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CNS Depressant effects and muscle relaxant activity of *Galphimia glauca* leaf methanol extract

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Abstract : Objective: This study assesses the depressant effects and muscle relaxant activity of *G. glauca* leaf methanol extract.
Methods: The leaf extract was administered in Swiss albino mice for one day to study depressant and muscle relaxant activity employing models like Sodium pentobarbital induced sleep test, Hole-board test, Open field test, Pentylenetetrazole induced convulsions, Picrotoxin induced convulsions, Grip strengthening test and Rota rod test.
Results: The LD_{50} of the leaf extract was found to be > 2000 mg/kg b.w. Mice treated with leaf methanol extract at 100, 200 and 400 mg/kg, b.w doses prolonged the sleeping time induced by sodium pentobarbital (40 mg/kg. b.w, i.p.). The leaf methanol extract at 400 mg/kg dose showed a significant ($P \leq 0.001$) sedative effect in the hole-board test and decreased spontaneous activity in mice and also delayed the onset of seizures induced by Pentylenetetrazole (90 mg/kg b.w, i.p) and Picrotoxin (10 mg/kg, b.w, i.p).The leaf extract showed significant ($P \leq 0.001$) effect on the ambulatory behaviour of mice in open field test. The leaf methanol extract also showed significant ($P \leq 0.05$) effects on muscle coordination in Grip strengthening and Rota rod test in mice ($P \leq 0.01$).
Conclusion: The results conclude that the *Galphimia glauca* leaf methanol extract has significant depressant and muscle relaxant effects compared to standard drugs.
Keywords : *Galphimia glauca*, Methanol extract, Convulsions, Rota rod test, Grip strength test.

1. Introduction

Galphimia glauca is found widely distributed in Deccan Plateau of southern India, mostly seen in Eastern Deccan Plateau (Telangana State). The climate of this region favours its habitat. It is an ever green plant belongs to family of Malpighiaceae. The plant is commonly known by the name "*Calderona amarilla*" and "*Flor estrella*"[1, 2]. The ethyl acetate fraction of plant's aerial parts is reported for anti-asthmatic activity which acts by inhibiting the LTD_4-induced airway smooth muscle contraction[3]. Ruiz et al., 2007, reported the anxiolytic effects of natural galphimines of the plant and their chemical derivatives in ICR mice that are exposed to the elevated plus-maze test[4]. Traditionally *G. glauca* is used in conditions of panic, phobia, stress anxiousness and it is as well used to produce calming effect on nerves[5]. Based on the traditional use of this plant

the present study is aimed to explore the CNS depressant effect and muscle relaxant activity of *Galphimia glauca* leaf methanol extract (GGLME) using *in vivo* models.

2. Materials and methods

2.1 Plant Material

The shrub *G. glauca* was collected from the medicinal garden existing in the School of Pharmacy, Anurag Group of Institutions. The leaves were collected in the time of November, 2014. The plant was identified and authenticated by taxonomist, Dr. E. Narsimha Murthy, Satavahana University, Karimnagar, Telangana State, India. A voucher copy is stored with the reference number No.333, in the Department of Pharmacognosy and Phytochemistry, School of Pharmacy.

2.2 Chemicals and drugs

All the chemicals were of analytical grade and purchased from SD Fine chemicals, Mumbai, India. Sodium pentobarbital used in this study was purchased from Sigma Chemicals Co., USA; Diazepam was procured from Natco Pharmaceuticals India Ltd, Pentylenetetrazole from Sigma-Aldrich, USA. Picrotoxin is received as gift sample from Sri Disha biotech, Hyderabad, India Ltd.

2.3 Preparation of the extract

G. glauca leaves were collected, dried in shade and powdered coarsely. Leaf powder of 150 g was subjected to Soxhlet extraction using 600 ml of methanol. The extract was collected and then concentrated to dryness and stored. The yield obtained for *G. glauca* leaf methanol extract (GGLME) was 12 %.

2.4 Animals

Swiss albino mice of six to eight weeks of age with 22.5 ± 2.5 g of either sex were used. Mice were acclimatized for seven days to the laboratory conditions. The animals were retained in 12 hour light/dark cycles at 22 ± 2 °C with 60 to 70 % relative humidity. Entire pharmacological studies were carried out randomly using six animals of either sex in each group. The experimental protocol was approved by the Institutional Animal Ethics Committee of the institute (IAEC), School of Pharmacy, Anurag Group of Institutions (the approval number: I/IAEC/LCP/032/2014/SM-13).

