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In-vitro and *in-vivo* Anxiolytic studies of *Passiflora alata*

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ABSTRACT
Passiflora alata, is increasingly recognized for its potential therapeutic applications, especially in the treatment of mood disorders. However, comprehensive studies evaluating its safety and anxiolytic efficacy are limited. This work aims to examine *P. alata* for anxiolysis using *in-vitro* and *in-vivo* assays. To determine the safety, acute toxicity tests were carried out at a dosage of 2,000 mg/kg body weight. The GABA receptor binding assay was used *in-vitro* to test *P. alata* leaf methanol extract (PLM) binding affinity. The Rotarod test was used to measure motor coordination, and the Open Field and Elevated Plus Maze (EPM) tests were used to determine the *in-vivo* anxiolytic efficacy. Acute toxicity testing revealed no harmful effects, indicating its safety at the studied dosage. Tests conducted *in vitro* demonstrated PLM's strong affinity for GABA receptors. PLM therapy reduced anxious behaviours in *in-vivo* tests, while the EPM test revealed a dose-dependent increase in anxiolytic effects. PLM at 400 mg/kg dosage showed notable anxiolytic effects. The Rotarod test findings, however, did not show any gains in motor coordination, pointing to a different mode of action than Diazepam. In conclusion, *P. alata* has promised anxiolytic activity, and the PLM extract, in particular, is safe at the dosages studied. The outcomes point to its potential as a complementary or substitute therapy for the treatment of anxiety. More research is required to understand its therapeutic processes and long-term efficacy fully.
Keywords: *Passiflora alata*, Anxiolytic, GABA, Elevated plus maze, Open field test

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INTRODUCTION

One of the most common mood disorders associated with stress is anxiety disorders, which are known to increase early mortality and disability greatly[1]. Over 20% of individuals will at some point in their lives, have one of these disorders, which are made worse by the complexity of modern life and result in varied degrees of anxiety. Notably, patients in developing countries have shown an association between anxiety disorders and chronic pain [2].

In the past, the main pharmacological intervention for acute anxiety episodes was benzodiazepines. Studies show that the GABAergic and serotoninergic systems are involved in anxiety [3]. Treatment for anxiety disorders has also demonstrated efficacy for selective serotonin reuptake inhibitors (SSRIs). Nevertheless, benzodiazepines have benefits, but their therapeutic utility is limited by side effects such as drowsiness, memory problems, muscle relaxation, and drug interactions. As a result, there is a constant need for novel, more potent, and better-tolerated medicines [4].

Using herbal remedies as potential complementary or alternative medicines is becoming more widely acknowledged. Research on medicinal plants has progressed worldwide, showcasing their pharmacological efficacy in various animal models for neurological conditions [5]. As a result, numerous herbal remedies have been the subject of extensive animal research to assess their potential for psychotherapy. These studies greatly benefit the development of innovative pharmacotherapies based on therapeutic plants and isolated active phytochemicals [6-7].

Passiflora alata is an evergreen plant that is native to Brazil and is an important vine with several medicinal applications. Some traditional healers use it for digestive and nerve-related problems. it is famous for its hypnotic and sedative properties. Phytochemicals include quadranguloside and oleanolic acid-3-sophoroside, β-carboline alkaloids like harmane and harmine, and steroids like β-sitosterol [8-11]. Pharmacologically *P. alata* proved to have immunomodulatory [12], antidiabetic [13], antitumor [14], antioxidant [15], and gastroprotective [16] activities.

With its complex phytochemical profile and established pharmacological activity, this study aims to investigate *P. alata* for its anxiolytic activity. It is in line with the larger quest for safer, more potent remedies for anxiety disorders, especially those made with natural ingredients.

