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RESEARCH ARTICLE

ANTIFUNGAL EFFICACY OF ALKALOIDS, FLAVONOIDS AND STEROIDS OF DIFFERENT PARTS OF *CARISSA CARANDAS* LINN

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ABSTRACT

Evaluation of the antifungal activity of extracts from different parts (root, stem & leaf) of *Carissa carandas* (Apocynaceae) Linn. was carried out. Different parts were collected, dried and extracted by using well established methods for alkaloids, flavonoids and steroids. Screening was done by using 'Disc Diffusion Assay' against *Candida albicans* (Yeast), *Aspergillus flavus* & *Tricophyton mentagrophyte* (fungi). Minimum inhibitory concentration, Minimum fungicidal concentration & Total activity were studied. Mean and Standard Deviation have also been calculated. *C. albicans* found to be the most susceptible organism while *T. mentagrophyte* found to be resistant organism for all the tested extracts. Alkaloid extract of stem showed best activity (IZ= 10 mm, AI= 0.43±0.02, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA= 65.6 ml/g) against *C. albicans* while free flavonoids (IZ= 11 mm, AI= 0.24±0.02, MFC= 0.312 mg/ml, MIC= 0.156 mg/ml, TA= 73.7 ml/g) & bound flavonoids (IZ= 10 mm, AI= 0.22±0.02, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA= 8 ml/g) of stem showed very good activities against *A. flavus*. The range of MFC & MIC was found to be 1.25-0.312 mg/ml & 0.625-0.156 mg/ml, respectively. Results reveal good antifungal potential of extracts. Hence, may be explored for formation of new antifungal drugs.

INTRODUCTION

In the developing countries synthetic drugs are not only expensive and inadequate for the treatment of diseases, but are also often with adulterations and side effects, as a result, different remedies evolved in different regions of the world (Britto *et al,* 2001). The World Health Organization has reported that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic health care needs. Hence, there is a need to search plants of medicinal value. Today, world is gradually turning to herbal formulations, which are known to be effective against a large repertoire of diseases and ailments. More importantly, they are not known to cause any notable derogatory effects and are readily available at affordable prices (Sharma *et al,* 2008). Plants used in the traditional medicine contain a vast array of active substances that can be used to treat many human diseases (Stary *et al,* 1998). Plant extracts have been proposed to be used as antimicrobial substances (Del Campo *et al,* 2000).

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To determine the potential and to promote the use of herbal medicines, it is essential to intensify the study of medicinal plants that find place in folklore (Awadh-Ali *et al.,* 2001). In the present investigation, *Carissa carandas* has been selected for the study. *Carissa carandas* (common name Karaunda) is a perennial shrub belongs to family Apocynaceae. It grows naturally in the Himalayas at elevations of 300 to 1800 meters, in the Siwalik Hills, the Western Ghats and in Nepal and Afghanistan. It flourishes well on lands with high temperatures. At present it is grown on a limited scale in Rajasthan, Gujarat, Bihar and Uttar Pradesh regions of India. Various medicinal properties viz. Stomachic, Anthelmintic, Cardio tonic, Lowering blood pressure are attributed to this plant. Other properties attributed are strengthening tendons, effective against remittent fever, earache and syphilitic pain.
Alkaloids are known to have pharmacological effects and are used in medications, as recreational drugs or in entheogenic rituals. Literature indicates that plant alkaloids have considerable biological activity (Cowan, 1999; Okunade *et al*., 2004). Phytochemicals like alkaloid, terpenoid, steroid, glycoside and tannin were identified in fruits and leaves of *Carissa carandas* (Verma *et al*., 2011). Flavonoids are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anti-cancer

activity (Del-Rio *et al*., 1997). It was reported that flavonoids can improve the blood circulation and lower the blood pressure (Blumenthal, 2003). Flavonoid, terpenoid, steroid, coumarins, glycoside were isolated from fruits of *Carissa carandas* (Brahmbhatt, 2012). Phenolic acids, flavanols, flavonols of *C. carandas* showed significant antifungal activity as they substantially inhibited all the tested fungal species (Siddiqui *et al*., 2011). Steroids are frequently used as signalling molecules, represents highly concentrated energy stores, along with phospholipids function as components of cell membranes. Triterpinoids (cyclic steroids) were isolated from leaves of *Carissa carandas* (Siddiqui *et al*., 2011). Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their antimicrobial activity may provide new antimicrobial substances. Review of the current literature reveals that no work has been carried out for extraction and screening of specific compound from selected plant. Hence, in the present work an extraction and screening for antifungal activity of extracts of *C. Carandas* has been undertaken.

MATERIALS AND METHODS

Different parts of *C.carandas* (leaf, stem and root) were collected in the month of April to June from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at Department of Botany, University of Rajasthan and voucher specimen no: RUBL 21130 was submitted to the Herbarium, Botany Department, University of Rajasthan.

