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ORIGINAL ARTICLE

Innovative Research on Isolation, Characterization, and Identification of Bioactivity in the Isolated Constituent from Methanol Extract of *Galphimia glauca* cav. Stems

Baba Shankar Rao Garige[1,2], Keshetti Srisailam[3], Vattikuti Uma Maheshwara Rao[4]

[1]Department of Pharmacognosy and Phytochemistry, School of Pharmacy, Anurag Group of Institutions, Medchal, [2]Centre for Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Hyderabad, [3]Department of Pharmacy, University College of Pharmaceutical Sciences, Satavahana University, Karimnagar, Telangana, [4]Department of Pharmacy, Teegala Ram Reddy College of Pharmacy, Hyderabad, India

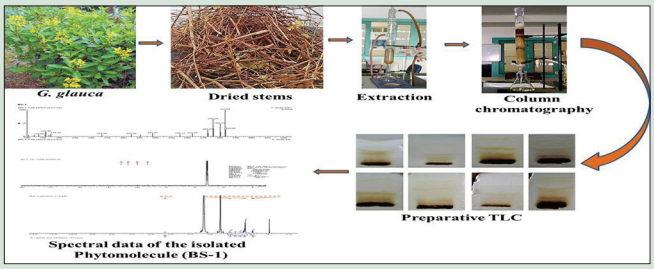
ABSTRACT

Background: *Galphimia glauca* Cav (GG) is naturalized in tropical and subtropical regions of the world including India. **Objective:** The present study has been opted to shed light on GG stems to isolate, characterize, and explore the analgesic and anti-inflammatory potential of the isolated phytomolecule using *in vivo* models. **Materials and Methods:** The bioactive fraction of the active stem methanol extract was subjected to column chromatography followed by preparative thin layer chromatography to separate the phytoconstituent. The isolated phytoconstituent was characterized and evaluated for toxicological studies, analgesic, and anti-inflammatory activity. **Results:** The isolated phytoconstituent "BS-1" was characterized by melting point, Rf value, IR spectra, mass spectra, ^{1}H-NMR spectrum, and ^{13}C NMR spectrum. The LD_{50} of BS-1 was found to be >2000 mg/kg. The results were significant ($P < 0.001$ and $P < 0.01$) in hot plate test and tail clip test. The central analgesic effect of BS-1 was further proved through reversal actions of naloxone. The peripheral analgesic actions exhibited by BS-1 were significant ($P < 0.001$) in formalin and writhing test when compared to control group. In carrageenan test, BS-1 exhibited significant ($P \leq 0.05$) dose-dependent activity on comparison of the high dose with respective low dose. The BS-1 exhibited significant ($P \leq 0.05$) anti-inflammatory activity, when correlated with the standard drug in cotton pellet induced granuloma test. **Conclusion:** The BS-1 exhibited significant analgesic and anti-inflammatory activity in central and peripheral models of analgesic activity and in acute and chronic models of anti-inflammatory activity.

Key words: Analgesic, anti-inflammatory, column chromatography, formalin test, *Galphimia glauca* Clav, hotplate test, isolation

SUMMARY

- Pain is an unpleasant sensory and emotional experience associated with tissue damage, whereas inflammation is a body defense reaction. The novel molecule coded as "BS-1" was isolated from *G. glauca* stem methanol extract (GGSME).
- The BS-1 isolated from methanol fraction (GGM) of GGSME exhibited significant activity in relieving both central and peripheral pain; in addition it was significant in treating acute and chronic inflammation. These results were significant and dose dependent.



Abbreviations Used: GG: *Galphimia glauca*, GGSME: *Galphimia glauca* stem methanol extract, GGH: *G. glauca* n-hexane fraction, GGC: *G. glauca* chloroform fraction, GGEA: *G. glauca* ethyl acetate fraction, GGM: *G. glauca* methanol fraction, IAEC: Institutional Animal Ethics Committee, CPCSEA: Committee for the Purpose of Control and Supervision of Experimental Animals, OECD: The Organization for Economic Co-operation and Development, WHO: World Health Organization, b.w: Body weight, i.p: Intraperitoneal, p.o: per oral, NSAIDS: Nonsteroidal anti-inflammatory drugs, IPE (%): Indicates percentage inhibition of paw edema, ^{1}H-NMR: Proton nuclear magnetic resonance, ^{13}C NMR: Carbon-13 nuclear magnetic resonance, LCP: Lalitha College of Pharmacy, AGI: Anurag Group of Institutions, BS-1: Novel isolated molecule.

Correspondence:
Dr. Baba Shankar Rao Garige,
Department of Pharmacognosy and Phytochemistry, Anurag Group of Institutions, (Formerly Lalitha College of Pharmacy), Venkatapur Village, Ghatkesar Mandal, Medchal - 501 301, Telangana, India.
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INTRODUCTION

India is a center of alternative systems of medicines. This is not only due to the sustained practice of traditional medicines in the country but also due to the universal recognition of these authentic therapies and medicines which are now the vast and fast-growing networks of health resources and hospitals of traditional medicines. India had a great long tradition of science and revolution to evolve its own native system of healthcare such as Ayurveda, Sidda, and Yoga. Over the time, Indian medicine has also interacted with diverse civilizations and assimilated other systems of medicine as well.

At present, the world is facing great challenges with lifestyle-related diseases and noncommunicable diseases.[1] Herbal drugs offer solutions to these problems. Among the natural sources of drugs, plants, in particular, serve as the primary source of lead molecules of therapeutic significance.[2] Till date, there have been a very large number of traditional medicinal plants whose actual potential is not yet explored.

Galphimia glauca Cav. (GG) is one such shrub which grows up to a height of 2–3 m, belonging to the family of *Malpighiaceae*.[3] This

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shrub is universally dispersed. In India, it is spread across all the states and seen growing abundantly in Deccan plateau regions of India.

The bush is usually recognized as "*Calderona amarilla*" and "*Flor estrella*".[4,5] The ethyl acetate extract of GG aerial parts were reported for its potential anti-asthmatic properties which act through inhibiting the LTD_4-induced muscle contraction.[6] Nader *et al*., 2006, reported the triterpenoids production in the liquid cultivated hairy root of GG.[7] Aguilar-Santamaría *et al*., 2007, reported the cytotoxic and toxicological effects of GG aqueous, methanol, and ethanol extracts.[8] Tortoriello *et al*., 2011, reported the chemical structures of the novel nor-seco-triterpene compounds isolated through bioguided separation from methanol extract prepared from GG aerial parts.[9]

The solid-phase extraction technique is used to separate phenolic acids from the shrub GG by making use of bismuth citrate and zirconium silicate powders as sorbants and their efficacy was resolved by using HPLC-DAD.[10] Galphimines: Galphimine-A, Galphimine-B and Galphimine-C were reported by Cardoso Taketa *et al*., 2004; Tortoriello *et al*., 2011.[9,11] Terpenoids: with trivial names galphin-A, galphin-B and galphin-C and one nor-friedelane with trivial name galphimidin along with Flavanol: quercetin and two sterols, sitosterol 3-O-β-d-glucoside and stigmasterol were isolated from the aerial parts of GG as mentioned by del Rayo Camacho *et al*., 2002.[12]

