ORIGINAL ARTICLE

In vitro evaluation of crude extracts of *Catharanthus roseus* for potential antibacterial activity

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Context: *Catharanthus roseus* (periwinkle) is an important medicinal plant, mentioned in Ayurveda, an ancient Indian Sanskrit literature. The plant is selected to evaluate the possibility for novel pharmaceuticals since most of the bacterial pathogens are developing resistance against currently available antibiotics.
Aims: To determine the antibacterial activity of crude extracts from different parts of *Catharanthus roseus* against several bacterial species of clinical significance.
Materials and Methods: Extraction of each plant part in appropriate solvent followed by evaluation of antibacterial activity by agar well diffusion assay against a total of six bacterial stains. Further, minimum inhibitory concentration(s) was evaluated for active crude extracts.
Results: Data indicated that the pattern of inhibition depends largely upon the extraction procedure, the plant part used for extraction, state of plant part (fresh or dry), solvent used for extraction and the microorganism tested. Dry powder extracts of all plant parts demonstrated more antibacterial activity than extracts prepared from fresh parts. Furthermore, extracts prepared from leaves were shown to have better efficacy than stem, root, and flower extracts. Organic extracts provided more potent antibacterial activity as compared to aqueous extracts. Among all the extracts, the ethanolic extract was found to be most active against almost all the bacterial species tested. Hot water and cold water extracts were completely inactive. Gram-positive bacteria were found more sensitive than Gram-negative bacteria.
Conclusions: The study promises an interesting future for designing potentially active antibacterial agents from *Catharanthus roseus*.

Key words: Agar well diffusion assay, antibacterial activity, Ayurveda, *Catharanthus roseus*, minimum inhibitory concentration, periwinkle

INTRODUCTION

Emerging and reemerging infections and spread of deadly, drug-resistant strains of organisms pose a challenge to the global public health for their treatment. Bacterial resistance to antibiotics is a major therapeutic problem and the pace at which new antibiotics are being produced is slowing.[1] Thus, the search for novel antimicrobial agents is of the utmost importance in the current world.[2] Global attention has been shifted towards finding new chemicals, specifically herbals, for the development of new drugs. These natural products can provide unique elements of molecular diversity and biological functionality, which is indispensable for novel drug discovery.[3]

Natural products or natural product-derived drugs comprise about 28% of all new chemical entities launched onto the market.[4] A large proportion of natural products in drug discovery has stemmed from the diverse molecular structures and the intricate carbon skeletons of natural products.[5]

Plants have proved to be significant natural resources for medicines; documentation of their use in medicine originates from ancient times. Ethnobotanical and ubiquitous plants provide a rich resource for natural drug research and development.[6] Medicinal plant-based drugs have the added advantage of being simple, effective and offering a broad spectrum of activity with greater emphasis on preventive action.[7] Indigenous systems of medicine and plant-based drugs could provide both concepts of therapy as well as therapeutic agents to complement modern medicine, especially in management of lifestyle and communicable diseases. Medicinal plant products could also prove useful in minimizing the adverse effects of various chemotherapeutic agents as well as in

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Received: 06-03-2008; **Accepted:** 10-06-2008

prolonging longevity and attaining positive general health.[8] The global interest in the medicinal potential of plants during the last few decades is therefore quite logical.

India is one of the richest countries in the world with regard to diversity of medicinal plants.[9] *Catharanthus roseus* L. (periwinkle) is an important member of the family Apocynaceae. This short-lived perennial with dark green and glossy leaves is native to Madagascar. This plant is frequently mentioned in Ayurveda (an ancient Indian literature) and has traditionally been used to treat diseases including cancer and diabetes. The plant has more than 70 types of alkaloids (mostly monoterpene indole alklaloids), and some are known to be effective in treating various types of cancers including breast and lung cancer, uterine cancer, melanomas, and Hodgkin's and non-Hodgkin's lymphoma.[10] The anticancer drugs vincristine and vinblastine are synthesized from alkaloids of *Catharanthus roseus*. The plant is also known for its antihypertensive and antispasmodic properties. Considering the medicinal value that this plant has already been shown to have, we evaluated the antibacterial potential in crude extracts of different parts (*viz.,* leaves, stem, root and flower) of this plant against clinically significant bacterial strains.

MATERIALS AND METHODS

Plant Material

Catharanthus roseus was collected in late July and early August of 2006 from semi-arid, unshaded land near the Jaipuria Institute in Vasundhara, Ghaziabad (NCR), India. The plant was taken to the laboratory and was authenticated by Prof. P. Kaushik. Plant materials (leaves, stem, root and flower) were washed separately first under running tap water, followed by sterilized distilled water.

