Phytochemical constituents, antimicrobial screening and acute toxicity studies of the ethanol extract of *Carissa edulis* Vahl. root bark in rats and mice

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Abstract

Background: The ethanol extract of *Carissa edulis* root bark was screened for its phytochemicals, antimicrobial activities and safety after single administration in rats and mice.

Methods: The extract was subjected to qualitative chemical screening of active chemical constituents and disc diffusion method was performed to determine the antimicrobial properties at different concentrations of 200, 100, 50 and 25 mg/ml. Lorke's method was employed in conducting the oral and intraperitoneal acute toxicity test.

Results: The results showed the presence of saponins, cardiac glycoside, terpenoids and carbohydrates while tannins, alkaloids, anthraquinones and flavonoids were not detected in the extract. At the various concentrations, the extract was active against the growth of *Salmonella typhi, Shigella dysenteriae* and *Streptococcus pyogenes* whereas *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aerogenosa, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Candida albicans* were resistant. The oral and intraperitoneal LD_{50} values were 3,807.9 mg/kg and 288.5 mg/kg, respectively, in both rats and mice.

Conclusion: The ethanol extract possessed some active principles having antibacterial properties and is safe following single oral administration but not via the intraperitoneal route.

Key words: Phytochemicals, Antimicrobial, $LD_{50,}$ Rats, Mice, *Carissa edulis*, Ethanol extract

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Introduction

Medicinal plants are the most common sources of remedies in traditional medicine, the most ancient way of curing diseases. These plants are also the sources of many conventional drugs such as morphine, vincristine and ergometrine.(1) Despite advances in health care delivery, medicinal plants still play important role in human and animal health care and about 60% of the world's population (and 80% of Africa's population) depend on herbal medicine for their primary health care.(2) Many of these plants have been screened for their phytochemical constituents and antimicrobial properties with the view to authenticate their folkloric uses and safety. (3-5) However, out of the 250,000–500,000 plant species on earth, only 1 to 10% has been studied for their potential medicinal values. (6)

The plant, *Carissa edulis* Vahl., has been reported to be used in managing disease conditions such as epilepsy, headache, toothache, cough, chest complaints, rheumatism, fever, sickle cell anaemia, gonorrhoea, syphilis, helminthoses and rabies.(7-9) The plant belongs to the family Apocynaceae and is distributed in tropical Africa and Asia. It is locally called "Carisse" in English, "Cizaki" or "Karen-kafo" in Hausa, "Behoni" in Fulfulde, "Andirim" or "Ndirim" in Babur-Bura and "Ndirma" in Marghi languages. Previous researches on the leaves and fruits of this plant yielded the presence of carbohydrates, tannins, flavonoids, saponins, cardiac glycosides, terpenes and steroids and the extracts had activities against some microorganisms.(4,10-11) This work was aimed at evaluating the ethanol extract of the root bark of the plant for its phytochemical constituents, antimicrobial activities and LD_{50} values in rats and mice.

Materials and Methods

Plants Source

The plant, *Carissa edulis* was obtained in the bush near Ngulde in Askira/Uba Local Government Area of Borno state. It was identified and authenticated by Prof S. S. Sanusi of the Department of Biological Science and a voucher sample (Vet212K1) was preserved at the Veterinary Pharmacology Laboratory, University of Maiduguri, Nigeria.

Extract Preparation

The root bark of the plant was collected, cleaned, crushed and then air-dried at room temperature for one week. It was pulverized using a mortar and pestle and 350 g of the ground herb was soaked overnight in petroleum ether. The residue from defatted samples was extracted in 95% ethanol for 24hr. The sample was filtered using Whatman filter paper No. 1 and evaporated to dryness under reduced pressure using a rotary evaporator (R201D PEC Medicals U.S.A.).

Phytochemical Screening

The plant extract was subjected to qualitative chemical screening for the identification of the various classes of phytochemical constituents using standard methods. [1,12] Carbohydrates, tannins, phlobatannins, cardiac glycosides, terpenoids, flavonoids, alkaloids and anthraquinones were sreened for.

