Research Article

Phytochemical Profiling and Assessment for In vivo Anti-inflammatory Activity of Cosmostigma racemosum (Roxb.) Wight Leaf Extracts

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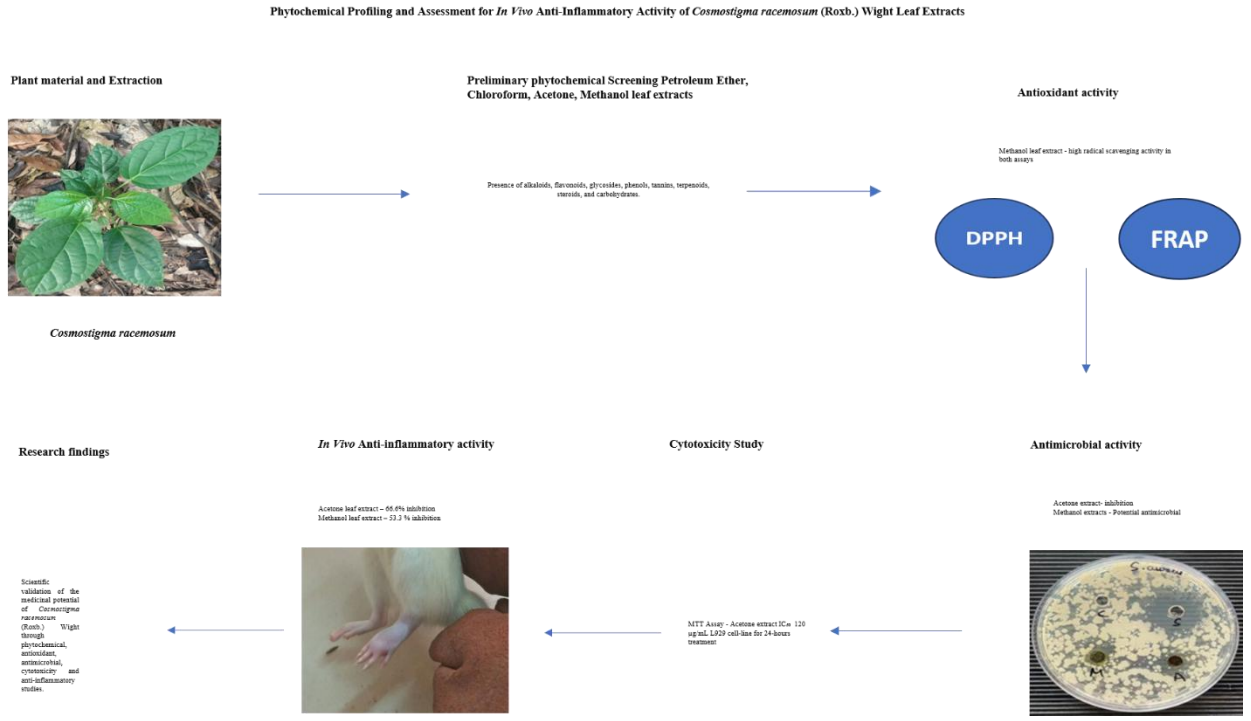
Abstract

Plants due to their diverse bioactive compounds including secondary metabolites with medicinal properties are significant source of therapeutic agents. Preliminary phytochemical screening of Cosmostigma racemosum (Roxb.) Wight leaf extracts were performed following standard protocols. The antioxidant potential of leaf extracts was evaluated using DPPH radical-scavenging and FRAP assays, the antimicrobial activity of acetone and methanolic extracts were assessed by agar well diffusion method, the cytotoxic potential of the extracts was appraised referring to MTT assay based on the viability of treated cells. Further acetone and methanol leaf extracts were assessed for in vivo anti-inflammatory property using the carrageenan-induced rat paw edema method. The present study revealed the diverse phytoconstituents including alkaloids, flavonoids, glycosides, phenols, tannins, terpenoids, steroids and carbohydrates in leaf extracts, exhibited promising antioxidant property, notable antimicrobial activity against the tested bacterial and fungal strains. At 200mg/kg body weight of Wistar rats, the acetone extract produced a remarkable reduction in paw edema compared with the methanol extract indicating anti-inflammatory potential. The antioxidant, antimicrobial and anti-inflammatory activities of Cosmostigma racemosum (Roxb.) Wight may be attributed to phytochemicals such as flavonoids and alkaloids supporting its strong therapeutic potential, warranting further research to identify the precise bioactive compounds responsible for its pharmacological effects.

