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Isolation, Characterization and Assessment for Central Nervous System Effects of Novel Phytomolecule from *Galphimia glauca* Cav. Stems

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The investigation had explored the *in vivo* central nervous system (CNS) effects of novel isolated phytoconstituent from *Galphimia glauca* stems. The isolated molecule coded as "BS-2" was subjected to toxicological, central analgesic and central nervous system depressant studies on Swiss albino mice and Wistar rats. The LD-50 of BS-2 was found to be > 2000 mg/kg. The mice treated with BS-2 at 12.5, 25 and 50 mg/kg doses exhibited significant effect in the hot plate test ($P < 0.5$) and tail clip test (83 % inhibition). Mice treated with BS-2 prolonged the time of sleep induced by sodium pentobarbital (40 mg/kg, i.p.). Throughout the hole-board experiment and open field test the BS-2 at 50 mg/kg exhibited a significant ($P \leq 0.001$) dose-dependent activity. The BS-2 was characterized by R_f value, melting point, IR spectra, mass spectra and ^{1}H NMR spectrum. The BS-2 showed potential *in vivo* central pain relieving and central nervous system depressant effects.

Keywords: Column chromatography, *Galphimia glauca*, Depressant effects.

INTRODUCTION

Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients, parts of plants, other plant materials or combinations. Herbs have their key role in traditional and complementary medicines. Currently the world is facing great challenges with non-communicable lifestyle related diseases like diabetes, heart diseases, hypertension, obesity, cancer, stroke, chronic obstructive pulmonary disease, cirrhosis, depression, heart attack, arteriosclerosis and migraine [1].

Traditional medicine comprises of medicinal aspirations of traditional knowledge that have been developed over generations within various societies before the era of modern medicine. According to WHO, the traditional medicine implies the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicine, spiritual therapies, manual techniques and exercises applied singularly or in combination to treat, diagnose and prevent illness and maintain wellbeing. Reaching the un-reached through traditional medicine, the Government of India had formed the Department of AYUSH with the prime objective of focusing its thrust mainly on growth, development, promotion and propagation of Indian traditional systems of medicines like Ayurveda, Unani, Siddha, Yoga, Naturopathy, Homeopathy and Sowa Rigpa. Natural sources of drugs provide solutions to most of these problems. Among them, plants are the primary source of many therapeutic lead molecules [2]. Till recently, there existed a vast list of traditional medicinal plants whose actual potential has not even been searched.

Galphimia glauca Cav. is a showy shrub with bright, colourful yellow petals, which belong to the family of the Malpighiaceae [3]. This shrub is spread across the states of India. It is found growing abundantly in the uplands of the Deccan plateau, Telangana State. The plant is usually recalled as "*Calderona amarilla*" and "*Flor estrella*". The plant is employed to treat inflammation, pain and nervous excitement [4]. The tea prepared from yellow leaves is drunk to relieve fever, coronary pain and to soothe the nerves. The aerial parts of *G. glauca* were reported to control asthma through inhibition of LTD_4 induced muscle contraction [5]. Tortoriello *et al.* [6] cited the chemical structures of the new nor-seco-triterpene molecules isolated from the methanol extract of *G. glauca* aerial parts.

The solid-phase extraction procedure is opted to isolate phenolic acids from *G. glauca* with the aid of zirconium silicate and bismuth citrate as sorbants and their efficiency was determined by using HPLC-DAD [7]. Galphimines *i.e.*, Galphimine-A, B and C were reported by Cardoasa Taketa *et al.* [8]. The ongoing phytochemical research work replaces the extracts with potential molecules having a significant biological activity, where efficient chemical molecules obtained from medicinal herbs.

To confirm the traditional use, in previous research work, we have disclosed that *G. glauca* leaf and stem methanol extracts showed potent analgesic and anti-inflammatory activities [9,10]. In other research work, we disclosed the CNS depressant effects and muscle relaxant properties of *G. glauca* stem and leaf methanol extract [11,12]. Observing the substantial results of the *G. glauca* stem methanol extract with CNS depressant properties through our earlier work, the present work was carried out as a continuation of earlier work in our research lab. This study is primarily focused on CNS depressant activity guided isolation and characterization of a new molecule form stems of *G. glauca* Cav.