Microbial Cultures

Laboratory isolates of *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis Salmonella typhi, Pseudomonas aerogenosa, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Shigella dysenteriae* species and *Candida albicans* were obtained from the Department of Veterinary Medicine laboratory, University of Maiduguri, Nigeria. The isolates were cultured separately on nutrient agar plate and incubated for 24hr. The medium (25 ml) was poured into sterile Petri dishes and allowed to solidify. A colony of each test organism was sub cultured on 10 ml nutrient broth and incubated at 37°C for 8hr. One milliliter of the broth culture was used to flood the agar plates.

Antimicrobial activity

The disc diffusion method as described by National Committee for Clinical Laboratory Standards was used to determine the growth inhibition of bacteria by the plant extract.[13] Discs containing different concentrations (200, 100, 50 and 25 mg/ml) of dissolved extract were prepared using sterile Whatmann filter paper No. 1, (6 mm in diameters). The discs were dried at 50°C. Over-night cultures of each of the bacterial or Candida isolates was diluted with sterile normal saline to give inoculum size of 10^6 cfu/ml. Nutrient agar medium was prepared, sterilized, cooled and poured into sterile Petri dishes to a depth of 4 mm (about 25 ml per plate) to solidify. Pure cultures of the test organisms were used to inoculate the Petri dishes. This was done by spreading the inocula on the surface of the prepared nutrient agar plate using sterile cotton swabs which have been dipped in the diluted suspension of the organism. The discs were then aseptically placed evenly on the surface of the inoculation and gently pressed down to ensure contact using a pair of forceps. The plates were finally incubated at 37°C for 24hr. Tetracycline (250 mg/ml) (Greenfield Pharmaceutical Co. LTD, JIANGSU Province, China) was used as positive control in each plate. Plates prepared using the same procedures without extract or antibiotic were equally set as negative control. The plates were examined after 24hr for clear zone of inhibition. Antimicrobial activity by the extract was measured using a transparent ruler and recorded as the difference in diameter between the clear zone and the disc (6 mm).

Animals

Wistar albino rats (=12) (124-220 g) and Swiss mice (=12) (16-35 g) were brought from Jos, Plateau State. They were kept in plastic rat cages and allowed to acclimatize to the laboratory environment for a minimum period of one week before the commencement of the experiments. They were fed with grower's mash (Vital Feeds Nig Ltd, Jos, Nigeria) and water was provided *ad libitum*. The experiments were conducted in compliance with the international guiding principles for biochemical research involving animals.[14]

Determination of Median Lethal Dose (LD_{50})

The median lethal dose (LD_{50}) was estimated using Lorke's method.[15] Adult rats and mice (twelve each) of both sexes were used. The rats were fasted for 24hr and deprived of water 12hr before administration of the extract while the mice were deprived of feed and water 4hr before administration. The test was conducted in two phases. In the first phase, 3 groups containing 3 rats or mice per group received 10, 100 and 1000 mg/kg (oral) of the extract respectively. They were monitored for 24hr for mortality and general behavior. Based on the outcome of the first phase, 3 groups containing 1 rat or mouse per group were used in the second phase. After administration, they were again monitored closely for the first 24hr; thereafter, all treated rats and mice were observed for 14 days. The experiment was repeated on other sets of rats and mice but now using the intraperitoneal routes. Post mortem examination was performed. The median lethal dose (LD_{50}) was calculated as the geometric mean of the least dose that kills a rat or mouse and the highest dose that does not kill any rat or mouse.

$LD_{50} = \sqrt{a \times b}$ where: a = least dose that kills a rat/mouse and

b = highest dose that does not kill any rat/mouse.

Results

Phytochemical Composition of Crude Ethanol Extract of *Carissa edulis* Root Bark

The extract yield of the crude ethanol extract of *Carissa edulis* root bark was 2.3% v/w, dark brown in color and readily soluble in water. The result of the phytochemical screening is presented in Table 1. Saponins, cardiac glycoside, terpenoids and carbohydrates were detected while tannins, alkaloids, anthraquinones and flavonoids were not detected in the extract.

