Original Research Article

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

ABSTRACT

Aims: This study evaluated and compared the yield, phytochemical composition and anti-inflammatory properties of methanol and aqueous crude extracts of the apical leaves of *Sida cuneifolia*.
Study design: analytical study design was used for yield and phytochemical composition while experimental study design was used for anti-inflammatory evaluation studies.

Place and Duration of Study: department of pharmacy laboratory Mbarara University of Science and Technology between August 2022 to September 2023.

Methodology: The yields of the extracts were determined and a qualitative phytochemical screening test was carried out to establish their composition. Acute dermal toxicity to determine the toxicity level of the ointment extract using the 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:
Aqueous extract had a higher percentage yield of 10.1% compared to the methanol extract (4.7%). Alkaloids, phenolic compounds, steroids, tannins, cardiac glycosides were present in methanol extract while alkaloids and cardiac glycosides were missing in aqueous extract
Methanol and aqueous extracts at different concentrations of (0.5. 1.0, 2.0 mg/mL) showed dose dependent significant *P=.05* 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 methanol extract at 2.0mg/mL was the highest (66.5%) among the extract dose levels, but lower than that of the standard. (76.66%)

The *Sida cuneifolia* ointment extracts of (0.5%, 2.5% and 5.0%) w/w showed significant (P=.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 showed more potent activity of 91.4% compared to that of the reference standard; Diclofenac gel 0.1%. (79.41%). Both the dose level of extract ointments (2.5% and 5.0%) w/w exhibited identical levels of percentage inhibition of (91.3%) at the end of 4 hours

Conclusion: The study provides scientific evidence for the ethno-medical use of apical leaves of S. cuneifolia, and this can be in the transformative development of ethnomedicine.

Keywords: *Sida cuneifolia, Anti-inflammatory, Phytochemical analysis, leaf extract, diclofenac gel, HBRC.*

1. INTRODUCTION

Sida cuneifolia is a small shrubby herb with yellow mallow flowers, five distinct petals, and five sepals. The leaves are notched at the top and the fruit consists of mericarps. *S cuneifolia* belongs to the family of malvacae. *S. cuneifolia* belong

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to the family of malvacae and the genus *Sida* which has about 200 species distributed in the tropical and subtopical regions of the world. [1]
Sida cuneifolia is an important in the plant in the preparation of indigenous drug in African herbal treatment. [2], [3]. it is a folk medicine in Uganda and other parts of Africa. Given its importance, there is no research to validate its use. [4]–[8]
Although some studies exist on antibacterial, antifungal activity and Phyto chemical analysis of leaves of *Sida cuneifolia* there are no reports on anti-inflammatory activity of the 'apical leaves' the plant part used in herbal preparations.
This study aims to evaluate the anti-inflammatory activity of the apical leaves of *Sida cuneifolia.* The findings of this study will contribute information to the development of anti-inflammatory drugs.

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2. MATERIAL AND METHODS

2.1 *Plant material*

The plant was collected from Sekajja institute of traditional medicine in Buyijja Mpigi district. The plant vochure 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.

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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 foreign matter then left to dry at 24*c for 1 hour in well ventilated room.
Extraction was done using blender maceration/blender extraction vertical method with distilled water for the aqueous extract and methanol 99% for methanol extract a ratio of 1.10 weight / volume. An industrial blender Philips HR2041 model was used. The fresh leaf aqueous extract and the methanol extracts were prepared by sieving using surgical cotton wool in a glass funnel to remove the tacky particles then through filter paper Whatman's no1 England. The aqueous extracts were freeze-dried using freeze drier bench top 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;

Percentage of extraction = (weight of extract (g)/weight of plant material) x 100

The dry extract was stored in an air-tight container and stored in a refrigerator below 10*c for subsequent experiments.

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2.3 Chemical and Instruments

Analytical grade chemicals used in this study were purchased from Abak chemical supply limited Kampala Uganda. Reference standard Acetyl salicylic acid and diclofenac gel 0.1% were purchased from Wilbert Pharmaceutical Limited Mbarara Uganda. DU-8200 single beam UV/VIS spectrometer was used for the in vitro study.

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2.4 Animal selection

Wister Albino mice of either sex aged 10 to 12 weeks and 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 +/- 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 size syringe size. And disposed for incineration. (*Uganda National Council for Science and Technology*, 2021; Govindaraghavan and Sucher, 2015)

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2.5 Acute Dermal Toxicity

Acute dermal toxicity was performed as per Organization for Economic Cooperation and Development's [11], [12] and the European Union Reference Laboratory for Alternatives to Animal Testing's EURL ECVAM guidance on the "Acute Toxicity – Dermal" test method (2017). 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 14days

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2.6 Establishment of anti-inflammatory activity

2.6.1 Invitro anti-inflammatory activity

HRBC membrane stabilization method was used was used to estimate anti-inflammatory activity as *Sida cuneifolia* an apical leaf. [13]–[15]. Blood was collected from a healthy volunteer and was mixed with equal with equal volumes of Alsevers solution. This blood solution was centrifuged at 3000 rpm and the packed cells were separated, then washed with isosaline solution and a 10% v/v suspension was made with isosaline. The HRBC suspension was used for the estimation of anti-inflammatory property. Different concentration of the extract, reference sample and control were separately mixed with 1ml of phosphate buffer,2ml of hyposaline, 0.5ml of HRBC suspension, all the assay mixtures were incubated at 37°C for 30minutes and centrifuged at 300rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560nm. Percentage protection was determined 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)