EXPERIMENTAL

The plant *Galphimia glauca* Cav. (GG) was collected from the lawn present in the campus of Anurag Group of Institutions. The stems were collected in June 2017, dried under shade and powdered. *Galphimia glauca* Cav. was identified and authenticated by Taxonomist, Dr. E.N. Murthy, Satavahana University, Karimnagar, India. A voucher copy is preserved with the number No. 333 at Department of Pharmacognosy and Phytochemistry, School of Pharmacy, Anurag Group of Institutions, Medchal District, India.

All the required chemicals were purchased from SD Fine chemicals, Mumbai, India. Morphine weas purchased from Troikaa Pharmaceuticals Inc. India. The sodium pentobarbital, diazepam and pentylenetetrazole were purchased from Sigma Chemicals Co., USA, Natco Pharmaceuticals, India Inc. and Sigma-Aldrich, USA, respectively. Picrotoxin and naloxone was received as generous gift sample from Sri Disha Biotech, Mallapur, Hyderabad, India and Samarth Pharma Inc.

Melting point was performed on a Lab India melting point apparatus [LabIndia, India]. JASCO FT/IR-4600 spectrophotometer was employed to record IR Spectra [JASCO, Mary's Court, Easton, USA]. A mass spectrum was recorded with Waters Acquity Xevo TQ MS, LC/MS/MS System [WATERS (INDIA) Pvt. Ltd, Bangalore, India]. ^{1}H NMR spectra was recorded on JEOL JNM-ECZ500R/S1 NMR spectro-photometer [JEOL (INDIA) Pvt. Ltd, New Delhi, India]. The DMSO-d_6 solvent was employed in ^{1}H NMR studies. Silica gel used for Column chromatography (# 230-400) was procured from Finar, India and Preparative TLC chromatography (# 350) was procured from SD Fine Chemicals, Mumbai, India. Aluminium based pre-coated plates were procured from Merck, Germany. Rotary evaporator was employed to recover solvent from fractions [Heidolph rotatory evaporator, Germany].

Preparation of the extract: 0.20 Kg of *G. glauca* stem powder was subjected to Soxhlet extraction employing 0.6 L of methanol. The *G. glauca* stem methanol extract (GGSME) was collected, dried and stored. The yield obtained was 0.030 Kg.

Animals: For this study, mouse (Swiss albino strain) and rats (Wistar strain) were used. The mice of 40-60 days old (22 ± 2.5 g) rats 84 to 98 days old (235 ± 25 g of either sex were employed. Animals were acclimatized for duration of ten days in the research lab. The animals were provided with necessities for the care, hygiene, nutrition and environment [temperature (22 ± 2 °C); relative humidity (45-55 %); light (fluorescent tube lights; noise (< 65 decibels)]. The studies were carried out randomly with six mice/rats of either sex in each group. The work protocol was approved by the Institutional Animal Ethics Committee of the institute (IAEC), School of Pharmacy, Anurag Group of Institutions (the protocol number: I/IAEC/LCP/032/2014/16).

Acute toxicity studies: According to The Organization for Economic Co-operation and Development (OECD) guidelines, 423-2d, acute oral toxicity studies were conducted [13].

Fractions of GGSME: In our previous experimental work carried out on stem extract, the stem methanol extract showed significant CNS depressant and muscle relaxant properties [12]. Hence, in the present research the active stem extract, GGSME was subjected to fractionation employing solvents of varying polarities (*n*-hexane, chloroform, ethyl acetate and methanol). For this purpose GGSME (0.03 Kg) was dissolved in methanol (0.075 L) and fractioned with 0.4 L each of *n*-hexane, chloroform, ethyl acetate and methanol. The fractions were concentrated, yield obtained was registered and coded for proper identification [GGH: *G. glauca n*-hexane fraction; GGC: *G. glauca* chloroform fraction; GGEA: *G. glauca* ethyl acetate fraction; GGM: *G. glauca* methanol fraction].

Phytochemical screening for GGSME fractions and BS-2: Phytochemical screening was performed to disclose the existence of various phytoconstituents in GGSME fractions and the new isolated molecule BS-2 [14]. The methanol fraction (GGM) showed positive results, disclosing the phytoconstituents present such as terpenoids, steroids, saponins, tannins, flavonoids and phenolic compounds, whereas the remaining fraction (*n*-hexane, chloroform and ethyl acetate fractions) showed clear negative results. The new isolated molecule (BS-2) belongs to the chemical class of terpenoid.

