

**Phytochemical, antibacterial and antioxidant studies of the leaves of
*Carissa spinarum***¹Dessale Feyissa and ¹Yadessa Melaku*.¹ Applied Chemistry Program, School of Applied Natural Sciences, Adama Science and Technology University, Ethiopia.

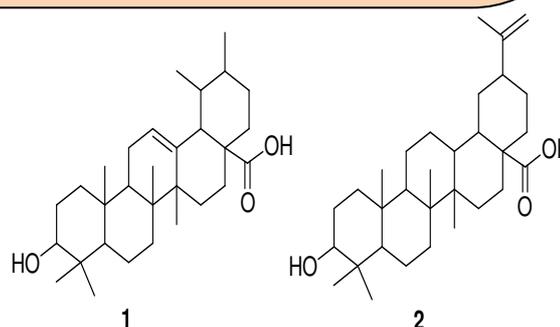
*Corresponding Author: E-Mail: yadessamelaku2010@gmail.com

Received: 16th Nov 2016, Revised and Accepted: 24th Nov 2016**ABSTRACT**

Carissa spinarum (Apocynaceae) is an indigenous plant to Ethiopia traditionally used against gonorrhoea, stomachache, rheumatism, chicken pox, and skin diseases. It is also utilized as wound healing and an antidote to snake-bite. With the aim to find out the active extract or constituents, the leaves of the plant was successively extracted with n-hexane, EtOAc and MeOH to furnish 2.2%, 3.6%, and 7.3% crude extracts, respectively. The EtOAc extract after silica gel column chromatography has led to the isolation of two triterpenes, namely, ursolic and betulinic acid. The structures of these compounds were elucidated using spectroscopic methods including IR, UV and NMR. Both compounds were not yet reported from this plant. The EtOAc extract of the leaves of *C. spinarum* was tested for its *in vitro* antibacterial activity against four strains of bacteria such as *E. coli*, *S. aureus*, *P. aeruginosa* and *P. mirabilis*. The EtOAc extract demonstrated modest antibacterial activity against *S. aureus* with an inhibition zone of 15 mm at 1.5 mg/mL compared to standard drugs. The antibacterial activity displayed by the leaves of *C. spinarum* is likely due to the presence of ursolic and betulinic acid. The radical scavenging activity of the EtOAc extract was also assessed using DPPH and found to inhibit the radical by 5%. The result is much lower than ascorbic acid which inhibit DPPH radical by 90%. The antibacterial activity displayed by the plant corroborates the traditional uses of this plant against bacteria.

Keywords: Phytochemical, Antibacterial, Antioxidant, *C. spinarum*, Ursolic acid, Betulinic acid.**1. INTRODUCTION**

Carissa spinarum belonging to the family Apocynaceae and genus *Carissa* is a small spiny evergreen shrub of tropical deciduous forest. It is an indigenous plant to Ethiopia locally called 'Agamsa' (Afan Oromo) and 'Agam' (Amharic) showing its wide distribution in the country [1]. *C. spinarum* is one of the most important medicinal plants to Ethiopia traditionally used for the treatment of various diseases. The roots and stem barks of this plant has been used in folk medicine for the treatment of wound healing, gonorrhoea, stomachache, headache, rheumatism [2], chicken pox, skin diseases and as an antidote to snake-bite [3]. The leave decoction of *C. spinarum* is also used for the treatment of diabetes, malaria and pneumonia [4]. Pharmacological studies revealed that the stem extracts of *C. spinarum* possess antioxidant, cardiotoxic, hepatoprotective and antipyretic [5].

**Figure - 1: Structures of ursolic acid (1) and betulinic acid (2) isolated from *C. spinarum*.**

Previous phytochemical studies show that the root and stem of *C. spinarum* contains caffeic acid, and carenone [6]. The roots and barks were shown to have sterols, terpenes and cardiac glycosides, whereas sesquiterpenes, phenolic compounds and lignans were reported from the stems. Two lignans viz carissanol and carinol were isolated from the stems of this plant [7-11]. While the roots and stem barks of *C. spinarum* has been

studied, to the best of our knowledge there is no published report on isolation of chemical constituents and evaluation of biological activities of the extracts of its leaves. The objective of this paper is therefore to present the results of the study of the chemical constituents, antibacterial and radical scavenging activities of the EtOAc extracts of the leaves of *C. spinarum*.