The current phytochemical research replaces the herbal extracts with molecules having a potential biological activity, where effective chemical compounds come from medicinal plants.[13] Conventionally, a tea made from the yellow leaves of the plant GG is used to relieve coronary pain as well as for soothing the nerves, bringing down the fever and emolliating injuries. It is a remedy for pain, inflammation, and nervous excitement.[14,15]

To relate the traditional use, in our earlier studies we have proved that the stem and leaf methanol extract exhibited significant anti-nociceptive and anti-inflammatory properties.[14,15] In other studies, we proved the central nervous system (CNS) depressant effects and muscle relaxant properties of GG leaf and stem methanol extract.[16,17] Seeing the important outcomes of the GG stem methanol extract (GGSME) in treating pain and inflammation through our previous work, the current study was carried out as an extension of previous work at our institute. This study was principally concentrated on analgesic and anti-inflammatory activity guided isolation and characterization of a novel molecule form GG stems.

MATERIALS AND METHODS

Plant material

The evergreen shrub GG was collected from the lawn present in the Anurag Group of Institutions. The fresh stems were collected in July 2016, shade dried and powdered. The shrub was identified and authenticated by taxonomist, Dr. E. Narsimha Murthy, Satavahana University, Karimnagar District, Telangana State, India. A voucher copy is stored with the reference number No. 333, in the Department of Pharmacognosy and Phytochemistry, School of Pharmacy.

Chemicals and drugs

The chemicals used for the current study were acquired from SD Fine Chemicals, Mumbai, India. Morphine was acquired from Troikaa Pharmaceuticals, Inc. Gujarat State, India. Carrageenan was obtained from Sigma-Aldrich, USA (Merck group). The drugs Diclofenac sodium and Naloxone were received as gift samples from Novartis India Inc., and Samarth Pharma Inc. India, respectively.

Instrumentation, apparatus, and general conditions

Melting point was determined on a LabIndia melting point apparatus (LabIndia, Maharashtra, India). IR Spectra was recorded on a JASCO FT/IR-4600 spectrophotometer (JASCO, Mary's Court, Easton, USA). A mass spectrum was obtained with Waters Acquity Xevo TQ MS LC/MS/MS System (WATERS [INDIA] Private Limited, Bangalore, India). ^{1}H and ^{13}C NMR spectra were recorded on JEOL JNM-ECZ500R/S1 NMR Spectrophotometer (JEOL [INDIA] Private Limited, New Delhi, India). Chemical shifts (δ) are cited in parts per million (PPM). The special grade solvent (DMSO-D6) was used for NMR studies. Silica gel (# 230–400) used for column chromatography was purchased from Finar, Gujarat, India and preparative thin layer chromatographic (TLC) chromatography (#350) was purchased from SD Fine Chemicals, Mumbai. Precoated aluminium-based plates were purchased from Merck, Germany. Solvents were recovered from fractions using rotary evaporator (Heidolph rotatory evaporator, Germany).

Preparation of the extract

GG stem powder of 0.20 kg was subjected to Soxhlet extraction using 0.6 L of methanol. The stem extract was collected and then concentrated to dryness and stored. The yield obtained for GGSME was 0.030 kg.

Animals

For the current study, both species, mouse (Swiss albino strain), and rat (Wistar albino strain) were used. The mice of 42–56 days old (22.5 ± 2.5 g) and rats of 84–98 days old (234 ± 24.8 g) of either sex were employed. Animals were acclimatized for 10 days in the working laboratory environment. The animals were maintained with hygiene, care, and nutrition under proper environmental conditions such as temperature (22°C ± 2°C), relative humidity (45%–55%), and light (fluorescent tube lights were used as a source of light). Twelve hours of darkness and 12 h of light were maintained with noise <65 decibels. The entire animal studies were performed randomly with six animals (mouse/rat) of either sex in an individual group. The study protocol was approved by the Institutional Animal Ethics Committee of the institute, School of Pharmacy, Anurag Group of Institutions (the protocol number: I/IAEC/LCP/032/2013/15).

Acute toxicity studies

According to the Organization for Economic Co-operation and Development (OECD) guidelines, 423-2d, acute oral toxicity studies were conducted.[18]

Fractionations of *Galphimia glauca* stem methanol extract

In our earlier pharmacological studies conducted on stem extract, the stem methanol extract exhibited significant anti-nociceptive, anti-inflammatory properties.[14] Hence, in the current study, the active extract, GGSME was subjected to fractionation using solvents such as n-hexane, chloroform, ethyl acetate, and methanol. For this purpose, 0.030 kg of GGSME was dissolved in 0.075 L of methanol and fractionated with 0.4 L each of n-hexane, chloroform, ethyl acetate, and methanol. The obtained fractions (GG n-hexane fraction [GGH], GG chloroform fraction [GGC], GG ethyl acetate fraction [GGEA], and GG methanol fraction [GGM]) were concentrated, and the percentage yield was recorded.

Phytochemical screening for the *Galphimia glauca* stem methanol extract fractions and BS-1

Phytochemical screening was carried out to explore the nature of phytoconstituents present in various fractions

(GGH, GGC, GGEA, and GGM) obtained from the GGSME and the novel isolated molecule BS-1.[19] The methanol fraction (GGM) exhibited clear and positive results exploring the nature of phytoconstituents present in it such as steroids, terpenoids, saponins, flavonoids, tannins, and phenolic compounds, whereas the n-hexane, chloroform, and ethyl acetate fractions exhibited negative results. The novel isolated molecule (BS-1) belongs to a chemical class of terpenoid.

Separation of phytoconstituents and characterization

Preparation of sample for separation

About 0.015 kg of the GGM obtained is dissolved in equal volumes of methanol and water (200 ml + 200 ml), extracted twice with ethyl acetate (1:1 ratio). The ethyl acetate fractions were pooled, concentrated to about 50 ml volume and then extracted with hexane in the ratio of 1:5. The hexane insoluble fraction was separated, concentrated to obtain in powdered form. It was then dissolved in a small quantity of ethyl acetate.

Fifty grams of silica powder was added to the above ethyl acetate solution to coat its surface. It was then subjected to vacuum evaporation in Heidolph rotary evaporator and then dried. The powdered material was packed properly and stored in a vacuum desiccator. The obtained silica powder was activated at 110°C for using in the column.

Column chromatography

The column employed for this purpose is a borosilicate column, measuring the length of about 75 cm, with an internal diameter of 3 cm. The Silica gel with 230–400 μm particle size was used for this study.

