

Phytochemical Investigation and Determination of Antibacterial Activity of Solvent Leave Extracts of *Carissa spinarum*

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Background: Among many traditionally used medicinal plants, *Carissa spinarum* (Agam) is a well-known indigenous plant in Ethiopia. It is used in its raw form to treat different diseases in different parts of the country. Therefore, the aim of this study is to investigate extraction, isolation, and determination of the antibacterial properties of the solvent leaf extract of *Carissa spinarum*.

Methods: In this study, 800 g of powdered leaves of *Carissa spinarum* were macerated with 2500 mL of methanol and yielded 58 g (7.25%, w/w) of gummy material. The extract was then further partitioned by using ethyl acetate and chloroform. The extracts were subjected to phytochemical screening test. The antibacterial activity of the three solvent leaf extracts of *Carissa spinarum* were evaluated using disc diffusion method. The methanol extract was subjected to column chromatography silica gel (60–200 mesh) by mixing methanol:petroleum ether (4:1). Then fractions were collected and investigated by TLC and finally identified using spectroscopy.

Results: The three extracts (methanol, ethyl acetate, and chloroform) of *Carissa spinarum* were presented to antimicrobial activity by disc diffusion method against four bacterial species using gentamycin and ampicillin discs as positive controls and DMSO as a negative control. All extracts had a relatively antibacterial effect with different extent zones of inhibition. However, the methanol extract showed superior antibacterial activity compared with DMSO and ethyl acetate and chloroform extracts. These could be due to variation of the phytoconstituents. The most probable structure of the compound isolated was 5-(2',3',4',6'-tetrahydroxy-5'-methoxycyclohexyloxy)-2,3,4 trihydroxypentanoic acid.

Conclusion: Data obtained from this study collectively indicated that the three solvent extracts of *Carissa spinarum* have a promising antimicrobial activity which supports the traditional claim of the plant for treatment of infection.

Keywords: antibacterial activity, *Carissa spinarum*, phytochemicals determination

Background

The role of plants and plant parts as sources of medicine is immense. Most of the drugs nowadays originate from plant sources. The use of plants for therapeutic purpose is probably the oldest phenomenon.¹ Extraction and structural elucidation of numerous active secondary metabolites from these plants have been developed to high activity profile drugs. Plants would be the main sources for a variety of traditional and modern medicines. According to a report from the World Health Organization (WHO), around 80% of population in developing countries relies on traditional medicines. The largest proportion of these essential medicines are primarily related to herbal extracts or their active principles like, leaves, flowers, roots, fruits, and seeds.² One plant species that is known for its medical use is *Carissa spinarum* (Agam).³

Traditional medicines are the leading foundation for biologically active remedies. There has been an increasing interest in medicinal plants as natural products in different parts of the world.⁴ The therapeutic significance of medicinal

plants relies on the presence of pharmacologically active secondary metabolites and their effect in the human body. Phytochemicals are bioactive chemicals of plant origin. Many species have been recognized to have medicinal value and valuable effect for the well-being of humans, such as, infected wounds, which are a common public health concern.⁵

Carissa spinarum has a wide variety of applications in traditional medicine. Traditionally the leaf part of *Carissa spinarum* is used to treat external parasites and inflammation. Moreover, the various parts of *Carissa spinarum* are used to treat numerous ailments in Ethiopia. For instance, it is useful in treatment of rheumatism, purgative and snake repellent, wounds, and has emerged as a good source of the traditional medicine for the treatment of, arthritis, microbial infection, epilepsy, viral infection, cancer diseases, among others.^{6–9}

In Ethiopia, there are numerous medicinal plants which are used simply in the raw form to treat different diseases. Among many traditionally used medicinal plants, *Carissa spinarum* (Agam) is one of the most well-known indigenous plant in Ethiopia in which the raw form is used to treat different diseases in different parts of the country.

Therefore, this study was mainly initiated by performing the process of crude extraction, phytochemical screening, antibacterial test and structural elucidation of the leaves of *Carissa spinarum*, which enables us to know the types of compounds found within this plant for use in the management of different ailments.