MATERIAL AND METHODS

Acute toxicity studies

The acute oral toxicity study was performed to determine the therapeutic index following OECD revised draft guidelines 423 by CPCSEA, India. Female Wistar rats fasted overnight and were administered test extracts orally in 5, 50, 300, and 2000 mg/kg doses. They were observed for four hours for behavioral, autonomic, and neurological symptoms and mortality, with body weights recorded. Behavioral changes, toxic symptoms, and mortality were monitored weekly, with body weights documented on the 7^{th} and 14^{th} days. As no lethality was observed, $1/5^{th}$ and $1/10^{th}$ of the highest dose were selected as therapeutic doses. The rats were observed for 14 days for long-term effects, and fatalities were noted if any [17].

In-vitro GABA receptor binding assay

Preparation of brain homogenate

Animals were sacrificed by decapitation to harvest cells needed for the *in-vitro* experiments. Rat brain was homogenized at 4 °C in 10 ml 10 mM Tris-HCl pH 7.4, 1 mM EDTA, and centrifuged for 15 min at 25 000 × g. The pellet was finally resuspended in 50 mM Tris-HCl pH 7.4, 4 mM $MgCl_2$ and 1 mM EDTA, frozen in liquid nitrogen, and stored at −80 °C until usage.

Estimation of total protein content

BCA assay kit was used to estimate the total protein content in the brain homogenate. The working solution was prepared by blending the 50 parts of reagent-1 to one part of reagent-2 and stored in the refrigerator for one day. The standard solution was prepared by dissolving the 1mg of the reagent 3 with 1ml of reagent 4 and stored at -20^0C.

From these standard solutions, 1mg/ml of BCA standard solution was diluted with saline to prepare various concentrations viz., 1, 2.5, 5, 7.5, 10, 15, 20µg/ml.

GABA receptor binding assay

The samples were transferred to GF/C filter plates and pre-soaked with 0.1% polyethyleneimine to end the test. After four ice-cold 200 µl Tris-HCl cycles at 50 mM pH 7.4, filter-bound radioactivity was measured using a Microplate Reader (Microbeta). The data show specific ligand binding to the receptor for the tested substances. Using GraphPad Prism, nonlinear regression analysis of the competitive curves was used to determine the IC_{50}, $B_{max,}$ and K_d values [18].

Elevated plus Maze (EPM) Test:

The Elevated Plus-maze is a well-established animal model and is currently the first-choice test for anxiolytic drugs. It has been validated for rats. It is based on the rat's inherent tension between a desire to discover a new environment and the propensity to stay away from a potentially risky place. Incorporating a variety of ethological characteristics has been suggested to improve this paradigm's usefulness more recently. In this study, we assessed the anxiolytic effects of *P. alata* leaf methanol extract (PLM) using the EPM model of anxiety [19].

The model most commonly used to evaluate the anxiolytic potential of new drugs is the EPM test. Two perpendicular open arms measuring 50 x 10 cm and two perpendicular enclosed arms measuring 50 x 10 x 40 cm were present in the elevated plus maze. The maze is 50 cm above the ground and made entirely of wood. The maze was situated in a room with sound attenuation and low light levels (25 lx). After thirty minutes, the rat was positioned on the maze's central platform facing the enclosed arm and watched for ten minutes. The time spent in open and enclosed arms and the number of entries into open and enclosed arms were used to evaluate the parameters. Every safety measure was implemented to guarantee the rat wouldn't experience any distress from outside sources. To prevent olfactory cues from influencing the subsequent rat, the maze was cleaned with wet tissue paper soaked in a 70% ethanol solution after every test [20].

All the animals were divided into three groups of five rats each.

