

Phytochemical Screening and Antimicrobial Effects of Leaf and Root Extracts of *Crotalaria Brevidens* on *Candida Albicans*, *Staphylococcus Aureus* and *Escherichia Coli* in Maseno (Kenya)

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Abstract: *Crotalaria brevidens* (slenderleaf) leaves and shoots are used as food and have medicinal properties when consumed by human beings. It also acts as an agent in promotion of suicidal germination of striga, a parasitic plant that is a major problem weed for maize and millet growers. In view of its medicinal importance, and there being increased tolerance of many microorganisms towards known antibiotics, there is a need to establish the anti microbial properties of extracts obtained from its roots, stem, leaf and other body parts against pathogenic microorganism. Even though this plant is reported to have immense medicinal value in treating stomach related ailments, malaria and many other tropical diseases, before this study little was known about the antimicrobial potentials of its roots, stem and leaves against three candidate microorganisms namely; *Candida albicans*, *staphylococcus aureus* and *E. coli*. This study was thus initiated to investigate (1) the antimicrobial effects of slenderleaf on *Candida albicans*, *staphylococcus aureus* and *E. coli*, and (2) establish the presence of phenols, steroids, glycosides, saponins, quinones, tannins, terpenoids and flavonoids in its crude leaf and root extracts. The plant roots and leaves used during these studies were collected, shade dried and blended to obtain a fine powder. Ethanol was used as the solvent to extract the pure components by dissolving 25g of leaves and 6g of roots separately in 150ml of ethanol in each case. After seven days, the extract was filtered and the filtrate put in a rotary evaporator to obtain a pure solid sample of the extract. A stock solution was made with 3g of the leaf extract that resulted by dissolving in 40ml distilled water making a concentration of 75mg/ml. the stock was diluted to 3.75mg/ml, 11.25mg/ml, 18.75mg/ml and 37.5mg/ml as 5%, 15%, 25% and 50% respectively. A control with distilled water (0%) was used. This was then replicated thrice to minimize variability and arranged in a completely randomized design. The screening of antimicrobial activity of crude extracts was done by measuring the zone of inhibition using agar diffusion method. Data obtained were subjected to analysis of variance (ANOVA) and means separated and compared using least significance difference (LSD) at ($p < 0.05$). There was a clear zone observed around the discs impregnated in the extract and transferred to the inoculated petri dishes. High inhibition was observed on *Escherichia coli* at a concentration of 37.5mg/ml. phytochemical screening showed presence of flavonoids, tannins, saponins, anthraquinones, phenols, terpenoids and cardiac glycosides as secondary metabolites. The crude extracts obtained in these studies clearly indicated antimicrobial properties against the three tested microorganisms, and therefore there is need to determine the main active components for studies that may lead to the discovery of new natural drugs.

Keywords: Slenderleaf, antimicrobial, pathogenic, phytochemical, microorganisms, natural drugs

I. Introduction

Slenderleaf is a plant in the family *Leguminosae/ Fabaceae* that is consumed in Africa [1], [2], [3]. The plant grows in grassland and bush land, often on termite mounds at roadsides, in cultivated lands, disturbed forests and near seasonally flooded areas. It grows from 500m-2700m above sea level and is propagated by seeds. In Kenya its leaves are widely used as a vegetable with bitter taste in western Kenya [2], [4] [5]. Slenderleaf has bluish-green leaves and grows to a height of 210cm, with bright yellow flowers and produce seed colored seeds that normally contain anthocyanins [6]. These plants also occur in the wild from northern Nigeria eastwards to Ethiopia and south to southern Tanzania. Presently about 4 billion people (approximately 60 - 80 %) of the world population and 90 % in Africa use herbal medicine from extracts of different plants for their primary health care [7] [8] [9][10], slenderleaf falling in this category.

Even though the medicinal uses of plants have long been the subject of human curiosity and need, the medicinal value of slenderleaf one of the most important African indigenous vegetable whose young leaves and shoots are consumed and contributes 100% of the daily dietary requirement for vitamin A, vitamin C, iron, calcium and 40% proteins when 100g of fresh weight are consumed [6]. Slenderleaf has medicinal Implications where it treats malaria and stomach related ailments n western Kenya, it is also used to treat boils, swellings and improves appetite [2].

