

In-vitro antibacterial activity and phytochemical analysis of leaf and stem bark crude extracts of *Croton macrostachyus* against selected pathogens, Wolaita Sodo, South Ethiopia

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Abstract

Background: Medicinal plants have long been used as antiseptic, antiallergic, antibacterial, and antiviral medicines to treat human ailments. *Croton macrostachyus* is one of the important medicinal plants that take an essential part in traditional African medicine, widely used to treat various disorders including diabetes, malaria, and dysentery.

Objective: The present research aimed to determine the in-vitro antibacterial activity of ethanol, methanol, and ethyl acetate extracts of *C. macrostachyus* leaf and stem barks against some selected human bacterial pathogens.

Methods: The leaf and stem barks extracts of *C. macrostachyus* were prepared by by maceration method using three different solvents such as ethanol, methanol, and ethyl acetate. The prepared crude extract was used to determine the antibacterial activity, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) using clinical laboratory standards institute guidelines. Qualitative phytochemical analyses were also performed using the standard protocol.

Results: The ethanolic, methanolic, and ethyl acetate crude leaf and stem bark extracts showed inhibitory actions against *E. coli*, *P. aeruginosa* and *S. aureus*. The gram-positive *S. aureus* was more susceptible to all the crude extracts. The inhibitory activity was concentration-dependent and the leaf crude extracts showed higher activity than stem bark extracts. The methanolic leaf crude extract showed the highest inhibitory activity when compared to ethanol and ethyl acetate. The MIC of methanolic, ethanolic and ethyl acetate crude leaf extracts ranged from 0.625-2.25 mg/ml against selected bacterial pathogens. The MBC results revealed that the methanolic, ethanolic, and ethyl acetate crude leaf extracts showed 1.25-5 mg/ml concentrations against selected bacterial pathogens. The qualitative phytochemical analysis results confirmed the presence of tannins, alkaloids, saponins, flavonoids, glycosides, and phenolic compounds in both ethanolic and methanolic crude leaf extracts and except alkaloids in the ethyl acetate crude leaf extracts. The phytochemical qualitative analysis of stem bark crude extracts results indicated that glycosides were present, alkaloids, flavonoids and phenolic compounds were absent in all three solvent extraction systems, tannins, and saponins were positive with ethanol and ethyl acetate, negative with methanol solvent extractions.

Conclusion: The present study concluded that crude leaf extracts showed better antibacterial activity than stem bark extracts against selected pathogens and phytochemical analysis of crude leaf extracts confirmed the presence of phytochemical constituents in the crude extracts prepared from *C. macrostachyus* and thus, supports the traditional usage of the plant against various diseases.

Introduction

Medicinal Plants are a source of naturally occurring nutrients and biologically active substances produced during their growth and accumulated from the environment. many medicinal plants are consumed as food and have been used in traditional medicine in a variety of therapeutic preparations,

also as medicinal resources [1]. Traditional medicine is described as the sum of all practice and knowledge whether or not it can be explained, employed in the diagnosis, prevention, and removal of physical, mental, or social imbalances and only depending on practical experience learning and observation passed down from verbally or in writing, through generation to generation [2].

In Ethiopia, up to 80% of people seek treatment through traditional medicine. The major reasons why medicinal plants are needed in Ethiopia are due to cultural traditions, community trust in traditional medicine's therapeutic benefits, accessibility issues to modern healthcare facilities, and their relative affordability. It's a common misconception that because medicinal plants are pure and natural, this equates to being harmless. Users of traditional medicines believe they are safe for human nutrition due to their long history of use. However, the absence of their regulation offers no such guarantee [3]. There is a wide variety of plants that are used by traditional healers [4].

C. macrostachyus is commonly known as a rush foil (English) and Bisana (Amharic). The name of Croton comes from the Greek word Kroton which means ticks because the seeds resemble ticks. The specific name *macrostachyus* is a contraction of two words, the Greek word *macro* meaning large and *stachyus* relating to the spike, hence, a species characterized by large spikes [5]. It is a deciduous, pioneer tree that belongs to the Euphorbiaceae family which has 300 genera and 8,000 to 10,000 species and most abundant plant in the tropics. It is native to Ethiopia, Eritrea, Kenya, Tanzania, Uganda, and Nigeria. It has a variety of medical uses, including the treatment of gastrointestinal diseases, cancer, typhoid, pneumonia, gonorrhea, malaria, rabies, wounds, diarrhea, hepatitis, jaundice, scabies, toothaches, and stomach pain. It is also utilized in ethno-veterinary medicine [6].

The rapid emergence of resistant bacteria is a worldwide issue endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives [7]. Many decades after the first patients were treated with antibiotics, bacterial infections have again become a threat [8]. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industries due to reduced economic incentives and challenging regulatory requirements [9]. However, many medicinal plants have been recognized as valuable resources of natural antimicrobial compounds as alternatives that can potentially be effective in the treatment of these problematic bacterial infections the Global prevalence of infectious diseases caused by bacteria is a major public health problem [10].

The bacterial agents include *S. aureus*, *E. coli*, *P. aeruginosa*, etc. cause several human infections [11]. The recent emergence of antibiotic resistance and related toxicity issues limit the use of antimicrobial agents [12] and is prompting a revival in research on the antimicrobial role of plants against resistant strains due to comparable safety and efficacy [13, 14, 15]. Medicinal plants would be the best source to obtain a variety of drugs. Therefore, the present study mainly focused on in-vitro antibacterial activities of leaf and stem bark extracts of *C. macrostachyus* against human bacterial pathogens.