Group I: Standard diazepam (1mg/kg per oral)

Group II: (PLM-200 mg/kg, per oral)

Group III: (PLM-400 mg/kg, per oral)

Open Field Test:

The study was carried out with minor adjustments using the methodology that Brown *et al*. had previously published. The device consists of plywood with dimensions of 72 x 72 x 36 cm. Transparent Perspex glass is used for one of the walls to guarantee that the rat being studied is visible to the viewer. Using a blue marker, the cardboard floor was divided into sixteen equal squares measuring 18×18cm. A black marker

was used to draw the central square. Transparent Plexiglas covered the cardboard. The animals were divided into three groups (Groups I -III); each group comprised five rats and treated with Diazepam (1 mg/kg body weight, p.o.), PLM (200 mg/kg body weight, p.o.), and PLM (400 mg/kg body weight, p.o.), respectively. After thirty minutes, each rat was put in its corner of the arena, and the behavior of each was observed for five minutes. Each rat's number of rearings and the number of squares it crossed were noted. To remove any odour cue, the instrument was cleaned with 70% ethyl alcohol between observations and allowed to dry. The area the animal explored was used to measure its level of locomotor activity [21].

Muscle relaxant activity using rota rod:

Rotarod apparatus was used to evaluate motor coordination produced by drugs in animals. Prior to the experiment, the rats were trained to become adept at staying on a diameter rod that rotated at 20 rpm for 300 s. The animals just needed two or three tries to pick up this skill. Rats were divided into three groups; each group comprised five rats. Different groups were treated with Diazepam (1 mg/kg, body weight, p.o.), PLM (200 mg/kg body weight, p.o.), and PLM (400 mg/kg body weight, p.o.), respectively. Then, the animals were placed in the four paws on the rotating bar, which is 2.5 cm in diameter and 25 cm high from the floor. The animals were observed for five minutes. The difference between the fall-off time of the rat before and after treatment was considered as an index of muscle relaxation. After administering the standard or test drug, the performance time was measured at 15 min intervals for 90 min (15, 30, 45, 60,75, and 90 minutes). Parameters: fall-off time was measured [22].

Statical analysis

The results of the experiments and observations were expressed as mean ± standard error mean (SEM). The statistical analysis was performed with GraphPad Prism version 9.0 software using appropriate tools to understand the significance.

RESULTS

Acute Toxicity Studies

Acute toxicity studies revealed *P. alata*'s safety, showing no toxicity symptoms at 2,000 mg/kg body weight for 14 days of observation. The 1/5th dose, i.e., 400 mg/kg body weight, is selected as the therapeutic dose for future *in-vivo* studies (Table 1).

Table 1. Acute toxicity response on body weight

Sample	Body Weights									Signs of toxicity	No. of deaths
	PSM			PLM			PLE				
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14		
Control	218.5± 2.71	219.8± 2.56	230.1± 2.91	219.5± 2.71	229.4± 2.56	230.1± 1.91	228.5± 1.71	239.8± 2.56	240.1± 1.91	None	0
5mg/kg	219 ± 2.74	217.8± 2.89	238.6± 2.43	212.8± 2.43	223.5± 2.60	236.5± 2.01	223.6± 1.1	233.1± 2.70	246± 2.84	None	0
50mg/kg	220.8± 2.09	222.3± 11.47	239.5± 2.56	229± 2.89	236.12± 3.70	235.66± 3.7	227.8± 1.0	235± 2.30	247.3± 2.71	None	0
300mg/kg	215.1± 1.31	223± 11.7	241± 2.6	211.5± 3.05	229.6± 3.16	240.66± 3.93	222± 1.44	239.8± 2.98	259.3± 2.73	None	0
2000mg/kg	215.6± 1.03	227.3± 2.51	247.6± 2.31	218± 2.71	227.8± 2.95	249± 2.44	229.8± 2.75	238.5± 2.59	251.8± 2.54	None	0

In-vitro GABA receptor binding assay

In the *in-vitro* GABA receptor binding assay, test compounds' affinity to GABA receptors is assessed by comparing their binding levels to those of a known anxiolytics like Diazepam. *P. alata* extracts were tested at various concentrations (1-20µg/ml) using rat brain homogenate with a protein concentration of 8.24mg/ml. The leaf methanol extract (PLM) showed high binding activity (27.97µg/ml), close to Diazepam (22.7µg/ml), while the leaf ethyl acetate extract (PLE) also showed significant binding (31.78µg/ml). The stem methanol extract (PSM) had the lowest capacity (37.69µg/ml), indicating leaves are more effective than stems for anxiolytic properties. This suggests the GABAA receptor's GABA site is the action mode for *P. alata*'s phytochemicals. PLM was chosen for further *in-vivo* studies at 200 & 400 mg/body weight.

