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Research Article

"*IN VITRO* ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *CARISSA OPACA* STAPF EX HAINES"

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ABSTRACT

Medicinal plants offer a valuable approach to take care of microbial infections. The present study was undertaken to evaluate the antimicrobial potential of Carissa opaca. This plant is currently used in ayurveda and traditional Indian medicine as cardiotonic, jaundice and hepatitis and also showing potent antioxidant activities. The antimicrobial property of extracts from roots of Carissa opaca were evaluated against Gram-positive, Gram-negative bacterial and fungal pathogens using the disk diffusion method. Among all the solvent extracts evaluated for its antimicrobial activity, ethyl acetate and acetone extracts showed good antimicrobial activity against bacterial and fungal pathogens with minimum inhibitory concentration (MIC) values ranging from 6.25-50.00 µg/mL and from 50-100.00 µg/mL, respectively. Both the extracts showed higher MIC values against bacterial pathogens compared to fungal pathogens. Thus, the current investigation will lead to fresh resource of new antimicrobials in the future.

Keywords: Carrisa opaca, Disk diffusion method, Antimicrobial activity, Minimum inhibitory concentration

INTRODUCTION

Human beings have been using plants for basic protective and curative health care since past. Current reports suggested that above 9,000 plants have recognized remedial purpose in different countries[1]. Medicinal plants are used in conventional population at different ancestral systems like Ayurveda, Chinese medicine, or the Japanese Kampo system. According to the World Health Organization (WHO), over 80% of the world's population, or 4.3 billion people, faith upon such conventional plant-based systems of medicine to provide crucial health care[2]. Medicinal plants are regularly used for the cure of various illnesses in India, as these are believed to have advantages over the conventionally used drugs that are pricey and known to have dangerous side effects[3]. Despite the being of powerful antibiotics, resistant and multi-resistant pathogens are continuously emerging, imposing the need for an enduring investigate and development of new drugs[4]. World Health Organization (WHO) has listed 21,000 plants, which are used for medicinal principle around the world. Among these 2500 species are from India, out of which 150 species are used commercially on a large scale. India is the largest producer of medicinal herbs and is called as the botanical garden of the world[5]. *Carissa opaca* Stapf ex Haines found in areas of India (Garhwal Himalayas) and Southeast Asia where it is grown for its fruit, as an adornment, and to be used as a living boundary marker[6]. Leaves of *C. opaca* contained tannin whereas the roots of *C. spinarum* were reported to contain caffeic acid, cardiac glycosides odorosides B, C, G and H, and evomonosid while its leaves produce urosolic acid and naringin[7,8]. The leaves and constituents of the flower oil of contain carissone, palmatic acid, benzyl salicylate, benzyl benzoate and α-farnesene[7,8]. The whole plant is used as cardiotonic and especially root used as purgative and cleaning wounds and animals[6]. This plant is useful in dysentery, stomachache and stimulant while leaf decoction is given to cure jaundice and hepatitis[11,15]. The chloroform and aqueous fractions of *C. opaca* fruit a high amount of total phenolic and flavonoid contents potent antioxidant activities[10]. A new germacrane derivative, carenone, was isolated from the stems of another *Carissa* species (*C. spinarum*) and leaves exhibited significant antibacterial activity against Gram negative organisms[12] and other species *C. lanceolata* R.Br. showed antimicrobial activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*[13]. Ahmad *et al*., 2009 found this plant cure fever and useful in eye disorders and fruit of the plant mixed with the roots of the *Mimosa pudica* is taken as an aphrodisiac[14]. Pharmacological studies have not yet been undertaken with *Carissa opaca* species, but lignans, sesquiterpenes of eudesmane type and several cardiac glycosides have been isolated from *Carissa* species[9]. The main objective of this study was to identify Indian subcontinental Himalayan plant for its antimicrobial activity which might serve as good candidates for the development of new antimicrobial agents and/or standardized phytomedicines.

MATERIAL AND METHOD

Plant material

Dried roots of *C. opaca* were collected from the hills of the Srinagar (Garhwal), India. The taxonomic identification of this plant was identified and confirmed by The Botanical Survey of India, Dehradun, Uttarakhand, India and a sample of the plant was stored as the voucher specimen.

