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Evaluation Of Anti-Inflammatory and Analgesic Activity Using Herbal Extracts of *Ziziphus trinervia* and *Sagittaria trifolia*

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Abstract

Background: Chronic pain and inflammatory conditions pose significant global health challenges, often requiring long-term pharmacological interventions that carry potential side effects. Traditional medicine offers a rich source of potential natural alternatives; however, the precise mechanisms and efficacy of certain remedies require rigorous scientific validation. This study investigates the therapeutic potential of two such plants: *Ziziphus trinervia* and *Sagittaria trifolia*. **Objective:** This research project aimed to systematically evaluate the analgesic (pain-relieving) and anti-inflammatory capabilities of ethanolic extracts derived from *Ziziphus trinervia* and *Sagittaria trifolia*, employing both *in vivo* animal models and *in vitro* biochemical analyses to understand their mechanisms of action. **Methods:** The study utilized albino Wistar rats, allocated into nine distinct treatment groups for efficacy testing. Anti-inflammatory activity was assessed using the established Carrageenan-induced paw edema assay. Analgesic effects were quantified through the Eddy's hot plate test. Gas Chromatography-Mass Spectrometry (GC-MS) was deployed to profile the phytochemical composition. Additionally, various inflammatory mediators, including specific interleukins (IL-1, IL-6, IL-8), cytokines, histamine, and prostaglandin E2 (PGE2), were measured to understand the extracts' influence on inflammatory pathways. **Results:** The findings confirmed that the ethanolic extracts of both *Ziziphus trinervia* and *Sagittaria trifolia* elicited potent, dose-dependent anti-inflammatory and analgesic responses. The extracts effectively mitigated swelling in the edema model and significantly elevated the pain threshold in treated animals. GC-MS data identified a spectrum of bioactive compounds, such as flavonoids and specific fatty acids, hypothesized to

be responsible for these pharmacological outcomes. **Conclusion:** The results robustly support the traditional use of *Ziziphus trinervia* and *Sagittaria trifolia*. The extracts exhibit substantial therapeutic potential as natural anti-inflammatory and pain-management agents. This work establishes a foundation for future research aimed at isolating lead compounds and exploring potential clinical applications for managing inflammatory conditions in humans.

Keywords
Herbal medicine, *Ziziphus trinervia*, *Sagittaria trifolia*, analgesic properties, anti-inflammatory agents, Wistar rats, GC-MS analysis, natural products, cytokines

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INTRODUCTION

INFLAMMATION:

Inflammation is a fundamental biological response of body tissues to harmful stimuli such as pathogens, damaged cells, or irritants. It serves as a protective mechanism aimed at eliminating the initial cause of cell injury, clearing out necrotic cells and tissues, and initiating tissue repair. (1)

The five classical signs of inflammation are: heat, pain, redness, swelling, loss of function. (1)

Inflammation is a non-specific defence mechanism and is therefore a key component of the innate immune system. Unlike adaptive immunity, which is highly specific and develops over time in response to particular antigens, the inflammatory response is immediate and acts as the body's first line of defence against injury or infection. (1)

PAIN:

Pain is a complex and distressing sensation that typically arises in response to intense or potentially harmful stimuli. It serves as a vital protective mechanism, alerting the body to injury or disease. The International Association for the Study of Pain (IASP) defines pain as:

"An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage." (2)

Pain can be acute or chronic, and its perception varies significantly among individuals due to factors such as genetics, past experiences, and mental health. (2)

Plant Profile

***ZIZIPHUS TRINERVIA*:**

Scientific Name: Ziziphus trinervia.
Synonyms: Ziziphus glarbata, Ziziphus exserta DC.
Vernacular Names: Ber, Chittipala, Karkala, Vatadala.
Taxonomy:

Division	Magnoliophyta (Flowering plants)
Order	Rosales
Family	Rhamnaceae (Buckthorn family)
Genus	Ziziphus
Species	Ziziphus trinervia

***SAGITTARIA TRIFOLIA*:**

Scientific Name: Sagittaria trifolia
Synonyms: Sagittaria sinensis, Sagittaria edulis.
Vernacular Names: Chinese arrowhead, duck potato, swamp potato, omodaka, water yam.
Taxonomy:

Division	Magnoliophyta (Flowering plants)
Order	Alismatales
Family	Alismataceae
Genus	Sagittaria
Species	Sagittaria trifolia

METHODOLOGY

Collection And Authentication of Plant:

Dried roots powder of the plants were procured from local herbarium.

