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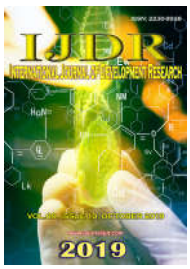

RESEARCH ARTICLE OPEN ACCESS

EVALUATION OF PHYTOCHEMICAL AND ANXIOLYTIC PROPERTY OF COW URINE BETEL VINE EXTRACT IN ALBINO MICE

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ABSTRACT

Objective: To study the phytochemical and anxiolytic property of Cow urine Betel vine extract in albino mice. **Methods:** The anxiolytic activity of Cow urine Betel vine extract at (250 and 500mg/kg, p.o) in mice was assessed by using elevated plus-maze and light –dark model. **Results:** The Cow urine Betel vine extract significantly reduces the duration of time spent in the closed arm in Elevated plus-maze model and decreased entries into dark chamber in the Light-Dark model at the doses of 250 and 500mg/kg respectively. **Conclusion:** The Cow urine Betel vine extract possess anxiolytic activity since it reduced the duration of time spent in closed arm and also reduced the number of entries into dark chamber produced by both Elevated plus-maze and Light-dark model.

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INTRODUCTION

Anxiety disorder is increasingly recognized as a highly prevalent and chronic disorder with onset during the teenage years, with an incidence of 18.1% and a lifetime prevalence of 28.8%. (Kessler, 2005). The disorder is associated with significant disability (including educational and occupational) which has a negative impact on the quality of life (Kasper, 1998). Pharmacotherapeutic approaches for the management of anxiety disorders include psychotropic drugs, but these agents are limited by their side-effect profile, the need for dietary precautions, and drug interactions (Baldessarini, 2001). Regular use of benzodiazepines causes deterioration of cognitive functioning, addiction, psychomotor impairment, confusion, aggression, excitement, anterograde amnesia, physical dependence, and tolerance (Suresh, 2006). These are some of the factors that caused interest in many researchers to evaluate new compounds from plant origin in the hope to identifying other anxiolytic drugs with fewer unwanted side effects. Various types of herbal medicines are used as anxiolytic agents in different parts of the world such as Citrus aurantium from Brazil-Indians, Afro-Brazilians and Caboclos, (Eliana, 2008) roots of kava plant from the topical pacific region, and the saponin-containing fraction of leaves of Albizia lebbeck from India are all known to have anxiolytic effects (Adnaik, 2009). The major obstruction in the application of herbal medicine into medical practice is the lack of sufficient scientific and clinical data and better understanding of efficacy and safety of the herbal products. *Piper betel (Betel vine)* is a Vedic plant, which is using as a remedy for various diseases. It is used in variety of decoction, in curing wounds, burns, impectigo, furuneloris, eczema, lymphangits and juice is beneficial stomatic. Kammaru (a variety of Piper betle) leaf has a good level of juice that heals pharyngitis, abdominal pain and swelling. Generally betel leaf cures urticaria and as per ayurvedic medicine, it recovers the loss of equilibrium between the three 'humours,' namely, Vatha, Pitha and Kapha. The roots and fruits are well-known for treatment of malaria, asthma (Nandkarni's, 2007 and Deshpande, 1970). In Ayurveda, betle leaf juice is commonly utilized as an adjuvant & combined with different other medicines most likely for better effects beside its separate use as medicine. In Susrta Samhita, tambool leaves have been described as aromatic, sharp, hot, acrid and valuable for voice, laxative, appetizer, beside this they soothe vata and aggravate

pitta (Kumar, 1999). In Unani system of medicine it is described to improve taste and appetite, tonic to brain, heart and liver, lessens thirst, clears throat and purifies blood. Cow urine is believed to have therapeutic value and used in many drug formulations. Essentially, Cow urine is used as disinfectant and for purification. With an approximate shelf life of around 5 years, this has proved to be the most effective natural antiseptic and disinfectant, when compared to the synthetic chemicals which are currently available to the consumers. Thus, it strengthens the fact that cow's urine is not a toxic effluent as 95% of its content being water, 2.5% urea and the remaining 2.5%, a mixture of minerals, salts, hormones and enzymes. In the rural villages in India, Cow's urine is being used since a very long time as an effective in neurological disease like anxiety and convulsion etc. Hence the combination of Cow urine and Betel vine will provide a synergestic effect in treating these neurological diseases the present study is conducted to evaluate the anxiolytic activity by using different models and studying the effect of plant on their exploratory behavior.

