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Research Article

ANTIBACTERIAL ACTIVITY OF *CROTON MACROSTACHYUS* AGAINST SOME SELECTED PATHOGENIC BACTERIA

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ABSTRACT

Croton macrostachyus is a multipurpose plant that has been used for various remedies as constituents of traditional medicine. Up to now the treatment of infectious diseases caused by bacteria is mainly depend on the use of commercial antibiotics. Currently, due to the emergence of multi-drug resistant strains and widely distributed infectious diseases caused by bacteria unable to treat by the existing drugs. Hence the aim of the present study was to evaluate the antibacterial activity of *C. macrostachyus* extracts. Agar well diffusion and broth dilution assay methods against selected pathogenic bacterial strains were employed. The bacterial strains were assessed for antibacterial susceptibility of three different extracts ethanol, methanol and chloroform. The highest antibacterial inhibition against *S. aureus* (ST) (32.00±1.00 mm) and *S. flexner* (30.67±0.58) was revealed by chloroform extracts while the least inhibition zone (7.00±1.00 mm) has shown against *E. coli*. The least MICs 3.75 mg/ml have shown by Chloroform extract against *S. flexner*, *S. pneumonia* and *S. aureus* (ST) and ethanol 7.5 mg/ml against *S. aureus* (ST) and methanol 15 mg/ml against *S. pneumonia* and *S. aureus*. MBCs of chloroform extract (7.5 mg/ml) against *S. pneumonia* and *S. aureus* (ST), ethanol extracts (15 mg/ml) against *S. aureus* (ST) and methanol extract (15 mg/ml) against *S. aureus* against were also seen. It clearly indicates that the crude chloroform extract of *Croton macrostachyus* showed highest antibacterial activity against all studied bacterial strains as compared to the two solvents used in this study. Thus, further study and characterization of active compounds of this chloroform plant extract is required.

Keywords: *Croton macrostachyus*, Antibacterial activity, MBC and MIC

INTRODUCTION

Use of natural products for curing human disease is an ancient and universal as medicine (Habtamu *et al.*, 2012). Most of the currently available drugs for treatment of different human and animal diseases obtained from natural products especially medicinal plants (Mishra and Tiwari, 2011; Microorganisms are frequently a cause of prevailing diseases, presenting a serious public health issues in a significant segment of the population as showed by both private and official health care systems (Nastro *et al.*, 2000 and Antonio *et al.*, 2007). Currently, about 80% of populations on developing countries use traditional medicine for their health care with modern pharmacopoeias containing at least 25% of drugs derived from plants and many others which are synthetic analogues build on prototype compounds isolated from plants (Giday *et al.*, 2007 and Dias *et al.*, 2012).

Neglected diseases are infectious diseases that primarily, though not exclusively, affect vulnerable populations in developing countries where poor sanitation and lack of access to health care foster disease transmission and vector proliferation (Laychiluh *et al.*, 2014). These diseases, which include diarrheal diseases, tuberculosis and typhoid causes 35,000 deaths per day in the developing world along with significant morbidity. The world emergence of *Escherichia coli*, *Klebsiella pneumoniae* and many other B-lactamase producers has become a major therapeutic problem (Haile *et al.*, 2008, Girish *et al.*, 2008).

Although the treatment of infectious diseases caused by bacteria is mainly based on the use of commercial antibiotics, however, currently due to the emergence of multi-drug resistant strains like *E.coli* and *K. pneumoniae* are widely distributed in hospitals and are increasingly being isolated from community acquired infections (Mathieu *et al.*, 2009, Naik *et al.*, 2010). Infectious diseases caused by bacteria unable to treat by the existing drugs and due to high cost of new generation antibiotics, low income people do not get equal service to these drugs (Akinsulire *et al.*, 2007). Screening of plant extracts is a good starting point for antimicrobial drug discovery (Shewit *et al.*, 2012). Higher plants remain a vital source of these new substances, especially in low resource countries (Songca *et al.*, 2013). Therefore, the aim of the present study is to indicate and evaluate the antibacterial activity of *C. macrostachyus* extracts with different extraction solvents.

MATERIALS AND METHODS

Study area, Period and type of study design

This study was conducted in Amhara Regional State, North Western Ethiopia, North Gondar Zone, at Gondar University, Department of Biotechnology (Molecular Biology Laboratory) from March 12, 2014 to May 30, 2014. The study design was experimental/interventional using appropriate method to determine antibacterial activity of *C. macrostachyus*.

