Research Article

Evaluation of Anxiolytic Activity of Ethanolic Extract of Tubers of *Gloriosa superba* Linn in Mice

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ABSTRACT

The clinical applications of well-known benzodiazepines as anxiolytic agents are limited because of their side effects. Therefore, the development of new pharmacological agents from medicinal plants is well justified. Among medicinal plants, *Gloriosa superba* Linn (Liliaceae) has been recommended for relief of anxiety in traditional folk medicines. Nevertheless, no pharmacological studies have so far evaluated it in this regard. The purpose of the present study was to investigate the anxiolytic effects of ethanolic extract of the tubers of *Gloriosa superba* Linn in mice. The ethanolic extract of the tubers of *Gloriosa superba* Linn (EEGS), after preliminary phytochemical screening was subsequently evaluated for antianxiety activity using Elevated Plus Maze (EPM) model and Rota-Rod Test (RRT) in Swiss albino mice. Diazepam was used as standard drug. Adult Swiss albino male mice were randomly divided into four groups (n = 6). Groups I and II received CMC (1%, w/v, p.o) and diazepam (1mg/kg, i.p), respectively, while groups III and IV received orally 80 and 120 mg/kg doses of the EEGS respectively. The mice were then individually placed in animal anxiety models such as Elevated Plus Maze (EPM) and Rota Rod Test (RRT) and are evaluated for various parameters. In EPM test, 80 and 120 mg/kg doses of the extract significantly increased the percent number of entries and time spent in open arms compared to control. In RRT model both doses of the EEGS significantly reduced (P<0.001) the fall of time of albino mice compared to that of control. The present study clearly demonstrated that the ethanolic extract exerts an anxiolytic effect on mice, and it could serve as a new approach for the treatment of anxiety.

Keywords: Anxiolytic; Elevated Plus Maze; *Gloriosa superba* Linn; Rota Rod Test.

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INTRODUCTION

Anxiety is an emotional state, unpleasant in nature, associated with uneasiness, discomfort and concern or fear about some defined or undefined future threat. Anxiety is a complex progressive behavioural and physiological alteration of the organism, which ultimately leads to wide variety of central nervous system (CNS) disorders, if remain untreated. Some degree of anxiety is a part of normal life. Treatment is needed when it is disproportionate to the situation and excessive. Some psychotics and depressed patients also exhibit pathological anxiety. Anxiety affects most of the population nearly one-eighth of the total population world-wide. Benzodiazepines, being major class of compounds used for treatment of anxiety, present a narrow margin of safety between the anxiolytic effect and unwanted side effects, has prompted researchers to evaluate new compounds specially plant based drugs having less undesirable effects.[1,2]

Despite significant advances in the understanding and management of neuropsychiatric disorders during the past few decades, anxiety and depression still remain leading causes of mortality. According to a World health report (WHO, 2001), 450 million people suffer from a mental or behavioural disorder, yet only a small portion of them receive even the most basic treatment.[3] Currently, the most widely prescribed medicines for anxiety disorders are benzodiazepines; however, the clinical use of these agents is limited by their side effects. In spite of their relative safety, benzodiazepines can lead to disturbing effects such as amnesia, dependence liability, and sedation, which cause considerable concern. Therefore, the exploration of new medications from traditional medicinal plants possessing anxiolytic effects without the complications of benzodiazepines would be of great importance for the relief of anxiety-like disorders. On the basis of these considerations, the tubers of the medicinal *Gloriosa superba* (Liliaceae) were selected for the present study.

Gloriosa superba (Liliaceae) is one of the herbaceous medicinal climber which is a striking tuberous plant with brilliant wavy edged yellow and red flowers that appears from November to March every year. In folklore medicine, it is used to treat cancer, gout, asthma, leprosy, arthritis, piles, ulcers and as abortifacient, anthelminthic and anti-inflammatory agents etc. The plant is one of the seven upavishas in the Indian medicine, which cure many ailments but may prove fatal on misuse. The tuberous root stocks of *Gloriosa superba* boiled with *Sesamum* oil

reduces arthritis pain in joints.[4,5,6] The high medicinal value of this plant could be due to the presence of many secondary metabolites which act as bioactive compounds against diseases. However, until now there have been no reports on the pharmacological evaluation of the anxiolytic effects of *G. superba.* Present study intended to analyze these active compounds in the tubers of *Gloriosa superba* responsible for its medicinal properties. Hence in the present study, the anxiolytic activity of ethanolic extract of tubers was investigated using animal models such as the Elevated Plus Maze (EPM) test and Rota Rod Test (RRT).

