ANTI-ANXIETY EFFECTS AND RELATED LOCOMOTOR ACTIVITY OF METHANOLIC EXTRACT AND FRESH JUICE OF *CLERODENDRUM INERME* LEAVES, IN WISTAR RATS

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ABSTRACT*: Aim of this study is to investigate the effects of Clerodendrum inerme leaves as anti-anxiety agent using different behavioral paradigms such as hole board/head dip apparatus, light & dark box and elevated plus maze (EPM). It was observed that C. inerme has mood elevation effect, increased rearing; it decreased anxiety and fear on wistar rats. After acute toxicity test C. inerme L. methanolic extract 200 mg/kg, 300 mg/kg, 400 mg /kg (CIM) and C. inerme L. 10 ml/kg and 15 ml/kg fresh juice of (CIF) were selected and tested for their anti-anxiety behavior. Results were compared with diazepam 1 mg/kg. The effects were found to be most potent with C. inerme L. 300 mg/kg and 400 mg/kg methanolic extract C. inerme L along with 15 ml/kg fresh juice C. inerme L which also showed decreased anxiety in rats. There were also motor alteration as number of transitions and rearing increased dramatically within the treated group. With 400 mg/kg CIM and 15 ml/kg CIF significant difference in head dips and rearing were observed when compared with diazepam ($p<0.001$) and ($p<0.001$) respectively which strongly contributes to the fact that leaves of C. inerme possess strong anxiolytic activity and inhibits fear. We further suggest phytochemical specific receptor activity so that its anti anxiety potential can be clinically utilized.*

Keywords: Clerodendrum inerme leaves, Anti-anxiety, rearing, Clerodendrum inerme fresh juice

INTRODUCTION

During past few decades herbal remedies gained popularity and their demand is increasing globally. As a matter of fact its acceptance is not only in far off undeveloped parts of the world but now there is an increasing demand in developed areas as well where conventional ways of treatment are deeply rooted[1]. Ethno-botanical research is very important for conservation and sustainable utilization of these medicinal plants. As we know there are many indigenous medicinal plants, which are known only to the local practitioners and their use is limited to their community. This information is never written anywhere but known only by their ancestors, as they inherit that information as a generation. based knowledge that needs to be documented and investigated not just for the benefit of mankind but for further scientific studies on safety of their use and investigation of their adverse effects. More such studies should be encouraged to preserve knowledge and rare uses of native medicinal plants. This present research is an attempt to preserve this ancient heritage and investigate efficacy and safety of C. inerme as an anxiolytic agent. C. inerme, C. phlomidis linn and C. multiflorum are extensively used in Ayurveda and Siddha system of medicine for nervous disorder [2].

Clerodendrum Inerme: This is commonly known as dum dum in pakistan. This indigenous plant has attractive white flowers 1.5-2 inches in length with long purple stamens extending beyond white petals of the flower and has oval fruits [3]. It iswidely distributed in coastal vegetations, edges of mangroves, along with swamps, lowland streams and sometimes in sandy areas. This plant belongs to the family Verbenaceae [4]. Many important uses of Clerodendrum genus has been reported in various indigenous systems of medicine. Most effective cures were against typhoid, jaundice, cancer, syphilis and hypertension [5]. These plants are sometimes the only source of cure in their native lands in remote areas which are far off from urban areas especially in India, Japan, China, Thai, and Korea. Isolation of Compounds and some chemical studies revealed that Clerodendrum contains phenolic, di- and tri terpenes, flavanoids steroids and volatile oils [6].

Chakraborthy suggests that very little effort is done to explore benefits of this plant [7]. Thorough investigations have been made to study pharmacological activities of Clerodendrum inerme and other species of this class. The aqueous extract of leaves of this plant has a hypotensive effect on the arterial pressure of rabbits (at higher doses ≥ 0.001 mg/ml) [8].