Materials and methods

Description of the study area

The study area “Larena” is found partly in Sodo Zuriya and partly in Humbo Woredas, which are part of the sixteen Woredas in the Wolaita Zone (Fig. 1). Geographically, the area lies at a latitude and longitude of 6°45'13.22"N and 37°45'17.12"E, respectively, and an elevation of about 1780m amsl. It is found at a distance of 390 km (to the south) from Addis Ababa via the Addis Ababa-Shashemene-Wolaita Sodo route, or 336km (to the south) from Addis Ababa via the Addis Ababa-Hosana-Wolaita Sodo route. It is 10 km to the South of Sodo town. The annual average temperature ranges from 25-35°C. The climate of the area is sub-humid with relatively high rainfall. According to the Ethiopian traditional way of climate zoning, it can be grouped as Weina Dega (Subtropical) highland climate.

Study design

An in vitro experimental study was conducted at Wolaita Sodo University, Ethiopia from January 2022 to April 2023. The leaf and stem bark plants were collected and the antibacterial activity of the plants was conducted in the Post Graduate Microbiology Laboratory, Department of Biology, Wolaita Sodo University. Antibacterial activities were determined by using agar well diffusion, minimum inhibitor concentrations, and minimum bactericidal concentrations (broth dilution) method. The extraction of plants was done by maceration methods using three different solvents; ethanol, methanol, and ethyl acetate. Negative control 2.5% DMSO and positive control Tetracycline (30 µg) were used to monitor the antibacterial activities of stem bark and leaf extracts of plants against human bacterial pathogens.

Collection of Plant samples and Authentication

The fresh plant leaf and stem bark samples of *C. macrostachyus* were collected in plastic bags and transported to the Department of Biology, Wolaita Sodo University. The little bit collected plant specimens were used for authentication by Dr. Melaku Wendaferash a botanist in the Department of Plant Biology, at Addis Ababa University, Addis Ababa, Ethiopia. The voucher specimens were deposited to the National Herbarium of Ethiopia with Code No. (GY001).

Preparation of Crude Extract

Plant extracts were prepared in accordance with methods described by Basri and Fan [16]. Briefly, the dried leaf and stem bark samples were ground to a fine powder using an electrical grinder. The powder was stored in an airtight container and kept in the refrigerator at 4 °C until used for further processing. The crude extracts of leaf and stem bark were prepared by using three different solvents such as Ethanol, ethyl acetate, and methanol. For the preparation of crude extracts, 80 grams of leaf and stem bark powdered was mixed with 400ml of solvent individually. All the flasks were marked properly with the name of the solvent and type of plant materials. The flasks containing the mixture were wrapped in aluminum foil to avoid contamination [17]. All the flasks were incubated under a shaker incubator at 170 rpm for 72 hrs. The resulting mixture after the incubation period was filtered with Whatman No1 and air-

dried for seven days. The resultant dried extracts were considered as crude extracts. The yield of the crude extracts was calculated and preserved with 10ml of DMSO at 4°C for further analysis [18. 19. 20].

Qualitative phytochemical screening

Qualitative phytochemical analysis of crude extract of *C. macrostachyus* was carried out to determine the presence of secondary metabolites such as alkaloids, glycosides, flavonoids, tannins, saponins, and phenolic compounds using standard protocols as described below.

Test for tannins:

In this test, 0.5 grams of powdered crude extract was added to the test tube containing 10ml of distilled water and mixed well. Then, the mixer was boiled and filtered in a hot condition. A few drops of 0.1% ferric chloride (FeCl_3) were added into the test tube containing filtered extract and observed for the color changes. The development of brownish green or a blue-black color in the test tube indicates the presence of tannins [20, 21].

Test for alkaloids:

In this test, about 0.5 grams of powdered crude extract was boiled with 10 ml of prepared acid alcohol mixer for 2 mins and the boiled crude extract solution was filtered after cooling. 5ml of filtrate was mixed with 2ml of diluted ammonia followed by chloroform 5ml and mixed gently. The resulting filtrate was mixed with Mayer's reagent as described by Adachukwu et al. [22]. The formation of a yellow-colored creamy precipitation with Mayer's reagent confirms the presence of alkaloids.

Test for saponin by Foam test:

In this method, 0.5gm of culture filtrate was mixed with 10 ml distilled in a test tube and shaken vigorously for 2 minutes. The formation of a foam persistent for 30 mins confirmed the presence of saponins [20, 21].

Test for flavonoids:

In this method, 2 ml of crude extract was mixed with a few drops of 10% Sodium hydroxide solution and incubated in a hot water bath for 10 minutes. The appearance of yellow color was changed into colorless after the addition of a few drops of Hydrochloric acid at 1% concentration indicating the presence of flavonoids [20, 22].

Test for Glycosides:

In this test, 2 ml of crude extract was mixed with 1 ml of glacial acetic acid and a few drops of ferric chloride. Finally, 1 ml of concentrated sulphuric acid was added slowly to the mixer. The appearance of a brown ring at the interface indicated the presence of glycosides [23].