Materials and Methods

Apparatus and Chemicals

In this study we used the following apparatus: funnel, oven, pipettes (different sizes), TLC plate, water bath, UV lamp, beakers, electronic balance, conical flask, electronic grinder, and test tubes, capillary tube, Whatman No. 1 filter paper, glass rod, cotton, spatula, gas jar, tongs, pencil, ruler, scissors, aluminium foil, test tube ranker, glove, stand, column chromatography, round bottom flasks (different sizes), measuring cylinder, rotary evaporator (RE-200), petri dish, refrigerator, test tube holder, dropper, lamp, incubator, IR spectrometer, ¹H-NMR spectrometer (400 MHz, Bruker), ¹³C-NMR spectrometer (100 MHz, Bruker) and DEPT-135 (100 MHz, Bruker). Further, chemicals used for this study include: solvents (petroleum ether, ethyl acetate, chloroform and methanol), sulphuric acid, hydrochloric acid, ferric chloride, magnesium, sodium hydroxide, sodium bicarbonate, copper sulphate, mercury chloride, potassium iodide, distilled water, glacial acetic acid, ammonia solution, acetic anhydride, phenolphthalein indicator, litmus paper, iodine crystal, silica gel (60–200 mesh), DMSO, Mueller–Hinton agar, denatured ethanol and standard tablets (ampicillin and gentamycin). All the instruments and apparatus were of analytical grade.

Collection and Authentication of *Carissa spinarum*

The healthy aerial parts of the leaves of *Carissa spinarum* were collected from Central North Gondar on January, 2021 (Figure 1). The collected plant material was authenticated by Dr Getnet Masresha a botanist from the Department of Biology at the University of Gondar and a voucher specimen TA/001/2021 was deposited in the University of Gondar herbarium. The plant material was splashed with distilled water to remove any dust material on it. The collected plant material was dried under shade immediately after collection. The shaded dried healthy leaves were powdered using a mechanical grinder in uniform powder size as shown in Figure 2. It was weighed and then the powdered sample was kept in a sealed container for extraction purposes to conduct this research.

Extraction of *Carissa spinarum*

The air dried powdered leaves of *Carissa spinarum* (800 g) were macerated with methanol for 72 h, slightly shaking twice a day at room temperature. The mixture was filtrated with Whatman No. 1 filter paper. The methanol extract was concentrated by rota evaporator (RE-200) until it became completely dry and it yielded 58 g of black gummy material (7.25% by w/w).

The methanol extract was further partitioned by using lesser polarity (ethyl acetate and chloroform).



Figure 1 The leaves of *Carissa spinarum*.



Figure 2 Powdered leaves of *Carissa spinarum*.

Phytochemical Screening

Preliminary phytochemical screening of secondary metabolites was carried out on the methanol, ethyl acetate, and chloroform extracts, according to the standard procedures described by many researchers.¹⁰

Test for Flavonoids

Two milliliters of each solvent leaf extract of *Carissa spinarum* solution was mixed with 1.5 mL of 50% methanol solution. Then the solution was heated and metal magnesium be added. Few drops of concentrated hydrochloric acid was added and the red color was observed for flavonoids.¹¹

Test for Alkaloids

About 0.5 g of each solvent leaf extract of *Carissa spinarum* was dissolved in 2N HCl and it was filtered. The filtrates were tested with different reagents. Mayer's test: the filtrate was treated with Mayer's reagent, and then the appearance of creamish precipitate indicate the presence of alkaloids.¹²

Tannins Test

About 0.5 g of each solvent leaf extract of *Carissa spinarum* was mixed in 10 mL of distilled water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added and observed for brownish green or blue black coloration.¹³

Test for Saponins

About 0.5 g of each solvent leaf extract of *Carissa spinarum* was diluted with 20 mL of distilled water in a test tube and boiled for 15 min. The formation of foam indicated the presence of saponins.¹⁴

Test for Steroids

Two milliliters of acetic anhydride was added to 2 mL each of solvent leaf extract of *Carissa spinarum* along with 2 mL concentration of sulphuric acid. The formation of blue or green that was detected for the presence of steroids.¹⁵

Test for Cardiac Glycosides

We dissolved 0.2 g of each solvent leaf extract of *Carissa spinarum* in 1 mL of glacial acetic acid containing and 1 drop of ferric chloride solution was added. This was then under layered with 1 mL of concentrated sulphuric acid. The formation of a brown ring obtained at the interface indicated the presence of cardiac glycosides.¹⁶

Test for Proteins

To 2 mL of the gram of each solvent leaf extract of *Carissa spinarum* solution 1 mL of 40% NaOH solution and 1 to 2 drops of 1% CuSO₄ solution was added. Formation of violet color indicated the presence of peptide linkage of the molecule.¹⁷

Test for Anthraquinones

About 0.5 g of each solvent leaf extract of *Carissa spinarum* was taken in a dry test tube and 5 mL of chloroform was added and shaken for 5 min. The mixture was filtered and added 10% of ammonia solution. A pink violet or reddish color observed for the presence of anthraquinones.¹⁸

Partitioning of the Methanol Extract by Solvents

The methanol extract showed many spots when checked by TLC; as a result it was further macerated by ethyl acetate (400 mL) with occasional shaking for 48 h and then chloroform (200 mL) for 48 h for decreasing the number of components. The methanol extract (residue), chloroform extract and ethyl acetate extract were concentrated with a rotary evaporator and afforded 40 g, 7 g, and 4 g, respectively. The extracts were kept in an airtight container and stored in a deep freezer until the commencement of the experiment.