MATERIALS AND METHODS

Plant material: Fresh leaves of the Piper betel (Betel vine) were collected from the nearest plantation and leaves were authenticated, shade dried and powdered to get moderate coarse powder. The dry powder was then extracted with cow urine at 40-45^{0}C by maceration process for 72 hrs. Later the cow urine extract was filtered and the filtrate was concentrated to a semi solid mass by using vacuum distillation apparatus. The extract is obtained was used for the phytochemical and pharmacological investigations. In the present study, oral administration of the Cow urine Betel vine extract at the doses of 250mg/kg and 500mg/kg significantly inhibits convulsion and anxiety and the results were compared with Standard Diazepam (4mg/kg) treated group.

Experimental animals: Swiss mice of either sex, 8-10 weeks old, weighing about 25-30 g were used in experiments. Animals were housed in polypropylene cages maintained under standard condition (12 hours light / dark cycle; 25 ± 30C, 45-65% humidity) and had free access to standard feed and water ad libitum. All the animals were acclimatized to laboratory condition for a week before commencement of experiment. All experimental protocols were reviewed and accepted by the Institutional Animal Ethical Committee (IAEC) prior to the initiation of the experiment.

Acute toxicity studies as per OECD guide lines 425: The Cow urine Betel vine extract was orally administered to different groups of mice (three mice per group) at doses of 5, 50, 300 and 2000 mg/kg body weight, respectively. The animals were observed for 48 hours to study their general behavior and to detect signs of discomfort and nervous manifestations. As even the mice receiving the highest dose of extract (5000 mg/kg body weight, p.o.) did not show any mortality, dose levels at 1/20th (250 mg/kg body weight, p.o.) and 1/10th (500 mg/kg body weight, p.o.) of this highest dose were selected for the anxiolytic activity.

Assessment of Anxiolytic acivity: The anxiolytic activity was examined by using the elevated plus maze (EPM) test, light and dark test (L and DT). The animals were divided into four groups, with each group consisting of six male mice. Group 1 received vehicle (normal saline); Group 2 received diazepam (1 mg/kg); groups 3 and 4 receive cow urine Betel vine extract (250 and 500 mg/kg).

Elevated Plus Maze: The EPMT apparatus consisted of four arms elevated 30 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. Two of the arms were enclosed with high walls (30 × 7 × 20 cm), and the other arms were connected via a central area (7 × 7 cm) to form a plus sign. The maze floor and the walls of enclosed arms were painted black. The room was illuminated with a 40-W lamp at the central platform. The animals were treated with vehicle, extract and diazepam orally, 60 min prior to the test. The experiment was performed between 0900 and 1400 hours, and the mice became accustomed to the dimly lit experimental laboratory for 30 min prior to behavioral testing. Each mouse was individually placed on the central platform facing toward an open arm. The frequency and duration of entries into the open and closed arms were observed for 5 min. An entry was counted when all four paws of the mouse entered an open or closed arm. Subsequently, the percentage of time spent (duration) in the open arms [100 × open/(open + enclosed)] and percentage of the number of open arm entries (frequency, 100 × open/total entries) were calculated for each animal. The apparatus was thoroughly cleaned after each trial (Carr, 2006).

Light and dark test: The L and DT apparatus consisted of open top wooden box. Two distinct chambers, a black chamber (25 cm long × 35 cm wide × 35 cm deep), painted black and made dark by covering its top with black plywood, and a bright chamber (25 cm long × 35 cm wide × 35 cm deep), painted white and brightly illuminated with 40-W white light source, were placed 25 cm above the open box. The two chambers were connected through a small open doorway, (7.5 cm long × 5 cm wide) situated on the floor level at the center of the partition. The mice were placed individually in center of the light box after 60 min of oral treatments and observed for 5 min (Ambavade, 2006).

Statistical analysis

All the values were expressed as mean ± S.E.M. The statistical analysis was carried out using one way ANOVA followed by Turkey's comparison test. A probability level of $P<0.05$ was considered moderately significant, $P<0.01$ is considered as significant and $P<0.001$ is considered as highly significant.

RESULTS

Phytochemical screening

Qualitative chemical Evaluation: The extract so obtained from the above process was subjected for the confirmation phytochemical constituents.

1. Detection of carbohydrates:

A Small quantity of the extract was dissolved in distilled water and filtered. The filtrate was subjected to the following tests.

1. Molisch's test 2. Fehling's test 3. Barford's test

1. **Molisch's test:** To 1 ml of the filtrate few drops of alcoholic alpha napthol was added and 2 ml of conc. Sulphuric acid was added slowly through the sides of the test tube. Purple colored ring was formed at

junction of the two layers, which indicates presence of carbohydrates.