Test for Phenols

In this test, 2 ml of crude extract was mixed with 5 ml of diluted ammonia and observed for color appearance. The appearance of a bluish-green color, a minute after the addition of diluted ammonia indicated the presence of the phenols [24].

Collection of bacterial culture and inoculum preparation

The American type culture collection bacterial strains such as *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* (ATCC27853), and *Staphylococcus aureus* (ATCC25923) were obtained from Ethiopia Biodiversity Institute, Addis Abeba, Ethiopia. All the above-mentioned cultures were checked properly for their purity and viability before use.

The loop full of purified bacterial colony was transferred into a test tube containing sterilized normal solution and standardized with sterile saline to a turbidity equivalent to 0.5 McFarland using a UV Visible spectrophotometer at 600nm according to the guidance of Roopashree *et al.* [25].

Different concentrations of crude extract preparation by serial dilution method

A Stock Solution at 200 mg/ml was prepared by reconstituting 1 g of each of the extracts in 5 mL of DMSO (Dimethyl Sulfoxide). Three sterilized test tubes with 2.5 ml of DMSO were arranged on a test tube rack and transferred 2.5 ml of crude extract stock solution to the first test tube and then successive two-fold serial dilutions were carried out to reach 100, 50, and 25 mg/ml concentrations. These three different concentrations of crude extracts were used to determine the antibacterial activity by well diffusion assay [20].

Another stock solution of crude extract at 20 mg/ml was prepared by reconstituting 0.1 g of each of the dried crude powder in 5 mL of DMSO. The prepared stock solution was used to prepare different concentrations of working solutions such as 10, 5, 2.5, 1.25, and 0.625 mg/ml using two-fold serial dilution [26].

Antibacterial activity of crude leaf and stem bark extracts of *C. macrostachyus*

Antibacterial activity of crude leaf and stem bark extracts was determined by agar well diffusion assay as described by Schillinger and Lucke [27] and Denebo *et al.* [20]. The sterilized Mueller Hinton agar (MHA) was transferred to the sterilized Petri dishes and allowed to solidify. After the solidification, perfect 6 mm diameter wells were prepared and 0.1 ml (10^{-7} CFU/mL) of the selected bacterial cultures were inoculated on the MHA. All the wells were filled with 50µl of the prepared crude leaf and stem bark extracts at different concentrations such as 100, 50, and 25 mg/ml. The antibiotic tetracycline (30 µg/disc) was used as a positive control, and 50 µL of DMSO solution was considered as a negative control. All the plates were incubated at 37°C for 24 h and then examined for the zone of inhibitions [28, 19, 20].

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The crude leaf and stem bark extracts at different concentrations such as 10, 5, 2.5, 1.25, and 0.625 mg/ml were used to determine the minimum inhibitory concentration (MIC) by broth dilution method [29, 30, 20], and minimum bactericidal concentration by re-culturing methods [31].

Data Analysis

The mean $\pm$ standard deviation (SD) values of the *C. macrostachyus* were compared using descriptive statistics. All statistical analyses (mean and standard deviation) were performed using the Statistical Package of Social Sciences (SPSS) version 20. Differences were considered statistically significant at a p-value of less than 0.05.

Results

The Yield of the crude leaf and stem bark extracts of *C. macrostachyus*

The yield of crude leaf and stem bark extracts of *Croton macrostachyus* is presented in Table 1. According to the data in Table 1, the high yield of methanolic leaf extract of *C. macrostachyus* was 2.1% (1.89 gm) on dry weight bases followed by ethanolic crude leaf extract and ethyl acetate crude leaf extract with a percentage value of 2.1% (1.74 gm) and 1.2% (0.97gm) respectively. Similarly, the methanolic extract of the stem bark of the experimental plant showed a high yield percentage (4.1%), and the lowest yield was obtained from the ethyl acetate stem bark crude extract (3.1%).

Table 1
Crude extract yield of *Croton macrostachyus*

S.No.	Name of the Solvent	Crude extract yield of <i>C. macrostachyus</i>			
		Yield of leaf crude extract (gm)	Yield of leaf crude extract (%)	Yield of stem bark crude extract (gm)	Yield of stem bark crude extract (%)
1.	Ethanol	1.74	2.1	3.19	3.9
2.	Methanol	1.89	2.3	3.33	4.1
3.	Ethyl Acetate	0.97	1.2	2.54	3.1

Qualitative phytochemical analysis of crude leaf and stem bark extracts of *C. macrostachyus*

The data presented in Table 2 shows the qualitative phytochemical analysis of the crude leaf and stem bark extracts of *C. macrostachyus* prepared by using three different solvent systems. The presented results in Table 2 reveal that the methanolic and ethanolic crude leaf extract was rich in various kinds of phytochemicals such as tannins, alkaloids, saponins, flavonoids, glycosides, and phenolics. The ethyl

acetate crude leaf extract contains most of the phytoconstituents except alkaloids. The phytochemical investigation of stem bark crude extracts showed that the phytochemicals such as alkaloids and flavonoids were absent. The phytochemicals such as tannins, saponins, and phenolics were positive in ethanolic and methanolic crude stem bark extracts. Most of the phytochemicals were absent in ethyl acetate crude stem bark extract (Table 2).