Preparation of Extracts

Alkaloids Extraction

Alkaloids were extracted from different parts of the selected plant by well established method (Ramawat *et al*., 2000).Finely powered sample (100g) of plant parts were extracted in 20ml methanol after shaking of 15 min. After filtration, filtrate kept for drying then residual mass were treated with 1% H_2SO_4 (5ml. 2 times).Extraction was then done in 10ml. Chloroform ($CHCl_3$) by using separating funnel. Organic layer of chloroform was rejected and aqueous layer was basified with 30% NH_4OH (P^H=9-10). Now again, extraction was done in 10ml. chloroform & organic layer of chloroform (lower layer) was collected in a flask and repetition of step was done with fresh chloroform. Extracts was then dried in vaccuo for further use.

Flavonoid extraction

Selected plant parts were separately washed with sterilized water; shade dried, and finely powdered using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan (1969). One hundred grams of each finely powdered sample was soxhlet extracted with 80% hot methanol (500ml) on a water bath for 24 h and filtered. Filtrate was re- extracted successively with petroleum ether (fraction I), diethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, where as diethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. The ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for removal of bound sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract obtained was washed with distilled water to neutrality. Diethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vaccuo and weighed. The extracts were stored at 4^oc and were re-suspended in their respective solvents to get 10mg/ ml concentration for antimicrobial assay.

Steroid Extraction

Steroids were extracted from different parts of the selected plant by well established method (Y. Tomita *et al,* 1970) after preliminary detection of steroids. Finely powdered sample (100g) of plant parts were extracted in petroleum ether for 2-4hr.After filtration, residual mass was treated with 15% ethanolic HCl for 4hr. Extraction was then done in ethyl acetate followed by washing in dis. water to neutralize the extract. Neutral extract was then passed over sodium sulphate to remove moisture contents and was dried in vaccuo. Chloroform was used for reconstitution of extract, filtered and dried for further use.

Selected Test Microorganisms

Three pathogenic bacteria were screened, viz., *Candida albicans* (MTCC no. 183), *Aspergillus flavus* (MTCC no. 277) and *Tricophyton mentagrophyte* (MTCC no. 7687). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Fungal strains were grown and maintained on Sabouraud Dextrose Agar medium. *Candida albicans* is major model of pathogenic yeast which is found in mouth, throat, intestine and genitourinary tract of human and considered as common constituent of bowel flora together with many bacterial species e.g. *E. coli*, *S. aureus* and *P. mirabilis*. It lives in 80% of the human population with no harmful effects, although overgrowth results in candidiasis which is often observed in immunocompromised individuals such as patients of cancer, transplant and AIDS. It is a causal agent of opportunistic oral and genital infections in humans (Enfert *et al*., 2007). Superficial and mycosis infections cause local inflammation and discomfort in human beings (P.G. Pappas, 2006). Candidiasis also known as 'thrush', which is usually occur in immune compromised people (Ryan *et al.,* 2004).

Aspergillus flavus is the second leading cause of invasive and non-invasive aspergillosis (Hedayati *et al*., 2007). The presence of *Aspergillus* in the air is a major risk factor for both invasive and allergic aspergillosis (Denning *et al.,* 1997). *A. flavus* can cause storage problems in stored grains. It also causes diseases in economically important crops, such as maize and peanuts and produce potent mycotoxins. It can also be a human pathogen, associated with aspergillosis of the lungs and sometimes causing corneal, otomycotic and nasoorbital infections. *Tricophyton mentagrophyte* is a cosmopolitan dermatophyte, belonging to a homogeneous group of fungi called the dermatophytes. The organism is found in soil, floor of swimming pools, hairs of wild boar, cats and dogs, farm animals, foot wears and from human toe webs without clinical lesions. It requires keratin for growth and can cause a variety of cutaneous (hair, nail and skin) infections in humans and animals hence considered to be anthropophilic or zoophilic in nature (Van Rooij *et al*., 2006; Sanchez-

Castellanos *et al*., 2007). It causes dermatophytosis in dogs, cats, cattle and especially in rodents (George *et al*., 1957; Houk *et al*., 1996 and Ajello *et al*., 1967).

Antimicrobial assay

'Disc Diffusion Assay' was performed for screening (Andrews, 2001). SD agar base plates were seeded with the fungal inoculum (inoculum size 1×10^7 CFU/ml). Sterile filter paper discs of Whatmann no.1 (6mm in diameter) were impregnated with 100µl of each of the extract of concentration 10mg/ml to give a final concentration of 1 mg/disc. Discs were left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in triplicate along with Ketoconazole (1mg/disc) for *T. mentagrophyte* and Terbinafine for *C. albicans* and *A. flavus* as standard drugs. The plates were kept at 4 C for diffusion of extract, thereafter were incubated at 27°C (*C. albicans* and *A. flavus* for 48 h & *T. mentagrophyte* for 5-7 days). Activity index for each extract was calculated Table 1 by the standard formula viz.

