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Antimicrobial activities of methanolic extract of *Carissa opaca* roots and its fractions and compounds isolated from the most active ethyl acetate fraction

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ABSTRACT

Objective: To study the antibacterial and antifungal activities of methanolic extract of roots of *Carissa opaca* and its fractions in hexane, chloroform, ethyl acetate, *n*-butanol and water, and the isolated compounds.

Methods: The zones of inhibition of the samples against test microorganisms were determined by agar well diffusion method. Minimum inhibitory concentrations of the samples were determined by agar well dilution method. Test microorganisms included four standard bacteria [*Bacillus subtilis* ATCC 6633 (*B. subtilis*), *Escherichia coli* ATCC 8739 (*E. coli*), *Pseudomonas aeruginosa* ATCC 9027 (*P. aeruginosa*), and *Staphylococcus aureus* ATCC 6538], two standard fungi [*Candida albicans* ATCC 10231 (*C. albicans*)] and *Aspergillus niger*, and six clinical isolates (*B. subtilis*, *E. coli*, *P. aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi* and *Enterobacter cloacae*). The most active fraction was investigated to isolate compounds. The chemical compounds isolated from the ethyl acetate fraction were identified by gas chromatography-mass spectrometer, high performance liquid chromatography and liquid chromatography-mass spectrometer.

Results: *E. coli*, *P. aeruginosa*, and *C. albicans* were the most susceptible. Less polar fractions exhibited stronger efficacy than polar ones, and ethyl acetate fraction proved to be the most potent. Zones of inhibition of hexane, chloroform and ethyl acetate fractions, and amoxil against *C. albicans* were 19.96, 22.01, 23.10 and 19.20 mm, respectively. Ethyl acetate faction was the most toxic to all the test microorganisms, with minimum inhibitory concentrations of 8.0, 7.8 and 7.78 μg/mL against *P. aeruginosa*, *C. albicans* and *B. subtilis*, respectively. Isolated compounds, limonene, 2'-hydroxyacetophenone, vanillin, naphthalenone, 2,3,3-trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone, 2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, β-sitosterol, vitamin E, rutin, quercetin, lupeol, epigallocatechin, showed considerable antimicrobial activities against test microorganisms.

Conclusions: The roots of *Carissa opaca* contain compounds with significant antimicrobial potential.

1. Introduction

While microbial infections are common ailments, their treatment is a serious problem due to continuous drug resistance that microorganisms soon develop against antibiotic drugs[1]. Efforts to discover new remedies are, therefore, crucial. Great hope rests with plants as they contain different types of primary and secondary metabolites with antimicrobial pharmacophores[2]. Over the past few decades, numerous studies have been conducted on plants to explore possible candidates for antibiotic drugs[3,4].

Carissa opaca Stapf ex Haines (*C. opaca*) (Family: Apocynaceae) is a wild, thorny shrub found in Himalayan mountainous regions of Indo-Pakistan subcontinent[5-7]. The local people use this plant as a remedy for a number of ailments. The leaves are known to be effective in asthma, jaundice and hepatitis[8,9]. The leaves, fruits and seeds of the plant have been found to possess good antimicrobial, antioxidant and anti-enzymatic activities[10,11]. The roots of *C. opaca* are employed to cure wounds and injuries, and are known to be purgative[12]. The plant has also been shown to

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possess compounds of classes of flavonoids, tannins, terpenoids and glycosides, which have bioactivities[13,14].

The purpose of the present study was to explore roots of *C. opaca* for possible antimicrobial compounds by using assay-guided isolation. Methanolic extract of the roots of the plant and its fractions in different solvents were, therefore, tested against a number of common pathogens. Compounds from the most active ethyl acetate fraction were isolated and studied for antimicrobial activity. Both standard strains and clinical isolates were used in the study.

2. Materials and methods

2.1. Chemicals and instruments

Solvents used for extraction and fractionation were of high performance liquid chromatography (HPLC) grade. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, Mueller-Hinton agar from Merck, and potato dextrose agar from Himedia. Standard antibiotic drugs amoxil and cefixime were obtained from Pharmagen, Lahore, Pakistan. Glass columns were purchased from Pyrex. Aluminium and glass thin-layer chromatography (TLC) plates were purchased from Merck. Gas chromatography-mass spectrometer (GC-MS) was from Agilent (7890A), and column was Hp-5mS. The initial temperature was kept at 60 ℃ and it was raised by 5 ℃ per min till 270 ℃ after which there was 10 ℃ rise in temperature till 310 ℃. HPLC from Shimadzu (SPd-6AV) was used for identification of isolated compounds. Liquid chromatography-mass spectrometer (LC-MS) model used was LCQ Advantage Max Thermo Finnegan; column was Thermo Hypersil Gold C18, 250 mm × 4.6 mm, 5 μm; mobile phase was methanol; water (80:20); total time of scan was 20 min; temperature was kept at 30 ℃, and the flow rate of mobile phase was 1 mL/min.