Figure 1:*Clerodendrum inerme*

Similarly aqueous extract of C. inerme leaves possess analgesic and antipyretic effects which was investigated using hot plate, tail flick method and tail immersion method [9]. Ethyl acetate and n-hexane extracts of stems and leaves of C. inerme and C. phlomidis are known for their antifungal properties. However leaf hexane extract C. inerme, inhibits the plant pathogenic fungi better than the human pathogenic fungi [10]. The methanolic extract of C. infortunatum (MECI) has anticancer potential was assessed for chemotherapeutic activity against ehrlich ascites carcinoma in tested mice [11]. Methanolic extract of C. inerme has known antioxidant and cytotoxic potential which suggests

that this plant can may have anticancer potential [12]. In old Chinese leaves decoction of this plant is used to treat pneumonia like influenza [13]. Antimicrobial activity of this plant leaves were reported in 2009 by Yln in China later in 2013 antibacterial activity against specific strains of pathogen were investigated [14]. Anti-arthritis activity of C. inerme glycosides were reported in rats in 2009 [15]. C. inerme leaves are potent neuro-protective agent effective in kainic acid induced neurotoxicity in rats [16]. Exact mechanism of anti-inflammatory action of ethanolic extract of leaves of this plant and its bio-assay guided extraction was demonstrated in 2015 [17].

MATERIAL AND METHODS

PLANT MATERIAL AND PREPARATION OF ITS EXTRACT:-

Leaves of Clerodendrum inerme, Verbenaceae, were collected locally and verified and authenticated from Centre for Plant Conservation, Herbarium University of Karachi voucher no 87668. Leaves were dried under shade and pulverized into crude powder. Methanolic extract was prepared by continuous extraction from Soxhlet apparatus. Heating mantle was set between 72-75˚C and round bottom flask was filled with methanol whereas thimble was filled with dried coarse powder of C. inerme leaves. Assembly was adjusted and subjected to continuous extraction for atleast 6 cycles. Concentrate in flask thus obtained was subjected to further evaporation by rotavapour. Fresh leaves juice was prepared extemporaneously by freshly picked leaves at 10 am in morning and placed in juicer blender (moulinex®) later stored in amber color bottle after filtration by muslin cloth. Methanolic extract and fresh juice was than subjected to preliminary phyto-chemical analysis for presence of alkaloids, flavanoids, terpenes, tannins and steroids with the help of test reagents for respective tests like shinoda test, wagner's reagent.

ACUTE TOXICITY STUDY:

Wistar rats were divided into test and control groups (n=6). The test group was given an increasing oral dose 1, 2 and 5 gm respectively of methanolic extract. Rats were allowed free access to food and water. Rats were observed for mortality and symptoms for next 48 hours. No mortality was observed up to 3 gm/kg. OECD guidelines were followed while performing acute toxicity testing for methanolic extract of clerodendrum inerme leaves [37].

1. ANIMALS

Male wistar rats weighing between (150-250 gm) were obtained from Dow University of Health & sciences and acclimatized for 14 days in Dr HMI institute of pharmacology and herbal sciences Hamdard university Karachi Pakistan. They were fed a standard pellet diet and water. They were maintained in a controlled laboratory environment (22± 5°C, 12 hr of light/dark cycles). All experimental procedures and paradigms were approved by the institutional animal ethical committee.

GENERAL PROCEDURES:-

PROTOCOL

The animals were subjected to battery of three tests in following order hole Board, plus maze and light & dark field [18]. Animal was observed for various activities in each paradigm for five minutes. The behavior tests were analyzed visually and manually recorded. Animals were divided in five treatment groups and three control groups i.e. for C. inerme methanolic extract (CIM) 200 mg/kg, 300 mg/kg, 400 mg/kg and for Clerodendrum inerme fresh juice(CIF) 10 ml/kg and 15 ml/kg were selected, for control groups diazepam 1mg/kg [19, 20] as positive control and tween80 5% and distilled water as negative control group were selected for study.

HOLE BOARD

It is an apparatus comprising of closed arena 16 grid holes in floor 2″ approx. Frequency of head dipping and duration of head dips provide measure of neophilia irrespective of locomotor activity [21, 22]. Apparatus is elevated from ground about 15 cm high.

Following parameters were considered:-

a) Number of head dips
b) Duration of head dips
c) Number of rearing
d) Duration of rearing

Significant results are graphically represented.

ELEVATED.PLUS MAZE (EPM):-

The plus maze is a plus shaped paradigm with four arms perpendicular to each other, the two open arms lie opposite to each other measuring 40x10x0.5 cm and it lies perpendicular to closed arms that measures 40x10x16 cm, with central podium.