Test organism and sample collection

Organisms used for this antibacterial activity tests were isolated from patient specimens visiting Gondar University

Teaching Hospital. *Staphylococcus aureus*, *Klebsiella pneumonia*, *Echerichia coli*, *Shigella flexner* and *Salmonella pneumonia* isolates and *Staphylococcus aureus* ATCC25923 reference strain were used. For this study, *C. macrostachyus* leaf part were collected and identified, voucher plant parts were stored at 4°C until used. The extracts have extracted from this leaf and tested for their antibacterial activity for the above selected bacteria isolated.

Preparation of plant extract

Plant leave materials were washed using tap water and distilled water respectively, air dried in shade at room temperature and powdered with grinder. The powdered plant materials of 84 gram of dried leaf powder was soaked separately in 504ml solvents of ethanol, methanol and chloroform for 48hrs, until complete extraction of the bioactive material achieved. At the end of 48 hrs, each extract was filtered through what man No.1 filtered paper and concentrated at room temperature. The dried extracts were stored in pre-weighed screw capped bottles and the yields of extracts have kept in refrigerator at 4°C until use. Each of 50 mg/ml concentrations of plant extracts were prepared by dissolving 500 mg/ml stock extracts and used for susceptibility test.

Susceptibility test

Microorganisms were isolated and collected separately from the sample specimen by culturing on tryptica soya agar at 37°C for 24 hrs by using streak plat method. Bacterial suspensions were prepared by taking 3-4 same well isolated colonies from tryptica soya agar and

transferred in to 4 ml Sterile Normal Saline Solution (SNSS). The suspensions turbidity was made until comparable to 0.5 McFarland standards. From these suspensions a volume of 60 µl were added on the surface of previously solidified Muller-Hinton Agar (MHA). The suspensions were uniformly distribute and streaked on MHA surface by Sterile Cotton Swab (SCS). This was achieved by rotating the Petri-plate to have equal distribution of cultures over MHA surface. On each plate, equidistance wells were made with a 6 mm diameter by sterilized borer 2 mm from the edge of the plate. Fifty micro liter of each plant extracts (50 mg/ml) was aseptically added and water as negative control was added in to separate well. This was followed by allowing the agar plate on the bench for 40 min prediffusion followed by incubation at 37°C for 24 hr. The tests were performed in triplicate. Tests that gave contradicting results were repeated again for an easy decision. The inhibition zone were compared with susceptible control strain *S. aureus* ATCC 25923, whose susceptibility pattern is known. Statistical significance (P value) was calculated by using one way analysis of variance (ANOVA).

Minimum Inhibitory Concentrations (MIC^s) and Minimum Bactericidal Concentrations (MBC^s)

The MICs of plant extracts were determined using an agar dilution method as described by (Bauer *et al.*, 1966). The tests were performed using broth dilution method. Broth containing test tubes were tightly closed, arranged in test tube rank 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 (50 mg/ml) were serially diluted in broth containing test tubes to bring 30 mg/ml, 15 mg/ml, 7.5 mg/ml and 3.75 mg/ml concentration respectively. Each of the different concentration extracts was aseptically introduced. The inhibition growths were observed after 24 hr 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. For MBC Broth containing test tubes that didn't show any bacterial growth at MIC were used. Small volumes of these broths were streaked on to the surface of tryptica soya agar medium by sterile wire loop. The medium was incubated at 37°C for 24 hr. The least concentration of plant extracts that effectively inhibit bacterial growth was recorded as MBC of extracts.

Data decoding and Data 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). Microsoft Excel was employed for analysis of MIC and MBC.

RESULT

Antibacterial susceptibility to Plant extracts

The antibacterial effects of *C. macrostachyus* crude extracts showed

effective bacterial growth inhibition against almost all tested bacterial organisms. The plant extracts showed as low 7 ± 1.00 mm to as high 32 ± 1.00 mm diameter inhibition zones (Table 1). Methanol extract has shown the least inhibition zones of (8.33 ± 1.53) mm against *E. coli* ($p=0.00$). Compared to methanol, ethanol showed slightly higher inhibition zone against all tested bacteria. Unlike methanol and ethanol extracts, chloroform extract has shown the highest inhibition zone against isolates of *K. pneumonia* (24.67 ± 0.58 mm), *S. Flexner* (30.67 ± 0.58 mm), *S. aureus* (24.33 ± 1.15 mm), *S. pneumonia* (25.33 ± 306 mm) and *S. aureus* (ST) (32 ± 1.00 mm). It also showed significantly the least growth inhibition zone (7.00 ± 1.00) against *E.coli* clinical ($p=0.00$).

