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Comparative Evaluation of the Anti-Inflammatory Properties of Methanol and Aqueous Crude Extracts of Apical leaves of *Sida cuneifolia*: An Ethnomedicinal Plant

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Authors' contributions

This work was carried out in collaboration among all authors. Author KI designed and wrote the study protocols. Authors AAA and SDC reviewed and made amendments of the manuscript. Author KI executed the experiments with supervision and monitoring by authors KI and AAA. Authors AAA and SDC managed the analyses of the study. Author KI wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to assess and compare the yield, phytochemical compositions, and anti-inflammatory properties of methanol and aqueous crude extracts derived from the Apical leaves of *Sida cuneifolia.*

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Study Design: An analytical study design was employed to evaluate yield and phytochemical composition, while an experimental study design was utilized for anti-inflammatory evaluation studies.
Place and Duration of Study: The study was conducted at Mbarara University of Science and Technology, specifically in the Department of Pharmacy Laboratory, spanning from August 2022 to September 2023.
Methodology: The extraction yields were determined, and phytochemical profiling conducted to establish the composition of the extracts. Acute dermal toxicity was assessed to determine *s. cuneifolia* ointment toxicity level using OECD guidelines No. 402. Anti-inflammatory activities were evaluated using the HRBC membrane stabilization model and carrageenan-induced paw inflammatory model *in-vitro* and *in-vivo*, respectively.
Results: The aqueous extract exhibited a higher percentage yield (10.1%) compared to the methanol extract (4.7%). The methanol extract contained alkaloids, phenolic compounds, steroids, tannins, and cardiac glycosides, while alkaloids and cardiac glycosides were absent in the aqueous extract.
Methanol and aqueous extracts, at different concentrations of (0.5, 1.0, 2.0 mg/mL), showed dose-dependent significant stabilization towards HRBC membranes of (54.6%, 59.9%, 66.5%), and (3.85%, 12.57%, 17.10%), respectively. The percentage of protection for the concentration of the methanol extract at 2.0 mg/mL was the highest (66.5%) among the extract dose levels but lower than that of the standard (76.66%).
Sida cuneifolia ointment dose levels of (0.5%, 2.5%, and 5.0%) w/w demonstrated significant *(P=0.05)* reductions in mice paw volume, with percentage inhibitions of (86.33%, 91.4%, and 91.4%). Dose levels (2.5% and 5%) w/w exhibited more potent activity of (91.4%) compared to that of the reference standard, Diclofenac gel 0.1% w/w. (79.41%). Both dose levels of extract ointments (2.5% and 5.0%) w/w exhibited identical levels of percentage inhibition (91.3%) at the end of 4 hours.
Conclusion: This study provides scientific evidence supporting the ethno-medicinal use of Apical leaves of *Sida cuneifolia*, suggesting its potential transformative development in ethnomedicine.

Keywords: Sida cuneifolia; anti-inflammatory; phytochemical analysis; leaf extract; diclofenac gel; HBRC.

1. INTRODUCTION

Sida cuneifolia, characterized by small shrubby herb morphology featuring yellow mallow flowers with five distinct petals and sepals, possesses notched leaves at the apex, and its fruit comprises mericarps. Belonging to the malvacae family and the *Sida* genus, which encompasses approximately 200 species distributed across tropical and subtropical regions globally.[1] *Sida cuneifolia* holds a notable position in the creation of indigenous remedies for African herbal treatments.[2,3] Widely utilized in folk medicine in Uganda and other African regions, its importance is underscored by the lack of research validating its traditional applications [4-8].

Despite existing studies on antibacterial, antifungal activity, and phytochemical analysis of *Sida cuneifolia* leaves, there is a notable absence of information regarding the anti-inflammatory properties of the specific plant part known as the 'Apical leaves,' commonly used in herbal preparations [9,10]. This study is designed to address this gap by evaluating the anti-inflammatory activity specifically in the Apical leaves of *Sida cuneifolia*. The outcomes of this research endeavor aim to contribute valuable insights to the development of anti-inflammatory drugs.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

The plant was collected from Sekajja institute of traditional medicine in Buyijja Mpigi district. The plant voucher specimen was prepared and identified as *Sida cuneifolia* Roxb. Ik/2023/001 by a plant taxonomist at the department of Biology Mbarara University of Science and Technology.

Ethical clearance was obtained from the Uganda National Council of science and technology NS659ES.

