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Comparative Study of the Antibacterial Activity of Leaves of Croton Macrostachyus and Aloe Vera

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Abstract
The antibacterial activity of the dichlomethane/methanol (1:1) leaf extracts of *Croton macrostachyus* and *Aloe vera* were compared. Antibacterial effects of crude extracts were performed using modified Kirby-Bauer disc diffusion and agar dilution techniques to determine the zone of inhibition and minimum inhibitory concentrations (MIC), respectively. The extracts were tested for the antibacterial activities against Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeraginosa*, *Escherichia coli* and *Salmonella S1-S2).* The results demonstrate that dichloromethane/methanol (1:1) leaf crude extract of *A. vera* has shown strong zone of inhibition against *S. aureus* (23±0.29mm) and *Salmonella* (20±1.26mm) compared to amoxicillin-clavulanic acid (20±0.29mm and 19±0.5mm, respectively). Leaf dichloromethane/methanol (1:1) extracts of *C. macrostachyus* sowed strong zone of inhibition against *Salmonella* (21±0.29mm) compared to Amoxicillin-clavulanic acid (20±1.26mm) and Trimethoprim (13±0.00mm). The dichloromethane/methanol (1:1) extracts of *A. vera* compared with *C. macrostachyus* demonstrated significant antibacterial activity against *S. aureus* and *Salmonella S1-S2*. These medicinal plants could be developed into affordable and safe standardized herbal products and may serve as a source of new molecules for broad-spectrum antimicrobial agents.
Keywords: Antimicrobial activity, *Aloe vera, Croton macrostachyus,* dichlomethane: methanol extract, Minimum inhibitory concentrations, Zone of inhibition

INTRODUCTION

Plants have been known and used since time immemorial to treat most of the diseases affecting human kind and animals, therefore scientists have found them to be a better choice for bioactive compounds. According to Ngule et al., (2013), about 80% of the individuals from developing countries use traditionally known plants as medicine. Medicinal plants have been studied for a longer time and found to have pronounced pharmacological uses such as antibacterial, antifungal, anti-inflammatory, anti-diabetic activity, anti-cancer and antioxidant activities. In addition they possess hepatoprotective, haemolytic, larvicidal, antihelmintic , pain relief activities (Arivoli and Tennyson, 2012, Hosahally et al., 2012).

The genus *Aloe* has been in use for several diseases, particularly connected with the digestive system; they have also been used for wounds, burns and skin problems. The term *Aloes* stand for the dried juice, which flows from transversely cut bases of its leaves. It is the best herbal answer to support the health and healing mechanisms of the body because it does not heal, rather it feeds the body's own systems in order for them to function optimally and be healthy. *Aloe* species have been used for long time in folk medicine for treatment of constipation, burns, killing bacteria, dermatitis, diseases connected with the digestive system; wounds and skin problems. The medicinal role of *Aloe* specie believed to be due to the synergistic action of different compounds constituent. Ethiopians have used traditional medicines for many centuries, the use of which has become an integral part of the different cultures where 80% of the people use medicinal plants and plant remedies selected over centuries.

In Ethiopia, *Aloe* spp. has been used in a wide range of skin and hair care products; also they form the basis of health drinks and tonics". In rural parts of the country, its mucilaginous fluid applied to cuts and wounds in order to prevent infections and bring about healing (Oda and Erena, 2017). The efficacy of *Aloe* liquid as an antibacterial agent is shown to have a wide range against Gram positive and Gram negative bacteria. The antimicrobial agents of *Aloe vera* gel was reported to effectively kill or greatly reduce or eliminate the growth of *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Propionibacterium acne, Helicobacter pylori* and *Salmonella typhi* (Rubina et al, 2009).

Croton species are among the most common traditional medicinal plants used in Africa, Asia, and South America for treatment of diabetes (Moshi et al., 2000), digestive problems (Yirga et al., 2011), malaria