MATERIALA AND METHODS

Collection of Plant material

The underground tubers of *Gloriosa superba Linn* were considered for the present study. The Plant specimens were collected during the month of June from its natural habitat from Botanical Garden, Palode, Thiruvananthapuram and from the Botanical Garden, Kuzhippallam, Thiruvananthapuram. Care was taken to select healthy plants and normal organs. The tubers were authenticated by the Botanist Dr P M Radhamany, Professor and Head, Department of Botany, University of Kerala, Karyavattom, Thiruvananthapuram, Kerala, India. The voucher specimen was deposited in Botany Department of the institution for further documentation. (Voucher no. KUBH6028).

Preparation of the Extract[7]

The tubers were washed thoroughly with running tap water to remove adhering soil. Then they were cut into smaller pieces and were dried in shades until they were free from moisture. The dried specimen were pulverized mechanically to a coarse powder. The air-dried, powdered tubers of *Gloriosa superba Linn* was freed from fatty substances by Soxhlet extraction with petroleum ether for 24 hours at room temperature. Defatted and dried plant materials were subjected to Continuous hot extraction in Soxhlet apparatus with ethanol. Extracts were filtered off and separated from solid materials using Whatman No. 1 filter paper. The extract so obtained was then evaporated under reduced pressure in a rotary evaporator at temperature 50^0C until all the solvent had been completely removed to get an extract sample.

Preliminary Phytochemical Screening

Freshly prepared extracts were subjected to preliminary phytochemical screening for various constituents such as carbohydrates, alkaloids, glucosides, steroids, saponins, flavanoids, tannins and phenolic compounds by standard reagents.

Experimental animals

Adult male Swiss albino mice weighing 25-30g were used for the study. The animals were procured from animal house, Sree Chithra Institute of Biotechnology, Thiruvananthapuram, Kerala. The animals were housed at a standard environmental condition (at 25±2^0C, humidity 60± 10% with 12 hrs light and dark cycles) with food and water ad libitum. The animals were acclimatized for a period of seven days before the study. All the experimental procedures and protocols used in this study were reviewed by Institutional Animal Ethics Committee. (IAEC No.01/05/2014/MCT). The animals were used according to the CPCSEA guidelines for the use and care of experimental animals. Acute toxicity studies along with gross behavioural studies of the ethanolic extract of *Gloriosa superba* were done in albino mice and LD_{50} of the drug was determined graphically.[8]

Evaluation of antianxiety activity

Experimental design

48 adult Swiss albino mice weighing 25-30gms were selected.24 animals were allocated for Elevated Plus Maize (EPM) test, and 24 animals to Rota Rod Test (RRT). In each model there were 4 groups of 6 animals each. The group 1 received vehicle (1% w/v CMC 10ml/kg p.o), group 2 received standard drug Diazepam (1mg/kg p.o.), group 3 and 4, received the ethanolic extract (80 and 120mg/kg p.o respectively) of tubers of *Gloriosa superba.*

Elevated Plus Maze [9-13]

The test was developed by Montgomery in 1958. It is one of the classical models to assess the anxiolytic effect of the drug. The test measures the total number of entries and time spent in the open arm which is an indication of anxiety level. Based on the number of entries and time spent in the open arm, the drugs can be classified as anxiolytic and anxiogenic agents.

The elevated plus maze consist of two open and two closed arms each of 50x10x40 cm dimensions, with an open roof arranged in such a manner that the two arms are opposite to each other. The maze is elevated to a height of 50 cm. The animals were placed individually in the centre of the maze, with head facing towards the open arm and noted the following parameters such as the Number of entries in open or closed arm (an arm entry is defined as the entry of four paws into the arm) and Average time spent in each arm for 5 minutes. After 5 mins the preference of the animal to open/closed arm, average time spent and number of entries to open arm in each group was compared.

Rota-rod apparatus [11,14,15]

In this classical method introduced by Dunham and Miya in 1975, drugs were evaluated for their potential to interfere with the motor coordination activity of mice or rats by observing the fall of time of these rodents when placed on a rotating rod for 5 mins.

The apparatus consists of a metal rod with 3 cm diameter attached to a motor with the speed that can be set between 5-30 rotations/minute. The rod is divided into 3 sections by plastic discs, so simultaneously 3 mice can be tested. The rod is placed at a height of about 50 cm above

the table top in order to discourage the animals from jumping off the roller.