The maze is elevated 50cm above around [16]. To analyze anti-anxiety parameter in this paradigm we observed following activities number of crossings, time spent in open arm (in sec) TOA, and time spent in closed arm TCA [23].

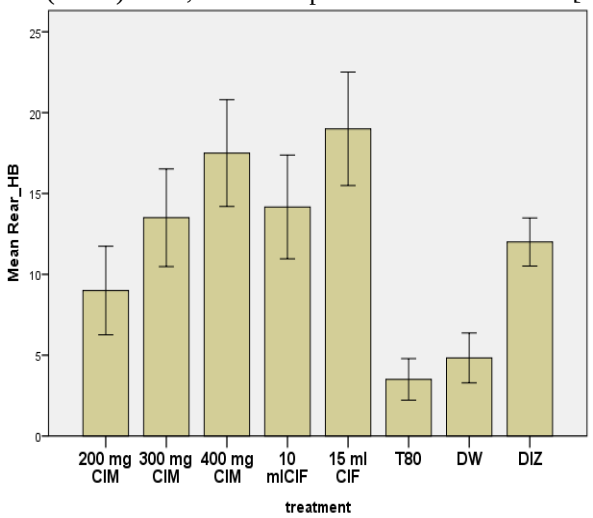

Figure 2: Mean rearing ± SEM in hole board. Note the effect on rearing on all treated groups which may be suggestive of fearless behavior

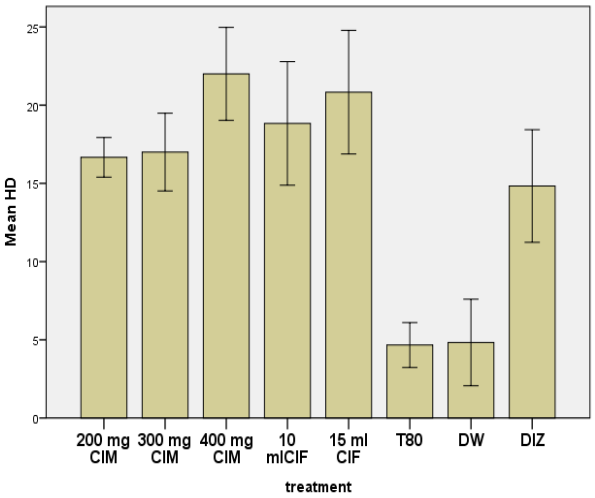

Figure 3: Mean head dips ± SEM in hole board apparatus.

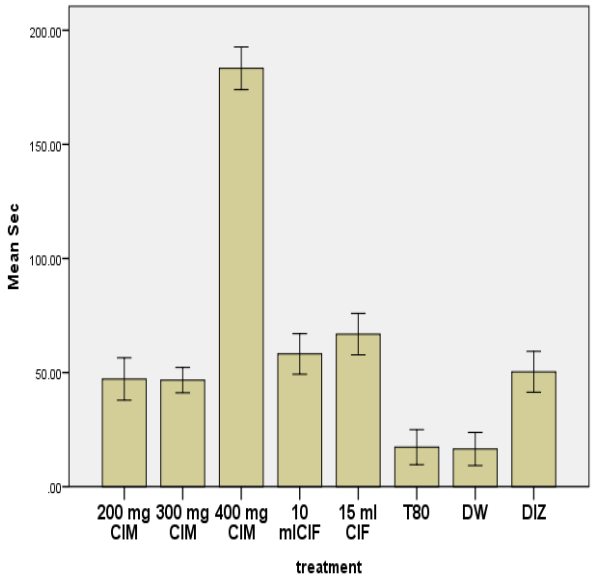

Figure 4: Time spent in light field ± SEM in LD box.

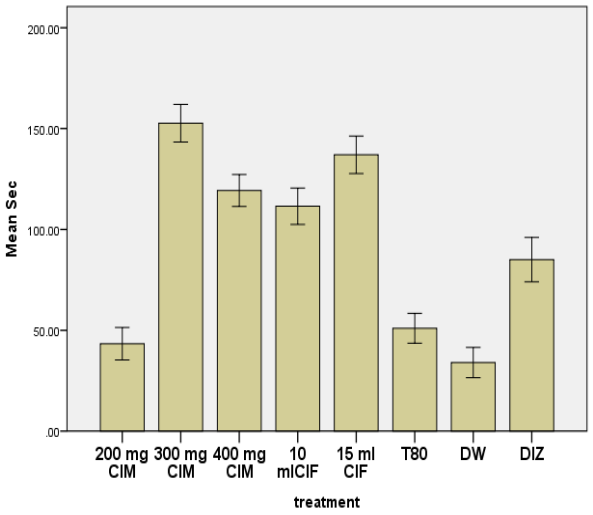

Figure 5: Time spent in open arm ± SEM in (EPM)

