ANXIOLYTIC AND MOTOR COORDINATION ACTIVITY OF ETHANOLIC AND AQUEOUS EXTRACTS OF DENDROPHTHOE FALCATA LEAVES IN MICE

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ABSTRACT: The aim of the present study was to evaluate the anxiolytic effects and motor activity of ethanolic and aqueous extracts of leaves of Dendrophthoe falcata (DF) in mice. Swiss albino mice were treated orally with ethanolic and aqueous extracts of DF leaves (250 mg/kg and 500 mg/kg) and distilled water along with alprazolam (1 mg/kg) intraperitoneally for seven days and then exposed to Elevated Plus Maze (EPM) and Light Dark Box (LDB). The effects of the plant extracts on motor coordination were evaluated by Rota rod test. We further investigated the mechanism of action underlying the anxiolytic-like effect of the extracts by pre-treating animals with antagonist of benzodiazepine (flumazenil, 3mg/kg) prior to evaluation using EPM and LDB. Mice treated with ethanol DF extract (250 mg/kg) showed significant increase in; percentage of open arm entries, time spent in open arms of EPM and decrease in motor coordination in Rotarod test. Furthermore, the ethanol DF extracts (250 mg/kg and 500 mg/kg) significantly increased the percentage of time spent and number of transitions in the Light box in LDB test, comparable to that of the alprazolam, indicating the anxiolytic effects of the substance. The result shows that the DF extracts might dose-dependently increase chloride ion influx, which was blocked by co-administration of negative allosteric modulator, flumazenil, suggesting a GABA(A) receptor mediated mechanism of action. Taken altogether, the present study demonstrates that the ethanolic leaves extract of DF has anxiolytic activity. The extract might act at the benzodiazepine recognition site of the GABA-benzodiazepine receptor complex.

INTRODUCTION: Anxiety disorders are the most common and prevalent behavioral disorders that can result in significant impairment of function and quality of life. Currently, the benzodiazepines and the SSRIs are the most commonly employed medicinal treatments for the common clinical anxiety disorders. Benzodiazepines are potent anxiolytics but can be associated with problematic sedation, memory problems, tolerance and discontinuation symptoms.

Novel compounds however seek to target synaptic and extrasynaptic GABA receptor subtypes in order to selectively control neuronal excitability in networks involved in anxiolysis. Research in the area of herbal psychopharmacology has revealed a variety of promising medicines that may provide benefit in the treatment of general anxiety and specific anxiety disorders. Dendrophthoe falcata (L.f) Ettingsh is one of the hemiparasitic plants that belong to the Loranthaceae family of mistletoes.

The whole plant is used in indigenous system of medicine as cooling, bitter, astringent, aphrodisiac, narcotic and diuretic and is useful in treating pulmonary tuberculosis, asthma, menstrual disorders, swelling wounds, ulcers, renal and vesical calculi and vitiated conditions of kapha and pitta. Dendrophthoe falcata is reported to contain biological active substances such as...
The plant has also shown the potential anti-convulsant activity in the previous research. Therefore, in this study, we investigated the anxiolytic effects of compound *Dendrophthoe falcata* in mice using the elevated plus maze (EPM), light/dark box (LDB) test, and also the motor incoordination activity was assessed using Rota-rod test. We also examined whether its anxiolytic effects are mediated by GABA(A) receptors through co-administration of the benzodiazepine antagonist flumazenil.

**MATERIALS AND METHODS:**

**Plant Material:**
The dried leaves of *Dendrophthoe falcata* (DF) were procured from Science Market, Ambala, Haryana during the month of November 2013. The leaves were authenticated by Dr. Sunita Garg (Chief Scientist), Raw Material Herbarium and Museum, Delhi (RHMD) CSIR-NISCAIR. (Reference no: NISCAIR/RHMD/Consult/2013/2353-134).

**Drugs and Chemicals:**
All standard chemicals used in this study were of analytical grade. Ethanol, manufactured by Merck, India was used as solvent along with distilled water. Alprazolam used as standard was obtained from Asian Pharmaceutical Pvt. Ltd, Nepal and Flumazenil INJ from Usan Pharmaceuticals Pvt. Ltd, India.

