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Meenakshi Fartyal*, Padma Kumar

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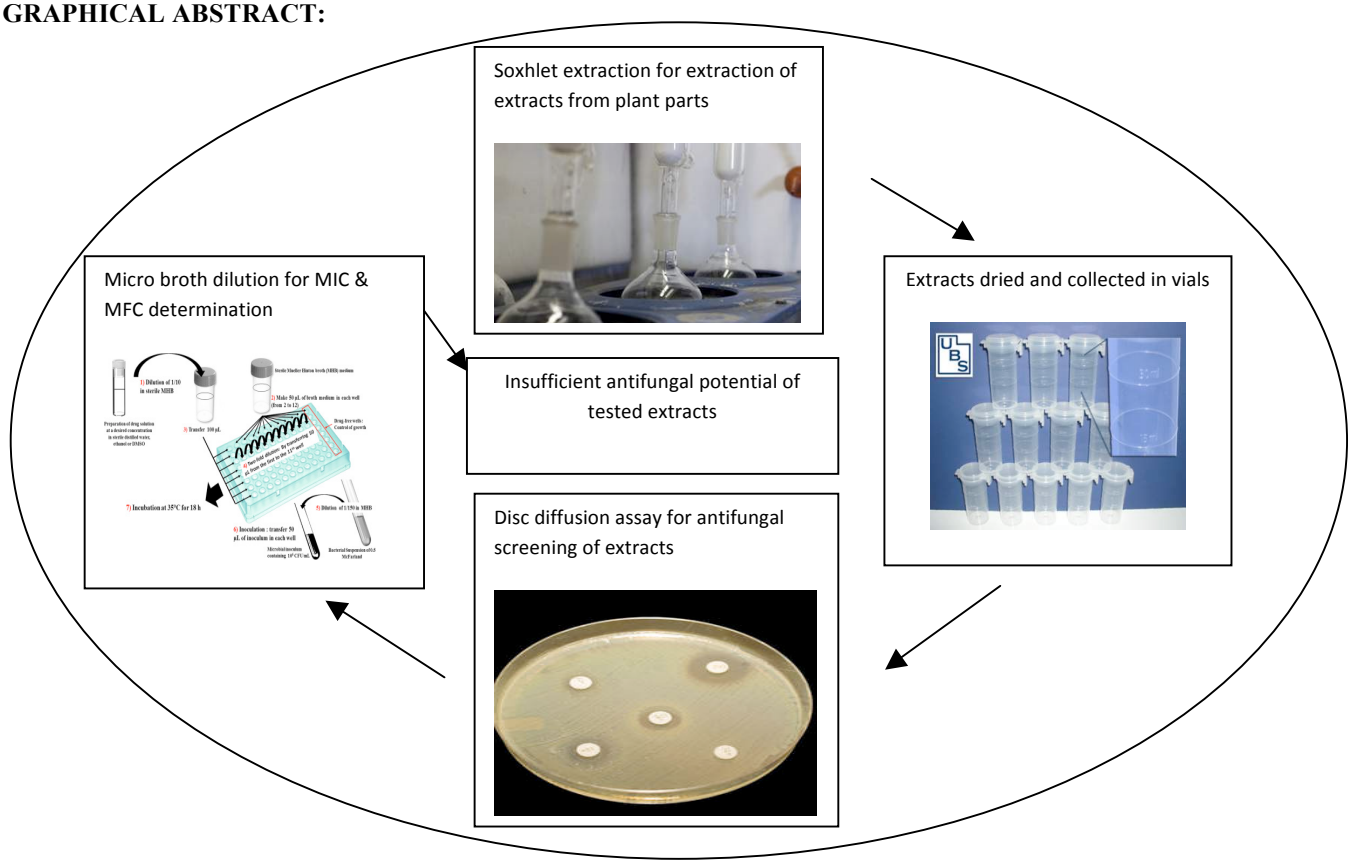
Insufficient Antifungal Potential of Crude Extracts of *Carissa carandas* Linn. & *Nerium oleander* Linn

Meenakshi Fartyal*[1], Padma Kumar[1]

[1]Laboratory of Plant Tissue Culture and Secondary Metabolites, Department of Botany, University of Rajasthan, Bapu nagar, Jaipur- 302004, India. Email*: mksh35@gmail.com

GRAPHICAL ABSTRACT:



ABSTRACT:

