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Evaluation of Carrageenan-induced Anti-inflammatory Activity of Ethanolic Leaf Extract of *Psychotria bisulcata* Wight &Arn. in Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Psychotria bisulcata Wight & Arn. (Rubiaceae) is a shrub widely used in traditional medicine for the treatment of rheumatoid arthritis, diabetes, infertility and impotence. The present research work is focused on *in vitro* and *in vivo* anti-inflammatory activity in ethanolic leaf extract of *P. bisulcata*. The ethanol extract was subjected to *in vitro* anti-inflammatory assays such as hypotonic solution-induced hemolysis, heat-induced hemolysis. The *in vitro* anti-inflammatory assays revealed that the ethanolic leaf extract of *P. bisulcata* showed a high level of inhibition in hypotonic solution-induced hemolysis in 250 µg/mL as 55.80±1.91and in heat-induced hemolysis as 62.23±4.23. The *in vivo*

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anti-inflammatory activity was assessed using the carrageenan-induced paw edema model, with diclofenac as the standard. When compared to the standard diclofenac, the ethanolic leaf extract of *P. bisulcata* showed considerable anti-inflammatory efficacy in a dose dependent manner, resulting in the reduction of paw edema volume. The rats treated with ethanolic leaf extract of 500 mg/kg showed better activity. The research discovers that *P. bisulcata* has substantial anti-inflammatory action, indicating that it has medicinal potential in the treatment of inflammatory ailments.

Keywords: Carrageenan, anti-inflammatory, Rubiaceae, Psychotria bisulcate.

1. INTRODUCTION

Medical plants are crucial to rural communities, especially in isolated developing nations with little health services. Plant-based medications, food supplements, health products, and cosmetics are becoming more popular in poor and developed nations due to their non-toxic, low-side effects, and accessibility. There is growing global interest in herbal remedies and laboratory research into the bioactive components and pharmacological capabilities to treat various ailments. Phytotherapy is herbal medicine treatment. Certain herbal therapies are peaked with synthetic pharmaceuticals, proving that ethnopharmacology and traditional medicine have brought many drugs to the international market.

The physiopathology of inflammation shows that free radicals, physical, chemical, and biological stress may cause inflammation, which causes pain, redness, swelling, heat, and malfunction. Uncontrolled and persistent inflammation can cause autoimmune, rheumatic, asthmatic, and inflammatory bowel illnesses (Bousquet et al. 2002,Flavell 2002). Pain and inflammation entail enzyme activation, inflammatory mediator release, fluid extravasation, cell migration, and tissue injury and healing (Madzhitov 2012). Traditional pain and inflammatory management approaches vary by country. Herbal treatments are utilized to treat pain and inflammation in under developed nations due to their affordability, accessibility and environmental benefits (Ullah et al. 2014).

Inflammation is defined as the local response of living mammalian tissue to injury due to an external factor. It promotes tissue repair by providing a protective response. Sometimes, inflammation seems to produce events that are quite serious and become chronic occurrences of rheumatoid arthritis and hay fever, which may be life threatening diseases (Tag et al. 2015). Inflammation manifests usually in the form of painful swelling associated with some changes in skin covering the site (Jain and Bari 2010). It involves a cascade of events elicited by numerous stimuli that include infectious agents, ischemia, thermal and physical injury, and antigen-antibody interaction (Poornima et al. 2012).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of acute and chronic inflammation, pain and fever. They are effective but associated with the reappearance of symptoms after discontinuation. Their use is associated with adverse effects like severe gastritis, peptic ulcer, nausea, vomiting, salt and water retention, worsening of renal function in renal or cardiac and cirrhotic patients, hypersensitivity, etc (Grosser et al. 2011). The risk of death as a result of use of NSAIDs is 1 in 10,000 for young adults aged from 16 to 45, and the risk increases tenfold for those over 75 years old. Further, synthetic drugs are very expensive to develop (Ahmad et al. 2010). On the contrary, many medicines of plant origin have been used for a long time without any adverse effects. It is therefore essential that efforts be taken to develop new drugs of plant origin that possess anti-nociceptive and anti-inflammatory effects, which will be economically feasible as well as contain fewer side effects.

