Original Article

Anxiolytic Potential of Chloroform Extract of *Ziziphus mauritiana* Lam. Leaves in Mice

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ABSTRACT

Introduction: With a tremendous spike in the cases of anxiety in recent years, scientists are looking for herbal drug alternatives for its treatment as all the conventional medication options available in the market have side effects and lead to dependence. Ethnopharmacological reports revealed the usefulness of *Ziziphus mauritiana* Lam. for anxiety and depression. Therefore, it was envisaged to explore this plant's possible anxiolytic activity. **Objectives:** The purpose of the present study was to evaluate the anxiolytic activity of *Z. mauritiana* leaves based on its ethno pharmacological relevance. **Materials and Methods:** Anxiolytic activity of the chloroform leaf extract of this plant was determined with the use of animal models elevated plus maze, mirrored chamber and light/dark box test. The chloroform extract was given orally to mice at 200 and 400 mg/kg and number of entries and time spent in the animal models was observed. The extract was then subjected to PTLC and a pentacyclic triterpenoid (RJ11) was isolated which was then characterized using spectroscopic techniques. Molecular docking studies of isolated compound RJ11 was done on $GABA_A$ receptors. **Results:** The chloroform extract showed significant anxiolytic effect in mirrored chamber, elevated plus maze and light/dark models at the dose of 400mg/kg. Molecular docking studies of isolated compound RJ11 on $GABA_A$ receptors revealed good binding affinity and interactions with central benzodiazepine binding site on $GABA_A$ that may be responsible for the anxiolytic effect of this plant. **Conclusion:** The chloroform extract of leaves of *Z. mauritiana* Lam. possess anxiolytic effect which may be attributed to binding affinity of pentacyclic triterpenoid present in this plant with $GABA_A$ receptors on its benzodiazepines receptor site.

Keywords: *Ziziphus mauritiana* Lam., Chloroform extract, Anxiolytic, Triterpenoid, Molecular docking study.

INTRODUCTION

Anxiety can be described as an emotion or feeling of intimidation, panic and discomfort.[1] According to a recent report by WHO, it was observed that one out of 13 people around the globe are suffering from anxiety making it the most common mental disorder and especially specific phobia, major depressive disorder and social phobia being the most prevalent.[2] Moreover, according to Anxiety and Depression Association of America (ADAA), around 75 % individuals in developing countries, diagnosed with a mental disorder are left untreated which eventually results in almost 1 million people taking their lives per annum.[3] To curb this massive issue, plethora of steps are being taken to prevent and treat the incidence of anxiety. Several effective medications are now available in the market which can help a person suffering from this disorder and among them Benzodiazepines (BDZ) or tranquilizers are the most extensively prescribed drugs.[4] BDZ are good option for relaxing the muscles and calming the mind in an overwhelming situation but they are addictive and has various side effects like dizziness, depression, problem in vision, headache and if stopped abruptly can also cause withdrawal symptoms.[5] Alternate therapies are therefore being sought and as a consequence of which scientists have developed a great interest in medicinal

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plants.[6] For the purpose of anxiety, herbal medicines in the form of aromatic oils are essentially used, but its high cost, low availability and tedious administration makes it hard for usage whereas herbal extracts can be a better option due to their ample availability and easy administration.[7] *Ziziphus mauritiana* Lam, a tropical fruit tree species which has a potential for treating sexual weakness, obesity, ulcers, cough, wound, diarrhea, burning sensation, stomatitis, skin diseases, insomnia, fever, and general debility.[8] An extensive range of biological activities i.e. anti-depressant, acetyl cholinesterase inhibition, anxiolytic and sedative have been revealed in the extract or isolated ingredients of the plants from this Ziziphus genus in the literature related to modern pharmacology.[9] On the basis of ethnopharmacological records, present study was designed to investigate the anxiolytic effects of the leaves of this plant.

MATERIALS AND METHODS

Plant Material

Ziziphus mauritiana Lam. fresh leaves were brought from a horticulture garden located in Hanumangarh, Rajasthan and the plant's taxonomic identification was validated by Guru Nanak Dev University's Department of Botanical and Environmental Sciences, Amritsar. In the Department's herbarium, a voucher specimen with the number S.R.BotSci/1139 has been deposited.