2.2. Microorganisms

Standard microorganisms were obtained from Pharmagen, Lahore. They included four bacteria *Bacillus subtilis* ATCC 6633 (*B. subtilis*), *Escherichia coli* ATCC 8739 (*E. coli*), *Pseudomonas aeruginosa* ATCC 9027 (*P. aeruginosa*), *Staphylococcus aureus* ATCC 6538 (*S. aureus*), and two fungi *Candida albicans* ATCC 10231 (*C. albicans*) and *Aspergillus niger* (*A. niger*). All the standard stains were purchased from KWIK-STICK. Clinical isolates, obtained from Children Hospital, Lahore, included *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. aureus*, *Salmonella typhi* (*S. typhi*) and *Enterobacter cloacae* (*E. cloacae*).

2.3. Collection of plant materials

Roots of *C. opaca* were collected from adjoining hills of Abbottabad (Pakistan) in March 2013. The plant was identified by Professor Ajaib Khan, taxonomist, GC University, Lahore, where a voucher specimen was deposited (GC-Herb Bot 2271). The roots were separated from the aerial parts of the plants, washed with distilled water and wiped with a clean piece of cloth. They were crushed and ground to obtain a finely divided powder.

2.4. Extraction and fractionation

Methanolic extract of the powder was obtained through cold maceration method by soaking the material in methanol for 15 days followed by filtration. The process was repeated thrice and the extracts were combined. The solvent was evaporated on rotary evaporator to obtain a dried crude methanolic extract. The methanolic extract was then suspended in distilled water in a separatory funnel and partitioned successively with hexane, chloroform, ethyl acetate, and *n*-butanol to obtain fractions in these solvents. This process left residual aqueous fraction at the end. The solvents were removed on rotary evaporator at low pressure to get dried fractions.

2.5. Study of antimicrobial activity

2.5.1. Sample preparation

The dried methanolic extract and its fractions were dissolved in DMSO to obtain stock solutions (50 mg/mL). From each stock solution, five dilutions were prepared in DMSO of 10, 20, 30, 40, and 50 mg/mL concentrations. Solutions of standard drugs were also prepared in the same manner.

2.5.2. Determination of zones of inhibition (ZOI)

Well-known agar well diffusion method was used according to a reported procedure[15]. For preparation of 1 L of Mueller-Hinton agar, 38 g of agar was suspended in 1 L of distilled water. Mixture was homogenized and autoclaved at 121 ℃ for 1 h. It was then placed in an incubator at 50 ℃ till used. Distilled water was added to a lyophilized culture. It was streaked onto a slant made of tryptic soy agar, and was allowed to incubate for 24 h at 32.5 ℃. After incubation, bacterial growth was observed, and 3 mL saline solution was added into the slant containing culture. It was shaken carefully to mix well. From this culture suspension, 2 mL suspension was taken out and its absorbance was standardized with 0.5% McFarland standard solution at 550 nm. Then, 2 mL of this diluted suspension was added into 100 mL of liquid agar whose temperature was maintained at 50 ℃ in order to make the seed agar. In case of spore-forming bacterium *B. subtilis*, incubation of 7 days was required for maximum formation of spores on the slant. Growth on the surface of slant was washed with 20 mL 0.9% saline solution and the mixture was heated at 70 ℃ for 30 min in order to kill the vegetative growth of the bacterium. From this culture suspension, 2 mL suspension was taken out and its absorbance was standardized with 0.5% McFarland standard solution at 550 nm. The rest of the procedure was the same. The Mueller-Hinton agar (21 mL) was poured into a 100-mm Petri plate and allowed to solidify. As soon as it was solidified, 4 mL seed agar was poured into it to set a thin layer of it on the base agar. The Petri plate was placed in a refrigerator for cooling for 1 h. Holes were made into it and were labelled. Plant extracts/fractions were filled into the holes. The plate was then incubated for 24 h, after which, it was observed for antimicrobial activity of the samples, and ZOI were

measured. The same method was used for all the plant samples, isolated compounds, and standard drugs. Each experiment was performed at least thrice to ensure maximum validity.