Regarding methanol extracts, the highest inhibition zone (24 ± 1.73 mm) was recorded against *S. aureus* (ST). While the least inhibition was seen against *E.coli* clinical isolate (8.33 ± 1.53 mm). Similarly, the maximum inhibition zone for ethanol extract was seen against *S. aureus* (ST) (25 ± 0.00 mm) while the minimum was against *E.coli* 13.33 ± 1.53 mm.

S. aureus (ST) has revealed higher inhibition susceptibility of (32 ± 1.00 mm) for chloroform extract $p=0.00$. The minimum inhibition zone (7 ± 1.00 mm) had shown against *E.coli* isolate $p=0.00$. Compared to commercial vancomycin antibiotics, 21.67 ± 0.58 , 20 ± 0.00 , 17 ± 0.00 and 24.33 ± 1.53 mm against, against *S. pneumonia*, *S. Flexner*, *S. aureus*, *K. pneumonia* and *S. aureus* (ST) consecutively, chloroform extract showed higher inhibition value of *K. pneumonia* (24.67 ± 0.58 mm), *S.*

flexner (30.67±0.58 mm), *S. aureus* (24.33±1.15 mm), *S. pneumonia* (25.33±3.06 mm) and *S. aureus* (ST) (32±1.00mm) respectively. However, chloroform extract showed low inhibition value (7±1.00 mm) against *E.coli* than vancomycin (13.67±3.05 mm). There was no any inhibition found for all tested bacteria with negative control.

Table 1. Growth inhibition zones of solvent extracts against selected clinical isolates bacterial pathogen.

The MIC values of plant extracts against tested bacteria have shown a range of 3.75mg/ml to 30mg/ml concentrations. Most of tested bacteria have shown MIC value of 15mg/ml, followed by 30mg/ml. The MIC value of chloroform extract ranged from 3.75mg/ml to 15mg/ml. Three of test bacteria *S. aureus* (ST), *S. pneumonia* and *S. Flexner* have shown the lowest MIC value of 3.75mg/ml while *S. aureus* showed high MIC (15mg/ml). Both *E.coli* and *K. pneumonia* have showed similar MIC of 7.5mg/ml.

S#No.	Isolates	Mean Inhibition Zones of Extracts			positive and Negative control	
		Ethanol	Chloroform	Methanol	Vancomycin	Water
1.	<i>E. coli</i>	13.33±1.53 ^a	7.00±1.00 ^a	8.33±1.53 ^c	13.67±3.05 ^a	0±0.0
2.	<i>K. pneumonia</i>	20.33±1.15 ^b	24.67±0.58 ^b	23.33±3.21 ^{bc}	21.00±1.00 ^c	0±0.0
3.	<i>S. Flexner</i>	23.67±1.15 ^c	30.67±0.58 ^c	21.33±1.15 ^{bc}	20.00±0.00 ^c	0±0.0
4.	<i>S. aureus</i>	20.00±0.00 ^b	24.33±1.15 ^b	20.00±0.00 ^b	17.00±0.00 ^b	0±0.0
5.	<i>S. pneumonia</i>	23.67±1.53 ^c	25.33±3.06 ^b	20.33±1.53 ^b	21.67±0.58 ^c	0±0.0
6.	<i>S. aureus (ST)</i>	25.00±0.00 ^c	32.00±1.00 ^c	24.00±1.73 ^c	24.33±1.53 ^d	0±0.0

The above values were mean of triplicate values in the form of mean plus standard deviation and values of the same column followed by different superscript letter are statistically different (p≤0.05).

DETERMINATION OF MIC^S AND MBC^S

DETERMINATION OF MICS

The MICs and MBCs of different concentrations of methanol, ethanol and chloroform extract were assessed as shown in (Figure 1).

In methanolic extract, have shown the MIC values as least as 15mg/ml and as high as 30 mg/ml. five of the test bacteria (83.3%), *E.coli*, *S. flexner*, *S. aureus*, *S. pneumonia* and *S. aureus* (ST) have shown the lowest MIC value of 15mg/ml and only *K. pneumonia* showed the highest MIC value 30 mg/ml. correspondingly the MIC value of ethanol extract was also ranged from 7.5mg/ml to 30mg/ml. four of the tested bacteria (66.7%), *E.coli*, *K. pneumonia*, *S. flexner* and *S. pneumonia* were showed the highest MICs 30 mg/ml While *S. aureus* and *S. aureus* (ST) showed low MIC values 15mg/ml and

