

Research Article



In-Vitro and *In-Vivo* Antibacterial Activities of *Croton macrostachyus* Methanol Extract against *E. coli* and *S. aureus*

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Abstract | An experimental trial was conducted to investigate the *In-vitro* and *In-vivo* antibacterial activity of *Croton macrostachyus* leaf extracts against *Escherichia coli* and *Staphylococcus aureus*. Antimicrobial susceptibility test using disc diffusion and broth micro-dilution test for the determination of minimum inhibitory concentration (MIC) were used to assess the antibacterial activity of methanolic extracts from the plant. Commercial antibiotic disc (tetracycline) was used as positive reference to determine the sensitivity of the bacterial strains. Seventy two (72) albino mice weighted between 35-45g were used for *In-Vivo* bacterial inhibitory activity of the plant extract. The mice were allotted in six (6) groups of twelve (12) animals. Clinical signs, body weight changes and post-mortem findings were registered during the study period. Phytochemical screening of *Cr. macrostachyus* leaf methanol crude extract showed presence of terpenoids, alkaloids, phenolic compounds, saponin, tannins and flavonoids. Methanol extract of *Cr. macrostachyus* showed a strong effect in inhibiting growth of tested isolates in *In-Vitro* and *In-Vivo*. Extracts from *Cr. macrostachyus* plant showed stronger antibacterial activity against *S. aureus* than *E. coli* with minimum inhibitory concentration values of 3.75mg/ml and 7.5mg/ml, respectively. The extract has shown the lowest inhibition zone (9.25±0.54mm) against *E. coli* and the highest inhibition zone (21.63±0.02) was seen against *S. aureus*. In addition, the extract was significantly better than the effect of tetracycline against *S. aureus* (P<0.05). Relatively higher number of organs with lesion and death of mice during the trial was registered in experimental groups, infected with *E. coli* than *S. aureus* infected ones, both treated with the plant extract. These findings present scientific evidences for the use of this plant as a remedy against disease caused by *S. aureus* and *E. coli* pathogens. Finally, recommendations were made for further investigation to be done for evaluating its particular therapeutic dose and optimize their application in patients.

Keywords | Antibacterial activity, *Croton macrostachyus*, *Escherichia coli*, *Staphylococcus aureus*

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INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care

(Owolabi et al., 2007). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento et al., 2000).

Plant products and their active constituents played an important role in plant disease control by combating growth and development of pathogens and including resistance in plants. Plant based natural constituents can be derived

from any part of the plant like bark, leaves, flowers, roots, fruits, seeds etc. (Gordon and David, 2001). As these plants and their products are known to possess various secondary metabolites, which showed significant inhibitory effect against the growth of pathogens, therefore, the plant and their products should be utilized to combat the disease causing pathogens.

Thus the wide spectra of antimicrobial research are geared towards the discovery and development of novel antibacterial and antifungal agents. The demand for plant based medicines, health products, pharmaceuticals, food supplement and cosmetics etc. are increasing in both developing and developed countries. Since these natural products are non-toxic, have less side effects and easily available at affordable prices they are recognized worldwide (Kalia, 2005).

Croton macrostachyus is a deciduous tree 3-25m high, although more commonly 6-12m; crown rounded and open with large spreading branches. Bark pale gray or gray-brown, finely reticulate, fairly smooth, and finely fissured with age; slash reddish; shoots densely and shortly hairy. Leaves large, green, turning to orange before falling, ovate, base rounded, apex acuminate, satellite hairy but more densely so beneath on long stems crowded at the ends of branchlets. Flowers creamy to yellow-white, sweetly scented, at least on separate shoots, in erect spikes, all over the tree, sometimes a few females accompanying the males, appearing only briefly with the flower spike turning down as fruits mature. The generic name is derived from the appearance of the seed, for 'croton' is based on the Greek word for a tick. The specific epithet is from the Greek macro- (large) and -stachyus (relating to a spike) hence "with a large spike". *C. macrostachyus* is common in secondary forests, on forest edges along rivers, around lakes, in moist or dry evergreen upland forests, woodlands, wooded grasslands or clump bush land and along roadsides. It is native to Ethiopia, Eritrea, Kenya, Nigeria, Tanzania and Uganda (Orwa et al., 2009).