2.2 Preparation of Plant Extract

The Apical leaves were excised at 0.5 cm of the harvested branch shoots that were about 10cm long. They were then washed for 10minutes with running water to remove impurities then left to dry in shade at room temperature for 1 hour so as to drain off excess water.

Extraction was conducted using the blender maceration/blender extraction vertical method with distilled water for the aqueous extract and 99% methanol for the methanol extract at a ratio of 1:10 (weight/volume). An industrial blender, Philips HR2041 model, was utilized. The fresh leaf aqueous extract and methanol extracts were prepared by sieving using surgical cotton wool in a glass funnel to eliminate tacky particles and then through filter paper, Whatman's no.1 England. The aqueous extracts were freeze-dried using a benchtop freeze dryer model FD-1LC to obtain dry extract, while the methanol extract was oven-dried at 40°C to obtain dry extract. Percentage yield was calculated using the formula below:

> Percentage yield of extraction = [Weight of extract (g) / (Weight of plant material (g)] x 100

The dry extract was stored in an airtight container and kept in a refrigerator below 10°C for subsequent experiments

2.3 Chemicals and Instruments

Analytical grade chemicals used in this study were purchased from Abak chemical supply limited Kampala, Uganda. Reference standards; Acetyl salicylic acid and Diclofenac gel 0.1% were purchased from Wilbert Pharmaceutical Limited Mbarara Uganda. The DU-8200 single beam UV/VIS spectrometer was employed in the *in-vitro* study, utilizing spectroscopic analysis techniques.

2.4 Animal Selection

Wister Albino mice of either sex aged 10 to 12 weeks, weighing between 20-35g were acquired from the animal facility of the Department of Pharmacy, Mbarara University of Science and Technology. Animals were kept under laboratory conditions 25 ^{0} C (+/- 2), 12 h light, standard cages with a floor area of 228 square inches housing 6 mice each.

Animals were provided with standard rodent diet; rodent pellets and water *ad libitum.* After 7 days, the animal was randomly selected for the different experimental groups and used for the acute dermal toxicity test and the *in-vivo* determination of anti-inflammatory activity. At the end of the experiment, the animals were sacrificed with one dose of Ketamine 80/kg and Xylazine 10 mg / kg intraperitoneally using a 25-gauge needle and a 1 ml syringe and disposed for incineration [11,12].

2.5 Acute Dermal Toxicity

Acute dermal toxicity was performed as per Organization for Economic Cooperation and Development's and the European Union Reference Laboratory for Alternatives to Animal Testing's EURL ECVAM guidance on the "Acute Toxicity – Dermal" test method (2017) [13,14]. A single ointment dose of 2000mg/kg body weight was administered to skins of the adult Wister albino mice observed for signs of toxicity such as changes in behaviour, body weight, clinical signs, and mortality half hourly for the first 6 hours, then once every hour for the first 24 hours, and then at once daily for 14 days.

2.5 Establishment of Anti-Inflammatory Activity

2.5.1 Invitro anti-inflammatory activity

The HRBC membrane stabilization method for estimating the anti-inflammatory activity of *Sida cuneifolia* Apical leaf was conducted following the methods outlined by Shinde et al. Sharma et al. and Yesmin et al. [15-17].

Blood from a healthy volunteer was combined with an equal volume of Alsevers solution. The resulting blood solution underwent centrifugation at 3000 rpm, separating the packed cells. These cells were then washed with isosaline solution, and a 10% v/v suspension was created using isosaline. The HRBC suspension served for anti-inflammatory property estimation. Various concentrations of the extract, reference sample, and control were individually mixed with 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of HRBC suspension. All assay mixtures underwent a 30-minute incubation at 37°C, followed by centrifugation at 300 rpm. After decanting the supernatant liquid, haemoglobin content was measured at 560 nm using a

Table 1. *Sida cuneifolia* ointment formulation table

Ingredients	Quantity (mg/g)	G	Percentage w/w
Herbal extract (concentration 1)	0.5		0.5
Herbal extract (concentration 2)	1.0		1
Herbal extract (concentration 3)	2.5		2.5
Petroleum jelly (base)		10	
Beeswax (base)		0.5	
Olive oil (carrier oil)		2.5	

spectrophotometer. Percentage protection was calculated using the formula below:

Percentage Inhibition = [(OD_control - OD_sample) / OD_control] x 100

Where;

OD_control - optical density of the control (in the absence of the test compound)
OD_sample- optical density of the sample (in the presence of the test compound)

The experiment was repeated in three replicates

2.5.2 Development of formulation

Petroleum jelly, known for its skin permeability and recognized as an inert/excipient carrier for bioactive compounds/extracts [18-21]. served as the base. Olive oil, combined with petroleum jelly and heated to 40°C, incorporated melted beeswax. After cooling to 27°C, the mixture was titrated into containers with varying proportions of the extract as detailed in Table 1. The solution was stirred thoroughly and left to cool and solidify. Subsequently, it was stored in the dark at 8°C for later use in experiments.