Keywords: Cosmostigma racemosum, phytochemical, antioxidant, antimicrobial, MTT assay, carrageenan.



GRAPHICAL ABSTRACT

1. Introduction

Inflammation is a localized protective response to infection, irritation or injury, characterized by redness, warmth, swelling, pain and mediated by factors such as histamine, bradykinin and prostaglandins [1]. There are two types of inflammation that are recognized; acute and chronic. In contradiction of chronic inflammation, which has a long-term, whole-body effect and causes mild inflammation through the body, the former is a short-term reaction with a localized effect and responds where a problem exists. These inflammations are known to cause diseases like multiple sclerosis, rheumatoid arthritis, chronic asthma and bronchitis [2]. Even though inflammation is essential to an individual's health, it can be uncomfortable and upsetting resulting in adverse effects. Inflammation is commonly treated using nonsteroidal anti-inflammatory medications such as celecoxib, naproxen, etoricoxib, ibuprofen, indomethacin and diclofenac [3, 4]. However, gastrointestinal, kidney, liver and cardiac toxicities are a few of the many adverse effects linked with these conventional drugs [5].

Medicinal plants continue to serve as a valuable source of natural remedies in primary health care and they have been employed in folk medicine for the treatment of gastrointestinal disorders, ulcers, inflammation and other diseases [6]. They habitually produce alkaloids, tannins, flavonoids, steroids, phenolic acids, saponins and terpenoids as secondary metabolites under stress which possesses a variety of pharmacological effects on a range of illness and conditions including inflammation [7].

Cosmostigma racemosum (Roxb.) Wight by habit is a woody climber generally called as milky weed belonging to Asclepiadaceae family. As per the information gathered from the local communities, the healthy leaf paste is commonly applied for inflammation treatment in cattle. Nevertheless, there is a dearth of empirical data to support their often-praised application [1]. The present study was conducted to investigate the phytochemical constituents and antioxidant activity of petroleum ether, chloroform, acetone and methanol extracts, antimicrobial property, cytotoxicity study and *in vivo* anti-inflammatory activity of acetone and methanol leaf extracts of *C. racemosum* by Carrageenan-induced paw oedema in Wistar rat's model to document the pharmacological efficacy of the study plant [8].

2. Materials And Methods

2.1 Plant material collection and authentication

The plant material was collected during the vegetative growth phase from Udupi district, Karnataka state. The plant was identified and deposited in ward herbarium at Department of Applied Botany, Mangalore University. Fresh, healthy leaves were selected and washed thoroughly under running tap water to remove the dust and any debris, shade-dried at ambient laboratory conditions (25°C-30°C) with good constant air circulation. Before being extracted further, the dried leaves were crushed into a coarse powder and stored at room temperature in airtight glass containers [7].

2.2 Preparation of successive extracts and preliminary phytochemical screening

Petroleum ether, chloroform, acetone and methanol solvents were utilized in a Soxhlet apparatus to extract the leaf sample successively [9]. Upon removing any residual solvents through rotary evaporator, the extracts were refrigerated for further analysis. Subsequently phytochemical screening in triplicate with appropriate positive and negative controls was performed with the leaf extracts of all solvents to detect the presence of carbohydrates, proteins, alkaloids, flavonoids, phenolic compounds, tannins, terpenoids, saponins and other secondary metabolites [10].

2.3 Antioxidant property

2.3.1 DPPH assay

The antioxidant potential of the leaf extracts was evaluated using DPPH radical-scavenging and ferric reducing antioxidant power (FRAP) assays [3 and 5]. A solution of 0.2 mM 1,1-diphenyl-2-picryl-hydrazyl (DPPH) in methanol was prepared. An aliquot of the sample (0.5 ml) taken in the concentration range of 10-500 µg/ml was added to 1ml solution of DPPH. After the incubation period of 30 min at 30°C temperature, at 517 nm, absorbance was measured against an appropriate blank. The DPPH radical was computed using the formula given below [11].

DPPH radical scavenging activity (%) = $[(Abs_{control} – Abs_{sample})] / (Abs_{control})] \times 100$

Where $Abs_{control}$ (Ao) = absorbance of DPPH radical + solvent;

Abs_{sample} = absorbance of DPPH radical + sample extract/standard.