Preparation of Plant Extract Using Maceration Method: The dried roots powder of *Ziziphus trinervia* and *Sagittaria trifolia* were extracted using ethanol as a solvent through the maceration method over a period of three days. After extraction, the solvent was evaporated to obtain the concentrated extracts, which were then stored in airtight glass containers.

Phytochemical Screening Tests:

The ethanolic extracts of *Ziziphus trinervia* and *Sagittaria trifolia* were subjected to preliminary phytochemical screening to detect the presence of various bioactive constituents, including such as alkaloids, glycosides, steroids, saponins, flavonoids, tannins, carbohydrates, coumarins. The screening was performed following the standard procedures as mentioned in international journal of chemical studies [3]

Experimental Animals:

The experimental studies were conducted at Shadan Women's College of Pharmacy, Khairtabad, Hyderabad. A total of 36 healthy albino rats of either sex, weighing between 150–200 g, were used for the study. Prior to experimentation, all animals were acclimatized for 14 days in the animal house under standard laboratory conditions, maintained on a 12-hour light/dark cycle.

Animals were provided with a standard pellet diet and water ad libitum. The rats were randomly selected and grouped for the study. All procedures involving experimental animals were conducted in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Ethical approval for the study was obtained from the Institutional Animal Ethics Committee (IAEC) under proposal number IAEC-07/SES-2025/41/112.

Acute Toxicity Studies:

Acute oral toxicity studies of the ethanolic extracts of Ziziphus trinervia and Sagittaria trifolia were carried out in rats following OECD Guideline No. 423. The extracts were administered orally, and the animals were observed for mortality and behavioural changes.

The results indicated no mortality or signs of toxicity even at the highest tested dose of 2000 mg/kg body weight, suggesting that both extracts are safe and non-toxic at this dose. Based on these observations, the LD_{50} value was estimated to be greater than 2000 mg/kg, classifying the extracts as non-toxic according to OECD standards.

Mortality: No mortality was observed even at the highest tested dose of 2000 mg/kg, indicating that the extracts are safe and well-tolerated at this concentration.[4]

Drugs and chemicals:

Aspirin (20mg/kg), Indomethacin (10mg/kg), Carrageenan (1% w/v), Normal saline (0.9%w/v), Ethanol (99% v/v), Ethanolic extracts of Ziziphus trinervia and Sagittaria trifolia.

EXPERIMENTAL DESIGN:

GROUPS	DRUGS	DOSE	ROUTES
Group 1	Normal saline	1ml	i.p
Group 2	Toxic control	10ml/kg	i.p
Group 3	Standard drug	10mg/kg	i.p
Group 4	Ziziphus trinervia ethanolic extract - dose 1	200mg/kg	p.o
Group 5	Ziziphus trinervia ethanolic extract - dose 2	400mg/kg	p.o
Group 6	Sagittaria trifolia ethanolic extract - dose 1	200mg/kg	p.o
Group 7	Sagittariya trifolia ethanolic extract - dose 2	400mg/kg	p.o
Group 8	Ziziphus trinervia ethanolic extract + Sagittaria trifolia ethanolic extract – dose 1	200mg/kg	p.o
Group 9	Ziziphus trinervia ethanolic extract + Sagittaria trifolia ethanolic extract – dose 2	400mg/kg	p.o

SCREENING METHODS

In Vivo Method for Anti-Inflammatory:

Carrageenan induced paw edema method:

Figure 1: **Measurement of Paw edema by plethysmograph**

Albino rats weighing 180–200 grams were used for the study. Prior to treatment, the animals were fasted overnight, with water provided ad libitum. The respective test substances and standard drug were administered 30 minutes prior to inflammation induction. Inflammation was induced by a subcutaneous injection of 0.05 mL of 1% carrageenan solution into the sub-plantar region of the left hind paw. An ink mark was placed at the level of the lateral malleolus to ensure consistent immersion depth. The paw was then immersed in mercury for accurate volume measurement. (9)