2.5.3 Anti-inflammatory activity of *Sida cuneifolia* Apical leaves using the carrageenan induced acute paw oedema in mice

The assay, following methodologies from Okokon and Nwafor and Winter et al. evaluated the anti-inflammatory activity of *Sida cuneifolia* using carrageenan-induced mice paw oedema. Mice (n = 6) were divided into five groups, and acute inflammation was induced by sub plantar administration of 0.1 ml of 1% (w/v) carrageenan in normal saline to the right hind paw. Paw oedema, expressed as the increment in paw thickness due to carrageenan, was measured.

After 15 minutes, the negative control and positive control groups received normal saline 0.9% and Diclofenac gel 1%, respectively, while the experimental groups received their respective *S. cuneifolia* ointment dose levels: 0.5%, 2.5%, 5.0% w/w. Paw volume was measured at 1h, 2h, 3h, and 4h post-treatment using a plethysmometer and the mercury displacement method, with data recorded.

Oedema volume was calculated as the difference between paw thickness at the respective time point and the baseline before carrageenan injection. The percentage inhibition of oedema volume between the treated and control groups was determined as follows:

Percentage inhibition = [(Vc- Vt) / Vc] x 100

where, Vc and Vt represented mean increase in paw volume in control treated group, respectively.

2.5.4 Statistical analysis

The values are expressed as mean +/- SEM. Statically analysis was performed using one-way analysis of variance (ANOVA), followed by Dennett's *t*-test to compare the treatment groups with the negative control group. *P =.05* was considered significant.

3. RESULTS

3.1 Extraction Yields

Aqueous extract had a better percentage yield of 10.1% compared to the methanol extract (4.7%)

3.2 Phytochemical Composition of *Sida cuneifolia* Apical Leaves

Preliminary phytochemical analysis revealed that both extracts have a variety of phytochemicals. However, the methanol extract had more

Phytochemical compounds than the than the aqueous as presented in the table.

Table 2. Phytochemicals screening for aqueous and methanol extracts of *S. cuneifolia* Apical leaves

Phytochemical constituents	Methanol Extract	Aqueous Extract
1.Alkaloids	+	-
2.cardiac Glycosides	+	-
3.Saponins	+/-	+
4. Steroids	+	-
6.Fixedoils/ fats	-	-
7.Phenolic compounds	+	+
8.Flavonoids	-	-
9.Proteins	-	-
10.Gums & mucilage	+	+
11.Carbohydrates	+	+
12. Tannins	+	+

+; Presence of phytochemicals, - Absence of phytochemical

3.3 *In-vitro* anti-inflammatory activity

The methanol and aqueous extracts at different concentrations (0.5. 1.0, 2.0 mg/mL) showed significant stabilisation towards HRBC membranes. Methanolic extracts had better protection compared to the aqueous extracts with similar concentrations. Increase in dose level resulted in increased percentage protection.

3.4 *In-vivo* Anti-inflammatory Activity

Sida cuneifolia ointment showed significant reductions in in mice paw volume and percentage inhibitions. Dose levels (2.5 % and 5%) w/w showed potent activity compared to the reference standard diclofenac gel of reference 0.1%. results were tabulated in Table 4 and changes in percentage in inhibition per hour up to 4 hours presented in Fig. 1.

Histopathological examination of representative animal paw tissue in the control, standard and treatment group of highest dose level (5%w/w *S. cuneifolia* ointment) showed marked infiltration of inflammatory cells (Mast cells, polymorphonuclear cells, plasma cells, and lymphocytes) and oedema as shown in Fig. 2. Inflammatory cell infiltration was less dense in the treatment group sample compared to the standard (diclofenac gel 0.1%) specimen as shown in Fig. 2.

