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IN VITRO ANTIBACTERIAL ACTIVITY OF CROTON MACROSTACHYUS FOR SKIN AND WOUND INFECTION

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ABSTRACT

The aim of this study was to investigate the in vitro antimicrobial activity, to determine the minimum inhibitory concentration (MIC) of the antimicrobial activity of the agent against some aerobic and anaerobic bacteria that usually found in skin and wound infections and identify the chemical composition of the extracts of Croton macrostachyus leaves. The antimicrobial activity of the cold water, hot water, ethanol and methanol extracts of leaf of Croton macrostachyus were tested against standard strains and clinical isolates of some aerobic and anaerobic bacteria using the agar plate disc diffusion technique. The result showed that all extracts of Croton macrostachyus leaf possesses a very strong broad spectrum antimicrobial activity against two gram positive (Staphylococcus's aureus ATCC-25923 and Streptococcus pyogenes) and two gram negative (Pseudomonas aeruginos ATCC-27853, and Escherichia coli ATCC - 25922). The cold water and the ethanol extract were more potent than the hot water and ethanol extract. Moreover, cold water and methanol extracts were significantly better than the effect of penicillin against E. coli, P.aeruginos and S. pyogenes ($P \leq 0.0000051$, $P \leq 0.0013$ and $P \leq 0.012$ respectively).The minimum inhibitory concentrations (MICs) values were determined by well diffusion technique. The cold water and methanol extracts had broad spectrum activity on gram positive and negative bacteria. The highest activity was shown in S. pyogens with the MIC values of 78µg/ml and least activity on S. aureus with the MIC values of 1250µg/ml of cold water

extracts showing the extract to be antimicrobial. Qualitative phytochemical analysis was done for various phytoconstituens. Results of the phytochemical screening indicated that Croton macrostachyus leaves contained flavonoids, carbohydrate, protein, tannins, and phenols. This study provides some scientific bases for the use of this plant as a remedy for skin and wound infections whose causative agents are some of the pathogens studied.

Key words: *Antibacterial activity, Croton macrostachyus, extracts, phytochemical tests, skin and wound infection.*

Introduction

Skin and wound infections are caused by mixed bacterial flora, aerobic and anaerobic, gram negative and gram positive bacteria of the endogenous, oral, gastrointestinal and skin flora(Black,2005). Some of the skin and wound infections can be potentially life threatening and may progress rapidly (Brook 2002). Most bacterial infections of the skin and wound are caused by, S*taphylococcus aureus*, S*treptococci pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Clostridium perferingers* and *Treponema perenue*. *Staphylococcus aureus* and *Streptococcus pyogenes* are the most common pyogenic (pus forming) pathogens (Mckane and Kandel 1996). Tan et al. (1998) showed that *S. aureus* is the common organism isolated from both primary and secondary pyodermis accounting for 67% and 46.7% of them. They also demonstrated that *S. aureus* had a high rate of resistance (89.5%) to penicillin and ampicillin.

According to Stulberg et al. (2002), the common skin infections include cellulites, erysipelas, impetigo /school sores, ecthyma, folliculitis, boils/ furuncles and carbuncles, acne, yaws, and staphylococcal scalded skin syndrome. Morbidity and mortality due to skin and wound infections widely vary depending on the clinical entity, age and immune status of the individual.

Croton macrostachyus of family *Euphorbiacea* is a tree to about 16 meter tall and widespread in tropical Africa. It 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 associated with Juniperus -Podocarpus habitats and also occurs in the warmer parts of the montane rain forests and semi-tropical rain forests (Royal Botanic Gardens,2009). It is native in Ethiopia, Eritrea, Kenya, Nigeria, Tanzania and Uganda.

Croton macrostachyus was found one of the common plants to have the highest diversity of medicinal applications by three socio-cultural groups of Ethiopia, namely the Amharas,

