PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE LEAF EXTRACTS OF *Carissa edulis*

Supported by tetfund TERTIARY EDUCATION TRUST FUND

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Received: March 29, 2021 **Accepted:** May 19, 2021

Abstract: *Carissa edulis* leaf extracts were tested against six clinical isolates of *Aspergillos fumigatus, Candida albicans, Trichophyton mentagrophytes, Escherichia coli, Staphylococcus aureus* and *Streptococcus pyogenes*. The extracts were obtained by successive extraction with hexane, ethyl acetate and methanol using Soxhlet apparatus. The results showed that the methanol extract had the highest activity among the three extracts. the methanol extract of *C. edulis* was active on five of the organisms that were tested against except for *A. fumigatus*, the ethyl acetate extracts of *C edulis* was active on only two fungi, that is *C. albicans* and *T. mentagrophytes* out of the six organisms, while the hexane extract of *C. edulis* was active on two bacteria which are *E. coli* and *S. aureus* only, *A. fumigatus* was not sensitive to all the extracts of *C. edulis*. *E. coli* was the most sensitive to the methanol extract with MIC 12.5 mg/cm³, *Staphylococcus aureus* and *Candida albicans* had MIC 25 mg/cm³, while *Streptococcus pyogenes* and *Trichophyton mentagrophytes* had MIC 50 mg/cm³. Therefore, *Carissa edulis* is a good antimicrobial agent against bacteria and fungi that cause skin infections.

Keywords: Antimicrobial, *Carissa edulis*, leaf extracts, phytochemicals

Introduction

Herbal medicine or phytomedicine is recognized as the most common form of alternative medicine (Ogbonnia *et al*., 2011). Over the years, the use of herbs in the treatment of illnesses has been very successful and its historic usage has been useful in drug discovery and development. Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases, this practice being an alternative way to compensate for some perceived deficiencies in orthodox pharmacotherapy (Zhu *et al*., 2002). Traditional healing systems around the world that utilize herbal remedies are an important source for the discovery of new antibiotics (Okpekon *et al.*, 2004); some traditional remedies have successfully been used against antibiotic-resistant strains of bacteria (Kone *et al.*, 2004). There is high resistance to the current existing drugs use in the treatment of microbial infection. Plants are the cheapest and safer alternative sources of antimicrobials (Sharif and Banik, 2006; Doughari *et al*., 2007). *Carissa edulis* (Vahl.) belongs to the family Apocynaceae. The plant is native in many countries such as Australia, Cambodia, Cameroon, Eritrea, Ethiopia, Ghana, Guinea, Japan, Kenya, Nigeria, etc. Many members are woody climbers found in the tropics and subtropics (Evans, 2009). Several classes of chemical constituents have been isolated from the genus *Carissa* (Dharani, 2010), such as, sesquiterpenes, cardiac glycosides, phenolic compounds and Lignans (Kirira *et al.*, 2006; Wang and Likhitwita, 2009).

The Fruit (ripe and unripe), and flowers of *Carissa edulis* are edible. The English name of the plant is carisse, other common names include; 'endelkoring-noeminoem' (Africana), 'agam' (Tigrigna and Amharic), 'emir' (Arabic) (Sofowora, 1986). The plant is commonly known among Hausa people in Northern Nigeria as 'cizaki' and in Somalia as 'adishawel' (Oliver, 1996). *C. edulis* has many traditional uses; its fruit is edible, while its pungent root is used in Ethiopia for a variety of medicinal purposes. These include rheumatism (Giday, 2001), headache, gonorrhoea, syphilis, rabies and as a diuretic (Addis *et. al.*, 2001). Thus there are no adverse effects that have ever been reported on *C. edulis* as herbal medicines. The folkloric uses of *C. edulis* in Kenya include fever, sickle cell anaemia and hernia (Yako, 1992; Ibrahim, 1997).

The plant *C. edulis* is reported in traditional medicine as a good source of medicine for treatment of skin infections, acotoparasitic diseases, abdominal problems, headache and sexually transmitted diseases (Omino, 1993). Previous researches on the leaves and fruits of the plant showed the presence of carbohydrates, tannins, flavonoids, saponins, cardiac glycosides, steroids and triterpenes (Ibrahim and Bolaji, 2002; Ibrahim *et al.*, 2005).

Materials and Methods

Collection, identification and preparation of plant material

The leaves of *Carissa edulis* was collected from Kudingi forest, Sabon Gari Local Government Area, Zaria, Kaduna State in the month of August 2016. It was taken to the Herbarium unit of the Department of Botany, Ahmadu Bello University, Zaria for proper identification and authentication and a voucher specimen number 601 was given. The leaves of the plant collected was dried under shade at room temperature, pulverized to powder using clean agate motar and pestle, the powder was stored in an air tight container for subsequent use.