B_{max} denotes a receptor's maximal ability to bind a ligand. From the results, PLM had the highest receptor binding capacity, followed by PLE and PSM, with B_{max} values for PLE, PLM, and PSM being 0.49, 0.53, and 0.42 respectively. The dissociation constant (Kd) measures the tightness of ligand-receptor binding, where

a lower value denotes tighter binding. PLM had the highest ligand affinity, followed by PLE, and PSM had the lowest, according to the K_d values of 15.95 for PLE, 14.94 for PLM, and 18.19 for PSM. PLM displayed greater binding capacity and affinity than the other samples, while PSM had the lowest B_{max} and K_d values. Diazepam, on the other hand, had the lowest K_d, indicating the greatest binding.

Table 2. Analysis of Ligand Bound Protein

Treatment	IC_{50}
Diazepam	22.7µg/ml
PLE	31.78µg/ml
PLM	27.97µg/ml
PSM	37.69µg/ml

Table 3. Analysis of B_{max} and K_d

	B_{max}	K_d
Standard (Diazepam)	0.63±0.12	12.65±1.32
PLE	0.49±0.54	15.95±1.22
PLM	0.53±0.63	14.94±1.47
PSM	0.42±0.72	18.19±1.33

Open field test

The open field test examines the behaviour in an open arena to assess its emotional condition. Lesser exploration and a preference for walls or corners indicate increased anxiety, whereas higher activity levels and exploration indicate lesser anxiety. This test aids in evaluating how our treatments affect the emotions of animals. Animals in the study received 200 mg/kg, 400 mg/kg, of PLM and 1 mg/kg of Diazepam, and their movements and rearings were observed. The use of Diazepam greatly boosted number of rearings and crossings. PLM produced 35.6±0.5 crossings and 15.2±0.58 rearings at 400 mg/kg, whereas at 200 mg/kg, it produced 28.4±0.75 crossings and 12.6±0.4 rearings. The medication with the highest activity was Diazepam, with 18.2±0.73 rearings and 56.2±0.58 crossings. The time spent and behavioural changes in the open field showed the anxiolytic ability of *P. alata*, whereas the oral administration of these extracts decreased aversion to the field and encouraged exploration. (Figure 1).

Table 4a. Effect of *P. alata* on the number of crossings in open field test

Treatment	No. of crossings	
	Before treatment	After treatment
Group I: Standard diazepam (1mg/kg per oral)	43.8±0.73	56.2±0.58**
Group II: (PLM-200 mg/kg, p.o)	45.11±0.76	28.4±0.75***
Group III: (PLM-400 mg/kg, p.o)	42.49±0.71	35.6±0.5*

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001, **P<0.01, *P<0.05

Table 4b. Effect of *P. alata* on the number of rearings in open field test

Treatment	No. of rearings	
	Before treatment	After treatment
Group I: Standard diazepam (1mg/kg per oral)	22±0.7	18.2±0.73**
Group II: (PLM-200 mg/kg, p.o)	21.34±0.69	12.6±0.4***
Group III: (PLM-400 mg/kg, p.o)	22.66±0.73	15.2±0.58***