According to (Agize et al., 2013), reports peoples of Dawro zone use different parts of *Cr. macrostachyus* for the treatment of different ailments in humans and animals like bleeding, liver problem, lymph inflammation; severe abdominal cramp, ascaris parasite; blackleg; malaria, stomach ache; diarrhea; headache, snake bite and rheumatism. Screening of plant extracts is a good starting point for antimicrobial drug discovery.

Despite their use, studies conducted on the traditional remedies used in animal health care in Ethiopia are inadequate when compared with the multiethnic cultural diversity and the diverse flora of Ethiopia. In most scenarios, similar to other forms of traditional knowledge, the ethno-veterinary medicinal plant knowledge, is not compiled

(Fullas, 2010), in Ethiopia is passed verbally from generation to generation and most researches in the area are survey based without experiment based scientific evaluations of the traditional remedies (Abebe, 2001).

Therefore, the present study was planned to observe the antimicrobial activity of methanolic extracts of *Cr. Macrostachyus* against two pathogenic bacterial strains in *In-vitro* and *In-vivo*.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

The fresh plant leaf samples of *Cr. macrostachyus* were collected and washed individually under running tap water to remove soil particles and other dirt. Leaves were air dried in the laboratory at room temperature for 15 days. The dried samples were ground well into a fine powder in a pestle and mortar (household flourmill). The powder was stored in air sealed polythene bags in the refrigerator at 4°C for further processing. A Voucher specimen is identified at the National Herbarium (Ethiopia), Department of Biology, Addis Ababa University, Faculty of Natural Sciences.

PREPARATION OF EXTRACT

300mg powder of air-dried leaf of *Cr. macrostachyus* was first soaked with n-hexane for 3 days. After filtration, the extract was concentrated under reduced pressure and temperature of 40°C using rotary evaporator to recover the solvent and the concentrated marc exhaustively extracted with methanol after soaking for 72 hours at room temperature. The extract was concentrated in rota-vapor and afforded 9mg yellow residue. When Thin Layer Chromatography (TLC) developed for the crude extract using chloroform: ethanol (6:4) solvent system showed six (6) colored spots under UV lamp.

The column was packed with 60mg silica-gel. 9mg of crude methanol extract was adsorbed on silica-gel with 25ml of petroleum ether. The adsorbed sample was then applied to the top of packed silica-gel in column chromatography using spatula. The column elution started with 30ml of pure diethyl ether, followed by increasing polarity of solvent system, diethyl ether, ethyl-acetate, ethanol, methanol, water, 33 fractions were collected each 30ml. then, some fractions were taken to spectroscopic analysis due to their amount and level of purity.

PHYTOCHEMICAL DETECTION

The extracts were subjected to phytochemical screening to test presence of metabolites such as terpenoids, Cardiac glycosides, anthraquinones. Alkaloids, phenolic compounds, saponin, tannins and flavonoids as described by (Iqbal, 2012).

BACTERIAL STRAINS

Both bacterial strains of animal and human pathogens used in the antimicrobial bioassay were procured from National Veterinary Institute, Debre Zeit.

The bacterial cultures one gram-negative, *E. coli*, and gram-positive bacteria, *S. aureus*, were maintained on nutrient agar in School of Veterinary microbiology laboratory, Wolaita Sodo University. Each bacterial culture were further maintained on the same medium after every 48 hours of transferring and stored at 4°C before use in experiments.

MEDIA PREPARATION AND ITS STERILIZATION

For agar well diffusion method (Bauer et al., 1996), antimicrobial susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 mg/L) was used for developing surface colony growth. The minimum inhibitory concentration (MIC) values were determined by serial micro dilution assay (Akinyemi, 2005). The suspension culture, for bacterial cells growth was done by preparing Nutrient Broth (w/v), was taken for evaluation. All the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min.