2.6.2 Development of formulation.

The base was petroleum jelly as it is potent in skin permeability, considered inert / excipient carrier of the bioactive compound / extract. [16]–[19]. Olive oil was mixed with petroleum jelly and heat up to 40^{0}c melted Bees wax added. It was allowed to cool to 27^{0} C and titrated into containers with different proportions of the extract as shown in Table 1, stirred to thoroughly mix and left to cool and solidify. It was then stored at 8 c in the dark for subsequent use in the experiments

Table 1. ***Base composition and formulated dose levels***

Base composition		Extract/base dose levels	
Petroleum jelly	10g	0.5mg/g	(0.5%w/w),
Olive oil (vegetable oil)	2.5g	1mg/g	(1% w/w),
Beeswax	0.5g	2.5mg/g	(2.5%w/w),

2.6.3 Anti-inflammatory activity of Sida cuneifolia apical leaves using the Carrageenan induced Acute Paw Oedema in mice

The assay followed previous work described in literature by [20]–[22]
anti-inflammatory activity of *Sida cuneifolia* was evaluated using carrageenan induced mice paw oedema. The mice were divided into five groups (n = 6); 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 of each mouse.
Oedema was expressed as the increment in paw thickness due to carrageenan administration.
After 15 minutes, the negative control and positive control groups received normal saline 0.9%and Diclofenac gel 1%, respectively, the experimental groups received their respective group ointment extract dose levels. ;0.5%, 2.5%, 5.0% w/w. The paw volume measured (in ml) at 1h, 2h, 3h and 4h after application of the respective treatments by mercury displacement method using a plethysmometer and data recorded. Oedema was expressed as the increment in paw thickness due to carrageenan administration. The oedema volume was obtained as the difference between the thickness of the paw (at respective time point) and before(baseline)of the injection of carrageenan
The percentage inhibition of the volume of oedema between the treated and control group was calculated as follows;
Percentage inhibition = [(Vc- Vt) / Vc] x100
where, Vc and Vt represented mean increase in paw volume in control treated group, respectively.

2.6.4 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 AND DISCUSSION

3.1. Extraction yields

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

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3.2. Phyto chemical composition of *Sida cuneifolia* apical leaves

Preliminary phytochemical analysis revealed that both extracts have a variety of phytochemicals. However, the methanol extract had more Phyto chemical compounds than the than the aqueous as presented in the table

Table 2. Phytochemical present in aqueous and methanol extracts of *S. cuneifolia* apical leaves

Phyto-constituents	Methanol	Aqueous
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

Table 3. *In vitro anti-inflammatory activity0f 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*

3.4. *In vivo anti-inflammatory activity*

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The ointment extracts of *Sida cuneifolia* 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 1 and changes in percentage in inhibition presented in graph 1
Histopathological examination of representative animal paw tissue in the control, standard and treatment group of highest dose level (5%w/w ointment extract) showed marked infiltration of inflammatory cells (Mast cells, polymorphonuclear cells, plasma cells, and lymphocytes) and oedema as shown in Figure 2. Inflammatory cell infiltration was less dense in the treatment group sample compared to the standard (diclofenac gel 0.1%) specimen as shown in figure 2

