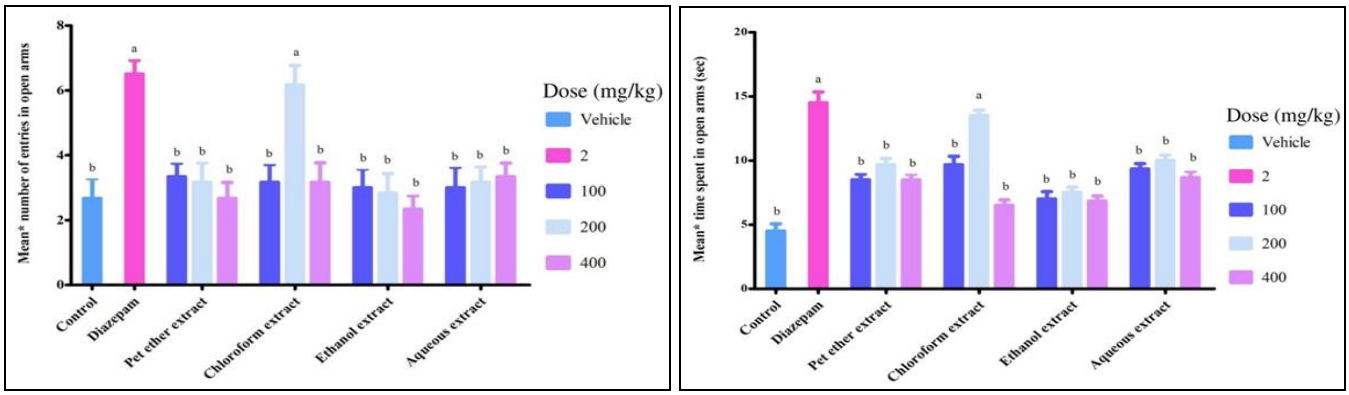

FIG. 1: ANTIANXIETY ACTIVITY PROFILE OF DIFFERENT EXTRACTS OF *E. NERIIFOLIA* LEAVES USING EPM

The data are expressed as mean ± SEM; *n=6; [a]$p<0.001$ *vs.* control; [b]$p<0.001$ *vs.* diazepam; one way ANOVA followed by Tukey's multiple range test.

TLC Fingerprint Profile: Twelve different spots were observed in the TL chromatogram of *E. neriifolia* while seventeen spots were observed in the TL chromatogram of *E. hirta.* **Table 2** and **3** show the R_f values of the different spots obtained along with their relative concentrations (calculated using the area under the curve) for *E. neriifolia* and *E. hirta*, respectively. **Plate 1A** and **B** shows the TL chromatograms while **Fig. 3** and **4** show the spectra of *E. neriifolia* and *E. hirta*, respectively.

TABLE 2: PERCENTAGE RELATIVE CONTENT OF DIFFERENT SPOTS IN TLC OF *E. NERIIFOLIA* LEAVES

Concentration (µl)	Spot	R_f	% Relative content*
4	1	0.08	0.28
	2	0.11	0.69
	3	0.14	3.88
	4	0.21	4.05
	5	0.26	5.06
	6	0.31	7.47
	7	0.36	8.64
	8	0.42	32.23
	9	0.52	9.96
	10	0.59	18.47
	11	0.72	2.14
	12	0.78	7.18

*Calculated based on area under curve

TABLE 3: PERCENTAGE RELATIVE CONTENT OF DIFFERENT SPOTS IN TLC OF *E. HIRTA* AERIAL PARTS

Concentration (µl)	Spot	R_f	% Relative content*
4	1	0.03	1.93
	2	0.07	0.26
	3	0.09	1.71
	4	0.12	1.46
	5	0.16	5.41
	6	0.22	1.31
	7	0.29	2.27
	8	0.34	2.92
	9	0.39	1.37
	10	0.43	1.90
	11	0.49	14.07
	12	0.58	31.11
	13	0.68	19.10
	14	0.74	0.95
	5	0.79	4.57
	16	0.86	8.35
	17	0.95	1.31

*Calculated based on area under the curve

Phytochemical Investigation: The bioactive chloroform extract of *E. neriifolia* showed the presence of flavonoids, steroids/terpenoids, phenols, and tannins while the bioactive ethanol extract of *E. hirta* was found to be positive for flavonoids and steroids/terpenoids **Table 4**.