2.3.2 FRAP assay

2.5mL of 0.2M phosphate buffer and 2.5 mL of freshly prepared 1% potassium ferricyanide (K3Fe (CN6)) were combined with varying quantities of the extract (0.2-1mL). Later the mixture was incubated for 20 minutes at 50°C. After adding 2.5 mL of 10% trichloroacetic acid (TCA), the mixture was centrifuged for 10 min at 3000rpm. 2.5 mL of the resulting supernatant, 2.5mL of methanol and 0.5mL of 0.1% FeCl3 was mixed in an aliquot. A spectrophotometer was used to measure the final mixture's absorbance at 700 nm. The reducing power was measured in milligrams of ascorbic acid equivalents per gram of extract (mg AAE/g) and the experiment was conducted in triplicate [11, 12]. The standard Error of the Mean (SEM) is calculated using the formula;

SEM = s/n

Where s = sample standard deviation and n=3 (number of replicates).

2.4 Antimicrobial property

Using the agar well diffusion method in compliance with standard microbiological procedures [13,14], the antifungal activity of acetone and methanol leaf extracts of *Cosmostigma racemosum* was evaluated against *Candida albicans*, *Aspergillus niger* and *Trichophyton rubrum,* while the antibacterial property was determined against *Staphylococcus aureus*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Escherichia coli.*

Soya bean casein-digested agar plates were infected with *S. aureus*, *B. cereus*, *K. pneumoniae* and *E. coli*, whereas, potato dextrose agar plates were inoculated with *C. albicans*, *A. niger* and *T rubrum* (90 mm). The 5 mm well on agar plates was filled with 25µl of acetone and methanol leaf extracts, 25µl each of standard Ciprofloxacin for *S. aureus*, *B. cereus*, *K. pneumoniae*, *E. coli* and Itraconazole for *C. albicans*, *A. niger* and *T rubrum* and distilled water as control. [14] The treated plates containing *S. aureus*, *B. cereus*, *K. pneumoniae*, *E. coli* was incubated for 24 hrs. in aerobic chamber at 37°C. For 24 to 48 hours, *C. albicans, A. niger* and *T. rubrum* were cultured in an aerobic chamber at 27°C. Afterward, the zone of inhibition surrounding the wells on the treated plates were examined [15].

2.5 Cytotoxicity study

The cytotoxic potential of the leaf extracts was evaluated against L929 mouse fibroblast cell line using MTT assay [16]. To obtain approximately 10,000 cells in 200µl, the cells were treated with trypsin, aspirated into a 15ml centrifuge tube, centrifuged at 300 x g (g = acceleration due to earth's gravity) and then resuspended in DMEM (Dulbecco's Modified Eagle's Medium). Following that, 200 µl of the cell suspension was transferred to each well of a 96-well micro titer plate and the plate was incubated for a 24hrs period at 37°C with 5% CO_2. The cells were subsequently incubated at 37°C with 5% CO_2 for further 24 hours after the medium was changed to acetone and methanol leaf extract solutions at concentrations of 20, 40, 60, 80 and 100 µl from stock. Following incubation, 100 µl of 10% MTT reagent (final concentration of 0.5mg/ml) was applied to each well after acetone and methanol leaf extracts were withdrawn. The plate was incubated for 3 hours at 37°C with 5% CO_2 environment. After removing the medium, the formazan crystal formed was dissolved by adding 100 µl of solubilization solution DMSO (Dimethyl Sulfoxide) [17]. A microplate reader was used to measure the absorbance at a wavelength of 570nm with a reference wavelength of 630nm. After adjusting for background and blank values, the percentage of cell growth inhibition was computed. The dose response curve was used to calculate the IC_{50} value. The cytotoxic activity of the extract was evaluated using the IC_{50} value in µL, which represents the concentration of the extract needed to inhibit 50% of cell viability [18].

2.6 *In vivo* anti-inflammatory potential

2.6.1 Experimental animals and ethical approval

Before being used in experiments, healthy adult male Wistar albino rats weighing between 150 and 200 grams were procured from the institutional animal facility and allowed to acclimate for seven days to conventional laboratory settings (temperature between 27°C and 29-degree Celsius, relative humidity between 50 and 70 percent and 12-hour light/dark cycle) [19]. The animals were kept in polypropylene cages with sterile husk bedding, a regular pellet meal and unlimited access to water. All experimental procedures were performed in accordance with CPCSEA guidelines and were approved by the Institutional Animal Ethics Committee (Ethical clearance certificate number: SCP/IAEC/F150/P184/2021). Animals were handled humanely. Randomization of animals into treatment groups and blinding of the observer measuring paw volumes were used to minimize bias [2, 20].