Rubiaceae, a complicated taxonomic family, produces several unique metabolites with tremendous biological potential, including indole alkaloids (Runguphan et al. 2009). Due to its psychoactive properties, chemical and pharmacological investigations have focused on the genus Psychotria, which comprises over 2000 species (Farias et al. 2009). Phytochemical research with this genus has characterized alkaloids, aglycones, heterosides of iridoids, triterpenes, and phenolic derivatives. The anti-inflammatory, antibacterial, and cytotoxic capabilities of compounds and extracts from this plant group are noteworthy (Calixto et al. 2016,Carvalho etal. 2017).

Psychotria sps are used to cure various diseases like anti-inflammatory, diabetes, infertility and

impotence. A literature review clearly indicated that there is no previous research work available on the phytochemical and pharmacology analysis of the aforesaid endangered and medicinally potential species. Hence, the present research work is focused on the *in vitro* and *in vivo* anti-inflammatory properties.

2. MATERIALS AND METHODS

2.1 Plant Identification

Psychotria bisulcata was collected from Kinnakorai, Ooty,Tamil Nadu, India during the month of October (2021). Then the plant was identified by The Botanical Survey of India,Coimbatore (Ref. No: BSI/SRC/5/23/2021/Tech.204).

2.2 Extraction of Plant Material

50 g of the air-dried powdered leaf material of *Psychotria bisulcata* were packed in small thimbles made from Whatmann No.1 filter paper separately and extracted ethanol using Soxhlet apparatus. The dried extract obtained with solvent was weighed. The percentage yield was calculated in terms of air-dried weight of the extract from the solvent. The stock solution of the extract obtained was prepared (1 mg/ml of respective organic solvents) and used for further analysis.

2.3 In vitro Anti-Inflammatory Activity

2.3.1 Membrane stabilization ability of various solvents leaf extracts of *P. bisulcate*

2.3.1.1 Hypotonic solution-induced hemolysis

To make Alsever's solution, dissolve 2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in distilled water and sterilize (Shinde et al. 1999). Retinal blood from Wistar albino rats was obtained. We combined the blood with an equal amount of sterilized Alsever's solution. Pack cells were washed three times with isosaline (0.9%, pH-7.2) and suspended in 10% (v/v) isosaline after centrifuging the blood at 3000 rpm for 10 minutes. The 4.5 ml reaction mixture contains 1 ml phosphate buffer (pH-7.4), 2 ml Hyposaline (0.45%), 1 ml plant extract (1 mg/ml), and 0.5 ml RBC suspension. The reference medication was diclofenac sodium. The reaction mixture without plant material was the control and phosphate buffer were the blank. We incubated the test solutions at 37˚C for 30 minutes and centrifuged again. Haemoglobin was measured at 560 nm in the supernatant solution.

The control absorbance is ‘Ac’ while the test absorbance is ‘At’.

$$\text{Percentage of inhibition} = \frac{Ac - At}{Ac} \times 100$$

Where ‘Ac’ is absorbance of the control and ‘At’ is absorbance of the test.

2.3.1.2 Heat-induced hemolysis

Each tube was gently mixed with 1.0 ml of 10% RBC and 1 ml of different solvent plant extracts (1 mg/ml). The positive control contained 1.0 ml of RBC and 1.0 ml of diclofenac sodium (10, 30, and 100 µg/ml). A negative control of 1.0 ml of 10% erythrocyte suspension and normal saline was used. The experiment was tripled. After heating at 56° C for 30 minutes, the solution cooled to ambient temperature and was centrifuged at 2500 rpm for 10 minutes. The supernatant was collected and analyzed spectrophotometrically (UVmini 1240, Shimadzu) at 560 nm to determine haemolysis. Formula for calculating hemolysis inhibition %

$$\text{Percentage of inhibition} = \frac{Ac - At}{Ac} \times 100$$

Where ‘Ac’ is absorbance of the control and ‘At’ is absorbance of the test (Wani and Mohammad 2018).