Extract Preparation

Extraction was performed by two different modes: (1) Extraction of fresh plant material without drying and (2) Extraction after drying each plant part.

Hot Water Extraction

10 g of each plant part was boiled in 100 ml distilled water with constant stirring for 30 min. The solution was allowed to cool to room temperature and then filtered using muslin cloth. The filtrate was centrifuged at 5000 rpm for 15 min. The supernatant was again filtered using Whattman's Filter No. 1 under strict aseptic conditions. The filtrate was collected in fresh sterilized glass tubes and stored at 4°C until use.

Cold Water Extraction

10 g of each plant part was macerated in pestle and mortar with 100 ml distilled water at room temperature and then filtered using muslin cloth. Filtrate obtained was again filtered using Whattman's Filter No. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized glass tubes and used within 24h for evaluation of antibacterial activity.

Organic Solvent Extraction

10 g of each plant part was thoroughly mixed with 100 ml organic solvent (ethanol and methanol). The mixture thus obtained was filtered through muslin cloth and then re-filtered by passing through Whattman's filter No. 1. The filtrate was then concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts were prepared by mixing well the appropriate amount of dried extracts with appropriate solvent to obtain a final concentration of 100 mg/ml. Each solution was stored at 4°C after collecting in sterilized glass tubes until use.

Dry Powder Extractions

Crude dry powder extracts were prepared by first air-drying the plant material and then powdered using sterilized pestle and mortar under strict aseptic conditions. The powder was further subjected for aqueous and organic solvent extraction protocols as described above.

Bacterial Strains

A total of six bacterial strains including both Gram-negative and Gram-positive bacteria (*Escherichia coli* MTCC-739, *Salmonella paratyphi* MTCC-735, *Klebsiella pneumoniae* MTCC-39, *Bacillus cereus* MTCC-430, *Bacillus subtilis* MTCC-736, and *Staphylococcus aureus* MTCC-740) were selected to assess susceptibility patterns against the extracts prepared in the present study. All strains were collected from the Microbial Type Culture Collection (MTCC), India. The bacterial cultures were maintained in nutrient agar slants at 37°C. Each of the microorganisms was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

Antibacterial Susceptibility Assay

Extracts obtained by various processes were evaluated for their potential antibacterial activities by the standard agar well diffusion assay.[11] All extracts were sterilized by sterile membrane syringe filter (pore size 0.45 μm, manufactured by Pall Life Sciences). Petri dishes (100 mm) containing 18 ml of Mueller Hinton Agar (MHA) were seeded with approximately 100 μl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 10^8 CFU/ml). Media was allowed to solidify. Wells of 6 mm diameter were cut into solidified agar media using a sterilized cup-borer. 100 μl of each extract was poured in the respective well and the

plates were incubated at 37°C overnight. The experiment was performed in triplicate under strict aseptic conditions to ensure consistency of all findings. The antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by each extract at the end of incubation period.

Sterilized distilled water and other solvents used in preparation of extracts were used as negative control. Tetracycline was used as a standard antibiotic (*i.e*. positive control) in the present study for a comparative analysis with the effectiveness of various plant extracts against selected microflora. The antibiotic (ALCYCLIN-500, manufactured by Alembic Limited) was procured from local chemist. On the basis of claim *i.e*. 500 mg/capsule; an appropriate amount of tetracycline powder was dissolved in sterilized distilled water to obtain a final concentration of 5 µg/ml; this solution was used as a standard antibiotic throughout the study.

Assessment of Minimum Inhibitory Concentration

Active extracts obtained by agar well diffusion assay were further subjected to determine the minimum inhibitory concentration (MIC) required for the bacteriostatic effects by standard two-fold microdilution broth methodology.[12] A stock solution of each active extract was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 8.0 µg/ml to 4096 µg/ml. A standardized inoculum for each bacterial strain was prepared so as to give an inoculum size of approximately 5 x 10^5 CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain using reflective viewer. All the chemical ingredients used in present study were of analytical grade and were purchased from Hi Media, India.

