Journal of Advances in Microbiology

7(1): 1-11, 2017; Article no.JAMB.37894
ISSN: 2456-7116

Antibacterial Activity of *Sida cuneifolia* Vollesen against *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author BK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors LAM, JMM and PJ managed the analyses of the study. Author TC managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2017/37894
Editor(s):
(1) Pongsak Rattanachaikunsopon, Professor, Department of Biological Science, Faculty of Science, Ubon Ratchathani University, Thailand.
(2) Akpaka, E. Patrick, Professor, Unit of Pathology & Microbiology,Faculty of Medical Sciences,The University of the West Indies St. Augustine, Trinidad & Tobago.
Reviewers:
(1) César Luiz Da Silva Guimarães, Federal University of Rondônia State, Brazil.
(2) Izharul Hasan, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/22245

Original Research Article

Received 31st October 2017
Accepted 23rd November 2017
Published 11th December 2017

ABSTRACT

Background: *Sida cuneifolia* is utilized traditionally to treat many ailments yet as far as we know its medicinal properties have not been scientifically tested locally in Kenya. The aim of the present study was therefore to investigate the antimicrobial properties of *S. cuneifolia* against *Staphylococcus aureus* and investigate the phytochemicals present in the leaves, roots and stem that are of medicinal importance.
Method: The plant was separated into root, leaves and stem bark. Water and ethanol were used for extraction of active ingredients. Antimicrobial bioassays and minimum inhibitory concentration (MIC) tests were done on *S. aureus*. Phytochemicals of medicinal importance were also determined using thin layer chromatography.
Results: Ethanol extracts had significantly higher activity than water. Roots showed higher inhibition than leaves and stem. The stem ethanol extracts had an MIC of 10^{-10} g/ml. Ethanol leaf and root extracts had all the five phytochemicals tested for (alkaloids, flavonoids, tannins, saponins and terpenoids). Alkaloids were absent in ethanol stem extracts while both alkaloids and flavonoids were absent in the stem and leaf water extracts.

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Conclusion: The results showed that the *S. cuneifolia* leaf and root ethanolic extracts could be used to treat ailments caused by *S. aureus*. It is recommended that further toxicological testing be done.

Keywords: Sida cuneifolia; antimicrobial activity; crude extracts; phytochemicals; Staphylococcus aureus.

1. INTRODUCTION

Although a large proportion of plants are used traditionally to treat many ailments, their antimicrobial activities have not been tested scientifically. A good number of plant extracts are known to have biological activities. At least 119 compounds have been derived from 91 plant species of which 77% of them yield important folklore medicines [1]. The medicinal value of these plants is found in chemical substances that produce physiological changes in the human body [2]. Plants used as traditional medicines offer an alternative solution in terms of the discovery of new plants that have antimicrobial properties as compared to antibiotics used as medicines in this era [3,4].

Majority of these plant species have originated from the tropical and subtropical flora. One of such plants is *Sida cuneifolia* which is a shrub that has a tough woody stem and can grow up to a meter tall if left undisturbed. It has yellow flowers with five distinct petals and five sepals. Its leaves are notched at the tip with smooth margins [5]. Traditionally, *Sida cuneifolia* has been used as a herbal remedy to manage up to 12 diseases [6]. In Busoga, Uganda, it is used to disinfect umbilical cord wounds [7]. Among the Sabaot people around Mt. Elgon in Kenya it is known as *Kupchuwet* and the roots are chewed to treat sore throat [8].