2.5 Acute Toxicity Studies

According to The Organization for Economic Co-operation and Development (OECD) guidelines, 423-2d, acute oral toxicity studies were conducted[6].

2.6 Phytochemical Screening

Phytochemical screening of the *G. glauca* leaf methanol extract (GGLME) was carried out using standard procedures[7, 8].

2.7 CNS depressant activity

2.7.1 Test for Sodium pentobarbital induced sleeping time

This test was described by Fujimori (1965) [9]. The hypnotic and sedative effects of GGLME in combination with sodium pentobarbital were evaluated. For this purpose mice were divided into groups as mentioned below. Group I served as negative control and received distilled water before the administration of the sodium pentobarbital (40 mg.kg. b.w, i.p.). Group II received diazepam (1 mg/kg, b.w, i.p.), served as positive control while Groups III to V received leaf extract sixty min before the administration of sodium pentobarbital. Each animal was placed on a table and observed for the uncoordinated movements to the sedative phase of the test. Loss of the righting reflex related to the hypnosis and the duration of the sleep was also observed and recorded. The time elapsed between the loss and recovery of the righting reflex was considered as the sleeping time[10].

Group I: Negative control, received distilled water [10 ml/kg, body weight (b. w.), per oral (p. o.)].

Group II: Positive control, treated with diazepam [1 mg/kg, (b. w.), intraperitoneally (i. p.)].

Group III-V was treated with GGLME [100, 200 and 400 mg/kg, b.w., respectively, (p.o)].

2.7.2 Hole-board test

This test was first described by Boissier et al. (1964) to evaluate specific components of behaviour of mice such as curiosity or exploration [11]. For this test the apparatus used is a wooden box of 50 cm x 50 cm x 30 cm with four equidistant holes (3 cm diameter) on the floor. Group I served as negative control received only distilled water. The standard (group II) received diazepam (2 mg/kg, b.w, i.p.) thirty minutes before performing the test. GGLME was administered to groups as mentioned in section 2.7.1. After sixty min, each animal is placed in the centre of the hole-board test apparatus and number of head-dips of mice into the holes, and the numbers of rears were recorded over a time period of five min. The floor of the apparatus was cleaned to remove the traces of earlier paths after each trial. A decrease in the number of head dips, the number of rears compared to the control was considered to indicate a sedative effect[12].

2.7.3 Open field test

This method was described by Barros et al. (1991)[13]. The open field is a non-conditioned anxiety test to register general motor activity, locomotion, rearing and the speed of locomotion. The test was conducted according to the procedure previously described by Lopez-Rubalcava et al (2006) with some changes [14]. The apparatus was made up of plywood measuring 60 cm x 60 cm x 40 cm. Transparent glass was used in the making to ensure that the animal under observation was visible. The floor made of cardboard was divided into 12 equal squares. The animals were grouped as mentioned in section 2.7.1. Group I served as negative control; Group II as a positive control received diazepam (1 mg/kg, b.w, i.p.) and groups III to V received *G. glauca* extract as mentioned in section 2.7.1. After thirty and sixty min post treatment of standard and extract administration, each animal was placed in the corner of the apparatus and the animal behaviour was monitored for five min session through video recording. The locomotion (The number of times the animal entered each square (counts per 5 min), rearing (frequency with which the mice stood on its hind legs) was recorded.

2.7.4 Effect on Pentylenetetrazole-(PTZ) induced convulsions in mice

This test was performed to evaluate the anticonvulsant activity of the extract as described by Loscher et al. (1991) [15]. The mice (s) used in this test were grouped as mentioned in section 2.7.1. Group I, Groups III to V were treated as mentioned in sec. 2.7.1. Group II received diazepam 1.0 mg/kg b.w; i.p. Thirty min after i.p injection and sixty min after oral administration convulsions were induced to all the groups by the administration of Pentylenetetrazole (PTZ) (90 mg/kg b.w, i.p). The period of time that elapsed between the administration of the pro-convulsive and the first myoclonus and tonic extension were visually evaluated over a period of forty min. The percentage of mice (s) that died within forty minutes was recorded. Animals that did not convulse within forty min after PTZ administration were considered protected.