Table 2
Qualitative phytochemical analysis of crude leaf and stem bark extracts of *C. macrostachyus*

S. No.	Phytochemicals	Leaf crude extract			Stem bark extract		
		Ethanol	Methanol	Ethyl acetate	Ethanol	Methanol	Ethyl acetate
1	Tannins	+	+	+	+	+	-
2	Alkaloids	+	+	-	-	-	-
3	Saponins	+	+	+	+	+	-
4	Flavonoids	+	+	+	-	-	-
5	Glycosides	+	+	+	+	+	+
6	Phenolic	+	+	+	+	+	-

The antibacterial activity of crude leaf extracts of *C. macrostachyus* against some selected pathogenic bacteria

The crude leaf extracts of the experimental plant *C. macrostachyus* exhibited diverse inhibition activity against the selected ATCC bacterial pathogens (Table 3, 4, 5); and the results were expressed in the form of a millimeter (mm) in diameter of the zone of bacterial growth inhibition. The data presented in Tables 3, 4, and 5 clearly showed that the selected bacterial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were susceptible to the ethanolic, methanolic, and ethyl acetate crude leaf extracts. However, there were significant differences ($p < 0.05$) in the mean diameter zone of inhibition between the extractions as well as between the bacterial pathogens.

In terms of ethanolic crude leaf extract, 100mg/ml concentration produced a higher zone of inhibitions against all the three selected bacterial pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* with 18.17 ± 0.65 mm, 20.07 ± 0.22 mm and 22.00 ± 1.33 mm respectively. The zone of inhibition produced by the ethanolic crude leaf extracts at 100 mg/ml was nearly similar to the zone developed by the positive control (tetracycline) against *P. aeruginosa* and *S. aureus* (Table 3).

Table 3

The antibacterial activities of ethanolic crude leaf extracts of *C. macrostachyus* against some selected pathogenic bacteria

Name of pathogenic Bacteria	Ethanolic crude leaf extracts of <i>C. macrostachyus</i> in different concentrations			Positive Control (Tetracycline 30 µg/disc)	Negative Control (DMSO)
	25 mg/mL	50 mg/mL	100 mg/mL		
	Mean Zone of Inhibition (ZOI) in mm				
<i>E. coli</i>	10.23 ± 0.38 ^b	13.92 ± 0.71 ^b	18.17 ± 0.65 ^c	26.85 ± 0.21 ^a	0
<i>P. aeruginosa</i>	12.05 ± 0.32 ^b	14.13 ± 0.23 ^b	20.07 ± 0.22 ^b	19.50 ± 0.5 ^b	0
<i>S. aureus</i>	15.25 ± 0.35 ^a	17.55 ± 0.32 ^a	22.00 ± 1.33 ^a	25.86 ± 1.54 ^c	0
Note: All results shown are the mean triplicates ± Standard deviation values. Means bearing a different letter of superscripts in a column differ significantly, but the same letters in superscripts indicate an insignificant difference at <i>P</i> < 0.05.					

In terms of methanolic crude leaf extracts, the lowest concentration 25mg/ml produced less zone of inhibition against selected bacterial pathogens, while 50mg/ml and 100mg/ml concentrations produced higher inhibition zones. The pathogenic bacterium *S. aureus* showed significantly more susceptibility (25.47 ± 0.90 mm) than the pathogenic bacteria *E. coli* (21.8 ± 0.83 mm) and *P. aeruginosa* (19.61 ± 0.33 mm). The methanolic crude leaf extracts at 100 mg/ml concentration produced a significantly similar zone of inhibition developed by the positive control (tetracycline) against *S. aureus* (Table 4). The ethyl acetate crude leaf extract of *C. macrostachyus* expressed a moderate level of antibacterial activity even at higher concentrations of the crude extracts against all three selected pathogenic bacteria (Table 5). The methanolic and ethanolic crude leaf extracts showed higher activity than ethyl acetate extraction against the selected bacterial pathogens.

Table 4

The antibacterial activities of methanolic crude leaf extracts of *C. macrostachyus* against some selected pathogenic bacteria

Name of pathogenic Bacteria	Methanolic crude leaf extracts of <i>C. macrostachyus</i> in different concentrations			Positive Control (Tetracycline 30 µg/disc)	Negative Control (DMSO)
	25 mg/mL	50 mg/mL	100 mg/mL		
	Mean Zone of Inhibition (ZOI) in mm				
<i>E. coli</i>	14.15 ± 0.17 ^b	18.17 ± 0.16 ^a	19.61 ± 0.33 ^c	26.85 ± 0.21 ^a	0
<i>P. aeruginosa</i>	14.27 ± 0.59 ^b	14.93 ± 0.45 ^b	21.8 ± 0.83 ^b	19.50 ± 0.5 ^b	0
<i>S. aureus</i>	12.59 ± 0.44 ^a	15.81 ± 0.48 ^b	25.47 ± 0.90 ^a	25.86 ± 1.54 ^c	0
Note: All results shown are the mean triplicates ± Standard deviation values. Means bearing a different letter of superscripts in a column differ significantly, but the same letters in superscripts indicate an insignificant difference at <i>P</i> < 0.05.					