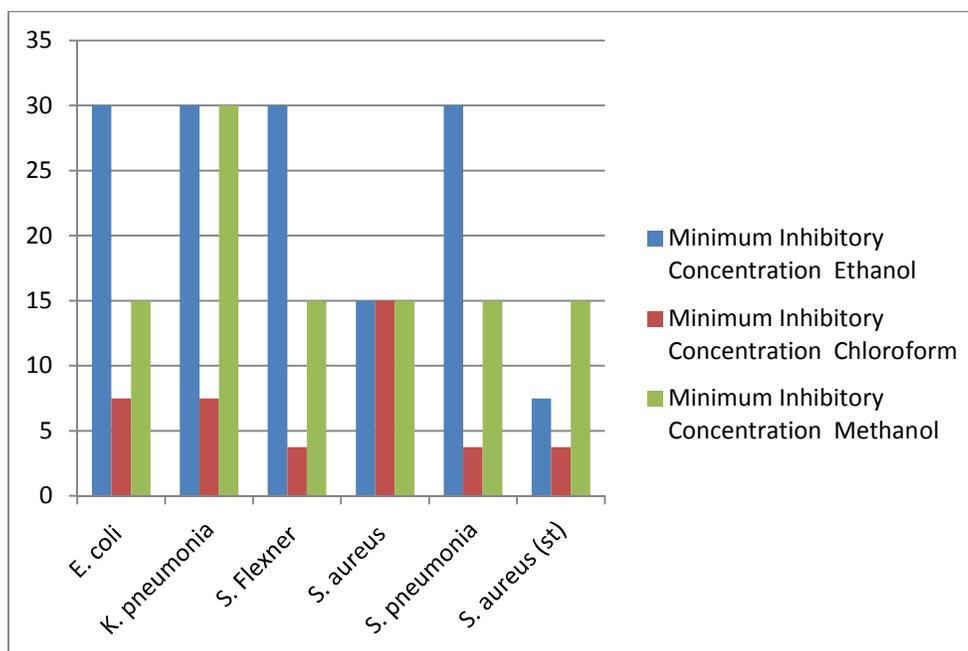


Figure 1: The MIC values of extracts against selected bacterial pathogen

7.5 mg/ml respectively. *S. aureus* clinical isolate has shown same MIC value (15 mg/ml) against all plant extracts. From the

DETERMINATION OF MBC^S

In the present study, least values of MIC Broths that didn't show any bacterial growth were used to determine MBC (Figure 2). The minimum MBC value was 7.5 mg/ml against *S. flexner*, *S. pneumonia* and *S. aureus* (ST) and maximum was 40mg/ml against *E.coli*, *S. flexner*, and *K. pneumonia*. Ethanolic extract showed the lowest MBC 15mg/ml against *S. aureus* (ST) and highest MBC value 40 mg/ml against three of tested bacteria *E.coli*, *K. pneumonia* and *S. flexner*.

MBC of 30 mg/ml, 30 mg/ml and 15mg/ml were also recorded against *S. aureus*, *S. pneumonia* and *S. aureus* (ST) respectively.

three plant extracts (chloroform, methanol and ethanol) the highest MIC value (30mg/ml) was showed by ethanolic extract.

Similarly, chloroform extract showed the lowest MBC of 7.5mg/ml. The highest MBC value 30 mg/ml has shown against *S. aureus*. The extract also showed MBC value of 15mg/ml against *E.coli* and *K. pneumonia* and lowest value of 7.5mg/ml against *S. flexner*, *S. pneumonia* and *S. aureus* (ST) respectively. Regarding methanolic extract, the highest MBC values were 40mg/ml against *K. pneumonia* and lowest 15mg/ml against *S. aureus* respectively. The MBC and MIC value were found to be similar for *S. aureus* methanol extract, while the remaining has shown different values for this test.