2.7.5 Picrotoxin induced convulsions in mice

This test was performed to evaluate the anticonvulsant activity of the extract as described by Reyes et al (2010) [16]. GGLME was administered to animals of Groups III to V as mentioned in sec 2.7.1. Group I received only distilled water. Groups II treated with Diazepam (1 mg/kg, b.w, i.p.). Thirty min after i.p injection and sixty min after oral administration convulsions were induced to all the groups by the administration of Picrotoxin (10 mg/kg, b.w, i.p). The presence or absence of clonic convulsions, as well as the latency to clonus and tonics seizures, was observed for 40 min fallowing the administration of picrotoxin. The percentage of mice protected from administration of picrotoxin was recorded

2.8 Muscle coordination test

2.8.1 Grip strength test

This test is used to assess muscular strength and neuromuscular functions in mice and rats. This method was described by Boissier and Simon (1960)[17]. Mice of either sex were placed on a thin metal rod which is fixed to stand at a height of fifty cm. All mice (s) which remain hanging to metal rod for about one min

duration were selected for this study. The mice were divided randomly into groups as mentioned in sec 2.7.1. Group II was treated with diazepam (1 mg/kg i.p), while the remaining groups were treated as mentioned in sec 2.7.1. The fall off time of the standard and test groups from the thin metal rod is compared with control group as a measure of relaxant activity.

2.8.2 Rota rod test

This test was performed as described earlier by Dunham and Miya (1956) to test drugs interfering with motor coordination [18]. This test is performed using Rota rod apparatus which is of four compartment model (V. J, Instruments, India Ltd.). Mice of either sex were allotted randomly into groups (n = 6, of either sex) were treated as mentioned in section 2.7.1. Group I and Groups III to V were treated as mentioned in sec. 2.7.1, while the Group II (positive control) received 1 mg/kg i.p of diazepam. Thirty min after i.p and sixty min after oral administration of standard and test extract, mice were kept on rota rod at a speed of 24 rpm/min. Mice of all the groups were subjected to Rota rod test and mice (s) which fell off from the rotating rod were noted. The difference in fall of time observed with negative control group and extract treated group is an index of muscle relaxant activity.

2.9 Statistical analysis

Numerical data was expressed as mean ± SEM (standard error of mean). Statistical analysis were performed with one-way analysis of variance (ANOVA), followed by Tukeys' multiple comparison test and p values ≤ 0.05 was considered to be statistically significant. The statistical analysis was carried out with Graph Pad Prism 5.0 software.

3. Results

3.1 Acute Toxicity Studies

The results showed no mortality/toxic symptoms in mice treated with leaf extract (2000 mg/kg) until the 14^{th} day of the treatment period according to the OECD 423-2d guidelines. Based on the results, we have selected 100, 200 and 400 mg/kg as low, moderate and high doses to assess the depressant and muscle relaxant effects.

3.2 Phytochemical Screening

In the Phytochemical Screening leaf methanol extract showed positive results for Amino acids, Carbohydrates, Protiens, Flavonoids, Tannins and Phenolic compounds.

3.3 CNS depressant activity

3.3.1 Test for Sodium pentobarbital induced sleeping time

This test is a classic pharmacological method for the screening of sedative-hypnotic drugs. The sedative and hypnotic actions of GGLME administered to mice in combination with sodium pentobarbital are shown in Fig 1. GGLME in combination with sodium pentobarbital exhibited significant ($P \leq 0.001$) synergistic sedative and hypnotic effects and these effects are in a dose-dependent manner. The duration of the sleeping time produced by sodium pentobarbital was significantly ($P \leq 0.001$) prolonged in a dose-dependent manner with GGLME (400 mg/kg, bw.)

