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IN *Vitro* ANTIBACTERIAL EFFECTS OF CHLOROFORM, METHANOL AND WATER EXTRACTS OF *CROTON Macrostachyus Stem* BARK AGAINST *Escherichia Coli* AND *Staphylococcus Aureus Standard* AND CLINICAL STRAINS

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ABSTRACT

In Ethiopia, different parts of *Croton macrostachyus* (*C. macrostachyus)* are used as a traditional medicine to take care of infectious diseases such as typhoid and measles, but there is no documented report on the antibacterial activity of stem bark of this plant in Ethiopia. *C. macrostachyus* stem bark was extracted using chloroform, methanol, and water extraction solvents and tested for their antibacterial activities against *Escherichia coli (E. coli)*, clinical isolates and standard, and *Staphylococcus aureus (S. aureus)*, clinical isolates and standard, using agar well diffusion and broth dilution methods. The positive control was Chloramphenicol, while dimethyl sulfoxide (DMSO) was served as negative control. The present study showed the potent antibacterial activity of the *C. macrostachyus* stem bark extract against all tested bacterial pathogens. The methanol extract of *C. macrostachyus* stem bark showed the highest zone of inhibition (17±1mm) against *S. aureus* (standard) and the lowest zone of inhibition (12±1) against *E. coli* (clinical isolate). In this study, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 500 & 500 mg/ml, 62.5 & 125 mg/ml and 250 & 500mg/ml were obtained for water, methanol, and chloroform extracts of *C. macrostachyus* stem bark against clinically isolated *E. coli* respectively. *C. macrostachyus* stem bark extracts have confirmed antibacterial effects, mainly on *E. coli* and *S. aureus*. Thus, *C. macrostachyus* stem bark could be effective for prevention of bacterial infections and may be considered as an option to antibiotic regimens. But further studies should be conducted with different extraction solvents and toxicity and phytochemical analysis must be performed on these plants to use as sources and templates for the synthesis of drugs.

Contribution/Originality: This study contributes in the existing literature by providing basic information for other researchers regarding the antibacterial potential of Ethiopian *C. macrostachyus* stem bark so that *C. macrostachyus* stem bark may be considered as an option to antibiotic regimens.

1. INTRODUCTION

Microbial diseases continue to be major threats to the world regardless of hard work and advancement in developing modern medicine. There are numerous reports on the isolation of bacteria that are known to be sensitive but became multi-resistant to routinely used drugs and other medications available on the market (Nascimento *et*

al., 2000). This is due to bacteria possess the genetic ability to acquire and transmit resistance traits against currently available antibacterial (Nega and Tigist, 2015).

The impact of bacterial diseases is especially important in developing countries such as Ethiopia where there is inadequate access to modern drugs and prices are mostly unaffordable when the latter are available. Extensive use of antibiotics often resulted in the development of resistant strains and these create a problem in the management of infectious diseases. Furthermore, side effects associated with antibiotics are often fewer when using medicinal plants (Nega and Tigist, 2015)

Medicinal plants have some prize over antibiotics such that there is superior patient tolerance, relatively less costly, agreement due to a long history of use and are renewable in nature (Vermani and Garg, 2002). Currently, the ever-increasing risk from drug-resistant bacteria calls for a universal effort to search for novel solutions that can also be based on the natural products from plants that are selected on the basis of documented ethnomedicinal use (Lulekal *et al*., 2014). Medicine from herbs is readily obtained in our widely varied vegetation, inexpensive and all plant parts carry the potential for introducing new templates into modern medicine (Jackie *et al*., 2016). Natural plant sources are usually the raw material for the most pharmaceutical company (Amin *et al*., 2016).

Croton macrostachyus is a deciduous tree belonging to the family *Euphorbiaceae*. The leaves are large and green, turning to orange before falling. It is also characterized by creamy to yellow-white colored flowers with green (when young) to grey (at maturity) fruits. *C. macrostachyus* is commonly named as 'Bisana' in Amharic, Ethiopia and it is an important medicinal plant in East Africa including Ethiopia (Abraham *et al*., 2016). It is traditionally used for the treatment of wounds (Teklehaymanot and Giday, 2007; Giday *et al*., 2009; Abraham *et al*., 2016) malaria, rabies, and gonorrhea (Giday *et al*., 2007) *Tineaversi color*, diarrhea, hepatitis, jaundice, and scabies (Teklehaymanot and Giday, 2007).