Animals were brought to the testing room one hour before starting the procedure. Before starting the actual experiment all the mice were trained by giving 3 trials, by placing them on the rotating rod at 5 rpm for a period of 60 sec each, at an interval of 10 mins between trials.

After 15 mins of the trial, mice from different treatment groups were evaluated for motor coordination at an interval of 30 ,60 and 90 minutes after administration of test and standard drugs. The time when each animal falls off from the rod for the first time and the number of falls were noted during the 5 min test period and was considered toxic if it fell from the rotarod three or more times. Apparatus was cleaned thoroughly between trials. All behavioural recordings were carried out with the observer blind to the treatment the mice had received.

Statistical Analysis

All observations were presented as Mean ± SEM and were analysed using One-way Analysis of variance (ANOVA) followed by Dunnett's post hoc test. P values lower than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The percentage yield of the extract (EEGS) was 9.4%w/w. The extract was pungent dark brown thick paste, partially soluble in water, sparingly soluble in dilute acid and completely soluble in dilute alkali.

Preliminary Phytochemical screening of the EEGS showed the presence of carbohydrates, alkaloids, steroids, flavonoids, tannins and phenolic compounds. The LD_{50} value of EEGS was found to be 1260mg/kg. Two different doses of the extract namely low($1/15^{th}$) and high ($1/10^{th}$) such as 80mg/kg and 120mg/kg were selected with respect to the LD_{50} dose for the present study. The gross behavioural studies showed mild sedation of animals. There were no autonomic and behavioural changes but CNS depression noted at higher doses.

Evaluation of anxiolytic effect- Elevated plus Maze Model

Total time spent in open arm

The total time spent in open arm in elevated plus maze in different groups of Swiss albino mice were shown in Table 1 and Figure 1.

Table 1: Evaluation of the total time spent in open arm by albino mice in elevated plus maze

Groups	Treatment	Total time spent in open arm (sec)
Normal Control	1% CMC 10ml/kg	6.33± 0.192
Standard	Diazepam (1mg/kg)	24.66± 0.304****
EEGS	80mg/kg	12± 0.333****
EEGS	120mg/kg	22.33± 0.192****

All values are Mean ± SEM for n=6. Statistical analysis is by One-way ANOVA followed by Dunnett's multiple comparison test. **** indicates P< 0.001 compared to the control group

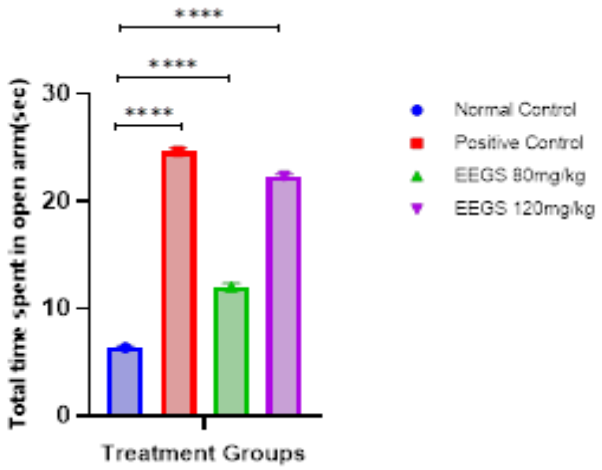

Figure 1: Evaluation of total time spent in open arm of EPM

The total time spent in open arms increased from 6.33± 0.192 (sec) in control group to 24.66 ± 0.304 in the standard drug, diazepam treated group. The total time spent by the groups treated with ethanolic extract at a dose of 80mg/kg is 12± 0.333 and 120mg/kg is 22.33± 0.192 (sec) respectively. The total time spent by the high dose extract treated group is comparable to the standard drug treated group. Both doses of the extract significantly(P<0.001) increased the total time spent in the open arm in the EPM model compared to the normal control group.

Number of entries into open arm

Figure 2 and Table 2 represent the number of entries of albino mice into open arm of the elevated plus maze. All values are expressed as Mean ± SEM for n=6. Statistical analysis is done by One-way ANOVA followed by Dunnett's multiple comparison test. *** indicates P< 0.001 compared to the control group and * indicates P<0.05 compared to that of control group.

Table 2: Evaluation of the number of entries of albino mice into open arm

Groups	Treatment	No: of entries into open ARM
Normal Control	1% CMC (10ml/kg p.o)	3.83± 0.28
Standard	Diazepam (1mg/kg p.o)	8.66± 0.05***
EEGS	80mg/kg	4.83± 0.925*
EEGS	120mg/kg	6.66± 0.991*

All values are Mean ± SEM for n=6. Statistical analysis is by One-way ANOVA followed by Dunnet's Multiple Comparison test. *** indicates P< 0.001 compared to the control group and * indicates P<0.05 compared to that of control group.