Isolation and characterization of phytoconstituents

Preparation of sample for isolation: *G. glauca* methanol fraction (GGM, 0.015 Kg) was dissolved in water and methanol (200 mL + 200 mL), extracted with ethyl acetate (1:1 ratio). The ethyl acetate fractions were pooled, concentrated and then extracted with hexane (1:5 ratio). The hexane insoluble fraction was concentrated and then dissolved in a small quantity of ethyl acetate. Silica powder (75 g) was admixed to the above ethyl acetate solution to coat its surface. It was then subjected to vacuum evaporation in Heidolph rotary evaporator and then dried and stored in vacuum desiccator until use. The obtained silica powder was activated at 110 °C for using in the column.

Column chromatography: The borosilicate column, measuring 75 cm length and 3 cm internal diameter was employed for this study. The silica gel with particle size 230-400 µm was used for this study.

Procedure: The column was washed, dried and rinsed with solvent before using. A small piece of cotton was placed at nozzle tip, which aids in filtration. The slurry was prepared by admixing silica gel (150 g) with chloroform, which was then employed in packing the column. The surface coated silica powder for the isolation of phytoconstituents was loaded into the column and the column was eluted with chloroform (400 mL) initially, followed by ethyl acetate in chloroform (0, 10, 20, 30, 40, 50, 52.5, 55, 57.5, 60, 62.5, 65, 67.5, 70, 72.5, 75, 77.5, 80, 90 and 100 %). 200 mL of each solvent preparation was used for elution. Each fraction was collected in 25 mL volume and labelled for its identification.

Thin layer chromatography (TLC): All the fractions were concentrated and subjected to TLC studies employing ethyl acetate and chloroform as mobile phase. Separated phytomolecules were visualized employing 10 % of H_2SO_4 in methanol as charring solution. The similar fractions identified were pooled and labelled.

Preparative TLC studies: The fractions **13-17** of column chromatography were employed for phytoconstituent separation. For this study, the silica gel slurry was prepared by mixing distilled water (1.5-2.5 parts) to silica gel (1 part) and stirred perfectly. The resultant is employed for coating the glass plates by adopting 'pouring method'. The slurry was poured in the middle of ten glass plates (2 × 4 inch) arranged in a row and then distributed uniformly, kept undisturbed for 10-15 min and then air dried for duration of 45 min. The plates were then activated at 110 °C for of 2 h in hot air oven. The sample was spotted and developed in a glass tank with mobile phase, 10 % methanol in chloroform. The separated molecules were cut, scraped and collected.

Characterization of the new isolated molecule (BS-2): The uniquely isolated new phytomolecule was coded as "BS-2". The BS-2 was characterized by its melting point, phytochemical screening, R_f value, IR spectra, ^{1}H NMR spectra and Mass spectra. The DMSO-D6 was used to dissolve BS-2 and NMR studies were conducted using JEOL USA spectrophotometer (JNM-ECZ500R/S1).

Pharmacological studies: Studies were carried out to evaluate the *in vivo* central analgesic effects and CNS depressant effects for the isolated molecule (BS-2) to explore its potential activities. The dose of 12.5, 25 and 50 mg/kg was administered orally for evaluating the studies.

Central analgesic activity

Hot plate method: This study was carried out on hot plate (V.J. Instruments, India) to evaluate the pain mediated centrally as stated previously by Ishola *et al.* [15]. The mice (n = 6) were placed on the hot plate which was set and maintained at temperature 55 ± 1 °C. The reaction time was registered with the time taken by the animal to blow/lick/jump off the surface of hotplate. Mice were grouped (I-VII) and pre-treatment response was registered. 60 min after oral and 30 min after intraperitoneal delivery, the post-treatment response was registered at 30, 60 and 90 min respectively.

Group I: Received distilled water [10 mL/kg, body weight (b.w.), per oral (p.o.)].

Group II: Treated with morphine [10 mg/kg, b.w., intraperitoneally (i.p)].

Groups III-V: Treated with BS-2 [12.5, 25 and 50 mg/kg, b.w., respectively, (p.o)].

To assess central opioid receptors involvement further, a study was performed. Individually, two groups (n = 6) of mice, Group VI and VII were pre-challenged with 5 mg/kg intraperitoneal administration of naloxone, 15 min earlier to oral and intraperitoneal administration of BS-2 (50 mg/kg) and morphine (10 mg/kg) respectively. The response of all animals were registered before and after the treatment as per the hot plate method [16].