In case of medicinal value, *C. macrostachyus* has many uses. Leaf extract is applied to the itchy scalp. A decoction of the leafy twigs mixed with *Justicia schimperiana* is taken to treat jaundice and smallpox. The traditional preparation can be taken with pepper, butter, and milk. The mixture of the leafy branches and roots is used as a mouthwash to treat a toothache. The leaves or young shoots of *C. macrostachyus* are eaten to treat fever and oedema and mashed leaves are used for hemorrhoids. *C. macrostachyus* stem bark maceration is drunk as an abortifacient and uterotonic, to expel a retained placenta. In addition to this stem bark is chewed to treat a toothache (Tesemma, 2007).

Several hydroalcoholic *C. macrostachyus* stem bark extracts against a clinical strain of *Neisseria gonorrhoeae* was reported by Mesfin (2007) with the MIC value of 125–250 mg/ml. There are negative as well as positive reports on the antibacterial activity of methanol extracts from *C. macrostachyus* leaves (Matu and Van Staden, 2003; Wagate *et al*., 2010; Jackie *et al*., 2016). Contrasting results could be attributed to the locality of plant species, parts used, time of collection, storage conditions, and methods of analysis (Suffredini *et al*., 2006; Jackie *et al*., 2016). Even though the majority of the Ethiopian population uses *C. macrostachyus* to treat different diseases traditionally, a single study was conducted regarding the antibacterial effect of *C. macrostachyus* leaf but there is no study conducted on the other parts of this plant in Ethiopia. Therefore, the objective of this study was to assess the antibacterial activity of chloroform, methanol and water extracts from *C. macrostachyus* stem bark against clinical and standard strains of *E. coli* and *S. aureus*.

2. MATERIALS AND METHODS

2.1. The Study Area

The study area is found in Gondar town which is located in the North-Western part of Ethiopia mainly in the Amhara Region. It is placed about 738 Km from Addis Ababa, the capital city of Ethiopia. Geographically Gondar has located 12^0 35' 07" North latitude and 37^0 26' 08" East longitudes and altitude range from 2000–2200m above sea level (David, 2011).

2.2. Collection of *Croton Macrostachyus* Stem Bark

Croton macrostachyus stem bark was collected from behind Tewodros campus student cafe, University of Gondar, Ethiopia. The plant material was transported to the Microbiology Laboratory of Biotechnology Department, the University of Gondar to assess the antibacterial activity of the chloroform, methanol, and water extracts of *C. macrostachyus* stem bark against the clinical and standard strains of *E. coli* and *S. aureus*.

2.3. Preparation of *Croton Macrostachyus* Stem Bark Extracts

This was carried out as previous demonstrated, with slight modifications, by Predrag *et al.* (2005). The freshly collected stem bark was cut into pieces and shade dried at room temperature for 10 days and milled using an electric blender. The powder was stored in the closed containers. Each of the *C. macrostachyus* powder was extracted with chloroform, methanol, and water. Twenty-five gram of powdered sample was mixed in a conical flask with 100ml of solvents of chloroform, methanol, and water separately for 48 Hrs, until absolute extraction of the bioactive component of the plant. After 48 Hrs, each of the extracts was filtered through gauge and then by Whatman no1 filter paper. The filtrates were then located in a rotary evaporator in order to remove the extraction solvents and collected in a sterile container for extra use. Extracts were kept at 4° C to keep the antibacterial property before they were used for agar well diffusion and broth dilution assay.

Each plant extract was then suspended in DMSO (Oxoid) to the concentration of 500 mg/ml to obtain the zones of inhibition. For the minimum inhibitory concentration measurements the stock solution (500 mg/ml) was serially diluted to provide the concentrations of 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.63 mg/ml, 7.81 mg/ml, 3.90 mg/ml, and 1.95 mg/ml.

2.4. Determination of Antibacterial Activity

Antibacterial activity of the extracts was analyzed using agar well diffusion assay according to the technique illustrated by Taye *et al.* (2011). Several Mueller-Hinton agar plates were made and labeled according to the extract and by the name of the bacteria, including a negative control (DMSO) and positive control (Chloramphenicol). A bacterial suspension was prepared in sterile normal saline with reference to the 0.5 McFarland Standards. The turbidity of the bacterial suspension was compared with 0.5 McFarland standard solutions, followed by culture of 100 µl of the bacterial suspension on Mueller-Hinton agar plates using a sterile glass rod spreader and allowed to remain in the incubator for 15 minutes to remove excess moisture. On each plate, equidistant wells were made with a 6 mm diameter sterilized cork borer, 2 mm from the end of the plate. One hundred microliters from each plant extract (500 mg/ml) were aseptically placed into a respective agar well. This was followed by incubation at 37° C for 24-48 Hrs. The inhibition zone around each well was recorded in millimeters (mm) and the assay was carried out three times for each extract to obtain consecutive results.