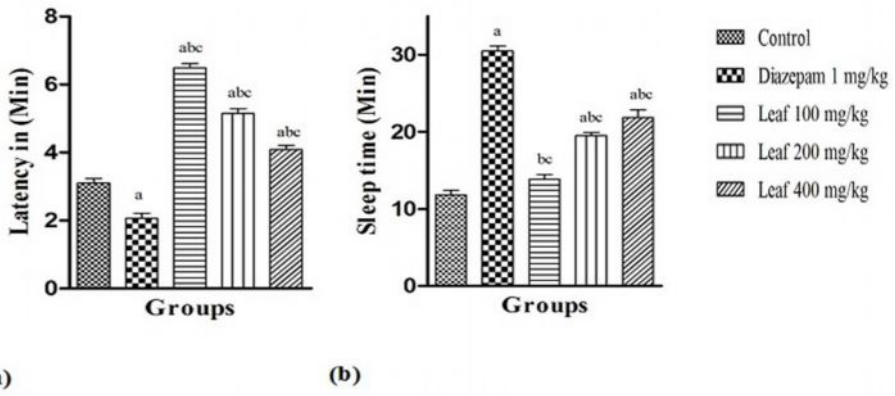

Fig1. Effects of *G. glauca* leaf methanol extract (GGLME) on Sodium pentobarbital induced sleep time
a) Latency of Sodium Pentobarbital induced sleep; **b)** Time of sleep induced by sodium pentobarbital.

[a]$P \leq 0.001$ indicates comparison with group I
[b]$P \leq 0.001$ indicates comparison with group II
[c]$P \leq 0.001$ indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts

3.3.2 Hole –board test

The sedative effect of GGLME was confirmed in the hole-board test. Fig. 2 represents the effects of both diazepam (2.0 mg/kg) and GGLME of varying doses on the performance of mice in the hole-board test. Treatment with extract (400 mg/kg) significantly decreased the head-dipping number (19.0 ± 0.7), showing p values ≤ 0.001 when compared to negative control group, and the number of rears (11.8 ± 1.2) relative to the negative control group.

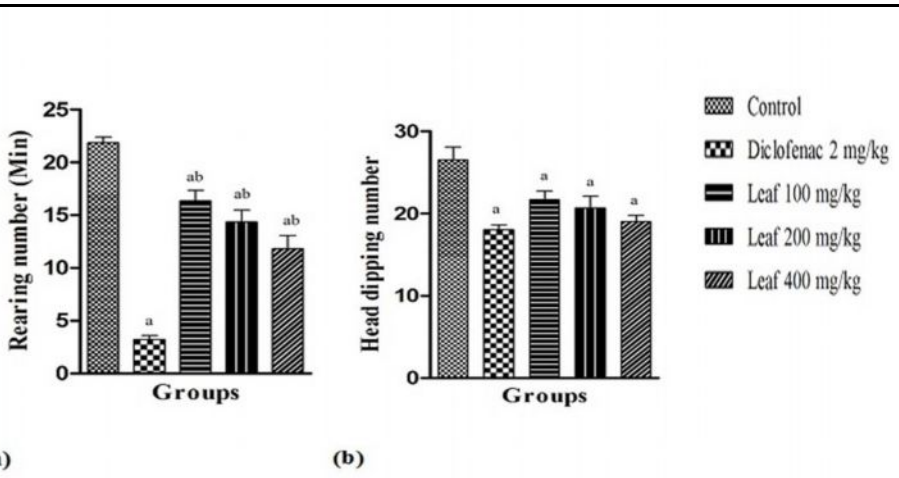

Fig. 2: Effects of *G. glauca* leaf methanol extract (GGLME) on a) Sedative behaviour (Rearing number); b) Sedative behaviour (Head dipping number)

[a]$P \leq 0.001$ indicates comparison with group I.
[b]$P \leq 0.001$ indicates comparison with group II

3.3.3 Open field test

The CNS depressant effect of GGLME was confirmed by the behaviour of mice in open field test. The extract significantly ($P \leq 0.001$) decreased the rearing and spontaneous ambulatory activity of the mice. The above results are shown in Fig 3.

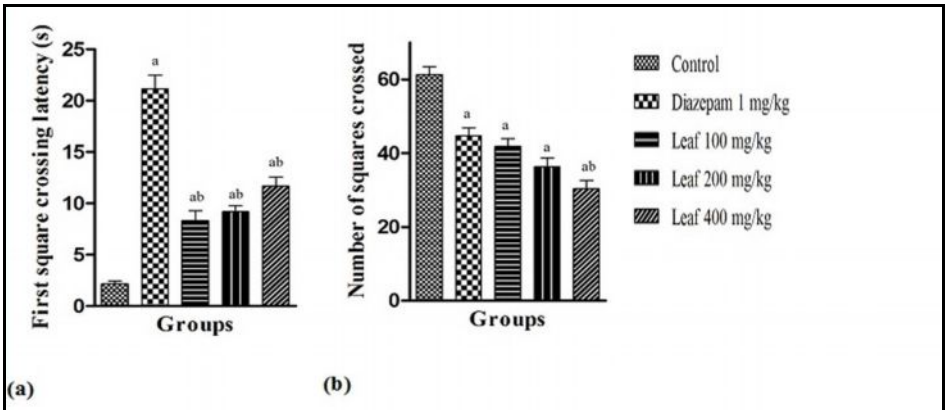

Fig. 3: Effect of *G. glauca* leaf methanol extract (GGLME) on the ambulatory behaviour of mice in open field test **(a)** First square crossing latency (s); **(b)** Number of squares crossed