Haffner's tail clip method: This model was adopted to prove the central analgesic effect as stated previously by Ishola *et al.* [15]. Initially the rats used were screened for inducing pain at tip of the tail with a metal artery clip. The rats, which did not dislodge the clip in 10 s were removed from the experiment. Remaining animals (n = 6) were groped (I-V) and pre-treatment response time was registered for individual animal. Groups I to V were treated as mentioned in above procedure of hot plate method.

After 1 and 0.5 h of oral and intraperitoneal treatment of the BS-2 and reference drug, the same procedure was repeated for recording the post-treatment response time.

$$\text{Inhibition (\%)} = \frac{\text{Post treatment latency} - \text{Pre treatment latency}}{\text{Cut off time} - \text{Pre treatment latency}} \times 100$$

CNS depressant activity

Test for sodium pentobarbital induced sleeping time: This sleeping time test was mentioned earlier by Fujimori [17]. The sedative and hypnotic action of BS-2 together with sodium pentobarbital was studied. For this study, mice were grouped as cited below. Group I was treated as negative control and received distilled water ahead of intraperitoneal administration of the sodium pentobarbital (40 mg/kg). Group II treated as positive control received intraperitionally diazepam (1 mg/kg), Groups III to V were treated with BS-2 1 h before the administration of sodium pentobarbital. Individual mice were monitored to record the uncoordinated movements to the sedative phase of the test. Loss of the righting reflex and the sleep period was also registered. The time period elapsed between the loss and recovery of the righting reflex was treated as the sleeping time [18].

Group I: Negative control, received per oral (p.o) distilled water [10 mL/kg]

Group II: Positive control, received diazepam intraperitoneal (1 mg/kg)

Group III-V was treated with BS-2 [12.5, 25 and 50 mg/kg]

Hole-board test: This test was reported by Boissier *et al.* [19] to evaluate behaviour of animals like curiosity and/or exploration. The wooden box of 50 cm × 50 cm × 30 cm with 4 equidistant holes with diameter 3 cm was employed for the study. The animals in group I received only distilled water. The animals in group II received diazepam (1 mg/kg, i.p.) 30 min ahead of performing the test. BS-2 was orally administered to groups as cited in procedure to test for sodium pentobarbital induced sleeping time. After 1 h, each mice was placed in the centre of the apparatus and overall count of head-dips and number of rears was recorded for a time 5 min. The floor of the apparatus was cleaned after each trial. A decrease in the

total count of head dips/dipping time of the head and the total number of rears compared to the control was regarded to indicate a sedative effect [20].

Open field test: This test was initially reported by Barros *et al.* [21]. This test is a non-conditioned anxiety test to register locomotion, the speed of locomotion, rearing as well as general motor activity. The test was carried out according to the procedure earlier described by Lopez-Rubalcava *et al.* [22] with some changes. The apparatus consist of plywood (60 cm × 60 cm × 40 cm) framed with glass to observe mice. The floor of cardboard was subdivided into 12 squares, which were equally spaced. The mice were grouped as cited in procedure to test for sodium pentobarbital induced sleeping time. The group I animals served as negative control; Group II received intraperitoneal diazepam (1 mg/kg) and groups III to V were treated with BS-2 as stated in test for sodium pentobarbital induced sleeping time. After 30 min and 60 min post treatment of standard and BS-2 administration, the behaviour of individual mice was registered through video recording for a period of 5 min after placing individual mouse in the corner of the apparatus. The locomotion [(The total number of times each mouse entered each square per 5 min], rearing (the frequency with which the mouse stood on its hind legs) was monitored and recorded [23].

Statistical analysis: The experimental results were disclosed as Mean ± SEM. Statistical analysis were performed with one-way analysis of variance (ANOVA), Tukey's multiple comparison test was followed to calculate the significance of results. The statistical analysis was performed using Graph Pad Prism 5.0 software.

RESULTS AND DISCUSSION

Acute toxicity studies: The GGSME, GGM and BS-2 did not register any toxicity and mortality in the dose range of 5, 50, 300 and 2000 mg/kg in mice and rat during the 2 weeks of study. Hence it can be classified as category 5 as stated in OECD-423, guidelines. So the GGSME, GGM and BS-2 were found to be non-toxic. Based on the yield obtained and acute toxicity results of the BS-2, the doses, 12, 25 and 50 mg/kg were chosen to evaluate the CNS activities.

Fractionations of GGSME: The active extract, GGSME was fractionated using *n*-hexane, chloroform, ethyl acetate and methanol. The yield obtained for fractions *n*-hexane (GGH), chloroform (GGC), ethyl acetate (GGE) and methanol (GGM) was registered as 6.6, 16.6, 20 and 56.6 % respectively.