Procedure

Column employed for this study was washed thoroughly, dried and rinsed with chloroform before using. A small cotton piece was placed in the column at nozzle tip, which aids in filtration. Silica gel about 150 g was used with chloroform to make a slurry. The prepared slurry was used for packing the column. After the column was packed with silica, the excess of solvent was drained out. The surface coated silica powder for the separation of phytoconstituents was loaded into the column, and a cotton piece was placed above it and the column was eluted with 400 ml of chloroform initially, followed by series of solvents with varying polarities. The solvent ethyl acetate in chloroform was employed for this purpose in varying percentages (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%). A volume of 200 ml of each solvent preparation was used for elution. Individual fractions were collected in 25 ml capacity and labeled for their identification.

Thin layer chromatographic studies

All the fractions collected were concentrated to about 10 ml and subjected to TLC studies using mobile phase (ethyl acetate: chloroform [80:20]), the plates were dried, and the separated compounds were visualized using charring solution (10% of sulphuric acid in methanol). The fractions that were identified as similar were pooled and labeled.

Preparative thin layer chromatographic chromatography

The combined fractions (13–17) of column chromatography were employed as a sample for separation of phytoconstituents. For preparative TLC, the adsorbent silica gel slurry was prepared by mixing 1.5–2.5 parts of distilled water to 1 part of silica gel and stirred perfectly. The resultant slurry was used for coating the glass plates. The technique adopted for coating the glass plates was "pouring method." Eight glass plates (labeled A-H) of size 2 inch × 4 inch were arranged in a row. The prepared slurry was poured at the center of glass plate and then uniformly distributed all over the surface. After leaving it for 10 min, the plates were air-dried for a period of 45 min. The plates were then activated in hot air oven at 110°C for of 2 h. The sample was spotted and developed in glass tank which was saturated earlier with the 10% methanol in chloroform mobile phase. The compounds which got separated were cut, scrapped, and collected separately.

Characterization of the novel isolated molecule (BS-1)

The uniquely isolated novel phytomolecule was coded as "BS-1." The BS-1 was characterized by its melting point, Rf value, phytochemical screening, IR spectral data, ^{1}H-NMR spectra, ^{13}C NMR spectra and mass spectral studies. The BS-1 was soluble in DMSO-D6, and NMR studies were carried out using JEOL USA Spectrophotometer (JNM-ECZ500R/S1).

Pharmacological studies on the novel isolated molecule (BS-1)

Studies were conducted to assess the *in vivo* anti-nociceptive and anti-inflammatory activities for the isolated molecule (BS-1) to explore its pharmacological significance. The dose range of 12.5, 25, and 50 mg/kg b.w was administered orally for assessing the studies.

Analgesic activity

Thermal stimulus model

Hot plate method

This test was carried out to assess the thermal stimulus-induced pain as described earlier by Ishola *et al*.[20] The mice were placed on the surface of the hot plate (V. J Instruments, India) which was heated and set constant at a temperature of 55°C ± 1°C. The reaction time was recorded as the time taken by the mice to lick/blow the hind or fore paw or jump out of the hot plate. All mice were grouped into six groups ($n = 6$) and pretreatment reaction time for all the animals was recorded. Sixty minutes after oral treatment and 30 min after i.p injection, the posttreatment reaction time was registered with time intervals of 30, 60, and 90 min, respectively.

- Group I received distilled water (10 ml/kg, body weight [b.w.], per oral [p.o.])
- Group II was treated with morphine (10 mg/kg, b.w., intraperitoneally [i.p])
- Groups III–V were treated with BS-1 (12.5, 25, and 50 mg/kg, b.w., respectively, [p.o]).

Evaluation of opioid receptors participation in the analgesic activity of the BS-1

This test was reported by Zakaria *et al*. to assess the central analgesic activity.[21] The BS-1 with a dose of 50 mg/kg given orally was evaluated for this test. Two groups of mice ($n = 6$) "i.e.,"; Group VI and Group VII were prechallenged with opioid antagonist, naloxone (5 mg/kg; i.p) 15 min earlier to the administration of morphine (10 mg/kg; i.p) and BS-1 (50 mg/kg; p.o), respectively. The reaction time of each mouse was registered before and after the treatment as per the procedure cited in thermal stimulus model.

Mechanical stimulus model

Haffner's tail clip method

This test is a model employed to prove the central analgesic activity as reported previously by Ishola *et al*.[20] The rats engaged in this study were at first screened for inducing pain at the tip of the tail by using a metal artery clip. The mice which failed to attempt to dislodge the metal artery clip in 10 s were discarded from the study. Animals were divided into V groups ($n = 6$) and pretreatment response time for each animal was registered. Groups I to V were treated according to the procedure of thermal stimulus model.

After 1 h and 0.5 h of oral and i.p. administration of the BS-1 and standard drug, the same procedure was perused for registering the posttreatment response time.

$$\text{Inhibition (\%)} = \frac{[\text{Posttreatment latency}]-[\text{Pretreatment latency}]}{[\text{Cut off time}-\text{Pretreatment latency}]}\times 100$$

Chemical stimulus model

Formalin test

This test was reported by Jimoh *et al.* to assess both central and peripheral pain.[22] The mice employed for this study were abstained from food overnight and were used for testing the formalin-induced pain. Groups I–V were treated according to the procedure of thermal stimulus model. Group VI received standard drug diclofenac sodium (20 mg/kg; i.p.). After 30 min of standard drug administration and 60 min after treatment with BS-1, formalin (20 µL of 2.5% solution) was injected subcutaneously into the right hind paw of an individual mouse. Each mouse was observed for pain responses in both phases (early phase [0–5 min] and in the late phase [15–30 min]), respectively. The time spent (sec) for biting/licking the hind paw was noticed and recorded.

$$\text{Inhibition (\%)} = \frac{\text{Reation time [control group]}-\text{reaction time [treated group]}}{\text{reaction time [control group]}}\times 100$$

Writhing test

The test was carried out to prove the peripheral analgesic activity as reported previously by William Carey *et al.*[23] The mice used for the test were divided into VII groups ($n = 6$) and kept on a fast overnight. Group I received water (10 ml/kg), Group II received standard diclofenac sodium (20 mg/kg; i.p.), whereas Group III to V received the BS-1 treatment in accordance with the procedure of thermal stimulus model. After 0.5 h of drug/BS-1 administration, all the mice were treated with 0.7% acetic acid (10 ml/kg; i.p.), and the numbers of writhing's were recorded for a duration of 0.5 h

$$\text{Inhibition (\%)} = \frac{\text{Number of writhes [control group]}-\text{Number of writhes [treated group]}}{\text{Number of writhes [control group]}}\times 100$$

Investigation of peripheral receptors involvement in the analgesic activity of the BS-1

This test was reported by Zakaria *et al.* to assess the peripheral analgesic activity.[21] Separately 2 groups of mice ($n = 6$), Group VI and Group VII were prechallenged with naloxone (5 mg/kg; i.p.) 0.25 h before the administration of diclofenac sodium (20 mg/kg; i.p.) and oral administration of BS-1 (50 mg/kg), respectively. 0.5 h later, the mice were subjected to writhing test and the results were recorded.

