Evaluation of antimicrobial activity of extracts of *in vivo* and *in vitro* grown *Vinca rosea* L. *(Catharanthus roseus*) against pathogens

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Abstract: The antimicrobial activity of *Vinca rosea* was evaluated against pathogenic bacterial strains (*Bacillus subtilis, B. licheniformis* and *Azotobacter* sp.) and fungal strains (*Asprgillus niger*, *Alternaria solani* and *Rhizopus oryzae*) using agar well diffusion method. Methanolic extracts of *in vivo* leaf, *in vitro* leaf, *in vitro* calluses of leaf, nodal and fruit explants were used and exhibited antimicrobial activity as indicated by minimum inhibitory concentration (MIC). *In vitro* extracts showed better results as compared to the *in vivo* extracts for both the antibacterial as well as the antifungal activity. Among all the extracts, maximum zone of inhibition (30.3 mm±0.58[a]) was formed by *in vitro* leaf callus extract concentration of 2.0mg/ml against *B. licheniformis.* Similarly in case of antifungal activity, maximum zone of inhibition (34.6mm ±0.57[a]) was formed by *in vitro* leaf callus extract and MIC value is 6.0mg/ml against *A. niger*. Hence these results clearly depicts that *V. rosea* possess a great strength to fight against the microbial activity and can be used against various infections.

Keywords: *Vinca rosea*, antimicrobial activity, lLeaf callus, *Asprgillus niger*, *Bacillus subtilis, Bacillus licheniformis.*

INTRODUCTION

Medicinal plant products are useful in minimizing the adverse effects of various chemotherapeutic agents, in prolonging life span and attaining positive general health (Kaushik *et al*., 2000). It has been evaluated that approximately 75% of the total world population has been using plant-derived medicines (Gaines, 2004). The WHO estimates that up to 80% of people still believe on traditional remedies such as herbs for their medicines. The phytochemical extracts of the plants can be used in allopathic medicine as they are a potential source of antiviral, antitumor and antimicrobial agents (Nair *et al*., 2005). The most popular and valuable anti-cancer agents such as paclitaxel and vinblastine are derived solely from plant sources (Katzung, 1995; Pezzuto, 1996). Medicinal plants are the most important source of life saving drugs for the majority of the world's population (Shrivastava and Singh, 2011).

Among the valuable medicinal plants *Vinca rosea* L. possess an important and significant place in the list of medicinal plants. *V. rosea* L. belongs to family *Apocynaceae* is an herbaceous shrub also known as Madagascar periwinkle or *Catharanthus roseus* worldwide. It is cultivated mainly for its alkaloids, which possess anticancerous, antimicrobial and antidiabetic activities (Jaleel *et al*., 2006). It has been used in folk medicine as an antidiabetic, diuretic and antidysenteric, an antihemorrhagic and for wound healing (Pahwa, 2009).

Various *in vitro* techniques as micro propagation from existing and adventitious meristems or organ, tissue and cell cultures provide a large amount of *V. rosea* plant material for the isolation of alkaloids having medicinal properties (Pietrosiuk *et al*., 2007). The antibacterial potential in crude extracts of different parts (i.e., leaves, stem, root and flower) of *C. roseus* against clinically significant bacterial strains was reported (Muhammad *et al*., 2009). The tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries (Sidhu, 2010).

C. roseus is an important antimicrobial plant for novel pharmaceuticals since most of the bacterial pathogens are developing resistance against many of the currently available antimicrobial drugs. The anticancer properties of *C. roseus* have been the major interest in all investigations. The antimicrobial activity has been checked against different microorganisms. The findings showed that the extracts from the leaves of this plant can be used as prophylactic agent in many of the diseases, which sometime are of the magnitude of an epidemic (Prajakta and Ghosh, 2010).

Keeping in view the medicinal importance of this plant, micropropagation technique was used for the growth of *in vitro* plants and evaluated the antimicrobial (antibacterial & antifungal) potential of the plant. The present research was focused to extraction of alkaloids with the help of organic solvents and their comparison of antimicrobial activity of both *in vivo* and *in vitro* plants.

MATERIALS AND METHODS

Surface sterilization of explants and micropropagation

The used explants for micro propagation and callogenesis were obtained from Botanical Garden of Lahore College for Women University and Green House of Lawrence

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Garden, Lahore, Pakistan. Explants were washed with running tap water and detergent. Then excised explants were surface sterilized with the 40% bleach (sodium hypochlorite) for 20 min. Then several rinses were given with autoclaved distilled water till the removal of smell of bleach. The explants were then cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with various concentrations of BAP and NAA for micropropagation and 2, 4- D and Kin for callogenesis. *In vivo* grown plants of *V. rosea* leaves, *in vitro* grown *V. rosea* leaves, leaf callus, nodal callus and fruit callus as explants were used for antimicrobial activity.