**Preparation of extracts:**
Dried leaves (250 g) were reduced to coarse powder by grinding. The coarsed powder were packed in Soxhlet apparatus and continuously extracted with ethanol at 60 – 70 °C for 48 hours. Similarly 200 g of powdered leaves were soaked in 1000 ml of distilled water and macerated for 72 hours at room temperature. The mixture was stirred every 18 hours. The extract was filtered to remove solid particles using Whatman’s filter paper No 1. The solvents used were then removed by distillation and the concentrated extracts were dried under reduced pressure at a temperature not exceeding 40 °C in rotatory evaporator. The brown residue of aqueous extract obtained was 9.46 % (w/w). The extracts were kept in petri dish and stored in temperature 4°C.

**Phytochemical Screening:**
Phytochemical screening was carried out for ethanol and water soluble fractions as per the standard methods.

**Animals:**
Swiss albino mice (20-25g) of either sex used in the present study were obtained from disease free animal house of Lala Rajpat Rai University, Hisar, India. Mice were grouped and housed in the animal house at Swift School of Pharmacy, Rajpura, Punjab. The temperature of animal house was controlled at 22 ± 2°C and humidity was maintained at 55 ± 5%. Also 12/12 h light/dark (7:00-19:00 h light) schedule was maintained.

Mice were allowed free access with food and water. The access to food and water was withdrawn night before the experiments and during the experiments. Mice are acclimatized to above mentioned environment for seven days before the commencement of experiments. Animals were divided into six groups. Five animals were used in each experimental group. The experimental protocol was duly approved by Institutional Animals Ethics Committee (IAEC) and CPCSEA via Reg. no. 1616/PO/a/12/CPCSEA (Protocol no. IAEC/SSP/13/PR-001).

**Evaluation of Anxiolytic activity:**

**Elevated Plus Maze test:**
The elevated plus-maze consisted of four arms that comprised of two open arms (30 × 6 cm in mice) and two closed arms (30 × 6 cm in mice) enclosed by 20 cm high walls. Each arm had a delimited central area of 6 × 6 cm. The entire maze was elevated to a height of 50 cm above the floor. Mice were orally pre-treated with DF, vehicle or alprazolam (i.p.), 30 min before placement on the EPM. To begin a test session, mice were placed in the center of the maze facing one of the open arms. An entry into an arm was defined as the animal placing all four paws over the line marking that area. The observed parameters were (i) time spent in the open...
arms and (ii) number of entries into the open arms during the 5 min test period. The percentage of open arm entries (100 × open/total entries) was calculated for each animal.

Light dark Box test:
The apparatus (45×21×21cm) consisted of two compartments with one third painted white and two thirds painted black, and these compartments were separated by a divider with a 3.5×3.5cm opening at floor level. The white compartment was illuminated by light source. After the drug treatment, each mouse was gently placed in the corner of the white area away from the dark chambers and monitored over a 5-min observation period. The number of transfers from one compartment to the other and the time spent in the illuminated side was measured.

Evaluation of Motor coordination:
Rota rod test:
The effect on motor coordination was assessed using Rota-rod apparatus (B. D. Instrumentation, Ambala Cantt, India). The apparatus consists of four compartments with rotating rod across each compartment adjusted with a motor whose speed was maintained at 25 rotations per minute. The extracts were administered orally 60 min before testing and alprazolam (1 mg/kg i.p.) was injected 30 min before assessing the test to each mouse. The control group were given distilled water. The fall in time was recorded before and after the treatment of different extracts and the standard drug.

Acute Oral toxicity study:
The acute oral toxicity of ethanol extract of D. falcata was evaluated in mice according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD). A single high dose of 5,000 mg/kg of crude extract was administered to both three male mice and three female mice in the treatment groups by the oral route. The crude extract was suspended in a vehicle (distilled water).

Following the fasting period, body weight of the mice were determined and the dose was calculated in reference to the body weight as the volume of the extracts solution given to the mice is 10 ml/kg. Another three male mice and three female mice were allotted distilled water and were regarded as the control groups. Food was provided to the mice approximately an hour after treatment. The mice were observed in detail for any indications of toxicity effect within the first 6 hours after the treatment period, and daily further for a period of 14 days. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.

Statistical analysis:
The results were analysed via analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. The data were expressed as the mean ± S.E.M. The differences were considered statistically significant when p<0.05.