2. **Fehling's test:** 1 ml of the extract was treated with 1 ml Fehling's solution I and 1ml II and then heated on water bath. Brick red colored precipitate indicates the presence of carbohydrates.
3. **Barfoed's test:** Small portion of the extract was treated with Barfoed's reagent. Red precipitate indicates the presence of carbohydrates.

Test for starch: A small amount of the powdered drug was treated with distilled iodine solution blue color indicates the presence of starch.

2. Detection of proteins and amino acids: A small quantity of extract was dissolved in few ml of water and was subjected to Million's, reagent, white precipitate shows the presence of proteins and amino acids.

a. **Biuret test:** To the solution of the extract equal volume of 5%w/v NaOH and four drops of 1%w/v $CuSO_4$ solution were added. Pink color indicates the presence of proteins.
b. **Ninhydrin test:** The solution of extract was treated with ninhydrin reagent. Purple color, indicates the presence of proteins.

3. Detection of phenolic compounds and tannins: The decoction was diluted with distilled water and filtered. The filtrate was treated with following reagent.

a. **Ferric chloride test:** The filtrate was treated with 5% of ferric chloride solution. Black colored precipitate indicates the presence of tannins and phenolic compounds.
a. **Test with lead acetate solution:** Few ml of filtrate was treated with lead acetate solution. White precipitate indicates the presence of phenolic compounds.
b. **Gelatin test:** To the filtrate of the extract, add 1ml of 1% solution of gelatin. White precipitate was seen, which indicates the presence of tannins.

4. **Test for phytosterols:** A small quantity of decoction was dissolved in 5ml of chloroform separately. Then the chloroform layer was subjected to,

a. **Salkowski test:** To 1ml of the above prepared chloroform solutions, few drops of conc. H_2SO_4 was added Red color in the lower layer, shows the presence of phytosterols.
b. **Libermann-Burchard's test:** The above chloroform solution was treated with few drops of conc. H_2SO_4 followed by 1ml of acetic anhydride solution green color shows the presence of phytosterols.

5. Test for fixed oils and fats:

a) **Spot test:** A small quantity of extract was pressed between two filter papers. Oil stain, shows presence of fixed oils.
b) **Saponification:** Few drops of 0.5N alcoholic potassium hydroxide were added to the solution of the extract along with a few drops of phenolphthalein. The mixture was heated on the water bath for about 1-2 hrs formation of soap or a partial neutralization of alkali indicates the presence of fixed oils and fats.

6. Test for alkaloids: A small amount of extract was stirred with few ml of dil Hcl and filtered. The filtrate was tested with various alkaloidal reagents such as Mayer's, Dragondraff's, Wagner's and Hager's.

a. **Mayer's test:** To the small amount of filtrate add few drops of Mayer's reagent. A white color precipitate indicates the presence of alkaloids.
b. **Dragondraff's test: (potassium bismuth iodide):** To the small amount of filtrate add few drops of Dragondroff reagent. An orange red color precipitate indicates the presence of alkaloids.
c. **Wagner's test:** To the small amount of filtrate add few drops of Wagner's reagent. A brown colored precipitate indicates the presence of alkaloids.
d. **Hager's test: (picric acid)** To the small amount of filtrate add few drops of Hager's reagent. A yellow crystalline precipitate indicates the presence of alkaloids.

7. Test for glycosides: A small amount of the extract was hydrolyzed with Hcl for one hour on a water bath and hydrolysate was subjected to

a. **Legal's test:** To 1 ml of the hydrolysate 1ml of pyridine, few drops of sodium nitroprusside solution was added and then the solution was made alkaline with NaOH solution. No pink color shows the absence of glycosides.
b. **Baljet's test:** To a solution of extract sodium picrate solution was added. No yellowish orange color was obtained showing the absence of glycosides.
c. **Bortranger's test:** Hydrolysate was treated with chloroform and the chloroform layer was separated. To this equal quantity of dilute ammonia solution was added. No pink color was observed in ammonical layer, confirms the presence of glycosides.
d. **Modified Bortranger's test:** The extract was boiled with few ml of dil HCl and 5ml ferric chloride solution. The contents are cooled and shaken with organic solvent. Organic layer was separated and to this equal volume of ammonia solution was added. The ammonical layer did not show pink color. In this test, addition of ferric chloride was added to break the C-C linkage of glycosides which is a stronger than C=O linkage.