For antifungal activity, growing medium consisted of potato dextrose agar and the temperature was maintained at 30.5 ˚C. The rest of the procedure was the same as mentioned above.

2.5.3. Determination of minimum inhibitory concentrations (MICs)

For the determination of MIC of a sample, agar well dilution method was used[16]. Mueller-Hinton agar was dissolved in distilled water with concentration 38 g/L, and autoclaved at 121 ˚C for 1 h. It was allowed to come to 50 ˚C before being poured into Petri plates for sample preparation. Different dilutions of each extract/fraction were prepared in this agar with concentrations 10-50 μg/20 mL. The content of each plate was mixed well and allowed to solidify. Stock solution of each extract/fraction was prepared in DMSO (5 mg/mL) and dilutions were made. Then, microbial cultures were transferred onto the content of each Petri plate with the help of a multipoint inoculator. Plates were then incubated for 24 h at 37.6 ˚C, after which they were observed, and MICs were noted.

2.5.4. Isolation and identification of compounds

Column and TLC was used to isolate compounds from ethyl acetate fraction (25 g). Silica gel was used as a stationary phase, and various solvent combinations were employed as mobile phase. The column yielded sub-fractions, EA1-EA5 upon elution with solvent systems of gradually increasing polarity. Each sub-fraction was then chromatographed on preparative TLC. Five compounds, F1-F5, were isolated from EA1; one compound, F6, was from EA2; two compounds, F7 and F8, were from EA3; two compounds, F9 and F10, were from EA4, and one compound, F11, was from EA5. The compounds were identified by GC-MS, HPLC and LC-MS.

2.6. Statistical analysis

All antimicrobial measurements were made in triplicate. Statistical analysis was done by using ANOVA, and statistical mean was calculated with ± SD.

3. Results

3.1. ZOI

ZOI of methanolic extract of *C. opaca* roots and its fractions was determined against a number of microorganisms. A comparison of the antimicrobial activity of plant extract/fractions with standard drugs was depicted in Figure 1.

3.2. MIC

MICs were determined against the standard microorganisms and the results are displayed in Figure 2. MIC values of methanolic extract and its fractions ranged from 7.8 to 48.8 μg/mL. *n*-Butanolic fraction was proved to be ineffective while aqueous faction, as well, displayed very little efficacy. Ethyl acetate faction was the most toxic to all the test microorganisms (Figure 2), with MICs of 8.0, 7.8 and 7.78 μg/mL against *P. aeruginosa*, *C. albicans* and *B. subtilis*, respectively.

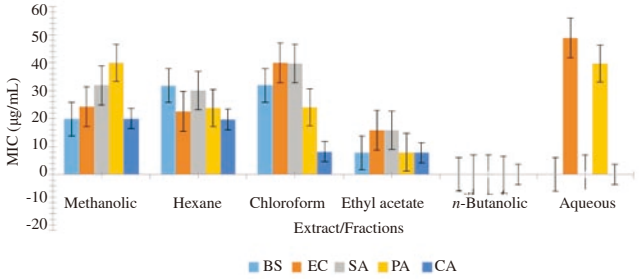

Figure 2. MIC values of methanolic extract of *C. opaca* roots and its fractions in various solvents (μg/mL) against four standard bacteria and a fungus.

BS: *B. subtilis*; EC: *E. coli*; SA: *S. aureus*; PA: *P. aeruginosa*; CA: *C. albicans* (n = 3).

3.3. Identification of chemical constituents

The chemical compounds isolated from the ethyl acetate fraction were identified by GC-MS, HPLC and LC-MS according to their