Table 5

The antibacterial activities of ethyl acetate crude leaf extracts of *C. macrostachyus* against some selected pathogenic bacteria

Name of pathogenic Bacteria	Ethyl acetate crude leaf extracts of <i>C. macrostachyus</i> in different concentrations			Positive Control (Tetracycline 30µg/disc)	Negative Control (DMSO)
	25mg/mL	50mg/mL	100mg/mL		
	Mean Zone of Inhibition (ZOI) in mm				
<i>E. coli</i>	10.76 ± 0.91 ^c	12.67 ± 0.56 ^b	13.59 ± 0.72 ^b	26.85 ± 0.21 ^a	0
<i>P. aeruginosa</i>	6.42 ± 1.2 ^b	11.45 ± 0.84 ^b	11.48 ± 0.95a	19.50 ± 0.5 ^b	0
<i>S. aureus</i>	12.74 ± 0.22 ^a	13.62 ± 0.76 ^a	14.14 ± 0.44b	25.86 ± 1.54 ^c	0
Note: All results shown are the mean triplicates ± Standard deviation values. Means bearing a different letter of superscripts in a column differ significantly, but the same letters in superscripts indicate an insignificant difference at <i>P</i> < 0.05.					

The antibacterial activity of crude stem bark extracts of *C. macrostachyus*

The data received from the antibacterial activity of ethanolic, methanolic, and ethyl acetate crude stem bark extracts are presented in Tables 6, 7, and 8. The prepared crude stem bark extracts using different solvent systems showed antibacterial activity against the selected bacterial pathogens.

Table 6

The antibacterial activities of ethanolic crude stem bark extracts of *C. macrostachyus* against some selected pathogenic bacteria

Name of pathogenic Bacteria	Ethanolic crude stem bark extracts of <i>C. macrostachyus</i> in different concentrations			Positive Control (Tetracycline 30µg/disc)	Negative Control (DMSO)
	25mg/mL	50mg/mL	100mg/mL		
	Mean Zone of Inhibition (ZOI) in mm				
<i>E. coli</i>	9.73 ± 0.36 ^b	11.50 ± 0.50 ^b	13.35 ± 0.61 ^b	26.85 ± 0.21 ^a	0
<i>P. aeruginosa</i>	9.01 ± 0.24 ^b	12.12 ± 0.27 ^b	13.92 ± 0.28 ^b	19.50 ± 0.5 ^b	0
<i>S. aureus</i>	11.69 ± 0.53 ^a	13.20 ± 0.57 ^a	15.15 ± 0.47 ^b	25.86 ± 1.54 ^c	0
Note: All results shown are the mean triplicates ± Standard deviation values. Means bearing a different letter of superscripts in a column differ significantly, but the same letters in superscripts indicate an insignificant difference at <i>P</i> < 0.05.					

There were no significant differences in the mean zone of inhibition between ethanolic and methanolic stem bark extracts, while the ethyl acetate crude stem bark extracts showed a significant decrease in the mean diameter inhibition zone. However, the methanolic crude stem bark extracts of *C. macrostachyus* at 100mg/ml concentration showed the highest activity against *S. aureus* (15.45 ± 0.45 mm), which was lower than the positive control (Tetracycline, 25.86 ± 1.54 mm) (Table 7). The least zone of inhibition was found to be with *E. coli* (6.49 ± 0.48 mm) at 100 mg/ml concentration of Ethyl acetate crude stem bark extracts (Table 8).

Table 7

The antibacterial activities of methanolic crude stem bark extracts of *C. macrostachyus* against some selected pathogenic bacteria

Name of pathogenic Bacteria	Methanolic crude stem bark extracts of <i>C. macrostachyus</i> in different concentrations			Positive Control (Tetracycline 30µg/disc)	Negative Control (DMSO)
	25mg/mL	50mg/mL	100mg/mL		
	Mean Zone of Inhibition (ZOI) in mm				
<i>E. coli</i>	10.66 ± 0.54 ^b	12.40 ± 0.47 ^b	14.01 ± 0.81 ^b	26.85 ± 0.21 ^a	0
<i>P. aeruginosa</i>	9.52 ± 1.80 ^b	11.14 ± 0.68 ^b	13.64 ± 0.43 ^b	19.50 ± 0.5 ^b	0
<i>S. aureus</i>	11.61 ± 0.47 ^a	13.95 ± 0.38 ^a	15.45 ± 0.45 ^b	25.86 ± 1.54 ^c	0
Note: All results shown are the mean triplicates ± Standard deviation values. Means bearing a different letter of superscripts in a column differ significantly, but the same letters in superscripts indicate an insignificant difference at <i>P</i> < 0.05.					

Table 8

The antibacterial activities of ethyl acetate crude stem bark extracts of *C. macrostachyus* against some selected pathogenic bacteria

Name of pathogenic Bacteria	Ethyl acetate crude stem bark extracts of <i>C. macrostachyus</i> in different concentrations			Positive Control (Tetracycline 30µg/disc)	Negative Control (DMSO)
	25mg/mL	50mg/mL	100mg/mL		
	Mean Zone of Inhibition (ZOI) in mm				
<i>E. coli</i>	4.72 ± 0.99 ^b	4.9 ± 0.60 ^b	6.49 ± 0.48 ^b	26.85 ± 0.21 ^a	0
<i>P. aeruginosa</i>	3.69 ± 0.38 ^b	4.85 ± 0.47 ^b	5.68 ± 0.73 ^b	19.50 ± 0.5 ^b	0
<i>S. aureus</i>	6.26 ± 0.62 ^a	8.20 ± 0.74 ^a	10.48 ± 0.77 ^a	25.86 ± 1.54 ^c	0
Note: All results shown are the mean triplicates ± Standard deviation values. Means bearing a different letter of superscripts in a column differ significantly, but the same letters in superscripts indicate an insignificant difference at <i>P</i> < 0.05.					