Staphylococcus aureus is a leading cause of both community-associated and hospital-acquired (nosocomial) bacteremia [9]. In sub-Saharan Africa, *S. aureus* bacteraemia is one of the major causes of morbidity and mortality [10] even with appropriate therapy*. Staphylococcus aureus* bacteremia is associated with serious complications like endocarditis, and with methicillin-resistant *S. aureus* (MRSA) [11]. The overall risk of nosocomial bacteraemia is 5·9 per 1000 admissions [12]. One major concern with *S. aureus* infections is methicillin resistance, which is now a big problem though it was rare some years ago [13]. The increasing resistance of this bacterial pathogen to various antibiotics has led to complications in the treatment of *S aureus* infections [14] and is also believed to be more resistant than Gram-negative bacteria [15]. Therefore, there is need for development of new antibiotics from ethnomedicinal plants that can be used to treat emerging resistant bacterial infections. The objective of this study was to evaluate the antimicrobial activity of ethanol and water based extracts of *S. cuneifolia* against *S. aureus,* and to determine the phytochemicals of medicinal importance in the different plant.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Plant Samples

Sida cuneifolia plants were collected from the field in Chepterit, Nandi County, Kenya by uprooting then identified [16]. The voucher specimen was deposited in the University herbarium. The plants were separated into leaves, stems and roots then chopped into smaller pieces, dried slowly under the shade for one week until moisture content of 13% was achieved. The plant parts were then packaged in clear plastic bags and transported in dry plastic re-sealable zipper storage bags back to KEMRI, Centre for Traditional Medicine and Drug Research (CTMDR). The plant materials were ground into fine powder using a mechanical grinder and passed through a sieve of 0.5 mm diameter and stored in a refrigerator (4-8℃).

2.2 Extraction

The solvents used to obtain crude extracts from the stored fine powder were hot water (70±1 ^{0}C) and 70% ethanol. Preparation of a stock solution with a concentration of 1 g/ml was prepared by dissolving 100 g of the ground samples into 100 ml of the respective solvents [17].

2.3 Ethanol Extraction

Fifty grams of the ground plant parts was placed in a 250 ml conical flask and 100 ml of 70% ethanol added into the flask and thoroughly shaken to mix well. The mixture was then left to settle for 24 hours.

After the 24 hours, the samples were filtered using 6 mm filter paper (Whatman no. 1). The filtrate was transferred into a round-bottomed flask. The flask was attached to a rotary evaporator until the ethanol evaporated leaving a thick paste. The paste was then transferred to vial and left to dry in front of a fan until it solidified indicating that all the ethanol had evaporated from the paste. The solid was stored in a refrigerator at 4℃ until required.

2.4 Hot Water Extraction

Fifty grams of the ground samples were mixed with 500 ml of water in a 1 litre conical flask and the mixture shaken until completely dissolved. The flask was placed in a shaking water bath (70±1℃) for one and a half hours.

After the incubation in the water bath, the mixture was removed and filtered using surgical cotton wool in a glass funnel. The filtrate was left to cool to below 10℃ then transferred into a 250 ml round bottom flask and was placed on a shallow hollow tray containing acetone and dry ice to freeze-dry the contents. The sample was further freeze-dried using Modulyo K4 freeze dryer (EDWARDS) until it was completely dry. The dried sample was removed from the flask, weighed and refrigerated at 4℃ for future use.

2.5 Source of Microorganisms

Pure isolates of the selected pathogen; *S. aureus* (ATCC 25923) was obtained from the Centre of Microbiology Research laboratory at the JICA-KEMRI.

2.6 Preparation of Media

Müller-Hinton agar (MHA) (OXOID, UK) was used to culture *S. aureus*. The agar was prepared by dissolving 38 gms of the agar in 1 litre of distilled water. The agar was sterilized at 121℃ for 15 min and the pH was adjusted to 7.1 using sterile 1N NaOH, then allowed to cool (50–55℃) before pouring into sterilize petri dishes [18].

A bacterial suspension of *S. aureus* was made from the primary culture by introducing it in sterile distilled water in a screw-capped test tube using a sterile plastic disposable loop. The test tube was shaken in order to evenly distribute of the inoculum in the tube. Using sterile swabs, the bacterial suspension was transferred by streaking onto the prepared MHA media and spread evenly. The plates were then incubated at 37℃ for 24 hours [19].