2.6.2 Acute oral toxicity

An acute oral toxicity limit test was performed according to OECD guideline 425. Male Wistar rats fasted overnight

provided with water *ad libitum* were administered a single oral dose of the extract at 2000mg/kg body weight by oral gavage. Animals were observed continuously for the first 4 hours for signs of toxicity (behavioral changes, autonomic or neurologic signs), then daily for 14 days for morbidity and mortality. Body weights and gross clinical observations were recorded on days 0, 7 and 14. No mortality or significant clinical toxicity was observed, hence, $1/10^{th}$ of the limit dose (200 mg/kg) was selected as the test dose for anti-inflammatory studies [3].

2.6.3 Evaluation of anti-inflammatory activity by carrageenan-induced hind paw edema model

Male Wistar rats of body weight range from 150g to 200g were randomly divided into four groups with six animals each and were fasted overnight before the experiment with free access to water. The animals were divided into four groups, with Group I serving as the normal control and receiving normal saline (5 ml/kg body weight) orally, while Group II served as test groups and received acetone and methanol extracts, respectively at 200mg/kg body weight orally [1, 2, 15]. The extracts were freshly prepared as fine homogenized suspensions in 2% (w/v) Tween-80 and administered one hour before sub plantar injection of 1% carrageenan in normal saline into the left hind paw [20].

Paw volume was measured immediately before carrageenan injection (time 0) and at 1, 2, 3 and 4 hours post-injection using a calibrated digital plethysmometer (Orchid Scientific Model PLM-01). The observer was blinded to treatment groups. Increase in paw volume (mL) at each time point was calculated relative to baseline (T0). Percentage inhibition of edema for each treated group at each time point was calculated as:

% Inhibition = 100 x (1-Vt/Vc)

Where Vt = mean paw volume (increase from baseline) of treated group, and Vc = mean paw volume (increase from baseline) of control group/ additionally, Area Under the Curve (AUC) for paw volume vs time (0-4hrs.) was computed to estimate overall anti-edematous effect [20, 21].

2.7 Statistical analysis

Every experiment was performed in triplicate. For each experiment group, n =6 is indicated and the data is expressed as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Dunnett's multiple – comparison test was used to establish statistical significance; $p < 0.001$ and was deemed statistically significant. [22].

3. Results

3.1 Preliminary phytochemical screening:

The preliminary phytochemical analysis indicated the presence of alkaloids, flavonoids, glycosides, phenols, tannins, terpenoids, steroids and carbohydrates [7]. The results presented in Table 1 demonstrate the presence of diverse classes of phytochemical constituents in the tested leaf extracts.

Table 1: Preliminary phytochemical screening of *Cosmostigma racemosum* leaf extracts

Phytochemicals	LEAF			
	Petroleum Ether	Chloroform	Acetone	Methanol
Carbohydrate	-	+	+	+
Protein	-	-	-	-
Alkaloids	-	-	+	+
Flavonoids	+	-	+	+
Saponin	-	-	-	-
Glycosides	+	-	+	-
Phenols	-	+	+	+
Tannins	+	-	+	+
Terpenoids	+	+	+	+
Steroids	+	+	+	+

(- Absent, + Present)

3.2 Antioxidant property

In the DPPH assay, the methanol extract exhibited the highest radical scavenging activity (89.34± 0.05%), followed by the acetone (88.35 ± 0.03%) and petroleum ether extract (85.64 ± 0.01%), whereas the chloroform extract showed the

lowest activity (19.73 ± 0.06%).

Similarly, the reducing power of *C. racemosum* leaf extracts justified the efficient antioxidant property in FRAP assay. Increase in absorbance of solution with increase of leaf extract concentration was observed [7, 10]. The methanol leaf extract exhibited the highest reducing activity with 0.171 ± 0.0003 value, while the chloroform extract showed the lowest activity 0.054 ± 0.0003. Petroleum and acetone leaf extracts showed 0.128 ± 0.0003 and 0.106 ± 0.0006 values respectively owing to possess strong radical scavenging potential, suggesting the antioxidant-active phytoconstituents in the leaf [12]. The data of DPPH and FRAP assays are shown in table 2.