[a]$P \leq 0.001$ indicates comparison with group I
[b]$P \leq 0.001$ indicates comparison with group II

3.3.4 Effect on Pentylenetetrazole-(PTZ) induced convulsions in mice

In this test, the latency time of myoclonus was delayed by diazepam (1.0 mg/kg), tonic seizures were prevented, and the incidence of death was decreased in comparison to the control group, whereas GGLME did not inhibit the appearance of myoclonic seizure but inhibited the tonic seizures ($P \leq 0.001$). However, GGLME at all test doses significantly delayed the onset of Pentylenetetrazole-induced convulsions. The percentages of dead animals were also reduced (20%) by GGLME at 400 mg/kg, bw. The results are shown in table 1.

Table 1: Effect of *G. glauca* leaf methanol extract (GGLME) on Pentylenetetrazole (PTZ) induced convulsions in mice

Group (s)	Dose mg/kg	Number of animals convulsed/used	Latency of tonic convulsions (mice)	Mortality (%)
I. Control	Distilled water	10/10	5.5 ± 0.3	100
II. Diazepam	1	0/10	-[a]	0
III. GGLME	100	3/10	13.3 ± 1.1[ab]	30
IV. GGLME	200	3/10	15.3 ± 1.2[ab]	30
V. GGLME	400	2/10	16.8 ± 1.3[ab]	20

[a]$P \leq 0.001$ indicates comparison with group I.
[b]$P \leq 0.001$ indicates comparison with group II

3.3.5 Picrotoxin induced convulsions in mice

GGLME administered significantly delayed the appearance of both clonic and tonic seizures and the extract reduced the mortality (10 %) induced by Picrotoxin. The results were shown in table 2.

Table 2: Effect of *G. glauca* leaf methanol extract (GGLME) on Picrotoxin induced convulsions in mice.

Group (s)	Dose mg/kg	Number of animals convulsed/used	Latency of tonic convulsions (mice)	Mortality (%)
I. Control	Distilled water	10/10	6.5 ± 0.2	100
II. Diazepam	1	0/10	-[a]	0
III. GGLME	100	2/10	12.5 ± 0.9[ab]	20
IV. GGLME	200	2/10	14 ± 0.8[ab]	20
V. GGLME	400	1/10	15.8 ± 0.8[ab]	10

[a]$P \leq 0.001$ indicates comparison with group I.
[b]$P \leq 0.001$ indicates comparison with group II
[c]$P \leq 0.001$ indicates the dose dependent activity on comparison of the high dose with respective low dose of the extracts.

3.4 Muscle coordination test

3.4.1 Grip strength test

The GGLME showed significant ($P \leq 0.01$) muscle relaxant activity with grip test, which is evidenced by the poor performance of the mice when subjected to hanging to thin metal wire. When compared to control group the percentage of animals loosing their catching reflex is found to be significant ($P \leq 0.01$) in GGLME. The results were shown in Fig 4.