Antibacterial Activity Evaluation

Inoculums Preparation and Standardization

Antimicrobial susceptibility test implies the capability of an antimicrobial agent to inhibit bacteria and fungal growth. In this study the antimicrobial activities (specifically antibacterial) of ethyl acetate, chloroform, and methanol leaf extract of *Carissa spinarum* were done by disc diffusion method using gentamycin and ampicillin disc as standard antibiotic, two gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus pneumoniae*) and two gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). Bacteria cultures used in this study were obtained from department of Microbiology Laboratory, University of Gondar. The media was prepared by dissolving 38 g of Muller–Hinton agar (MHA) powder in 1000 mL of distilled water, heated, well shaken and allowed to boil. It was then put in autoclave at 121°C for about 15 min to sterilize the media. Then the media was allowed to cool and poured in 12- plates and was put on a level surface. Finally the media was allowed to solidify, kept in an upright position in the incubator avoiding contamination from the hood.



Figure 3 Zone of inhibition shown by methanol fraction of leaves of *Carissa spinarum*.

Procedure for Performing the Disc Diffusion Test

After the preparation of the Muller–Hinton agar plates as per manufacturer instructions, the cultured bacteria were cleaned on the top of the pre-leveled media (100 μ L) and allowed to dry for 10 min. A sterilized cork borer was used to bore holes on the plates and 100 mg/mL of each extracts (ethyl acetate, chloroform, and methanol) was introduced in the holes by using a gentamycin disc (10 μ g/disc) and an ampicillin disc (10 μ g/disc) as standard. The petri dishes were then nurtured at 37°C for 24 h. At the end of incubation period, the antibacterial activity of each sample was done in triplicates and manipulated by quantifying the diameters of the average inhibition zone by millimeter including the wells and discs as shown in Figures 3–5.

Purification and Isolation of the Major Component

Out of the three extracts, the methanol extract showed better inhibition zone and it was subjected to column chromatography silica gel (60–200 mesh) by mixing the suitable solvents methanol:petroleum ether (4:1). A total of 24 fractions were collected and investigated by TLC. Fractions 6 to 20 showed similar R_f value and were mixed together. Then the solvent was removed completely. From these fractions brown gummy material was obtained. It was checked by TLC using different solvents and showed only a single spot. Finally it was sent to the Department of Chemistry, Addis Ababa University for spectroscopic analysis leveled as TA.

Results

Preliminary Examination

One drop of methanol solution of compound TA was put on blue litmus paper and changed the color to slightly red. Further one drop of phenolphthalein indicator was added to the compound and it became colorless with PH value of 4.8. Finally compound TA was found to be soluble in dilute NaOH as well as dilute NaHCO₃ with formation of bubbles in the latter case. Therefore, the above tests indicate that compound TA contains the carboxylic group.

Phytochemical Screening Test Result

Phytochemical analysis of the solvent leaf extracts of *Carissa spinarum* revealed the presence of various secondary metabolites which are summarized in Table 1.