2.4 *In vivo* Studies

2.4.1 Animals and management

Healthy mature man The Small Animal Breeding Station (SABS) College of Veterinary and Animal Sciences, Kerala Agricultural University, Mannuthy, Thrissur, Kerala, India, supplied 150–180 g Wistar albino rats. They were kept in polypropylene cages at conventional laboratory settings (25 ± 2°C, 60-70 % humidity, 12 hours of light/dark cycles). All animals were kept together for at least 7 days before pharmacological tests. Adlibitum water and commercial rat pellet diet (Sri Ram Animal foods, Coimbatore) were supplied. Before the trial, the rats were acclimated to lab settings for a week. All laboratory animal research followed Institutional Animal Ethics Committee rules (Reg. No. IAEC/KASC/2022-23/06 dated 11.05.2022).

2.5 Toxicity Studies

2.5.1 Acute toxicity

An acute oral toxicity study was performed as per Organizationfor Economic Co-operationfor Development (OECD 423) guidelines for the testing of chemicals. Swiss albino rats (n=6) were selected for the acute toxicity study. The animals were fasted overnight with access to water without food, after which the *P. bisulcata* leaf ethanol extract was administered orally at a dose level of 250, 500 and 1000 mg/kg body weight, and the animals were observed continuously for the first 2 hours for morbidity and up to 24 hours for mortality. If mortality was observed in 4 out of 6 animals, then the dose administered was identified as a toxic dose. If mortality was observed in two animals, then the same dose was repeated again to confirm the toxic dose.

2.6 Evaluation of Anti-Inflammatory Activity

2.6.1 Carrageenan-induced acute hind paw edema in rats

The anti-inflammatory properties of *P. bisulcata* ethanol leaf extract against carrageenan-induced acute paw edema in rats were assessed using (Winter et al. 1962). Four groups of six male Wistar albino rats (150-180 g) were maintained. Despite fasting overnight, they had free water. The left hind paw was marked beyond the tibio-tarsal junction. The initial paw thickness of each rat was measured by a digital vernier calliper. Animals were divided into four groups consisting of 6-rat groups:

Group I: Control (Untreated).

Group II: Positive control administrated with 0.6% CMC and 10 mg Diclofenac, a commercially available standard anti-inflammatory drug/kg body weight of the rat.

Group III: Administrated with 0.6% CMC and 250 mg ethanol leaf extract of *P. bisulcata*/kg body weight of the rat.

Group IV: Administrated with 0.6% CMC and 500 mg ethanol leaf extract of *P. bisulcata*/kg body weight of the rat.

After one hour, control and plant extract-treated groups received 0.1 ml of 1% (w/v) carrageenan (suspended in normal saline) in the left sub-plantar area. To compare non-inflammed paws (the right paw) was used as a reference. The left leg paw thickness was measured after 30 minutes, 1 hour, and 6 hours following carrageenan administration for the control and plant extract treated rats. Thus, 6 hours of readings were taken. Edema was measured with a computerized Vernier calliper.

2.7 Statistical Analysis

The statistical analysis was performed by one-way ANOVA and the student's t-test. The results were expressed as means ± SE to show variations in the various experiments. Differences are considered significant at $P < 0.05$.