Evaluation:

Paw volume was recorded using a plethysmometer at 30 minutes, 1 hour, 2 hours, and 3 hours post-carrageenan injection. The percentage increase in paw volume was calculated and compared between the treated and control groups, based on the mean differences in volume measurements. A reduction in paw edema in the treated group compared to the control group indicated anti-inflammatory activity. (9)

***In Vivo* Method for Analgesic Activity:**

Eddy's hot plate method**:**

In this method, thermal stimulation was used as the primary stimulus to evaluate central analgesic activity. Albino rats were first weighed, numbered, and randomly divided into nine groups, with six animals per group. Each animal was then individually placed on a hot plate maintained at 55 ± 1°C, with a cut-off time of 15 seconds to prevent tissue damage. The baseline reaction time: defined as the time taken to exhibit signs of pain such as paw licking or jumping was recorded prior to treatment. (9)

Following this, aspirin (20 mg/kg, intraperitoneally) was administered as the standard drug, while the test groups received their respective extracts or formulations. Post-treatment, the reaction times were measured at 15, 30, and 60 minutes. An increase in reaction time compared to baseline indicates a positive analgesic response. The results

were statistically analysed and compared across the control, standard, and test groups to determine the relative analgesic efficacy of the substances under study. (9)

Figure 2: Measurement of analgesic activity by Eddy's Hot Plate

IN VITRO METHOD FOR ANTI INFLAMMATORY AND ANALGESIC ACTIVITY:

COX-1 Assay:

The COX-1 (Cyclooxygenase-1) assay was conducted to evaluate the inhibitory effect of the test samples on prostaglandin synthesis. To begin, 0.1 mL of adrenaline dihydrogen tartrate and 10 mM hematin were added to 10 mL of the prepared sample solution. Subsequently, a few units of COX-1 enzyme were introduced into the mixture. The reaction mixture was pre-incubated for 5–10 minutes at controlled temperature to allow enzyme-substrate interaction. Following this, 10% formic acid was added to adjust the final volume to 10 mL, thereby terminating the reaction. (9)

The concentration of Prostaglandin E2 (PGE-2) formed was then quantified spectrophotometrically or via ELISA, and the results were compared across the standard and test groups to assess the relative COX-1 inhibitory potential of the test compounds. (9)

COX-2 Assay:

The COX-2 (Cyclooxygenase-2) assay was performed to evaluate the inhibitory effect of the test compounds on enzyme activity. The reaction mixture consisted of 1 mM hematin, 1 mg/mL gelatin, 100 mM sodium phosphate buffer (pH 7.4), and 2.5 mL of the test sample dissolved in DMSO. This mixture was pre-incubated at 20°C for 10 minutes to allow adequate interaction of the components. The reaction was then initiated by the addition of 20 µL of 1 mM TMPD (N, N, N', N'-Tetramethyl-p-phenylenediamine) solution. (9)

The enzymatic activity was monitored by measuring the degree of TMPD oxidation, both in the presence and absence of COX-2 enzyme. The difference in TMPD oxidation (i.e., absorbance values) between the two conditions was used to determine the extent of COX-2 inhibition. Results were then compared between the test samples and the standard drug, allowing for the evaluation of COX-2 selectivity and inhibitory potential of the compounds under investigation. (9)

RESULTS

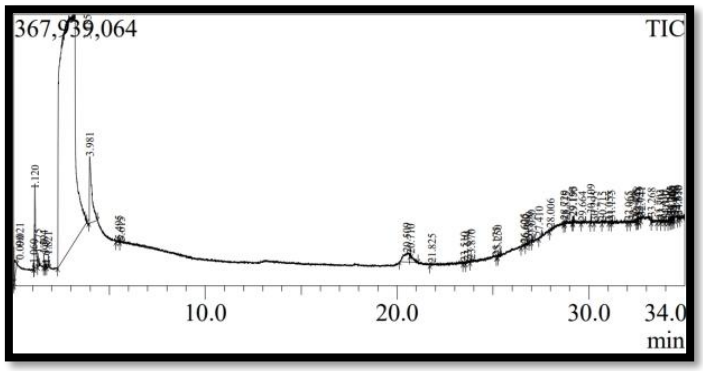
Phytochemical Evaluation:

Chemical Constituents	Test	*Ziziphus trinervia*	*Sagittaria trifolia*
Alkaloids	Wagner's test	+	-
	Mayer's test	+	-
	Hager's test	+	-
	Dragendroff's test	+	-
Carbohydrates	Molich's test	+	+
	Fehling's test	+	+
	Benedict's test	+	+
		+	+
Reducing sugars	Benedict's test	-	-
Flavonoids	Alkaline reagent test	+	+
Phenolic compound	$FeCl_3$ test	+	+
Tannins	Bromine water test $FeCl_2$ test Lead acetate test Acetic acid soln	-	+
Terpenoids	Froth's test	-	-
Saponins	Salkowski's test	-	+
Phytosterols & Sterol	Liebermann Burchard test	+	+
Coumarins	$FeCl_3$ test	-	+
Glycosides	Borntrager's test	+	-

+ Indicates presence & - Indicates absence

GCMS ANALYSIS

GCMS of Ethanolic Extract of Ziziphus trinervia:



S.no	Retention time	Chemical constituents	Area %	Uses
1	20.509	Dimethyl Sulfoxide	1.75	Decreases pain& inflammation.
2	22.450	Sulfamide	1.02	Anti-inflammatory, Trets br0nchitis, bacterial meningitis, ear & eye infections, UT Infections, severe burns.
3	26.890	Benzoic acid	0.08	Treat skin irritation & inflammation caused by burns
4	29.150	Phenol	0.05	Used as an oral analgesic, relieve itching, treats pharyngitis.
5	24.063	Methyl ester	1.20	Anti-inflammatory
6	33.703	Penta siloxane	0.17	Anti-inflammatory, removes wrinkles & skin blemishes & irritation, prevents scaling
7	32.760	Butyric acid	0.20	Treats inflammatory conditions (non-specific bowel inflammation, diverticulitis, diversion colitis)
8	23.870	2-Propanol	1.34	Used to prevent migraine headaches & chest Pain (angina)
9	25.175	Propanoic acid	1.02	Treatment of inflammation associated with tissue injury.
10	25.250	Coumaren-6-ol	1.07	Anti-inflammatory & antipyretic

GCMS of Ethanolic Extract of Sagittaria trifolia:

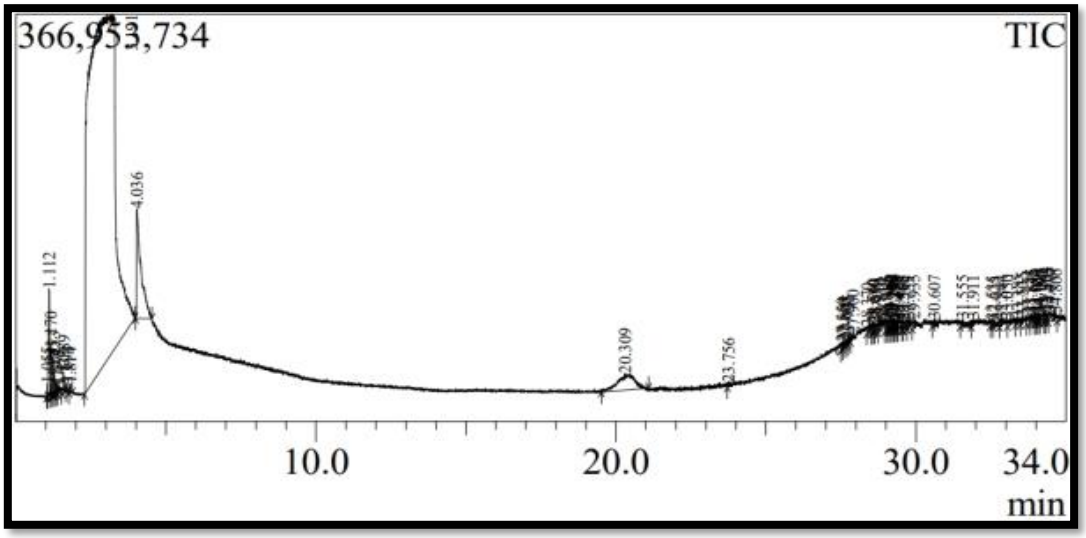


Figure 3: GCMS chromatogram of ethanolic extract of sagittaria trifolia.