8. Test for flavonoids: The extract was dissolved in ethanol and then subjected to the following tests.

A. **Ferric chloride test:** To the small quantity of methanol solution of extract few drops of neutral ferric chloride was added. Blackish red color shows the presence of flavonoids.
b. **Shinoida's test:** To the alcoholic solution a small piece of magnesium ribbon was added along with conc. HCl. Formation magenta color shows the presence of flavonoids.
c. **Fluorescence test:** Alcoholic solution was seen under ultra violet light. Green color fluorescence, indicates the presence of flavonoids.

d. **Reaction with alkali and acid:** With sodium hydroxide solution the extracts gave yellow color. Extract gave orange color with conc H_2SO_4 indicating the presence of flavonoids.
e. **Zinc, HCl reduction test:** To a small quantity of extract, a pinch of zinc dust was added. Then add few drops of conc. HCl. Magenta color indicate the presence of flavonoids.
f. **Lead acetate solution:** To a small quantity of extract a few drops of 10% lead acetate solution was added. Yellow precipitate shows the presence of flavonoids.

9. Detection of Saponins: The extract was diluted with 20ml of distilled water and it was agitated in a graduated cylinder for 15minutes. A one centimeter layer of foam was formed, indicates the presence of saponins.

10. Detection of Coumarins: A small amount of extract dissolved in alcohol and exposed to UV light, it does not show green fluorescence.

a. Small quantities of extract was dissolved in alcohol and add ferric chloride solution, it does not show green color indicates the absence of fluorescence.

Anxiolytic activity

Elevated Plus Maze: Solvent as cow urine extract shows significant antianxiety activity. Elevated plus maze model of anxiety to evaluate the anxiolytic effects in Betel vine with cow urine. In elevated plus maze apparatus, diazepam treated rats showed significant increase ($P < 0.001$) in the number of open arm entries, time spent in open arms and reduction in the time spent in closed arm. The extract of betel vine leaves 500mg/kg, markedly increased the percentage of average time spent by the animals in the open arms.The prominent antianxiety effect has been observed in betel vine extract 500mg/kg as compared to control.

Light - Dark Model: Anxiety disorders are one of the most prevalent and highly comorbid psychiatric conditions. Light-Dark model also provided anxiolytic effect because *Betel vine* leaves in cow urine extract at a dose of 500 mg/kg shown significantly increased the entries in light chamber and decreased the time spent and entries in the dark chamber in a similar fashion, Diazepam increased the time spent and entries in light chamber. The selected dose statistically showed significant anxiolytic activity and standard drug Diazepam (4mg/kg) exhibited significant anxiolytic activity.

Table 1. Effect of *Betel vine with cow urine* on Elevated plus maze model

Group	Dose (mg/kg b.w)	No. of entries		Time spent in sec	
		Open Arm	Closed Arm	Open Arm	Closed Arm
Control	-	4±0.5	10.3±1.2	71±5.3	223.8±6.3
Standard (diazepam)	2	10.6±0.9***	6.6±0.4**	155.1±5.1***	142.7±5.6
Test I (mg/kg)	250	6.1±0.7**	6.8±0.5*	127.2±4.6***	164.2±3.6***
Test II (mg/kg)	500	9±0.5***	6.9±0.7*	134.2±5.0***	159.7±8.4***

Note: Data was analysed using one way ANOVA followed by pairwise comparision. Values are expressed as mean ± S.E.M. *n*=6, $^{***}P < 0.001$, $^{**}P < 0.01$and $^{*}P < 0.05$.

Table 2. Effect of *Betel vine with cow urine* on Light Dark model

Group	Dose (mg/kg b.w)	No. of Entries		Time spent in sec	
		Light chamber	Dark chamber	Light chamber	Dark chamber
Control	-	3.16±0.4	5.0±0.5	91.3±3.0	209.2±3.8
Standard (Diazepam)	2	7.5±0.7***	2.6±0.4***	171.3±3.4***	125.3±5.2***
Test I	250	5.5±0.6*	1.5±0.3***	120.8±6.3***	167.3±5.6***
Test II	500	5.6±0.6*	1.6±0.3***	149.5±4.9***	154.8±5.9***

Note: Data was analysed using one way ANOVA followed by pairwise comparision. Values are expressed as mean ± S.E.M. *n*=6, $^{***}P < 0.001$, $^{**}P < 0.01$and $^{*}P < 0.05$ ns=not significant.

