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Bioactivity of crude extracts of *Nerium oleander* Linn. extracted in polar and non polar solvents

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Abstract

Today, the 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. The present study was designed to evaluate the antibacterial activity of crude extracts of *Nerium oleander* (Apocynaceae) Linn. Crude extracts from different parts (root, stem and leaf) of the plant were extracted using polar (Water and Methanol) and Non-polar (Petroleum ether) solvents and were screened for antibacterial activity by 'Disc Diffusion Assay' against three Gram negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Agrobacterium tumifaciens*) and two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). Minimum inhibitory concentration, Minimum bactericidal concentration and total activity of extracts against each sensitive pathogen have also been evaluated. Mean and Standard Deviation have also been calcultated. *B. subtilis* found to be the most susceptible organism followed by *K. pneumoniae*, *S. aureus* and *E. coli*. Pet ether extract of root (IZ=24 mm, MIC=0.078 mg/ml, MBC=0.039 mg/ml, TA=141.02 ml/g) and stem (IZ=20 mm, MIC=0.156 mg/ml, MBC= 0.078 mg/ml, TA= 16.02 ml/g) showed the best antibacterial activity against *B. subtilis* and *K. pneumoniae* respectively. Methanolic and water extract of leaf, stem and root, Pet ether extract of stem, Methanolic and water extract of stem and Pet ether extract of root and stem also showed very good activities against *B. subtilis*, *E. coli*, *K. pneumoniae* and *A. tumifaciens* respectively. The range of MIC and MBC was found to be 1.25-0.078 mg/ml and 0.625-0.039 mg/ml respectively. Results reveal the great antimicrobial potency of tested extracts.

Keywords: Pet ether extract, Methanolic extract, Water extract, Minimum inhibitory concentration, Minimum bactericidal concentration, Total activity.

Introduction

Medicinal plants are an indispensable part of the traditional medicine practiced all over the world because of low cost, easy access and ancestral experience.[1] During the past decade, traditional system of medicine has become increasingly important as they are considered to be safe and have long lasting effect. In many developing countries a large part of the population relies on herbal medicines. Phytomedicines have maintained popularity for historical and cultural reasons and have attracted attention as an alternative therapy.[2, 3] Irrespective of the decline in the use of plants as herbal medicines after the advent of antibiotics in 1900, the importance of botanicals in the evolution of medicines remains unchallenged. Revival of interest in plant derived drugs is mainly due to the current widespread belief that 'Green Medicine' is safe, cheap and more dependable than costly synthetic drugs with adverse side effects, thus making

natural therapeutics as an attractive option of synthetic pharmaceuticals.[4] Many researchers have focused the investigation of natural products and plant extracts as a source of new bioactive molecules.[5, 6]

In recent years, pharmaceutical industries have spent a lot of time and money in developing natural products, extracted from medicinal plants to produce more cost effective remedies that are affordable to a common man. WHO has acknowledged increasing awareness of herbals and recently defined traditional medicine (including herbal drugs and medicinal plants) as comprising therapeutic practices that have been in existence almost for several thousands of years before the development and spread of modern medicine and are still in use.[7]

Nerium oleander (comman name Kaner) is an evergreen shrub belongs to the family Apocynaceae. It is native to southern Europe, and is widely cultivated and naturalized in Asia, Europe and North America. It is four metres in height, occurs along watercourse, gravely place and damp ravines, widely cultivated particularly in warm temperate subtropical regions where it grows outdoors in parks, gardens and along road sides. Various medicinal properties viz. Cardiotonic, Analgesic, Antidiabetic, Anti-inflammatory, Antibacterial, Anticancer/ Antineoplastic, Antifungal, Depressant, Antimitotic, Insecticidal, Larvicidal are attributed to this plant. Other properties attribute are 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, and venereal disease and also cause abortions. The present investigation was undertaken to find out the antibacterial potential of crude extract of different parts of *N. oleander* against some Gram positive and Gram negative bacteria.

Antimicrobial activity of methanolic and aqueous extract of Nerium sp. have been reported against *Escherichia coli, Sterptococcus uberi* and *Staphylococcus aureus*.[8] Ethanol, methanol and acetone extract of leaves of Nerium sp. exhibited antimicrobial activity against *Klebsiella, Pseudomonas, Alkaligen* except *Acinetobacter* sp.[9, 10] Antimicrobial activity of aqueous and ethanolic extract of Nerium sp. had been reported against various pathogenic micro-organisms.[11] 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*.[12] Antimicrobial activity has been screened in Nerium flower (essential oil) against various pathogenic organisms.[13] Aqueous extract of Nerium sp. exhibit antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Salmonella typhi, Pseudomonas aeruginosa, Candida albicans*.[14, 15]

Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant crude 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 selected plant in such a way. Hence, in the present work an extraction and screening for antibacterial activity of the crude extracts of different parts of *N. oleander* has been undertaken.

Material and Methods

Different parts of *N. oleander* (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 21176 was submitted to the Herbarium, Botany Department, University of Rajasthan.