Shinashas and Agew-Awis mainly for the treatment of malaria (Gidey et al. 2006), for the treatment of malaria, venereal diseases, cough, diabetes, constipation, tape worms and hepatitis. The dichloromethane extract of the stem bark of *Croton macrostachyus* contain a triterpenoid and a tetratrpenoid (Zelalem, 2007). Similarly, the **c**rude chloroform and n-butanol fractions of *Croton macrostachyus* were effective against clinical isolates of *N. gonorrhoeae* as evaluated *in vitro* by agar dilution method with minimum inhibitory concentrations between 250-500 µg/ml(Mesfin Tefera, 2007). Kamanyi et al. (2009) reported that oral administration of aqueous and methylene chloride/methanol extracts of the stem bark *Croton macrostachyus* at the doses of 150, 300 and 600 mg/kg possess analgesic and anti-inflammatory properties in mice and rat model indicating that *Croton macrostachyus* is a potent source of analgesic and anti-inflammatory principles against pain induced by acetic acid, formalin and pressure and inhibited acute inflammation induced by carrageenan, histamine and formalin. Nyunia et al. (2009) stated that *Croton macrostachyus* used by Luhya Community of Kenya for the treatment of sore throat, flu, TB, uvula, haemostatics, stomachache, measles, sexually transmitted diseases, dysentery, diarrhea and malaria.

Edeoga et al. (2005) explained that 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 constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds. Phytochemical screening of the medicinal plants showed the presence of flavonoids, terpenoids, saponins, tannins and reducing sugars (Ayoola et al. 2008). The properties of flavonoids are their antioxidative, vasodilatory and antimicrobial traits (Krasteva et al., 2004)

Therefore, the purpose of this study was to evaluate the antimicrobial property of this medicinal plant. The finding of the study will enable to choose the most appropriate antimicrobial chemicals used in skin and wound infections.

Materials and Methods

Collection of plant material

Fresh leaves of *Croton macrostachyus* were collected on March 02, 2011 from Ambo University Campus, in Western Showa zone, with latitude and longitude of 8°59′N 37°51′E and an elevation of 2101 meters above sea level. The study was conducted from March 2011 to June, 2011 at the laboratory of Biology department, Ambo University, Ethiopia.

Preparation of Extracts

The fresh *Croton macrostachyus* leaves were washed; sun dried and ground using electrical grinder. Forty grams of the ground leaves were separately soaked in 300ml of different solvents (petroleum- ether, chloroform, acetone, distilled water, ethanol and methanol) at room temperature for about 48 hours and filtered using Whatman No. 1 filter paper. The same amount of the ground leaves was soaked in boiled water and filtered using Whatman No. 1 filter paper. The filtrates were then transferred into bottles. The petroleum- ether, chloroform, acetone, ethanol and methanol extracts were dried using rotary vacuum evaporator below 40^{o}C and the water extracts were dried using a freeze dry system using lyophilizer. The dried extracts were then stored in labeled sterile screwed –capped bottles at 5^{O}C in air tight vials until use.

Test organisms and Reference antibiotic

Antibacterial test was carried out by agar disc and agar diffusion method adopted from Iroha et al. (2008). Antibacterial activity of the plant was tested against four species of bacteria two gram positive (*Staphylococcus's aureus ATCC-25923* and *Streptococcus pyogenes)* and two gram negative *(Pseudomonas aeruginos ATCC-27853, and Escherichia coli ATCC -25922)*. The bacteria were obtained from Ethiopian Nutrition and Health Research Institute, Addis Ababa Ethiopia. *Streptococcus pyogenes* was cultured in nutrient broth supplemented by 5% defibrinated sheep blood and other bacteria were cultured in nutrient broth for 24 hours and the fresh inoculums were taken for the test.

Reference antimicrobial susceptibility test discs of penicillin, ampicillin, tetracycline and erythromycin were OXOID product, Oxoid limited, United Kingdom.

Inoculation Procedure

The antibacterial activity of the plant extracts were evaluated by agar disc diffusion method using Mueller Hinton Agar medium modified from Tayler et al. (1995) , Panthi and Choudhory (2006). For *S. pyogenes* the Mueller Hinton agar was supplemented with 5% (v/v) sterilized sheep blood. The Mueller Hinton agar plates were spread with a 0.5 McFarland $BaSO_4$ turbidity standard of 100 µl inoculums of each test organism. The antimicrobial activity testing was done in six replications.

Preparation of Antimicrobial Discs

Each of the 0.5gm dried extracts was re-suspended in 2 ml of the original solvents and poured in to sterilize petri dishes. In each extract 40 sterilized Whatman No. 1 filter paper discs (about 6 mm diameter) were soaked in each extract. Equal number and same sized filter paper discs absorbed the same volume of extracting solvents for negative control. The discs allowed to dry and there after the discs were impregnated on spread agar plates and incubated for 20 hours at 35 ^{O}C. Penicillin, ampicillin, tetracycline and erythromycin discs were used for positive control to compare with the tested plant extracts. Microbial growth inhibitions were determined by measuring the diameter of zone of inhibitions and the mean value are presented.