Plant derived products are present in 14 of 15 therapeutic categories of pharmaceutical preparations that are currently recommended by medical practitioners and they form an important part of the health-care

system [11]. Some medicinal plants have been used for a wide range of purposes such as food preservation, pharmaceutical, alternative medicine and natural therapies for thousands of years. It is generally considered that compounds produced naturally, rather than synthetically, will be biodegraded more easily and therefore be environmentally acceptable. Thus, natural antioxidants, antibacterial, antifungal, cytotoxic, antiviral agents and nutrients have gained popularity in recent years and their use and positive image among consumers are spreading. In recent years, multiple drug resistance in both human and plant pathogenic microorganisms have developed due to indiscriminate use of commercial antimicrobial drugs commonly used in treatment of infectious diseases [12]. In Kenya the leaves are used to cure stomach-aches, swellings and other diseases, it has been reported to have several agronomic advantages that include: ability to produce seeds under tropical conditions and is reported to perform well in nitrogen stressed soils due to their ability to fix nitrogen in the atmosphere, drought tolerance and intercropping suitability as a fodder crop and a green manure [5] [3] [2].

Slenderleaf is rich in vitamins, minerals, trace elements, dietary fiber and proteins [13] [14][15][16]. The composition of this vegetable per 100g edible portion is: water 74.5g, protein 8.8g, calcium 222mg, iron 0.8mg [17]. Effectively the vegetable is important in food security, during times of drought or poor harvest and are also vital for income generation. Withstanding their value as food, the vegetables also serve as source of medicines hence important in their ecological, agronomical and cultural values [18] [19][20]. It has a bitter taste which is attributed to presence of alkaloids and phenolic compounds [1][21] hence the need to carry out phytochemical screening to investigate the phytochemicals present. The nutritional and antioxidant potential of Slenderleaf is of health or nutritional significance and thus should help encourage its consumption [22], [23].

The development of drug resistance in human pathogen against commonly used antibiotics has necessitated the need for finding potential new compounds with therapeutic uses [24]. There have been increased resistant strains of clinically important pathogens which have led to the emergence of new bacterial strains that are multi-resistant [25]. The non-availability and high cost of new generation antibiotics with limited effective span have resulted in morbidity and mortality. Economic circumstances and widespread belief in the effectiveness of many traditional therapies may have also contributed to the high dependence on herbal medicine. On average, herbalists charge between Ksh. 20 and Ksh. 150 (US\$ 0.20 –US\$ 1.50) for cases, and often the patients pay in kind. It has been reported by workers [26] that in Sub-Saharan Africa, 480 million people (60%) do not have access to modern health care and pharmaceuticals. Peoples' economic status, population growth and prices of pharmaceutical drugs are factors driving communities into using herbal medicines [27]. Hence a need to look into effective antimicrobial agents among materials of plant origin arose such as slenderleaf with an aim of discovering potential active ingredients that can be used as alternative sources of new antimicrobial drugs [28][29].

Studies of antimicrobial effects of other species within the genus *Crotalaria* like *C. burhia* and the closely related *C. ochroleuca* have been done but hardly on slenderleaf. However, studies on traditional medicines, indigenous leafy vegetables have shown its therapeutic effects hence advocating for the initiation of this research to determine (1) whether crude root and leaf extracts of *Crotalaria brevidens* has antimicrobial effects on *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli*? and (2) confirm the occurrence of selected phytochemicals since the presence of active phytochemicals in some herbs used in curing many diseases affecting people in African communities has been documented [30] before this studies. Therefore, there was an increased need to do phytochemical screening on slenderleaf to justify the medical value implicated on it.

II. Materials And Methods

Collection of plant materials

Plant material of slenderleaf was obtained from a randomly selected farm near Maseno Girls Primary School, approximately one kilometer from Maseno University. The collected plant material was cleared of adhered soil particles in the field by shaking and placed inside polythene paper bags where it was transported to the university Botany laboratory. Running tap water was employed to get rid of the adhering soils and other external microorganisms. The plant roots and leaves were allowed to dry under shade for 8days [8].

Preparation of plant extracts

To prepare plant extract, ethanol was used as the solvent. Dried leaves were ground by use of an electric blender. 25g and 6g of leaves and roots powder respectively obtained were extracted in 150ml of ethanol each in two separate flasks for a period of 7 days at room temperature of 25⁰c. The extract was then filtered using Whatmann No. 1 filter paper and filtrate transferred to Rotary Evaporator. Ethanol evaporated at 78⁰C leaving behind a pure solid sample of the extract on the base of the round bottomed flask. The leaf and root extract obtained (plate 1) was used for testing antimicrobial activity. 3g of the extract was dissolved in 40ml of distilled water to make stock solution (75mg/ml). Dilutions were then made as 5 % (3.75mg/ml), 15 % (11.25mg/ml), 25 % (18.75mg/ml) and 50 % (37.5 mg/ml).