Anti-inflammatory activity

Carrageenan induced paw edema model

This test was performed according to the procedure described earlier by Kumar *et al.* to study the process of acute inflammation.[24] The rats were grouped into V groups ($n = 6$). Group I received water (10 ml/kg), Group II was treated with saline for the initial 7 days and with diclofenac sodium on the day of treatment (20 mg/kg; i.p.). Groups III–V were treated with appropriate doses of BS-1 for 7 days in sequence as cited in the procedure of Thermal stimulus model. The animals were kept on fast overnight and on the 8th day, i.e., 1 h after the dose of diclofenac sodium and BS-1 of varying doses, the paw edema was induced to animals of all the groups by introducing carrageenan into subplantar region of right hind paw (0.1 ml, 1% w/v in saline). The change noticed in the paw volume was registered at different time intervals (1st, 2nd, 3rd, and 4th h) in both diclofenac sodium and BS-1-treated groups before and after carrageenan challenge test by employing digital Plethysmometer (V. J Instruments, Maharastra, India).

$$\text{Reduction in edema (\%)} = \frac{[\text{Mean edema in control group}]-[\text{Mean edema in treated group}]}{[\text{Mean edema in control group}]}\times 100$$

Cotton pellet induced granuloma test

The method described by Aziz *et al.* was adopted in this study to explore chronic anti-inflammatory effects.[25] The rats used in this test were grouped into V groups ($n = 6$) and kept on fast overnight. Sterilized cotton pellets weighing 20 mg each were used. The animals were anesthetized by injecting urethane (1.5 g/kg; i.p.), and the skin incision was made on the dorsal side of the rats, and a sterilized cotton pellet was inserted subcutaneously, then finally the incision performed was closed employing surgical suture. Drug treatment was administered in a sequence for 7 days. Group I received water (10 ml/kg), Group II was treated with standard diclofenac sodium (20 mg/kg; i.p.), whereas Groups III to V were treated with relevant doses for 7 days in sequence as cited in the procedure of Thermal stimulus model. On day eight, the animals were anesthetized, and cotton pellets were taken out and foreign tissues was removed and dried for 1 day at 60°C and the dry weights were registered. The transudative and granuloma weights were registered and the percentage inhibition of granuloma tissue formation was determined with the formula mentioned below.

$$\text{Inhibition (\%)} = \frac{\text{Granuloma tissue weight [control group]}-\text{Ganuloma tissue weight [Treated group]}}{\text{Granuloma tissue weight [Control group]}}\times 100$$

Statistical analysis

The results were reported as a mean ± standard error of the mean statistical analysis were carried out with one-way analysis of variance (ANOVA), followed by Bonferroni post test and Tukey's multiple comparison test to calculate the significance of results. All the statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc. La Jolla, USA).

RESULTS

Acute toxicity studies

The GGSME, GGM, and BS-1 did not exhibit any toxic symptoms and also mortality in the range of 5, 50, 300, and 2000 mg/kg in rodent species (mice and rat) during the 14 days of study. Hence, it can be classified as category 5 according to OECD-423, guidelines. Therefore, the GGSME, GGM, and BS-1 were found to be nontoxic to rodents. Based on the toxicity results and the obtained yield of the BS-1, the appropriate doses, 12 mg/kg (low dose), 25 mg/kg (moderate dose), and 50 mg/kg (high dose) were chosen to assess the pharmacological studies.

Fractionations of *Galphimia glauca* stem methanol extract

The active extract, GGSME was fractionated using solvents such as n-hexane, chloroform, ethyl acetate, and methanol. The percentage yield obtained for n-hexane fraction (GGH), chloroform fraction (GGC), ethyl acetate fraction (GGE), and methanol fraction (GGM) are 6.6% 16.6%, 20%, and 56.6%, respectively.

Separation of phytoconstituents and characterization

Column chromatography

The bioactive fraction (GGM) was subjected to column chromatography. All fractions collected in 25 ml capacity and labeled for their identification. The scheme of phytoconstituents separation is cited in Figure 1.

Thin layer chromatographic studies

All the fractions of 25 ml capacity were collected from column chromatography and subjected to TLC studies. From the TLC results, the fractions containing phytoconstituents with identical Rf values were pooled and concentrated to dryness (Fraction No: 1-20).

Preparative thin layer chromatographic chromatography

The Preparative TLC was performed using column fractions (13–17) having identical phytoconstituent. The separation of phytoconstituents carried out with Preparative TLC. The separated fractions (1–4) were cut and collected separately from glass plates labeled A-H. The fraction cut 3 containing single, separated molecule was boiled with 400 ml of methanol in RBF and vacuum filtered. The filtrate was concentrated and subjected to hexane treatment. The hexane insoluble portion was concentrated to obtain novel isolated molecule (BS-1). The yield obtained was 856 mg. The TLC of the isolated novel molecule (BS-1) was performed in 10% methanol in chloroform mobile phase.

Characterization of the novel isolated molecule (BS-1)

The BS-1 belongs to terpenoid. The melting point of the BS-1 is 146.8°C–148.2°C. The Rf value was found to be 0.4. The IR spectra cited in Figure 2 showed a stronger broadband, i.e., 3241.1 cm-1, and the signals at 1466 cm-1, 1561 cm-1, 2918.7 cm-1, and 2850.3 cm-1 with an intense absorption peak at 1728 cm-1. The mass spectra cited in Figure 3 exhibited the presence of the base peak at 717.64; molecular ion peak appeared at 717.64 and M + 1 peak at 718.64. The ^{1}H-NMR spectrum, cited in Figure 4 of the constituent showed –OH proton at δ 5.28 ppm. The spectra contained signals due to $-CH_2$ and $-CH_3$ protons with a wide range from δ 0.761–2.02 ppm, 0.76–0.86 (6H), 1.14–1.31 (33H), 1.39–1.46 (4H), 1.70–1.72 (2H), and 1.89–2.02 (6H). The ^{13}C NMR, cited in Figure 5 indicated 113.833 (C-1), 107.905 (C-2), 100.109 (C-3), and 90.930 (C-4).