Determination of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs**)** was performed by well diffusion technique modified from Iroha et al. (2009).The Mueller Hinton agar (25 ml) was poured into each sterilized petri dish. Blood was added for the test of *Streptococcus pyogenes.* The plates were seeded with 0.1 ml of the equivalent to standard McFarland No. 0.5, of indicator each bacterium cultured for 24 h. The inoculum was spread evenly over plates with glass spreader. The seeded plates were allowed to dry in the incubator at 37° C for 20 minutes. Wells of 8 mm diameter were bored on the agar plates using sterile cork borer and 100 µl of each extract was introduced in the wells. A stock solution of 5mg/ml of cold water and methanol extracts were prepared by two folds serial dilution to give 5000, 2500, 1250, 625,312, 156 and 78 µg/ml. The inoculated plates were incubated at 35 ^{O}C for 20 hours and zone of inhibition was measured using vernal caliper in millimeters

Phytochemical detection

Phytochemical screening of the leaves of *Croton macrostachyus* was carried out according to Edeoga et al. (2005) , Ayoola et al. (2008), Venkatesan et al. (2009), Hassen(2009) and Cai et al. (2011) with some modification.

Teat for flavonoids

For the water extracts, the plant samples were in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration andwas observed indicating a positive test for flavonoids.

For alcoholic extracts Shinoda test was performed. To an alcoholic solution of each of the extract, three pieces of magnesium chips were added followed by a few drops of concentrated hydrochloric acid. Appearance of a red to purple color indicates the presence of flavonoids.

Test for Proteins

Proteins were detected by Ninhydrin Test. To the 30mg /ml of the extracts 1.5% ninhydrin reagent was added and boiled for few minutes. Formations of a deep blue color the extracts showing that the plant extracts contain amino acids (proteins).

Test for Phenols

Each of 30mg of the extracts was dissolved in 1 ml methanol – aqueous solution and 2 drops of 1% ferric chloride solution were added. Formation of blue or purple color indicates the presence of phenols.

Test for tannin

To one ml of each extract solution 5 drops of acetic acids were added and then 3 drops of 1% ferric chloride reagent. The formation of brownish color indicates the presence of tannins.

To test for carbohydrate

To 30 mg/ml of the extracts, three drops of 10% diluted HCL were added to each extracts and heated for some time and neutralized by three drops of 10% sodium hydroxide solution. Then five drops of Fehling solution were added to each extracts and heated in water bath. Formation of red precipitate indicates that the extracts contain carbohydrates.

Results

The result of *in vitro* antibacterial activities of *C. macrostachyus* leaves is shown in Table 1 below. The cold water extract of *C. macrostachyus* showed the maximum zone of inhibition against the *E.coli ATCC -25922 (*22.0±2.1) which is much greater than the standard antibiotics (penicillin, ampicillin, tetracycline and erythromycin) used in this study. The methanol, ethanol and hot water extracts showed activity against gram positive and negative bacteria, where as the solvent controls did not show any activity against the microorganisms used in this study. Acetone extracts exhibits minimum activity against the four bacteria but had no significant difference compared to penicillin discs. The petroleum ether and chloroform extracts were found to have no inhibitory effect against the bacteria.

Commercial antibiotic discs (penicillin, ampicillin, tetracycline and erythromycin) were used as positive reference to determine the sensitivity of the strains.

The effect of the four extracts were significantly more effective than penicillin and ampicillin discs (P=0.0000051, 0.0013, 0.011 and 0.0016 respectively) for *E.coli, S. aurus P.aeruginosa* and *S.pyogenes.*

S. aureus ATCC-25923 was found sensitive to penicillin, ampicillin, tetracycline and erythromycin (average zone of inhibition ≥23±0.71) whereas, *E. coli ATCC -25922, P .aeruginos ATCC-27853 and S. pyogenes* were found resistant to the antibiotics(average zone of inhibition ≤ 14.5±2.12).