Separation and characterization of phytomolecules

Column chromatography and TLC studies: The active fraction (GGM) was subjected to column chromatography. All fractions were collected in 25 mL volume and labelled. All the fractions of column chromatography were subjected to TLC studies. From the results, the fractions with identical R_f values were grouped and concentrated (Fraction No. 1-20).

Preparative TLC: The preparative TLC was performed on glass plates labelled A-H to separate the phytomolecules from column fractions (**13-17**) possessing similar phytomolecules using solvent methanol: chloroform (4:6). The separated fractions (**1-4**) were cut and collected from glass plates. The fraction cut **3** consisting of single molecule was then boiled with methanol (400 mL) and vacuum filtered. The obtained filtrate was concentrated followed by hexane treatment. The hexane insoluble fraction was concentrated to collect novel phytomolecule (BS-2). The yield obtained was 945 mg. The TLC of BS-2 was performed in methanol in chloroform mobile (10 %).

Characterization of BS-2: The BS-2 was identified as terpenoid. Its melting point is 196.8 °C and R_f value is 0.4. The IR spectrum revealed the presence of -OH, -C=C-, -CH_3, -C=O and -C-H at 3433.6, 2921.6, 2851, 1614 and 1384 cm^{-1}, respectively. The mass spectra (Fig. 1) showed the presence of base peak at 701.4 (100 %), [M+1] peak at 701.4, daughter ion at 679.4 (80 %). The ^{1}H NMR spectra disclosed the aliphatic group in range from 0.8 to 3.8 ppm, -OH at 4.18 ppm and aromatic protons at 6.2 to 7.5 ppm.

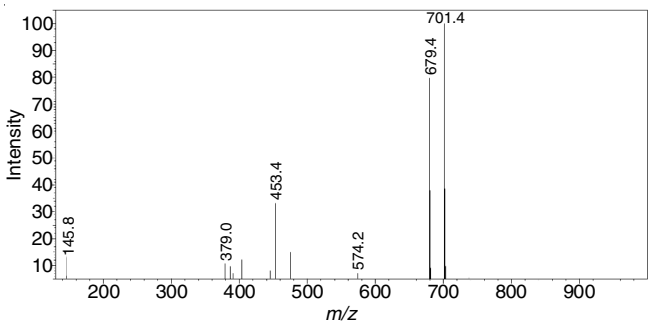

Fig. 1. Mass spectra of BS-2

Central analgesic activity

Hot plate method: The analgesic effects of BS-2 are shown in Table-1. The activity of BS-2 administered with low, medium and high dose was significant ($P \leq 0.5$) when compared with

TABLE-1
EFFECT OF BS-2 ON CENTRAL ANALGESIC ACTIVITY (HOT PLATE METHOD) IN MICE

Group	Dose (mg/kg)	Reaction time after administering control/BS-2			
		0 min	30 min	60 min	90 min
I (Distilled water)	10 (mL/kg)	3.3 ± 0.2	3.4 ± 0.2	3.3 ± 0.1	3.3 ± 0.1
II (Morphine)	10	3.3 ± 0.3	8.1 ± 0.2 [a]	10.0 ± 0.3 [a]	13.1 ± 0.1 [a]
III (BS-2)	12.5	3.5 ± 0.3	5.4 ± 0.2 [a, b]	7.1 ± 0.1 [a, b]	10.7 ± 0.2 [a, b]
IV (BS-2)	25	3.8 ± 0.1	6.1 ± 0.2 [a, b, c]	8.0 ± 0.1 [a, b, c]	11.4 ± 0.2 [a, b]
V (BS-2)	50	3.7 ± 0.2	7.5 ± 0.3 [a, c]	9.5 ± 0.3 [a, b, c]	12.9 ± 0.1 [a, b,c]
VI (Naloxone + Morphine)	(5 + 10)	3.6 ± 0.5	3.5 ± 0.4 [b]	3.4 ± 0.7 [b]	3.4 ± 0.1 [b]
VII (Naloxone + BS-2)	(5 + 50)	3.7 ± 0.1	3.6 ± 0.6 [b]	3.6 ± 0.4 [b]	3.6 ± 0.3 [b]

BS-2: Novel isolated molecule; Values are expressed as mean ± SEM.; n = 6; the statistical significance done by ANOVA, followed by Tukey's multiple comparison tests and is represented by a symbol. [a]$P < 0.001$, indicates comparison with group I. [b]$P < 0.5$, indicates comparison with group II. [c]$P < 0.5$, indicates the dose dependent activity on comparison of the high dose with respective low dose of the BS-2.

reference standard morphine (10 mg/kg) and with control group ($P \leq 0.001$).