Preparation of Extracts

Fresh leaves of *Ziziphus mauritiana* Lam. were dried in shade and coarsely powdered. Powdered leaves were subjected to soxhlet extraction with solvents of increasing polarity, including petroleum ether (60-80°C), chloroform, and methanol, for at least 48 hrs. The powder was air-dried and weighed again before each extraction. Each extract was concentrated by distilling off the solvent using rota evaporater and then evaporated to dryness on the water-bath. Extracts were weighed and the percentage was calculated in terms of the air-dried weight of the plant material.[10]

Phytochemical Screening

The various extracts of *Z. mauritiana* Lam. were subjected to qualitative chemical evaluation.[11]

Animals

Swiss albino mice (25-30 gm) were obtained from the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. The animals were given standard laboratory feed and water. All animals used in the study were naïve to the elevated plus-maze, light/dark paradigm, and mirror chamber tests. The experiments were accomplished in a semi soundproof laboratory. The biological studies were succeeded according to the guidelines of the Institutional Animal Ethics Committee. (Protocol Number: IAEC/ GNDU/ 2013/ 20).

Animal Models used for Anti-anxiety Activity

To evaluate the anxiolytic effect of the chloroform extract, mirror chamber, light/dark box and elevated plus-maze (EPM) animal models were used[12-13] and the experimental protocol is given in Figure 1.

Statistics

The data obtained from different groups were statistically analysed using one way analysis of variance followed by Tukey's test. Each experiment was performed in triplicate. Results were expressed as Mean ± SEM. $p \leq 0.05$ was considered to be statistically significant.

Isolation and Characterization of the Compound

Chloroform extract was dissolved in chloroform for loading on PTLC plates, and placed in a PTLC chamber with hexane and ethyl acetate as the solvent system (70: 30). Three bands were observed on the PTLC plate which were scraped off, then dissolved in chloroform and filtered. The pure compound isolated by PTLC was then confirmed by spectroscopic analysis and chemical tests. Isolated compound was characterized by spectroscopic techniques (^{1}H & Mass). ^{1}H NMR Spectra was recorded on 500 MHz, Bruker Avance II 400 NMR Spectrophotometer SAIF, Panjab University Chandigarh. Chemical shifts in ^{1}H NMR were reported in with the number of protons, multiplet (d-doublet, t-triplet, dd-double doublet), and coupling constants (J) in Hz (Hertz) in the solvent specified, using tetramethylsilane as an internal standard. HRMS was

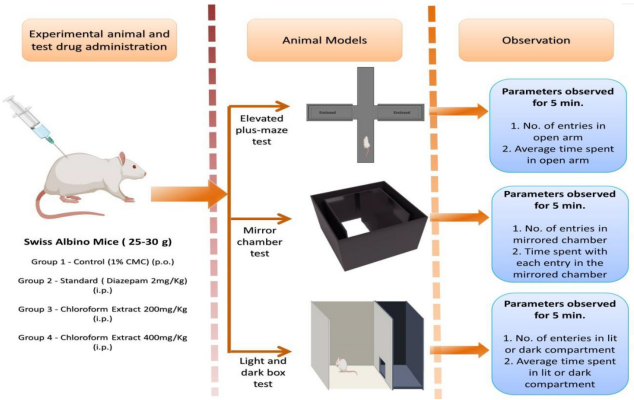

Figure 1: Experimental protocol for evaluation of anxiolytic activity of *Z. mauritiana* in EPM, mirror chamber and light and dark models.

recorded on micrOTOF-Qii Bruker Daltonik LC–MS/MS High Resolution Mass Spectrometer.

Molecular Docking Studies

Molecular docking studies were performed to explore the interactions of RJ11 with $GABA_A$ receptor. For that cause CryoEM structure of $GABA_A$ receptor in complex with diazepam (PDB entry: 6HUP; resolution, 3.58 Å), was employed.[14] ChemDraw Ultra (2013) was used to create the RJ11 structure, and the MM2 force field in Chem3D Ultra software was used to reduce energy.[15] Docking investigations were carried out using AutoDock Tools 1.5.6 (MGLTools). To acquire accurate ionization and toutomeric states of residues, the protein structure was prepared. Initially, all heteroatoms were removed, including water, and polar hydrogens were added. Following that, Kollman charges and solvation parameters were assigned, as well as Gasteiger charges. The grid box was designed to include all of the essential residues that interact with co-crystallized diazepam. The energy scoring grid was prepared with coordinates values of x = 119.6639, y = 151.7378 and z = 114.3583. Parameters of Lamarckian genetic algorithm followed during docking process were: 100 runs, elitism = 1, mutation rate = 0.02, population size = 150, crossover rate = 0.80, root mean square cluster tolerance = 2.0 Å, 27,000 generations and energy evaluations of 25,000,000 for each run. Accuracy of docking protocol was validated through docking co-crystalized diazepam in its binding domain. The program was capable to dock reference ligand diazepam (which was completely overlapped the co-crystalized diazepam) on its site, suggesting the reliability of protocol. RJ11 was docked into the binding domain of diazepam. Top best pose emerged from docking study with dock score of -9.5 Kj/mol was selected for discussion. Outputs were analyzed using Discovery Studio Visualizer 2021.[16-17]