Table 1. Diameter of Inhibition zone (mm) of bacterial growth by cold water, hot water, methanol and ethanol extracts of leaves of *Croton macrostachyus*

Bacterial strain	Extract	Zone of Inhibition in mm		
		Experiment Mean ± STDEV	Control Mean ± STDEV	Differences between experimental and control group P-value t-test
E.coli	Cold water Extract	22.00 ± 2.10	6.33 ± 0.52	0.0000000
	Hot water extract	14.67 ±	6.33 ± 0.52	0.0000001
	Ethanol Extract	15.33 ± 1.97	6.00 ± 0.00	0.0000002
	Methanol Extract	13.33 ± 2.34	6.50 ± 0.55	0.0000193
S.aureus	Cold water Extract	18.67 ± 1.75	6.33 ± 0.52	0.0000000
	Hot water extract	10.50 ± 1.22	6.40 ± 0.55	0.0000359
	Ethanol Extract	10.83 ± 1.33	6.50 ± 0.55	0.0000118
	Methanol Extract	12.83 ± 1.33	6.33 ± 0.52	0.0000003
	Cold water Extract	14.67 ± 1.21	6.17 ± 0.41	0.0000000
	Hot water extract	12.33 ± 1.97	6.67 ± 0.52	0.0000229
	Ethanol Extract	8.67 ± 0.82	6.67 ± 0.82	0.0008546
P. aeruginosa	Methanol Extract	14.50 ± 1.87	6.33 ± 0.52	0.0000006
	Cold water	17.33 ± 5.05	7.50 ± 1.05	0.0004383

S. pyogenes	Extract			
	Hot water extract	8.83 ±0.75	7.17 ± 0.75	0.0016464
	Ethanol Extract	14.33 ±1.03	7.83 ± 0.75	0.0000650
	Methanol Extract	14.33 ± 1.03	7.67 ± 1.03	0.0000003

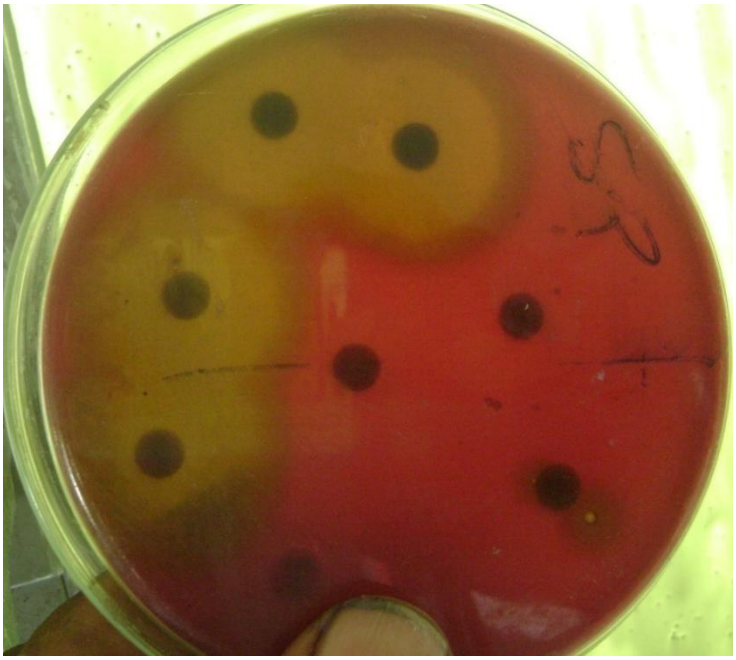

Figure1. Zone of inhibition of cold water extract in inverted view of Mueller Hinton agar (5% blood) plates inoculated with *Streptococcus pyogenes(* Left experimental groups and right are negative control groups)

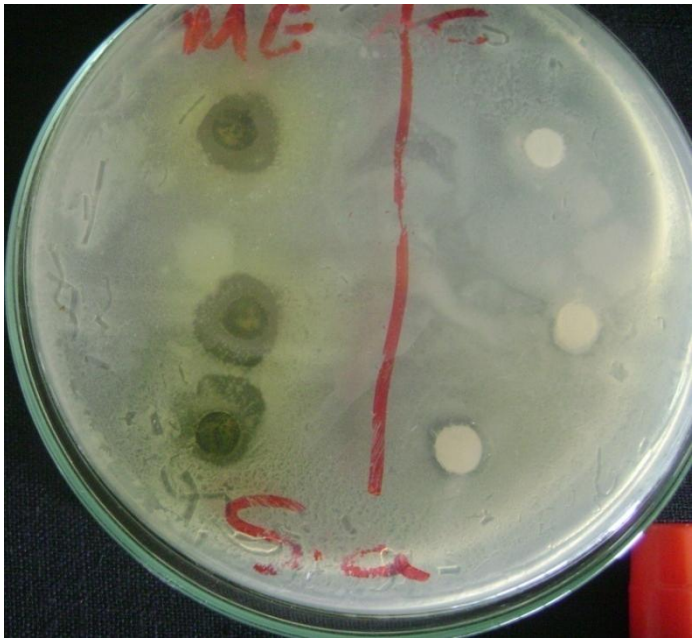

Figure2. Zone of inhibition of methanol extract in inverted view of Mueller Hinton agar plates inoculated with *Pseudomonas aeruginosa(* Left experimental groups and right are negative control groups)