Objective: To evaluate the antifungal potential of crude extracts from different parts of *Carissa carandas* Linn. (Leaf, stem & root) and *Nerium oleander* Linn. (Leaf, stem & root). **Material & methods**: Different parts of plants were collected, dried and then extracted by using the soxhlet extraction method in different polar and non-polar solvents (Water, Methanol & petroleum ether). Extracts were then screened for antifungal activity using a 'Disc Diffusion Assay' against *Candida albicans* (Yeast), *Aspergillus flavus* & *Tricophyton mentagrophyte* (fungi). Minimum inhibitory concentration, Minimum fungicidal concentration & Total activity were studied. The Mean and Standard Deviation were calculated. **Results**: The results indicate that all the tested extracts were found to have no antifungal activity against the tested microorganisms. **Conclusion**: The tested extracts did not have, or had too little, antifungal activity. Hence, may not be explored as promising sources of new antimicrobial drugs.

KEYWORDS: Disc diffusion assay, Minimum fungicidal concentration, Minimum inhibitory concentration, Polar and non polar solvents, Total activity.

Introduction

India with its richness and diversity can be considered as the paradise of medicinal plants. Since prehistoric times, nearly all cultures, both ancient and modern, have used plants as natural resources for medicinal purposes. WHO [1] reported that 80% of the global population relies on traditional therapies which involve the use of plant extracts or their active constituents. Developing countries depend on plants as the source of medicine thus; traditional medicine plays a major role in health care [2]. Since there has been an increase in the use of synthetic drugs leading to many side effects and undesirable hazards, there is a worldwide trend to go back to natural resources (mainly traditional plants) which are both culturally acceptable and economically viable. Medicinal plants dominate indigenous/alternative systems of medicine and are common elements in Ayurveda, Chinese, Homeopathy, Naturopathy, Oriental and Native American medicine. In the present investigation, *Carissa carandas* & *Nerium oleander* have been selected for the study.

Carissa carandas (common name Karaunda) is a perennial shrub belonging to the family Apocynaceae [3]. 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 land with high temperatures. Presently it is grown on a limited scale in the Rajasthan, Gujarat, Bihar and Uttar Pradesh regions of India. Various medicinal properties like stomachic, anthelmintic, cardiotonic, and lowering blood pressure are attributed to this plant. Other properties attributed are strengthening tendons, effectivity against remittent fever, earache and syphilitic pain [4].

Nerium oleander (common name Kaner) is an evergreen shrub belonging to the family Apocynaceae [5]. It is native to Southern Europe and is widely cultivated and naturalized in Asia, Europe and North America. It is four meters in height, occurs along watercourse, harsh and damp ravines; is widely cultivated particularly in warm temperate subtropical regions where it grows outdoors in parks, gardens and along roadsides. Various medicinal properties like cardiotonic, analgesic, antidiabetic, anti-inflammatory, antibacterial, anticancer/antineoplastic, antifungal, depressant, antimitotic, insecticidal, and larvicidal are attributed to this plant. Other properties attributed are the inhibition of Nuclear factor-kappa B (NF-/cB) activation, muscle stimulation, effective against asthma, seizures, cancer, menstrual pain, skin problems, warts, epilepsy, leprosy, malaria, ringworm, indigestion, venereal diseases, and causing abortions [6].

The microorganisms selected for the study are *Candida albicans***,** *Aspergillus flavus* & *Tricophyton mentagrophyte. Candida albicans* is a major model of pathogenic yeast which is found in the mouth, throat, intestine and genitourinary tract of humans and is considered a 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, transplants and AIDS. It is a causal agent of opportunistic oral and genital infections in humans [7]. Superficial and mycosis infections cause local inflammation and discomfort in human beings [8]. Candidiasis also known as 'thrush', usually occurs in immunocompromised people [9]. *Aspergillus flavus* is the second leading cause of invasive and non-invasive aspergillosis [10]. The presence of *Aspergillus* in the air is a major risk factor for both invasive and allergic aspergillosis [11]. *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 naso orbital infections.

Tricophyton mentagrophyte is a cosmopolitan dermatophyte, belonging to a homogeneous group of fungi called the dermatophytes. The organism is found in soil, floors of swimming pools, hairs of wild boar, cats and dogs, farm animals, footwear 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 it is considered to be anthropophilic or zoophilic in nature [12, 13]. It causes dermatophytosis in dogs, cats, cattle and especially in rodents [14, 15, 16].

Antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Aspergillus niger, Candida albicans* was seen in aqueous, ethanol, methanol, chloroform and acetone extracts of *C. carandas* [17]. Unripe roots and fruits of *C. carandas* exhibited antimicrobial activity in their methanol and petroleum ether extract [18]. Antimicrobial activity of ethanolic extracts of the fruits of *C. carandas* have been reported against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Bacillus subtilis, Escherichia coli, Proteus vulgaris* and *Proteus mirabilis* [19].

Antimicrobial activity of methanolic and aqueous extracts of *Nerium* sp. has been reported against *Escherichia coli, Streptococcus uberi* and *Staphylococcus aureus* [20]. Ethanol, methanol and acetone extracts of leaves of *Nerium* sp. exhibited antimicrobial activity against *Klebsiella, Pseudomonas, Alkaligen* excluding *Acinetobacter* sp.[21, 22]. Antimicrobial activity of aqueous and ethanolic extracts of *Nerium* sp. has been reported against various pathogenic micro-organisms [23]. Chloroform, ethanol and methanol extracts of root, bark and leaves of *Nerium oleander* exhibit antimicrobial activity against *Bacillus pumulis, Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Aspergillus niger*[24]. Antimicrobial activity has been screened in *Nerium* flower (essential oil) against various pathogenic organisms [25]. Aqueous extract of *Nerium* sp. exhibits antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Salmonella typhi, Pseudomonas aeruginosa, and Candida albicans* [26].

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 not so much work has been carried out for extraction and screening of specific compounds from selected plants. Hence, in the present work an extraction and screening for antifungal activity of crude extracts of *C. Carandas* & *N. oleander* has been undertaken.

Materials and methods

Different parts of *C.carandas* (leaf, stem and root) & *N. oleander* (leaf, stem, root and flower) were collected in the months of April to June from the western parts of India (Jaipur, Rajasthan). Plants were identified by a senior taxonomist at the Department of Botany, University of Rajasthan and voucher specimen no: RUBL 21130 (*C. carandas*) & RUBL 21176 (*N. oleander*) were submitted to the Herbarium, Botany Department, University of Rajasthan.

Preparation of Extracts:

Extraction in polar and non polar solvents:
Powder of all the plant parts were prepared in different round bottom flasks in different solvents. 20 g of powder was put in each flask and water, methanol and petroleum ether were used as solvents. Dried material and solvents were taken in a 1:10 ratio. Those were kept at the soxhlet unit for 24 hours. Then the extracts were filtered. The filtrates were subjected to evaporation to obtain dried extract. The percentage yield of each dried plant extract was calculated.

*Selected Test Microorganisms***:**
Three pathogenic bacteria were screened: *Candida albicans* (MTCC no. 183), *Aspergillus flavus* (MTCC no. 277) and *Tricophyton mentagrophyte* (MTCC no. 7687). The pathogens were procured from 'The Institute of Microbial Technology' IMTECH (Chandigarh, Punjab, India). Fungal strains were grown and maintained on Sabouraud Dextrose (SD) Agar medium.

Antimicrobial assay:

'Disc Diffusion Assay' was performed for screening [27]. Sabouraud Dextrose agar base plates were seeded with fungal inoculum (1×10^7 CFU/ml). Sterile filter paper discs of Whatmann No.1 (6mm in diameter) were impregnated with 100µl each of the extract of a 10mg/ml concentration giving a final concentration of 1mg/disc. Discs were left to dry in vacuo so as to remove residual solvent, which might have interfered with the determination of antimicrobial activity. 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 for 1h at 4°C for extract diffusion and were incubated thereafter at 27°C (*C. albicans* and *A. flavus* for 48 h & *T. mentagrophyte* for 5-7 days). Inhibition zone (IZ) values were measured & Activity index (AI) for each extract was calculated by the standard formula:

Activity index = IZ produced by the extract/ IZ produced by standard

Where, IZ = inhibition zone (in mm)

Mean and Standard deviation was also calculated for each extract (n=2). [Table I]

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

The Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against the test pathogens. The 'Micro Broth Dilution' method was followed for the determination of MIC values [8]. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make a final concentration of 10mg/ml. Two fold serially diluted extracts were added to broth media in 96-wells of microtiter plates. Thereafter 100µl of fungal inoculum (1×10^7 CFU/ ml) was added to each well. Fungal suspensions were used as a negative control, while broth containing the standard drug was used as a positive control. Micro titer plates were then incubated at 27°C for 48 h. Each extract was assayed in duplicate and each time two sets of microplates were prepared, one was kept for incubation while another was kept at 4°C for comparing the turbidity in the wells of micro plates. 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. The lowest concentration of extract showing no visible growth on sub culturing was taken as MFC. [Table II].