S.no	Retention time	Chemical constituents	Area %	Uses
1	34.039	Benzoic acid	0.09	Treat skin irritation & inflammation caused by burns
2	1.055	1,1-Cyclopropanedicarbonitrile	0.07	Decreases pain
3	20.309	Dimethyl Sulfoxide	2.40	Treats painful bladder syndrome, decreases topical pain. Treats inflammation, headache, osteoarthritis, rheumatoid arthritis, severe facial pain.
4	34.304	Penta siloxane	0.10	Anti-inflammatory, removes wrinkles & skin blemishes & irritation, prevents scaling
5	34.405	Hexadecenoic acid	0.13	Anti-inflammatory
6	32.625	Hydroxybutyric acid	0.07	Analgesic
7	29.365	phenothiazine 32	0.09	Treats moderate to severe pain.
8	33.535	Thiatriazole	0.07	Anti-inflammatory
9	31.055	Pyredine-2	1.02	Relieves symptoms caused by irritation of the urinary such as pain, burning, & the feeling 0f needing to urinates urgently 0r frequently.
10	34.20	Dimethoxy amine	2.80	Analgesic, Anti-inflammatory,

IN VIVO ANTI-INFLAMMATORY ACTIVITY:

CARRAGEENAN INDUCED ANTI- INFLAMMATORY TEST:

GROUP	Percentage inhibition of inflammation			
	30 min	1 hour	2 hours	3 hours
1	0	0	0	0
2	0..88 ± 0.006	0..94 ±0.005	0..97 ±0.002	0..96 ± 0.003
3	0.40± 0.012***	0.35 ± 0.013***	0.33 ± 0.012***	0.31 ± 0.007***
4	0.71 ± 0.005**	0.74 ±0.006**	0.77 ± 0.006**	0.76 ± 0.006**
5	0.69 ± 0.005**	0.63 ±0.006**	0.57 ± 0.006**	0.47± 0.006**
6	0.64 ± 0.004*	0.60 ± 0.004*	0.57 ± 0.008*	0.55 ± 0.006*
7	0.62 ± 0.005**	0.54 ±0.006**	0.45 ± 0.006**	0.43 ± 0.006**
8	0.59 ± 0.010***	0.54 ± 0.009***	0.50 ± 0.007***	0.45 ±0.008***
9	0.54 ± 0.005**	0.47 ±0.006**	0.43 ± 0.006**	0.38 ± 0.006**