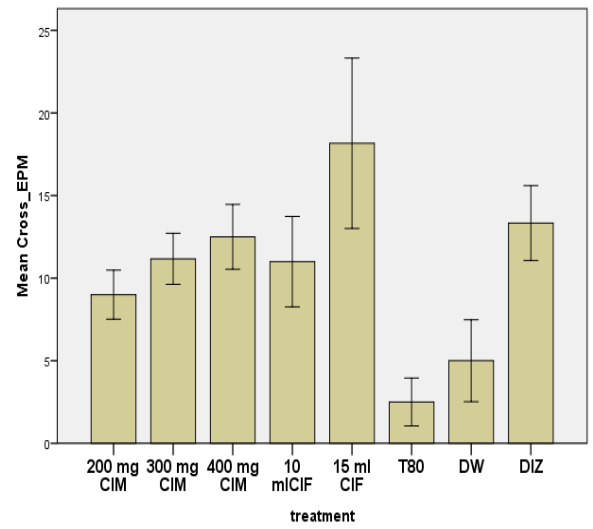

Figure 6: Mean number of crossings in elevated plus maze (EPM)± SEM.

Table 1: Overall mean results ± SEM

Sr no	Drug category	Rearing	Head Dips	Duration of head dips in sec	Crossings in EPM	Time spent in open arm	Crossings in L/D	Time spent in Light area
1	**Distilled water**	5±2	5±1	18±7	5±2	34±7	2±1	17±7
2	**5 % Tween 80**	5±1	5±1	25±9	3±1	51±7	2±0.9	17±7
3	**Diazepam**	12±1	15±3	61±8	13±2	85±9	7±2	50±8
4	**200mg Methanolic extract C.I.**	9±3	16±1	27±6	9±1	43±8	8±2	47±9
5	**300mg Methanolic extract C.I.**	14±3	17±2	61±9	11±1	152±9	5±1	47±5
6	**400mg Methanolic extract C.I.**	18±3	22±3	67±8	13±2	119±8	10±1	180±8
7	**10ml Fresh juice C.I.**	14±3	19±4	70±8	11±3	112±9	5±1	58±8
8	**15ml Fresh Juice C.I.**	19±3	21±4	81±7	18±5	137±8	5±1	66±8

LIGHT AND DARK ACTIVITY BOX

Light and dark apparatus is specifically designed for rats. The original maze is divided into two equal parts (26x27x28cm) half of the portion is transparent walls and half dark compartment provided a door between the compartments (10x10cm) which permits rat to go from one area to other a light source was placed above the box. Each rat was on the rampage in light compartment and watched for 5 minutes. Time spent in light (TSL) and dark area (TSDA) and crossing between compartments is recorded [24].

DATA ANALYSIS

Data was analyzed by one way ANOVA followed by Scheffe test using SPSS 19. The results were considered significant when ($p < 0.05$).

PRELIMINARY PHYTOCHEMICAL RESULTS: The principals found to be present are steroids, flavones, tannins, phenols, trirepenes, quinones and alkaloids.

Results:-

Results which were found significantly different from positive control i.e. diazepam are mentioned in results as highly significant difference was found with negative control i.e. distilled water and tween 80 (5%).

1. Elevated plus maze (EPM): For number of crossings in EPM one way ANOVA shows significant difference between groups {F=22.6 (7,40) p <0.001} (Figure 6).