(Alshawsh et al., 2009; Mesfin et al., 2009), insomnia and head-ache (Bum et al., 2012), hemorrhoids and ulcers (Antonio, 2007). The genus has been reported to have a number of biological activities for instance anti-hypertensive, anti-inflammatory, antimalarial and anti-viral (Mbiantcha et al., 2013; Prozesky et al., 2001).. In Ethiopia, people use the stem and roots of the plant for diarrhea treatment and decoction of the leaf to treat malaria (Giday et al., 2007). *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. Recent study by Sendeku et al. (2015) indicated that the methanol and ethanol extracts from leaves of *C. macrostachyus* showed antibacterial activity. According to (Agize et al., 2013), reports peoples of Dawro zone uses different parts of *Cr. macrostachyus* for the treatment of different ailments in humans and humans like bleeding, liver problem, lymph inflammation; severe abdominal cramp, ascaris parasite; blackleg; malaria, stomach ache; diarrhea; headache, snake bite and rheumatism. Therefore, this study was basically intended to conduct comparative study of the antibacterial activity of leaf crude extracts of *Aloe vera and Croton macrostachyus.*

Materials and Methods

Plant material collection

The leaves of *Croton macrostachyus* were collected from South Region, Sidama zone, Wondo Genet Woreda area located 248km from Addis Ababa. The leaves of *Aloe vera* were collected from Chuko area in Sidama zone, located 325 km from Addis Ababa**.** Both plant materials were collected from November to December, **2016** and authenticated by Reta Regassa botanist from Department of Biology, Hawassa College of Teachers Education. The voucher specimen (No.CM 001 for *Croton macrostachyus* and AV001 for *Aloe vera*) were deposited at Hawassa College of Teachers Education Herbarium for future reference.

Preparation of crude methanol extracts of plant samples

The collected specimens were chopped to reduce their size, air dried and grounded into fine powder with the help of mechanical grinder. The powder of leaf samples, *Aloe vera* 578g and *Croton macrostachyus* 623g were extracted exhaustively by cold percolation with dichloromethane/Methanol (CH_2Cl_2/CH_3OH) (1:1) three times for 24hrs while shaking at speed of 230r/min with a temperature of 28.0^{O}C. The plants samples were further extracted with methanol (100%). The extracts of the *A. vera* and *C. macrostachyus* were concentrated using rotary evaporator at 40^{o}C under reduced pressure and air dried and weighted to yield 58.4 g (10.1%) dark brown and 65.1 g (10.5) deep green crude extracts of plant samples, respectively.

Phytochemical screening tests

Preliminary phytochemical screening tests were done in leaf crude extracts of *C. macrostachyus* and *A. vera to* investigate the presence of secondary metabolites such as alkaloids, flavonoids, terpenes, saponins, tannins, phenolic compounds, and anthraquinones using standard procedures of **(**Pradeep et al, 2014).

Test for alkaloids

1mL of 1% HCl was added to 3mL of the test extract in a test tube. The mixture was heated for 20min, cooled and filtered. Then 1mL of the filtrate was tested with 0.5mL Wagner's, Hager's and Mayer's reagents. Formation of reddish brown precipitate for Dragendorff's and Wagner's reagents, yellow precipitate for Hager's and cream precipitate for Mayer's indicate the presence of alkaloids (Pradeep et al, 2014).

Test for flavonoids

Flavonoids wase determined by Mg-HCl reduction test. A piece of magnesium ribbon (powder) and 3 drops of conc. hydrochloric acid was added to 3mL of the test extract. A red coloration indicates the presence of flavonoids. Five milliliters of dilute ammonia solution was added to 5mL of the aqueous filtrate of extract followed by the addition of 1mL concentrated H_2SO_4. A yellow coloration indicates the presence of flavonoids. The yellow color has been disappeared on standing (Pradeep et al, 2014).

Test for terpenoids (Salkowski test)

About 5mL of the extract was mixed with 2mL of chloroform and then 3mL of concentrated H_2SO_4 was added. A reddish brown coloration at the interface confirms the presence of terpenes (Pradeep et al, 2014).

Test for tannins

Dried extract (0.2g) was boiled in 10mL of distilled water in a test tube and then filtered. Addition of 0.1% $FeCl_3$ solution results in a characteristic blue, blue-black, green or blue-green color which confirms the presence of tannins (Pradeep et al, 2014).

Test for saponins
Dried extract (0.2g) was boiled in 2mL of distilled water on a water bath and then filtered. A fraction of aqueous filtrate about 1mL was mixed with 2mL of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously. Formation of an emulsion confirms the presence of saponins (Pradeep et al, 2014).