Test microorganisms

In vitro antimicrobial studies were carried out on two bacterial strains i.e. *Escherichia coli* and *Staphylococcus aureus*, together with the imperfect fungal yeast species *Candida albicans*. The fungal yeast, the gram negative bacteria (*Escherichia coli*) and gram positive bacteria *Staphylococcus aureus* used in these studies were obtained from the Maseno University botany laboratory and maintained on Sabbaraud's agar medium.

Culture media preparation

Nutrient Agar (NA) and Potato Dextrose Agar (PDA) used in these studies were prepared according to manufacturer's instructions, autoclaved and dispensed at about 25ml per plate in 12x12cm Petri dishes. These set plates had a thorough overnight incubation to ensure they are sterile before use.

Determination of Antimicrobial Bioassays using Disc diffusion method

Each labeled medium plate was uniformly inoculated with *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* by using pour plate method in a form that lawn growth can be observed. Susceptibility testing was carried out by measuring the inhibitory zone diameters on the Agar medium using convectional paper by disc method. Circular discs, 6mm diameter each were cut from Whatman No.1 filter paper using a paper punch and each dipped in a known concentration of the extracts for about 2 minutes, then 3 discs were gently transferred to each of the Petri dish of the inoculated agar media. They were left at the set up station to avoid mishandling and after 24hrs they were incubated at 27^oc for another 24hrs. After this, the inhibitory zone distances were measured and rounded off to the nearest whole numbers (mm) for analysis. The measurements were measured by a ruler. The treatments were replicated three times to minimize variability [8].

To measure the MIC values, various concentrations of the stock, 5%, 15%, 25%, 50% were assayed against the test microorganism. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible growth [31, [32].

Phytochemical extraction method

Extract preparation: the dried leaves powder (25gm) were extracted in soxhlet apparatus by using 25ml of ethanol as the solvent for 48hrs and then concentrated by evaporation. These prepared extracts were used for phytochemicals analysis. All chemicals used in the study were of analytical grade. Phytochemical screening was done as earlier described [8], [33], [34] and [35].

Determination of alkaloids:

Two grams of the extract were extracted by warming it for 2 minutes with 20ml of 1% H₂SO₄ acid in a 50ml conical flask on a water bath, with intermittent shaking. It was then centrifuged and the supernatant was pipetted off into a small conical flask. One drop of Meyer's reagent was added to 0.1ml supernatant in a semi-micro tube. A cream precipitate indicates the presence of alkaloids [8].

Determination of flavonoids:

Five milliliters of dilute ammonia solution were added to a portion of the aqueous filtrate of the extract followed by addition of concentrated H₂SO₄. A yellow coloration indicates the presence of flavonoids [8][33].

Determination of tannin.

Tannin was determined by the Folin-Denis colorimetric method as earlier described [33]. About 0.5 g of the dried powdered samples was boiled in 20ml of water in a test tube and then filtered through Whatman No. 42 filter paper. A few drops of 0.1% ferric chloride was added. A brownish green or a blue-black coloration indicated the presence of tannins [35].

Determination of phenols:

Ferric chloride test was carried out where the extract was diluted to 5ml with distilled water. To this, a few drops of neutral 5% Ferric chloride solution was be added. A dark green or a blue-black color indicated the presence of phenolic compounds [34].

Determination of steroids.

Two ml of acetic anhydride was added to 0.5 g ethanol extract of each sample with 2ml H₂SO₄. Color changes from violet to blue or green in some samples indicate the presence of steroids [33].

Determination of saponin.

About 2 g of the powdered sample was boiled in 20ml of distilled water in a water bath and filtered. Ten milliliters of the filtrate was mixed with 5ml of distilled water and shaken vigorously to form a stable persistent froth. The froth was mixed with 3 drops of olive oil and shaken vigorously, and then was observed for the formation of emulsion [8].

Test for terpenoids:

Five milliliters of each extract was mixed with 2ml of chloroform, and concentrated sulphuric acid was carefully added to form a layer. A reddish brown coloration forming at the interface indicates presence of terpenoids [33].

Test for anthraquinones:

Powdered plant material was boiled with 10% HCl for a few minutes, then filtered and allowed to cool. This was then partitioned against equal volume of chloroform. Formation of rose-pink color upon addition of 10% aqueous ammonium solution, indicate the presence of anthraquinones [33].