Activity index = IZ produced by the extract/ IZ produced by standard [IZ = inhibition zone (in mm)]

Determination of Minimum Inhibitory Concentration (MIC) & Minimum Fungicidal (MFC) Concentration

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against the test pathogens. 'Broth micro dilution' method was followed for determination of MIC values (Barsi *et al*., 2005). Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter 100µl inoculum (1×10^7 CFU/ ml) was added to each well. Fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 27^0C for 48 h. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4^0C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum fungicidal concentration (MFC) was determined by sub culturing 50 µl from each well showing no apparent growth.

Table 1. Antifungal activity of extracts of *Carissa carandas* Linn. against some pathogenic fungi

Plant part	Extract	Microorganisms					
		C. albicans		*A. flavus*		*T. mentagrophyte*	
		IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI
Leaf	A1	-	-	7	0.17±0.01	-	-
	S1	8	0.27±0.01	-	-	-	-
	E1	7	0.58±0.01	-	-	-	-
	E2	7.5	0.62±0.01	-	-	-	-
Stem	A2	10	0.43±0.01	7	0.17±0.01	-	-
	S2	8	0.27±0.01	-	-	-	-
	E1	7	0.54±0.01	11	0.24±0.02	-	-
	E2	8	0.61±0.01	10	0.22±0.02	-	-
Root	A3	-	-	-	-	-	-
	S3	8	0.27±0.01	-	-	-	-
	E1	-	-	-	-	-	-
	E2	7.5	0.58±0.01	8	0.18±0.01	-	-

A1, A2, A3 = Alkaloid extract of respective plant parts,
S1, S2, S3= Steroid extract of respective plant parts,
E1, E2= Free & Bound flavonoids of respective plant parts,
IZ=Inhibition zone in mm (value: including 6mm diameter of disc),
AI= Activity index (IZ developed by extract/IZ developed by standard),
(-) = no activity,
±=SEM.

Table 2. MFC and MIC of active extracts of *Carissa carandas* Linn. against some pathogenic fungi

Plant parts & Extracts		Leaf				Stem				Root			
Microorganisms	MFC & MIC (mg/ml)	A1	S1	E1	E2	A2	S2	E1	E2	A3	S3	E1	E2
C. albicans	MFC	-	1.25	1.25	1.25	0.625	1.25	1.25	1.25	-	1.25	-	1.25
	MIC	-	0.625	0.625	0.625	0.312	0.625	0.625	0.625	-	0.625	-	0.625
A. flavus	MFC	1.25	-	-	-	1.25	-	0.312	0.625	-	-	-	1.25
	MIC	0.625	-	-	-	0.625	-	0.156	0.312	-	-	-	0.625
		-				-							
T. mentagrophyte	MFC	-	-	-	-	-	-	-	-	-	-	-	-
	MIC		-	-	-		-	-	-	-	-	-	-

A1, A2, A3 = Alkaloid extract of respective plant parts,
S1, S2, S3 = Steroid extract of respective plant parts,
E1, E2= Free & Bound flavonoids of respective plant parts,
MIC= Minimum inhibitory concentration (in mg/ml),
MFC=Minimum fungicidal concentration (in mg/ml),
(-) = no activity.

Least concentration of extract showing no visible growth on sub culturing was taken as MFC. Table 2.

Total activity (TA) determination

Total activity is the volume up to which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g. (Eloff, 2004) Table 3.

TA= amount of extract from 1gm of dry plant material/MIC of the extract

ml/g), bound flavonoids of leaf (IZ= 7.5 mm, AI= 0.62±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 7.2 ml/g), free flavonoid of stem (IZ= 7 mm, AI= 0.54±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 18.4 ml/g) and bound flavonoids of root (IZ= 7.5 mm, AI= 0.58±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 1.6 ml/g) showed satisfactory activities against *C. albicans* while alkaloid of leaf (IZ= 7 mm, AI= 0.17±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 73.6 ml/g) and alkaloid of stem (IZ= 7 mm, AI= 0.17±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 32.8 ml/g) showed satisfactory activity against *A. flavus*. Among all the tested extracts, free& bound flavonoids of stem, alkaloid of

Table 3. Quantity & Total activity of extracts of *Carissa carandas* Linn

Plant part	Extract	Quantity of extract mg/g dwt	Total Activity(ml/g)		
			C. albicans	*A. flavus*	*T. mentagrophyte*
Leaf	A1	46	-	73.6	-
	S1	50.5	80.8	-	-
	E1	12	19.2	-	-
	E2	4.5	7.2	-	-
Stem	A2	20.5	65.6	32.8	-
	S2	28.5	45.6	-	-
	E1	11.5	18.4	73.7	-
	E2	2.5	4	8	-
Root	A3	35.5	-	-	-
	S3	16	25.6	-	-
	E1	3.5	-	-	-
	E2	1	1.6	1.6	-

A1, A2, A3 = Alkaloid extract of respective plant parts,
S1, S2, S3 = Steroid extract of respective plant parts,
E1, E2= Free & Bound flavonoids of respective plant parts,
TA= total activity (extract per gm dried plant part/MIC of extract).