2.7 Antimicrobial Bioassays

Bioassay tests were done at the Mycology Laboratories, Center for Microbiology Research - Kenya Medical Research Institute (KEMRI). The sensitivity testing of the extracts were determined using Kirby Bauer disk method. The extracted solids were first reconstituted by using 1ml of Dimethyl sulphoxide (DMSO) in 1 gm of the extract before running antimicrobial assay [20].

Sterile 6 mm paper disks were impregnated with 15 µl of the reconstituted extract. The controls Chloramphenicol and DMSO (Dimethyl sulphoxide) were also impregnated on the discs. All extract-impregnated discs were transferred using a sterile forceps and placed on the prepared culture media in a laminar flaw chamber. The culture media was incubated at 37℃ for 24 hours. The plates were then examined for any antibacterial activity by the extracts.

The diameter of the zones of inhibition were measured using a ruler and recorded in mm. Chloramphenicol (30 µg disc $^{-1}$) was used as the standard antibacterial agent and was treated the same way as the crude extract. The analysis was done in triplicates; blank (DMSO), extract, and standard (chloramphenicol). The antimicrobial activity in terms of percentage was calculated by applying the RIZD (relative inhibition zone diameter) expression [21].

$$\%RIZD = \frac{[IZD\ Sample - IZD\ negative\ control\ (D)]}{IZD\ Standard\ (Chloramphenicol)} \times 100\%$$

Where: D is Plain disc with DMSO

%RIZD is Percentage of relative inhibition zone diameter

IZD is Inhibition zone diameter (mm)

2.8 Minimum Inhibitory Concentration (MIC)

MICs were not determined for plant parts that did not register any antibacterial activity. The MICs were determined using serial dilutions and the dilutions in the consecutive microtiter plates were diluted from the original crude extract. The wells of the plate had 100 µl of DMSO, the reconstituting solution for the serial dilutions. Concentrations were made of 1000 µg/ml, 10

µg/ml, 0.01 µg/ml, 0.001 µg/ml and 0.0001 µg/ml. The solutions in the microtiter plates were impregnated onto sterile paper discs and placed on plates that had been inoculated with the bacterial cultures. The dilutions from the wells on the discs were subjected to bioactivity test using disc diffusion method to determine the zones of inhibition [22].

2.9 Phytochemical Analysis

Phytochemical compounds that were present in the hot water and ethanol extracts of *S. cuneifolia* were tested on silica gel plates via thin layer chromatography (TLC) to determine the presence of tannins, terpenoids, alkaloids and flavanoids. A system of solvents constituting ethanol and petether in a ratio of 7:3 was prepared. The silica gel plate was then placed into a beaker containing the solvent system. The solvent moved up via capillary action and resulting bands were visualized in a UV chamber [23]. Saponins were determined separately by the Frothing test [24].

2.10 Tannins Test

To test for the presence of tannins, the TLC plate was sprayed with ferric chloride-potassium ferricyanide reagent. A blue or green color on spots on the plate was indicative of the presence of tannins [25].

2.11 Terpenoids Tests

The presence of terpenoids was determined by spraying the TLC plates with 1% vanillin sulphuric acid reagent followed by gentle heating. If the spots on the TLC plate turned purplish, it indicated the presence of terpenoids [26].

2.12 Alkaloids Test

Alkaloids' presence was determined by spraying the TLC plates with Dragendorff's reagent that is composed of potassium bismuth iodide prepared from basic bismuth nitrate ($Bi(NO_3)_3$), tartaric acid, and potassium iodide (KI). If the spots turned orange, it would indicate the presence of alkaloids [27].

2.13 Flavonoids Test

The test for flavonoids involved heating the TLC plate over a steam bath (40–50℃) for 2 min, followed by placing the plate on a bottle with dilute ammonia to expose the plate to ammonia fumes. A yellow coloration would be a positive test for flavanoids [28].