2. MATERIALS AND METHODS

2.1. Plant material

The leaves of *C. spinarum* were collected in June, 2016 from Gelila, Limu Woreda, Eastern Wollega, Oromia, Ethiopia. The plant was authenticated by Prof. Legesse Negash and voucher specimen number DF002 was deposited in the National Herbarium of Addis Ababa University.

2.2. Instruments and apparatus

Melting points were recorded using Digital melting point apparatus. Analytical thin layer chromatograms were run on a 0.2 mm thick layer of silica gel GF254 (Merck) coated on aluminium plate. Column chromatography was performed using silica gel (230-400 mesh) Merck. NMR spectra were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz. The ultraviolet visible spectra were taken on T60 UV-Visible spectrophotometer (200-600 nm). Infrared (IR) spectra were obtained on Perkin-Elmer 65FT ((IR ν_{\max} KBr (4000-400) cm^{-1}) infrared spectrometer using KBr pellets.

2.3. Phytochemical Screening

Chemical screening for the presence of secondary metabolites including tannins, saponins, alkaloids, terpenoids, flavonoids, cardiac glycosides, anthraquinones and phenols were done according to the previous protocols reported in the literature [12-14].

2.4. Extraction and isolation

The powdered leaves of *C. spinarum* (235 g) were successively extracted with n-hexane (800 mL), EtOAc (1 L) and MeOH (1.2 L) each for 2 days at room temperature, filtered, and concentrated to afford 5.21 g (2.2%), 7.2 g (3.06%), and 17.3 g (7.362 %) dark green solids of the corresponding solvent extracts, respectively. The EtOAc extract (6.2 g) was adsorbed and subjected to silica gel (150 g) column chromatography using n-hexane for packing. The column was eluted with n-hexane:EtOAc of increasing polarities to afford 27 fractions which were combined based on their TLC profile to give six combined fractions. Volumes of 100 mL each were collected. Fr1-8, Fr9-14, Fr15-16, Fr17-22, Fr23-24 and Fr25-27 were eluted with hexane:EtOAc 4:1, 3:2, 1:1, 2:3,

1:9, and 0:1, respectively. Fractions eluted with Hexane:EtOAc (1:1) was recrystallized in hexane to furnish compound **1** (31 mg) while compound **2** (15 mg) was obtained from hexane:EtOAc (1:9)

Compound 1: Pale yellow solid (31 mg); M.p 277-280°C; IR ν_{\max} KBr (4000-400) cm^{-1} , 3435 (OH), 2936 (C-H), 1709 (-COOH), 1630 (C=C), 1384 (C-C); UV/Vis showed no absorption maxima; $^1\text{H-NMR}$ (400 MHz, CDCl_3 , δ_{H} (ppm)): 0.92, 0.93, 1.28, 1.29, 1.64, 0.77 and 0.79 (21H, all CH_3), 1.62 (*m*, 2H), 1.27 (*m*, 2H), 1.94 (*m*, 2H), 1.94 (*m*, 1H), 2.11 (*m*, 2H, H), 2.19 (*m*, 2H), 2.19 (*m*, 1H), 2.35 (*m*, 2H), 2.36 (*m*, 1H), 2.38 (*m*, 1H), 3.25 (*m*, 1H), 1.95 (*m*, 2H), 1.63 (*t*, 2H), 1.94 (*m*, 2H), 4.13 (*m*, 1H) and 5.27 (*m*, 1H). $^{13}\text{C-NMR}$ (100MHz, CDCl_3 , δ_{C} (ppm)): 39.0 (C-1), 28.1 (C-2), 79.0 (C-3), 39.4 (C-4), 55.2 (C-5), 18.2 (C-6), 32.9 (C-7), 39.1 (C-8), 47.9 (C-9), 37.0 (C-10), 16.9 (C-11), 125.8 (C-12), 137.9 (C-13), 41.9 (C-14), 29.3 (C-15), 24.1 (C-16), 47.5 (C-17), 52.6 (C-18), 38.8 (C-19), 38.7 (C-20), 31.5 (C-21), 36.9 (C-22), 29.7 (C-23), 15.4 (C-24), 14.1 (C-25), 17.0 (C-26), 23.5 (C-27), 177.9 (C-28), 22.6 (C-29), 21.1 (C-30).