Antimicrobial Properties of Crude Ethanol Extract of *Carissa edulis* Root Bark

The antimicrobial activity of the extract presented in Table 2 shows that the extract at the concentration of 200mg/ml inhibited *Salmonella typhi, Shigella dysenteriae* and *Streptococcus pyogenes* with zones of inhibition of 10, 12 and 11mm respectively whereas *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aerogenosa, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Candida albicans* were resistant. At 100mg/ml, the zones of inhibition of these three susceptible organisms were 8, 9 and 9mm respectively and at 50mg/ml only *Shigella dysenteriae* and *Streptococcus pyogenes were* inhibited (7mm in both). At 25mg/ml, all tested organisms were resistant. The control wells (tetracycline 250mg/ml) produced zones of inhibition in all the tested organism ranging from 23mm in *Klebsiella pneumoniae* to 16mm in *Staphylococcus aureus* and *Candida albicans.*

Acute Toxicity Study

The result of the acute oral toxicity test of the crude ethanol extract of *Carissa edulis* root bark in rats and mice using Lorke's method is presented in Table 3 while the result of the intraperitoneal route is presented in Table 4. The median lethal dose (LD_{50}) was 3,807.9 mg/kg using the oral and 288.5 mg/kg using the intraperitoneal route in both rats and mice. None of the animals treated orally showed clinical signs of toxicity except death at the dose of 5000mg/kg in both species (Table 3). However, in those treated intraperitoneally death was preceded by depression. In rats at the dose of 370 and 600 mg/kg intraperitoneal, death occurred within 48hr and 24hr respectively while in mice using the same dose and route, mortality was recorded within 72hr and 48hr respectively (Table 4).

Table 1: Phytochemical screening of crude ethanol extract of *Carissa edulis* root bark

Phytochemical constituent	Test	Inference
Carbohydrate	Molisch's	-
	Fehling's	+
Tannins		-
Alkaloids	Mayer's	-
	Dragendorff's	-
Anthraquinones	Free anthraquinones	-
	Combined anthraquinones	-
Saponin	Froth	+
Cardiac glycoside	General test	+
Terpenoids		+
Flavonoids		-

"+" = present, "-" = not detected

Table 2: Diameter (mm) of zones of inhibition for ethanol extract of *Carissa edulis* root bark against tested micro organisms

Organism	Extract 200mg/ml	Extract 100mg/ml	Extract 50mg/ml	Extract 25mg/ml	Tetracycline 250mg/ml
Salmonella typhi	10mm	8mm	Resistant	Resistant	20mm
Shigella dysenteriae	12mm	9mm	7mm	Resistant	22mm
Escherichia coli	Resistant	Resistant	Resistant	Resistant	21mm
Klebsiella pneumoniae	Resistant	Resistant	Resistant	Resistant	23mm
Pseudomonas aerogenosa	Resistant	Resistant	Resistant	Resistant	19mm
Proteus mirabilis	Resistant	Resistant	Resistant	Resistant	20mm
Staphylococcus aureus	Resistant	Resistant	Resistant	Resistant	16mm
Streptococcus pyogenes	11mm	9mm	7mm	Resistant	21mm
Bacillus subtilis	Resistant	Resistant	Resistant	Resistant	18mm
Candida albicans	Resistant	Resistant	Resistant	Resistant	16mm

Table 3: Oral acute toxicity test of the crude ethanol extract of *Carissa edulis* root bark in rats and mice

Phase	Species (No.)	Dosage (mg/kg)	Clinical signs	Mortality
1	rat (3)	10	None	0/3
1	rat (3)	100	None	0/3
1	rat (3)	1000	None	0/3
2	rat (1)	1600	None	0/1
2	rat (1)	2900	None	0/1
2	rat (1)	5000	None	1/1, 72hr
1	mouse (3)	10	None	0/3
1	mouse (3)	100	None	0/3
1	mouse (3)	1000	None	0/3
2	mouse (1)	1600	None	0/1
2	mouse (1)	2900	None	0/1
2	mouse (1)	5000	None	1/1, 96hr