Extraction

Extraction of the plant material was done using the method described by (Kokate *et al.*, 2003). The pulverized plant material (1 kg) was extracted with 2.5 dm^3 of n-hexane, ethyl acetate and methanol successively in a Soxhlet apparatus. The extracts obtained were concentrated via rotary evaporator to recover some solvent and final evaporation to dryness of the extracts was done via the water bath after which each extract was weight and stored in desiccator for subsequent use.

Phytochemical screening

The leaf extracts (hexane, ethyl acetate and methanol) were subjected to phytochemical screening in order to identify the phytochemical constituents present in the extract; this was carried out according to methods described by Sofowora (2008) and Evans (2009).

Test for carbohydrates

(i) Molisch's test: To 0.5 g of the extracts (hexane, ethyl acetate and methanol) was separately dissolved in 5 cm^3 of distilled water each and filtered. The filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube, followed by 1 cm^3 of concentrated H_2SO_4 down the side of the test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates (Evans, 2009).

(ii) Fehling's Test 1: To 0.5 g of the extracts (hexane, ethyl acetate and methanol) was separately dissolved in 5 cm^3 of distilled water each and filtered. The filtrates were hydrolysed

with dilute HCl, neutralized with alkali and heated with mixture of equal volume of Fehling's A & B solutions. Formation of brick red precipitate indicates the presence of combined reducing sugars (Evans, 2009).

(iii) Fehling's test 2: To 0.5 g of the extracts (hexane, ethyl acetate and methanol) was separately dissolved in 5 cm^3 of distilled water each and filtered, 2 cm^3 of the filtrates was added equal volume of Fehlings solution A and B and boiled in a water bath for two minutes, a brick red precipitates indicates the presences of free reducing sugar (Evans, 2009).

Test for tannins

(i) Ferric chloride test: To 0.5 g of the extracts (hexane, ethyl acetate and methanol) was dissolved in 5 cm^3 of water each and filtered. Two drops of ferric chloride solution was added to the filtrate. Appearance of blue-black (hydrolysable tannins) or green or blue-green (condensed tannins) precipitate indicates the presence of tannins (Evans, 2009).

(ii) Lead sub-acetate test: To 0.5 g of the hexane extract, 2 cm^3 of ethanol was added followed by three drops of lead sub-acetate solution; appearance of whitish-yellow precipitate indicates the presence of tannins. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2009).

Test for anthraquinones

(i) Bontrager test: To 0.5 g of the hexane extract, 10 cm^3 of chloroform was added and shaken. This was then filtered and 5 cm^3 of 10% ammonia solution was added to the filtrate. The presence of pink or cherry red colour in the upper layer indicates the presence of anthracenes. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2009).

(ii) Modified Borntrager's test: To 0.5 g of the (hexane, ethyl acetate and methanol) was boiled with 10 cm^3 of aqueous sulphuric acid and filtered while hot. The filtrate after cooling to room temperature was shaken with 5 cm^3 chloroform, the chloroform layer was separated and half of its volume, 10% ammonium hydroxide was added. A pink, red or violet colouration in the ammonia phase (upper phase) is an indication for the presence of combined anthracene or anthraquinone derivatives (Evans, 2009).

Test for flavonoids

(i) Shinoda test: To 0.5 g of the (hexane, ethyl acetate and methanol) each was dissolved in water, 2cm^3 of 50% methanol. Pieces of magnesium chips and 3 drops of hydrochloric acid was added, a pink, rose or red colouration indicated the presence of flavonoids (Evans, 2009).

(ii) Sodium hydroxide test: To 0.5 g of the (hexane, ethyl acetate and methanol) was dissolved in water and filtered. 2 cm^3 of 10% aqueous sodium hydroxide solution was then added. The solution was observed for the presence of yellow colour. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was used as an indication for the presence of flavonoids (Evans, 2009).

Test for saponins

(i) Frothing test: To 0.5 g of the hexane extract was dissolved in 10 cm^3 of water and shaken vigorously for 30 seconds and allowed to stand for one hour, the occurrence of frothing column of honey comb-like of at least 1 cm in height and persisting for at least 30 min indicates the presence of saponins. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

Test for cardiac glycosides

(i) Keller-Killiani test: To 0.1 g of the hexane extract was dissolved in glacial acetic acid containing ferric chloride and 1 cm^3 of sulphuric acid was added to the solution. The appearance of reddish-brown colouration at the interphase indicates the presence of deoxy-sugar. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

(ii) Kedde's test: To 0.1 g of the hexane extract was treated with 1 cm^3 of 2% solution of 3, 5- di-nitro-benzoic acid in 95% alcohol. The solution was made alkaline by the addition of 5% NaOH. The presence of purple-blue colour indicates the presence of cardenolides. The procedure above was repeated for ethyl acetate and methanol extracts (Evans, 2009).