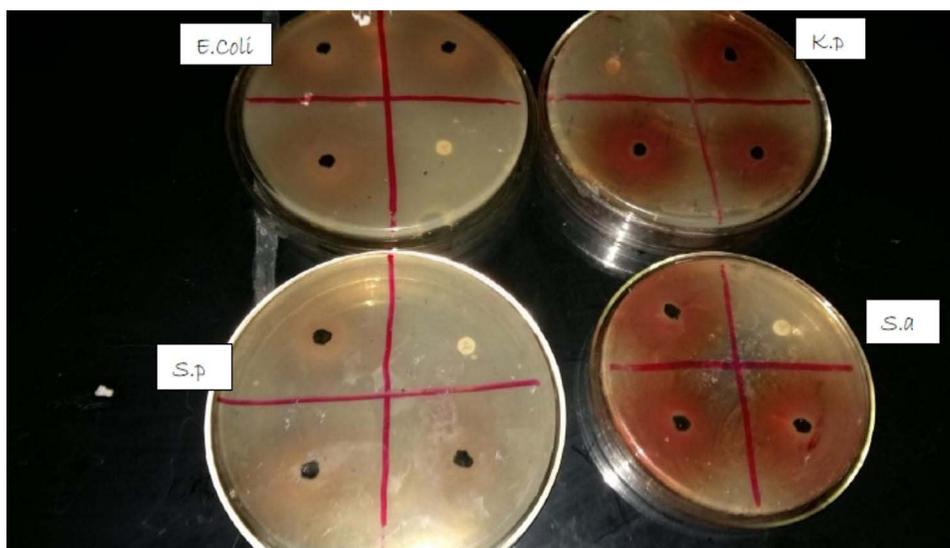


Figure 4 Zone of inhibition shown by ethyl acetate fraction of leaves of *Carissa spinarum*.

Antibacterial Activity of the Extracts of Ethyl Acetate, Chloroform, and Methanol Against Bacterial Strains

Evaluation of antibacterial activity of the crude extracts of ethyl acetate, chloroform and methanol of the leaves of *Carissa spinarum* were done by disc diffusion method using a gentamycin disc (10 µg/disc) and an ampicillin disc (10 µg/disc) as standard antibiotic; two gram-positive bacteria (*S.aureus* and *S. pneumoniae*) and two gram-negative bacteria (*E. coli* and *Klebsiella pneumoniae*) species in triplicate. The antibacterial activity of each sample was determined by measuring the average diameter of inhibition zone in mm including the wells and discs as shown in Table 2; Figures 3–5.

Characterization of the Major Component of Methanol Leaves Extract of *Carissa spinarum*

The characterization of the major component of compound TA was done using spectroscopic methods such as, IR, ¹H-NMR, ¹³C-NMR and DEPT-135 spectra.

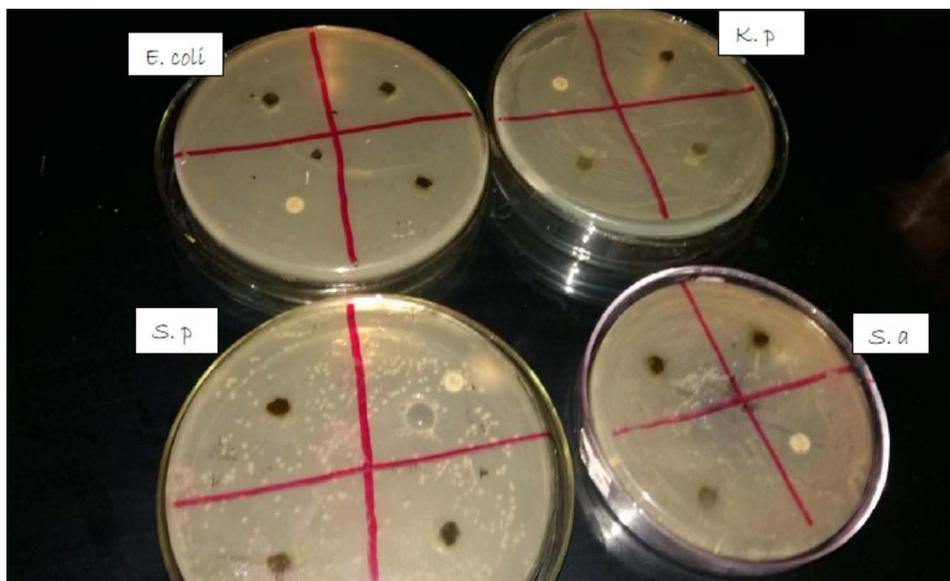


Figure 5 Zone of inhibition shown by chloroform fraction of leaves of *Carissa spinarum*.

Table 1 Phytochemical Screening Test of Different Solvent Leaf Extracts of *Carissa spinarum*

Metabolites Tested	Extracts		
	Methanol	Ethyl Acetate	Chloroform
Alkaloids	+	+	+
Saponins	+	+	–
Tannins	+	+	–
Phenols	+	+	+
Terpenoids	+	+	–
Flavonoids	+	+	+
Cardiac Glycosides	+–	–	–

Note: (+) present and (–) absent.