3. RESULTS

3.1 Membrane Stabilization Ability of Various Solvents Leaf Extract of *P. bisulcata*

3.1.1 Hypotonic solution-induced hemolysis

Fig. 1 shows the findings of the anti-inflammatory properties of *P. bisulcata* leaf extracts as determined by membrane stabilisation assay, which are shown in percentage inhibition ability. Among the present study, ethanol leaf extract of *P. bisulcata* exhibited maximum inhibition activity in 250 µg/mL as 55.8±1.91 and lowest in 50 µg/mL as 19.47±0.21. It possesses significant anti-inflammatory activity comparable with the standard drug.

3.1.2 Heat-induced hemolysis

Animal erythrocytes are involved in *in vitro* anti-inflammatory activity studies through the ARBC membrane stabilization ability as it has the similarity with that of the lysosomal membrane. Fig. 2 shows the potential of the ethanol leaf extract of *P. bisulcata* in membrane stabilization ability. The study found that the *P. bisulcata* ethanol leaf extract had stronger inhibitory action in 250 µg/mL as 62.23±4.23 and lowest in 50 µg/mL as 25.42±1.77.

3.2 *In vivo* Studies in Animal Model

3.2.1 Acute oral toxicity

The ethanol leaf extract of *P. bisulcata* was subjected to an acute toxicity study using Wistar albino rats. The animals were monitored for 24 hours after administration of the extract doses at 500, 1000 and 2000 mg/kg body weight of the animal. The parameters like alertness,

grooming, touch and pain response, tremors, convulsions, righting reflex, gripping strength, lacrimation, etc. were observed, and the results are presented in Table 1. None of the mortality was noted on the administration of ethanol leaf extract of *P. bisulcata* up to 2000 mg/kg body weight of the rats. Hence it was found to be non-toxic in animals.

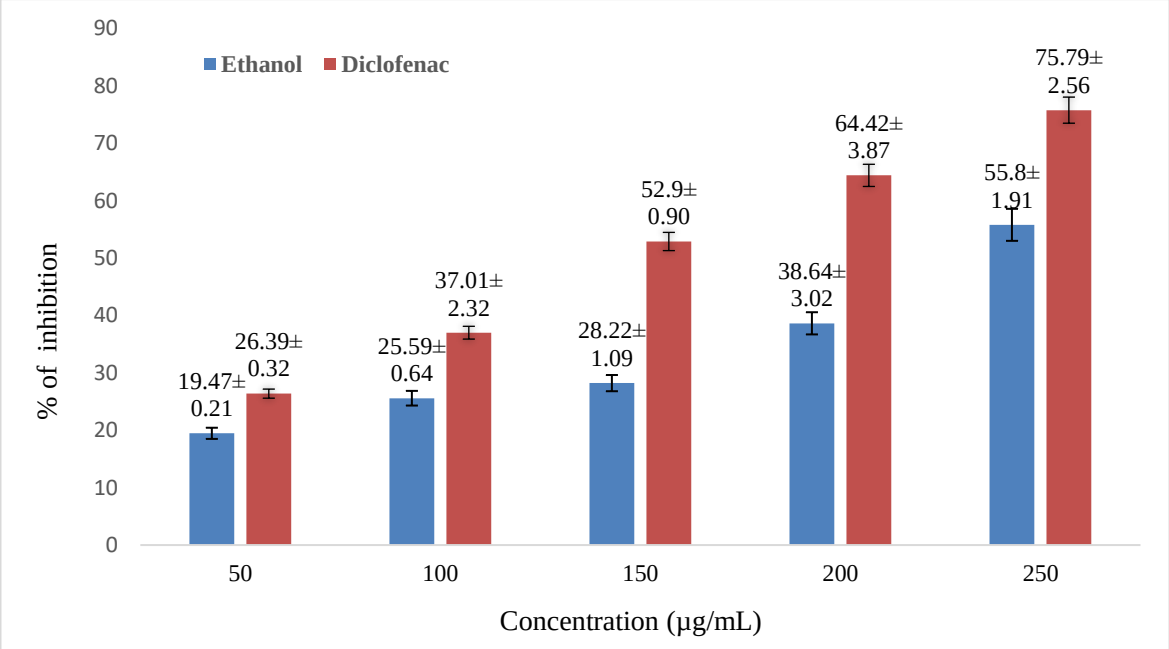

Fig. 1. Hypotonic solution-induced hemolysis inhibition abilities of the various solvent leaf extracts of *P. bisulcata*