Compound 2: White solid (15.1 mg); IR ν_{\max} KBr (4000-400) cm^{-1} , 3435(OH), 2928(C-H), 1710(-COOH), 1630 (C=C), 1469 (C-C); UV/Vis showed no absorption maxima; $^1\text{H-NMR}$ (400MHz, CDCl_3): 0.66, 0.79, 0.90, 1.03, 1.22, and 1.26-1.27 (18H, all CH_3), 1.27 (*m*, 2H), 1.30 (*m*, 2H), 1.30 (*m*, 2H), 1.41 (*m*, 2H), 1.41 (*m*, 2H), 1.44 (*m*, 2H), 1.47 (*m*, 2H), 1.47 (*m*, 2H), 1.50 (*t*, 2H), 1.53 (*m*, 2H), 1.78 (*m*, 1H), 1.78 (*m*, 1H), 1.83 (*m*, 1H), 1.89 (*s*, 1H), 1.92 (*m*, 1H), 4.32 (*m*, 1H) and 4.35 (*m*, 2H); $^{13}\text{C-NMR}$ (100MHz, CDCl_3): δ_{C} (ppm) 38.8 (C-1), 24.2 (C-2), 77.3 (C-3), 38.9 (C-4), 52.8 (C-5), 18.4 (C-6), 36.7 (C-7), 39.2 (C-8), 47.4 (C-9), 36.9 (C-10), 23.2 (C-11), 23.7 (C-12), 38.8 (C-13), 40.4 (C-14), 28.7 (C-15), 33.1 (C-16), 55.2 (C-17), 47.2 (C-18), 138.6 (C-20), 38.6 (C-21), 27.9 (C-22), 27.4 (C-23), 16.5 (C-24), 17.3 (C-25), 17.4 (C-26), 15.6 (C-27), 178.80 (C-28), 21.53 (C-30).

2.5. Studying of antibacterial activities

The anti-bacterial activity of ethyl acetate extract of the leaves of *C. spinarum* was done *in vitro* using the American Test Culture Collection (ATCC) bacterial strains. Four bacterial strains were obtained from Oromia Public Health Research, Capacity Building and Quality Assurance Laboratory Center, Adama, Ethiopia. Three Gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35032, *Proteus mirabilis* ATCC 25923) and one gram-positive (*Staphylococcus aureus* ATCC 25923) were selected for the study. The antibacterial activities were determined using well diffusion method against different strains of bacteria [15].

Ciprofloxacin and Tetracycline were used as a positive control in the study.

2.5.1. Media preparation

Mueller Hinton agar was prepared by dissolving the solid media in distilled water. The solution was then sterilized in autoclave at 121 °C for 15 minutes, cooled then poured in Petri dishes. The solution was then left to solidify.

2.5.2. Inoculation and incubation

Antibacterial activity was done on Mueller Hinton agar. 1 mL of bacteria suspension was uniformly spread on the sterile Mueller Hinton Agar Petri dish. 0.1 g of the sample was dissolved in 1 mL of chloroform. 0.5 mg/mL and 1.5 mg/mL concentrations of the sample were prepared. 6 mm-diameter wells were cut from the agar using a sterile cork-borer and both concentrations of the sample were placed in the wells. The petri dish was then placed in an incubator for 24 hours at 37 °C. At the end of incubation period, the inhibition diameter was measured and expressed in millimeters. 3 drops of each bacterial suspension were applied on the Petri dish to compare with 1 drop of chloroform applied on it. Ciprofloxacin and Tetracycline antibiotic standards were used as a positive control group reference drug. Chloroform was used as a negative control group. Control for each bacterial strain, Ciprofloxacin, Tetracycline and chloroform before sample action was at 6 mm. Antibacterial activity was determined by measuring the inhibition zone diameter (mm) against each test organism.

2.6. DPPH assay

The radical scavenging activities of the EtOAc extract of the leaves of *C. spinarum* were done according to [16]. A stock solution of 12.5 mg/mL of the plant extract was prepared. 1 mL from the stock solution was mixed with 4 mL of

0.004 % of 2,2-diphenyl-1-picryl hydrazyl (DPPH) in methanol in a brown vials. After an incubation period of 30 min at 37°C in an oven, the absorbance was determined against a blank at 517 nm¹⁷. The percent of DPPH discoloration of the samples was calculated according to the formula [18]:

$$(\%) \text{ inhibition} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ control}} \times 100$$

Where A control was the absorbance of the DPPH solution and A sample was the absorbance in the presence of plant extract. Samples were analyzed in triplicate. Ascorbic acid was used as positive control.