RESULTS

Antimicrobial efficacy of secondary metabolites (Alkaloid, steroid & flavonoid) were assessed by using IZ, AI (Table 1), MIC, MFC (Table 2). Quantity of extract per gram of plant material was also calculated (Table 3). In present investigation, 12 extracts were tested against three pathogenic organisms, including one yeast *C. albicans* and two fungi *A. flavus*& *T. mentagrophyte.* Among all the tested extracts free flavonoid & alkaloid extract of root found to be inactive against all the tested pathogens. *T. mentagrophyte* found to be resistant pathogen as no extract showed activity against it. *C. albicans* found to be most susceptible organism as 9 out of 12 extracts showed activity against it. Best activity was observed in alkaloid extract of stem (IZ= 10 mm, AI= 0.43±0.02, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA= 65.6 ml/g) against *C. albicans* while in free flavonoids (IZ= 11 mm, AI= 0.24±0.02, MFC= 0.312 mg/ml, MIC= 0.156 mg/ml, TA= 73.6 ml/g) & bound flavonoids (IZ= 10 mm, AI= 0.22±0.02, MFC= 0.625 mg/ml, MIC= 0.312 mg/ml, TA= 8 ml/g) of stem against *A. flavus*. Steroid extract of leaf (IZ= 8 mm, AI= 0.27±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 80.8 ml/g), bound flavonoids of stem (IZ= 8 mm, AI= 0.61±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA=4 ml/g), steroid of stem (IZ= 8 mm, AI= 0.27±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 45.6 ml/g) and steroid of root (IZ= 8 mm, AI= 0.27±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 25.6 ml/g) showed good activities against *C. albicans* while bound flavonoid of root showed good activity (IZ= 8 mm, AI= 0.18±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 1.6 ml/g) against *A. flavus*. Free flavonoid of leaf (IZ= 7 mm, AI= 0.58±0.01, MFC= 1.25 mg/ml, MIC= 0.625 mg/ml, TA= 19.2 stem and bound flavonoid of root found to be the most active substances as they showed activities against 2 of the 3 pathogens. Plant extracts, which had shown activity in diffusion assay, were evaluated for their MIC & MFC values (Table 2). The range of MFC & MIC of extracts recorded was 1.25-0.312 & 0.625- 0.156, respectively. In present study, lowest MIC value 0.156 mg/ml was recorded against *A. flavus*, indicating significant antimicrobial efficacy of tested extracts. Quantity of extract obtained per gram from plant parts & TA was calculated and recorded (Table 3). TA indicates the volume at which extract can be diluted without losing ability to kill microorganisms. High value of TA were observed against *C. albicans* (80.8 ml/g) followed by *A. flavus* (73.6 ml/g).

DISCUSSION

Extracts from different parts of *C. carandas* have previously been studied for their antifungal activity but, still meager work has been carried out as far as the antifungal activity of secondary metabolite extracts is concerned. Most of the research has been restricted on determination of IZ of extracts without calculating AI, MIC, MFC and TA. Determination of MIC and MFC has now become an inevitable step in antimicrobial studies in order to establish their antimicrobial activity so as to explore them at industrial level for production of drugs, which could replace the existing ones. Hence, most of the studies carried out so far could only reveal their antimicrobial activities, but are not helpful for establishing them as antifungal. Result of the present study indicated that the extracts of all the parts (leaf, stem & root) of *C. carandas* have antifungal activity against two of the selected fungus.

The results were in agreement with the findings of previous studies. Furthermore, it may help to discover new chemical classes of antifungal that could serve as selective agents for the maintenance of human health and provide biochemical tools for the study of infectious diseases.

Conclusion

Present study concluded that among all the tested extracts of different plant parts, flavonoid extract was found to be the most active substances while steroid extract was found to be the least active. *C. albicans* was recorded as the most susceptible organisms while *T. mentagrophyte was* completely resistant throughout the study. Lowest MIC value 0.156 mg/ml was recorded against *A. flavus*, indicating significant antimicrobial efficacy of the tested extracts. High values of TA (80.8 ml/g) were observed against *C. albicans*, indicating strong antimicrobial potential, even in the diluted forms of the extracts. Therefore, study may be beneficial for further studies related to the production of novel antifungal drugs.

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