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 [29]. [Table III]

Total Activity= Amount of extract from 1gm of dry plant material/ MIC of the same extract

Statistical analysis: The results were expressed as mean ± (standard deviation) SD (n = 2)

Table I: Antifungal activity of crude extracts of *Carissa carandas* Linn. & *Nerium oleander* Linn. against some pathogenic fungi

Plant & Plant parts	Extract	Microorganisms					
		Candida albicans		*Aspergillus flavus*		*Tricophyton mentagrophyte*	
		IZ(mm)	AI	IZ(mm)	AI	IZ(mm)	AI
1. *C. carandas*							
Leaf	**P1**	-	-	-	-	-	-
	M1	10.5	1.31±0.01	-	-	-	-
	W1	-	-	-	-	-	-
Stem	**P2**	8	1±0.01	-	-	-	-
	M2	-	-	-	-	-	-
	W2	-	-	-	-	-	-
Root	**P3**	-	-	-	-	-	-
	M3	-	-	-	-	-	-
	W3	-	-	-	-	-	-
2. *N. oleander*							
Leaf	**P4**	-	-	-	-	-	-
	M4	7	0.47±0.01	-	-	-	-
	W4	10	0.67±0.01	-	-	-	-
Stem	**P5**	-	-	-	-	-	-
	M5	-	-	-	-	-	-
	W5	-	-	-	-	-	-
Root	**P6**	-	-	-	-	-	-
	M6	-	-	-	-	-	-
	W6	-	-	-	-	-	-

P1, P2, P3, P4, P5, P6 = Petroleum ether extract of respective plant parts,

M1, M2, M3, M4, M5, M6 = Methanolic extract of respective plant parts,

W1, W2, W3, W4, W5, W6 = Water extracts of respective plant parts,

IZ=Inhibition zone in mm (value: including 6mm diameter of disc), mm= millimeters,

AI= Activity index (IZ developed by extract/IZ developed by standard),

(-) = no activity, ±=SEM.

Table II: MIC and MFC of active crude extracts of *Carissa carandas* Linn. & *Nerium oleander* Linn. against some pathogenic fungi

Plants & Plant parts	Extracts	Microorganisms and their MIC & MFC					
		Candida albicans		*Aspergillus flavus*		*Tricophyton mentagrophyte*	
		MFC(mg/ml)	MIC(mg/ml)	MFC(mg/ml)	MIC(mg/ml)	MFC(mg/ml)	MIC(mg/ml)
1. *C. carandas*							
Leaf	**P1**	-	-	-	-	-	-
	M1	0.625	0.312	-	-	-	-
	W1	-	-	-	-	-	-
Stem	**P2**	1.25	0.625	-	-	-	-
	M2	-	-	-	-	-	-
	W2	-	-	-	-	-	-
Root	**P3**	-	-	-	-	-	-
	M3	-	-	-	-	-	-
	W3	-	-	-	-	-	-
2. *N. oleander*							
Leaf	**P4**	-	-	-	-	-	-
	M4	1.25	0.625	-	-	-	-
	W4	0.625	0.312	-	-	-	-
Stem	**P5**	-	-	-	-	-	-
	M5	-	-	-	-	-	-
	W5	-	-	-	-	-	-
Root	**P6**	-	-	-	-	-	-
	M6	-	-	-	-	-	-
	W6	-	-		-	-	-

P1, P2, P3, P4, P5, P6 = Petroleum ether extract of respective plant parts,

M1, M2, M3, M4, M5, M6 = Methanolic extract of respective plant parts,

W1, W2, W3, W4, W5, W6 = Water extracts of respective plant parts,

MIC= Minimum inhibitory concentration,

MFC= Minimum fungicidal concentration,

mg/ml= milligram per milliliter, (-) = no activity

Table III: Quantity & Total activity of crude extracts of *Carissa carandas* Linn. & *Nerium oleander* Linn.