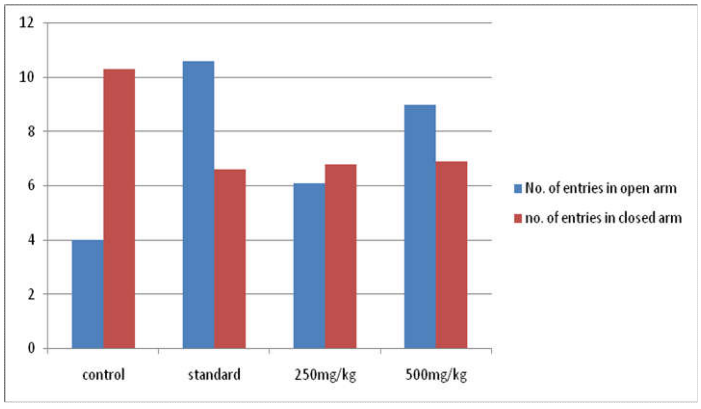

Figure 1. Histogram showing effect of *Betel vine with cow urine* shows No. of entries in the Elevated plus maze model

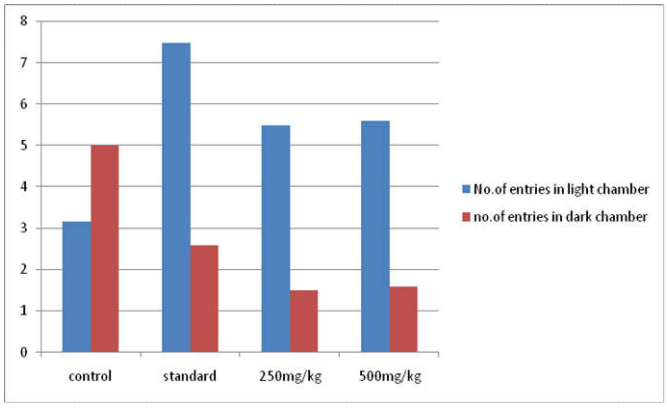

Figure 2. Histogram showing effect of *Betel vine with cow urine* extract shows No. of entries of the Light Dark model

DISCUSSION

Anxiety, like all emotions, has cognitive, neurobiological and behavioral components. It is a negative emotion that occurs in response to perceived threats that can come from internal or external sources and can be real or imagined (Moser, 2007). The incidence of anxiety in the community is very high and associated with lot of morbidity (Rauniar, 2007) the pharmacological activity of the above extract could be used to treat anxiety type of disorders. The present work has shown that anxiolytic activity by the Cow urine Betel vine extract as assessed by Elevated Plus Maze and Light and dark model. The EPMT is used to evaluate psychomotor performance and emotional aspects of mice. Results obtained on the elevated plus maze after treatment with Cow urine Betel vine extract (250 and 500 mg/kg) revealed anxiolytic activity, since increases in open arm entry parameters are the most representative indices of anxiolytic activity (Lister, 1990). Time spent on the central platform appears to be related to decision making and/or risk assessment, and the total arm entries is a contaminated measure reflecting changes in anxiety or in general activity (File, 2001). The anxiolytic-like activity was also observed in the light and dark model. Light and dark model is an ethological-based approach-avoidance conflict test and it is sensitive to drugs that affect anxiety. In this test, the number of transitions between the light and dark compartments as well as the time spent in the light side are recognized as anxiety indices, despite the transition parameter being highly dependent on locomotor activity (Bourin, 2003). Mice treated with Cow urine Betel vine extract (250 and 500 mg/kg) showed increase in the time spent in the light compartment and no changes in the numbers of shuttle crossings, confirming the activity upon the main anxiolytic parameter. The observed anxiolytic effect of Cow urine Betel vine extract may be due to the agonistic effect on GABA/benzodiazepine receptor complex, or antagonize the 5-HT1B receptor or agonize the 5-HT1A receptor (Nishikava, 2004 and Millan, 1997). Earlier reports on the chemical constituents of plants and their pharmacology suggest that plants containing flavonoids, alkaloids, phenolic acids, essential oil, saponins and tannins possess activity against many CNS disorders (Bhatacharya, 1997). The extract revealed the prrsence of tannic acid, gallic acid, alkaloids, sterols, flavonoids, glycosides, hydrolyzable tannins and high–molecular-weight polyphenolic compounds. It is possible that the mechanism of anxiolytic action of Cow urine Betel vine extract could be mediated by synergistic action of these phytochemicals. The results obtained in this study suggest that the Cow urine Betel vine extract possesses anxiolytic properties. Thus, the extract has potential clinical applications in the management of anxiety.

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