After sterilization media was allowed to cool to 50°C in a water-bath. Pouring of about 20ml agar into pre-labeled sterile Petri dishes was made. They were then allowed to set at room temperature and were dried so that no drops of moisture remain on the surface of the agar.

ANTIMICROBIAL SUSCEPTIBILITY TEST**DISK DIFFUSION ASSAY**

The antibacterial of the plant extracts was initially determined using the disc diffusion assay (Anon, 1997). Each dried plant extract was dissolved and prepared as described by (Gangoue-Pieboji et al., 2009).

E. coli and *S. aureus* were over night cultured (24hrs) at 37°C on nutrient agar for the preparation of cell suspensions. *E. coli* and *S. aureus* cell suspensions adjusted to 0.5 McFarland standards (10⁸CFU/ml) were prepared and 0.1ml of the suspension was poured on Muller Hinton agar (MHA) (Oxoid, UK) and dispersed using a cotton swab to ensure robust cell growth. Plates were then allowed to dry for 5 minutes prior incubation.

The sterile filter paper discs (Whatman No. 3, diameter 6 mm) were soaked in 30µl of plant extract for 30 minutes. The extract-soaked filter paper discs were then placed on the inoculated MHA plates, allowed prior incubation to stand for 30 minutes at room temperature to permit proper diffusion of the extract. All plates were incubated at 37°C for 24hrs, and the resulting inhibition zones were measured in millimeter. This experiment was done in triplicate

and the antimicrobial activity was expressed as the mean of inhibition diameters (mm) produced by the plant extract. As positive controls, discs containing Tetracycline 1µg were used.

MEASUREMENT OF MINIMAL INHIBITION CONCENTRATION (MIC)

The minimum inhibitory concentration is defined as the lowest concentration which able to inhibit any visible bacterial and fungal growth on the culture plates (Shahidi, 2004). According to National Committee for Clinical Laboratory Standards (NCCLS) agar dilution method (Sambrook and Russell, 2001), the MIC of *Cr. macrostachys* extract was tested with some modification.

The MICs of plant extracts were determined using an agar dilution method as described by (Bauer et al., 1996). The tests were performed using broth dilution method. Broth containing test tubes were tightly closed, arranged in test tube rack and autoclaved under 15 psi pressures at 121°C for 15 min. The broths were allowed to cool until the temperature is equitable to room temperature. The extract solutions (50mg/ml) were serially diluted in broth containing test tubes to bring 30mg/ml, 15mg/ml, 7.5mg/ml and 3.75mg/ml concentration, respectively. Each of the different concentration extracts was aseptically introduced. The inhibition growths were observed after 24hr incubation at 37°C. The presence of growth was evaluated by comparing turbidity of culture containing test tubes with the negative control. The lowest concentration, at which there was no turbidity, was regarded as MIC value of the extract (Sambrook and Russell, 2001; Ondruschka and Asghari, 2006). After a 24-hour incubation period, the inhibition zone diameters (mm) were measured.

The experiment was performed in triplicates and the interpretation of antibacterial properties was conducted according to (Adithepchaikarn et al., 2008). Inhibition zones >15mm were categorized as strong activity, from 10-15mm as moderate activity and <10mm as weak activity.

ANTIBACTERIAL ACTIVITIES OF PLANT EXTRACT IN-VIVO AND EXPERIMENTAL DESIGN

The experiment was done by using 72 Albino mice, their age ranged between (10-14) weeks with weight ranged between (35-45) grams purchased from Ethiopian Public Health Institute, Addis Ababa. Mice were housed in plastic cages 20×50×75cm dimensions (12 mice/cage), placed in a special highly sanitized housing room with optimum conditions included temperature, light and ventilation belongs to the School of Veterinary Medicine, Veterinary Microbiology Laboratory for two weeks for adaptation. Standard rodent diet (Commercial feed pellets) and sterilized water were freely available.