2.5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MICs of plant extracts was determined using a broth dilution method as described by Bauer *et al.* (1966). The extract stock solution (500 mg/ml) was serially diluted in a broth to provide the concentrations of 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml, 15.63 mg/ml, 7.81 mg/ml, 3.90 mg/ml, and 1.95 mg/ml. One hundred microliters of inoculums (1.5×10^6 CFU/ml) were then inoculated into each tube. The growths inhibition was observed after 24 Hrs of 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.

The MBC was determined by sub-culturing samples from the wells with concentrations above the MIC value on new Mueller Hinton agar plates. The MBC was regarded as the lowest concentration of the extract related to no bacterial culture. Each assay was carried out in triplicate.

2.6. Data analysis

The data was analyzed using Statistical Package for Social Sciences Software (SPSS) version 20. The inhibition zones of the extracts on the pathogens were compared using one way ANOVA.

3. RESULT

3.1. Antibacterial Activity of *Croton Macrostachyus* Stem Bark Extract

The antibacterial effects of *C. macrostachyus* stem bark extracts showed antibacterial activity against all the tested bacterial strains. The diameter of the zone of inhibition was wide-ranging ranging from (7.7±0.6 mm) to (17±1.0 mm) diameter (Table 1). All the water, chloroform, and methanol extracts of *C. macrostachyus* stem bark caused 7.7±0.6 mm to 17±1 mm inhibition zones. The bacteria, which were inhibited with a zone diameter of 17±1 mm, were *S. aureus* (standard) (with methanol extracts) and the lowest inhibition zone (7.7±0.6 mm) was found against *E. coli* (clinical) which is extracted by methanol. The antibiotic Chloramphenicol (positive controls) was regularly high. DMSO, which was the negative control, did not show inhibitory activity.

Table-1. Antibacterial activity of the chloroform, methanol and water extracts of *Croton. macrostachyus* stem bark against clinical and standard strains of *S. aureus* and *E. coli*.

Antibacterial	Extraction Solvent	Diameter of inhibition (mm)			
		E. coli		*S. aureus*	
		clinical	*standard*	*clinical*	*standard*
C. macrostachyus	Chloroform	12 ± 1.0^{a}	13.0 ± 1.0^{ab}	11.0 ± 1.1^{a}	16.0 ± 3.6^{b}
	Methanol	12 ± 1.0^{a}	15.0 ± 1.5^{b}	12.7 ± 1.0^{a}	17 ± 1.0^{b}
	Water	7.7 ± 0.6^{a}	10 ± 1.0^{a}	8.67 ± 0.6^{a}	10 ± 1.0^{a}
Chloramphenicol		39±1.0	42±1.0	42±1.0	39±1.0

Values are means of triplicate determinations; Values within the same column followed by different superscripts are significantly different at ($P< 0.05$).

3.2. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC value of *C. macrostachyus* stem bark extracts against the tested bacteria showed variety from 62.5 mg/ml (Methanol extract of *C. macrostachyus* both on clinical and standard strains of *E. coli*) to 500 mg/ml (water extract on the same bacteria). The methanol extract of *C. macrostachyus* stem bark demonstrated least MIC value 62.5 mg/ml against *E. coli* (both on clinical and standard strains) whereas water extract showed 500 mg/ml against *E.* coli (clinical). *S. aureus* (clinical and standard) and *E. coli* (standard) showed comparatively efficient MIC value 125 mg/ml in chloroform and methanol extracts (Table 2).

The MBC values, which were determined by sub-culturing the samples having dilution values of greater or equal to MIC values, were illustrated in Table 2. The MBC values of the extracts varied from 125 mg/ml (Methanol extract against *E. coli*, both clinical and standard) to 500.00 mg/ml (water extract against the growth of *E. coli* (standard and clinical) and chloroform extract against *E. coli* and *S. aureus* (clinical).

Table-2. MIC and MBC (mg/ml) of the chloroform, methanol and water extracts of *C. macrostachyus* stem bark extracts against clinical and standard strains of *S. aureus* and *E. coli*.

Bacteria	Methanol		Chloroform		Water	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (clinical)	62.5	125	250	500	500	500
E. coli (standard)	62.5	125	125	250	250	500
S. aureus(clinical)	125	250	250	500	125	250
S. aureus(standard)	125	250	125	250	250	250

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration;

4. DISCUSSION

The antibacterial effect of *C.* macrostachyus stem bark was tested using the agar well diffusion and broth dilution methods. Each of the extracts tested in the present study displayed antibacterial activity on all bacterial strains tested. Though, differences were observed between antibacterial activities of the extracts. These differences could be due to the variations in the chemical composition of these extracts.