Plants & Plant parts	Extracts	Quantity of extract (mg/g.d.wt.)	Total Activity(ml/g)		
			Candida albicans	*Aspergillus flavus*	*Tricophyton mentagrophyte*
1. *C. carandas*					
Leaf	**P1**	24.17	-	-	-
	M1	117.5	376.60	-	-
	W1	68.75	-	-	-
Stem	**P2**	12.67	20.27	-	-
	M2	66	-	-	-
	W2	28	-	-	-
Root	**P3**	12.67	-	-	-
	M3	77	-	-	-
	W3	25	-	-	-
2. *N. oleander*					
Leaf	**P4**	7	-	-	-
	M4	95	152	-	-
	W4	46	147.43	-	-
Stem	**P5**	2.5	-	-	-
	M5	33.5	-	-	-
	W5	31.5	-	-	-
Root	**P6**	11	-	-	-
	M6	47.5	-	-	-
	W6	38	-	-	-

P1, P2, P3, P4, P5, P6 = Petroleum ether extract of respective plant parts,

M1, M2, M3, M4, M5, M6 = Methanolic extract of respective plant parts,

W1, W2, W3, W4, W5, W6 = Water extracts of respective plant parts,

TA= total activity (extract per g dried plant part/MIC of extract),

mg/g.d.wt.= milligram per gram dry weight of extract,

ml/g= milliliter per gram.

Results:

The study screened the antifungal activity of extracts of selected plant parts on *Candida albicans* (Yeast), *Aspergillus flavus* & *Tricophyton mentagrophyte* (fungi), using the *in vitro* technique, "Disc Diffusion Assay". The water, methanol and petroleum ether extracts (18 extracts) of the selected plant parts (6 parts) were assessed for their antifungal activity in terms of the zone of inhibition in mm against selected microorganisms [Table I]. In the present study, all the tested extracts showed no activity or very little activity at the tested concentration of 1 mg/ml. Nevertheless, the highest antifungal activities were recorded for methanolic extracts of leaves of *C. carandas* (IZ= 10.5mm, AI= 1.31±0.01) followed by water extracts of leaves of *N. oleander* (IZ=10mm, AI= 0.67±0.01) against *C. albicans*. Petroleum ether extracts from the stem of *C. carandas* (IZ= 8mm, AI= 1±0.01) & methanolic extracts of leaves of *N. oleander* (IZ= 7mm, AI= 0.47±0.01) also showed some activities against *C. albicans*. Such low values of IZ indicate the insufficient antifungal potential of the extracts. The extracts that showed activity in the disc diffusion assay were evaluated for their MFC and MIC values by using a 96-well broth dilution method and are tabulated in Table II. The lowest MFC & MIC values of the extracts that showed confined activity were 0.625 mg/ml & 0.312 mg/ml, respectively. TA values were also calculated [Table III]. The highest TA value recorded for the methanolic extract of leaves of *C. carandas* (376.6ml/g), indicated low efficiency of the extract also in diluted form. The remaining extracts exhibited no activity. *Aspergillus flavus* and *Tricophyton mentagrophyte* were found to be completely resistant throughout the study.

Discussion:

Due to indiscriminate use of antimicrobial drugs, the microorganisms have developed resistance to many antifungal drugs and very few successful drugs are now available for the treatment of fungal infections. This has created immense clinical problems in the treatment of infectious diseases. Hence, continuous research for getting new & potent antifungal agents is the need of the present scenario, either by designing and synthesizing new agents, chemically or through the search of new natural sources. Ever since the importance of the distribution of pharmacologically active principles in higher plants was understood and acknowledged, the importance of such plant−derived medicines in modern therapeutic practice has paved the way for the development of new drug leads that are safe, cost- effective and eco- friendly. The present investigation is an effort towards this direction. In the present study, *C. carandas* & *N. oleander* have shown insignificant antifungal potential against all the three tested microorganisms. This can be due to the lack of ability of the tested extracts to inactivate or suppress the mechanism followed by the particular fungus responsible for their activity or due to the absence of some specific compound in these extracts accountable to kill or inhibit the growth and activity of fungus. However, the studied extracts had relatively high MIC and MFC values and thus cannot be considered as good antifungal agents.

Conclusion

C. carandas & *N. oleander* crude extracts were found to be inefficient to work as antifungal agents with high values of MIC and MFC or no activity. To conclude, it is necessary to emphasize the significance of more comprehensive studies that will detect new potent molecules underlying the action of crude extracts that allow for new discoveries. Essentially, in this study, these extracts lacking of significant antifungal activity will be useful to avoid any study repeated in this direction in the future.

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