The BS-2 showed its central analgesic effects with its highest dose (50 mg/kg), which was found significant ($P \leq 0.5$) with morphine. It was confirmed when naloxone treated groups reversed the pain reliving property. The study results are cited in Table-1.

Haffner's tail clip test: The study results are illustrated in Fig. 2. The effect of BS-2 that was given orally with 12.5, 25 and 50 mg/kg showed dose dependent inhibition of pain, with 80 % inhibition at higher dose when compared with standard group.

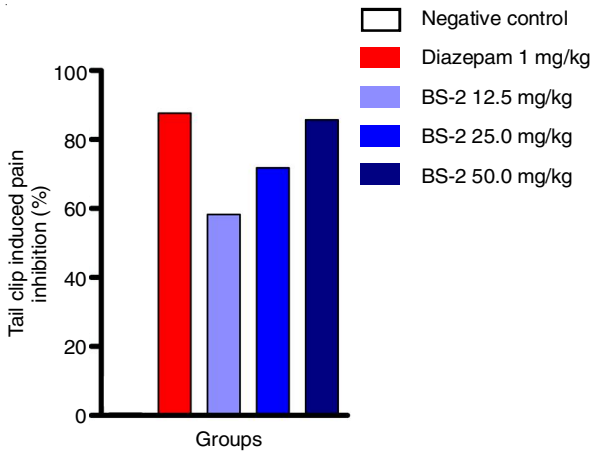

Fig. 2. Effect of BS-2 on Tail clip induced pain in rats (Haffner's tail clip test)

CNS depressant activity

Test for sodium pentobarbital induced sleeping time: The sedative and hypnotic actions of BS-2 are shown in Fig. 3. The BS-2 exhibited a significant ($P \leq 0.05$), dose dependent and synergetic actions in combination with sodium pentobarbital. The sleep time of sodium pentobarbital was significantly ($P \leq 0.05$) extended in a dose-dependent manner with BS-2 at higher dose (50 mg/kg).

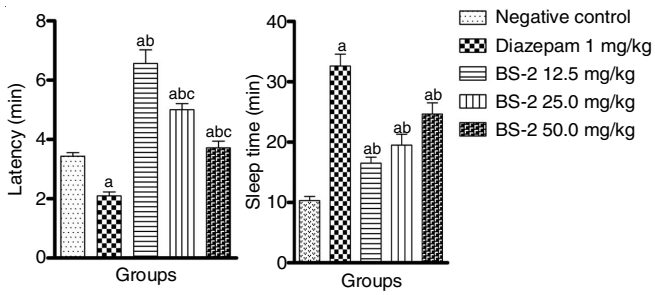

Fig. 3. Depressants effect of BS-2 on sodium pentobarbital induced sleep in mice; Values are expressed as Mean ± SEM.; n = 6; the statistical significance done by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests and is represented by a symbol, $^{a}P \leq 0.05$ indicates comparison with group I, $^{b}P \leq 0.05$ indicates comparison with group II, $^{c}P \leq 0.05$ indicates the dose dependent activity in comparison of the high dose with respective low doses of BS-2

Hole-board test: The sedative action of BS-2 was confirmed in this test. Fig. 4 disclosed the effects of BS-2 and diazepam

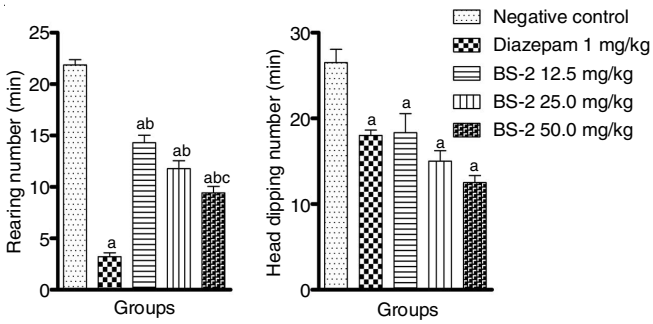

Fig. 4. Effect of BS-2 on hole-board test in mice; Values are expressed as Mean ± SEM.; n = 6; the statistical significance done by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests and is represented by a symbol. $^{a}P \leq 0.01$ indicates comparison with group I, $^{b}P \leq 0.001$ indicates comparison with group II, $^{c}P \leq 0.001$ indicates the dose dependent activity in comparison of the high dose with respective low doses of BS-2

(1.0 mg/kg) of different doses on the performance of animals in this test. Treatment with BS-2 (50 mg/kg) significantly ($P \leq 0.01$) lowered the total head-dipping number (13.8 ± 0.4) and number of rears, when correlated with group control.