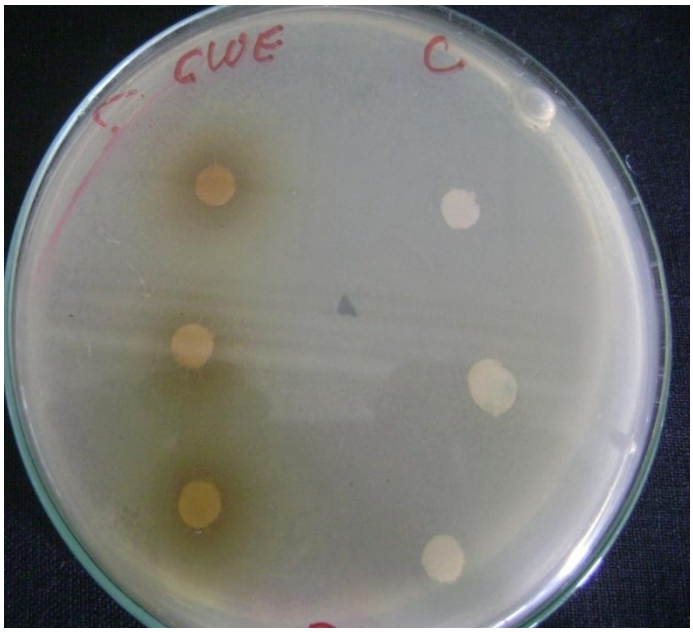

Figure 3. Zone of inhibition of cold water extract in inverted view of Mueller Hinton agar plates inoculated with *E.coli (* Left experimental groups and right are negative control groups)

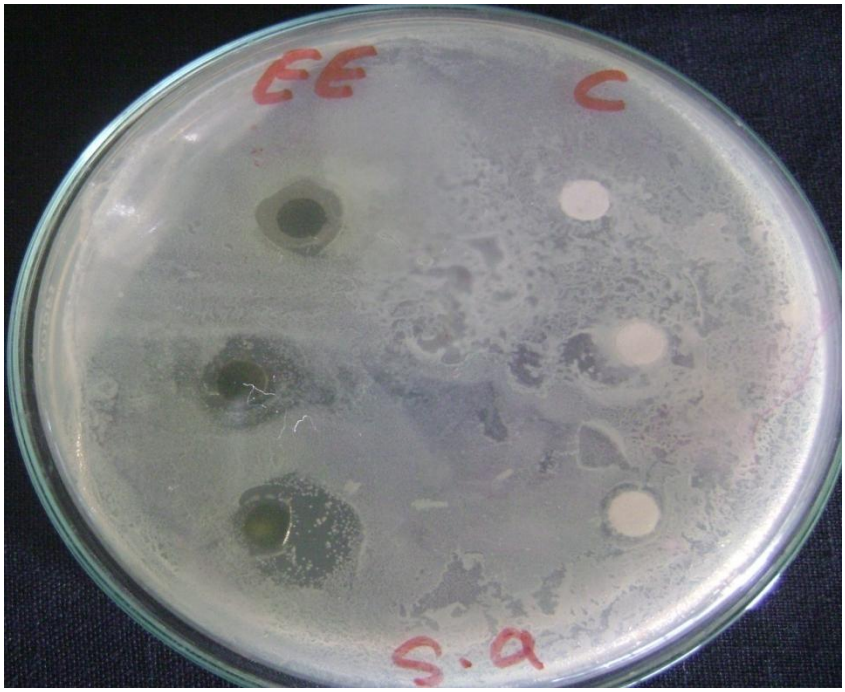

Figure4. Zone of inhibition of ethanol extracts in inverted view of Mueller Hinton agar plates inoculated with *Pseudomonas aeruginosa(* Left experimental groups and right are negative control groups)

The mean zones of inhibition of the extracts were compared with penicillin. The Cold water and methanol extracts were significantly better than the effect of penicillin against *E. coli, P.aeruginos and S. pyogenes* ($P \leq 0.000005$, $P \leq 0.001$ and $P \leq 0.01$ respectively).