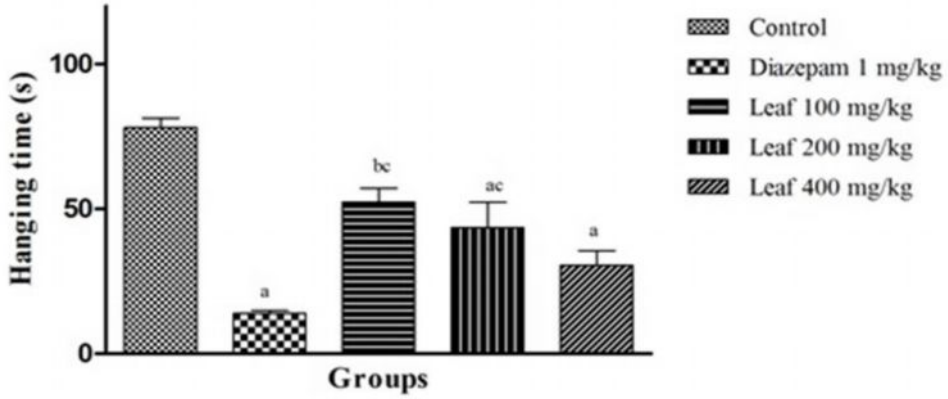

Fig 4: Effect of *G.glauca* leaf methanol extract (GGLME) on Grip test in mice

[a]$P \leq 0.001$ indicates comparison with group I.
[b]$P \leq 0.01$ indicates comparison with group I
[c]$P \leq 0.01$ indicates comparison with group II

3.4.2 Rota rod test

The experimental results are reported in Fig 5. The percentage animals falling from the Rota rod in both the standard group (Diazepam, 1mg/kg) and GGLME showed significant ($P < 0.001$) reduction in time spent by the animals in Rota rod test when compared with the control group.

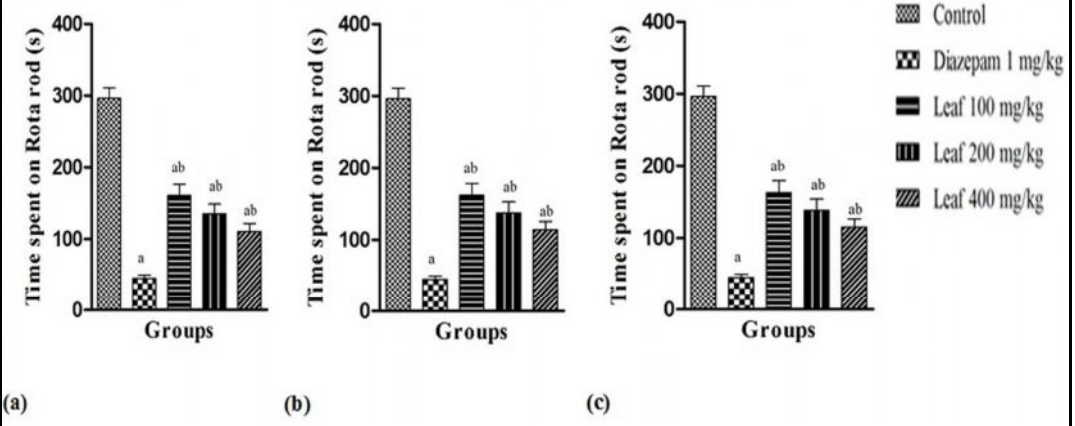

Fig 5: Effects of *G. glauca* leaf methanol extract (GGLME) on muscle coordination in rota rod
Time spent (s) by the mice on rota rod: **a)** after 30 sec; **b)** after 60 sec; **c)** after 90 sec

[a]$P \leq 0.001$ indicates comparison with group I
[b]$P \leq 0.05$ indicates comparison with group II

4. Discussion

The plant *G. glauca* was earlier reported for its anti-anxiety effects [19]. Ethno medical uses assume that the above activity may be mediated through its effect on the CNS. Based on these findings in this study we attempted to evaluate the possible CNS depressant effects and muscle relaxant activity on methanol extract prepared from leaves of the plant. Extensive research in this class of drugs has flooded the market with synthetic drugs with more side effects causing cognitive dysfunctions, physical dependence, and tolerance [20].

Anxiety is a personal emotional state of fear felt by feelings of tension, worried thoughts, nervousness and physical changes like increased blood pressure showing its effect on person's physical and mental health. Depressants are used for relaxation that slows normal brain function and hence used in the treatment of various conditions of anxiety, stress, panic attacks, sleep disorders, sedatives and hypnotics, anticonvulsants and as well in soothing the nerves.