IR Spectrum of Compound TA

The IR spectrum ([Appendix 1](#) and [2](#)) showed broad and strong bands at 3400 cm^{-1} and 3230 cm^{-1} indicating the presence of hydroxyl group (–OH). In addition to this the bands show the presence of hydrogen bonding (dimer) that partially obscure the C–H stretching bands which is a characteristic feature of carboxylic acids. The bands at 2884 cm^{-1} and 2830 cm^{-1} are assigned to C–H stretching; the bands at 1700 cm^{-1} to carbonyl group (C=O), the bands at 1235 cm^{-1} and 1055 cm^{-1} to C–O stretching and finally the bands at 1455 cm^{-1} and 1375 cm^{-1} for methylene and methyl group bending absorption respectively. The IR absorption of compound TA in comparison with estimated values is summarized in [Table 3](#).

¹H-NMR Spectrum of Compound TA

In ¹H-NMR spectrum of compound TA ([appendix-2](#)), the signals are not well resolved due to similar chemical shifts between two groups of protons, ie second-order spectrum. The –COOH proton absorption is not also observed, which could be off the chart to the left. Strong hydrogen bonding may also cause the peak to become broadened with very low intensity. In some cases the absorption may also disappear. For the characterization compound TA major signals are considered and others are omitted as impurities. Accordingly the signals at: 4.17 (C₂H), 2.0 (C₂OH), 3.7 (C₃H), 2.0 (C₃OH), 3.6 (C₄H), 2.0 (C₄OH), 3.62 and 3.4 (C₅H₂), 2.9 (C₁H), 3.6 (C₂H), 2.0 (C₂OH), 3.2 (C₃H), 2.0 (C₃OH), 3.6 (C₄H), 2.0 (C₄OH), 2.9 (C₅H), 3.80 (C₆H), 2.0 (C₆OH), and 3.2 (C₁H₃) are assigned as described in [Table 4](#).

Table 2 Antibacterial Activities of the Methanol, Chloroform, and Ethyl Acetate Leaf Extracts of *Carissa spinarum* Using Disc Diffusion Techniques

Extracts	Control	Concentration	Average Zone of Inhibition (Mean ±SEM in mm)			
			<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. pneumoniae</i>	<i>S. aureus</i>
Methanol		100 mg/mL	11.0±0.333 ^{a,b}	11.0 ± 0.23 ^b	11.0± 0.57 ^b	13.0± 0.68 ^{a,b}
	Gentamycin	10 µg/disc	14.0±0.577	13±0.13	12±34	20.0± 0.57 ^{a,b}
Ethyl acetate		100 mg/mL	10.0±0.333	10.0±0.72	10.0±0.24 ^{a,b}	9.0± 0.32
	Gentamycin	10 µg/disc	13.0±0.34	12.67±0.333	11.23±0.33	20.0± 0.54 ^{a,b}
Chloroform		100 mg/mL	8.67±0.22	7.0±0.41	9.0±0.27	8.0± 0.17
	Gentamycin	10 µg/disc	12.0±0.31	11.31±0.33	10.0±0.65	20.0± 0.19 ^b

Notes: Values expressed as mean ±SEM for n=3. The mean comparisons for different extracts and control (gentamycin 10 µg) were performed by one-way ANOVA followed by Tukey's HSD post hoc multiple comparison test. Where, compared to ^aethyl acetate, ^bchloroform, p<0.05.

Table 3 IR Spectrum of Compound TA

Group	IR Absorption (cm ⁻¹) of Compound TA	Estimated Value (cm ⁻¹)
-OH	3400 and 3230	3400–2400
C-H stretch	2884 and 2830	3000–2840
C=O	1700	1780–1650
CH ₂ bending	1465	1465
CH ₃ bending	1375	1375
C-O stretch	1235 and 1055	1300–1000

¹³C-NMR and DEPT-135 Spectra of Compound TA

The carbon-13 spectra of compound TA ([Appendix 3](#) and [4](#)) were not run properly since there is a difference in the number of carbon atoms of ¹³C-NMR (9 carbon atoms) and DEPT-135 (12 carbon atoms). In principle the numbers of carbon atoms in the DEPT-135 should have been less than that of ¹³C-NMR if quaternary carbon atoms are present. So, for the characterization of compound TA the carbon atoms from DEPT-135 spectrum are considered. In addition from many instances the peak intensity of carbonyl carbon is very weak and sometimes it may not appear unless enhanced. So from the DEPT-135 spectrum 12 carbon atoms are observed and out of these 9 are methine (CH), 1 methylene (CH₂), 1