The mice were grouped into six (6) groups of twelve (12) animals each. The six groups were injected subcutaneously as follows:

Group I (-ve Control I)	Injected with 0.1ml normal saline
Group II (-ve Control II)	Injected with 0.1 ml of plant extract
Group III (+ve control I)	Injected with 0.1 ml of E. colisuspension, treatment not given(1×10^8 CFU/ml)
Group IV (+ve control II)	Injected with 0.1ml of S. aureus suspension, treatment not given (1×10^8 CFU/ml)
Group V (Experimental I)	Injected with 0.1 ml of E. coli suspension and treated (1×10^8 CFU/ml)
Group VI (Experimental II)	Injected with 0.1 ml of S. aureus suspension and treated (1×10^8 CFU/ml)

Infection was induced by a method adapted from (Amiri et al., 2011). Bacterial inoculum used to induce infection was (1×10^8 CFU/ml) of *E. coli* and *S. aureus* suspensions. 0.1 ml of each dilution was infused into the rats by intra-muscular route and the rats had been watched for symptoms. Starting from second to five day the Groups V and VI were injected with plant extract (the dose given was depended on MIC, and the dose depended to the weight of the body 40mg/kg/day) and all clinical outcomes were recorded over a period of two weeks. After five days the blood was taken from the tested mice for bacterial count (a loopful), then was diluted in 0.5ml normal saline, then 0.1 of it inoculated on nutrient agar and manitol salt agar for identification of the inoculated bacteria.

CLINICAL SIGNS

Animals were continuously observed for clinical signs; fecal consistency, dehydration, unusual behavior, depression, death, swelling at injection site and any change in activity of the mice were recorded daily throughout the experimental.

BODYWEIGHT CHANGES

Weight of the animals was measured using electronic weighing balance and differences were assessed according to feed requirement and comparison between treated and control animals. These measurements were done a first week before inducing infection, after a week post treatment.

BLOOD COLLECTION

1 ml of blood from the treated animals was drawn from the vein of treated animals 24hrs after treatment each day and inoculated on already prepared on Petri dishes with nutrient agar. The inoculated plates were incubated at 37°C for 24hrs. The counts of emerged colonies were used to evaluate the efficacy of treatment.

POST-MORTEM FINDINGS

Animals were sacrificed by surgical knife when dies during experiment or at the end of the experimental day, and vital organs like lungs, heart, kidneys, GIT, muscles, spleen are examined for pathological lesions in Microbiology laboratory, Veterinary school.

STATISTICAL ANALYSIS

The data collection instrument was experimental through basic laboratory technique. Data's like susceptibility was analyzed using SPSS software package version 20.0. Statistical Significance (P-value) was calculated using analysis of variance (ANOVA) using a significant level of (P<0.05). Microsoft Excel was employed for analysis of minimum inhibitory concentration.

RESULTS AND DISCUSSIONS

PHYTOCHEMICAL SCREENING

The qualitative phytochemical screening reveals the presence of alkaloids, terpenoids, saponins, flavonoids, phenolic compounds and tannins. The results were shown in (Table 1) below.

Table 1: Phytochemical constituents of the leaf methanol extracts of *Cr. macrostachyus*

Constituents	Methanolic Leaf extract
Alkaloids	+
Terpenoids	+
Saponins	+
Flavonoids	+
Cardiac glycosides	-
Phenolic compounds	+
Anthraquinones	-
Tannins	+

+ Present, - Absent

ANTIBACTERIAL ACTIVITY

The results of present study showed that the methanol extract of *Cr. macrostachyus* inhibited the growth of tested isolate strongly; this may be due to presence of the phytochemical groups as mentioned in (Table 2).

According to the findings, *S. aureus* was found to be more

sensitive to the extract than *E. coli*. Methanol extract has shown higher inhibition zones of (21.03 ±0.02 mm) against *S. aureus* (p < 0.02) and the lesser inhibition zones of (9.25±0.54 mm) against *E. coli*. The observed difference in antibacterial activities of extracts between *E. coli* and *S. aureus* bacterial strains may be attributed to the difference in the outer membrane of both isolates. There was no any inhibition found for all tested bacteria with negative control (Table 2).