RESULTS

The test solution, standard drug and vehicle were administered 45 min before the test and behavior of the animals was observed for 5 min in elevated plus-maze, light/dark box and mirror chamber. The effect of Chloroform extract (200 and 400 mg/kg respectively) on the elevated plus-maze test was significant ($p < 0.05$), as compared to control but less effective than the standard drug diazepam (Figure 2). The present data showed that chloroform extract (200 and 400 mg/kg) increased the time spent in the light area as compared to control, suggesting that the extract possess anxiolytic properties (Figure 3). Similar results were observed in mirrored chamber model where there was significant

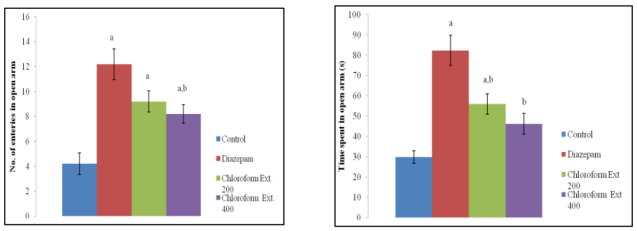

Figure 2: Anti- anxiety activity of chloroform extract of leaves of *Z. mauritiana* in EPM. A: Number of entries in open arm(s), B: Time spent in open arm(s). Values were expressed as Mean ± SEM, *n*=6. Data was analyzed by one-way ANOVA followed by Tukey's test a= *p*< 0.05 vs control; b= *p*<0.05 vs standard.

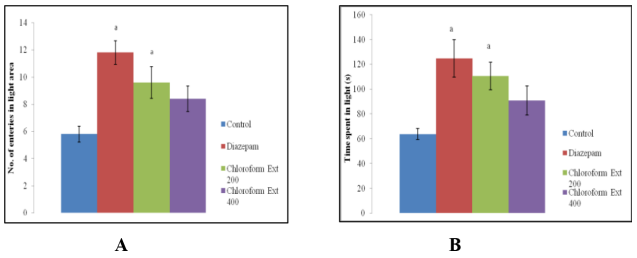

Figure 3: Anti-anxiety activity of chloroform extract of leaves of *Z. mauritiana* in light/dark box text. A: Number of entries in light area, B: Time spent in light area. Values were expressed as Mean ± SEM, *n*=6. Data was analyzed by one-way ANOVA followed by Tukey's test a= *p*< 0.05 vs control; b= *p*<0.05 vs standard.

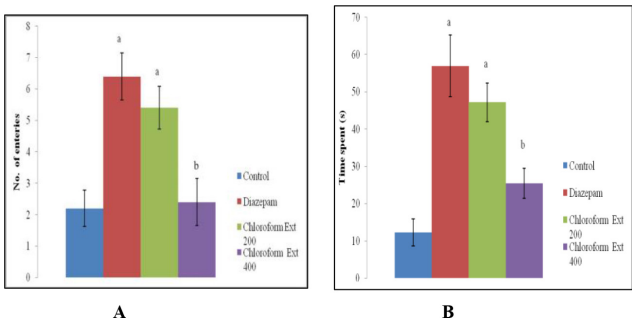

Figure 4: Anti-anxiety activity of chloroform extract of leaves of *Z. mauritiana* in mirrored chamber. A: Number of entries in mirror chamber, B: Time spent in mirror chamber. Values were expressed as Mean ± SEM, *n*=6. Data was analyzed by one-way ANOVA followed by Tukey's test a= *p*< 0.05 vs control; b= *p*<0.05 vs standard

increase in number of entries and time spent in mirror chamber by the chloroform extract (Figure 4).