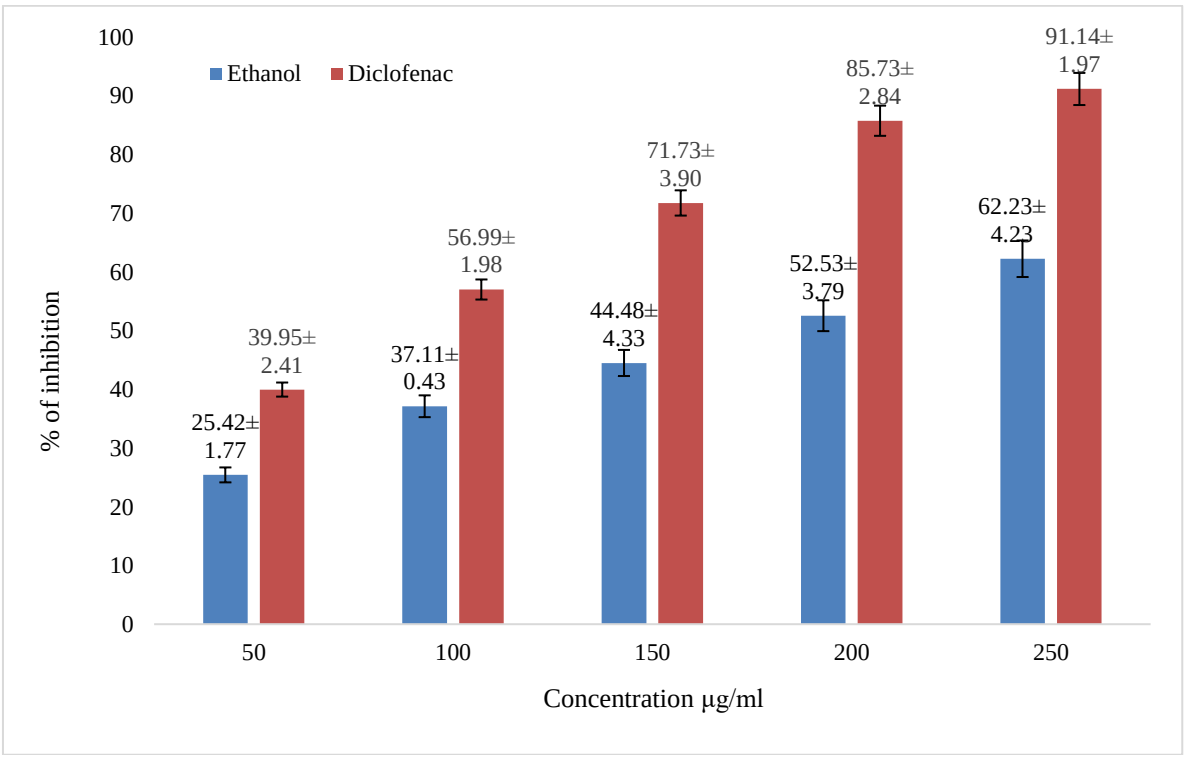

Fig. 2. Heat-induced hemolysis activity of *P. bisulcata*

Table 1. Acute toxicity study of ethanol extract of *P. bisulcata* leaves

Observations	*P. bisulcata* (mg/kg body weight)			
	Before	After		
		500	1000	2000
Alertness	N	N	N	N
Grooming	N	N	N	N
Touch Response	P	P	P	P
Pain Response	P	P	P	P
Tremors	N	N	N	N
Convulsions	A	A	A	A
Righting Reflex	N	N	N	N
Gripping Strength	N	N	N	N
Pinna Reflex	N	N	N	N
Corneal Reflex	N	N	N	N
Pupils	N	N	N	N
Urination	N	N	N	N
Salivation	A	A	A	A
Skin Colour	N	N	N	N
Lacrimation	A	A	A	A
Hyper Activity	A	A	A	A