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

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

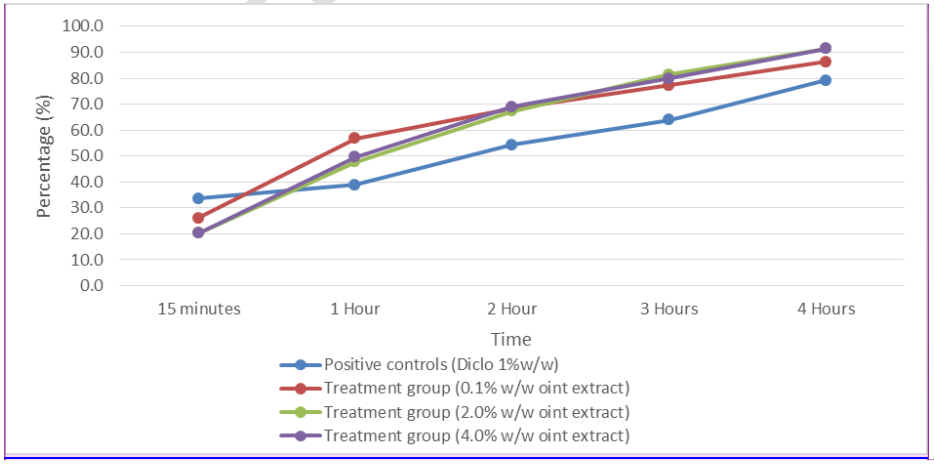

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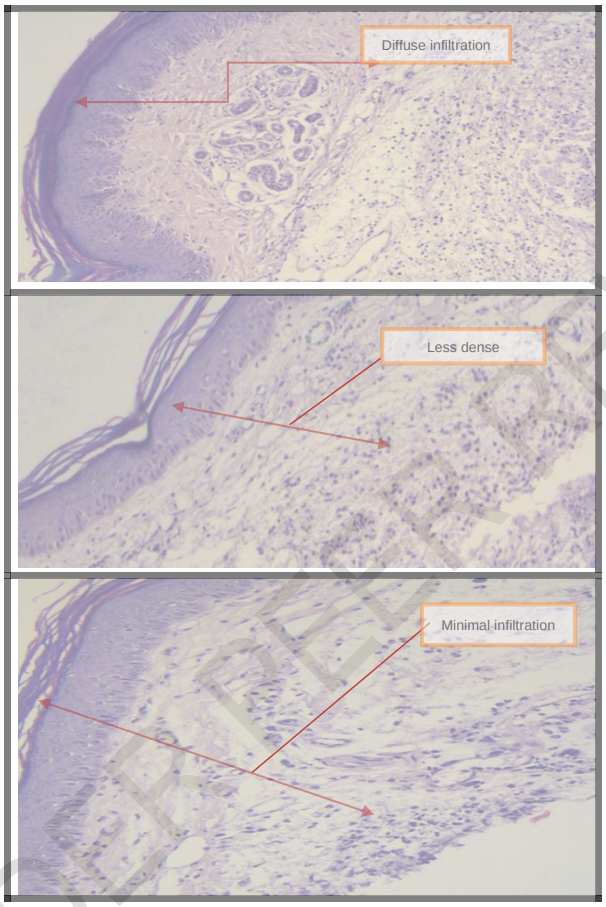
Figure. 1. Percentage inhibitions of ointment extract dose level (0.5%, 2.5%, 5.0%) w/w at a one hourly time interval up to 4 hours



3.5. ***Figure 2.*** *Histological sections of paw tissues; Photomicrograph 'a'- control, 'b'- standard,'c'-ointment extract*

4. Discussion

Various plant parts that contain biologically active chemical components are employed in the treatment, or control of various disease conditions.[23]–[27]. *Sida cuneifolia apical leaves are used in folk medicine to manage inflammatory by topical application* [6], [28]

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 with different forms of polarities were used to obtain extracts from fresh apical leaves of Sida cuneifolia. Water extracted higher amount of yield compared to methanol. On the one hand, the high yield lessens plant destruction and promotes protection of the species, however, qualitative phytochemical screening, alkaloids, phenolic compounds, steroids, tannins, cardiac glycosides were present in methanol extract while alkaloids and cardiac glycosides were missing in aqueous extract; a disparity which can be attributed to the solvent nature, yet these were present in methanol extract to the variation in polarity of the solvent which influence the nature of compounds that are extracted. A similar difference was observed in phytochemical preliminary studies on leaves of Sida cuneifolia

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The HRBC membrane stabilisation method was chosen because erythrocyte membrane is an analogue to the lysosomal membrane [14], [14], [15] *and its stabilisation implies that the extract can also stabilize lysosomal membranes. Stabilization of lysosomal membranes is important in limiting the inflammatory response by preventing release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases which cause further tissue inflammation and damage upon extracellular release.* [13], [29]. *Though the exact mechanism of membrane stabilisation is not known yet, hypotonicity- induced hemolysis may arise from cell shrinkage due to osmotic loss of intracellular electrolytes and fluid components. the extract may inhibit the processes which may stimulate od enhance the efflux of these intracellular components.* [27], [30], [31]

The results indicate that the apical leaf extract have at various concentrations has significant anti-inflammatory property and that methanolic extract were most potent at a concentration of 2.0mg/mL with a percentage inhibition of 66.5% hence on that basis qualified to be the candidate for the in vivo study

Development of edema in paw of the mice after injection of the carrageenan is due to release of histamine, serotonin, prostaglandin, and the like (georgewill, anti-inflammatory 2010) winter 1962, it is well known that carrageenan induced paw edema 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], [32]–[35]

Our results revealed that the administration of the ointment extract inhibited edema from the first hour and during all phases of inflammation. Two treatment dose levels of the extract 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 finding suggests that the extract ointment has anti-inflammatory properties. [26]

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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. [26], [36]

In addition, natural extract like this S. cuneifolia extract ointment could possess antioxidant properties which help mitigate oxidative stress associated with inflammation. and at the same time influence immune cell behavior such as macrophage and neutrophil migration during acute phase of inflammation leading a reduction of inflammatory cell infiltration into the tissues. [35]

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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. [37]

This histological evidence aligns with the quantitative data, further strengthening the conclusion that the ointment extract 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 in mice

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These findings authenticate the folk use lore information on the anti-inflammatory properties of the apical leaf properties of Sida cuneifolia.
Further studies are recommended to isolate the bioactive compounds responsible for this property and identify their possible mechanism of action.

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. is attached to this document is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal.

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

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DEFINITIONS, ACRONYMS, ABBREVIATIONS

HRBC: human Red blood Cell
REC; Research Ethics commit