2.14 Saponins Test

To test for the presence of saponins, 20 ml of sterile water was added to 1 g of the extracts in a tube then shaken vigorously for 15 minutes and left to stand for 10 minutes. A thick persistent froth of about 1 centimeter thick indicated the presence of saponins [24].

3. RESULTS

3.1 Antimicrobial Activity of Ethanol and Aqueous Extracts of *S. cuneifolia*

The crude extracts of the various plant parts showed considerable antimicrobial activity against *S. aureus.* While only the aqueous root extracts exhibited antimicrobial activity, ethanol based extracts from all plant parts were effective against *S. aureus* (Table 1).

Significant differences in antimicrobial activities were observed between the extracts of the different plant parts (Table 2). Also the antibacterial activity depended upon the type of solvent used for extraction. There were no significant differences in antimicrobial activity between the stem and leaf water extracts. Aqueous extracts of the root, stem, leaf and ethanol based extracts of the roots all displayed significant differences in their antimicrobial activities. Roots performed the best among the extracts with an average zone of inhibition of 8.33 mm (Table 2) and RIZD of 8.64% when extracted using ethanol (Fig. 1). This was followed by stem ethanol extracts with a zone of inhibition of 8 mm and a RIZD of 7.40%. Aqueous extracts of the stem and leaves performed poorly registering no activity. Ethanol extracts performed better than the hot water extracts.

The positive control surpassed the aqueous and the ethanol extracts in antimicrobial activity and displayed the highest activity against *S. aureus*.

The minimum inhibitory concentrations of the *S. cuneifolia* plant part extracts against *S. aureus* are shown in Table 3. The MIC values of different plant extracts were found in the range of 0.01-1000 µg/ml. Ethanol root and leaf extracts showed the least MIC at a concentration of 0.01

μg/ml followed by ethanol stem extracts with a MIC of 10 μg/ml. Aqueous-based root extracts had a MIC of 1000 μg/ml. The aqueous extracts of leaves and stems showed no antibacterial activity, their MICs were therefore not determined.

3.2 Phytochemical Composition in *S. cuneifolia* leaf, Stem and Roots

Table 4 shows the phytochemicals present in the stem, leaf and root and leaf extracts. Ethanol-based extracts of leaf and root had all the five phytochemicals that were tested for. Terpenoids and saponins were found in all the plant parts irrespective of the extraction solvent. Alkaloids and flavonoids were absent in the stem, leaf and root water extracts, while alkaloids were also absent in the ethanol stem extracts.

4. DISCUSSION

The antibacterial activity of aqueous and ethanol-based extracts on leaves, stem and roots of *S. cuneifolia* were tested on *S. aureus.* All the extracts showed varying degrees of antibacterial potential. Also the antibacterial activity of all extracts depended largely upon the solvent used in extraction. Ethanol-based extracts displayed significantly higher activity than the aqueous extracts. To the best of our knowledge, no scientific study has reported antibacterial activity of *S. cuneifolia* on *S. aureus.* Furthermore, this study showed that the antibacterial activity of *S. cuneifolia* depends on the extraction procedure.

Results of the present study showed that the ethanol-based extracts of the roots displayed the highest activity against *S. aureus*. The roots showed a higher activity against *S. aureus* when both water and ethanol were used as a solvent during extraction. Other studies have reported high phytochemical concentrations in roots [29]. Furthermore, ethanol-based extracts of the roots were also found to contain all the five phytochemicals tested for in this study. Moreover, the ethanol root extracts also had the lowest MIC. The absence of alkaloids and flavanoids in aqueous extracts could be the reason for lower or no inhibitory activity or no effect of the hot water extracts of roots, stem and leaves. Either the compounds were not extracted or must have been lost during boiling of the plant material. Moreover, these plant active compounds are volatile and can be lost during boiling [30]. Also, ethanol as a solvent was better than water because of its polarity and can dissolve both polar and non-polar compounds [31] because of its ability to dissolve many hydrophilic and lipophylic components. It is also miscible with water, is volatile and has a low toxicity to the bioassay used therefore useful as an extract. This implies that ethanol extracted more active phytoconstituents compared to water making it a better solvent for the extraction of most active plant compounds with medicinal properties [32-36].