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001, **P<0.01, *P<0.05

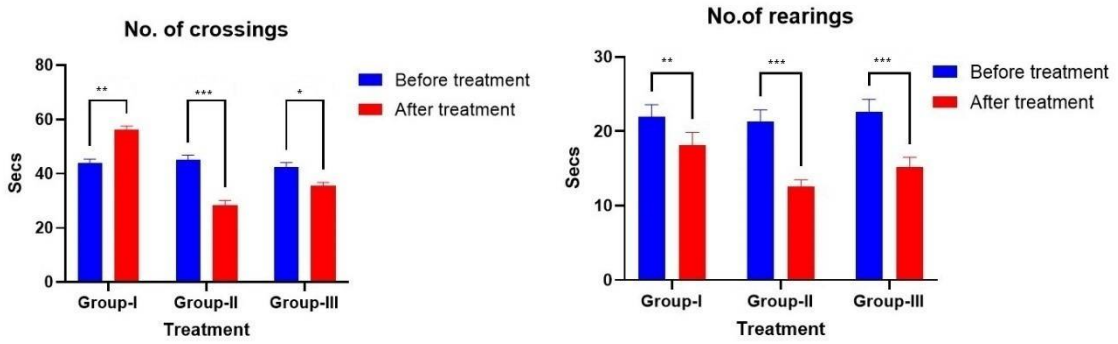


Figure 1. Effect of *P. alata* on the number of crossings and rearings in open field test

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001, **P<0.01, *P<0.05

Elevated Plus Maze

The EPM test assesses unconditioned anxiety in animals by observing behavior on a platform. Animals with high anxiety typically spend more time in the closed arms and less in the open arms, while those with low anxiety do the opposite [23]. The animals treated with standard Diazepam have increased the number of entries in the open arms and with more spent time. Before treatment animals have spent 46.2±0.47sec in open arms and produced a 10.6±0.46 entries. They spent the majority of the time in closed arms (230.09±0.56sec). The PLM-treated groups exhibited an anxiolytic effect, explored the open arm with 14.6±0.36 ($p<0.05$) entries, and spent 93±0.73secs ($p<0.001$) at a lower dose (200 mg/kg body weight). Whereas the higher dose (400 mg/kg body weight) significantly increased the time spent in the open arms (120±0.92sec, $p<0.001$) with increased entries (18±0.49, $p<0.001$) (Table 5 & 6).

Similarly, the animals have spent more time in the closed arms in the anxiety, but treatment with the standard and the PLM has motivated them to move towards the open arms and decreased the number of entries and time spent in the closed arms. PLM at lower dose has decreased the number of entries in the closed arm and exhibited 4.8±0.33 ($p<0.001$) entries, and spent 180.52±0.89 secs ($p<0.05$). At the higher dose (400 mg/kg body weight) the time spent in the closed arms (147.59±1.13sec, $p<0.001$) and number of entries (6±0.63, $p<0.001$) were significantly decreased. The results evidence that the oral administration of PLM has an anxiolytic effect in a dose-dependent manner and comparable with the standard Diazepam (Figure 2).

Table 5a. Effect of *P. alata* on the of time spent in open arms

Treatment		Group I: Standard diazepam (1mg/kg per oral)	Group II: (PLM-200 mg/kg, p.o)	Group III: (PLM-400 mg/kg, p.o)
Time Spent	Before treatment	46.24±0.47	48.11±0.49	44.39±0.46
	After treatment	130±0.58***	93±0.73***	120±0.92***

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001

Table 5b. Effect of *P. alata* on the number of entries in open arms

Treatment		Group I: Standard diazepam (1mg/kg P oral)	Group II: (PLM-200 mg/kg, p.o)	Group III: (PLM-400 mg/kg, p.o)
No. of entries	Before treatment	10.60±0.46	11.03±0.47	10.18±0.44
	After treatment	25.2±0.33***	14.6±0.36*	18±0.49***