Preparation of Extracts

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

Selected Test Microorganisms

Five 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, 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[16] and pneumonia. Some virulent strains cause serious illness

or death in the elderly, the very young or the immunocompromised.[17, 18] Intestinal mucosa associated *E. coli* is observed in increased numbers in the inflammatory bowel diseases, Crohn's diseases and ulcerative colitis.[19, 20] *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 and toxin mediated diseases e.g. food poisoning, toxic shock syndrome (TSS) and scaled skin syndrome. In infants its infection can cause a severe disease Staphylococcal scalded skin syndrome (SSSS).[21] 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. pneumoniae* causes 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

The disc diffusion assay was performed for screening.[22] 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 (6 mm in diameter) were impregnated with 100µl of each of the extract of concentration 10 mg/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 or Gentamycin (1mg/disc) as standard drug for bacteria. The plates were kept at 4^{o}C for the diffusion of extract, thereafter were incubated at 37^{o}C 24h. Activity index for each extracts was calculated. [Table 1] by the standard formula viz.

Table 1: Antimicrobial activity of extracts of *Nerium oleander* 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	P1	10.	0.31±0.01	10	0.34±0.04	7	0.70±0.01	-	-	-	-
	M1	5	0.28±0.01	14	0.47±0.07	7	0.70±0.01	13.5	0.48±0.01	-	-
	W1	9.5	0.34±0.01	18.5	0.62±0.01	-	-	8	0.28±0.01	12.5	0.45±0.01
Stem	P2	11.	0.54±0.01	8.5	0.28±0.01	7	0.47±0.01	20	0.71±0.10	15	0.50±0.01
	M2	5	0.37±0.01	16.5	0.55±0.01	8	0.54±0.01	16	0.57±0.01	-	-
	W2	16	0.27±0.01	15	0.50±0.04	12	0.80±0.01	18.5	0.66±0.01	-	-
Root	P3	11	-	24	0.80±0.04	7	0.70±0.01	13	0.44±0.01	14.5	0.48±0.01
	M3	8	-	16.5	0.55±0.01	-	-	8	0.27±0.01	10	0.34±0.01
	W3	-	-	14	0.47±0.01	-	-	-	-	-	-

IZ=Inhibition zone in mm (value: including 6mm diameter of disc), AI= Activity index (IZ developed by extract/IZ developed by standard), P1, P2, P3=Pet ether extracts of respective plant parts, M1, M2, M3=Methanolic extracts of respective plant parts, W1, W2, W3=Water extracts of respective plant parts, (-)= no activity, ±=SEM.

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 against the test pathogens. 'Broth micro dilution' method was followed for the determination of MIC values.[23] 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. Micro titer plates were then incubated at 37°C for 24 h. Each extract was assayed in duplicate and for each plant extract showing antimicrobial activity 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.

Table 2: MIC and MBC of active extracts of *Nerium oleander* against different pathogens

Plant parts		**Leaf**			**Stem**					**Root**
Microorganism		**P1**	**M1**	**W1**	**P2**	**M2**	**W2**	**P3**	**M3**	**W3**
E. coli	MIC	0.625	0.625	0.312	0.156	0.625	1.25	-	-	-
	MBC	0.312	0.312	0.156	0.078	0.312	0.625	-	-	-
B. subtilis	MIC	0.625	0.312	0.156	1.25	0.312	0.312	0.078	0.312	0.312
	MBC	0.312	0.156	0.078	0.625	0.156	0.156	0.039	0.156	0.156
S. aureus	MIC	1.25	1.25	-	1.25	1.25	0.312	1.25	-	-
	MBC	0.625	0.625	-	0.625	0.625	0.156	0.625	-	-
K. pneumoniae	MIC	-	0.312	1.25	0.156	0.312	0.156	0.312	1.25	-
	MBC	-	0.156	0.625	0.078	0.156	0.078	0.156	0.625	-
A. tumifaciens	MIC	-	-	0.312	0.156	-	-	0.156	0.625	-
	MBC	-	-	0.156	0.078	-	-	0.078	0.312	-

P1, P2, P3= Pet ether extracts of respective plant parts, M1, M2, M3= Methanolic extracts of respective plant parts; W1, W2, W3= Water extracts of respective plant parts; MIC= Minimum inhibitory concentration; MBC= Minimum bactericidal concentration, (-)= no activity.

Table 3: Quantity and Total activity of extracts of *Nerium oleander*

Plant part	**Extract**	**Quantity of extract mg/g dwt**	**Total Activity (ml/g)**				
			E. coli	***B. subtilis***	***S. aureus***	***K. pneumoniae***	***A. tumifaciens***
Leaf	**P1**	7	11.2	11.2	5.6	-	-
	M1	95	152	304.48	76	304.48	-
	W1	46	147.43	294.87	-	36.8	147.43
Stem	**P2**	2.5	16.02	16.02	2	16.02	16.02
	M2	33.5	53.6	53.6	26.8	107.37	-
	W2	31.5	25.2	25.2	100.96	201.92	-
Root	**P3**	11	-	141.02	8.8	35.25	70.51
	M3	47.5	-	152.24	-	38	76
	W3	38	-	121.79	-	-	-

P1, P2, P3= Pet ether extracts of respective plant parts; M1, M2, M3= Methanolic extracts of respective plant parts; W1, W2, W3= Water extracts of respective plant parts; TA= total activity (extract per gm dried plant part/MIC of extract).