Isolation of Compound

From Chloroform Extract, a RJ11 phytoconstituent was isolated which was identified by spectroscopic analysis coupling with chemical tests. The isolated compound showed single spot-on TLC plate. The compound was of yellow color and its structure is shown in Figure

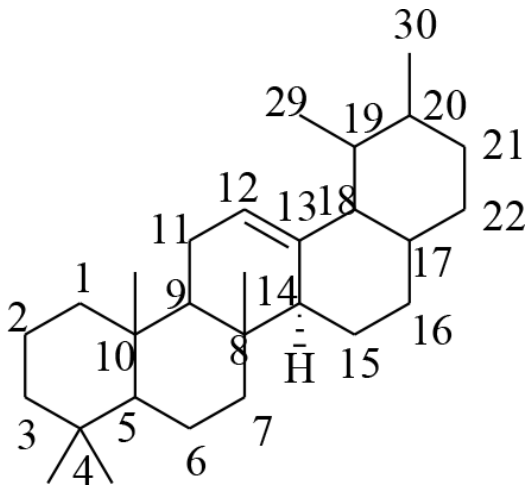

Figure 5: 2D representation of RJ11.

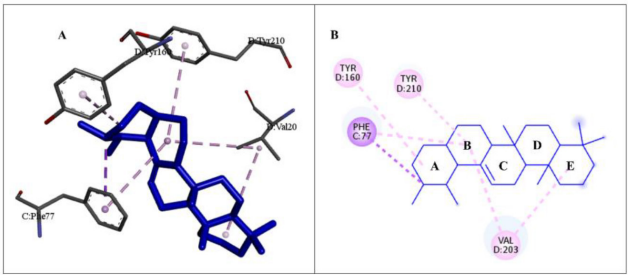

Figure 6: Molecular docking of RJ11 with $GABA_A$ receptor: A. 3D view of RJ11interactions with $GABA_A$ receptor; B. 2D view of RJ11interactions with $GABA_A$ receptor.

5. The structure of the RJ11 was elucidated by ^{1}H NMR and Mass spectroscopy. The spectral data was in agreement with those reported in literature:[17a]

^{1}H NMR (500 MHz, CDCl3): 5.27-5.29 (^{1}H, m), 2.10-2.04 (3H, m), 1.56-1.39 (22H, m), 1.42-0.95 (20H, m). HRMS-MS M^+ *m/z*: 382. 2371.

Docking Study

The overall binding mode of RJ11 with residues of the binding site of diazepam in $GABA_A$ receptor suggests that RJ11 is well positioned in the cavity and is stabilized by various electrostatic interactions. Major interactions of RJ11 with $GABA_A$ receptor rinclude π−σ, π−alkyl and hydrophobic alkyl interaction. Val203 is found to make hydrophobic alkyl interactions with RJ11. Ring A and B of RJ11 are sandwiched between Tyr210 and Phe77 through π−alkyl interactions with both residues. π−σ interaction has been observed between ring A and Phe77. Tyr160 is also forming π−alkyl interaction with Ring A of RJ11 as shown in Figure 6. The overall interactions suggest that RJ11 is sufficiently loaded with small, rigid, and planar groups representing optimum scaffold capable for $GABA_A$ receptor.

DISCUSSION

Different kinds of human diseases are increasing day by day due to hectic daily schedule and bad food habits. Anxiety is becoming a major disease as a consequence of mental stress exerted by daily busy life. Various treatments have been reported in medical science for curing anxiety such as Allopathy, Ayurvada and Homeopathy.[18] People now a days prefer herbal drugs compared to conventional drugs as these are considered to be safe, inexpensive and have relatively less adverse effects. A variety of medicinal plants have been exposed to extensive chemical analysis, resulting in the extraction of pure bioactive compounds that have been pharmacologically assessed for anxiolytic activity.[19] The present study deals with the evaluation of anti-anxiety effect of the leaves of *Z. mauritiana*. The plant was evaluated with respect to the anxiolytic effect produced by oral administration of Chloroform extract. The anti-anxiety activity of chloroform extract of the plant at a dose of 200 mg and 400 mg was evaluated employing widely used models, elevated plus-maze, and light/dark box test and mirror chamber. These models were chosen as they are effective, cheap, and simple, less time consuming, requires no preliminary training to the mice and does not cause much discomfort to the animals while handling. Extracts separately suspended in CMC vehicle, were administered orally to mice and the activity was compared with that observed in the control group as well as with the group treated with the standard anxiolytic drug diazepam.

Significant increase over control in the, number of entries and time spent in open arm in the elevated plus maze, time spent in the light area in light/dark box test and increase in the number of entries and time spent in the mirrored chamber clearly indicates anxiolytic effect of the chloroform extract in the present study.