N – Normal, A – Absent, P – Present

The ethanol leaf extract of *P. bisulcata* was subjected to acute toxicity studies using Wistar albino rats weighing 150-180 g. The animals were monitored for 24 hours after administration of the extract at 500, 1000 and 2000 mg/kg body weight of the rats to determine the lethal dose. None of the mortality was noted on the administration of ethanol leaf extract of *P. bisulcata* up to 2000 mg/kg body weight of the rats, which was found to be non-toxic in animals. Hence, it was considered safe to administer to the animals according to OECD guidelines. Though the higher concentration of the extract did not cause any toxic effect, the doses 250 and 500 mg/kg body weight were selected for the hepatoprotective and anti-inflammatory studies owing to avoid later effects on the biological systems. Because it is believed that longer exposure to the higher concentration of any chemical may cause some ill effects on the test animals.

3.3 *In vivo* Anti-Inflammatory Activities

3.3.1 Carrageenan-induced hind paw edema in rats

Evaluation of Carrageenan-induced acute hind paw edema and the ability of the ethanol leaf extract of *P. bisulcata* against the paw edema in rats is given in the Fig. 3 & Table 2. Carrageenan-induced paw edema remained as such even after 6 hours of its administration through injection on the sub plantar region of the rat's hind paw.

The mean increase of the paw thickness of the negative control rat (3.54 mm) was higher than the ethanol leaf extract of *P. bisulcata* (2.02 and 1.97 mm in 250 and 500 mg/kg respectively) and the standard drug, Diclofenac (1.62 mm) administered to rats (positive control). The inhibition percentage of paw edema was higher in Diclofenac administered rats (54.23%) than the ethanol leaf extract of *P. bisulcata* administered rats at 500 (44.35%) and 250 mg/kg body weight of the rats (42.93%). Thus, dose- dependent results were detected. Though dose- dependent results were observed, significance at 1% level ($p < 0.01$) was recorded on positive control and 500 mg/kg ethanol leaf extract of *P. bisulcata* administered rats. Whereas, 5% level significance ($p < 0.05$) was noted on 250 mg/kg of ethanol leaf extract of *P. bisulcata* administered rats.

4. DISCUSSION

The results of the current study *in vitro* and *in vivo* anti-inflammatory activity of ethanolic leaf extracts of *Psychotria bisulcata* were assessed against hypotonic solution- induced hemolysis and Heat- induced hemolysis. The extracts anti-inflammatory efficacy increased substantially with concentration. The *in vivo* anti-inflammatory properties of ethanolic leaf extracts of *P. bisulcata* on Wistar rats in carrageenan-induced paw edema model, revealed the dose dependent reduction in paw edema volume. The most significant reduction was recorded at 500 mg/kg.

Comparatively, the extract displayed similar or enhanced efficacy in reducing inflammation compared to the standard anti-inflammatory drug diclofenac.

The RBC membrane is a two-dimensional (2D) structure and compromises its integrity, comprised of a cytoskeleton with a lipid bilayer, tethered together via band-3 proteins at the spectrin-ankyrin binding sites and glycophorin at the acting junctional complexes. Any defects in the cytoskeleton integrity of the RBC or blood disorders such as sickle cell disease, haemolytic anaemia, thalassemia, spherocytosis, and elliptocytosis occur (Aidoo et al. 2021). There is structural analogue between the erythrocyte membrane and lysosomal membrane. Lysis occurs in the lysosomal membrane during inflammation and releasing enzyme components that cause a variety of illnesses (Labu et al. 2015).