3.5 Histopathological Analysis

Histopathological examination of paw tissues revealed that the control group exhibited significant infiltration of inflammatory cells and edema, including mast cells, polymorphonuclear cells, plasma cells, and lymphocytes. In contrast, the treatment group with the highest dose level (5%w/w *S. cuneifolia* ointment) and the standard group (Diclofenac gel 0.1%) showed reduced inflammatory cell infiltration and edema, supporting the observed anti-inflammatory effects and indicating a greater reduction in the treatment group compared to the standard group. The inflammatory cells observed included polymorphonuclear cells, plasma cells, and lymphocytes as shown on the photomicrographs.

4. DISCUSSION

Various plant parts that contain biologically active chemical components are employed in the treatment, or control of various disease

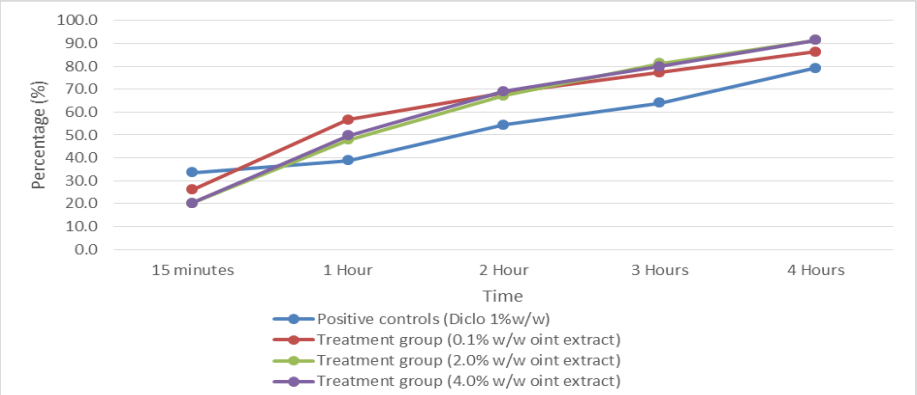

Fig. 1. A graph showing Percentage inhibitions of *S. cuneifolia* ointment dose level (0.5%, 2.5%, 5.0%) w/w at a one hourly time interval up to 4 hours

Table 3. *In-vitro* anti-inflammatory activity of Apical leaf extract of the concentration of *S. cuneifolia*

Treatment /dose level	Volume	Mean Abs +/-SEM	Percentage inhibition
ASA/standard 0.5mg/ml	100ul	.124+/-.001*	76.66
M1 2.0mg/ml	100ul	.210+/-.003*	66.5
M2 (1mg/ml)	100ul	.173+/-.001*	59.9
M3(0.5mgml)	100ul	.235+/-.002*	54.6
AQ10-0.5mg/ml	200ul	.493+/-.001*	3.85
AQ21mg/ml	200ul	.447+/-.002*	12.57
AQ3 2mg/ml	200ul	.425+/-.001*	17.10

**P=.05 with control*

conditions.[22-26]. *Sida cuneifolia* Apical leaves are used in folk medicine to manage inflammatory by topical application [9,27].

In this study the phytochemical composition and anti-inflammatory properties of *S. cuneifolia* Apical leaves were evaluated based on folk lore information using the HRBC membrane stabilization model and carrageenan induced paw inflammatory model *in-vitro* and *in-vivo* respectively.

Two solvents, water and methanol, differing in polarity, were utilized to extract compounds from fresh Apical leaves of *Sida cuneifolia*. Water yielded a higher quantity compared to methanol, a factor beneficial for plant preservation and species protection. However, qualitative phytochemical screening revealed alkaloids, phenolic compounds, steroids, tannins, and cardiac glycosides in the methanol extract, while the aqueous extract lacked alkaloids and cardiac glycosides. This disparity, can be attributed to the solvent's polarity, highlighting the influence of solvent nature on the extracted compounds. Similar differences were noted in preliminary phytochemical studies on *Sida cuneifolia* leaves by Nalubega, Nyanzi, and Nakavuma, Kipng'etich et al. [9,10].

Inflammation is a common phenomenon and it is a reaction of living tissue towards injury.

The HRBC membrane stabilization method was chosen based on the erythrocyte membrane's analogy to the lysosomal membrane [15,17]. The stabilization of HRBC membranes suggests the potential of the extract to stabilize lysosomal membranes, crucial for limiting inflammatory responses. This prevents the release of lysosomal components, such as bacterial enzymes and proteases, known to exacerbate tissue inflammation and damage when extracellularly released [16,28,29]. While the exact mechanism remains unknown, it is suggested that extracts may inhibit processes stimulating osmotic loss, thus preventing hypotonic hemolysis [26,30-33].