Minimum Inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of crude leaf extracts of *C. macrostachyus*

The MIC results of the crude leaf and stem bark extracts of *C. macrostachyus* presented in Table 9 showed that all the selected bacterial pathogens were very susceptible to the minimum inhibitory concentrations ranging from 0.625–2.5 mg/ml. The minimum inhibitory concentration of ethanolic crude leaf extracts of *C. macrostachyus* was 0.625mg/ml for *S. aureus*, and 1.25 mg/ml for *E. coli* and *P. aeruginosa*. In terms of methanolic crude leaf extracts, the minimum inhibitory concentration was 0.625 mg/ml for *S. aureus*, *E. coli* and *P. aeruginosa*. The minimum inhibitory concentration of ethyl acetate crude leaf extracts was 1.25 mg/ml for *S. aureus*, *E. coli*, and *P. aeruginosa*. The minimum inhibitory concentration of crude stem bark extracts ranged from 1.25–2.5 mg/ml. In terms of ethanolic and methanolic crude stem bark extracts of *C. macrostachyus* was 1.25 mg/ml for *S. aureus*, *E. coli* and *P. aeruginosa*. The minimum inhibitory concentration of ethyl acetate crude stem bark extract was 1.25 mg/ml for all three selected bacterial pathogens (Table 9).

Table 9
Minimum Inhibitory concentration of crude extracts of *C. macrostachyus*

S. No.	Nature of the crude extracts	Name of the bacterial pathogens		
		E. coli	P. aeruginosa	S. aureus
		mg/mL		
1.	Ethanollic leaf	1.25	1.25	0.625
2.	Methanollic leaf	0.625	0.625	0.625
3.	Ethyl acetate leaf	1.25	1.25	1.25
4.	Ethanollic stem bark	1.25	1.25	1.25
5.	Methanollic stem bark	1.25	1.25	1.25
6.	Ethyl acetate stem bark	2.5	2.5	2.5

The data obtained through the determination of MBC of crude leaf and stem bark extracts are presented in Table 10. The ethanolic crude leaf extract showed a bactericidal effect at 1.25 mg/ml for *S. aureus* and 2.5 mg/ml for both *E. coli* and *P. aeruginosa*. In terms of methanollic leaf crude extract showed bactericidal effect at 1.25 mg/ml for all the three selected pathogens. The MBC of ethyl acetate crude leaf extracts was 2.5 mg/ml for both *E. coli* and *P. aeruginosa*, and 1.25 mg/ml for *S. aureus*. The MBC of ethanolic, methanollic and ethyl acetate crude stem bark extracts ranged from 2.5 to 5 mg/ml for gram-negative bacterial pathogens. In terms of Gram-positive bacteria *S. aureus*, the ethanolic and methanollic stem bark crude extracts at 1.25 mg/ml concentration showed a bactericidal effect, and ethyl acetate stem bark crude extract at 5 mg/ml concentration showed a bactericidal effect (Table 10).

Table 10
Minimum Bactericidal concentration of crude extracts of *C. macrostachyus*

S. No.	Nature of the crude extracts	Name of the bacterial pathogens		
		E. coli	P. aeruginosa	S. aureus
		mg/mL		
1.	Ethanollic leaf	2.5	2.5	1.25
2.	Methanollic leaf	1.25	1.25	1.25
3.	Ethyl acetate leaf	2.5	2.5	1.25
4.	Ethanollic stem bark	2.5	2.5	1.25
5.	Methanollic stem bark	2.5	2.5	1.25
6.	Ethyl acetate stem bark	5	5	5

Discussion

Nature has given us very rich botanical wealth, and a large diversity of plants worldwide. Plants have been proven as a potential source of medicines to cure and prevent human-related diseases. Microbial infections are the most important cause of diseases and mortality globally. Antimicrobial resistance is a serious threat to public health worldwide and it leads to the development of multidrug-resistant microorganisms that compromise the susceptibility rate to the available antibiotics [32, 33, 34]. Therefore, the search for novel, safe, and effective antimicrobial agents from medicinally important plants is inevitable.

The present study aimed to investigate the antibacterial activities of crude leaf and stem bark extracts of *C. macrostachyus* prepared by three different solvent systems against selected bacterial pathogens. The methanolic crude leaf and stem bark extract yield was higher than the yield obtained from the ethanolic and ethyl acetate solvent extraction. The methanolic crude leaf and stem bark extracts yield was 2.1% (1.89gm) and 4.1% (3.3gm) respectively. The yield results indicated that the polarity of the solvents determined the yield. The high polarity solvent increased the yield [35]. The yield percentage of the crude methanolic extracts of two different plants *Pulicaria crispa* and *Pulicaria undulata* was studied and reported as 22.6% and 23%, respectively, (w/w) on dry weights bases. The reason for the difference in the percentage yield of methanolic extract was due to differences in environmental conditions [36].