Generally, suppression of hypotonicity and heat-induced lyses of human RBCs are used to measure the mechanism of anti-inflammatory effects, as the human RBC membranes resemble lysosomal membranes (Kardile et al. 2016). When the lysosomal enzyme in RBC is ruptured either by a hypotonic solution or by heat that makes excessive fluid collection in these

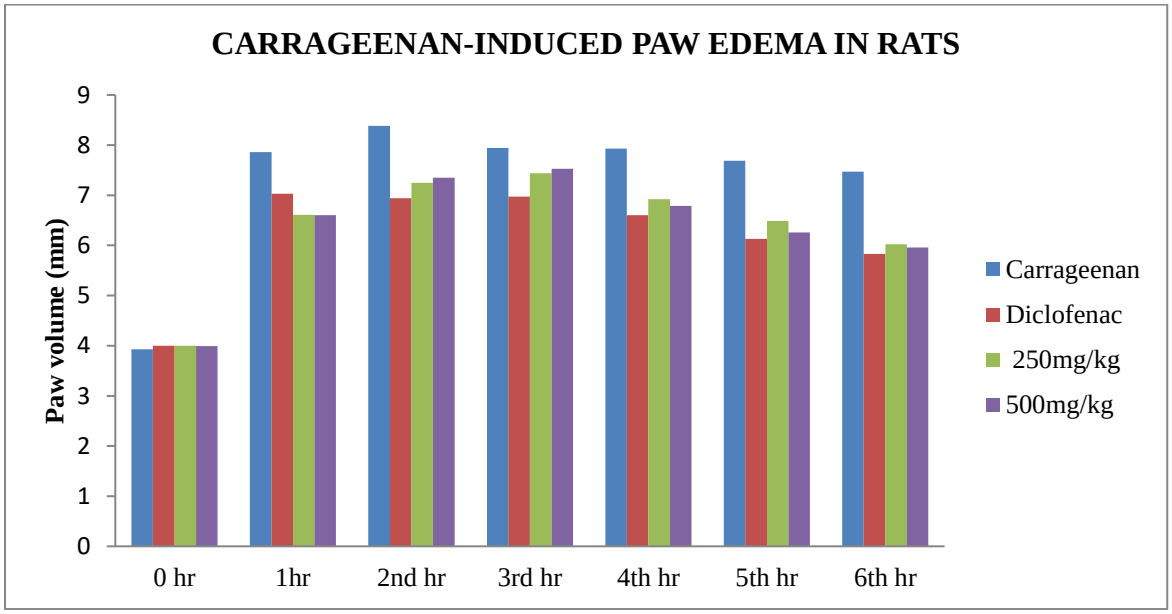

Fig. 3. Ability of ethanol leaf extract of *P.bisulcata* on the reduction of Carrageenan induced paw edema calculating from 0 to 6th hour

Table 2. Ability of the ethanol Leaf Extract of *P. bisulcata* on the Reduction of Carrageenan-Induced Paw edema

Group	Paw volume after induction with carrageenan increase in paw volume (Mm) after carrageenan injection (mean ± SEM)/Percentage inhibition of edema			
	Initial Paw volume(mm)	Final Paw volume(mm)	Difference	Percentage (%)
Control (0.5% CMC)	3.26±0.143	3.25 ±0.112	-	-
Carrageenan 1% (w/v)	3.93±0.111	7.47±0.395	3.54	-
Diclofenac 10mg/kg	4.21±0.22	5.83±0.145***	1.62	54.23 %
Ethanol leaf extract 250mg/kg	4.00±0.19	6.02±0.244**	2.02	42.93 %
Ethanol leaf extract 500mg/kg	3.99±0.105	5.96±0.206**	1.97	44.35 %

*Values are expressed as the mean ± S.D. Statistical significance (p) calculated by one way ANOVA followed by Dunnett's. ns- not significant **P< 0.05 calculated by comparing treated group with control group.*

cells. Extracellular lysosomal component leakage results in tissue inflammation and injury. It makes the cells more vulnerable to subsequent damage caused by the free radicals evolved from the lipid peroxidation (Sikder 2010). Thus, the lysosomal membrane stabilization regulates the inflammatory response (Yoganandam et al. 2010).

