Available online at www.elixirpublishers.com (Elixir International Journal)

Bio Technology

Elixir Bio Tech. 74 (2014) 26997-27001

ISSN: 2229-712X

Evaluation of antimicrobial efficacy of flavonoids, alkaloids and steroids of Carissa *carandas* linn. against some pathogenic bacteria

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ARTICLE INFO

Article history:

Received: 13 July 2014;

Received in revised form:

21 August 2014;

Accepted: 6 September 2014;

Keywords

Flavonoids,

Alkaloids, Steroids,

Minimum inhibitory concentration,

Minimum bactericidal concentration &

Total activity.

ABSTRACT

Present work deals with assessing antimicrobial activity of *C.carandas* against multidrug resistant pathogenic bacteria. Leaf, stem and root were collected, dried and extracted using standard methods for flavonoids, alkaloids and steroids. Extracts were screened by using 'Disc Diffusion Assay'. Minimum inhibitory concentration, Minimum bactericidal concentration, Total activity, Mean and Standard Deviation were calculated. *S.aureus* was found to be the most susceptible organism followed by *B.subtilis* and *E.coli*. Bound Flavonoid of roots showed the best activity against B.subtilis (IZ= 15mm, MIC= 0.312mg/ml, MBC=0.156mg/ml, TA=3.20ml/g). Results reveal that extracts of *C.carandas* have good antimicrobial potential and may be exploited for antimicrobial drugs

Introduction

Medicinal plants are considerably useful and medicinally very important as they contain pharmaceutically active constituents which are used in the treatment of many human diseases (Stary *et al*., 1998). Plant extracts have been developed and proposed for use as antimicrobial substances (Del Campo *et al*., 2000). Many of the plant materials used in traditional medicine are readily available in rural areas which are relatively cheaper than modern medicine (Mann et al.,2008).Thus it is important to characterize different types of medicinal plants for their antioxidant and antimicrobial potential (Bajpai *et al*., 2005). Due to a rapid increase in the rate of infections, antibiotic resistance in microorganisms and due to side effects of synthetic antibiotics, medicinal plants are gaining popularity over these drugs (Bapu *et al*., 2009). The antimicrobial activities of medicinal plants can be attributed to the secondary metabolites such as alkaloids, flavonoids, steroids, tannins, terpenoids that are present in these plants (Sher A, 2009). Antimicrobial activities of many plants have been reported by the researchers (Ateb *et al*., 2003). In the present study *Carissa carandas* has been selected for the study.

Carissa carandas (comman 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, Cardiotonic, Lowering blood pressure are attributed to this plant. Besides, it cares strengthen tendons, remittent fever, earache and syphilitic pain. The present investigation was undertaken to find out the antibacterial potential of flavonoids, alkaloids and steroids of different parts of *C.carandas* against some Gram positive and Gram negative bacteria.

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 S *et al*.2011).Literature related to antimicrobial activity of alkaloids of *Carissa carandas* was not yet found.

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 MR. 2012). Phenolic acids, flavanols, flavonols of *C. carandas* showed significant antibacterial activity as they substantially inhibited all the tested fungal species (Siddiqui R. *et al*. 2011).

Steroids and their metabolites 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 BS *et al*.2011). Literature related to antimicrobial activity of steroids of *Carissa carandas* was not yet found.

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 antibacterial activity of the flavonoids, alkaloids and steroids of *C.carandas* has been undertaken.

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Material 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:

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^{o}c and were re-suspended in their respective solvents to get 10mg/ ml concentration for antimicrobial assay.

Alkaloids Extraction:

Alkaloids were extracted from different parts of the selected plant by well established method (Ramawat K.G. *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 was 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.

Steroid Extraction:

Steroids were extracted from different parts of the selected plant by well established method (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., *Escherichia coli* (MTCC no.46), *Bacillus subtilis* (MTCC no. 121), *Staphylococcus aureus* (MTCC no. 3160), *Klebsiella pneumonae* (MTCC no.4030) and *Agrobacterium tumifaciens* (MTCC no. 431). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Bacterial strains were grown and maintained on Muller-Hinton Agar medium.