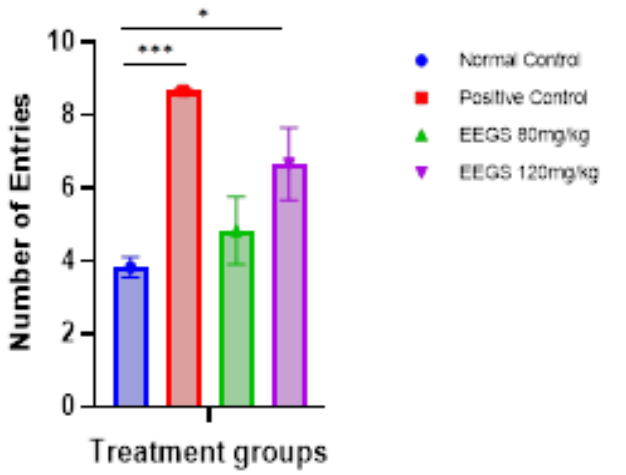

Figure 2: Evaluation of the number of entries of albino mice into open arm of EPM

The number of entries in the open arm of EPM by the Control group is 3.83± 0.28 and the standard drug treated group is 8.66± 0.05. The result showed that the standard drug diazepam significantly increased (P<0.001) the total number of entries in the open arm of EPM compared to the normal control group. The low dose and high doses of EEGS treated groups also significantly(P<0.05) increased the number of entries in the open arm of EPM compared to the normal control group. The number of entries of low and high doses of EEGS are found to be 4.83± 0.925 and 6.66± 0.991respectively. This indicates the dose dependent anxiolytic activity of the extract in albino mice.

The EPM test is principally based on the behaviour that exposure of animals to an elevated maze alley evokes an approach-avoidance conflict and rodents consistently spend greater time in the closed arms when placed in mazes comprising of open and closed arms. Based on these assertions, EPM tests are reliable means of identifying selective anxiolytic effect of drugs and used as a tool in the investigation of the psychological and neuro-chemical basis of anxiety. The EPM test has been validated pharmacologically, physiologically and behaviourally, and has become one of the most widely used behavioural tests for anxiety. Anxiety is induced by a fear due to height in rodents when placed on the EPM. The ultimate manifestation of anxiety is exhibited by preference to remain at safer places and a decrease in the motor activity. Treatment of mice with the EEGS resulted in significant alterations on the behavioural responses measured in the EPM test

Evaluation of Anxiolytic Activity-Rotarod Test

The figures 3,4,5 and Table 3 indicate the fall of time in rotarod apparatus in different treatment groups of Swiss albino mice at different time intervals. All values are expressed as Mean ± SEM for n=6. Statistical analysis is done by One-way ANOVA followed by Dunnett's multiple comparison test. a* indicates P< 0.001 compared to the control group.

Table 3: Evaluation of the fall of time of albino mice in Rotarod apparatus at different time intervals after treatment

Groups	Treatment	Time (sec) of Animals Remained without Falling from ROD		
		30 min	60 min	90 min
Normal Control	1%CMC (10ml/kg p.o)	276.05± 4.73	273.051± 5.562	270± 2.635
Standard	Diazepam (1mg/kg p.o)	91.83± 1.862a*	143± 1.633a*	178.43± 6.774a*
EEGS	80mg/kg	181± 2.438a*	205.66± 1.91a*	226± 3.127a*
EEGS	120mg/kg	107± 3.153a*	145± 1.394a*	149.5± 2.761a*

All values are Mean ± SEM for n=6. Statistical analysis is by One-way ANOVA followed by Dunnett's multiple comparison test. a* indicates P< 0.001 compared to the control group.