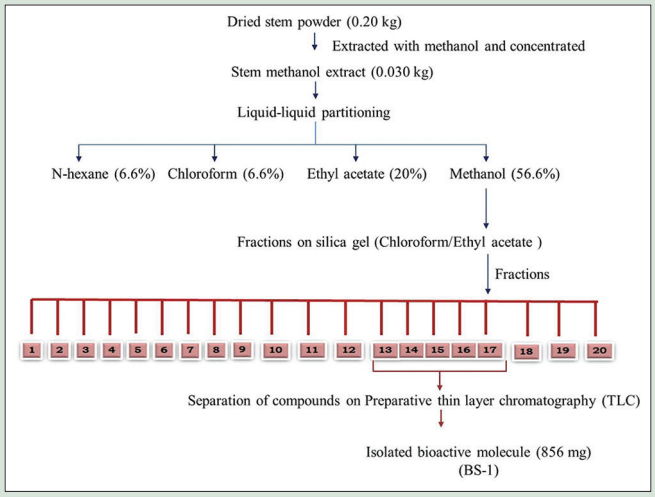

Figure 1: The scheme of phytoconstituents separation from stem methanol extract

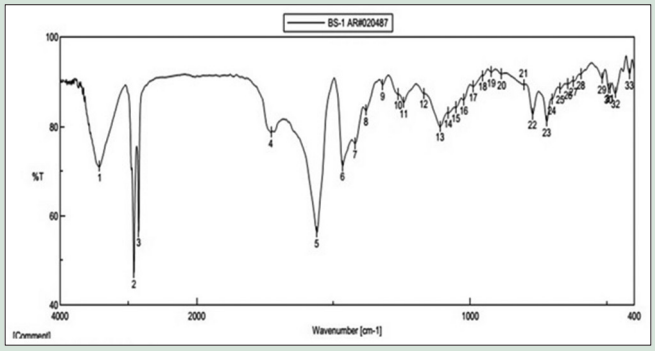

Figure 2: Infrared spectra of novel isolated molecule (BS-1)

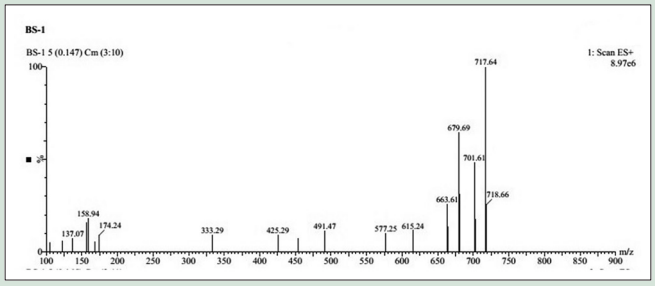

Figure 3: Mass spectra of novel isolated molecule (BS-1)

Pharmacological studies of the novel isolated molecule (BS-1)

Analgesic activity

Thermal stimulus model

Hot plate method

The placement of animals on the hot plate elicited nociceptive reactions. The anti-nociceptive effects of BS-1 are cited in Figure 6. The activity of BS-1 that was administered orally with low, moderate, and high dose was significant ($P \leq 0.05$) when correlated with morphine (10 mg/kg) and with the control group ($P \leq 0.001$).

Evaluation of opioid receptors participation in the analgesic activity of the BS-1

The BS-1 exhibited its central analgesic actions with its highest dose (50 mg/kg) which was found significant ($P \leq 0.05$) with standard drug morphine. It was proved when naloxone administered groups reversed the pain inhibition property. The results are cited in Figure 6.

Mechanical stimulus model

Haffner's tail clip test

The results are illustrated in Figure 7. The activity of BS-1 administered orally with 12.5 and 25 and 50 mg/kg doses was significant ($P \leq 0.01$) when correlated with control group and dose-dependent ($P \leq 0.05$).

Chemical stimulus model

Formalin test

The effect of BS-1 in inhibiting the licking and biting response was significant in initial and late phases of pain. This test disclosed the dose-dependent actions ($P \leq 0.01$) of BS-1 acting at two phases. The BS-1 at all tested doses showed significant ($P \leq 0.001/P \leq 0.05$) analgesic activity in comparison with standard drugs (morphine and diclofenac sodium. The results are cited in Table 1.

Writhing test

The effect of BS-1 on the writhing response in animals is tabulated in Table 2. When correlated with negative control group BS-1 administered orally with 12, 12.5, and 50 mg/kg was significant ($P \leq 0.001$) in decreasing the number of writhing in mice. The BS-1 exhibited 80.25% inhibition at 50 mg/kg dose.

Investigation of peripheral receptors involvement in the analgesic activity of the BS-1

The BS-1 exhibited its peripheral actions with its highest dose (50 mg/kg). It was proved when naloxone administered groups exhibited negative

response on abdominal constriction in mice. The results are cited in Table 1.

Anti-inflammatory activity

Carrageenan induced paw edema model

The accessed results are cited in Figures 8 and 9. The BS-1 at a higher dose of 50 mg/kg inhibited paw edema dose-dependently ($P < 0.05$) with percentage inhibition of 73.41% and 85.02% at 3rd and 4th h, respectively. The anti-inflammatory effect of BS-1 at 25 and 50 mg/kg was comparable with the standard drug at respective time points.

Cotton pellet induced granuloma test

The granulomatous tissue formation is related to the chronic inflammatory process. The BS-1 at a higher dose reduced the transudative weight to 123.8 mg and granuloma formation to 82.75% when correlated with diclofenac sodium which exhibited 92.9 mg and 79.8% reduction in transudative weight and granuloma formation. The accessed results are cited in Table 3.

DISCUSSION

Many of the effective chemical compounds have come from medicinal plants. It is therefore important that the efforts have to be made to tap the real potential of natural sources of medicines. In our previous work, we have explored the analgesic and anti-inflammatory properties of GG stem and leaf parts. The GGSME showed significant analgesic and anti-inflammatory activity than leaf extract.[14,15] The importance of the current study is to isolate, characterize, and evaluate the phytomolecule responsible for relieving pain and inflammation from the crude methanol extract of GG stems.

The GGSME was subjected to fractionation with solvents of varying polarities. The fractions n-hexane, chloroform, ethyl acetate, and methanol were collected and subjected to phytochemical screening to explore the phytoconstituents present. The study results showed the presence of significant phytoconstituents such as steroids and terpenoids, tannins, and phenolic compounds, flavonoids and saponins in methanol fraction (GGM) when correlated with remaining fractions.

Based on the results, the active fraction GGM was subjected to column chromatography. The solvent system for performing column chromatography is selected based on literature, phytochemical screening of GGM, the polarities of the expected compounds and finally through TLC trial experiments. The solvent composition consisting of chloroform and ethyl acetate is employed for column chromatography; further, the polarity of the selected solvent system is slowly raised for better elution. All the fractions of the column were subjected to TLC studies. The fraction numbers 13, 14, 15, 16, and 17 containing compounds with identical Rf values were pooled and then subjected to preparative TLC studies to separate the bioactive phytoconstituent employing 10% methanol in chloroform as mobile phase The separated phytoconstituent (BS-1) was further purified by hexane treatment.