E.coli is one of the most frequent causes of many common bacterial infections including bacteremia, urinary tract infection (UTI), traveler's diarrhea, neonatal meningitis (Veneir *et al*., 2007) and pneumonia. Some virulent strains cause serious illness or death in the elderly, the very young or the immunocompromised (Hudault *et al*., 2001; Nataro and Kaper, 1998). Intestinal mucosa associated *E. coli* is observed in increased number in the inflammatory bowel diseases, Crohn's diseases and ulcerative colitis (Toder, 2007; Rolhin *et al*., 2007).*S.aureus* is the most common hospital acquired pathogen and cause staph infections which is responsible for various diseases including: mild skin infections e.g. folliculitis, invasive diseases e.g. wound infections and bacteremia etc., and toxin mediated diseases e.g. food poisoning, toxic shock syndrome (TSS) and scaled skin syndrome etc. In infants its infection can cause a severe disease Staphylococcal scalded skin syndrome (SSSS) (Curran and Al-Salihi, 1980). Recently, the serious emergence of antibiotic resistance staph occurred with a specific strain is Methicillin-Resistant *Staphylococcus aureus* (MRSA) and research being done to investigate hospital acquired MRSA. *B.subtilis* bacteria are nonpathogenic. They can contaminate food; however, they seldom result in food poisoning. *K.pneumonae* cause destructive changes to human lungs inflammation and hemorrhage with cell death (necrosis) .The range of clinical disease includes pneumonia, thrombophlebitis, UTI, cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, meningitis and bacteremia and septicemia. *Agrobacterium tumefaciens* is a tumor producing pathogenic bacteria and do not benefit the plant. Economically, this pathogen is a serious pathogen of walnuts, grape vines, and stone fruit.

Antimicrobial assay:

'Disc Diffusion Assay' was performed for screening (Andrews JM., 2001). MH agar base plates were seeded with the bacterial inoculum (inoculum size 1×10^8 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 streptomycin (1mg/disc) as standard drug for bacteria. The plates were kept at 4°C for diffusion of extract, thereafter were incubated at 37°C for 24h. 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

Where, IZ = inhibition zone.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal (MBC)/ 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 (1×10^8 CFU/ ml) was added to each well. Bacterial suspensions were used as negative control, while broth containing standard drug was used as positive control.

Table 1: Antimicrobial activity of extracts of Carissa carandas against some pathogenic bacteria

Plant part	Extract	Microorganisms									
		E.coli		B.subtilis		S.aureus		K.pneumonae		A.tumifaciens	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI	IZ	AI
Leaf	E1	-	-	9	0.32±0.04	9	0.45±0.06	-	-	9	0.32±0.04
	E2	8.25	0.39±0.01	9.5	0.34±0.02	9.5	0.48±0.03	-	-	-	-
	A1	7.25	0.30±0.01	-	-	-	-	-	-	-	-
	S1	-	-	-	-	-	-	-	-	-	-
Stem	E1	-	-	9	0.32±0.04	9	0.60±0.09	12	0.60±0.01	-	-
	E2	8.25	0.34±0.01	-	-	9	0.40±0.06	-	-	-	-
	A2	9.25	0.33±0.01	-	-	-	-	-	-	-	-
	S2	-	-	-	-	8	0.8± 0.03	-	-	8.5	0.28±0.02
Root	E1	-	-	8.5	0.30±0.02	9.5	0.43±0.03	-	-	-	-
	E2	-	-	15	0.50±0.01	8.5	0.53±0.04	-	-	-	-
	A3	9.25	0.30±0.01	-	-	-	-	-	-	-	-
	S3	-	-	12	0.40±0.01	9	0.75±0.02	-	-	-	-

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

Table 2: MIC and MBC of active extracts of Carissa carandas against different pathogens

Plant parts		Leaf				Stem				Root			
Microorganisms		E1	E2	A1	S1	E1	E2	A2	S2	E1	E2	A3	S3
E.coli	MIC	-	0.625	1.25	-	-	0.625	0.625	-	-	-	0.625	-
	MBC	-	0.312	0.625	-	-	0.312	0.312	-	-	-	0.312	-
B.subtilis	MIC	0.625	0.625	-	-	0.625	-	-	-	0.625	0.312	-	0.312
	MBC	0.312	0.312	-	-	0.312	-	-	-	0.312	0.156	-	0.156
S.aureus	MIC	0.625	0.625	-	-	0.625	0.625	-	1.25	0.625	1.25	-	0.625
	MBC	0.312	0.312	-	-	0.312	0.312	-	0.625	0.312	0.625	-	0.312
K.pneumonae	MIC	-	-	-	-	0.312	-	-	-	-	-	-	-
	MBC	-	-	-	-	0.156	-	-	-	-	-	-	-
A.tumifaciencs	MIC	0.625	-	-	-	-	-	-	1.25	-	-	-	-
	MBC	0.312	-	-	-	-	-	-	0.625	-	-	-	-

E1 = Free flavonoids, E2= Bound flavonoids,
A1, A2, A3= Alkaloids of respective plant parts,
S1, S2, S3= Steroids of respective plant parts,
MIC= Minimum inhibitory concentration,
MBC=Minimum bactericidal concentration,
(-)= no activity.