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.[24] [Table 3]

Result

Antimicrobial potency of crude (Pet ether, methanolic and water) extracts was assessed by IZ, AI (Table-1), MIC and MBC (Table-2). Quantity of extract per gram of plant was also calculated (Table-3). In the present investigation total 9 extracts were tested and all were found active against at least one of the tested pathogens. *B. subtilis* was observed to be the most susceptible organism followed by *E. coli*, *S. aureus* and *K. pneumoniae*. Best activity was observed in pet ether extracts of the root against *B. subtilis* (IZ=24mm, MIC=0.078 mg/ml, AI=0.80±0.04). Water extract of the leaf (IZ=18.5mm, MIC=0.156 mg/ml, AI=0.62±0.01), Methanolic extract of the stem (IZ=16.5mm, MIC=0.312 mg/ml, AI=0.55±0.01) and Methanolic extract of the root (IZ=16.5mm, MIC=0.312 mg/ml, AI=0.55±0.01) showed very good activity against *B. subtilis*. Pet ether extract of the stem showed very good activity against *E. coli* (IZ=16mm, MIC=0.156 mg/ml, AI=0.54±0.01). Pet ether extract of the stem (IZ=20mm, MIC=0.156 mg/ml, AI=0.71±0.10), methanolic extract of the stem (IZ=16mm, MIC=0.312 mg/ml, AI=0.57±0.01) and water extract of the stem (IZ=18.5mm, MIC=0.156 mg/ml, AI=0.66±0.01) showed very good activity against *K. pneumoniae*. Methanolic extract of the leaf, water extract of the stem and root and pet ether extract of the stem and root also showed good activity against *B. subtilis* and *A. tumifaciens* respectively. Among all the crude extracts, pet ether extracts found to be most bioactive and pet ether crude extract of stem were active against all the five tested bacteria. MIC and MBC values (Table-2) were evaluated for plant crude extracts which had shown activity in diffusion assay. The range of MIC and MBC of extract recorded was 1.25-0.078 mg/ml and 0.625- 0.039 mg/ml respectively. In present investigation, lowest MIC value 0.078 mg/ml was recorded against *B. subtilis*, showing significant antimicrobial potential of test extract and higher susceptibility of *B. subtilis*. TA and Quantity of extract obtained per gram from plant parts was calculated and tabulated (Table-3). TA indicates the volume at which extract can be diluted, without losing ability to kill microorganisms. High values of TA were observed against *B. subtilis* and *K. pneumonae* followed by *E. coli* and *A. tumifaciens*, which proves the potential to inhibit the growth of test microorganisms, even at low concentration. Maximum TA values calculated were 304.48ml/g against *B. subtilis* as well as against *K. pneumoniae* and 147.43 ml/g against *E. coli* and *A. tumifaciens*.

Discussion

Due to indiscriminate use of antimicrobial drugs, the microorganisms have developed resistance to many antibiotics. This has created an immense clinical problem in the treatment of infectious diseases.[25] This situation has risen to an alarming level. Hence, a continuous research for getting new antimicrobial agents is the need of the present scenario. Present study is an effort towards this direction, in which *N. oleander* has shown antibacterial potential against all the five tested pathogens which are the major causative agents of various human diseases. Although the plant (*N. oleander*) has been studied previously, for its antimicrobial activity but only restricted to the determination of IZ and that too without AI, MIC, MBC and TA evaluation. Hence could not explore the preparation of antibiotics. Such studies could only indicate their antimicrobial potential but can't replace the existing antibiotics. In present investigation IZ, AI, MIC, MBC and TA have been evaluated for each extract to determine their antimicrobial activity. The activity of plant extracts against both Gram positive and Gram negative bacteria may be an indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. From this study, it can be concluded that crude extracts of different parts of plant exhibited potential bactericidal properties. Present investigations together with previous studies provide support to the antibacterial properties of *N. oleander*. Therefore, it can be used as an antibacterial supplement in the developing countries towards the development of new therapeutic agents. Further pharmacological and clinical studies are required to understand the mechanism and the actual efficacy of these plant extracts in treating various infectious diseases. The demonstration of broad spectrum of antibacterial activity by *N. oleander* may help to discover new chemical classes of antibiotic substances that could serve as alternatives or second line treatment for infectious disease and their control.

Conclusion

Results of the present study indicate that almost all the tested crude extracts of *N. oleander* have potent antibacterial activity and may be exploited for future

antimicrobial drugs. Crude extracts of all the three parts of *N. oleander* have activity against both gram-positive and gram-negative bacteria indicative of the presence of broad spectrum antibiotic compounds. The results of the antibacterial activity of the study were in agreement with the findings of previous studies. Furthermore, it may help to discover new chemical classes of antibiotics that could serve as selective agents for the maintenance of human health and provide biochemical tools for the study of infectious diseases.

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