The phytochemical test of BS-1 revealed that it belongs to the chemical class of terpenoid. The BS-1 was characterized by IR, ^{1}H-NMR, ^{13}C NMR,

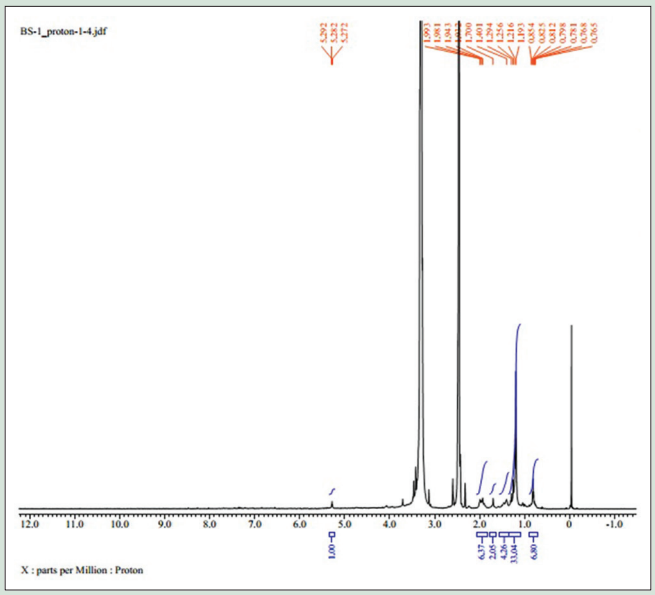

Figure 4: ^{1}H-NMR spectra of novel isolated molecule (BS-1)

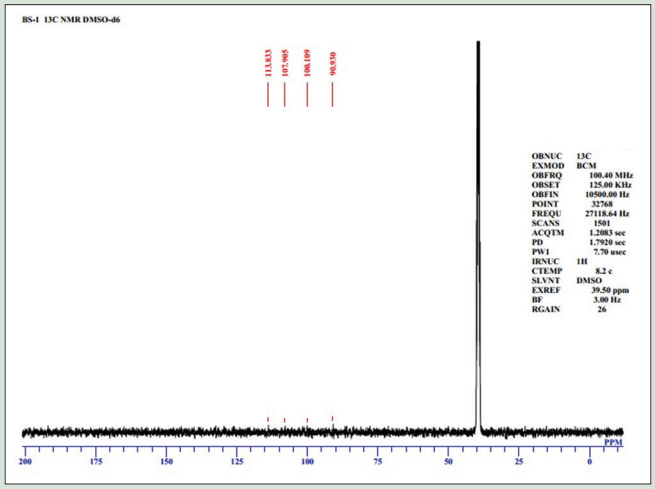

Figure 5: ^{13}C NMR spectra of novel isolated molecule (BS-1)

Table 1: Analgesic effect of novel isolated molecule (BS-1) on formalin-induced pain in mice

Group (s)	Dose (mg/kg)	Paw licking time (s)			
		Early phase (0-5 min)	Inhibition (%)	Late phase (15-30 min)	Inhibition (%)
I (distilled water)	10 (ml/kg)	173.5±4.5	-	112.5±3.1	-
II (morphine)	10	36.5±2.9[a]	78.96	4.2±0.2[a]	96.26
III (BS-1)	12.5	79.3±3.5[a,b]	54.29	36.0±1.6[a,b,c]	68
IV (BS-1)	25	48.1±2.5[a,c,d]	72.27	24.5±2.6[a,b,c,d]	78.2
V (BS-1)	50	34±2.2[a,c,d]	80.40	3.5±0.1[a,c]	96.8
VI (diclofenac sodium)	20	88.1±1.7[a]	49.22	12.1±1.1[a]	89.2

Values are expressed as mean±SEM; (n=6); the statistical significance done by two-way (ANOVA); followed by Bonferroni posttests and is represented by a symbol. BS-1: Novel isolated molecule. [a]P<0.001 indicates comparison with group I; [b]P<0.001 indicates comparison with group II; [c]P<0.05 indicates comparison with group VI; [d]P<0.01 indicates the dose dependent activity on comparison of the high dose with respective low dose of the BS-1. BS-1: Novel isolated molecule; ANOVA: Analysis of variance; SEM: Standard error of the mean

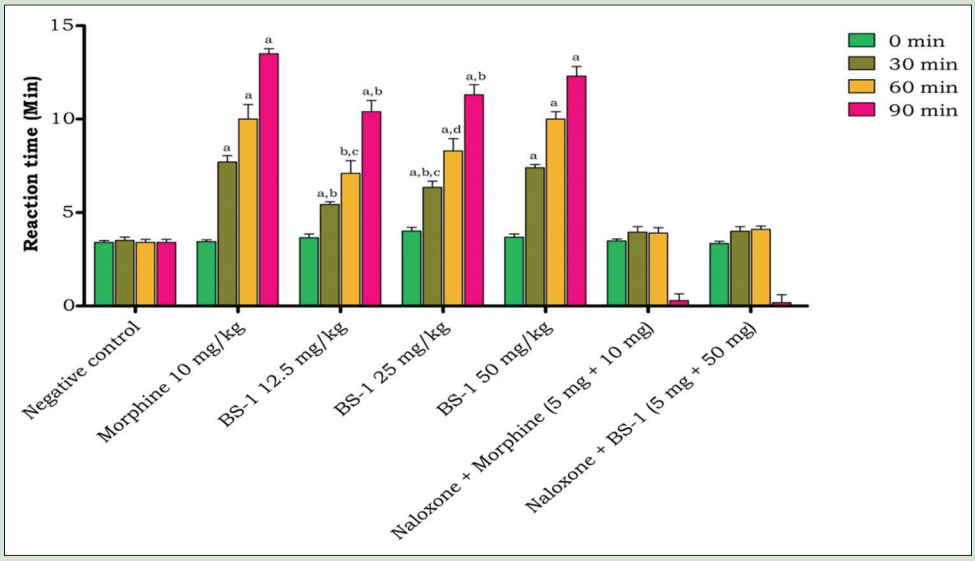

Figure 6: Analgesic effect of novel isolated molecule (BS-1) on thermal stimulus induced pain in mice. Values are expressed as mean ± standard error of mean; $n = 6$; the statistical significance done by two-way analysis of variance (followed by Bonferroni post test) tests and is represented by a symbol. $^{a}P \leq 0.001$ indicates comparison with negative control; $^{b}P \leq 0.01$ indicates comparison with morphine; $^{c}P \leq 0.05$ indicates the dose dependent activity on comparison of the high dose with respective low dose of the BS-1. BS-1: Novel Isolated molecule

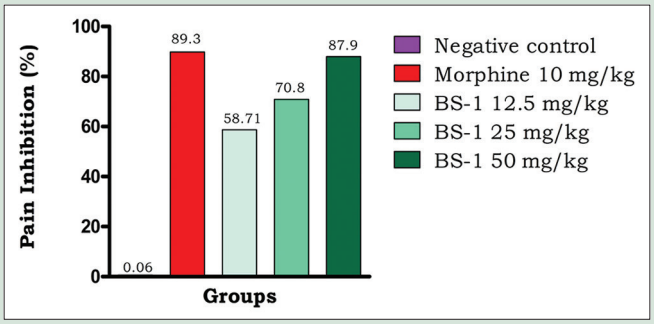

Figure 7: Analgesic effect of novel isolated molecule (BS-1) on Tail clip-induced pain in rats (Haffner's tail clip test)