Table 2: Antioxidant property of *Cosmostigma racemosum* Leaf extracts

Solvent	DPPH radicle scavenging activity (%)
Petroleum Ether	85.64 ± 0.01
Chloroform	19.73 ± 0.06
Acetone	88.35 ± 0.03
Methanol	89.34 ± 0.05

DPPH (2,2-diphenyl-1-picrylhydrazyl radicle scavenging) assay

Solvent	FRAP radicle scavenging activity (%)
Petroleum Ether	0.128± 0.0003
Chloroform	0.054 ± 0.0003
Acetone	0.106 ± 0.0006
Methanol	0.171 ± 0.0003

FRAP (Ferric reducing antioxidant power) assay

3.3 Antimicrobial efficacy of *Cosmostigma racemosum* acetone and methanol leaf extracts

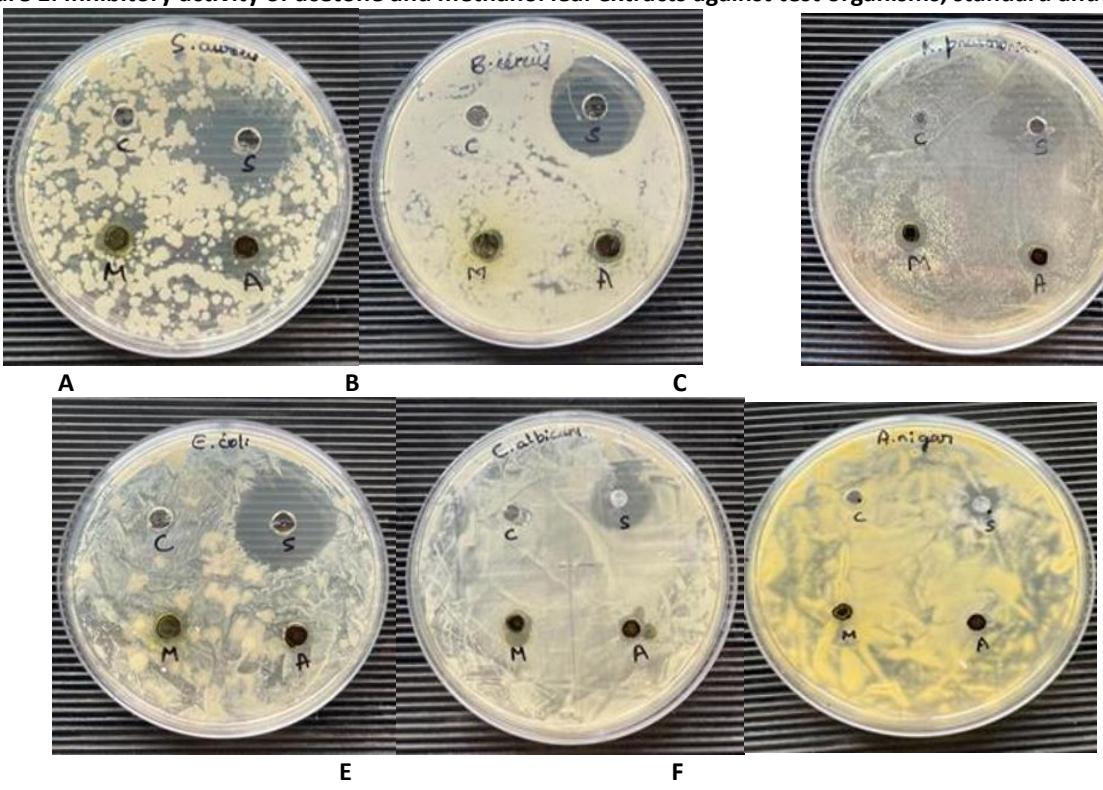
The investigated bacteria were susceptible to the antibiotic activity of acetone and methanol extracts. While both extracts had comparable action against *Klebsiella pneumoniae* (10 mm), acetone extract showed higher antibacterial activity against *Staphylococcus aureus* (11 mm) and *Bacillus cereus* (10 mm). while methanol extract demonstrated action against *Escherichia coli* (9 mm) and acetone extract was inert [12,13,14]. With inhibition zones of 7 mm and 9 mm respectively, acetone and methanol extracts had moderate activity against *Candida albicans*; however, neither extract was effective against *Aspergillus niger* or *Trichophyton rubrum*. Table 3 and Figure 1 show the outcome.