In the study GGLME exerted depressant effects on the CNS. Although the leaf extract did not induce sleep, the animals treated with the extract were found to be awoke, calm and relaxed. Nevertheless, administration of GGLME at single doses of 100, 200 and 400 mg/kg sixty min before the administration of Sodium pentobarbital resulted in decreased sleeping latency and increased sleeping time. The effects of plant extract are compared to the effect of standard drug diazepam, a classical benzodiazepine. The GGLME showed its ability in potentiating pentobarbital-induced hypnosis. This action may be due to its effects on the central mechanisms that are involved in the regulation of sleep or it may be due to inhibition of pentobarbital metabolism [21, 22]. The sedative effects of drugs are generally evaluated by measuring the sleep time which is induced by Sodium pentobarbital in laboratory animals [22, 23]. In our study results the GGLME prolonged sodium pentobarbital induced hypnosis which could be an indication of CNS depressant activity. It is well known that these CNS depressant effects are mediated through the GABA/benzodiazepine receptor complex [24] or it may be assumed that the GABAergic system participates in the GGLME induced enhancement effects of pentobarbital. Furthermore the depressant effect of GGLME is confirmed by its reduction in the exploratory behaviour and locomotor activity of mice. Diazepam is employed as a standard drug in behavioral pharmacology [25].

The response of the animal towards unusual environment is measured using hole-board. This test is widely employed to assess the anxiety, emotional status, and the response towards stress [26] and decreased number of head dips which is an indicative of CNS depressant effects [27]. In this test GGLME treated mice showed decrease number of head dips indicating the lowered levels of anxiety, and enhanced exploratory behaviour in them.

Open field test provides simultaneous measure of locomotion, exploration and anxiety [28]. Locomotion and rearing is a response to the levels of excitability of CNS. Through this test the CNS depressant actions of

the GGLME is further confirmed by the locomotion (decreased in the number of times the animal had crossed the squares) and decreased exploration response of the mice (rearing and object sniffing).

In Pentylenetetrazole (PTZ) and Picrotoxin induced convulsion test in mice, the seizure onset is the time taken from the injection of PTZ/Picrotoxin to the first myoclonic jerks of the forelimbs, which is considered the first sign of the beginning of a seizure activity [29, 16] The animals employed in this study were observed for various seizure stages which include Confusion/tremor, head twitches, individual jerks/jumps, orofacial seizure, clonic seizure, tonic seizure, straub tail and death. Idiopathic generalized epilepsy includes myoclonic seizures, absence seizures and generalized tonic-clonic seizures. In these types of epilepsy no nervous system abnormalities (brain and spinal chord) other than the seizures have been identified as of yet [30]. The generalized seizures include both clonic and tonic seizures. The clonic seizures are a rigid extension of the forlimbs/hindlimbs with or without loss of posture, while the clonic seizure consisted of rhythmic contractions of forelimbs /hindlimbs. GGLME exhibited a significant protection against convulsions induced by Pentylenetetrazole-(PTZ). It increased the threshold of clonic seizures induced by PTZ and delayed the progression to tonic convulsion. However, GGLME at all tested doses of 100, 200 and 400 mg/kg restricted the incidence of death (10-30%) in both PTZ and Picrotoxin induced convulsions. Agents that reduce $GABA_A$ synaptic functions/reduction in GABA mediated opening of the chloride ion channel/excitatory amino acids like glutamate and aspartate are considered as hypotheses for seizures. PTZ /Picrotoxin show its effect by inhibiting GABA activity. The standard drug diazepam acts by inhibiting the PTZ/Picrotoxin induced seizure by enhancing the action of GABA-A receptor thus facilitating the GABA-A receptor mediated opening of chloride ion channels [31]. The activity of GGLME may be due to the above said mechanisms.

Skeletal muscle relaxant is used in the treatment of muscle spasm and spasticity [32]. The test for muscle coordination was studied employing Grip strength test and Rota rod test. The muscular strength and neuromuscular functions in mice is assessed using grip strength test [17]. Loss of grip strength is measured as a muscle relaxant activity [33]. The activity of drugs interfering with motor coordination is evaluated employing rota rod test. The mice on revolving rod are allowed to spend time on it. The animal which spends less time indicates the muscle relaxant effect of the tested compound [18]. Many CNS depressive drugs are active in this test [31]. Our experimental findings are similar to that of standard drug diazepam used in the study of Grip strength test and Rota rod test which concludes that the GGLME may act similarly to that of diazepam.

5. Conclusion

The study concludes that *G. glauca* leaf methanol extract (GGLME) has significant CNS depressant effects and muscle relaxant properties against all the tested models. The results support the traditional claims of the plant in treatment of phobia, panic, stress, anxiety and it is as well used in producing calming effect on nerves. This suggests the biological constituents present in plant are worthy of further investigation and structure elucidation.

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