Open field test: The CNS effect of BS-2 was confirmed by noticing the way each mouse behaved in open field test. The BS-2 significantly ($P \leq 0.001$) lowered the rearing cum spontaneous ambulatory activity of mice. The experimental results are represented in Fig. 5.

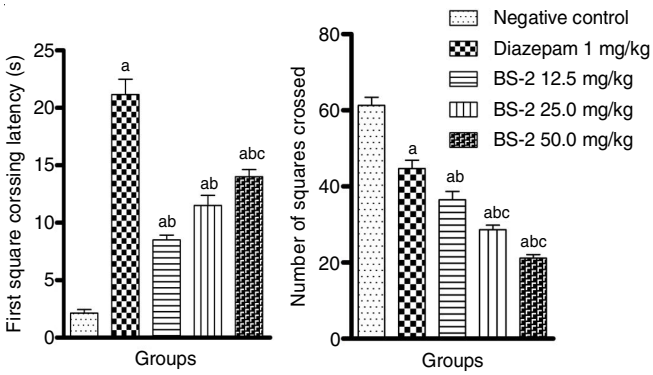

Fig. 5. Effect of BS-2 on the ambulatory behaviour of mice in open field test; Values are expressed as Mean ± SEM.; n = 6; the statistical significance done by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests and is represented by a symbol, $^{a}P \leq 0.001$ indicates comparison with group I, $^{b}P \leq 0.001$ indicates comparison with group II, $^{c}P \leq 0.05$ indicates the dose dependent activity in comparison of the high dose with respective low doses of the BS-2

Many of the substantial molecules of therapeutic potential have obtained from medicinal plants. It is therefore vital to tap the potential of natural drugs. In earlier research, we have proved the anti-inflammatory and analgesic activities of *G. glauca* leaf and stem parts. The *G. glauca* stem methanol extract (GGSME) exhibited potential anti-inflammatory and analgesic activity compared to leaf extract [9,10]. The emphasis of this study is to isolate, characterize and evaluate the therapeutic effects of novel phytomolecule from GGSME.

The GGSME was fractioned with *n*-hexane, chloroform, ethyl acetate & methanol and then subjected to phytochemical screening to disclose the phytoconstituents. The results

explored the phytoconstituents like phenolic compounds and tannins, terpenoids and steroids, saponins and flavonoids in methanol fraction (GGM) when compared with the remaining fractions.

Based on the phytochemical screening results, the active fraction (GGM) was subjected to column chromatography. The solvent system for column chromatography was chosen based on the results of phytochemical screening, literature, polarities of expected phytomolecules and TLC studies. The column elution was started initially with chloroform and ethyl acetate and then gradually the polarity of solvent system was enhanced for effective elution. TLC's were performed for all the fractions, among them the fractions 13, 14, 15 16 and 17 contained similar constituents identified with R_f values. These fractions were combined and subjected to preparative TLC using solvent methanol: chloroform (4:6).

The separated phytomolecule (BS-2) was purified with hexane treatment and identified as terpenoid by chemical test. The IR spectrum, revealed the presence of -OH, -C=C-, -CH_3, -C=O and -C-H at 3433.6, 2921.6, 2851, 1614 and 1384 cm^{-1}, respectively. The mass spectrum revealed the presence of base peak at 701.4 (100 %), [M+1] peak at 701.4, daughter ion at 679.4 (80 %). The results of proton NMR revealed the aliphatic group at range from 0.8 to 3.8 ppm, -OH at 4.18 ppm and aromatic protons at 6.2 to 7.5 ppm.

The central analgesic and CNS depressant activities were performed for the novel isolated molecule BS-2. The *in vivo* analgesic activity was carried out employing central pain models like the hot plate test and Haffner's tail clip test models [15]. The sodium pentobarbital induced sleep test, Hole-board test and Open field tests were performed to prove the CNS depressant effects. The LD-50 of BS-2 was registered to be > 2000 mg/kg. The result was significant ($P < 0.5$) in hotplate and tail clip tests (83 % inhibition of pain) confirming the central analgesic actions. The results proved significant ($P < 0.05$) in a dose dependent manner. The central actions were further disclosed treatment with naloxone. The BS-2 also exhibited actions similar to morphine in inhibiting the pain.