Table 3: Inhibitory activity of *C. racemosum* acetone and methanol leaf extracts against test compounds and organisms.

Test Organisms	Test Compounds	Concentration per well (µg/ml)	Zone of inhibition (mm)
Staphylococcus aureus	Control	-	-
	Ciprofloxacin (Standard)	2.5	25
	Acetone (25µl)	2500	11
	Methanol (25µl)	2500	09
Bacillus cereus	Control	--	--
	Ciprofloxacin (Standard)	2.5	23
	Acetone (25µl)	2500	10
	Methanol (25µl)	2500	09
Klebsiella pneumoniae	Control	-	-
	Ciprofloxacin (Standard)	2.5	22
	Acetone (25µl)	2500	10
	Methanol (25µl)	2500	10
Escherichia coli	Control	-	-

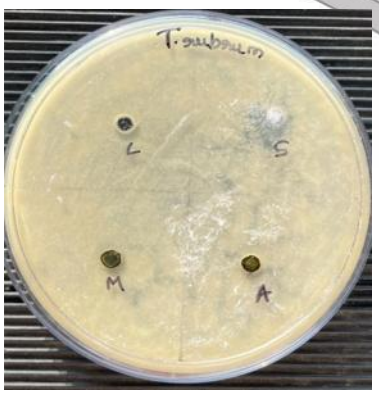
	Ciprofloxacin (Standard)	2.5	25
	Acetone (25µl)	2500	-
	Methanol (25µl)	2500	09
Candida albicans	Control	-	-
	Itraconazole (Standard)	2.5	20
	Acetone (25µl)	2500	07
	Methanol (25µl)	2500	09
Aspergillus niger	Control	-	-
	Itraconazole (Standard)	25	13
	Acetone (25µl)	2500	-
	Methanol (25µl)	2500	-
Trichophyton rubrum	Control	-	-
	Itraconazole (Standard)	25	14
	Acetone (25µl)	2500	-
	Methanol (25µl)	2500	-

Figure 1: Inhibitory activity of acetone and methanol leaf extracts against test organisms, standard and control



A **B** **C**

D **E** **F**



G

(*Staphylococcus aureus* – A, *Bacillus cereus* – B, *Klebsiella pneumoniae* – C*, Escherichia coli* - D, *Candida albicans* – E, *Aspergillus niger* – F and *Trichophyton rubrum* – G).

3.4 MTT Assay:

Petroleum ether, chloroform, acetone and methanol leaf extracts of *C. racemosum* exhibited varying degrees of cytotoxic activity against L929 cell-line following 24 hours of exposure [17]. Among the tested extracts, acetone showed the highest cytotoxic potential with an IC_{50} value below 100µg/ml, followed by chloroform (199. 65µg/ml), methanol (298.21 µg/ml) and petroleum ether (382.77 µg/ml).

Table 4: The IC_{50} values of the test compounds for L929 cell-line for 24-hour treatment

C. racemosum **Leaf extract**	**L929 cell line** **IC_{50} (in µg/ml) 24hr**
Petroleum ether	382.77µg/ml
Chloroform	199.65µg/ml
Acetone	72.36µg/ml
Methanol	298.21µg/ml

3.5 *In vivo* anti-inflammatory efficacy:

Employing a carrageenan-driven paw edema model in Wistar rats, the anti- inflammatory effect of acetone and methanol leaf extracts was assessed [23]. The control group, which received 1ml of Tween-80, experienced a paw edema volume of 0.15±0.004 ml. Diclofenac sodium (10 mg.kg), typical drug, significantly reduced paw edema to 0.04±0.004 ml or 73.3% inhibition. Among the leaf extracts, the acetone extract at 200mg/kg showed the best anti-inflammatory impact with 66.6% inhibition and a drop in paw edema volume to 0.005±0.007 ml, however its effect was less influential as that of the standard drug. At the same dosage the methanol extract displayed a paw edema volume of 0.007±0.003 ml, corresponding to 53.3% inhibition. When compared to methanol extract, acetone leaf extract substantially decreased the rat's paw volume ($p<0.001$) for 4 hours following carrageenan administration [1,2]. Table 5 displays the variations in percentage inhibition of acetone and methanol leaf extracts of *C. racemosum* against the standard drug; diclofenac sodium.