Oral $LD_{50} = \sqrt{a \times b}$ = oral $LD_{50} = \sqrt{2900 \times 5000} = \sqrt{14500000}$ = 3,807.9 mg/kg

Table 4: Intraperitoneal acute toxicity test of the crude ethanol extract of *Carissa edulis* root bark in rats and mice

Phase	Species (No.)	Dosage (mg/kg)	Clinical signs	Mortality
1	rat (3)	10	None	0/3
1	rat (3)	100	None	0/3
1	rat (3)	1000	Depression	3/3, 24hr
2	rat (1)	140	None	0/1
2	rat (1)	225	Depression	0/1
2	rat (1)	370	Depression	1/1, 48hr
2	rat (1)	600	Depression	1/1, 24hr
1	mouse (3)	10	None	0/3
1	mouse (3)	100	None	0/3
1	mouse (3)	1000	Depression	3/3, 24hr
2	mouse (1)	140	None	0/1
2	mouse (1)	225	None	0/1
2	mouse (1)	370	Depression	1/1, 72hr
2	mouse (1)	600	Depression	1/1, 48hr

Ip $LD_{50} = \sqrt{225 \times 370} = \sqrt{83250} = \underline{288.5 \text{ mg/kg}}$

Discussion

The phytochemical analysis of the crude ethanol extract of the *Carissa edulis* Vahl. root bark revealed the presence of saponins, cardiac glycosides and terpenes. These three phytochemicals have been reported to influence physiological and pharmacological activities of the body. Previous work on the leaves of this plant revealed the presence of tannins, flavonoids, cardiac glycosides and terpenes.[11] Saponins are known to produce anti-inflammatory activity and erythrocyte haemolysis.[16-17] Cardiac glycosides have strong activity on the heart and some have been used in the treatment of congestive heart failure. Cardiac glycosides may also have

pesticidal properties.[18] Thymoquinone which is a triterpene saponin has been shown to be effective in disease conditions such as cancer, asthma, atherioscleriosis and diabetes.[19]

The extract has activity against three important organisms, *Salmonella typhi, Shigella dysenteriae* and *Streptococcus pyogenes.* The antibacterial activities could be due to the presence of terpenes in the extract as this secondary metabolite is known to possess antimicrobial properties.[20] Previous work has reported antibacterial activities of different parts of the plant such as leaves, stem and fruit. It has been reported that the aqueous extracts of the fruit and leaves of *C. edulis* possessed antibacterial activities against *S. aureus, B. subtilis, E. coli* and *P. aeruginosa* where as these organisms were resistant in this study.[4] The contrasting result might be due to the difference in the chemical compositions of the root bark from that of the fruit and leaves or this might arise from the different solvents for extraction. The concentrations of the extracts used to test activity against the organisms were different.

The acute toxicity study on the ethanol extract of *C. edulis* root bark indicated that the oral and intrapiretoneal median lethal dose (LD_{50}) were 3,807.9 mg/kg and 288.5 mg/kg, respectively, in both rats and mice. Previous work obtained similar result (282.8 mg/kg) using intrapiretoneal route in mice but reported a higher value (above 5000 mg/kg) using the oral routes in mice.[9] Any substance with oral LD_{50} above 1000 mg/kg in rats is regarded as being of low toxicity or relatively safe.[21] Thus this extract is safe when taken orally. However the intraperitoneal routes with LD_{50} values of 288.5 mg/kg would be regarded as toxic. This is also based on classification that any substance with intraperitoneal LD_{50} in rats between 50 and 500 mg/kg is considered toxic.[21] In another classification, substance like this extract with oral LD_{50} values is between 0.5 -5 g/kg is regarded as moderately toxic (Hodge and Sterner, 1949; OECD, 2001; Saganuwan, 2012).[22-24]

Conclusion

The crude ethanol extract of *Carissa edulis* root bark contains saponins, terpenes and cardiac glycosides and possess antibacterial activities. The oral and intraperitoneal LD_{50} values of

3,807.9 mg/kg and 288.5 mg/kg, respectively, in both rats and mice indicated that the extract is safe following single oral administration but toxic via the intraperitoneal route.

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