Table 2: Growth inhibition zones of *Cr. macrostachyus* methanol extract against bacterial strains

Bacterial strain	Mean Inhibition Zones of Extracts	Positive Control	Negative Control
	Methanol	Tetracycline	Water
<i>E. coli</i>	9.25±0.54	14.83±2.07	0±0.0
<i>S. aureus</i>	21.03±0.02*	16.75±1.85*	0±0.0

These figures were mean of triplicate values in the form of mean plus standard deviation and values of the same column followed by *superscripts are statistically significant (p≤0.05).

Gram-negative bacteria possesses high permeability barrier for numerous antibiotic molecules similarly for these extracts. Their periplasmic space also contains enzymes, which are capable of breaking down foreign molecules (Shan et al., 2007) and appears to be less susceptible to plant extracts than gram positive bacteria.

In this study, Methanolic extracts of *Cr. macrostachyus* showed minimum inhibition zones at the concentration of 7.5mg/ml and 3.75mg/ml against *E. coli* and *S. aureus*, respectively, in agar well diffusion method. This result disagrees with previous reports by (Sendeku et al., 2015), 15mg/ml against *S. aureus* and 50mg/ml against *E. coli* in agar well diffusion method. This difference may be due to difference in experimental protocols and source of bacterial strains. The increased concentration may be linked with previous exposure of isolates for antimicrobial drugs, which may be the cause for enhanced resistance. This may happened through unreasonable prescription, presence of

antibiotics without prescription and widespread traditional self-medication.

Methanol extracts of this plant also showed highest inhibition zones compared to the positive control (tetracycline) against *S. aureus*. This might indicate the ingredients of the plant extract are more potent than the antibiotics.

RE-IDENTIFICATION OF THE TEST ORGANISM

According to results presented in (Table 3), too numerous to count number of bacteria in group VI was zero, and this result gives a shiny way for using a drug against gram positive bacteria. On the other hand it must take in consideration that the plant extract may have a side effect for the patient where it observed that the mice of group II (Table 4) affected locally (in place of injection); therefore it is very necessary to use plant extract has a strong inhibition against bacteria and a little side effect for the host.

(Table 3) presents number of bacteria units recorded in mice blood samples. Accordingly, was decreased in group VI, where the *S. aureus* infected mice of this group were treated with methanol extract of *Cr. macrostachyus*, followed by group V infected with *E. coli* and treated with methanol extract of *Cr. macrostachyus*.

CLINICAL SIGNS

The effect of this plant extract against bacteria in *in-vivo* gave a good result as bacterial inhibitory agent and also resulted in death of mice as shown in (Table 5), (in case of comparing group VI with group V). This may be interpreted that the administrated dose of tested extract was little as affected dose, or may at this dose the immunity system of mice be compromised (may be a toxic for the host), so it appeared from studies that the tannins can cause several disorders in mice and rats such as liver cancer (this is in contrast with the positive results of methanol extract of tested plant on laboratory animals), or the mice may need to other course of administrating the plant extract. Table 5 illustrates the clinical symptoms which were noted on all groups of mice during laboratory experimental

Table 3: Bacterial count from mice blood samples

Bacterial Count (CFU)		Mice Groups					
		Group I (-ve control I)	Group II (-ve control II)	Group III (+ve control I)	Group IV (+ve control II)	Group V (Experimental I)	Group VI (Experimental II)
Low	Low	12	12	0	0	4 (33.3%)	6 (50%)
	Moderate	0	0	4 (33.3%)	3 (25%)	6 (50%)	6 (50%)
	Too numerous to count	0	0	8 (66.6%)	9 (75%)	2 (16.6%)	0
Total		12	12	12	12	12	12

Table 4: Distribution of lesions in tissues of experimental mice on post mortem meat examinations

		Mice Groups					
		Group I (-ve control I)	Group II (-ve control II)	Group III (+ve control I)	Group IV (+ve control II)	Group V (Experimental I)	Group VI (Experimental II)
Lesioned organ	Liver	0	3	0	4	0	0
	GIT	0	1	6	0	1 (8.3%)	0
	Lungs	0	0	0	6	0	1 (8.3%)
	Kidneys	0	0	5	0	3 (25%)	1 (8.3%)
	Others (heart, muscle, spleen, soft tissues etc)	1	2	1	2	1 (8.3%)	2 (16.7%)
	Absent (no lesion found)	11	6	0	0	7 (58.3%)	8 (66.7%)
Total		12	12	12	12	12	12