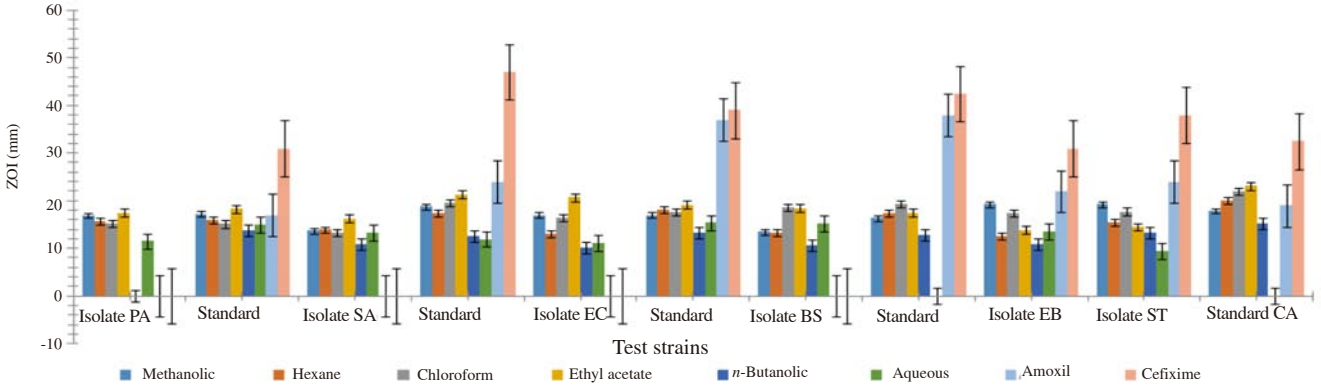

Figure 1. Comparison of ZOI of methanolic extract of *C. opaca* roots and its fractions in different solvents against standard and isolated bacteria, a standard fungus and two standard antibiotic drugs.

Concentration of each sample and drug was 40 mg/mL.

EC: *E. coli*; SA: *S. aureus*; PA: *P. aeruginosa*; BS: *B. subtilis*; EB: *E. cloacae*; ST: *S. typhi*; CA: *C. albicans* (n = 3).

Table 1

Compounds identified in ethyl acetate fraction of methanolic extract of *C. opaca* roots by using GC-MS, HPLC and LC-MS.

Eluate from column	Compound isolated by TLC	Techniques used for identification	RT on GC-MS, HPLC
EA1	F1, Limonene	GC-MS	7.22
	F2, 2'-hydroxyacetophenone	GC-MS	10.40
	F3, Vanillin	GC-MS	11.36
	F4, Naphthalenone	GC-MS	19.72
	F5, 2,3,3-Trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	GC-MS	21.48
EA2	F6,1, 2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	GC-MS	24.64
EA3	F7, β-Sitosterol	GC-MS	22.12
	F8, Vitamin E	GC-MS	23.67
EA4	F9, Rutin	HPLC	6.29
	F10, Quercetin	HPLC	6.53
EA5	F11, Lupeol	HPLC/GC-MS	26.16
	F12, Epigallocatechin	LC-MS	

RT: Retention time (min).

Table 2

Antimicrobial activity of compounds isolated from the ethyl acetate fraction of methanolic extract of *C. opaca* roots in terms of ZOI (mm).

Compound code	Name of compound	*E. coli*	*B. subtilis*	*P. aeruginosa*	*A. niger*	*C. albicans*
F1	Limonene	11.25 ± 0.15	-	10.65 ± 0.27	12.21 ± 0.59	-
F2	2'-hydroxyacetophenone	11.15 ± 0.34	12.62 ± 0.24	11.50 ± 0.32	11.34 ± 0.18	12.21 ± 0.14
F3	Vanillin	15.21 ± 0.15	11.42 ± 0.17	12.16 ± 0.35	14.28 ± 0.25	12.61 ± 0.48
F4	Naphthalenone	11.50 ± 0.75	-	12.62 ± 0.36	-	-
F5	2,3,3-Trimethyl-2-(3-methylbuta-1,3-dienyl)-6-methylenecyclohexanone	12.50 ± 0.75	-	12.09 ± 0.43	12.45 ± 0.37	-
F6	1,2-Benzenedicarboxylic acid, mono (2-ethyhexyl) ester	14.53 ± 0.38	-	-	13.06 ± 0.31	-
F7	β-Sitosterol	13.90 ± 0.29	12.01 ± 0.27	14.24 ± 0.14	14.36 ± 0.29	11.50 ± 0.76
F8	Vitamin E	14.62 ± 0.27	-	-	12.67 ± 0.41	11.25 ± 0.32
F9	Rutin	11.56 ± 0.16	11.12 ± 0.63	14.56 ± 0.27	-	-
F10	Quercetin	14.17 ± 0.11	13.01 ± 0.27	13.44 ± 0.18	-	-
F11	Lupeol	12.27 ± 0.35	12.71 ± 0.25	11.10 ± 0.31	12.21 ± 0.07	11.50 ± 0.75
F12	Epigallocatechin	12.35 ± 0.21	11.09 ± 0.37	-	-	-
Standard drug	Amoxicillin	16.98 ± 0.14	15.12 ± 0.67	15.02 ± 0.28	12.53 ± 0.38	13.57 ± 0.31

nature. These known compounds were isolated from this plant for the first time. Table 1 summarizes the techniques used for identification of these compounds. Compounds F1-F8 and F11 were identified on GC-MS. Rutin and quercetin, which are well known flavonoids were identified by comparing their HPLC profiles with standard compounds. Compound F12 was identified by LC-MS.