Test for steroids/triterpenes

(i) Liebermann-Burchard test: 1 cm^3 of acetic anhydride was added to 0.1 g of the hexane extract dissolved in 1 cm^3 of chloroform. Concentrated sulphuric acid was then added gently by the side of the test tube to form lower layer and at the junction of the two liquids, formation of reddish brown or violet brown ring, the upper layer bluish green or violet indicates the presence of steroids or triterpenes. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

(ii) Salkowski test: 2 cm^3 of chloroform was added to 0.1g of the hexane extract and 1 cm^3 of concentrated sulphuric acid was carefully added to the side of the test tube to form a lower layer. A reddish brown coloration at the interphase indicated the presence of steroidal nucleus. The procedure above was repeated for ethyl acetate and methanol extracts (Sofowora, 2008).

Test for alkaloids

To (1.0 g) of the Hexane extract was stirred with 20 cm^3 of 1% aqueous hydrochloric acid on water bath and filtered. The filtrate was basified with concentrated NH_4OH and extracted with chloroform. The chloroform layer was then extracted with 5 cm^3 of 1% HCl. The aqueous layer was divided into four portions for the following tests: To the first portion, 1 cm^3 of freshly prepared Dragendorff's reagent was added drop-wise and observed. To the second portion 1 cm^3 of Mayer's reagent was added drop-wise and observed. To the third, 1 cm^3 of Wagner's reagent was also added. The fourth portion was used as control. Appearance of orange-red, yellowish or cream colour and brown or reddish–brown precipitates respectively indicates the presence of alkaloids. Ethyl acetate and methanol extracts were treated similarly (Evans, 2009).

Antimicrobial activity

The antimicrobial activity of the leaf extracts of *Carissa edulis* was carried out on some fungi and bacteria that causes skin infections. The test organisms were clinical isolates of bacteria: *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes* and fungi: *Aspergillus fumigatus, Candida albicans, Trichophyton mentagrophytes* that were obtained from Microbiology laboratory, Ahmadu Bello University Teaching Hospital, Shika, Zaria.

Preparation of media plates for the antimicrobial sensitivity testing

The culture media used for the analysis were Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth (SDB). The media were used for sensitivity test, determination of minimum inhibitory concentration. (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) All media were prepared according to manufacturer's instruction and sterilized by autoclaving at 121°C for 15 min.

The standardized inoculum was prepared using normal saline for both the bacteria and fungi, the spore suspension was prepared with normal saline and 0.05% Polysorbates 80, and the isolates were flooded on sterilized Mueller Hinton agar plates for bacteria and Sabouraud dextrose agar plates for the fungi. Four wells were punched on each inoculated agar plate with a sterile cork borer of 6.0 mm diameter. The wells were properly labelled according to different concentrations of the extract prepared which were 100, 50, 25 and 12.5 mg/cm^3, respectively. Each well was filled up with 100 µl of the

extract aseptically. Positive controls were set up using ciprofloxacin for bacteria and fluconazole for fungi. Negative controls were also set up. The inoculated plates with the extract were allowed to stay on the bench for one hour and this was to enable the extract to diffuse on the agar. The plates were incubated at 37°C for 24 h (plates of Mueller Hinton agar) while the plates of Sabouraud dextrose agar were incubated at 30°C temperature for 4 days. At the end of incubation period, the plates were observed for any evidence of inhibition which appeared as a clear zone that is completely devoid of growth around the wells (zone of inhibition). The diameter of the zones were measured using a transparent ruler calibrated in millimetre and the results was recorded (EUCAST, 2000; Azoro, 2002; Alikwe *et al.*, 2013).

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the extract was determined by using two fold serial dilution methods with melted Mueller Hinton agar and melted Sabouraud dextrose agar at 40°C used as a diluent. The agar dilution method involves the incorporation of double strength concentrations of the extract into a double strength of agar medium (molten agar medium), using double dilutions starting from (100, 50, 25, 12.5, 6.25, 3.25, 1.56, 0.78, 0.39, 0.19 mg/cm³) followed by the inoculation of a standardized microbial inoculum onto the agar plate surface on a sterile filter paper disc. The MIC end point was recorded as the lowest concentration of the extract that completely inhibits growth after 24 h of incubation period at 37°C for bacteria and 4 days incubation period at 30 °C for the fungi (EUCAST, 2000; Azoro, 2002; Alikwe *et al.*, 2013).