TABLE 4: RESULTS OF PHYTOCHEMICAL SCREENING OF CHLOROFORM EXTRACT OF *E. NERIIFOLIA* AND ETHANOL EXTRACT OF *E. HIRTA*

Phytoconstituent	*E. neriifolia*	*E. hirta*
Fixed oils and fats	-	-
Saponins	-	-
Steroids and terpenoids	+	+
Flavanoids	+	+
Coumarins	-	-
Phenols and tannins	+	-
Alkaloids	-	-
Glycosides	-	-
Proteins and amino acids	-	-
Carbohydrates	-	-

+: present, -: absent

A **B**

PLATE 1: TLC FINGERPRINT PROFILE OF METHANOL EXTRACTS OF *E. NERIIFOLIA* LEAVES (A) AND *E. HIRTA* AERIAL PARTS (B)

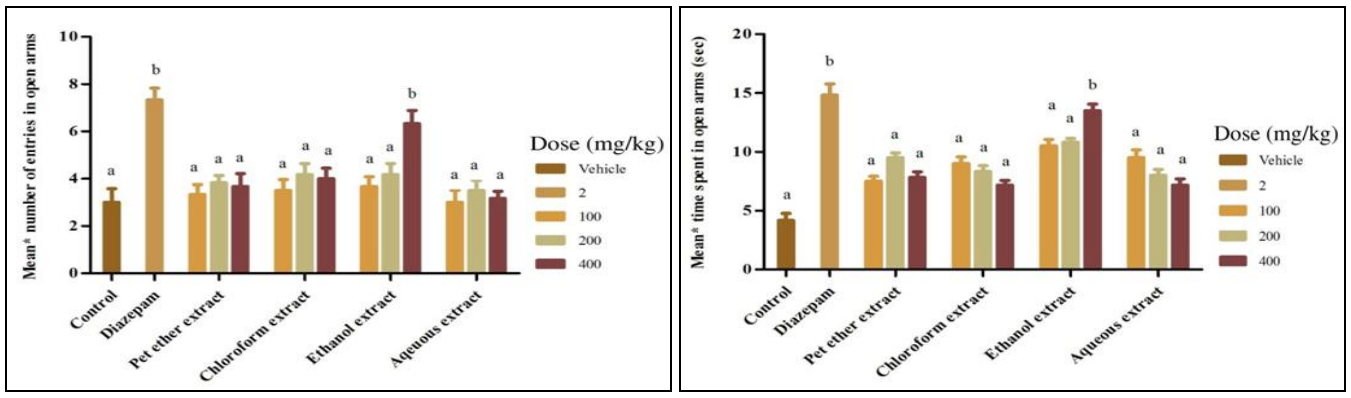

FIG. 2: ANTIANXIETY ACTIVITY PROFILE OF DIFFERENT EXTRACTS OF *E. HIRTA* AERIAL PARTS USING EPM

The data is expressed as mean ± SEM; *n=6; [a]p<0.001 *vs.* control; [b]p<0.001 *vs.* diazepam; one way ANOVA followed by Tukey's multiple range test.