The opioids bind to target receptors in the CNS. The primary classes of opioid receptors include μ receptors like μ_1 and μ_2, kappa receptors like κ_1 and κ_2 and delta receptors like δ_1 and δ_2 respectively. The central drugs show their effects through modulation of spinal receptors like μ_2, κ_1 & δ_2 and supraspinal receptors like μ_1, κ_3, δ_1 & σ_2, respectively [24]. Opioid are G-protein coupled receptors, which cause decrease of adenylcyclase activity resulting in reduced formation of the cAMP. Opioids exhibit both presynaptic and post synaptic actions resulting in closure and opening of Ca^+ and K^+ channels. The results explain the central analgesic effects of BS-2 through the involvement of central mechanisms as discussed above. Similar type of results was reported by Zakaria *et al.* [16].

In this study BS-2 showed CNS depressant effects. The administration of BS-2 with 12.5, 25 and 50 mg/kg, b.w. 60 min before the treatment of sodium pentobarbital resulted in decreased sleep latency and enhanced sleep time. The sedative action is a central nervous system depressant effect and the sedative actions are generally assessed by checking sodium pentobarbital induced sleep time in study animals [17,18].

The pentobarbital works by depressing the reticular activating system, which regulates wakefulness, sleep, level of arousal and coordination of the eye movements. Sleep latency was decreased and the total number of awakenings was reduced.

The time spent in REM sleep and slow wave sleep was reduced, while the non-REM sleep was enhanced. There was an enhanced reaction time and the animal could not be aroused easily. These depressant actions are due to their affinity for $GABA_A$ receptors and AMPA receptor, thereby inhibiting the glutamate effects and thus the overall effect results in producing CNS depressant actions [24,25]. The BS-2 in a dose-dependent reinforced sodium pentobarbital-induced hypnosis assuming that the GABAergic system participates in the BS-2 induced enhancement actions of pentobarbital.

Diazepam was used to produce sedative-hypnotic actions. It enhances sleep time by reducing sleep latency and night time awakenings. It exhibits GABAergic actions by binding to $GABA_A$ receptors (both α and γ subunits) causing opening of chloride ion channel indirectly and enhances membrane hyperpolarization. The actions of BS-2 are found equally similar to that of diazepam [25].

Hole-board test is used to assess the response of animals to conditions like emotion, anxiety and stress [26]. The reduced head dips count is a proof of CNS depressant activity [27]. In this evaluation test, BS-2 treated mice demonstrated a significant ($P \leq 0.001$) lowered number of head dips implying the enhanced exploratory behaviour and lowered anxiety levels.

The open field test is used to assess locomotion, exploration and anxiety [28]. Rearing and locomotion is the response to intensified levels of CNS excitability. This experiment proved the dose dependent depressant actions of BS-2 by the locomotion (cut down in the total number of times the animal had crossed each squares) and exploration (cut down in object rearing and sniffing) [25]. The BS-2 lowered the ambulatory and rearing activities of the animals.

The diazepam is given in medical care for diverse disorders like generalized anxiety, phobia, panic, stress and obsessive compulsive disorder [29,30]. In-hole board test diazepam exposed its actions in lowering anxiety, achieving the state of quiet and calmness and improved the exploratory behaviour of animals. In open field test, diazepam was more effective in managing the hyperexcitability of the CNS.

GABA is the principal CNS inhibitory neurotransmitter and diazepam increases the GABAergic inhibition effect at diverse levels of the CNS. $GABA_A$ α_2, α_3 and α_5 subunits mediate anxiolytic effects of benzodiazepines, considering that α_1 receptor mediate the sedative actions [30]. The activity of BS-2 noticed might be due to the above discussed mechanisms.

Conclusion

The isolated novel phytomolecule (BS-2) belongs to terpenoid. It showing significant analgesic activity might be by way of central sites and it also exhibited significant CNS depressant properties. This work aids in the reciprocity of research and assists clinical use. The research will be further more extended for getting the probable structure of phytomolecule with spectral data like ^{13}C NMR, 2-D NMR (HSQC, HMBC, COSY, NOESY *etc.*).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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