Carrageenan-induced hind paw oedema has been recorded as a well-replicated and investigated model for identifying the anti-inflammatory activity of natural products (Antonisamy et al. 2017). It is a test used to study both steroidal and non-steroidal anti-inflammatory drugs (NSAID) as it involves several mediators (Panthong et al. 2009). Studies have proved that CAT, SOD, and GPx levels and the protein expression levels of Nrf2 and HO-1 were reduced in carrageenan-induced edema. On administration of carrageenan injection in the hind paw of animals, there is a release of several inflammatory mediators that take place in three phase's initial, secondary and tertiary phases (Meira et al. 2014). The initial phase prolongs from the stage of injection to $1^{1/2}$ hrs where histamine and serotonin are released (DiRosa et al. 1971). second phase initiates after $1^{1/2}$hrs and prolongs to $2^{1/2}$ hrs where bradykinin is released, and in the third phase that leads up to 6 hrs were the mediator prostaglandins are suspected to be released and it leads up to 6 hrs after induction of inflammation (Oyedemi et al. 2010). To focus on inflammatory processes, the third phase is one of the most interesting ones where maximal vascular responses are determined and leukocytes migrate to the site of inflammation. It is already identified that prostaglandins are modulators of inflammatory responses and they plays a key role in the inflammatory mechanism (Panthong et al. 2009). In *P. bisulcata* leaf ethanol extract, the inhibition percentage and rate in reduction of inflammation were found to be higher than the related species and standard drug diclofenac.

Though potentially active chemicals are found in plants, very few numbers are used in the majority of pharmacological or harmful activities. Depending upon the growing environmental and soil conditions of the plants, which are growing and the seasons that are existing during their growth determine synthesizing concentration of the compounds in them (Steenkamp et al. 2004). In addition, any parts of the plant may contain poisonous principles (leaves, seeds, roots or bark). Only a few investigations are carried out to screen the toxicity level in the plants, which may limit their applications in conventional medicines. (Priya et al. 2013) published many biological activities of *Psychotria* species. The toxicity profile of a specific chemical must always be clinically assessed. Because certain poisonous chemicals used in the *in vitro* condition may not be toxic under the *in vivo* condition in which they are subjected to the biomodulation process during the metabolism. Repetitions of toxicity studies are inevitable during daily clinical observations as well as in the ultimate end point observations (Féres et al. 2006). None of the appreciable changes during food or water consumption were noticed. Any changes in the body weights, especially weight reduction of more than 10%, can cause unfavourable conditions in them. Even they cannot withstand until the end of the experiment period (Raza et al. 2007,Teo et al. 2022). Another simple way of toxicity measurement is the estimation of their body and organ weights. The lack of distinct variations between the body and organ weights of the treated and untreated animals lends credibility to the tested ethanol leaf extract of *P. bisulcata*.

5. CONCLUSION

In this study, Psychotria species showed amazing *in vitro* and *in vivo* characteristics, with *P. bisulcata* being the greatest producer of different chemical compounds. The investigation supports the anti-inflammatory effect of the ethanolic leaf extract of *P. bisulata*. The study also indicates the potent anti-inflammatory action of ethanolic leaf extract of *P. bisulcata*, as evidenced by a dose-dependent decrease in paw edema volume in Wistar rats. The study findings are on the need for natural anti-inflammatory drugs with significant effectiveness and fewer adverse effects as alternatives to pharmaceutical interventions.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

ETHICAL APPROVAL

All laboratory animal research followed Institutional Animal Ethics Committee rules (Reg, No: IAEC/KASC/2022-23/06 dated 11.05.2022).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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