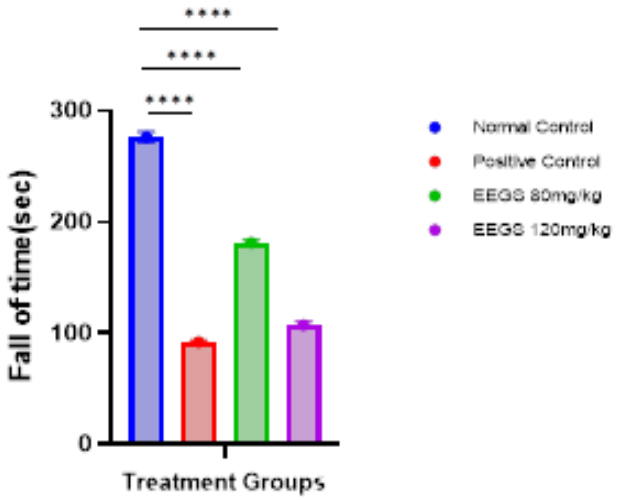

Figure 3: Evaluation of fall of time in RRT after 30 minutes of treatment

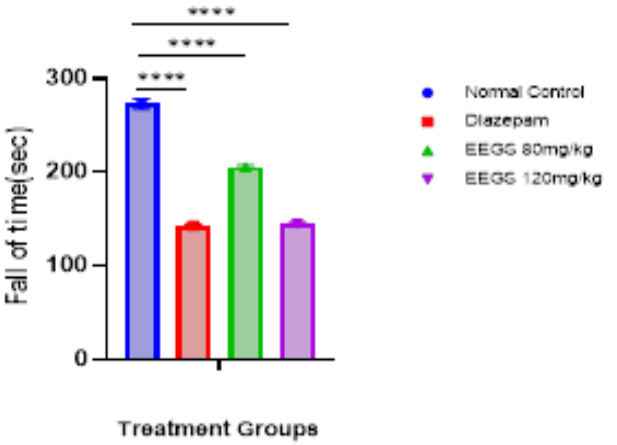

Figure 4: Evaluation of fall of time in RRT after 60 minutes of treatment

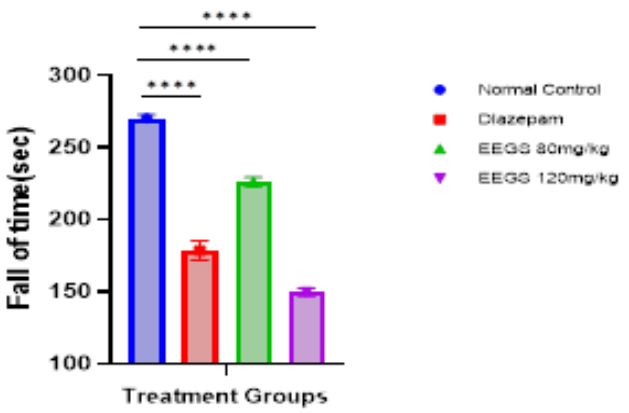

Figure 5: Evaluation of fall of time in RRT after 90 minutes of treatment

In Rotarod test the time spent by the animals on the revolving rod was tested at different intervals after the drug treatments. The assessment of motor incoordination effect of each treatment group was compared with the control group at that particular time interval period. The results showed that the standard drug diazepam significantly reduced the time spent by the animals on revolving rod when tested 30 minutes ,60minute and 90 minutes after drug administration as compared to control. The motor incoordination effect of diazepam was highly significant (P<0.001) when compared to normal control group. After 30 minutes of treatment, the fall of time of the control group was 276.055± 4.73 sec and that of the standard drug treated group was 91.83± 1.862sec.At this time interval the fall of time of low dose EEGS and high dose EEGS treated groups were found to be 181± 2.438 and 107± 3.153 seconds respectively. The extract at these dose levels significantly reduced the fall of time of albino mice from the rotarod after 30 minutes of drug treatment compared to the control group.

The fall of time of mice from the rotarod after 60 minutes of treatment was 273.051± 5.562 for normal control group. The fall of time for standard drug, low and high dose extract treated groups were found to be 143± 1.633, 205.66± 1.91 and 145± 1.394 seconds respectively. The fall of time of mice from the rotarod after 90 minutes of treatment was 270± 2.635 seconds for normal control group. The fall of time for standard drug, low and high dose treated groups were found to be 178.43± 6.77, 226± 3.127 and 149.5± 2.761a seconds respectively. The drug treatment significantly (P<0.001) reduced the fall of time at these time intervals. It was observed that as the time interval was increased the fall of time was also increased in all the treated groups of animals.