Table 5: Clinical symptoms of control mice, mice treated with *Cr. macrostachyus* extract during experimental trial

		Mice Groups					
		Group I (-ve control I)	Group II (-ve control II)	Group III (+ve control I)	Group IV (+ve control II)	Group V (Experimental I)	Group VI (Experimental II)
Clinical Findings	Diarrhea	0	0	0	0	2 (16.6%)	0
	Dehydration	0	0	0	1	0	1 (8.3%)
	Weight loss	0	1	0	0	1 (8.3%)	0
	Death during trial	0	0	8 (66.6%)	10 (83.3%)	4 (33.3%)	1 (8.3%)
	Others (less activity, depression, large wound in place of injection)	1	3 (25%)	4	1	1 (8.3%)	2 (16.6%)
	Normal activity	11	8	0	0	4	8
Total		12	12	12	12	12	12

trial. It appeared from (Table 5) the death rate in experimental group infected with *E. coli* and get treatment was higher (33.3%) than mice infected with *S. aureus* and taking the treatment. Death rate was decreased from 66.6% to 33.3%, when death rate of positive control I or Group III (Injected with 0.1 ml of *E. coli* suspension, treatment not given) compared with the death rate of experimental group I or Group V (Injected with 0.1 ml of *E. coli* suspension, treated with plant extract).

On the other hand, according to table 5 death rate was decreased from 83.3% to 8.3%, when death rate of positive control II or Group IV (Injected with 0.1 ml of *S. aureus* suspension, treatment not given) compared with the death rate of experimental group II or Group V (Injected with 0.1 ml of *E. coli* suspension, treated with plant extract).

Thus, it demonstrated that the ingredient of the plant extract was better effective against *S. aureus* than *E. coli*. According, to this table (5) negative control groups that take

plant extract had showed sluggish activity and depression which might indicative minimum side effects of the extract

POSTMORTEM FINDINGS

The result demonstrated lesion distribution recorded in all groups of mice in different organs. According to this table (Table 4), relatively more organs were found with lesion in experimental group I/ Group V (group infected with *E. coli* and take treatment) than experimental group II/ Group VI (mice infected with *S. aureus* and take treatment).

According to table 4, major lesions were recorded in group VI (experimental II) in soft tissues and circulatory organs. This finding is in agreement with reports by (DeLeo and Chambers, 2009) which dictates *S. aureus* is the leading cause of bacterial infections involving the bloodstream,

lower respiratory tract, skin and soft tissue in many countries. The most prevalent gram-negative pathogens, such as *E. coli*, cause a variety of disease in humans and animals,

particularly urinary tract infections (Iroha et al., 2010) and a strong correlation between antibiotic resistance development has been observed over the past half-century (Songca et al., 2013; Jacopy, 2009).

CONCLUSION AND RECOMMENDATIONS

Despite the existence of excess information regarding the prolonged and uneventful local use of this plant, scientific evidences regarding their efficacy are less abundant. The result and discussion of this study, clearly indicated that *Cr. macrostachyus* has ample potential to inhibit two human and animal pathogenic bacteria as it were seen from its strong inhibition against tested organisms. *Cr. macrostachyus* shows much promise in the development of phyto-medicine having antimicrobial properties and the drug derived from *Cr. macrostachyus* may have the possibility of alternative medicinal source because of their antibacterial activity. This study also indicated that methanol extract of this plant has highest capability to antibacterial activity against *S. aureus* even than tetracycline. In general, it can be concluded that methanol extract of *Cr. macrostachyus* is a strong inhibitor for bacterial growth in vitro and in vivo. Therefore, it is recommended to identify the active ingredients of the antibacterial agent and obtaining chemotherapist agent in different drug formulations therefore be used for enteric and systemic infections caused by *S. aureus* and *E. coli*.

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AUTHORS' CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

There is no conflict of interest.

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