Cardiac glycosides:

Five ml of extract was treated with 2ml of glacial acetic acid containing a drop of FeCl₃ solution. This was then underplayed with 1ml conc. H₂SO₄. A brown ring of the interface indicates a deoxy-sugar characteristic of cardenolides [33].

III.

Results And Discussions

Disc diffusion Assay

The antimicrobial activities of *Crotalaria brevidens* extracts against the examined microorganisms in the present study had their potency assessed by the presence or absence of inhibition zones and zone diameter. It was observed that more condensation of ethanol extracts resulted in more effective antimicrobial responses. This meant that with increase of the concentrations of the extracts are used, antimicrobial effects will increase.

Table1: Analysis of variance for zone of inhibition

Source	DF	Type II SS	MS	F value	Pr>f
Microbe	2	18.0751111	9.0375556	4.61	0.0161
Treatment	4	556.9764444	139.2441111	71.01	<.0001
Microbe vs. treatment	6	575.0515556	95.8419259	48.87	<.0001

Table 2: Mean data of *Crotalaria brevidens* extract and concentrations

VARIABLES	GROWTH INHIBITION DIAMETER
Microbe	
<i>Staphylococcus aureus</i>	6.6600ab
<i>Escherichia coli</i>	7.6933a
<i>Candida albicans</i>	6.1733b
LSD	0.5601
Extract concentration	
5%	7.2111b
15%	8.3222ab
25%	8.9667a
50%	9.7111a
Control	0.0000c
LSD	0.9001

*mean followed by same letter are not significantly different at P<0.05

Phytochemical screening for Alkaloid, tannins, saponins, cardiac glycosides, anthraquinones, phenols, terpenoids steroids and flavoids in root and leaf extracts.

When the extracts (plate 1) were screened for the presence of active secondary metabolites it was clear that the presence of alkaloids, tannins, saponins, cardiac glycosides, anthraquinones, phenols, terpenoids and flavoids was confirmed, while steroids were not detected in the leaf extract after screening. In the root extract presence of alkaloids, tannins, anthraquinones, phenols, terpenoids and flavonoids was confirmed, while steroids, saponins, cardiac glycosides were not detected. These results as obtained are summarized in the Table 3 below



Plate 1: Root and leaf extract respectively

Table 3: Phytochemical analysis

Secondary metabolite	leaf extract	root extract
Alkaloids	+	+
Tannins	+	+
Steroids	-	-
Saponins	+	-
Cardiac glycosides	+	-
Anthraquinones	+	+
Phenols	+	+
Terpenoids	+	+
Flavonoids	+	+

: + : present, - : absent

The leaves extract had inhibitory effect against various concentrations as shown in Table 1 and 2. The zone of growth diameter inhibition was more pronounced on *Escherichia coli*, followed by *Staphylococcus aureus* and finally *Candida albicans* (Table 4, Table 5 and Table 6).

Table 4- Zone of inhibition on *Staphylococcus aureus*

Percentage concentration	r ₁ (mm)	r ₂ (mm)	r ₃ (mm)	Average (nearest mm)
0%	0.0	0.0	0.0	0.0
5%	6.3	7.3	7.0	7.0
15%	8.0	9.5	7.0	8.0
25%	10.0	11.1	9.7	10.0
50%	7.0	8.0	9.0	8.0

Table 5- zone of inhibition on *Escherichia coli*

Percentage concentration	r ₁ (mm)	r ₂ (mm)	r ₃ (mm)	Average (nearest mm)
0%	0.0	0.0	0.0	0.0
5%	6.3	8.0	8.0	7.0
15%	9.0	10.1	7.5	9.0
25%	8.7	8.5	8.7	9.0
50%	14.0	14.2	12.4	14.0

Table 6- zone of inhibition on *Candida albicans*

Percentage concentration	r ₁ (mm)	r ₂ (mm)	r ₃ (mm)	average (nearest mm)
0%	0.0	0.0	0.0	0.0
5%	7.0	7.0	8.0	7.0
15%	8.3	8.0	7.5	8.0
25%	7.5	8.5	8.0	8.0
50%	8.3	7.5	7.0	8.0

However there was no significance difference in the means of *Staphylococcus aureus* and the other two microbes. There was also no significant difference in extract concentration of 15% compared to 5% and 25% although the means were significantly different from the control showing that the extract was able to hinder growth of respective microorganisms. This would be attributed to presence of chemical compounds in slenderleaf extract.