RESULTS

Results of the present investigation reveal the antibacterial nature of extracts of different parts (*viz*., leaf, stem, root, and flower) of *Catharanthus roseus*. Each plant part was extracted in its dry powder form [Table 1] and fresh state [Table 2] using both organic and aqueous solvents. Data obtained demonstrates that the antibacterial activity of plant parts depends largely upon the extraction procedure, type of solvent used for extraction, and the bacterial strains tested for susceptibility assay. Data indicate that extracts prepared from dried parts exhibited better antibacterial activities than those extracts prepared from fresh plant part. Almost all parts of the plant showed antibacterial potential in dried form [Table 1] while only leaf and root extracts showed zone(s) of inhibition in their fresh states [Table 2]. The dry leaf extracts were found to have maximum inhibition, followed by root, stem and flower extracts. Leaf extracts showed zone(s) of inhibition ranging from 5.24 mm to 21.83 mm in

Table 1: *In vitro* antibacterial activity of dry powder extracts of *Catharanthus roseus*

Extraction type		Effective zone of inhibition* (in mm diameter)					
		E. coli	*S. paratyphi*	*K. pneumoniae*	*B. cereus*	*B. subtilis*	*S. aureus*
Leaf extracts	Ethanol	5.24	6.97	15.38	-	5.39	21.83
	Methanol	-	8.20	7.84	-	-	17.45
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Stem extracts	Ethanol	13.66	4.10	-	-	7.24	15.30
	Methanol	-	-	4.43	-	-	11.16
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Root extracts	Ethanol	6.61	7.45	13.28	9.44	10.98	16.56
	Methanol	-	5.64	3.12	-	-	11.32
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Flower extracts	Ethanol	6.14	11.32	8.21	7.73	-	15.04
	Methanol	-	-	-	-	-	-
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Positive control	Tetracycline	35.62	33.29	22.64	26.71	22.97	30.46
Negative control	Distilled water	-	-	-	-	-	-
	solvents	-	-	-	-	-	-

*****The table lists values of the observed zone of inhibition (in mm diameter) excluding the diameter of well (6 mm) for extracts prepared by dried powder of respective part in agar well diffusion assay. Assay was performed in triplicate and results are expressed in terms of the average of the three values. In each well, the sample size was 100 µL. Tetracycline (5 µg/ml) was used as positive control while distilled water and solvents were used as negative control. *E. coli - Escherichia coli, S. paratyphi - Salmonella paratyphi, K. pneumoniae - Klebsiella pneumoniae, B. cereus - Bacillus cereus, B. subtilis - Bacillus subtilis, S. aureus - Staphylococcus aureus*

Table 2: *In vitro* antibacterial activity of extracts prepared from fresh parts of *Catharanthus roseus*

Extraction type		Effective zone of inhibition* (in mm diameter)					
		E. coli	*S. paratyphi*	*K. pneumoniae*	*B. cereus*	*B. subtilis*	*S. aureus*
Leaf extracts	Ethanol	7.22	8.17	5.12	-	5.39	9.23
	Methanol	-	7.10	3.42	-	-	11.25
	Hot water	-	8.44	10.91	-	-	12.21
	Cold water	-	-	-	-	-	-
Stem extracts	Ethanol	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Root extracts	Ethanol	4.21	8.22	6.50	-	3.18	11.16
	Methanol	5.66	6.26	7.42	-	19.56	14.78
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Flower extracts	Ethanol	-	-	-	-	-	-
	Methanol	-	-	-	-	-	-
	Hot water	-	-	-	-	-	-
	Cold water	-	-	-	-	-	-
Positive control	Tetracycline	35.62	33.29	22.64	26.71	22.97	30.46
Negative control	Distilled water	-	-	-	-	-	-
	solvents	-	-	-	-	-	-

*****The table lists values of the observed zone of inhibition (in mm diameter) excluding the diameter of well (6 mm) for extracts prepared from fresh parts of the plant in agar well diffusion assay. Assay was performed in triplicate and results are expressed in terms of the average of the three values. In each well, the sample size was 100 µL. Tetracycline (5 µg/ml) was used as positive control while distilled water and solvents were used as negative control. *E. coli - Escherichia coli, S. paratyphi - Salmonella paratyphi, K. pneumoniae - Klebsiella pneumoniae, B. cereus - Bacillus cereus, B. subtilis - Bacillus subtilis, S. aureus - Staphylococcus aureus*

diameter, while flower extracts were found to be virtually inactive in inhibition of microbial strains. Organic extracts were found to be more inhibitory than aqueous extracts. Ethanol was found to be a more suitable solvent for the maximum extraction of active metabolites than methanol. Gram-positive bacteria were found more susceptible as compared to Gram-negative species. *Staphylococcus aureus* was found most susceptible, as it was inhibited by almost all the organic extracts except that of fresh stem and fresh flower. Other Gram-positive bacteria (*Bacillus cereus* and *Bacillus subtilis*) were shown to have moderate-to-mild resistance, as they were not inhibited to such an extent. Ethanolic extracts were found to have variable inhibitory patterns against the Gram-negative bacteria with maximum inhibition of *Klebsiella pneumoniae* followed by *Escherichia coli*. Control experiments using sterile distilled water and solvents used for extraction (negative control) showed no inhibition of any bacteria.