Results of this study also show that phytochemical compounds are deposited in specific parts of the plant [37] in varying concentrations. Alkaloids and flavanoids, though present in ethanol extracts of stem and roots, were absent in aqueous root and stem extracts. Flavonoids have been reported to have antimicrobial activities [13]. The significance of the extraction solvent is highlighted here since the root aqueous extract with alkaloids and flavonoids could not give the extract the expected antimicrobial activity. This high inhibition by the root ethanolic extract against the tested bacteria indicates that the plant part can be used for treatment of *S. aureus* infections upon purification and isolation of its active compounds. The inactivity recorded for the root aqueous and leaf ethanolic extracts also demonstrates the importance of using the appropriate part of a plant in phytomedical work. For instance, although all the five phytochemicals were present in the ethanol-based extracts of the leaves, their activity was significantly lower than the ethanol-based stem extracts that lacked alkaloids that had very good antimicrobial activity.

Also, we used the entire stem that showed high antimicrobial activity when extracted using ethanol. Crude preparations of whole plant parts which contain both the active and non-active components have been known to have higher efficacy than semi-crude or pure plant substances [38]. Previous studies on the stem bark of *S. cuinefolia* showed activity against *S. aureus*, when extraction was done using distilled water, ethanol and diethyl ether [39].

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation and thus the lower the MIC the stronger and more efficient the extract is [40]. This shows that the root extracts can inhibit growth or kill pathogens at lower concentrations

therefore saving on costs. Although, dose-mortality studies were not performed in the present study, it is probable that if the concentration of the extracts is increased higher mortality of the pathogen may be achieved [41].

MRSA risk is higher when there is an increased use of penicillins, tetracyclines and cephalosporins, though it can be regulated by change in antibiotic use from time to time with the help of natural products. Use of highly concentrated antibiotics is responsible for a strong positive dose–response relationship between antibiotic use and MRSA, this being a significant positive dose–response relationship to MRSA [42]. A lower of concentration would ensure that no antimicrobial substances are wasted after their use into their environment. Moreover, resistance develops when bacteria in the environment is exposed to antibiotics which were not meant to be targeted [43]. The roots having the best MIC would therefore be used in low concentrations thus avoiding the possibility of excess antimicrobial substances in the environment reducing the loss of non-target organisms.

With all these emerging problems, there is need for newer methods to combat the ever-changing issues bringing about resistance. Scientific evidence and the traditional use of plants as medicines has provided the basis for indicating which plant extracts can be useful for managing medical conditions. Various publications have documented the antimicrobial activity of plant extracts from wild plants and trees to vegetables that are being used as food in our homes [44].

Staphylococcus aureus infections remain a significant problem in hospitalized patients [45]. All pathogens can easily share genes for antimicrobial resistance [46]. Resistance involves mutation of a bacterial gene on the chromosome or transfer of a resistance gene from other organisms via conjugative mobilization where plasmids physically combining with co-resident conjugative plasmids by recombination between regions of homology or mobilization by donation where the plasmid encodes for resistance [47, 48]. Strains of *S. aureus* that are resistant to chloramphenicol have an inducible enzyme that deactivates the drug by acetylation in the presence of acetyl coenzyme A [49]. Plasmid analysis and Phage typing are also evidence of the spread of chloramphenicol resistant *S. aureus,* by acquiring either of two chloramphenicol R-plasmids. Four epidemic strains have been identified in patients and hospital reservoirs. This has shown that there is rapid resistance developing against chloramphenicol thus with the good results from the roots, there might be reason for further development of the roots as a drug for there has been no resistance recorded [50].