Among the three tested microbial strains, the bacteria, *Escherichia coli* and *Staphylococcus aureus* were found to be more sensitive to the extracts than the fungi *Candida albicans*. The antibacterial activity was more pronounced on *Escherichia coli*, the gram negative than *Staphylococcus aureus*, and the gram positive

bacteria. This shows that despite morphological differences in terms of structural constitutions such as presence of an outer phospholipidic membrane with lipopolysaccharide components in the Gram negative *Escherichia coli*, the extract inhibition was more pronounced thus concluding that *Crotalaria brevidens* crude extracts is able to inhibit growth of *Escherichia coli*. These results agrees with those of earlier workers [37] testing the antimicrobial activity of root extract of *Crotalaria burhia*, another *Crotalaria* species which stated that bacterial strains were found to be more sensitive than fungi. However, the results differed on which bacteria was inhibited the most (Table 4 and 5). His results showed that *Escherichia coli* growth was inhibited more than *Staphylococcus aureus* which is contrary (vice versa) to our results.

Antibacterial activity of the extract showed broad spectrum of activity against bacterial strain *Escherichia coli* and *Staphylococcus aureus* at 50% and 25% concentrations respectively (Table 4 and 5). However on *Candida albicans* it ranged from 15%-50% concentrations showing antifungal activity.

Similar antimicrobial activity of different extracts from the leaves of *Crotalaria burhia* tested against pathogenic microorganisms that cause diarrhea, thrush/skin infections, genital infections and effective results on all the tested pathogens were obtained in this case even though these two plants are different species of *Crotalaria*. The information gained from these studies clearly confirm that these two *Crotalaria* species have the same effect on *Staphylococcus aureus*, *Candida albicans* and *Escherichia coli* and therefore their extracts have good potentials for use as future use as anti microbial agents . Despite the species differences between these plants the antimicrobial effects of which are tested are the same, differences may exist in their efficacy on different microorganisms considering that these differences depend on the individual different chemical contents of the plants and thus such differences are caused by factors such as structure of soil, daily and seasonal changes that occur during the collection of plant material, physiological period growth, part of the plant studied, extraction process, solvent material and type of microorganisms used as earlier observed [36], [12].

IV.

Conclusions And

Recommendations

The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive compounds are alkaloids, flavonoids, tannins and phenolics compounds [8], Alkaloid, tannins, saponins, cardiac glycosides, Anthraquinones, phenols, terpenoids and flavonoids were confirmed to be present in the crude extract, while steroids were not detected on the leaf extract. The preliminary phytochemical screening of *Crotalaria brevidens* root and leaf extract in these study, indicated presence of the group of compounds which included: alkaloids, flavonoids, tannins, saponins, cardiac glycosides, phenols, anthraquinones and terpenoids (Table 3). Many compounds belonging to these secondary metabolites groups have been reported to their antimicrobial activities [33], which therefore might explain why *C. brevidens* has good medicinal properties.

There is a need for isolation and purification of the active agents present in the extract of *Crotalaria brevidens*, since this may lead to possible discovery of new natural drugs serving as chemotherapeutic agents for treatment and control of antibiotic-resistant bacteria. This study will be a base to investigation of advanced purification and effect mechanism of its active compounds.

Crotalaria brevidens extracts investigated during this studies have shown good ability and possess antimicrobial activity against *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli*. The extensive use of this herbal drug by local people in treating various types of infections such as stomach disorders and oral thrush might therefore be justified by their antimicrobial activities against different strains of bacteria and fungi which are known to be responsible for causing various reproductive and digestive system infections.

The results also indicate that scientific studies carried out on leaf of *Crotalaria brevidens* having traditional claims of effectiveness might warrant fruitful results.

Recommendations for future research

- Further studies are needed to isolate and determine actual active components of these crude extract for future development of new natural drugs for infectious diseases.
- More research should be done using large quantities of roots so as to attain a quantifiable amount of extract which would guarantee the extraction of enough powder for phytochemical testing and antimicrobial assaying. This would help to counter the issue of extract being left in very minute quantities in the flask after rotary evaporation.
- There is a need for studies to be conducted to establish why certain secondary metabolites are not accumulated in the leaf or stem, yet others are found on both.
- There is need for more research to be done to explain more clearly the effect brought about by the variations in diameter of zone of inhibition as from a concentration of 25% going higher up to probably 100%. Such information would provide knowledge that would help explain why at 25% extract concentration would be more effective than at 50%.

- There is also need to do many studies about antimicrobial activities of *C. brevidens* flowers, shoots and barks to ascertain their significance.
- Moreover, there is need to do some more studies of *C. brevidens* root on concentrations above 50% to check whether the change in negative deviation as from 25% as evidenced in the experiment is justifiable.

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