The qualitative phytochemical analysis of ethanolic, methanolic, and ethyl acetate crude leaf and stem bark extracts of *C. macrostachyus* revealed the presence of tannins, saponins, flavonoids, glycosides and phenolics, alkaloids was absent only in ethyl acetate crude leaf extract. In terms of stem bark extracts, the obtained results revealed the presence of phytochemicals except alkaloids and flavonoids in ethanolic and methanolic crude stem bark extracts. Most of the phytochemicals were absent in ethyl acetate crude stem bark extract. All the above-mentioned phytoconstituents are known to have numerous pharmacological activities like anti-oxidant, antitumor, antimalarial, antiallergic, antiinflammation, and also protecting against bacterial, viral, and fungal infections [37, 38, 39, 40, 41, 42, 43, 44]. The presence of phytochemicals such as saponin, flavonoids, steroids, glycosides, and alkaloids in medicinal plants makes it used as an anticancer, anti-inflammatory, antispasmodic, and analgesic agent [45, 46]. The secondary metabolites such as tannins, saponins, flavonoids, glycosides, phenolics, and alkaloids in the crude extracts were responsible for chemotherapeutic effects exhibited by medicinal plants in both *in vitro* and *in vivo* [47].

The ethanolic, methanolic, and ethyl acetate crude extracts of the leaf and stem bark of *C. macrostachyus* exhibited different activities against selected bacterial pathogens. The results indicated that all the selected bacterial pathogens were susceptible to ethanolic, methanolic, and ethyl acetate crude leaf and stem bark extracts. There were significant differences in the mean diameter inhibition zone between gram-positive and gram-negative bacterial pathogens. The highest zone of inhibition in diameter ($25.47 \pm 0.90\text{mm}$) was observed by methanolic crude leaf extracts against *S. aureus* at 100mg/ml, followed by *P. aeruginosa* and *E. coli* with $21.8 \pm 0.83\text{ mm}$ and $19.61 \pm 0.33\text{ mm}$ in diameter

zone of inhibition respectively. Similarly, the crude leaf extract of *Calpurnia aurea* exhibited stronger antibacterial activity against gram-positive than gram-negative bacterial pathogens. The bacterial pathogen *S. aureus* was more susceptible than *E. coli* [35]. The methanolic crude extracts of *P. crispa* and *P. undulata* showed the highest zone of inhibition against gram-positive bacterial pathogen *S. aureus* and a moderate level of antibacterial activity against gram-negative bacterial pathogens [36].

Furthermore, the present study results are in line with the previous findings that the crude extract prepared by methanol showed the best antimicrobial activities from Ghana and Pakistan [48, 49]. The ethyl acetate crude leaf and stem bark extracts produced significantly less in the zone of inhibition at 100mg/ml concentration against both gram-positive and gram-negative bacterial pathogens. This contradicts the argument presented by Kebede and Shibeshi [50]. According to Kebede and Shibeshi, the antimicrobial activities of ethyl acetate crude leaf extracts of *R. communis* showed the highest activity against pathogenic bacteria and *Candida albicans*. The present study exhibited that the ethanolic, methanolic, and ethyl acetate crude stem bark extracts exhibited significantly lesser antibacterial activity than leaf crude extracts. This result contradicts the results of Ogueke et al. [51] who reported that the ethanol and methanolic crude stem bark extract of *A. boonei* showed the highest zone of inhibition against *E. coli*, *S. aureus*, *S. typhi*, *P. aeruginosa*, *S. cerevisiae*, and *Kluyveromyces* sp. The antibacterial activity of stem bark extract of *C. macrostachyus* is similar to previous findings of Minyamer and Belay [52].

The MIC and MBC results revealed that the methanolic crude leaf extracts of *C. macrostachyus* showed the highest activity at 0.625mg/ml – 1.25mg/ml against *E. coli*, *P. aeruginosa*, and *S. aureus*. The MIC and MBC of ethanolic and ethyl acetate crude leaf extracts ranged from 0.625 mg/ml to 2.5mg/ml. Similarly, the methanol leaf extract of *A. acuminata* and *P. granatum* had the lowest MIC value, 0.67 mg/ml, and the lowest MBC value 1.51 mg/ml against *E. faecalis*, *K. pneumoniae* and *P. aeruginosa*. The MIC and MBC values against gram-positive bacterium *S. aureus* and gram-negative bacterium *E. coli* obtained in this study with the ethanolic, methanolic, and ethyl acetate crude leaf and stem bark extracts of *C. macrostachyus* were lower than that reported by Minyamer and Belay, [52] and Adesokan et al. [53].