In the present study, chloroform, methanol, and water extracts of *C.* macrostachyus stem bark were evaluated for their antibacterial activity against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria, which are the major important human pathogenic microorganisms. Antibacterial activity of each plant extract was tested by agar well diffusion and broth dilution (MIC) methods. The extracts from *C. macrostachyus* stem bark persuaded growth inhibition against all the studied bacterial pathogens. Our results illustrated that between the bacterial strains there was variation in susceptibility to extracts. This may be due to the antibacterial effect of the extract depends on the bacterial strain and the extraction solvent used to extract the phytochemicals which contain antibacterial effect from the medicinal plant.

In this study, *C. macrostachyus* stem bark extracted by methanol has shown the highest inhibition zone (17 ± 1) against *S. aureus* (standard) and the lowest zone of inhibition were recorded in *E. coli* (clinical). It is reported that Gram-positive bacteria are more susceptible to antibiotics since they have only an outer peptidoglycan layer which, is an ineffective barrier (Karou *et al.*, 2005; Lulekal *et al.*, 2014). But Gram-negative bacteria have an outer phospholipidic membrane that makes the cell wall impermeable to lipophilic solutes, whereas the porines contain a selective barrier to hydrophilic solutes with an elimination limit of about 600 Da (Karou *et al.*, 2005). In addition to this periplasmic space of Gram-negative bacteria contains enzymes, which are able to break strange molecules and become to be less susceptible to plant extracts than the Gram-positive ones. Several research findings supported this justification, therefore extracts from some medicinal plants were found to be more effective against Gram-positive bacteria than Gram-negatives (Kelmanson *et al.*, 2000; Masika and Afolayane, 2002). The lowest inhibition zone was recorded against *E. coli* which is the clinical isolate; this may be due to development of resistance in the clinical isolate.

Chloroform extract of the *C. macrostachyus* stem bark was the second strong extract for its antibacterial activity and this is in agreement with Taye *et al.* (2011). But *C. macrostachyus* water extract had lower activity against all bacteria tested. This indicates, in comparison to water, the active ingredient which inhibits the growth of bacteria may dissolve better in methanol. However, Sendeku *et al.* (2015) reported chloroform extract from *C. macrostachyus* leaves exhibiting significant antimicrobial activity. Furthermore, water extract from leaves of *P. acerifolium* had been reported to have tough antimicrobial activity against various gram positive and gram negative human pathogenic bacteria (Thatoi *et al.*, 2008) and as stated by Dabur *et al.* (2007) the water extracts of *A. nilotica*, *J. zeylanica*, *L. camera* and *S. asoca*, were found to be the most active against different bacteria as well as fungal pathogens. It is clear that the effectiveness of the extracts largely depends on the type of solvent used to extract the phenolic compound from plants. The organic extracts provided more powerful antimicrobial activity as compared to the water extracts. This observation clearly points out the survival of non-polar residue in the extracts which have higher bactericidal and bacteriostatic abilities. Thatoi *et al.* (2008) mentioned that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily solubilized in organic solvents. Similar results showing that the alcoholic extract having the best antimicrobial activity is also reported by Antarasen and AmlaBatra (2012) *in Melia azedarach* leaf extracts

The antimicrobial analysis using the MIC value is been used by many researchers. In the present study, the MIC value of the active *C. macrostachyus* stem bark extracts obtained was lower than the MBC values suggesting that the extracts were bacteriostatic at lower concentration but bactericidal at higher concentration (Maji *et al.*, 2010; Antarasen and AmlaBatra, 2012). Minimum inhibitory concentration values of 62.5–500 mg/ml was found in *C. macrostachyus* stem bark extracts in this study. However, Jackie *et al.* (2016) reported MIC value range from 125-

500mg/m of *C. macrostachyus* ethanol extract against selected human pathogens.When testing methanol extracts of *C. macrostachyus* leaves and roots Wagate and colleagues found MICs from 15.6 to 250 mg/ml against three bacteria, *E. coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa* (Wagate *et al*., 2010).

5. CONCLUSION

From our investigation, it is concluded that the active antibacterials present in the stem bark of *C. macrostachyus* were methanol and chloroform-soluble. The active ingredients contained in extract of chloroform are quite effective against standard strains of *S. aureus* and *E. coli* along with activity against the remaining whereas the activity in methanol extract showed efficacious results against all the tested organisms.Further studies should be conducted with different extraction solvents and toxicity and phytochemical analysis must be performed on these plants to use as sources and templates for the synthesis of drugs to control disease-causing bacteria.

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