Extracts found to have inhibitory effects were further tested for determination of minimum inhibitory concentration (MIC) by two-fold broth micro-dilution method against susceptible bacterial species [Table 3]. Data indicated that dry powder extracts showed more inhibition than fresh extracts of each plant part. Leaf extracts were found to have very low MIC values as compared with stem, root and flower extracts. Furthermore, ethanolic extracts showed consistently better antibacterial activity than methanolic extracts. The best MIC value (256 µg/ml) for the dried leaf extract was seen against *Staphylococcus aureus* while it was 1024 µg/ml for *Klebsiella pneumoniae* and 4096 µg/ml for *Salmonella paratyphi*, though end-points were not reached in case of *Escherichia coli* and *Bacillus subtilis*. Fresh leaf extracts had MIC values more than 2048 µg/ml against all species of bacteria. *Staphylococcus aureus* was inhibited at 512 µg/ml by both dry stem and dry root extracts, but the MIC was much higher for this bacterium for fresh root or dry flower extracts. Gram-negative bacteria were found most resistant, as end points observed were much higher (ranging from 1024 µg/ml to > 4096 µg/ml) against almost all extracts.

All antibacterial activities were observed to be concentration-dependent. The efficacies of all extracts were less than that of the standard antibiotic, tetracycline.

DISCUSSION

Herbal medicines are a valuable and readily available resource for primary health care and complementary health care systems. Undoubtedly, the plant kingdom still holds many species of plants containing substances of medicinal value that have yet to be discovered; though large numbers of plants are constantly being screened for their antimicrobial effects.[13-15] These plants may prove to be a rich source of compounds with possible antimicrobial activities, but more pharmacological investigations are necessary.

Table 3: Minimum inhibitory concentration of active extracts of *Catharanthus roseus*

Extraction type		Minimum inhibitory concentration* (µg/ml)				
		E. coli	*S. paratyphi*	*K. pneumoniae*	*B. subtilis*	*S. aureus*
Dry leaf extracts	Ethanol	>4096	4096	1024	>4096	256
	Methanol	ND	4096	4096	ND	512
Fresh leaf extracts	Ethanol	4096	4096	>4096	4096	2048
	Methanol	ND	4096	>4096	ND	2048
Dry stem extracts	Ethanol	1024	>4096	ND	4096	512
	Methanol	ND	ND	>4096	ND	2048
Dry root extracts	Ethanol	4096	4096	1024	2048	512
	Methanol	ND	4096	>4096	ND	2048
Fresh root extracts	Ethanol	>4096	4096	4096	>4096	2048
	Methanol	>4096	4096	4096	256	1024
Dry flower extracts	Ethanol	4096	2048	4096	ND	1024

*Minimum inhibitory concentrations (µg/ml) of active extracts screened from agar-well diffusion assay against sensitive bacterial species by microbroth dilution assay standardized by NCCLS, **ND-Not detected, *E. coli - Escherichia coli, S. paratyphi - Salmonella paratyphi, K. pneumoniae - Klebsiella pneumoniae, B. cereus - Bacillus cereus, B. subtilis - Bacillus subtilis, S. aureus - Staphylococcus aureus*

The present study reveals the antibacterial potential of crude extracts of different parts of *Catharanthus roseus*. It is clearly demonstrated that extracts prepared from dried parts revealed better antibacterial activity than those extracts prepared from fresh plant part. Almost all parts of the plant showed antibacterial potential in dried form while only leaf and root extracts showed zone(s) of inhibition in their fresh states. Extracts from leaves of this plant demonstrated maximum antibacterial activity thus; it should be further studied to determine the active component(s). In a similar study, the leaf extracts of this plant was found to have significant antibacterial activity against *Xanthomonas campestris*.[16] Furthermore, Gram-positive bacteria were found to have more susceptibility as compared to Gram-negative bacterial species. This is probably due to the differences in chemical composition and structure of cell wall of both types of microorganisms. The present study also confirms the use of organic solvents in the preparation of plant extracts as compared to aqueous extracts. The polarity of antibacterial compounds make them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial species. Data also showed that some antimicrobial substances could only be extracted by organic solvents, suggesting that organic solvents are clearly better solvents of antimicrobial agents.[17]

The results also confirm the validity of the use of this plant as medicine in ancient medicinal traditions. The results of the present study supports the traditional usage of the studied plants and suggests that some of the plant extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. The most active extracts can be subjected to isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation by several methods such as NMR, Mass Spectrometry, UC-MS, LC-MS etc.

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Source of Support: Nil, **Conflict of Interest:** None declared.

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