Preparation of plant extracts

Plant materials were washed, air dried and used immediately for the extraction and stored in air tight containers. 100 grams of the dried roots of plant material of *C. opaca* were taken for extraction and separately crushed to a powder form using sterilized mortar and pestle. These crushed materials loaded into the wider part of the extractor by packing it in a thimble made up of filter paper. Menstruum (solvent) is placed in the round-bottomed flask and boiled. Repeated vaporization and condensation of the menstruum helps in percolation of the active constituents of the dried crushed form material in the round-bottomed flask through the siphon until the material exhausted. Seven solvents were used on the basis of their polarity and temperature of the mantle regulated according to the boiling point of respective solvents. The boiling range and the solvents used during extraction are given below: Petroleum ether (60^0C-80^0C), Benzene (75^0C - 78^0C), Chloroform (30^0C-35^0C), Ethyl acetate (77^0C), Acetone (80^0C), Ethanol (75^0C) and Distilled water (80^0C-100^0C). The powder was dissolved in the solvents used for extraction. The various extracts of *C. opaca* collected in different solvents through soxhlalation is subjected to distillation through Liebig's condenser where from the solvent gets separated from the respective active principle remains in the round bottomed flask. The extracts are collected and stored at 4^0C. The extracts were used after vacuum drying of extracts and dissolved in 1% dimethyl sulphoxide (DMSO). Plant extracts were tested for antibacterial activity against nine bacterial and six fungal pathogens by disk diffusion methods.

Testing of antimicrobial activity of plant extracts

The *in vitro* antibacterial and antifungal assays were carried out by adopting the modified disk diffusion method[16]. For the antimicrobial assay, the dried extracts were dissolved in 1% dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ mL and sterilized by filtration through a 0.45-µm membrane filter. Mueller-Hinton Agar was inoculated with (overnight, 12 h) bacterial

cell suspension (200 µL in 20 mL medium, 5 ×10^5 CFU/mL). Sterile filter paper disks of 6-mm diameter were impregnated with 20 µL extracts (equivalent to 100 µg/ mL/disk) and after complete evaporation, the disks were placed on the surface of the inoculated agar plates. G, gentamicin (10µg/disc); P_G, penicillin (10unit/disc); M, methicillin (5µg/disc); V, vancomycin (30 µg/disc) and Fc, cyclohexamide (30µg/disc) were used as reference antibiotics against bacterial and fungal pathogens, respectively. Negative controls were done using paper discs loaded with 20 µL of the solvent (DMSO). The plates were incubated at 37°C for 18 h. Similarly, Sabouraud Dextrose Agar was inoculated with yeast overnight cell suspension and incubated at 28°C for 48 h. At the end of the incubation period, the antimicrobial activities were evaluated by measuring the zone of inhibition. The minimum inhibitory concentration (MIC) was done according to the method described previously[16,17]. If extracts showed an MIC value ≤12.5 µg/mL, the antimicrobial activity was considered as good; from >12.6 to 49 µg/mL, the antimicrobial activity was moderate; from >50 to 99 µg/mL the antimicrobial activity was weak; over >100 µg/mL the extract was considered inactive.

Microorganisms used

The following microorganisms were used; Bacterial cultures: *Bacillus cereus*, *Staphylococcus aureus*, *Corynebacterium spp., Alcaligenes faecalis*, *Escherichia coli, Escherichia coli* (MTCC 1687*), Klebsiella pneumoniae, Pseudomonas aeruginosa* (MTCC 424), *Salmonella typhi,* and *Proteus vulgaris. S. aureus* (1A) methicillin and vancomycin resistant *Staphylococcus aureus* (VRSA) was isolated from a gangrene patient of the Government Hospital, Dehradun, Uttarakhand, India. Fungal cultures were: *Aspergillus flavus, Aspergillus niger, Alternaria solani, Candida albicans* (MTCC 227), *Penicillium monotricales ,Saccharomyces cerevisiae and Saccharomyces cerevisiae (*MTCC 3980). The spore suspension of fungal culture was made by harvesting the spores having the total spore near about 10^9 - 10[10] CFU/ml. All the MTCC cultures were gifted from Ranbaxy Lab Ltd., Poanta Sahib (H.P.), India

RESULTS AND DISCUSSION

The results of the antimicrobial activity of petroleum ether, benzene, chloroform, ethyl acetate, acetone, ethanol and aqueous extracts of *Carissa opaca* are summarized in Table 1 (inhibition zone diameter in mm). This plant has been thoroughly studied for its antimicrobial activity and the ethyl acetate and acetone extracts showed highest zone of inhibition against all tested bacterial pathogens.