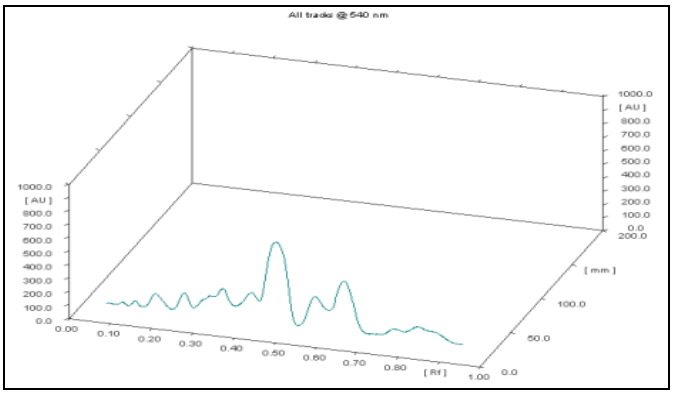

FIG. 3: SPECTRA OF TL CHROMATOGRAM OF *E. NERIIFOLIA* LEAVES

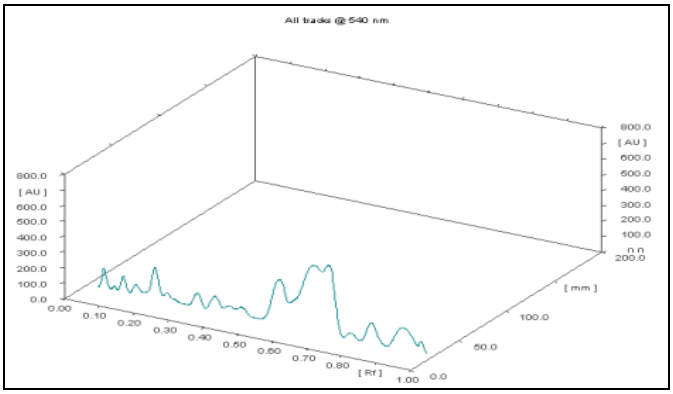

FIG. 4: SPECTRA OF TL CHROMATOGRAM OF *E. HIRTA* AERIAL PARTS

DISCUSSION: Despite the traditional use of *E. neriifolia and E. hirta* for treating nervous disorders, systematic scientific reports on the evaluation of its anti-anxiety effects are not available. Elevated plus maze model is considered to be etiologically similar to the anxiety observed clinically in human beings [15]. An anxiolytic agent increases both the frequency of entries and the time spent in open arms of the EPM and is thought to act via the GABA-A (γ-aminobutyric acid type A) receptor complex, justifying the use of diazepam as a positive control in the study [14]. Anti-anxiety agents are expected to increase the motor activity, which is measured by some entries and time spent by the animal in the open arm.

Results of the present study indicate that among all the extracts, the chloroform extract of *E. neriifolia* and ethanol extract of *E. hirta* showed a significant dose-dependent anti-anxiety activity at 200 mg/kg and 400 mg/kg, respectively which was statistically comparable to diazepam. Besides this, a dose-dependent decrease in the activity was observed, which might be due to mild sedation at higher doses. The fingerprint profile of both the plants was developed because it is one of the most basic, reliable and important parameters for herbal drug standardization. The optimum resolution was obtained using toluene: ethyl acetate: formic acid (9.5:0.5:0.1) for *E. neriifolia* and toluene: ethyl acetate: formic acid (8:2.5:0.1) for *E. hirta* on precoated silica plate after spraying with 0.5% anisaldehyde. Relative % content of each constituent separated was calculated based on AUC.

Phytochemical investigation of both extracts showed the common presence of flavonoids, steroids, and terpenoids indicating that these might be responsible for their anti-anxiety effect. Also, previous biochemical and pharmacological reports have shown that flavonoids have significant effects on the CNS [17], primarily due to their affinity for the central benzodiazepine receptors [18]. Thus, the anti-anxiety activity of the two extracts might be due to the flavonoids present in them.

CONCLUSION: Results of the present study indicate that the chloroform extract of the *E. neriifolia* leaves and *E. hirta* aerial parts have significant anti-anxiety activity at a dose of 200 and 400 mg/kg, respectively. Further, studies are underway to isolate the active constituent(s), elucidate their structure, and establish the mechanism of anti-anxiety activity.

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CONFLICT OF INTEREST: None

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