Preparation of plant extracts for bacterial activity
Before conducting antibacterial activities of the crude extractions of *Aloe vera* and *Croton macrostachyus* stock solution of each crude extract was prepared by diluting 200 milligrams of *C. macrostachyus* and 256 milligrams of *A. vera* crude extracts in 2 mL of dichloromethane to prepare a concentration of 100 mg/mL and 128mg/mL respectively based on the weight of the crude extracts which are measured using analytical balance. To determine the minimum inhibitory doses of the extract, six serial dilutions were done in micro tubes to prepare a final concentration of ranging from 3.25-100mg/mL (1:100; 1:50; 1:25; 1:12.5; 1:6.25; and 1:3.25) for leaf crude extract of *C. macrostachyus* and from 4-128mg/mL (1:128; 1:64; 1:32; 1:16; 1:8 and 1:4) for leaf crude extract of *A. vera*.

Selection of bacterial strain
In this study *Staphylococcus aureus* strain *(with reference number of ATCC29465)* from gram positive bacteria and *Pseudomonas aeraginosa (with reference number of ATCC27919)* and *Escherichia coli (with reference number of ATCC927)* from gram negative bacteria were used as test bacteria to evaluate the antibacterial activity of these two selected plants. These control pathogenic organisms were obtained from South Ethiopia Regional Public Health Laboratory. The same types of bacteria were also obtained from patient samples admitted to Hawassa University Comprehensive and Specialized Hospital to detect the effect of plant products. *Salmonella*, a gram negative bacteria was also used in this study. It is also obtained from patients stool culture.

Preparation of culture media for agar dilution method
Muller Hinton agar was prepared by weighing powder using a sensitive analytical balance and dissolved in distilled water, boiled on hot plate until it dissolved completely and sterilized by using Autoclave. Appropriate amount of different dilutions of the plant extracts were added to the prepared agar and distributed to the petridish. After drying the mixture of the media at room temperature in petridish, control organisms (*Staphylococcus aureus, Pseudomonas species* and *Escherichia coli*) and same type of organisms isolated from patients of Hawassa University Comprehensive and specialized hospital using a method of culturing and biochemical testing, were spread on the media which contain different concentrations of both plant extracts and incubated at 37 ºC for 48 hrs.

Preparation of bacterial suspension and determination minimum inhibitory concentration (MIC)
The minimum inhibitory concentration is defined as the lowest concentration which can inhibit any visible bacterial and fungal growth on the culture plates (Shahidi, 2004). The minimal inhibitory concentration (MIC) value of the plant extracts (*Croton macrostachyus* and *Aloe vera*) was determined using various dilutions. A standardized inoculums size of the study organisms were prepared from controls and organisms isolated from patients by sticking using sterile wire loop 3-5 single colonies of the organisms from subculture media into nutrient broth and adjusted the turbidity standard with 0.5 McFarland through observation. The concentration of the inoculums was taken as 0.5 McFarland's standards (Baker et al, 1983). The McFarland standard was prepared by dissolving 0.5 g of $BaCl_2$ in 50 ml of water to obtain a 1% solution of Barium chloride (w/v) and then mixed with 99.5 ml of 1% sulphuric acid solution. Organisms which were adjusted with the standard were transferred into the Muller Hinton agar media which is prepared based on the manufactures instruction and finally the antibacterial activity of the selected plant extracts with different dilutions were determined by observing the growth of bacteria. The lowest concentration at which there was no turbidity was considered as MIC value of the plant crude extracts (Sambrook and Russel, 2001).

Determination of antibacterial activity (zone of inhibition) using agar disc diffusion method
Each of the 0.5gm dried extracts was re-suspended in 2 ml of the original solvents and poured in to sterilize petri dishes. Amoxicillin-clavulanic acid and Trimethoprim were used for positive control to compare with the tested plant extracts. Whatman filter paper No. 1 was used to prepare discs which are approximately 6 mm in diameter. The discs were placed in a petridish and sterilized in a hot air oven. The loop used for delivering the antibiotics was made of 20 gauge wire and has a diameter of 2mm. The discs were placed in a sterile condition with in the sterilized petridish. Amoxicillin-clavulanic acid with a dose of 20 µg and Trimethoprim with a dose of 5 µg were used as standard antibiotic drug (antimicrobial agents-positive control) (CLSI, 2017). The prepared discs were

impregnated with diluted antibiotic solutions and placed on the surface of Muler Hinton Agar using a sterile pair of forceps. The plates were then incubated aerobically at 37°C for 48 hours. The diameter of the zone of inhibition produced by each concentrations of the plants extracts and that of the antibiotic disc was measured using a ruler after 48 hrs and recorded accordingly (CLSI, 2006) and the mean value are recorded.