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001, *P<0.05

Table 6a. Effect of *P. alata* on the time spent in closed arms

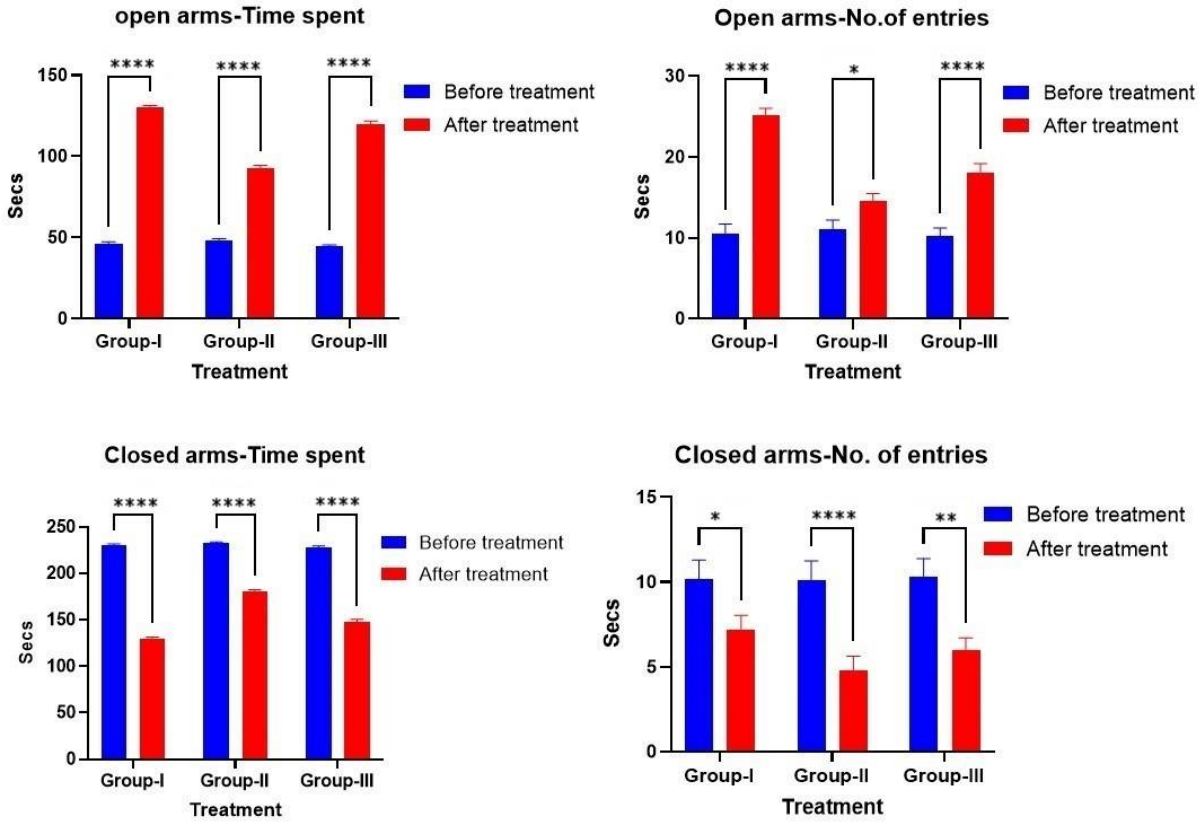
Treatment		Group I: Standard diazepam (1mg/kg per oral)	Group II: (PLM-200 mg/kg, p.o)	Group III: (PLM-400 mg/kg, p.o)
Time Spent	Before treatment	230.09±0.56	232.48±0.57	227.83±0.56
	After treatment	129.34±0.68***	180.52±0.89***	147.59±1.13***

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001

Table 6b. Effect of *P. alata* on the number of entries in closed arms

Treatment		Group I: Standard diazepam (1mg/kg per oral)	Group II: (PLM-200 mg/kg, p.o)	Group III: (PLM-400 mg/kg, p.o)
No. of entries	Before treatment	10.20±0.44	9.79±0.42	10.61±0.45
	After treatment	7.2±0.34*	4.8±0.33***	6±0.28**