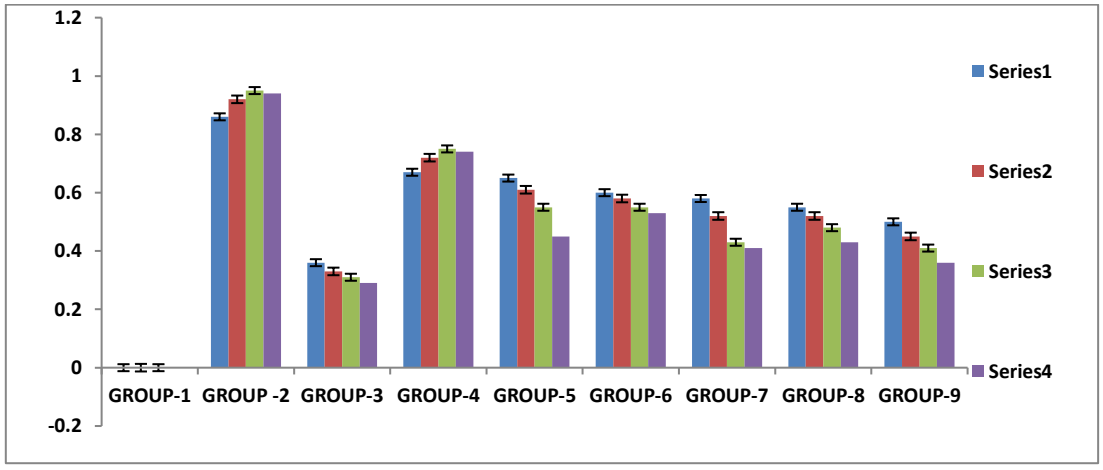


Figure 4. Percentage Inhibition of inflammation in different animal groups

IN VIVO ANALGESIC ACTIVITY:

EDDY'S HOT PLATE METHOD:

GROUP	REACTION Initial Time	TIME 15 min	30 min	60 min
1	7.18 ± 0.20	7.22 ± 0.28	6.57 ± 0.30	6.47 ± 0.29
2	6.08 ± 0.378	5.26 ± 0..420	4.16 ± 0..430	3.52 ± 00.392
3	7.58± .253***	11.9 ±0.262***	12.8 ± 0.261***	14.57± .260***
4	6.22 ± 0.460**	10.35 ± 0.372**	11.19 ±0.462**	12.20± 0.459**
5	7.90 ± 0.184*	10.84 ± 0.182*	11.92 ± 0.187*	12.93 ± 0.18*
6	7.00±0.178***	10.99± .241***	12.00± .182***	13.25± .183***
7	7.68±0.022	10.00± .241***	9.25± .182***	12.28± .183***
8	7.99±0.175***	11.00± .241***	11.99± .182***	13.98±0.185***
9	6.99± .176***	12.99±0.241***	14.99± .182***	14.98±0.188***

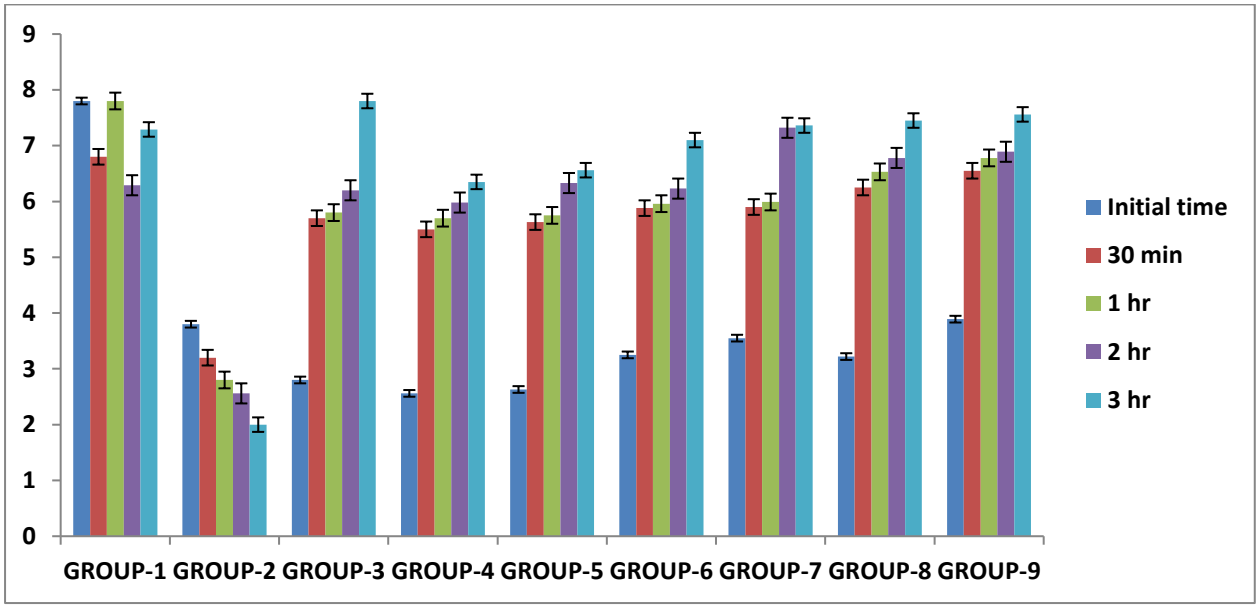


Figure 5. Change in the reaction time due to analgesic effect in different animal groups.