5. CONCLUSION

In this study, the results showed that root and stem extracts of *S. cuneifolia* have the potential to treat and control nosocomial or any other ailments caused by *S. aureus*. The scientific validation of the plant species may therefore help in discovering new drugs to tackle *S. aureus* infections. It is recommended that further phytochemical testing should be done to identify the active chemical compounds that might be of medical importance. Toxicological tests should also be done to determine the presence or amount of poisonous material in the plant that can harm the human body; and animal and animals trials to determine the effect of the drug in a living body.

6. DECLARATIONS

6.1 Availability of Data and Material

The datasets on Zone of inhibition used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Data on phytochemical and MIC generated during this study are included in this published article and its supplementary information files.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Table 1. Antimicrobial activity of ethanol and aqueous crude extracts of *S. cuneifolia* (Source: Author)

	Aqueous			Ethanol			Controls	
	Stem	Leaves	Roots	Stem	Leaves	Roots	Positive	Negative
S.aureus	-	-	+	++	+	++	+++	-

- .no activity; +, active; ++, medium activity; +++, high activity.

Table 2. Zones of inhibition against *S. aureus* using ethanol and water extracts of *S. cuneifolia* (Source: Author)

Zone of inhibition (mm)	Extracts							
	Water			Ethanol			Controls	
	Stem	Leaves	Roots	Stem	Leaves	Roots	Positive	Negative
	6 ± 0^{a}	6 ± 0^{a}	6.33 ± 0.57^{b}	8 ± 1.73^{d}	7 ± 1.0^{c}	8.33 ± 1.15^{e}	27 ± 1.0^{f}	6 ± 0^{a}

Means with different letters are significantly different at P≤0.05

Table 3. Minimum Inhibitory Concentration (µg/ml) of extracts of *C. cuneifolia* plant parts against *S. aureus* using different solvents (Source: Author)

	Water			Ethanol		
	Stem	Leaves	Roots	Stem	Leaves	Roots
Minimum inhibitory Concentration (µg/ml)	-	-	1000	10	0.01	0.01

Table 4. Phytochemical composition in *S. cuneifolia* plant parts based hot water and ethanol extraction methods (Source: Author)

	Aqueous			Ethanol		
	Stem	Leaves	Roots	Stem	Leaves	Roots
Terpenoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Alkaloids	-	-	-	-	+	+
Flavanoids	-	-	-	+	+	+
Saponins	+	+	+	+	+	+

+ – Present, – absent

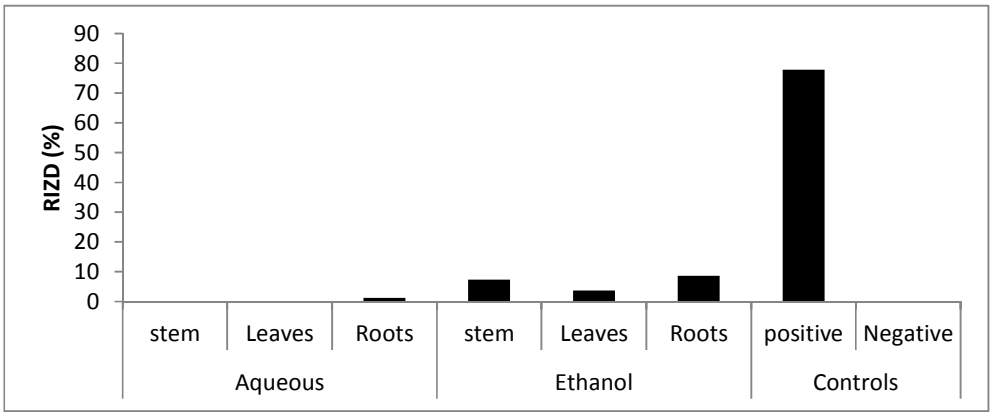

Fig. 1. Antimicrobial activity in percentage with controls as RIZD% (Source: Author)

Peer-review history:
The peer review history for this paper can be accessed here:
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