Table 5: Effect of Leaf extract of *Cosmostigma racemosum* on Carrageenan-induced rat paw edema at 4 hours

Group	**Dose (mg/kg)**	**Edema volume (ml)**	**% inhibition**
Control Tween-80	1 ml	0.15±0.004	-----
Standard (Diclofenac Sodium)	10 mg/kg	0.04±0.004	73.3
Acetone extract	200 mg/kg	0.05 ± 0.007	66.6
Methanol extract	200 mg/kg	0.07 ± 0.003	53.3

4. DISCUSSION:

Medicinal herbs have been widely used for centuries to cure and avert various ailments and continue to constitute an integral component of traditional healthcare systems worldwide [1, 24]. The antioxidant efficacy of the different solvent extracts varied considerably in both DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays. The methanol leaf extract exhibited the highest reducing activity, indicating that it contains a higher concentration of compounds capable of reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) compared with the lowest reducing activity exhibited in chloroform extract. The relatively high FRAP values of the methanol, petroleum ether and acetone extracts suggest the occurrence of flavonoids, phenolic compounds in *Cosmostigma racemosum* (Roxb.) Wight leaf with significant free-radical scavenging and reducing properties [11, 25].

The acetone and methanol leaf extracts of *C. racemosum* exhibited selective antimicrobial property. The leaf extracts produced inhibition zones of 9 mm and 11 mm, respectively against *Staphylococcus aureus* while the standard Ciprofloxacin (2.5 µg/ml) produced 25mm zone of inhibition, 9 mm and 10 mm inhibition zones against *Bacillus cereus* and 23 mm zone in Ciprofloxacin, 10mm inhibition zones in both extracts against *Klebsiella pneumoniae,* whereas the standard antibiotic produced a zone of 22 mm and the methanol extract showed an inhibition zone of 9 mm against *Escherichia coli,* while the acetone extract showed no inhibition zone at 25mm [12, 13, 16].

The methanol and acetone leaf extracts produced inhibition zones of 9 mm and 7mm, respectively against *Candida albicans,* while itraconazole (25 µg/ml) produced an inhibition zone of 20 mm. Neither extract showed any apparent antifungal activity against *Aspergillus niger* or *Trichophyton rubrum*, while the standard itraconazole produced inhibition zones of 13 mm and 14 mm respectively. The observed inhibitory zones show that the leaf extract contains antimicrobial components, even if the activity in acetone and methanol leaf extract was less than that of the conventional antibiotics. [9]. These findings support the ethnomedicinal use of the plant in the treatment of bacterial infections and highlight its potential as a source of natural antibacterial agents, justifying further studies on isolation, characterization and mechanism of action of the active constituents.

Due to the presence of flavonoids, alkaloids and terpenoids, the acetone leaf extract exhibited the strongest cytotoxic activity against the L929 cell line after a 24-hour treatment, as seen by its lower IC_{50} 72.36 µg/ml value compared to methanol leaf extract with 298.21µg/ml. The methanol and petroleum ether extracts, on the other hand, demonstrated higher IC_{50} values, suggesting relatively less cytotoxic effects on normal fibroblast cells. The present study reveals that the acetone extract's greater activity demands for additional exploration into its possible antiproliferative qualities, the higher IC_{50} values indicate that these extracts have better cellular tolerance. Overall, the results show that the extracts cytotoxicity activity varies depending on the extraction solvent and may be impacted by the phytochemical contained in each fraction [17,18].

The observed anti-inflammatory activity of *C. racemosum* may be linked to decreased vascular permeability and inhibition of inflammatory mediators such as prostaglandins and histamine. [26]. The significant reduction in paw oedema after four hours suggests possible interference with prostaglandin- mediated inflammation, while phenolic compounds and tannins may contribute by stabilizing cell membranes and reducing mediator release [27]. The traditional usage of *Cosmostigma racemosum* for inflammatory diseases suggests its potential as a safe and effective natural anti-inflammatory agent, especially in light of the drawbacks and adverse effects of synthetic nonsteroidal anti-inflammatory medications (NSAIDs) [28].

5. Conclusion:

The present research establishes the therapeutic relevance of *Cosmostigma racemosum* (Roxb.) Wight through systematic phytochemical, *in vitro* and *in vivo* evaluations and offers comprehensive scientific evidence to support the traditional medicinal claims associated with plant.

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