For time spent in open arm, one way ANOVA shows significant difference between groups {F=175.5 (7,40) p <0.001} while 300mg CIM, 400mg CIM, 15 ml CIF mean values increased significantly{-67.6, -34.3, -26.5, -52.0 }respectively (p<0.001)when compared with diazepam. (Figure 5)

2. Light and dark box: For time spent in light area one way ANOVA showed significant difference between groups {F=262(7,40) p value <0.001} post hoc scheffe test showed that mean value significantly increased in 400mg CIM group when compared with diazepam{-133.3, (p<0.001)} figure 4. For Crossing in light and dark field one way ANOVA shows significant difference between groups {f=19.06(7,40) p<0.001} when compared

3. Hole board apparatus: For head dips one way ANOVA showed significant difference between groups {f=33.71 (7,40) p < 0.001}most significant rise in mean value was observed with 400mg CIM {-7.1 (p<0.05)} when compared with diazepam see figure 3. In rearing One way ANOVA showed significant difference between groups {f=29.1 (7,40) p value <0.001} most significant increase in mean values were observed with 15 ml CIF { -7.0, (P<0.05)} when compared with diazepam (figure 2). Post hoc Scheffe test showed no significant results when compared with other treated groups.

DISCUSSION:

Many stress relieving Siddha poly-herbal formulations contain Clerodendrum leaves. A case study showed that a teenage girl suffering from intractable chronic motor tic disorder responded dramatically within an hour, to fresh grounded juice of C. inerme leaves. After a year of treatment her hematology, liver and renal functions turned normal [25]. This investigation strongly suggests its efficacy in Tourette syndrome which is marked by motor tics attention deficit hyperactivity disorder (ADHD) and obsessive compulsive disorder (OCD) [26]. Ethanolic extract of C. inerme Leaves were found to be effective against methamphetamine induced hyperlocomotor activity at doses of 100-300 mg/kg without affecting the spontaneous locomotor activity. This kind of activity is not only effective in motor tic disorders it is also suggested that it may help to prevent attention deficient hyperactivity disorder, schizophrenia or other psychiatric disorders like obsessive compulsive disorder [27]. Overall this plant extract alleviates animal behavior. In present study this is clearly exhibited by leaf extract of C. inerme in other behavioral paradigms in doses of 300 mg/kg and 400 mg/kg as rats tend to spend more time in lightened area when tested in light and dark box.

Neophilia is attraction of animal towards object or place simply because it is novel or in other words it is a curiosity based approach towards novality. Neophobia is aversion or strong dislike that animal show towards approaching new objects and place or in other words fear based avoidance towards novel stimulus [28, 29].This neophobia is strongly inhibited by C. inerme fresh juice as the animal tends to rear and number of head dips increased in all treated groups i.e 200 mg/kg CIM, 300 mg/kg CIM, 400 mg/kg CIM, 10 ml/kg CIF and 15 ml/kg CIF, which is comparable with diazepam (p<0.05). Anxiolytic agents strongly depress neophobia, present study showed that neophobia is inhibited by C. inerme leaves.

Neophilia is marked by locomotion, escape, curiosity or inspection in the new environment whereas neophobia is more likely marked by aversion, avoidance, proactive behavior and burying objects [18]. Overall novelty is defined as an outcome of an approach avoidance conflict [30]. In state of anxiety animal freeze or immobilize whereas in case of anxiolytic agents this response increases i.e. animal tends to escape or inspect the new environment [31]. Rearing in rats is momentarily raising forelimbs off the ground and standing on its hind leg indicates a response in novelty. Rearing in novel environment depends on animal state of fear, higher the fear state lesser the rearing and vice versa [32]. Rearing was significantly increased in all treated groups which show animal fearless behavior. It also suggests in this case that sympathetic stimulation may not be involved as animal is showing more fearless behavior.

Another possible way by which the animal is becoming fearless is most probably due to the involvement of HPA axis [33]. The rise of glucocorticoids majorly increases the stressful condition in the body along with reversible damage to hippocampus [34]. When Anxiolytic drug works, it prevents the rise of glucocorticoid levels additionally do not sedate the animal.

Hypoglycemia is also reported by extracts and fresh juice 15 ml/kg that is suggestive of involvement of HPA axis [3]. Certain phenols and flavanoids are suggested to be responsible for its mood elevation and anxiolytic ability but this needs a thorough investigation [35].

CONCLUSION:-

In conclusion C. inerme leaves are potentially anxiolytic in nature which is responsible for mood elevation without producing sedative effects and makes animal more fearless in novel environment however exact mechanism is yet to be established. It is strongly suggested that further investigations maybe performed to fully explore the receptor mechanism involved, using receptor blockers so we can develop better understanding of this plant.

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