3.4. Antimicrobial activity shown by isolated compounds

Antimicrobial activities of some of the isolated compounds with sufficient quantities were determined. The antimicrobial potential, in terms of ZOI, exhibited by the isolated compounds and standard drug amoxicillin is given in Table 2. *E. coli* was found the most susceptible toward vanillin by exhibiting ZOI 15.21 mm, while *B. subtilis* showed high sensitivity against quercetin (ZOI, 13.01 mm). *P. aeruginosa* was found the most susceptible toward rutin (ZOI, 14.56 mm). β-Sitosterol was more active against *A. niger* (ZOI, 14.36 mm) than the drug amoxicillin (12.53 mm). The fungus *C. albicans* (12.61 mm) was the most susceptible toward vanillin.

4. Discussion

Quest for more effective and safer antimicrobial therapies is necessitated by the resistance that pathogens soon develop against current drugs. Plants being reservoirs of various types of bioactive molecules are target of extensive research worldwide[1,3,17]. In the present work, methanolic extract of *C. opaca* roots, obtained by cold maceration method, and its fractions in different solvents were subjected to antimicrobial study against a number of standard microorganisms and clinical isolates.

Methanolic extract of *C. opaca* roots and its fractions showed antimicrobial activities in a dose-dependent manner against the test microorganisms. Methanolic extract exhibited significant ZOI against *E. coli*, *P. aeruginosa*, and *C. albicans*. Its potency against *P. aeruginosa* and *C. albicans* was comparable to that of standard drug amoxillin. Non-polar fractions showed even better toxicity against *C. albicans* with ZOI of hexane, chloroform and ethyl acetate fractions, and amoxil being about 19.96, 22.01, 23.10 and 19.20 mm, respectively. Notably, aqueous fraction was totally ineffective against this fungus. Anti-fungal compounds of this plant have, therefore, appeared in less polar solvents and are of hydrophobic nature. Ethyl acetate fraction showed efficacy against *P. aeruginosa* equal to that of amoxillin. This extract, therefore, may provide a lead for an antibiotic for this pathogen, and the extract itself may be recommended for topical application. Its effectiveness against *S. aureus* and *E. coli* was also significant. In general, less polar fractions were better antimicrobial agents than their more polar counterparts. Chloroform fraction showed good activities against *S. typhi* and *E. cloacae*. The antimicrobial activities of the extract/fractions were, as a whole, dose-dependent, which increased with

the increase in concentration.

The isolated compounds showed comparable ZOI with the standard drug amoxicillin. Vanillin, vitamin E and quercetin exhibited activity against *E. coli* almost equal to amoxicillin. Interestingly, the phthalate ester F6 also showed very good activity against *E. coli*. Phthalates have been reported to show antimicrobial activity in other studies as well[18]. Beta-Sitosterol exhibited remarkable activity against *P. aeruginosa* and *A. niger*. Vanillin and rutin were highly active against *A. niger* and *P. aeruginosa*, respectively. The results indicated that the isolated compounds contributed towards the overall antimicrobial properties exhibited by ethyl acetate fraction of methanolic extract of *C. opaca* roots. As long as we could explore the literature, these compounds, although known, were isolated for the first time from this plant, hence, a valuable addition to the phytochemical information on this plant is still needed.

Methanolic extract of roots of *C. opaca* and its fractions in various solvents showed moderate to high antimicrobial activities against test microorganisms. Ethyl acetate fraction displayed especially notable efficacy. *P. aeruginosa* and *C. albicans* were the most susceptible. Toxicity of ethyl acetate faction was comparable to that of amoxillin against *C. albicans*. The chemical compounds like vanillin, quercetin, rutin, vitamin E and β-sitosterol were found the most active against tested strains. Further investigation into this fraction has great prospects to yield exploitable natural products for future drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

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