Table 2: Analgesic effect of novel isolated molecule (BS-1) on acetic acid induced pain in mice (writhing test)

Group (s)	Dose (mg/kg)	Acetic acid induced writhing	
		Number of writhings	Inhibition (%)
I (distilled water)	10 (ml/kg)	23.3±1.2	-
II (diclofenac sodium)	20	5.0±0.2[a]	78.54
III (BS-1)	12.5	10.6±0.4[a,b]	54.50
IV (BS-1)	25	8.2±0.2[a,b,c]	64.80
V (BS-1)	50	4.6±0.4[a,b,c]	80.25
VI (naloxone + diclofenac sodium)	(5+20)	5.3±0.3[a]	77.25
VII (naloxone + BS-1)	(5+50)	4.7±0.2[a]	79.82

Values are expressed as mean±SEM; (n=6); the statistical significance done by one-way (ANOVA); followed by Tukey's multiple comparison tests and is represented by a symbol. $^{a}P<0.001$ indicates comparison with Group I; $^{b}P<0.01$ indicates comparison with Group II; $^{c}P<0.001$ indicates the dose dependent activity on comparison of the high dose with respective low dose of the BS-1. BS-1: Novel isolated molecule, ANOVA: Analysis of variance, SEM: Standard error of the mean

and mass spectra. The melting point of BS-1 was 146.8°C–148.2°C. The IR spectra showed a strong, but the broad, band at 3241.1 cm-1 indicating the presence of the –OH group, the signal at 2918.7 cm-1 and 2850.3 cm-1 perhaps due to aliphatic –CH stretching vibrations. An intense absorption at 1728 cm-1 is due to the presence of the ester carbonyl group. The signal at 1561 cm-1 is due to C-O-C stretching. The signal at 1466 cm-1 is due to C-O stretching. Mass spectroscopy is an important tool in structure elucidation of unknown compounds, where it is the only technique for molecular weight determination, through which we can predict the molecular formula. In mass spectra, the base peak, molecular ion peak and M + 1 peak were at 717.64, 717.64, and 718.64, respectively.

NMR spectroscopy is a research technique that exploits the magnetic properties of certain atomic nuclei. This type of spectroscopy determines the physical and chemical properties of atoms or the molecules in which they are contained. The ^{1}H-NMR spectrum of BS-1 showed –OH proton at δ 5.28 ppm. The spectra contained signals due to $-CH_2$ and $-CH_3$ protons with a wide range from δ 0.761–2.02 ppm, 0.76–0.86 (6H), 1.14–1.31 (33H), 1.39–1.46 (4H), 1.70–1.72 (2H) to 1.89–2.02 (6H). It is evident from the ^{1}H-NMR spectrum that the isolated molecule contains an alcohol group and the aliphatic functional group. The ^{13}C NMR, indicated 113.833 (C-1), 107.905 (C-2), 100.109 (C-3), and 90.930 (C-4). The spectra indicates the presence of a carbonyl group at δ 113.833 ppm, C = O group at δ 107.905 ppm and duplicate CH_3 groups at δ 90.930 ppm. The obtained results revealed the presence of a steroidal nucleus in the Novel isolated molecule (BS-1).

The analgesic and anti-inflammatory activities were carried out for the isolated molecule BS-1. The evaluation of *in vivo* analgesic activity was carried out by employing both central and peripheral pain models. The thermal stimulus-induced pain (hot plate test) and mechanical stimulus-induced pain (Haffner's tail clip test) models were employed for proving the central analgesic effects.[26] The chemical stimulus-induced pain (formalin test and writhing's test) models were employed for proving the peripheral analgesic actions,[27] whereas Formalin test is a model for both central and peripheral mechanisms.

The result was significant ($P < 0.001$ and $P < 0.01$) in a hotplate and tail clip tests suggesting the central analgesic effects. The results were

Table 3: Anti-inflammatory effect of BS-1 on cotton pellet-induced granuloma test in rats

Group (s)	Dose (mg/kg)	Granuloma wet weight (mg)	Granuloma dry weight (mg)	Transudative weight (mg)	Granuloma weight (mg) (mg/mg cotton)	Inhibition of granuloma (%)
I (distilled water)	10 (ml/kg)	176.3±3.8	60.6±5.03	115.7±5.7	2.03±0.14	-
II (diclofenac sodium)	20	121.2±3.9[a]	28.3±2.4[a]	92.9±4.2[a]	0.41±0.02[a]	79.80
III (BS-1)	12.5	107.7±5.4[a,c]	38.17±3.3[a]	69.53±6.1[a,b,c]	0.9±0.02[a,b,c]	55.6
IV (BS-1)	25	126.3±3.1[a,c]	33±3.0[a]	93.3±3.9[a,d,c]	0.65±0.02[a,c]	67.98
V (BS-1)	50	150.8±4.2[a,b]	27±1.5[a]	123.8±5.0[b]	0.35±0.01[a]	82.75

Values are expressed as mean±SEM; (n=6); the statistical significance done by one-way (ANOVA); followed by Tukey's multiple comparison tests and is represented by a symbol. BS-1: Isolated phytoconstituent; [a]P<0.01 indicates comparison with group I; [b]P<0.05 indicates comparison with group II; [c]P<0.05 indicates the dose dependent activity on comparison of the high dose with respective low dose of the BS-1. ANOVA: Analysis of variance; SEM: Standard error of the mean

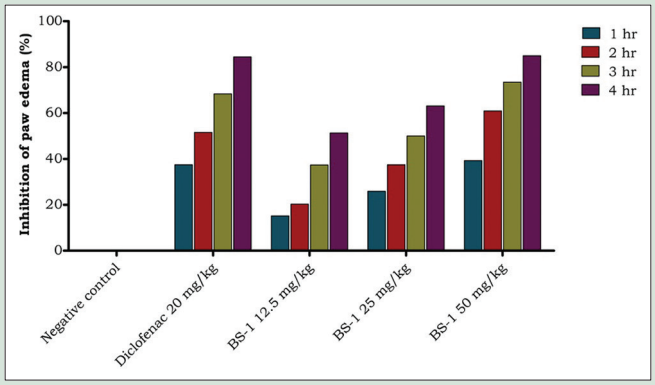

Figure 8: Anti-inflammatory effect of novel isolated molecule (BS-1) on Carrageenan-induced paw edema test in rats changes in paw edema volume after administration of control/standard/BS-1 (h); Percentage inhibition of paw edema: (a) After 1st h; (b) After 2nd h; (c) after 3rd h; (d) after 4th h. BS-1: BS-1: Novel isolated molecule

significant (P < 0.05) in a dose-dependent way. The central effect was further proved through reversal actions of naloxone. The BS-1 also exhibited significant (P < 0.001) peripheral analgesic actions in formalin and writhing test when compared to control group. In writhing's test BS-1 exhibited its dose-dependent activity ($P \leq 0.001$), along with its significant effects ($P \leq 0.001$) in decreasing the abdominal writhing's which was seen comparable with diclofenac sodium, which suggest the peripheral actions of BS-1.[28] In the formalin test, both morphine and BS-1 inhibited the early phase as well as the late phase of pain, besides in fact the diclofenac sodium solely inhibited the late inflammatory phase, suggesting both central and peripheral actions of BS-1.[22,29] The above results disclose the BS-1 analgesic effects through the involvement of central and peripheral mechanisms.