Table 4 ¹H-NMR Spectrum of Compound TA in Comparison with Estimated Value

Proton	Observed ¹ H-NMR Chemical Shift of Compound TA in (DMSO)	Estimated Value
C ₁ -OOH	Off chart to the left	12
C ₂ -H	4.17	4.17
C ₂ -OH	2.0	2.0
C ₃ -H	3.7	3.7
C ₃ -OH	2.0	2.0
C ₄ -H	3.6	3.62
C ₄ -OH	2.0	2.0
C ₅ -H ₂	3.62 and 3.4	3.64 and 3.39
C ₁ '-H	2.9	2.95
C ₂ '-H	3.6	3.57
C ₂ '-OH	2.0	2.0
C ₃ '-H	3.2	3.33
C ₃ '-OH	2.0	2.0
C ₄ '-H	3.6	3.57
C ₄ '-OH	2.0	2.0
C ₅ '-H	2.9	2.95
C ₆ '-H	3.80	3.81
C ₆ '-OH	2.0	2.0
C ₁ '-H ₃	3.2	3.24

Table 5 ^{13}C -NMR and DEPT-135 Spectra of Compound TA in Comparison with Estimated Value

Position Carbon	Observed ^{13}C -NMR Chemical Shift of Compound TA (DMSO)	Estimated Value	DEPT-135
1	174	173.2	Quaternary
2	74.11	73.9	CH
3	72.65	73.1	CH
4	70.39	70.0	CH
5	71.08	70.4	CH ₂
1'	81.51	78.4	CH
2'	70.87	70.2	CH
3'	72.48	73.0	CH
4'	69.58	69.9	CH
5'	82.74	80.3	CH
6'	68.41	67.7	CH
1''	57.87	57.7	CH ₃

methyl (CH₃) and 1 quaternary. Accordingly the peaks are assigned as follows, at: 174 (C-1), 74.11 (C-2), 72.65 (C-3), 70.39 (C-4), 71.08 (C-5), 81.51 (C-1'), 70.87 (C-2'), 72.48 (C-3'), 69.58 (C-4'), 82.74 (C-5'), 68.41 (C-6') and 57.87 (C-1''). The ^{13}C -NMR and DEPT-135 chemical shift of compound TA along with estimated values are summarized in Table 5. The spectroscopic data suggest that the most probable structure of compound TA can be: -5-(2', 3', 4', 6'-tetrahydroxy-5'-methoxycyclohexyloxy)-2, 3, 4-trihydroxypentanoic acid as shown in Figure 6.

Discussion

Worldwide progression of resistance to the existing antimicrobials and adverse effects of those drugs used in the management of communicable ailments inspire the investigation of medicinal plants and plant derived phytochemicals. Several studies have been investigated about the antimicrobial activities of numerous medicinal plants. For instance, essential oils extracted from *Ruta graveolens*, *Origanum vulgare* and *Myrtus communis* showed antifungal activity against resistant strains of pityriasis versicolor.¹⁹⁻²¹ Essential oils isolated from lavender showed antibacterial activity against multidrug resistant strains of *Pseudomonas aeruginosa*.²² Moreover, extracts from medicinal plants showed activity against the tested organism. For instance, oils extracted from *Zingiber officinale* (ginger), *Psidium guajava*, *Cyamopsis tetragonoloba* (Gaur) L and *Leucas cephalotes* exhibited antiviral activity against the tested viral strains.²³⁻²⁷ Furthermore, phytochemicals extracted from *Allium cepa* exhibited antiparasitic activity against strains of *Entamoeba gingivalis*.²⁸

Therefore, this study was executed to evaluate the phytochemicals and determine the antibacterial activity of leaf extracts of *Carissa spinarum*.

The methanol extract of *Carissa spinarum* showed higher zone inhibition against the tested bacterial species compared with the negative control (DMSO), ethyl acetate, and chloroform extract. This variation in growth inhibition between the methanol extract and the vehicle might be due to the existence of antibacterial secondary metabolites in the extract, which are in line with the previous reports on antimicrobial activity of medicinal plants.²⁹ Moreover, the qualitative phytochemical screening test result of solvent extracts of *Carissa spinarum* revealed the presence of steroids, tannins, saponin, flavonoids, and terpenoids. These phytochemicals could be the reason for antimicrobial activity of the *Carissa spinarum*. The previous study by Singh and Verma revealed that phytochemical such as alkaloids had antibacterial activity against the tested bacterial species.³⁰ The cardiac glycosides isolated from the methanol dried