All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001, **P<0.01, *P<0.05



All values are expressed as Mean ± SEM. The 'paired t' test was used for the individual groups to compare before and after the treatment. ***P<0.001, **P<0.01, *P<0.05

Figure 2. Effect of *P. alata* on the number of entries and time spent in open and closed arms

Rotarod test

The rotarod test evaluates motor coordination and peripheral neuromuscular function in rats, measuring their ability to balance on a rotating rod. A decrease in the time before falling off the rod suggests muscle relaxation, potentially indicating a calming effect or anxiety reduction. This test is instrumental in assessing the impact of treatments on the peripheral neuromuscular system and balance [24]. Diazepam has successfully improved motor coordination in rats and has a threshold time of 60 minutes. Surprisingly, PLM either at low or higher concentrations, has not exhibited statistically significant improvement in the early minutes for the experimental animals (Table 7 & figure 3).

Table 7. Effect of *P. alata* on the number of fall times

	Group I: Standard diazepam (1mg/kg p.o)		Group II: (PLM-200 mg/kg, p.o)		Group III: (PLM-400 mg/kg, p.o)	
	Before Treatment	**after treatment**	**Before Treatment**	**after treatment**	**Before Treatment**	**after treatment**
15 minutes	130.48±1.48	108±2.27*	129.18±1.47	125±1.42ns	135.75±1.54	121±1.38**
30 minutes	127.87±1.45	97.±2.03***	133.04±1.51	123±1.4*	126.59±1.44	119±1.35*
45 minutes	131.71±1.5	92±1.93***	130.39±1.49	117±1.33**	137.03±1.56	114±1.3**
60 minutes	126.59±1.44	90±1.9 ***	131.71±1.5	116±1.32***	125.33±1.42	113±1.28**
75 minutes	127.86±1.45	89±1.88***	126.58±1.44	113±1.28***	133.03±1.51	109±1.24***
90 minutes	131.70±1.5	89±1.86***	137.02±1.55	112±1.27***	130.38±1.48	108±1.23***

All values are expressed as Mean±SEM, statistical analysis by student's 't' test, ***P<0.001, **P<0.01, *P<0.05 indicates statistically significant.