The opioids bind to specific receptors in the CNS and other tissues. The important classes of opioid receptors include mu receptors (μ_1 and μ_2), kappa receptors (k_1 and k_2) and delta receptors (δ_1 and δ_2). The centrally acting drugs exhibit their actions through modulation of spinal receptors (μ2, κ1, and δ2) and supraspinal receptors (μ1, κ3, δ1, and σ2).[30] Opioid receptors are G-protein coupled receptors and cause a decrease of adenylcyclase activity leading to reduced formation of the cAMP. They mediate two types of actions. A presynaptic action results in closure of Ca^+ channels, while the postsynaptic activity results in the opening of K^+ channel leading to reduced neuronal excitability.

Peripheral analgesics inhibit COX enzymes in the peripheral tissues by blocking the synthesis and or releasing mediators of pain like cell-derived mediators such as (Vasoactive amines [histamine, 5HT and neuropeptide]), eicosanoids (PGD_2, PGE_2, PGF_2-α, PGI_2, and TXA_2), lysosomal components (Granules of neutrophils, granules of monocytes and tissue macrophages), platelet activating factor, cytokines, and free radicals.[31]

The results disclose that the BS-1 may exhibit its analgesic effects through the involvement of central and peripheral mechanisms as cited above. Similar kind of results was earlier reported by Zakaria *et al.*[21]

The assessment for acute and chronic phases of inflammation was carried out employing carrageenan-induced paw edema model and cotton pellet induced granuloma test, respectively. Two phases were involved in inducing paw edema, the initial phase, and the late phase. In the initial phase substances such as serotonin, kinins, and histamine are released, whereas prostaglandins are released in the later phase of inflammation.[25]

The BS-1 exhibited significant inhibition of paw edema ($P \leq 0.001$) when compared to control group at the 1st, 2nd, 3rd, and 4th h of treatment. The experimental results showed significant ($P \leq 0.05$) dose-dependent activity and the results were also comparable ($P \leq 0.001$) with the diclofenac sodium. In the chronic inflammation model, the BS-1 exhibited significant ($P \leq 0.01$) inhibitory effects in both transudative phase and proliferative phase of inflammation when compared to the control group. The BS-1 exhibited significant ($P \leq 0.05$) activity, when correlated with the standard drug.

The mediators of inflammation may include histamine, 5HT, prostaglandins (PGD_2, PGE_2 and PGF_2-α), prostacyclins (PGI_2), thrombaxane A_2 (TXA_2), leucotrienes (LTC_4, LTD_4, LTE_4 and LTB_4), cytokines (interleukin-1 [IL-1], IL-6, IL-8, IL-12 and IL-17), tumor necrosis factor (TNF-α and TNF-β), interferon (IFN-γ), free radical like superoxide oxygen (O'_2), H_2O_2, OH', nitric oxide (NO), kinins, clotting factor XII, fibrinolytic, and anaphylatoxins (C3a, C4a, C5a, C5b, C6, C7, C8 and C9).[30] In chronic inflammatory conditions, the macrophage stimulation was induced by IL-1α, IL-1 β, IL-2, and TNF-α. In addition, macrophage proliferation was induced by multiplication of small blood vessel, the proliferation of fibroblasts and M-CSF (Macrophage colony stimulating factor). The IL-1 and IL-6 are active in acute inflammation, while IL-12 and IL-17 are active in chronic inflammation. The acute and chronic anti-inflammatory effects of BS-1 perhaps arbitrated through the above-discussed mechanisms.

CONCLUSION

The novel isolated molecule (BS-1) belongs to the chemical class of terpenoid. It exhibited significant analgesic activity through central and peripheral sites, and it also exhibited significant anti-inflammatory properties. This study facilitates the exchange of research and supports clinical use. The studies will be further extended for spectral data like 2-D NMR (HSQC, HMBC, COSY, NOESY etc.).

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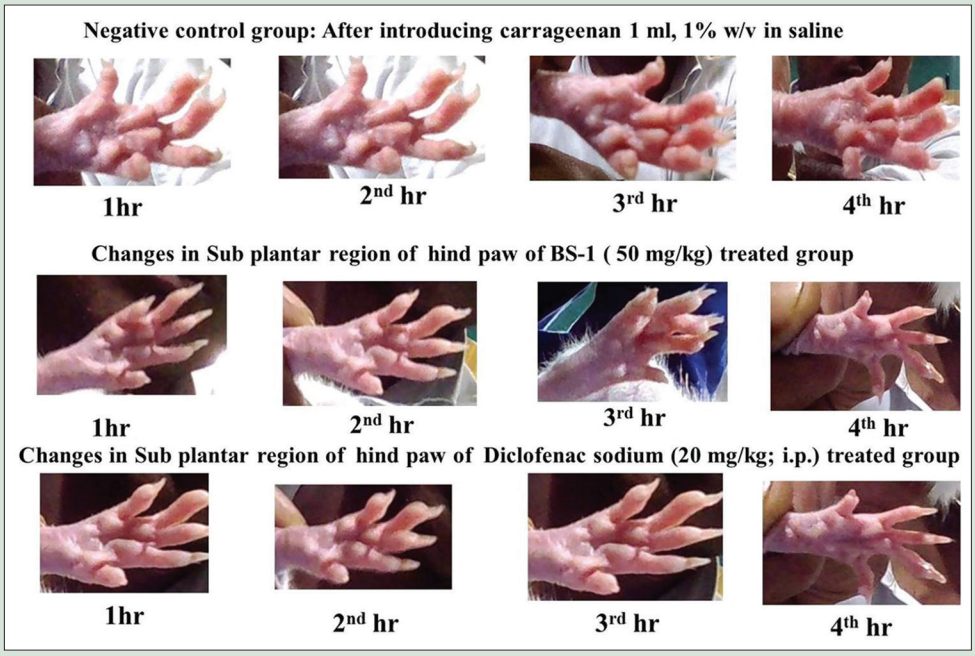

Figure 9: Anti-inflammatory effects novel isolated molecule (BS-1) on Carrageenan induced paw edema test in rats

would like to be thankful to Dr. VVLN Prasad for his guidance in the column chromatography and preparative TLC work. We also thank Dr. G. Kiran for his help in spectral interpretation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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