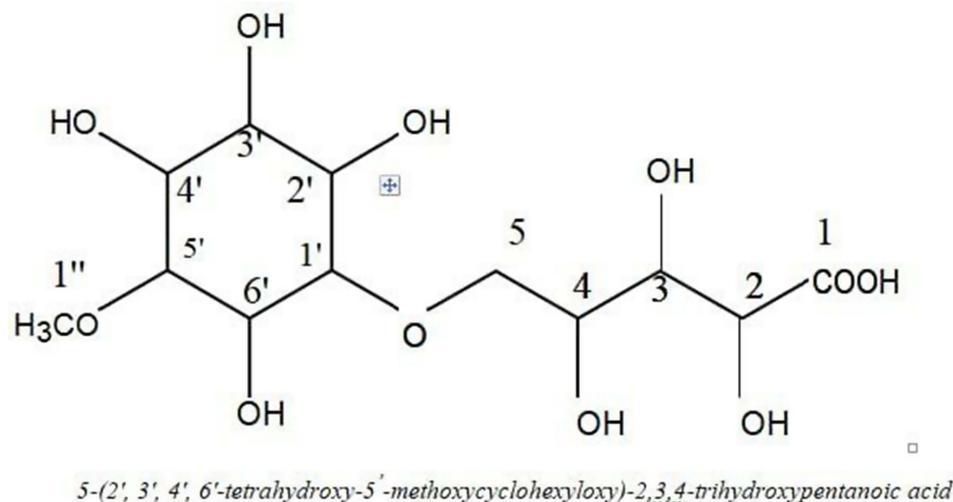


Figure 6 Proposed structure of compound TA.

stems extract of *Carissa spinarum* revealed antimicrobial activity.³¹ Moreover, the methanol root extract of *Carissa spinarum* showed wound healing and antimicrobial activity as reported by Sanwal and Chaudhary.⁹ Furthermore flavonoids, tannins, saponins, and steroids were identified in methanol leaf extract of *Carissa spinarum*.³² Therefore, the antibacterial activity of the methanol leaf extract of *Carissa spinarum* could be due to a synergistic effect of these phytochemicals.³³

The ethyl acetate and chloroform extracts of the 80% methanol extract of *Carissa spinarum* also exhibited antibacterial activity in all tested bacterial species with a different extent of zone of inhibition. The vehicle used for dissolving these extracts did not show any zone of inhibition in all tested bacterial species and was taken as a negative control. This shows the existence of antibacterial phytochemical in the extracts. An investigation reported by Clarence et al revealed that different extracts of *Carissa spinarum* had potential antibacterial activity against infections caused by pathogens such as *S. aureus* and *E. coli*,³⁴ which is in line with our finding.

The zone of inhibition in all solvent extracts of *Carissa spinarum* was significantly smaller than the standard (gentamycin) against *S. aureus* and *E. coli* even at maximal concentration of the extracts. However, the inhibitions of against *K. pneumoniae* and *S. pneumoniae* were comparable to gentamycin at a concentration of 100 mg/mL. Variation of the antibacterial activity among extracts and the standard might be the difference in the potential phytoconstituents essential for antibacterial activity.³⁵

Ethyl acetate extract of *Carissa spinarum* showed larger zone of inhibition against *E. coli*, *K. pneumoniae*, *S. pneumoniae*. Besides, the presence of alkaloids, saponins, tannins, flavonoids, phenols, and terpenoids from the phytochemical test result support the broad antibacterial activity of ethyl acetate extract. Furthermore, the phytoconstituents found in ethyl acetate extract might relatively polar compared with the chloroform leaf extract of *Carissa spinarum*. Pooled evidence from different reports revealed that numerous phytoconstituents were isolated from solvent extracts of various parts of *Carissa spinarum* and have been studied for their antimicrobial activity.³⁶

The chloroform extract displayed a somewhat lesser area zone of inhibition compared with the methanol and ethyl acetate leaf extracts of *Carissa spinarum*. This might be because of the lack of some potential phytoconstituents as indicated in our phytochemical screening test (Table 1).