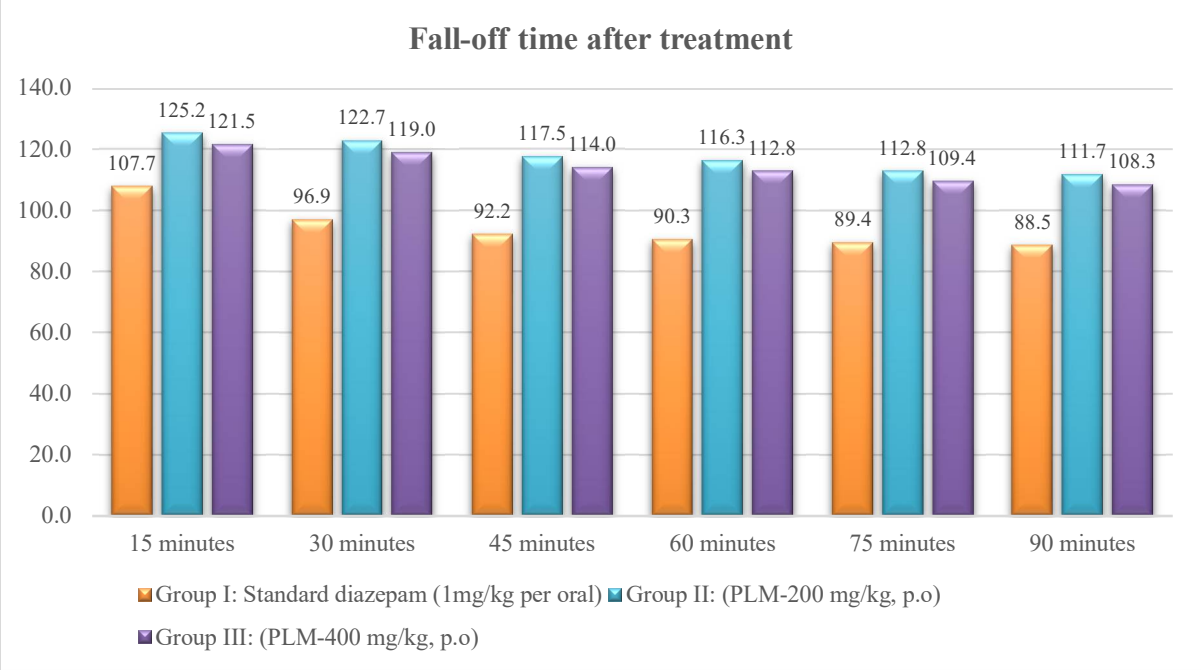


Figure 3. Effect of *P. alata* on the number of fall times

DISCUSSION

The acute toxicity studies of *P. alata* have been pivotal in establishing its safety profile. Our study demonstrated no toxicity symptoms at a dosage of 2,000 mg/kg body weight, suggesting its safety for consumption. The absence of toxicity aligns with previous studies, such as Boeira *et al.*, 2010, who reported safety up to 4800mg/kg body weight in Wistar rats. Our results, with no symptoms at 2000mg/kg, are consistent with these findings, confirming the safety of *P. alata* leaves [25].

In-vitro and *in-vivo* studies on *P. alata* extracts provide significant insights into their anxiolytic potential. The in-vitro GABA receptor binding assay revealed that the leaf methanol extract (PLM) has a higher binding activity, indicating a strong affinity with GABA receptors, key in anxiety modulation. This is reflected in its lower K_d and higher B_{max} values, suggesting its potential in anxiety reduction, similar to studies on *Leonurus cardiaca* and *Leonurus japonicus* extracts by Rauwald *et al.*, 2015 [26].

This rise in explorative behaviors, indicated by increased crossings and rearings, indicates a decrease in anxiety levels, affirming the anxiolytic potential observed in the *in-vivo* study. Similar results were observed in *P. edulis* aqueous extract on Wistar rats with significant anxiolytic behaviour [27].

The Elevated Plus Maze (EPM) test showed a dose-dependent increase in open arm exploration, indicating reduced anxiety. The higher PLM dose (400 mg/kg) exhibited a more significant anxiolytic effect, echoing dose-dependent findings in *P. incarnata* flowers reported by Grundmann *et al*., 200p [28].
Contrastingly, the Rotarod test results differed. *P. alata* extracts did not significantly enhance early motor coordination, unlike Diazepam. This suggests a variance in *P. alata*'s action or receptor specificity, as also observed in studies with *P. incarnata* by Elsas *et al*., 2010 [29].
A comprehensive consolidation of *in-vitro* and *in-vivo* studies supports *P. alata*'s anxiolytic potential, especially the PLM extract. The consistent results across behavioral tests signify its efficacy in anxiety modulation, though with potential differences in neuromuscular effect compared to standard anxiolytics like Diazepam. This underlines the need for further research to explore *P. alata*'s mechanisms and its potential as an alternative or adjunctive therapy in anxiety management.

CONCLUSION

The study evaluated the anxiolytic activity of the leaf and stem extracts of *P. alata* through *in-vitro* GABA receptor binding assay and *in-vivo* open field and elevated plus maze tests. According to the results of the *in-vitro* experiment, the methanol (PLM) and ethyl acetate (PLE) extracts of the leaves exhibited the greatest binding activity to the GABA receptors. The *in-vivo* experiments showed that the methanol leaf extract (PLM) had dose-dependent anxiolytic efficacy, with the higher dose exhibiting more pronounced effects. The findings imply that *P. alata* might be a possible source of phytochemicals that reduce anxiety.

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