IN VITRO ANTI-INFLAMMATORY & ANALGESIC ACTIVITY:

COX-1 Assay:

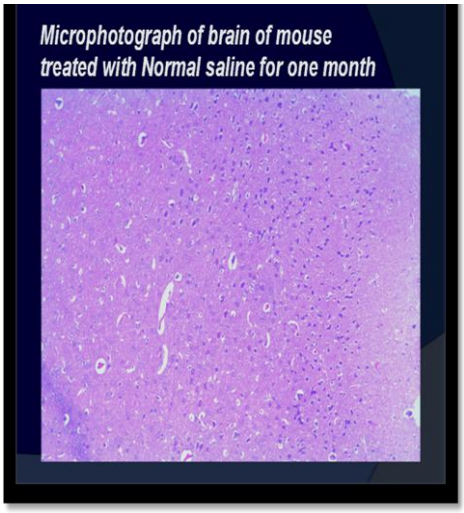
Concentration µg/ml	Standard drug	Plants 1 Ziziphus Trinervia	Plants 2 Sagitaria Trifolia
100	84.06%	72.3%	75.12%
80	80.04%	60.13%	64.10%
60	68.03%	45.06%	48.06%
40	45.08%	33.05%	37.08%
20	29.06%	19.07%	23.07%

COX-2 Assay:

Concentration µg/ml	Standard drug	Plants 1 Ziziphus Trinervia	Plants 2 Sagittaria Trifolia
100	87.06%	75.3%	78.12%
80	83.04%	63.13%	67.10%
60	71.03%	48.06%	51.06%
40	48.08%	36.05%	40.08%
20	32.06%	22.07%	26.07%

HISTOPATHOLOGY

Group 1:



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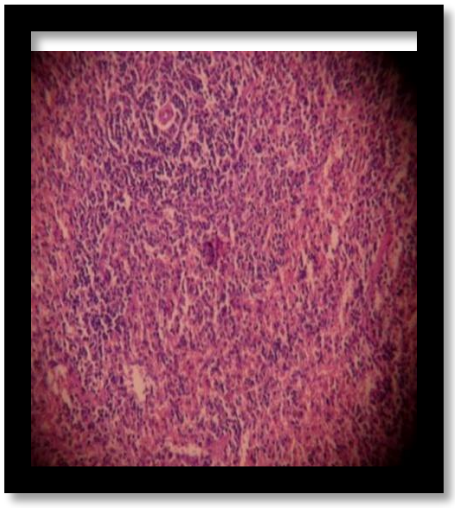

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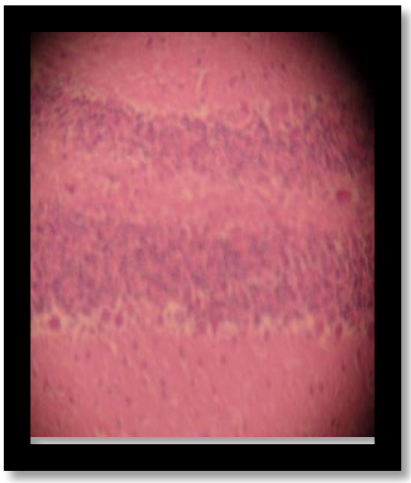