Conclusion

The leaf and stem bark of the medicinal plant *C. macrostachyus* was subjected to different solvent systems such as ethanolic, methanolic, and ethyl acetate for the preparation of crude extracts. The methanol solvent systems yielded the highest quantity of crude extracts whereas the ethanol and ethyl acetate yielded less. The qualitative phytochemical analysis showed that the methanolic and ethanolic crude leaf extracts contained all the expected essential secondary metabolites such as tannins, alkaloids, saponins, flavonoids, phenolics, and glycosides, whereas ethyl acetate crude leaf extract contained all the secondary metabolites except alkaloids. The methanolic, ethanolic, and ethyl acetate crude stem bark extracts contained fewer phytochemical constituents when compared to leaf crude extracts. The methanolic and ethanolic crude leaf extracts exhibited the best antibacterial activities against all the selected bacterial strains in a concentration dependent manner. The more susceptible

bacterial pathogen was *S. aureus*. The stem bark crude extracts showed antibacterial activity against all the selected bacterial pathogens but significantly less than the leaf crude extracts. The MIC and MBC of the methanolic crude leaf extract against all the selected bacterial pathogens were 0.625 mg/ml and 1.25 mg/ml respectively. Therefore, the present study has concluded that the methanol solvent can be used for the preparation of crude leaf extracts and exhibited more antibacterial activity than crude stem bark extracts. The present study also supports the traditional usage of *C. macrostachyus* leaf as medicine for the treatment of bacterial infections.

Declarations

Declarations: All authors have read, understand, and have complied as applicable with the statement on “Ethical responsibilities of Authors” as found in the instructions for authors.

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Authors contribution:

KRISHNA MOORTHY SIVALINGAM: Contributed to conceptualization, Sample collection, laboratory work and writing original draft; GETAYAWUKALYOHANIS: Contributed to Sample collections and laboratory work; GEDIF MESERET: Review and editing; EYOB CHUKALO: Review and editing; SENAPATHY MARISENNAYYA: Review and editing.

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Figures

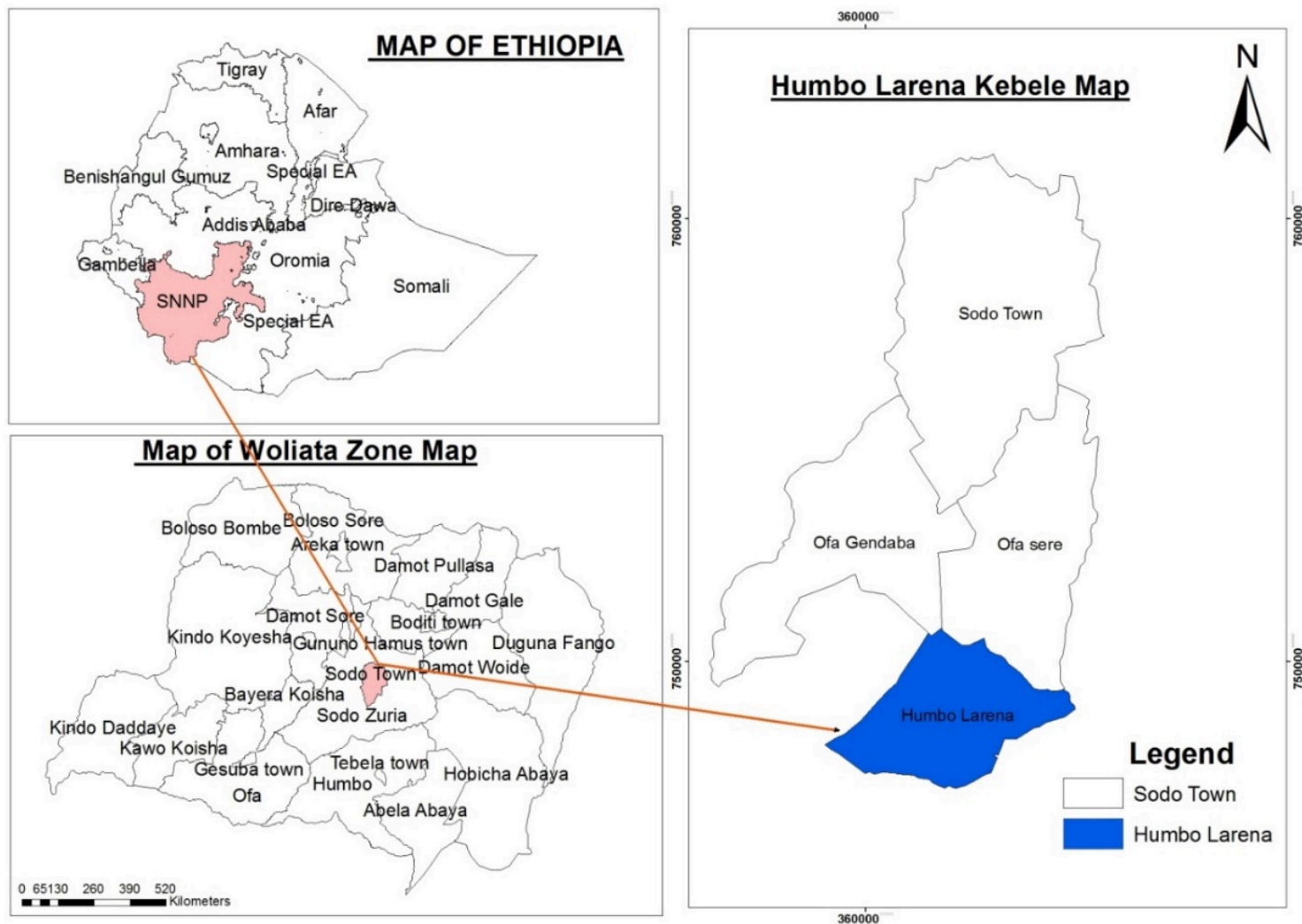


Figure 1

A map of the study area (*Source:* Ethiopia GIS version 10.8 software, 2021)