Results

Phytochemical screening tests

Phytochemical screening tests were carried out to detect the constituents present in *C. macrostachyus* leaf crude extract. Metabolites such as alkaloids, flavonoids, tannins, saponins, terpenoids and phenols were detected however anthraquinones were not detected in the extract (table 1).

Table 1. Phytochemical Observation Inferences

Phytochemical	Observation	Inferences
Tannins	Dark-green color	Presence of tannins
Saponins	Stable foam/frothing	Presence of saponins
Flavonoids	Yellow color decolorizes	Presence of flavonoids
Terpenoids	Reddish brown color	Presence of terpenoids
Alkaloids	Precipitation was observed	Presence of alkaloids
Phenols	Blue-green color observed	Presence of phenols
Anthroquinones	No violet/pink color	Absence of anthroquinones

Phytochemical screening tests in leaf crude extract of *Aloe vera*

Phytochemical screening test was also carried out to detect the constituents present in *A. vera* leaf crude extract. Metabolites such as alkaloids, flavonoids, tannins, saponins, terpenoids, phenols and anthraquinones were detected (table 2).

Table 2. Phytochemical Observation Inferences

Phytochemical	Observation	Inferences
Tannins	Dark-green color	Presence of tannins
Saponins	Stable frothing	Presence of saponins
Flavonoids	Yellow color disappear	Presence of flavonoids
Terpenoids	Reddish brown color	Presence of terpenoids
Alkaloids	Precipitation was observed	Presence of alkaloids
Phenols	Blue-green color observed	Presence of phenols
Anthroquinones	Violet/pink color was observed	Presence of anthroquinon

Minimum inhibition concentration (MIC) of the plant crude extracts

The minimal inhibitory concentration (MIC) value of the plant crude extract of was determined using agar dilution method. The plant crude extracts of *C. macrostachyus* has shown few inhibition effects on bacterial organisms isolated from patients admitted to Hawassa University Comprehensive and Specialized Hospital at the concentration of 25mg/dl on the other hand no inhibition effect of the plant crude extract has been seen on bacterial strains obtained from Regional Public Health Laboratory. The plant crude extracts of *A. vera* has shown few inhibition effects on bacterial strains obtained from Regional Public Health Laboratory at the concentration of 64mg/ mL and on bacterial organisms isolated from patients admitted to Hawassa University Comprehensive and Specialized Hospital at the concentration of 32mg/ml (table 3).

Table 3. Minimum inhibition concentration (MIC) of the plant crude extracts

Organisms	Minimum inhibition concentration (MIC) in mg/mL		
		C. macrostachyus	*A. vera*
Escherichia coli	Control organisms	NI	64
	Isolated from patients	25	32
Staphylococcus aureus	Control organisms	NI	64
	Isolated from patients	25	32
Pseudomonas aeraginosa	Control organisms	NI	NI
	Isolated from patients	25	NI

NI = No inhibition

The experiment was performed in triplicates and the interpretation of antibacterial properties was conducted according to standard protocols (Adithepchaikarn et al., 2008). Inhibition zones >15mm were categorized as

strong activity, from 10-15mm as moderate activity and <10mm as weak activity (table 4). Results obtained from the present study showed that *Aloe vera* leaf crude extracts exhibited high zone of inhibition against *S. aureus* (20±1mm) and on *Salmonella* (19±0.76mm) whereas moderate zone of inhibition against *E.coli* (15±1mm) and low against *P. aeruginosa* (11±1.53mm). The crude extract of *C. macrostachyus* showed high zone of inhibition in *Salmonella* (19±0.5mm), moderate zone of inhibition in *S. aureus* (15±0.29mm) and *P. aeruginosa* (14±0.58mm) and low in *E.coli* (10±0.38mm).

Table 4. Antibacterial activities of dichloromethane/methanol (1:1) crude extracts (100µg/mL), and antibiotics (Amoxicillin–clavulanic acid, 20µg/mL and Trimethoprim, 5µg/ml) against control organisms tested by agar diffusion assay

Bacterial Species (control organisms)	Zone of inhibition in mm*			
	C. macrostachyus	*A. vera*	Amoxicillin-clavulanic acid	Trimethoprim
Escherichia coli	10±0.38	15±1	18±0.76	15±0.29
Staphylococcus aureus	15±0.29	20±1	20±0.58	16±0.58
Pseudomonas aeruginosa	14±0.58	11±1.53	19±0.29	14±0.5
Salmonella S1-S2	19±0.5	19±0.76	16±0.76	13±0.29

*Values are mean inhibition zone (mm) ± S.D (standard deviation) of the three replicates, inhibition zone diameter in mm, including disc diameter (6 mm), and test medium: Muler Hinton Agar.