Table 3: Quantity & Total activity of extracts of Carissa carandas

Plant part	Extract	Quantity of extract mg/g dwt	Total Activity(ml/g)				
			E.coli	B.subtilis	S.aureus	K.pneumanae	A.tumefaciencs
Leaf	E1	1.2	-	1.92	1.92	-	1.92
	E2	4.5	7.2	7.2	7.2	-	-
	A1	46	36.8	-	-	-	-
	S1	50.5	-	-	-	-	-
Stem	E1	11.5	-	18.4	18.4	36.85	-
	E2	2.5	4	-	4	-	-
	A2	20.5	32.8	-	-	-	-
	S2	28.5	-	-	22.8	-	22.8
Root	E1	3.5	-	5.6	5.6	-	-
	E2	1	-	3.20	0.8	-	-
	A3	35.5	56.8	-	-	-	-
	S3	16	-	51.28	51.28	-	-

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

Micro titer plates were then incubated at 37°C for 24 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°C 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 bactericidal concentration (MBC) was determined by sub culturing 50 µl from each well showing no apparent growth. [Table2]. Least concentration of extract showing no visible growth on sub culturing was taken as MBC.

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 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g. (Eloff JN.,2004) [Table 3]

Results:

Antimicrobial potency of Alkaloids, flavonoids (Free and Bound) and Steroids were assessed by IZ, AI (Table-2), MIC & MBC (Table-1). Quantity of extracts per gram of plant material was also calculated (Table-3). In the present investigation total 12 extracts were tested, among which 'steroidal extract of leaf' was found inactive against any of the tested pathogens whereas other 11 extracts were active against at least one of the tested pathogens. *A.tumifaciens* & *K.pneumonae* were observed to be very resistant organism as very few of the tested extracts showed activity against them. The most susceptible organism in the investigation was *S.aureus* against which 8 out of 12 extracts showed good activity. Free flavonoid (F.F.) of stem showed the best activity against *K.pneumonae* (IZ=12mm, AI= 0.60±0.010, MIC= 0.312mg/ml) whereas bound flavonoids (B.F.) showed good activity against *E.coli* (IZ= 9.25mm,AI= 0.34±0.010,MIC= 0.625mg/ml). B.F. of roots showed best activity against *B.subtilis* (IZ= 15mm, AI=0.50±0.010, MIC= 0.312mg/ml) whereas F.F. of roots showed good activity against *S.aureus* (IZ= 9.5mm, AI= 0.43±0.010, MIC= 0.625mg/ml).All the three alkaloids showed activity only against *E.coli* whereas the steroids of root showed best activity against *B.subtilis* (IZ= 12mm,AI= 0.40±0.010,MIC= 0.312mg/ml). Leaf steroid showed no activity against any pathogen while stem steroid showed good activity against *S.aureus* & *A.tumifaciens*. Among all the extracts, flavonoids found to be the best bioactive compounds in *C.carandas* plant. MIC & MBC values (Table2) were evaluated for plant extracts which had shown activity; in 'Disc Diffusion Assay'. The range of MIC & MBC of extracts recorded was 1.25-0.312mg/ml & 0.625-0.156mg/ml respectively.

In present investigation lowest MIC value (0.312mg/ml) was recorded against *B.subtilis* & *K.pneumonae,* indicating significant antimicrobial potential of test extracts. Quantity of extract obtained per gram of plant parts & TA calculated was recorded (Table-3). TA indicates the volume up to which the extract can be diluted, without losing ability to kill microorganism. High values of TA were observed against *E.coli* (56.80ml/g) followed by *B.subtilis* (51.28ml/g), *S.aureus* (51.28ml/g) & *K.pneumonae* (36.85ml/g).

Discussion:

In present scenario, MDR (Multi Drug Resistance) is a problem of global concern. Several pathogens responsible for many human diseases, are becoming resistant to the existing antibiotics, hence efficiency of these medicines has deteriorated in a big way & their use is no more effective. On the other side high cost of available antibiotics and increase in the incidence of new & re-emerging infectious diseases makes a continuous and urgent need to discover new antimicrobials with less or no side effects, cost effective and have ability to affect the wide range of pathogens. Present study is an effort towards this direction. *C.carandas* had previously been studied for antibacterial activities, but still the literature available is meager. Antimicrobial activity against various pathogens have been recorded in aqueous, ethanol, methanol, chloroform & acetone extracts of *C.carandas* (Salar RK. *et al*.,2010) Chloroform, ethanol, acetone, dichloromethane, toluene & ethyl acetate extract of leaf & fruit of *C.carandas* found to exhibit antimicrobial activity against *S.auerus, K.Pneumonae* & *E.Coli* (Verma S. *et al*. ,2011) Antimicrobial activity of ethanolic, extract of fruits of *C.carandas* have also been reported against *S. aureus, S. epidermidis, S. pneumonae, B. subtilis, E. coli, P. vulgaris* & *P. mirabilis* (Israr F. *et al* . 2012).

However, *C.carandas* so far has not been studied out for flavonoids, Alkaloids & steroids. Mostly the crude extracts have been screened of all three parts (Root, leaf & stem), without MIC, MBC & TA determination. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence can't replace the existing antibiotics. In the present investigation IZ, AI, MIC, MBC & TA have been evaluated for each extract. Extracts recorded for low MIC values, indicate strong bio efficacy of the plant. The findings of the present investigation offer a scientific evidence to support the ethno-medicinal use of the plant and recommend to explore the plant for an alternative medicine for diseases caused by pathogen under study.

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