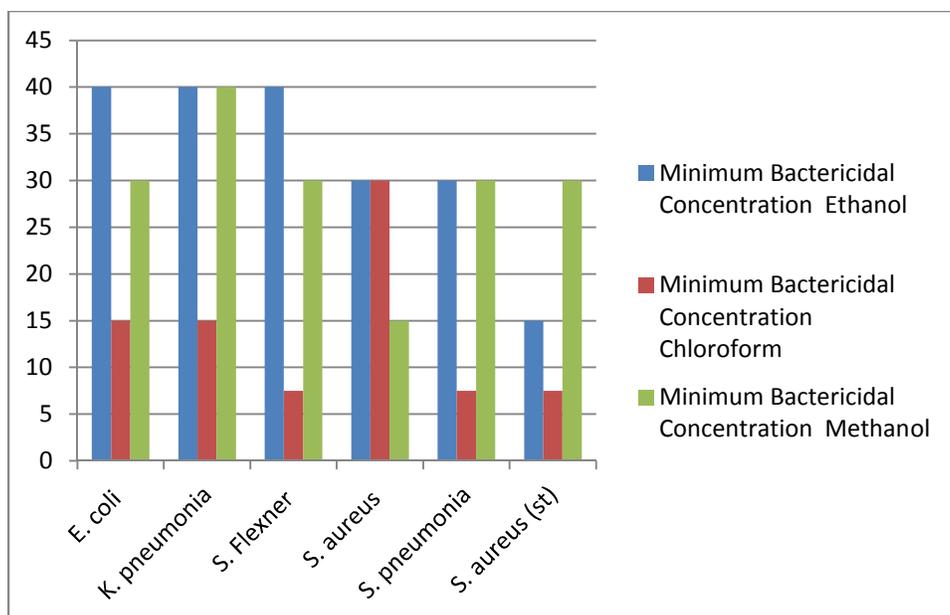


Figure 2: The MBC values of solvent extracts against tested bacterial pathogen.

DISCUSSION

Ethnobotanical investigations have been found to offer important clues in the identification and development of traditionally used medicinal plants in to modern drugs. The present study clearly indicated that ethanol, methanol and chloroform extract of *C. macrostachyus* could able to inhibit all test bacteria. However, the degree of their inhibition pattern is different this may be because of the difference in bacterial strain and the kind of solvent used.

In this study, chloroform extract has shown the lowest inhibition zone against *E.coli* organism and the highest inhibition zone was seen in *S. aureus* (ST). The observed difference in antibacterial activities of extracts between *E.coli* and *S. aureus* (ST) clinical isolate may be attributed to the difference in the outer membrane of both isolates. 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 (Duffy and Power, 2001) and appears to be less susceptible to plant extracts than gram-positive bacteria.

With compared to between methanol and ethanol, ethanol showed slightly higher inhibition zone. A similar to this study, a study of two different solvents evaluated by Amsalu *et al.*, 2011, indicated that ethanol shown the most effective solvent that significantly reduced radial growth of the pathogen compared to methanol and similarly in another plant species (*A. sativum*) reduced the radial growth of the pathogen by 83 %. The methanol extract of the plant showed the least antibacterial activity for all solvents used in this study. In another previous report the methanol extracts of other plant *A. schimperi* leaves

showed strong antibacterial activity against 10 bacterial strains (Biruhalem *et al.*, 2001). In this study, Methanolic extracts of *C. macrostachyus* showed minimum inhibition zones at the concentration of 50 mg/ml against *E.coli* in agar well diffusion method. But study in Kenya, by Cyrus *et al.*, 2009, found effective growth inhibition at a concentration of 15.6 mg/ml against *B. cereus* and 250 mg/ml against *P. Aeruginosa*. This result also disagrees with previous reports of Endale *et al.*, 2014, 1000mg/mL against multidrug resistance mycobacterium tuberculosis bacterium. This difference may be due to difference in bacterial isolates. 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. In other plant specimens of ethanol extract *A. schimperi* showed minimum inhibition zones at concentration of 125mg/ml against *S. aureus* and *P. aeruginosa* similarly (Biruhalem *et al.*, 2001). Chloroform extracts of this plant showed highest inhibition zones compared to the positive control (vancomycin) except *E.coli*. The most susceptible bacterium for all solvent extracts in study was *S. aureus* (ST) and the most resistant bacterium was clinical isolates of *E.coli*. The highest and the lowest MIC of this plant were observed in ethanol and chloroform extracts. However, *S. aureus* clinical isolate have got the same MIC for all extracts. The observed difference may be due to the difference of the nature of the

solvent and the bacteria type (clinical vs standard) that were used in this study.

Of all microorganisms used in this study, *S. aureus* (ST) had the highest susceptibility with chloroform extracts. This is an indication that the extract could be a good first line basis for drug production with high potency against infection by this bacterium. Methanolic extract MIC and MBC value were found to be similar for *S. aureus*. In this study most of tested bacteria showed MIC value of 15 mg/ml, followed by 30 mg/ml. This is an indication that most part of the bioactive compounds, which is found in this plant is non polar.

CONCLUSION

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 *C. macrostachyus* has ample potential to inhibit many human pathogenic bacteria as it were seen from its strong inhibition against tested organism. Currently due to the emergence in antibiotic resistant infections, the search for new alternative drugs to treat infections is entirely necessary and in this regard *C. macrostachyus* can give an alternative source for design of novel drugs. This study also indicated that chloroform extract of this plant has highest capability to antibacterial activity except *E.coli*, followed by ethanol and methanol extracts even than vancomycin.

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