Antibacterial activities of the plant crude extracts were also performed against bacteria isolated from infected patients admitted to Hawassa University Comprehensive Specialized Hospital. In this study standard antibiotics were also used as a positive control and methanol as a negative control (table 5).

Table 5. Antibacterial activities of dichloromethane/methanol (1:1) extracts (100µg/mL), and antibiotics (Amoxicillin–clavulanic acid, 20 µg/ml and Trimethoprim, 5µg/ml) against organisms isolated from patients.

Bacterial Species isolated from patients	**Zone of inhibition in mm***			
	C. macrostachyus	*Aloe vera*	Amoxicillin –clavulanic acid	Trimethoprim
Escherichia Coli	15±0.58	16±0.29	19±0.5	15±0.87
Staphylococcus aureus	17±0.29	23±0.29	20±0.29	12±0.58
Pseudomonas aeruginosa	12±0.00	13±0.58	19±0.76	15±0.29
Salmonella S1-S2	21±0.29	20±1.26	17±0.76	13±0.00

*Values are mean inhibition zone (mm) ± S.D (standard deviation) of the three replicates, inhibition zone diameter in mm, including disc diameter (6 mm), and test medium: Muler Hinton Agar

The results of the findings demonstrate that dichloromethane/methanol (1:1) leaf crude extracts of *A. vera* have shown strong zone of inhibition in *S. aureus* (23±0.29mm), *Salmonella* (20±1.26mm), and *E. coli* (16±0.29mm) compared to positive controls (20±0.29mm and 17±0.76mm for Amoxicillin-clavulanic acid, respectively). Leaf crude extracts of *C. macrostachyus* have presented high zone of inhibition against *Salmonella* (21±0.29mm) and *S. aureus* (17±0.29mm) compared to 20±1.26mm and 13±0.00mm zone of inhibition for Amoxicillin-clavulanic acid and Trimethoprim, respectively. The results obtained from the present study on antibacterial activities of the crude extracts using agar diffusion method revealed that the plants (*C. macrostachyus* and *A. vera*) exhibited potential antibacterial activity against all bacterial species in parallel with the standard drugs. However the plant extracts have demonstrated different antibacterial activities against tested organisms. *A. vera* compare to *C. macrostachyus* demonstrated significant antibacterial activity against *S. aureus* and *Salmonella S1-S2*.

Discussion

The use of medicinal plants as traditional medicines in the treatment of bacterial and other numerous human diseases has been demonstrated in many research works. In this study, the antibacterial activity of dichloromethane/methanol (1:1) *C. macrostachyus* and *A. vera* leaf crude extracts were compared using the agar disc diffusion method against gram positive bacteria (*S. aureus*) and gram negative (*P. aeruginosa* and *E. coli)* that are common etiology of skin and wound infections. The findings revealed that *Aloe vera* crude extracts exhibited high zone of inhibition against *S. aureus* (20±1mm) and *Salmonella* (19±0.76mm) and moderate zone of inhibition against *E. coli* (15±1mm) and *P. aeruginosa* (11±1.53mm) whereas *Croton macrostachyus* leaf extracts showed high zone of inhibition against *Salmonella S1-S2* (19±0.5mm) and *S. aureus* (17±0.29mm) and moderate zone of inhibition against *E.coli* (10±0.38mm).

In this study, the dichloromethane/methanol (1:1) leaves extracts of *A. vera* demonstrated significant antibacterial activity against *S. aureus* and *Salmonella S1-S2*compared to *C. macrostachyus*. High antibacterial activity of *Aloe ve*ra compare with *Croton macrostachyus* against *S. aureus* and *E. coli* might be attributed to the presence of anthraquinones which are absent in *Croton macrostachyus*. *A vera* can promote wound healing due

to the presence of these anthraquinones which possess antibacterial, antifungal and antiviral activities. The high antibacterial activity of the dichloromethane/methanol (1:1) leaves extracts of *C. macrostachyus* against *Salmonella S1-S2* compared to positive controls (Amoxicillin-clavulanic acid and Trimethoprim) can also be taken as a promising findings to further examine this plant to treat *Salmonella S1-S2* infections.

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