The findings suggest that the Apical leaf extract exhibits significant anti-inflammatory properties at various concentrations. Notably, the methanolic extract demonstrated the highest potency at a concentration of 2.0 mg/mL, achieving a substantial percentage inhibition of 66.5%. Based on these results, the methanolic extract qualifies as a promising candidate for further *in-vivo* studies.

Development of oedema in paw of the mice after injection of the carrageenan is due to release of histamine, serotonin, prostaglandin, and the like [22]. It is well known that carrageenan induced paw oedema is characterized by biphasic events with involvement of different of inflammatory mediators. In the first phase (2h after carrageenan injection) histamine and serotonin play a role, while in the second phase 3-4hafter injection kinin and prostaglandins are involved [30,34–37].

Findings suggest that the administration of the *S. cuneifolia* ointment inhibited oedema from the first hour and during all phases of inflammation. Two treatment dose levels of the ointment produced anti-inflammatory effect (91.3%) surpassing that of standard control diclofenac gel 0.1%. Diclofenac is a well-established NSAID and its efficacy in reducing inflammation is widely recognised; the findings suggests that the ointment has anti-inflammatory properties [25].

Table 4. Anti-inflammatory activity by *S. cuneifolia* ointment in induced paw oedema study

Experimental group	(mean+/- SEM) Time (Hours)						Percentage inhibition at 4 hours
	Before	15 min after carrageenan injection (0)	1	2	3	4	
Negative cont. (NS 0.9%)	0.56+/-0.001	0.61+/-.001	0.65+/-.001	0.69+/-.001	0.68+/-.001	0.70+/-0.001	-
Positive cont. (Diclofenac gel 0.1% w/w)	0.40+/-0.001	0.59+/-.001	0.62+/-.001	0.59+/-.001	0.55+/-.001	0.50+/-.001*	79.14
Treatment group 1 extract (0.5%w/w)	0.38+/-0.001	0.53+/-.001	0.50+/-.001	0.47+/-.001	0.44+/-.001*	0.41+/-.001*	86.33
Treatment group 2 (2.5%w/w)	0.41+/-0.001	0.61+/-.001	0.55+/-.001	0.51+/-.001	0.46+/-.001*	0.43+/-.001*	91.37
Treatment group 3 (5%w/w)	0.42+/-0.001	0.55+/-.001	0.55+/-.001	0.51+/-.001*	0.4+/-.0017*	0.44+/-.001*	91.37