Determination of minimum bactericidal/fungicidal concentration (MBC/MFC)

The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bactericidal/fungicidal concentration (MBC/MFC) of the extract. A sterilized wire loop was used to pick up the filter paper disc which showed no sign of growth and was placed in a test tube containing MHB or SDB prepared with 0.3% egg lecithin and 1% polysorbate 80 with a clear turbidity. The test tubes were covered and incubated at 37°C for 24 h and 30 °C for 4 days for the bacteria and fungi respectively. At the end of the incubation period, the tubes were examined/observed for the presence or absence of growth using turbidity as a criterion, the tubes that had no visible sign of growth (turbidity) were considered to be the minimum bactericidal/fungicidal concentration (MBC/MFC) and the result was recorded, this was to determine whether the antimicrobial effects of the extract was bacteristatic/fungistatic or bactericidal/fungicidal (EUCAST, 2000; Alikwe *et al.*, 2013).

Results and Discussion

Phytochemical screening of leaf extracts of *C. edulis* is shown on Table 1 below. The hexane extract indicated positive test to only steroids/triterpenes, the ethyl acetate extract showed positive test to carbohydrates, tannins, flavonoids, saponins, cardiac glycosides and steroids/triterpenes. While the methanol extract indicated positive test to carbohydrates, tannins, flavonoids, saponins, cardiac glycosides, steroids/triterpenes and alkaloids. The antimicrobial activities of the hexane, ethyl acetate and methanol extracts of *Carissa edulis* were tested on six clinical isolates of *Aspergillus fumigatus, Candida albicans, Trichophyton mentagrophytes, Escherichia coli, Staphylococcus aureus* and *Streptococcus pyogenes*. From the results obtained, methanol extracts of *Carissa edulis* had the highest activities against most of the organisms among the three extracts, It was also observed that the higher the concentration, the higher the activities of the extract. *E.coli* was the most sensitive to the methanol extract with MIC 12.5 mg/cm³ and *Staphylococcus aureus* and *Candida albicans* had MIC 25 mg/cm³, while *Streptococcus pyogenes* and *Trichophyton mentagrophytes* have MIC 50 mg/cm³, *A. fumigatus* was not sensitive to all the extracts of *C.edulis*. The results for the zones of inhibition, minimum inhibitory concentrations and the minimum bactericidal/fungicidal concentrations for the hexane, ethyl acetate and methanol extracts of *Carissa edulis* were presented in Tables 2 – 7.

Table 1: Phytochemical constituents of leaf extracts of *C. edulis*

TEST	INFERENCE		
	Hexane Extract	Ethyl acetate Extract	Methanol Extract
Carbohydrates			
Molischs test	-	+	+
Fehlings test	-	+	+
Tannins			
Ferric chloride	-	+	+
Lead acetate test	-	+	+
Goldbeaters skin test	-	+	+
Anthraquinones			
Borntrager	-	-	-
Modified Borntrager	-	-	-
Flavonoids			
Shinoda	-	+	+
Sodium Hydroxide	-	+	+
Saponins			
Frothing	-	-	+
Haemolysis	-	-	+
Cardiac glycosides			
Keller- kiliani	-	+	+
Kedde test	-	+	+
Steroids/Triterpenes			
Liebermann-Burchard	+	+	+
Salkowski test	+	+	+
Alkaloids			
Dragendorff	-	-	-
Mayers	-	-	-
Wagners	-	-	-

(- = Absent) (+ = Present)

Table 2: Zones of inhibition produced by hexane, ethyl acetate and methanol extracts of *Carissa edulis* and standard antimicrobial agents in MHA (mm)

Organisms	HE 10mg/100µl	EE 10mg/100µl	ME 10mg/100µl	CF 10µg/100µl
E. coli	13	0	20	27
S. aureus	18	0	18	30
S. pyogenes	0	0	12	21

HE - Hexane Extract, EE - Ethyl acetate Extract,
ME - Methanol Extract, CF - Ciprofloxacin 10 µg/ml,
MHA - Muellar Hinton Agar

Table 3: Zones of inhibition produced by hexane, ethyl acetate and methanol extracts of *Carissa edulis* and standard antimicrobial agents in SDA (mm)

Organisms	HE 10mg/ 100µl	EE 10mg/ 100µl	ME 10mg/ 100µl	FC 2.5µg/ 100µl
A. fumigates	0	0	0	13
C. albicans	0	9	19	27
T. mentagrophytes	0	12	14	33