RESULTS:
Phytochemical screening:
A preliminary phytochemical screening of the extracts revealed the presence of proteins, carbohydrates, glycosides, steroids, triterpenes, flavonoids, tannins and phenolic compounds.

Assessment of Anxiolytic activity:
Elevated Plus Maze test:
A one-way ANOVA indicated significant differences among the groups in the time spent in open arms and the percentage open arm entries (p<0.01) in EPM. Treatment with ethanol extract at a dose of 250 mg/kg, resulted in a significant increase (p<0.05) as compared to control group, in the time spent by mice in the open arm of the Elevated Plus Maze.

As expected, alprazolam also showed significant increase in the time spent in open arm (Fig 1). Also doses of 500 mg of the same extract and two different doses (250 mg/kg and 500 mg/kg) of water extract showed anxiolytic activity compared to control group but it was not significant.

Percentage of open arm entries in EPM by the group treated with ethanol extract (250 mg/kg) showed higher as compared to control groups (Fig 2). Alprazolam treated group however showed the maximum number of entries. Fumazenil (3mg/kg) co-administered to all treatment groups except...
control, showed no significant differences in the time spent in open arm and the percentage open arm entries (p>0.05) among all groups tested (Fig 3 and 4).

**Light Dark Box Test:**
As shown in Fig 5 and 6, one-way ANOVA revealed significant differences among the five groups in both the time spent in the light compartment and the number of transitions between compartments (p<0.01). Compared with the control group of mice, treatment with ethanol extract of DF at doses of 250 and 500 mg/kg significantly increased the time spent in the light compartment and the number of transitions between compartments (both p< 0.05).

After the mice treated with DF extracts and alprazolam were co-administered with Flumazenil, the time spent in the light compartment and the numbers of transitions between light and dark compartments were reduced (p > 0.05) as shown in Fig 7 and 8.
increase the percent decrease in fall off time to that of normal control group (p < 0.05). Also the ethanolic extract 250 mg/kg showed significant alteration in motor coordination compared to that of normal control group of mice (p < 0.05) (Fig 9). Mice treated with aqueous extract also showed considerable alteration in motor effect compared to that with control group but the data are insignificant (p > 0.05).

Acute Oral toxicity study:
During acute toxicity studies, DFEE (5000 mg/kg, p.o.) neither produced any abnormal effect nor moribund stages. No mortality was observed for the period of 14 days among the animals.

**DISCUSSION:** The present study is based on the in vivo method of analysis of behavioural outcomes of extracts obtained from leaves of *Dendrophthoe falcata* (DF) on the central nervous system of mice focused on anxiety and motor effect. The motor coordination or the muscle relaxation is the important pharmacological actions of antianxiety agents (especially benzodiazepines)\(^{14}\). The present study is focused on the anxiolytic characteristic together with motor coordination of DF extracts using the behavioural model of anxiety; Elevated Plus Maze (EPM) and Light Dark Box (LDB), which are the fundamental and precise animal models used for the experiment.

Studies have shown that plants are source of therapeutically important phytoconstituents such as alkaloids, carbohydrates, triterpenes, phytosterols and flavonoids\(^{16}\). In the present study, extracts were prepared using two different solvents; water
and ethanol, so as to obtain more possible soluble constituents based on the polarity of a solvent. This study has shown that the ethanolic extract of DF contains more of the pharmaco-logically active secondary metabolites such as flavonoids, triterpenoids, alkaloids and phenolics.

This could be because most of these secondary metabolites being organic in nature were soluble in ethanol which is an organic (moderately polar) solvent and not in water, a more polar solvent. Hence successful extraction of phytochemical compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure.

The EPM model was chosen since it is known to be effective, economical, simple, less time consuming and required no preliminary training for mice and does not cause much discomfort to them while handling. In the present study, alprazolam showed significantly increase in the time spent in open arm and the percentage open arm entries in the Elevated Plus Maze test. This increase in the proportion of the time spent and entries into the open arms of the maze indicates the reduction of anxiety. In a highly comparable manner, ethanolic extract of DF at a dose of 250 mg/kg, significantly increased the time spent and entries into the open arms of the maze. Taken altogether, our research suggested that ethanolic extract of DF has anxiolytic properties comparable to that of alprazolam.