**P=.05 with control paw volumes significant from normal control,*

** P < 0.05; Mean ± S.E.M = Mean values ± Standard error of means of six experiments*

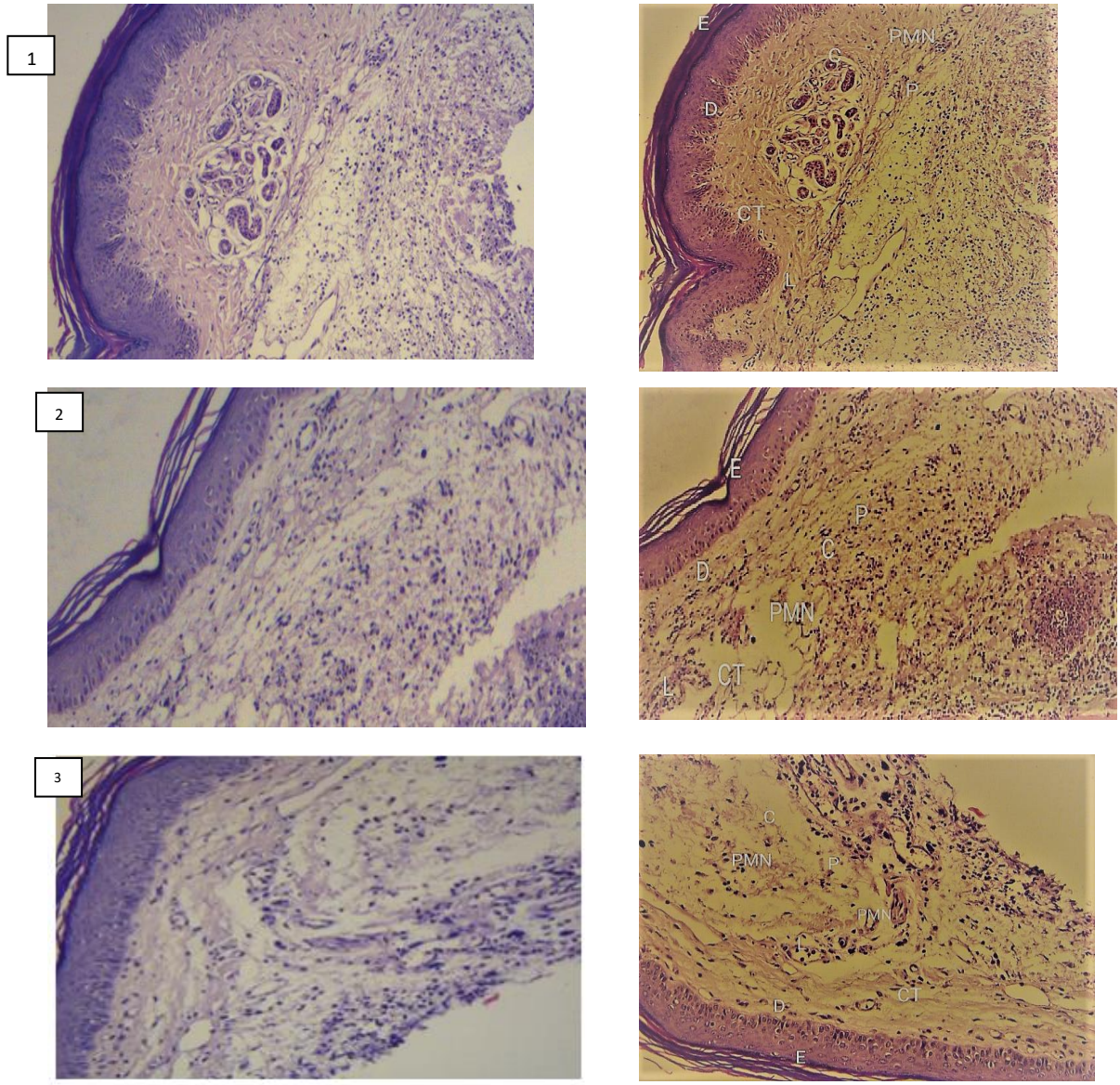

Fig. 1. Photomicrographs of mice paw tissues for their respective treatment groups
1 -control group, 2- standard group, S. cuneifolia ointment 5% w/w. (E -epidermis, D- dermis, CT- connective tissue, L- lymphocytes, PMN- polymorphonuclear cell, P- plasma cells

The significant inflammatory effect may be due to the inhibition of different aspects and chemical mediators of inflammation like cytokines (IL-1) and prostaglandins (PGE-2)) by phytochemical compounds; glycoside steroid, polyphenols and tannins contained in the extract [25,38].

In addition, natural extract like this *S. cuneifolia* ointment could possess antioxidant properties which help mitigate oxidative stress associated with inflammation. and at the same time influence immune cell behaviour such as macrophage and neutrophil migration during acute phase of inflammation leading a reduction of inflammatory cell infiltration into the tissues [37,38]

Both extract ointments 2.5%w/w and 5.0% w/w exhibited identical levels of percentage inhibition (91.3%). These observations suggest potential

saturation effect, when increasing the dose beyond a certain point may not yield significant additional reduction in oedema [39].

This histological evidence aligns with the quantitative data, further strengthening the conclusion that *S. cuneifolia* ointment has anti-inflammatory properties**.**

5. CONCLUSION

It is concluded that Methanol Apical leaf extracts possess significant anti-inflammatory activity in the HRBC membrane stabilization test and carrageenan-induced paw oedema assay in mice.

These results validate the traditional folk knowledge about the anti-inflammatory characteristics of the Apical leaf properties found in *Sida cuneifolia*. Additional research is advised to isolate the specific bioactive compounds responsible for these properties and elucidate their potential mechanisms**.**

CONSENT

All authors declare that written informed consent was obtained from the individual who voluntarily donated 5ml blood that was used in the invitro study. An approved copy of the consent by Mbarara University REC.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), as well as Uganda national guidelines on animal use and teaching were followed. All experiments have been examined and approved by the appropriate ethics committee”

The research Proposal and consent form for a blood donor was approved by Mbarara University of Science and Technology Research Ethics Committee under reference No. MUST-2022-728.

The protocols for experiments involving animal use were approved by the Institution Animal Care and Use Committee of College of Veterinary Medicine, Animal Resources and Biosecurity Makerere University. Kampala Uganda under reference No. under reference No. SVAR_IACUC/ 134/2022

A research permit from Uganda National Council of science and Technology was issued under No. NS659ES

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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