HE - Hexane Extract, EE - Ethyl acetate Extract,
ME - Methanol Extract, FC - Fluconazole 25 µg/ml,
SDA - Sabouraud Dextrose Agar

Table 4: Minimum inhibitory concentration (MIC) of test antimicrobial agents (*Carissa edulis* extracts) and standard agents against test bacteria (mg/cm³)

Organism	HE mg/cm³	EE mg/cm³	ME mg/cm³	CF µg/ml
E. coli	50.00	>100.00	12.50	0.31
S. aureus	50.00	>100.00	25.00	0.16
S. pyogenes	>100.00	>100.00	>100.00	1.25

HE - Hexane Extract, EE - Ethyl acetate Extract,
ME - Methanol Extract, CF - Ciprofloxacin 10 µg/ml

Table 5: Minimum inhibitory concentration (MIC) of test antimicrobial agents (*Carissa edulis* extracts) and standard agents against test fungi (mg/cm³)

Organisms	HE mg/cm³	EE mg/cm³	ME mg/cm³	FC µg/ml
A. fumigates	>100.00	>100.00	>100.00	5.00
C. albicans	>100.00	50.00	25.00	0.63
T. mentagrophytes	>100.00	50.00	50.00	0.63

HE - Hexane Extract, EE - Ethyl acetate Extract,
ME - Methanol Extract, FC - Fluconazole 25 µg/ml

Table 6: Minimum bactericidal/fungicidal concentration (MBC/MFC) of test antimicrobial agents (*Carissa edulis* extracts) and standard agents against test bacteria and fungi

Organisms	H E mg/cm³	E E mg/cm³	M E mg/cm³	CF µg/ml
E. coli	>100.00	>100.00	25.00	1.25
S. aureus	100.00	>100.00	50.00	0.31
S. pyogenes	>100.00	>100.00	100.00	2.50

HE - Hexane Extract, EE - Ethyl acetate Extract,
ME - Methanol Extract, CF - Ciprofloxacin 10 µg/ml,
FC - Fluconazole 25 µg/ml.

Table 7: Minimum bactericidal/fungicidal concentration (MBC/MFC) of test antimicrobial agents (*Carissa edulis* extracts) and standard agents against test bacteria and fungi

Organisms	H E mg/cm³	E E mg/cm³	M E mg/cm³	FC µg/ml
A. fumigates	>100.00	>100.00	>100.00	10.00
C. albicans	>100.00	>100.00	50.00	1.25
T. mentagrophytes	>100.00	100.00	100.00	2.50

HE - Hexane Extract, EE - Ethyl acetate Extract,
ME - Methanol Extract, CF - Ciprofloxacin 10 µg/ml,
FC - Fluconazole 25 µg/ml

The phytochemical screening of the leaf extracts showed the presence of secondary methabolites which are tannins, saponins, flavonoids, cardiac glycosides, steroids/triterpenes and alkaloid. The hexane extract show the presence of only one compound, the methanol extract contained more compounds than the ethyl acetate extract. The presence of these compounds could be said to be responsible for the antimicrobial activity of the plant. The antimicrobial activities of the hexane, ethyl acetate and methanol extracts of *Carissa edulis* were tested on six clinical isolates of *Aspergillus fumigatus, Candida albicans, Trichophyton mentagrophytes, Escherichia coli, Staphylococcus aureus* and *Streptococcus pyogenes*. The results showed that the methanol extract had the highest activity compared to the three extracts and this could be as a result of many compounds with different polarites that are present in the plant. the methanol extract of *C. edulis* was sensitive to five of the organisms that were tested against except for *A. fumigatus*, the hexane extract of *C. edulis* was active on two bacteria which are *E. coli* and *S. aureus* only, the ethyl acetate extracts of *C edulis* was active on only two fungi, that is *C. albicans* and *T. mentagrophytes* out of the six organisms. *E. coli* was the most sensitive to the methanol extract with MIC 12.5 mg/cm³ and *A. fumigatus* was not sensitive to all the extracts of *C. edulis*, *Staphylococcus aureus* and *Candida albicans* have MIC 25 mg/cm³, while *Streptococcus pyogenes* and *Trichophyton mentagrophytes* have MIC 50 mg/cm³.

Conclusion

The hexane, ethyl acetate and methanol extracts of *Carissa edulis* possess antimicrobial activity, but the methanol extract was observed to have the higher activity compared to other extracts, therefore this plant can be useful in antiseptic and disinfectant formulation as potential antimicrobial agent. The results support the traditional use of the plant in treatment of skin infections.

Conflict of Interest

The authors declare that there is no conflict of interest reported in this work.

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