The present work demonstrated the anxiolytic effects in anxiolytic models like Elevated Plus Maze and Rotarod test methods. The EPM model is based on exploration in a novel environment leading to the generation of approach avoidance conflict behaviour, which is highly sensitive to the $GABA_A$ benzodiazepine receptor complex. This animal model is considered one of the most widely validated tests for assaying sedative and anxiolytic substances such as the benzodiazepines. In EPM rodents, mice will normally prefer to spend much of their allotted time in the closed arms. This preference appears to reflect an aversion towards open arm that is generated by the fears of the open spaces. The fear due to height induces anxiety in the animals when placed on the EPM. The ultimate manifestation of anxiety and fear in the animals is exhibited by decrease in the motor activity and preference to remain at safer places.[24] Anxiolytic agents are expected to increase the motor activity, which is measured by the time spent by the animal in the open arms. Anxiolytic compounds, by decreasing anxiety, increase the open arm exploration time as well as the number of entries into the open arm.

The Rota rod test is a classical animal model used to evaluate peripheral neuromuscular blockade and the motor coordination[11]. A deficit in motor coordination would very likely affect performance in the behavioral tests. The Rota rod test is widely used to evaluate the motor coordination in rodents. When a mouse is repeatedly placed on a rod or cylinder which is rotating at a constant speed, the animal gradually learns to walk on it, adapting itself to the rotation speed. After ingestion of a central depressant, however, the animal easily falls from the rod. This test was first introduced by Dunham and Miya for assaying the drug effects on the motor activity. Since then, the effects of various central depressants, investigated by this test have been reported.

The ideal anti-anxiety drugs would suppress all the symptoms like irritability, uneasiness, jumpiness, feelings of apprehension, rapid or irregular heartbeat, stomach ache, nausea, faintness, and breathing problems associated with it, without causing any unwanted effects. Benzodiazepines (BZDs) are the major classes of compounds used in anxiety and they remain the most commonly prescribed treatment for anxiety. Ligands upon binding to the BZDs, $GABA_A$ receptor complex exert pharmacologically and clinically important profiles including anxiolytic, anti-convulsion, muscle-relaxation, sedation and reduction of neuronal oxidative metabolism. However, the clinical uses of BZD are limited by their side effects such as drowsiness and loss of coordination. fatigue and mental slowing or confusion and vertigo. The recognition of anxiolytic effects of nonbenzodiazepine azapirones agents, which act as 5-HT_{1A} partial agonists, such as buspirone, gepirone, and ipsapirone and their therapeutic role in clinical anxiety and mood disorders has further focused attention on the 5- HT_{1A} receptor. Although the azapirones interact with other neurotransmitter systems such as the dopaminergic and noradrenergic, they display nanomolar affinity for 5- $HT1_A$ receptor sites. It is estimated that, about 43% of anxiety sufferers use some form of complementary therapy. The most popular treatments include herbal medicines. Similarly, anxiety disorders are amongst the most common reason for people to try herbal medicines. Therefore, the development of new medications possessing anxiolytic

effect without the complication of BZD would be of great importance in the treatment of anxiety-related disorders. Medicinal plants are a good source to find new remedies for these disorders.[16]

The rationale for herbal medicine is likely to be based on the activity of plant or its chemical constituents, to interact in the context of the gut and body tissues, to affect bioavailability.

The tubers of the plant *Gloriosa superba Linn* is commonly used in folklore in different disorders. The tubers contain various phytoconstituents such as alkaloids, phenolic compounds, steroids and flavonoids. Flavonoids have recently increased the importance because they have been identified as a new type of ligand with in-vivo anxiolytic properties. Flavanoids are a group of polyphenolic compounds, have been demonstrated by a number of groups to be centrally active, possessing efficacies for a number of receptor systems in the CNS. Flavonoids with anxiolytic activity have been described in many plant species used in folk medicine such as Passiflora coerulea.[18] This effect has been attributed to the affinity of flavonoids for the central benzodiazepine receptors.[19-21] Furthermore a sedative effect on the central nervous system has been shown for quercetin and isoquercetin glycosides in mice.[22]

In this study, we observed that the EEGS at the dose of 80 and 120mg//kg/p.o induced significant increase in both the number of entries and time spent in the open arm whereas the number of entries and time spent in the closed arms were reduced in the EPM model. In RRT model both doses of the EEGS significantly reduced($P<0.001$) the fall of time of albino mice compared to that of control. However, further studies are required to identify the phytoconstituent responsible for the observed anxiolytic effect of ethanolic extract at these dose levels to explain the anxiolytic mechanism.

CONCLUSION

The present study demonstrated that the ethanolic extract of tubers of *Gloriosa superba Linn* possess the anxiolytic activity. The flavonoids present in this extract may be responsible for its anxiolytic activity. Furthermore, there is need to find out the exact mechanism by which the plant exerts the anxiolytic activity.

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