3. RESULTS AND DISCUSSION

3.1. Extraction yield

The ground leaves of *C. spinarum* were successively extracted with n-hexane, EtOAc, and MeOH to afford 2.2% hexane, 3.6% EtOAc and 7.3% MeOH extracts. The TLC profile of the EtOAc extract was found promising with the spots visualized after dipping in vanillin-H₂SO₄ followed by heating on heating mantle.

3.2. Phytochemical screening

Results of the phytochemical screening (Table 1) revealed that the n-hexane extract of the leaves of *C. spinarum* contains alkaloids, terpenoids, flavonoids and glycosides whereas tannins, saponins, anthraquinones and phenols were not detected. On the other hand, the EtOAc extract contains tannins, alkaloids, terpenoids, glycosides and anthraquinones. The presence of these secondary metabolites in the leaves of this plant is significant as they are reported as a remedy for the treatment of a wide array of diseases. The traditional uses of this plant against gonorrhoea, stomachache, headache and as wound

Table - 1: Phytochemical analysis of n-hexane and EtOAc extracts of the leaves of *C. spinarum*.

Phytochemical	Tests/methods	n-hexane extract	EtOAc extract
Tannins	Ferric Chloride test	-	+
	Gelatin test	-	+
	Potassium hydroxide test	-	+
Saponins	Froth test	-	-
Alkaloid	Wagner's test	+	+
Terpenoids	Salkowski Test	+	+
Flavonoids	Sodium Hydroxide Test	+	-
	Salkowski Test	+	-
Glycosides	Borntrager's test	+	+
Anthraquinones	Bontrager's test	-	+
Phenols	10% FeCl ₃	-	-

+ indicates presence and - indicates absence

healing activity are likely due to the presence of terpenoids in the extracts. Hence, the results support the use of the leaves of *C. spinarum* for the treatment of diseases by local herbalists or traditional healers.

3.3. Antibacterial Activities

The EtOAc extract was assessed for its antibacterial activity using disc diffusion method. The results of zone of inhibition in diameter against the four tested pathogens (*E. coli*, *S. aureus*, *P. aeruginosa* and *P. mirabilis*) and the standard antibiotics are summarized in Table 2. Results showed that none of the bacterial strains had shown positive result at the sample concentration of 0.5 mg/mL. However, the extract displayed zone of inhibition from 6-15 mm at 1.5 mg/mL for all bacterial strains used in this study. Strong activity was observed against *S. aureus* with zone of inhibition of 15 mm at 1.5 mg/mL compared with the other three strains of bacteria (Figure 2). The result is modest compared with ciprofloxacin and tetracycline using as standard drugs.

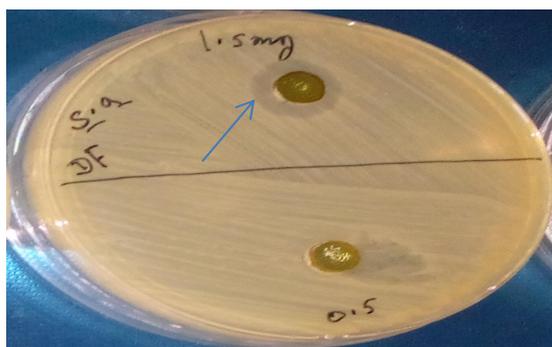


Figure - 2: Antibacterial activity of the EtOAc extract of leaves of *C. spinarum* against *S. aureus* using disc diffusion method.

The leaves of *C. spinarum* are traditionally used to treat various diseases caused by bacteria. Hence, the result obtained in the present study supports the traditional uses of *C. spinarum* against diseases caused by bacteria. Many literature reports showed that ursolic and

betulinic acids are used as antibacterial agents¹⁵⁻¹⁶. Hence the antibacterial activity displayed by the leaves of this plant is likely ascribed to the presence of ursolic and betulinic acids.

3.4. Antioxidant Activities

DPPH radical scavenging assay is a simple method for finding antioxidants by recording absorbance at 517 nm^[17]. A decrease in absorbance is observed when the radical is scavenged by antioxidants. The DPPH assay indicated that the EtOAc extract of *C. spinarum* leaves displayed low free radical scavenging activity with percent inhibition of 5% at 2.5 mg/mL. Many literatures showed that phenolics and flavonoids are among secondary metabolites responsible for scavenging radicals since most of them have hydroxyl on aromatic ring. This agrees very well with the present study as the chemical screening test revealed the absence of phenolics and flavonoids in the EtOAc extract of the leaves of *C. spinarum*. Therefore, the extract of the leaves of *C. spinarum* was not significant to decrease the free radical compared to ascorbic acid using as standard drug.