This anxiolytic-like activity of ethanolic DF extract in Elevated plus maze was further supported by the findings in Light dark box test. In the Light Dark Box test, animals usually try to spend more time in dark box compared to light box out of fear of exposure to the new environment. Transitions have been reported to be an index of activity exploration because of habituation over time spent in each compartment as reflection of aversion. Previous studies reported that benzodiazepines can increase the number of transfer and the time spent in light box indicating anxiolytic activity.

In our study, both aqueous and ethanol extracts showed considerable increase in time spent in the lighted box, number of crossing and the time latency, with the decrease in time spent in dark box. But the data claimed that the ethanolic extract (250 and 500 mg/kg) showed significant (p < 0.05) increase in the time spent in light area and number of transitions. Hence the results signified the ethanolic extract possessed an anxiolytic activity.

Rota rod test was first used to screen the neurotoxicity profile of anticonvulsants and later used to calculate motor dysfunction produced by centrally acting drugs to determine possible alterations in the motor coordination ability of the animal, based on the assumption that an animal with normal motor efficiency is able to maintain its equilibrium on a rotating rod.

In this test, the difference in the fall of time from the rotating rod between the control and standard (alprazolam) treated animal was taken as an index of muscle relaxation. Our observations, gave a conclusion that the mean time duration on rotating rod was significantly decreased (p < 0.05) in animals treated with ethanol extract of DF at a dose 250 mg/kg as compared to control group, indicating muscle relaxant activity and decline in motor coordination. Hence the muscle relaxant property of benzodiazepine class of drugs can also be acquired by *Dendrophthoe falcata* at a same dose that produce anxiolytic effect.

During the acute toxicity studies, ethanolic extract of *Dendrophthoe falcata* at dose (5000 mg/kg) orally, neither produced any abnormal effect nor moribund stages. Moreover, no death was observed for next 14 days among the experimented animals. The extract was found out to be safe with no toxicity at provided dose.

Previous research showed that the phytochemicals present in plants like flavonoids, terpenes are responsible for the anxiolytic activity. In this study phytochemical screening of the plant showed the presence of psychoactive chemicals such as flavonoids, triterpenes and phytosterols in the leaves. Hence further research on the comprehensive study of structure and activity of above mentioned phytoconstituents may be performed in order to investigate the possible compound or specific functional group responsible for anxiolytic and other neurobehavioural activities.
Results show that the anxiolytic effects of DF are dose-dependent. The plant extract is effective at lower dose (250 mg/kg) than at higher dose (500 mg/kg). Due to the limitations of the present study we cannot fully explain the exact reason behind this result and that we can only infer based on previous studies. Some studies on the anxiolytic effects of plant extracts, reported somewhat similar results (less effects on higher doses). This may attributed to the complex pharmacokinetic and pharmacodynamic system of plant extracts with the different organ system of the animal$^{24,25}$.

Plant extracts are composed of various components mostly with unknown pharmacological and dose-response data. It is possible that with higher dose complex interactions occur (probably between DF and other body systems), which then altered the anxiolytic effects of the extract. It would be beneficial to investigate the individual effect of the extract in order to elucidate the agents responsible for its anxiolytic effects. However, the present study is limited, thus further studies are needed to adequately address these issues. Nevertheless, the present results can be helpful for therapeutic decisions and with respect to toxicology.

The mechanism underlying the anti-anxiety like effect was accessed by pre-treating animals with flumazenil (Bzd antagonist) intraperitoneally to mice 30 min before testing in EPM and LDB$^{26}$. We observed that the anxiolytic-like effects of compound were significantly reduced by pre-treatment with flumazenil. These results further support the hypothesis that the anxiolytic activity of compound present in DF extract is mediated via GABA-benzodiazepine receptors. Further study in the anxioreceptive mechanism of GABA(A) subunits is needed to explore the exact mechanism of action.

**CONCLUSION:** Hence the study reveals, the ethanol extract of *Dendrophthoe falcata* leaves possesses the anxiolytic effect possibly mediated via GABA-benzodiazepine receptors. The phytoconstituents like alkaloids, triterpenes and flavonoids perhaps contains the active components responsible for the activity. This study could be base for the further study on efficacy and safety of plant in neuropharmacology.

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