Previous literature reports that *C. carandus* and *C. lanceolata* were evaluated for its antimicrobial activity. The leaf extract of *C. carandus* showed good antibacterial activity against six gram negative and gram positive bacterial pathogens but chloroform extract was found to be an excellent activity against *P. vulgaris* and *S. aureus*[18]. Carindone, carrissone and dehydrocarissone compounds were isolated from *C. lanceolata* and showed antibacterial activity having a MIC less than 0.5 mg/ml against *S. aureus* and *E. coli*[19]. Since these two plants and including *C. opaca* (used in the present study) belongs to the same family (Apocynaceae) may contain same active principle i.e. carindone, carrissone and dehydrocarissone, alkaloid and flavanoids. In the present investigation highest antibacterial activity of ethyl acetate and acetone extracts are shown against *Corynebacterium* spp., *A. faecalis*, *B. cereus*, *P. vulgaris*, *S. typhi*, *K. pneumoniaec*, *P. aeruginosa* (MTCC 424), *S. aureus* and *S. aureus* (1A) with an inhibition zone diameter ranging 14mm to 38mm**.** Sensitivity of amongst the tested Gram-positive bacteria, *B. cereus* and *Corynebacterium* spp. were found to be the most sensitive, while *S. aureus* was the less sensitive bacteria. In case of Gram-negative bacteria, *A. faecalis* was the most sensitive, while *S. typhi* and *E. coli* (MTCC 1687) was the less sensitive strain. Our study supported Srinivasan *et al*., 2001 (20) that gram-negative bacteria were less susceptible than gram-positive bacteria to the extracts[20]. None of the extracts were showed antifungal activity except acetone and ethyl acetate extracts and there were major differences in the minimum inhibitory concentration (MIC) on the antibacterial and antifungal activity observed in the acetone and ethyl acetate extracts.

Similarly antifungal activity against some fungal pathogens viz. *A. flavus, A. niger, A. saloni, C. albicans* (MTCC 227), *P. monotricales, S. cerevisiae* and *S. cerevisiae* (MTCC 3980) with an inhibition zone diameter ranging from 10 to 25 mm are shown (Table 1).

MIC results shows against bacterial pathogens were; ethyl acetate extract presented a good activity against *S. aureus, E. coli* (MTCC 1687), *P. aeruginosa* (MTCC 424) and *K. pneumoniae*, with MIC values of 12.5 µg/ml, respectively but moderate against *B. cereus*, *Corynebacterium* spp., *A. faecalis* and *P. vulgaris*, with 25 µg/ml, respectively and weak against *S. typhi, Corynebacterium* spp., *A. faecalis*, with MIC values 50 µg/ml. The acetone extract showed good activity against *E. coli* (MTCC 1687) and *P. aeruginosa* (MTCC 424), *B. cereus*, *Corynebacterium* spp., *A. faecalis, S. typhi* and *P. vulgaris,* respectively with MIC value 6.25 µg/ml and moderate against *S. aureus and K. pneumoniae* with MIC value 50 µg/ml (Table 2).

MIC results of fungal pathogens indicated that ethyl acetate and acetone extracts presented moderate to weak activity and higher MIC values. However MIC results of bacterial pahogens showed good antibacterial activity and lower MIC values (Table 2) and results indicate that this plant is less active against fungal cultures. The response of antimicrobial agents as well as the extract depends on the inhibition of various cellular process, followed by an increase in plasma membrane permeability and finally ion leakage from the cells [21].