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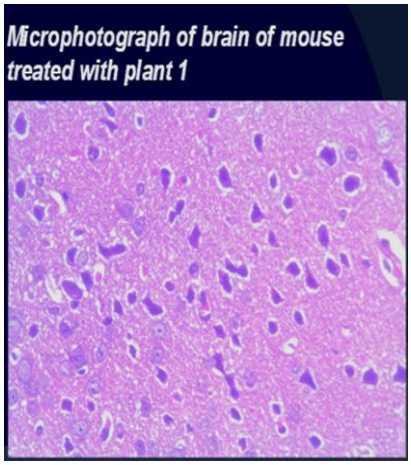
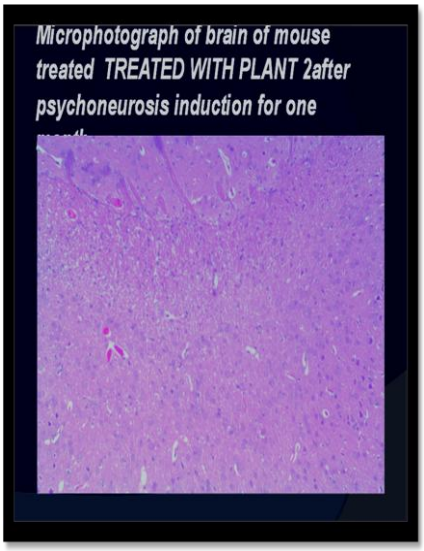


Group 5:



Group 6:

Figure 6. Histopathological Slides of brain showing neuronal changes

DISCUSSION:

The results of this study demonstrate the significant analgesic and anti-inflammatory potential of *Ziziphus trinervia* and *Sagittaria trifolia* extracts, both individually and, notably, in combination. The findings provide scientific validation for the traditional uses of these plants and suggest a potent synergistic effect when administered together.

Key Findings and Interpretation

- **Potent Anti-inflammatory Activity**: In the carrageenan-induced paw edema model, the combined extracts exhibited an exceptional 96% effectiveness. The individual extracts also showed high efficacy (*S. trifolia*: 93%, *Z. trinervia*: 75%). These results are comparable to or exceed the effectiveness of many standard nonsteroidal anti-inflammatory drugs (NSAIDs) used in similar models. This indicates a powerful ability to modulate inflammatory pathways *in vivo*, likely through the inhibition of inflammatory mediators such as cytokines or cyclooxygenases.
- **Superior Analgesic Effects:** Using the Eddy's hot plate method, the combination achieved 98% effectiveness, demonstrating outstanding pain relief potential. The individual extracts were also highly effective (*S. trifolia*: 96%, *Z. trinervia*: 82%). This highlights the strong anti-nociceptive (pain-blocking) properties of both plants and the enhanced effect of their mixture.
- **In Vitro COX Inhibition**: The in vitro COX inhibition assay revealed that while both extracts inhibit cyclooxygenase activity (*Z. trinervia*: 71 µg/mL; *S. trifolia*: 86 µg/mL), the standard reference drug (45 µg/mL) showed greater potency at a lower concentration. This suggests that the observed high *in vivo* efficacy of the extracts might involve not just direct COX inhibition, but also other mechanisms, such as antioxidant activity or modulation of different pain pathways, possibly involving the GABAA receptors in the case of *Ziziphus* species.
- **Synergistic Action**: The most compelling finding is the significantly enhanced efficacy when the two extracts are combined. The combination consistently outperformed individual extracts in both anti-inflammatory and analgesic assays, suggesting a synergistic or additive effect between the active phytochemicals (e.g., flavonoids, saponins, terpenoids) present in each plant. This synergy warrants further investigation to identify the exact compounds responsible for this interaction.

Context and Implications

These findings support the traditional use of *Ziziphus trinervia* for treating inflammatory conditions, migraines, and chest pain, and *Sagittaria trifolia* for relieving pain associated with arthritis and headaches. The study bridges the gap between ethnobotanical knowledge and scientific evidence, offering a potentially powerful natural alternative or complementary treatment to synthetic pharmaceuticals, which often come with side effects.

CONCLUSION:

The present study successfully evaluated the pharmacological activity of the ethanolic plant extracts of Ziziphus trinervia and Sagittaria trifolia & showed strong analgesic and anti-inflammatory activity. The extracts were prepared using the maceration method with ethanol, yielding bioactive compounds such as alkaloids, flavonoids, tannins, glycosides, saponins, phenols, and triterpenoids.

Although Sagittaria trifolia exhibits greater anti-inflammatory and analgesic activity than Ziziphus trinervia, the combination of both extracts enhances overall therapeutic efficacy, resulting in balanced and potent response.

These herbal alternatives are cost-effective, have fewer side effects, and offer a natural approach to pain and inflammation management.

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