3.5. Structural Elucidation of Isolated Compounds

Compound 1: Compound 1 was obtained as a white solid (31 mg) recrystallized from hexane. Its TLC showed one spot at R_f 0.70 with n-hexane:ethyl acetate (1:1) as a mobile phase. The UV/Vis spectrum showed no absorption maxima indicating the absence of conjugated chromophore in the compound. In the IR (KBr) spectrum of **compound 1**, the absorption band at 3435 cm^{-1} indicated the presence of hydroxyl group. The presence of C-H stretching of methyl group is evident at 2936 cm^{-1} . The absorption band at 1709 and 1630 cm^{-1} were ascribed to C=O and C=C stretching, respectively. Furthermore, the presence of C-C stretching is evident at 1384 cm^{-1} .

The proton (Appendix 1), ¹³C-NMR (Appendix 2) and DEPT-135 spectra of compound **1** were compared with the literature reported for ursolic acid and found in good agreement^[18-19].

Table - 2: Inhibition zone of the EtOAc extract, positive and negative controls

Extract/control	Concentration of extract and standard antibiotic (mg/mL)	Zone of inhibition (mm)			
		<i>E. coli</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>
EtOAc extract	0.5	6	6	6	6
	1.5	6	15	6	6
Ciprofloxacin		31	32	30	33
Tetracycline		24	27	26	25
Chloroform		0	0	0	0

No inhibition zone was observed for CHCl_3 used as the negative control

Compound 1 (Figure 1) was not yet reported from *C. spinarum*. Ursolic acid was previously reported from *Rosmarinus officinalis*, *Glechoma hederaceae*, *Ilex paraguariensis*, *Ichnocarpus frutescens*, *Phoradendron juniperinum*, *Syzygium claviflorum*, *Hyptis capitata* and so on. It is a constituent of several herbal medicines marketed in Asia and worldwide for inflammatory conditions. Its anti-bacterial, anti-fungal, anti-cancer, anti-platelet aggregation and anti-mycotic [15] properties were well documented.

Compound 2: Compound 2 was obtained as white solid (15.1 mg). Its TLC showed one spot at R_f 0.64 with n-hexane:ethyl acetate (1:1) as a mobile phase. The UV-Vis spectrum showed no absorption maxima indicating absence of conjugation in the structure of the molecule. In the IR (KBr) spectrum of the compound 2, the presence of O-H stretching is evident at 3435 cm⁻¹. Stretching vibrations of due to aliphatic C-H groups were observed at 2928 cm⁻¹. The diagnostic signal at 1710 cm⁻¹ due to the presence of C=O stretching of carboxyl group. The presence of C=C is evident at 1630 cm⁻¹. The absorption band at 1469 cm⁻¹ showed the presence of C-C stretching.

The NMR spectral analysis of compound 2 was in agreement with the literature reported for betulinic acid [20-24] (Figure 1). This compound was not yet reported from the genus *Carissa*. Betulinic acid is a known triterpenoid isolated from various organs and species of plants, including flowering *Eugenia*. This metabolite shows inhibitory activity on growth of human melanoma cells, and replication of the AIDS virus. It has also antibacterial property [16]. Therefore the antibacterial activity displayed by the plant is likely due to the presence of betulinic acid.

4. CONCLUSION

In conclusion, the phytochemical study conducted on the leaves of *C. spinarum* after silica gel column chromatography has led to the isolation of two triterpenes: ursolic acid and betulinic acid. Both of these compounds were not been reported from this species. Antibacterial study of the ethyl acetate extract of the plant exhibited modest activity against *S. aureus* compared to standard drugs. The presence of ursolic and betulinic acid in the extract of the leaves of *C. spinarum* adds one positive attributes to the plant as these compounds were reported to possess a wide spectrum of activity including anti-bacterial, anti-fungal, anti-cancer, anti-platelet aggregation and anti-mycotic. Furthermore the absence of phenolics and flavonoids may accounts for the low radical scavenging activity of the EtOAc extract of the leaves of *C. spinarum*.

Therefore the results obtained in the present study corroborate the traditional use of this plant against diseases caused by bacteria.

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