From the bacterial strain tested, *S. aureus* exhibited smaller vulnerability to chloroform leaf extract of *Carissa spinarum*. This might be the inherent drug-resistant properties of the bacterial strain, which hinders the antibacterial activity of the phytoconstituents.³⁷

In the present study, gram-positive bacteria (*S. pneumoniae* and *S. aureus*) were more vulnerable to the all solvent leaf extracts of *Carissa spinarum*. The average zone of inhibitions of the all solvent leaf extracts were larger for gram-

positives compared with gram-negatives at the same concentration. This variation might be due to the presence of special bacterial cell membrane structures in gram-negative strains such as lipopolysaccharide, which prevent the accumulation of phytoconstituents inside the bacteria cell.³⁸

Largely, the presence of alkaloids in *Carissa spinarum* extracts of might possess antibacterial activity. The promising antibacterial action of alkaloids from medicinal plants might due to alteration of bacterial cell structures or inhibition of macromolecules such as bacterial enzymes and other target proteins.³⁹

The current finding, as well as previous reports, revealed the presence of tannins in the extracts of *Carissa spinarum*.³⁶ The antibacterial effect of this phytoconstituent could be due to their detrimental effect on bacterial cell membrane and by promoting uncoupling oxidative phosphorylation that might lead to death of the bacteria.³⁹ The other phytoconstituents identified from the methanol and ethyl acetate leaf extract of *Carissa spinarum* are terpenoids. The possible antimicrobial activity of terpenoids could be related to its disruptive action on bacterial cell structures through causing them to malfunction.^{40,41}

Flavonoids are also the other phytoconstituent from solvent extract of *Carissa spinarum*. The potential antibacterial action of flavonoids encompasses, formation of complex with bacterial cell walls and precipitation of bacterial cell machinery materials such as proteins and genes.^{42,43}

Moreover, all leaf extracts of *Carissa spinarum* were rich in saponins. Previous reports revealed that saponins had multiple antibacterial effects. This effect might be probably due to membrane disruption and leakage of cell components.^{39,44}

Furthermore, the most active leaf extract of *Carissa spinarum* (methanol extract) of was purified and isolated for characterization of the major component present in the extract. The isolated compound was trihydroxypentanoic acid. This isolated compound might contribute to the superior antibacterial activity of methanol leaf extract of the *Carissa spinarum*. This finding is in line with a study done by Ruchira et al in which many compounds were isolated and assessed for their numerous pharmacological activity such as antiherpetic, cytotoxic, antioxidant, and antibacterial effects.³¹ In addition, another report by Mudiganti and Anisha investigated the presence of several compounds that are isolated from the various solvent extract of *Carissa spinarum* plant through GC-MS analysis and their potential medicinal values are under investigation.³²

In general, diverse speculations are set for antimicrobial mode of action of a herbal extract, depending on the phytoconstituent they comprise. The presence of the bioactive compound from a herbal extract leads to death of bacterial via several modes which include disruption of functional and structural proteins that are essential for the survival of the bacteria. Consequently, the overall antimicrobial effect of *Carissa spinarum* could be due to the existence of a higher quantity of phytoconstituent or due to synergistic action from different phytoconstituents.

In conclusion, findings from the current study collectively revealed that the methanol, ethyl acetate, and chloroform extracts of the leaves of *Carissa spinarum* have antibacterial activity against the tested bacterial species. All extracts of *Carissa spinarum* exhibited antibacterial action with a different spectrum of activity. The methanol fraction revealed the maximum antibacterial activity. The antibacterial activity of *Carissa spinarum* because of the existence of phytoconstituents (alkaloids, terpenoids, polyphenols, saponins, and flavonoids), can work either independently or synergistically. Furthermore, a compound was isolated and characterized with the help of IR, ¹H-NMR, ¹³C-NMR and DEPT-135 spectroscopic techniques. The IR, ¹H-NMR, ¹³C-NMR and DEPT-135 spectroscopic data in comparison with estimated value means that the most probable structure of isolated compound was 5-(2', 3', 4', 6' tetrahydroxy-5'-methoxycyclohexyloxy)-2,3,4-trihydroxypentanoic acid.

Abbreviations

¹³C-NMR, carbon-13 nuclear magnetic resonance; ¹H-NMR, proton nuclear magnetic resonance; DEPT, distortionless enhancement by polarization transfer; DRC, Democratic Republic of Congo; IR, infrared spectroscopy; MHA, Mueller-Hinton agar; NMR, nuclear magnetic resonance; TA, trihydroxypentanoic acid; TLC, thin layer chromatography; TM, traditional medicine; TMPs, traditional medicinal practitioners; UoG, University of Gondar; WHO, World Health Organization; DMSO, dimethyl sulfoxide; PPM, parts per million; Rf, retention factor; MS, mass spectrometry; 2D-NMR, two-dimensional nuclear magnetic resonance.

Ethics Approval

Ethical clearance and permission was obtained from University of Gondar Research and Ethical Review Committee.

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Author Contributions

All authors made a significant involvement to the work stated, that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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The authors report no conflicts of interest in this work.

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