Table 1: Antimicrobial activity of *C. opaca* extracts [a]

Microorganisms	Gram (+) / (-)	A	B	C	D*	E*	F	G	Standards [b] (G/P_G/M/V)
Bacterial pathogens	G (+)	18	14	14	28	28	20		30
*Bacillus cereus**	G (+)	22	21	-	32	28	-	-	28
Staphylococcus aureus (1A)	G (+)	21	22	-	32	26	-	-	26
*Staphylococcus aureus**	G (+)	24	16	14	28	28	20	-	22
Corynebacterium sp. *	G (-)	14	16	14	30	28	18	-	20
*Alcaligenes faecalis**	G (-)	-	-	-	20	18	19	-	30
Escherichia coli	G (-)	-	-	-	22	19	-	-	23
Escherichia coli	G (-)	-	-	-	38	38	18	-	26
(MTCC 1687*) **	G (-)	-	-	-	32	30	22	-	25
Klebsiella pneumoniae Pseudomonas aeruginosa*	G (-)	-	-	-	28	28	-	-	24
(MTCC 424)	G (-)	-	-	-	20	22	18	-	26
*Salmonella Typhi**	-	-	-	-	14	20	-	-	**(Fc)**
Proteus vulgaris	-	-	-	-	12	25	-	-	12
Fungal pathogens	-	-	-	-	15	25	-	-	14
Aspergillus flavus	-	-	-	-	14	25	-	-	18
Aspergillus niger	-	-	-	-	10	15	-	-	10
Alternaria solani	-	-	-	-	15	15	-	-	10
Candida albicans (MTCC 227)	-	-	-	-	9	13	-	-	20
Penicillium monotricales								-	9
Saccharomyces cerevisiae									
Saccharomyces cerevisiae (*MTCC 3980)*									

[a]Values are in inhibition zone diameter (mm) and Each estimation is average of triplicate.

[b]Standards: G, gentamicin (10µg/disc); P_G, penicillin (10unit/disc); M, Methicillin(5µg/disc); Vancomycin, (30 µg/disc), according to CLSI guidelines.

A=petroleum ether, B=benzene, C=chloroform, D=ethyl acetate, E=acetone, F=ethanol and G= aqueous extract: each extract has concentration of 100µg/disc.

Fc. Cyclohexamide (30 µg/disc) used as fungicide.

NT = Not tested, (-) = No inhibition, R= Resistant, *= For further MIC determination.

Table 2: MIC of *C. opaca* ethyl acetate and acetone extracts against bacterial cultures

Microorganisms	Ethyl acetate[a] (µg/disc)	Acetone [a] (µg/disc)	Standards [b] (G/P_G/M/V) For Ethy acetate (In mm)	Standards [b] (G/P_G/M/V) For Acetone (In mm)
Bacterial pathogens				
Bacillus cereus	25	25	25	20
Staphylococcus aureus	12.5	50	24	19
Corynebacterium spp.	25	25	26	21
Alcaligenes faecalis	25	25	22	16
Escherichia coli	12.5	6.25	19	18
(MTCC 1687)	12.5	6.25	23	17
Pseudomonas aeruginosa (MTCC	12.5	50	21	23
424)	50	25	22	25
Klebsiella pneumoniae	25	25	25	21
Salmonella typhi	50	50	**Standards (Fc)**	**Standards (Fc)**
Proteus vulgaris	100	50	16	11
Fungal pathogens	50	100	17	19
Aspergillus flavus	100	50	11	13
Alternaria solani	50	100	13	13
Candida albicans (MTCC 227)			14	16
Penicillium monotricales				
Saccharomyces cerevisiae (MTCC 3980)				

[a]Each estimation is average of triplicate.

[b]Standards: G ,gentamicin (10µg/disc); P_G, penicillin (10unit/disc); M, methicillin(5µg/disc); V, vancomycin (30 µg/disc), according to NCCLS guidelines.

Fc. Cyclohexamide (30 µg/disc) used as a fungicide.

CONCLUSION

Although this study investigated the *in vitro* antimicrobial activity, the results showed that the extracts *Carissa opaca* possessed good antibacterial activity, confirming the great potential of this plant for the production of bioactive compounds and could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant pathogens. *In vivo* data may be helpful in determining the real potential usefulness of these plants for the treatment of infectious diseases.

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