A bioactive phytoconstituent was further isolated from the chloroform extract by preparative thin layer chromatography and its characterization was done by ^{1}HNMR, Mass spectroscopy which confirmed the presence of triterpenoids. Docking study suggested that the compound RJ11 is well positioned in the benzodiazepine site of $GABA_A$ receptor and is stabilized by various electrostatic interactions. The overall interactions suggest that RJ11 is sufficiently loaded with small, rigid, and planar groups representing optimum scaffold capable to modulate $GABA_A$ receptor. Terpenoids are one of the largest classes of secondary metabolites which are majorly found in plants which are known to possess anti-inflammatory, antiplasmodial, astringent, diuretic, anticancer, digestive,

antioxidant and other medicinal properties.[20-21] Earlier studies suggest that the anti-anxiety and sedative effect might be due to presence of triterpenoid with possible mechanism of facilitating inhibitory action of GABA neurotransmitter.[22-24] Pentacyclic triterpenoids like, asiatic acid, oleanane, ursane and alpha-amyrin exibit pharmacological activity against neurodegenerative disorder and are known to possess neuroprotective activity.[25-28] The oral and intraperitoneal administration of betulinic acid (pentacyclic triterpenoid) has been reported to produce anti-anxiety activity in animals. A pharmaceutical formulation containing betulinic acid has also been patented as a therapy or preventative for anxiety.[29]

The phytoconstituent in the chloroform leaf extract of *Z. mauritiana* was identified as pentacyclic triterpenoid and docking studies showed that this triterpenoid exerts its anxiolytic effect by facilitating inhibitory action of GABA neurotransmitter.

CONCLUSION

The study explored the anxiolytic effect of the chloroform leaf extract of *Z. mauritiana* and the results justified the ethno-medicinal claim of its anti-anxiety effect. From the results and the literature, it can be concluded that pentacyclic triterpenoid phytoconstituent might be responsible for the anxiolytic effect of this extract but further evaluation is required to study its mechanism of action.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PTLC: Preparative Thin Layer Chromatography; **GABA:** Gama Amino Butyric Acid; **WHO:** World Health Organization; **ADAA:** Anxiety and Depression Association of America; **BDZ:** Benzodiazepines; **EPM:** Elevated Plus Maze; **ANOVA:** Analysis of variance; **NMR:** Nuclear Magnetic Resonance; **DMSO:** Dimethyl Sulfoxide; **LC-MS:** Liquid chromatography–mass spectrometry; **PDB:** Protein Data Bank; **TLC:** Thin Layer Chromatography; **CMC:** Carboxy Methyl Cellulose.

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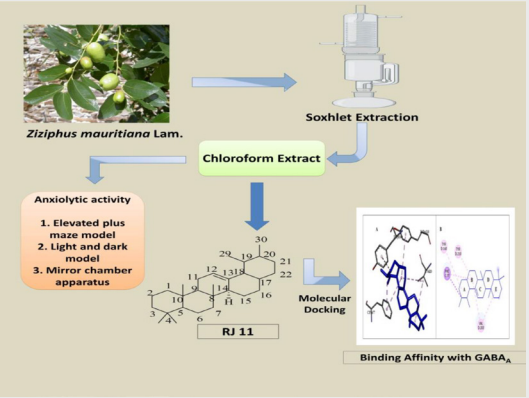
PICTORIAL ABSTRACT



SUMMARY

- People nowadays prefer herbal medications over conventional pharmaceuticals since they are seen to be safer, less costly, and have less side effects.
- A wide range of medicinal plants have been subjected to rigorous chemical investigation, yielding pure bioactive substances that have been pharmacologically evaluated for anxiolytic effect. The aim of this study was to determine the anti-anxiety effect of leaves of *Z. mauritiana*.
- The anti-anxiety effect of the plant's chloroform extract at doses of 200 and 400 mg/kg was assessed using elevated plus-maze, light/dark box test, and mirror chamber models.
- Significant increase in the number of entries and time spent in open arm in the elevated plus maze, time spent in the light area in light/dark box test and increase in the number of entries and time spent in the mirrored chamber clearly indicates anxiolytic effect of the chloroform extract in the present study.
- It can be concluded from this study that chloroform extract of leaves of *Z. mauritiana* Lam. Possess anxiolytic effect which may be attributed to binding affinity of pentacyclic triterpenoid present in this plant with $GABA_A$ receptors on its benzodiazepine's receptor site.

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