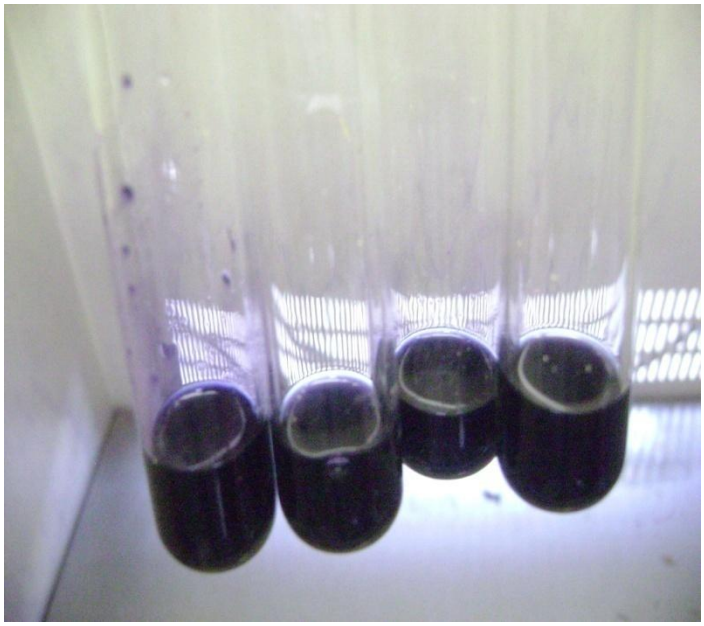

Figure 5. Ninhydrin test of cold water, hot water, methanol and ethanol extract of *Croton macrostachyus* leaves

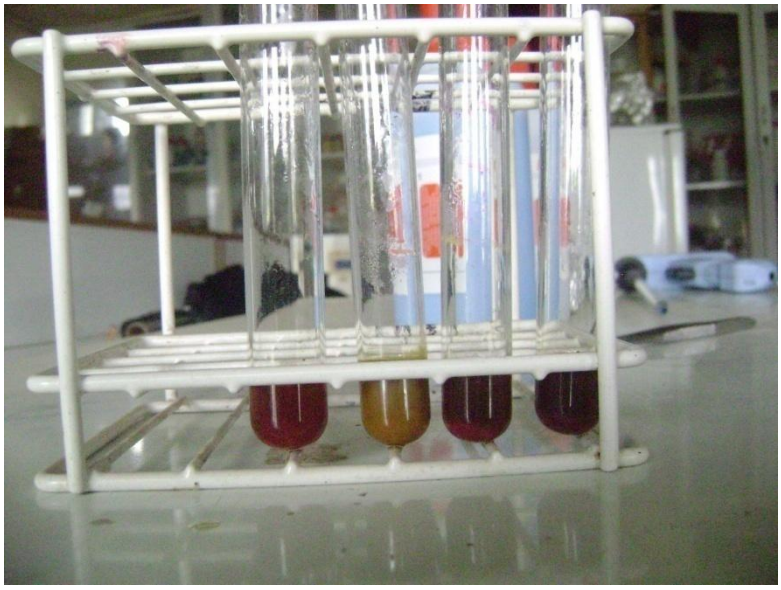

Figure 6. Fehling test of cold water, hot water, methanol and ethanol extract of *Croton macrostachyus* leaves

Table 2. Minimum Inhibitory Concentration (MIC) of cold water and methanol extracts of leaves of *Croton macrostachyus*

		Diameter of zone of inhibition in mm by different Concentration of extracts					
Test Organism	Extract	5000 µg/ml	2500 µg/ml	1250 µg/ml	625 µg/ml	312 µg/ml	78 µg/ml
E.coli	Cold water Extract	18	18	15	12	-	-
	Methanol Extract	15	14	14	10		
S.aureus	Cold water Extract	14	13	12	-	-	-
	Methanol Extract	16	15	15	14	-	-
P. aeruginosa	Cold water Extract	14	13	13	12	-	-

	Methanol Extract	15	14	12	12	-	-	
	Cold water Extract	17	16	16	14	12	12	
S. pyogenes	Methanol Extract	17	17	16	17	16	12	

Minimum inhibitory concentrations (MICs) of the cold water and methanol extracts of the leaves of *Croton macrostachyus* against the test organisms are shown in Table 2 above. The minimum inhibitory concentration of cold water and methanol extracts ranged from 78 µg/ml to 625 µg/ml .The MICs for both extracts were also similar (625 µg/ml) for the standard strain of E.coli, *S. aureus*, and *P. aeruginosa* where as the methanol extract had greater effect on the clinical isolates of *S.pyogenes.*

Table 3. Phytochemical constituents of the leaf extracts of *Croton macrostachyus*

Constituents	Cold water extract	Hot water extract	Methanol extract	Ethanol extract
Carbohydrates	+	++	+	+
Proteins	+	+	+	+
Flavoids	+	++	+	+
Phenols	+	+	+	+
Tannin	+	+	+	+
Alkaloids	-	-	+	_

* + + appreciable amount, + moderate amount, - not detectable

The qualitative phytochemical screening reveals the presence of carbohydrates, proteins, flavonoids, phenols, and tannins. Alkaloids are present in methanol extracts but absent in water and ethanol extracts. The results were shown in table 3 above.

Discussion

In this study, Petroleum ether, Chloroform, acetone, cold water, hot water, methanol and ethanol extracts of leaves of *Croton macrostachyus* were tested against two gram positive bacteria (*S. aureus* and *S. pyogenes*) and two gram negative (*P. aeruginosa* and *E. coli)* that are common etiology of skin and wound infections using the agar disc diffusion method. The cold water extracts of the medicinal plant was found to have potent antimicrobial activity against , *E. coli, S.*

aureus, S. pyogenes and *P. aeruginosa* with average inhibition zone of 22.0, 18.67, 17.33 and 14.67mm respectively. Following to cold water extract, the methanol extract had also a profound effect against the test bacteria used in this study. Acetone extracts exhibits minimum activity against the four bacteria. The petroleum ether and chloroform extracts were found to have no inhibitory effect against the bacteria. This indicates that the active ingredients of the plant compounds are polar. In comparison to cold water, methanol and ethanol extracts, the hot water extract had the least antibacterial activity. This may be the antibacterial agent is not heat resistant.

The MIC result of this study is between 78 µg/ml to 625 µg/ml. The result is in agreement to Vanden and Vlietinck(1991) who stated that in most plant extracts the compounds responsible for the biological activity are present within a range of 1 – 0.001%. Similarly, Mesfin Tefera (2007) used with minimum inhibitory concentrations between 250-500 µg / ml of crude water and alcoholic extracts of *Croton macrostachyus* against *N. gonorrhoeae*. The minimum inhibitory concentration of cold water and methanol extracts used in this study is less concentrated than used by Panthi and Chaudhary (2006). They used 1 mg/ml of antibacterial extract material against *S. aureus, P. aeruginosa* and *E coli*. Thus, it is possible to say that the methanol and cold water extracts of leaves of *C. macrostachyus* contains potent antibacterial compounds.

The diameter of the inhibition zones from methanol extracts in the concentration of 78 µg/ml was 12mm against the gram positive anaerobic bacterium, *S. pyogenes.*

Results of the phytochemical screening of *Croton macrostachyus* leaf extract as shown in Table 3 indicates that *Croton macrostachyus* leaves contained carbohydrates, proteins, phenols, tannins, flavonoids and slight alkaloids. In agreement to this Kmanyi et al. (2009) found that the *Croton macrostachyus* CH2Cl2/CH3OH and aqueous extracts contained alkaloids, phenols, terpernoids, saponins and flavonoids. The presence of these phytochemicals in *Croton macrostachyus* leaf extract is an indication that *Croton macrostachyus* has curative effects and therefore can be used as alternative medicine.

From the above results it can be concluded that water and methanol extracts of *Croton macrostachyus* leaves has great potential antibacterial compounds especially for the treatment of skin and wound infections caused by antibiotic resistant bacteria. Moreover, this study shows that *Croton macrostachyus* show much promise in the development of phytomedicines having

antimicrobial properties and the drugs derived from *Croton macrostachyus* may have the possibility of use in medicine because of their antibacterial activity.

It is therefore recommended that phytochemical substances present in *Croton macrostachyus* leaves should be isolated and purified to obtain their maximum therapeutic potentials and pharmacological assay on the plant.

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