Extraction procedures

Soxhlet extraction

Soxhlet apparatus was used for the extraction of alkaloids from *in vivo* grown *V. rosea* leaves. The weighed fresh *in vivo* plant material was crushed in pestle and mortar first and then placed in the extraction thimble. The weighed amount was placed in an extraction chamber, which was suspended above the flask containing the solvent methanol and below a condenser. The flask was heated and the methanol evaporated and moved into the condenser where it was converted into a liquid that trickled into the extraction chamber containing the plant material. The extraction chamber was designed so that when the solvent surrounding the sample exceeded a certain level it flowed and trickled back down into the boiling flask. At the end of the extraction process, the flask containing the methanol extract was removed and methanol was evaporated by using rotary evaporator.

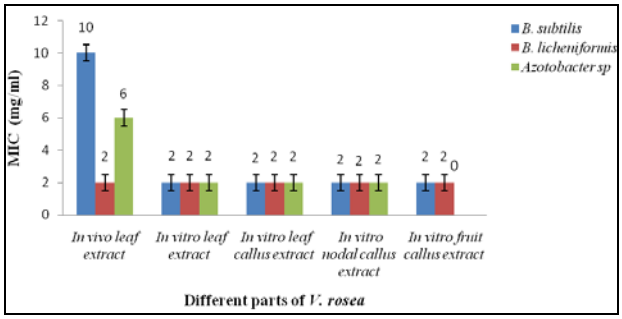

Fig. 1: MIC values of antibacterial activity of different parts of *V. rosea*

Extraction by filtration method

The samples from *in vitro* grown *V. rosea* leaves, leaf, nodal and fruit calluses were extracted by simple filtration method. The weighed amount of each sample was first crushed separately in pestle and mortar and then they were filtered in their respective flasks.

Microorganisms

The tested microorganisms were i.e. *Bacillus subtilis*, *Bacillus licheniformes* (gram positive bacteria) and *Azotobacter sp.* (gram negative bacteria) for antibacterial activity. Similarly three strains i.e. *Aspergillus niger*, *Alternaria solani* and *Rhizopus oryzae* were used for testing antifungal activity. The tested microorganisms were obtained from germplasm bank of Institute of Industrial Biotechnology, Government College University (GCU), Lahore, Pakistan. The microorganisms were cultured on nutrient agar slants. The cultures were maintained by subculturing periodically and preserved at 4ºC prior to use.

Assay for antimicrobial activity

Antimicrobial activity was tested by agar well diffusion method (Mukherjee *et al*., 1995). Different concentrations of the *Vinca* alkaloids were prepared in organic solvent i.e. methanol by using serial dilution method. 0.5ml of the 24h fresh cultures were poured into 35ml sterile molten nutrient agar in sterile petri plates and allowed to be solidified. After solidification, 7 mm wells were made using sterile borer. The wells were then filled with 0.1ml of the sample extract. The antibacterial assay plates were incubated at 37ºC for 24h and antifungal assay plates were incubated at 25ºC for 48h. After incubation, the zones of inhibition were measured. Each experiment was carried out in triplicate and mean diameter of the inhibition zone was recorded.

Minimum inhibitory concentration (MIC)

The extract which showed antimicrobial activity in agar well assay was subjected to MIC assay (Jones *et al*., 1985). In order to determine MIC, serial dilutions of the extract were prepared with concentration ranged from 2 mg/ml to 20 mg/ml and zone of inhibitions were checked. Minimum inhibitory concentration (MIC) as the lowest concentration of plant extracts inhibiting the growth of the organisms was determined.

STATISTICAL ANALYSIS

All experiments were arranged in a Completely Randomized Design and data were analysed using the Costat software. Data (mean ± SD) were collected from five experiments each with four replicates The data thus generated were analysed through one way analysis of variance (ANOVA) and the treatments' means were compared for significance by Duncan's New Multiple Range (DMR) test at 0.05% P.

RESULTS

Antibacterial activity

Antibacterial activity of different parts of *Vinca rosea* was observed, MIC values and their zones of inhibitions were presented in fig. 1 and table 1 respectively. Extracts of *in vivo* leaf, *in vitro* leaf, *in vitro* leaf callus, *in vitro* nodal callus and *in vitro* fruit callus were tested against three bacterial strains i.e. *Bacillus subtilis, Bacillus licheniformis and Azotobacter sp*. The organic solvent used for extraction of all above-mentioned samples is methanol.

Table 1: Comparison of antibacterial activity of different extract concentrations from different parts of *V. rosea*.

S. No.	Extract concentrations (mg/ml)	Tested Microorganism	*In vivo* leaf zone of inhibition (mm)	*In vitro* leaf zone of inhibition (mm)	Leaf callus zone of inhibition (mm)	Nodal callus zone of inhibition (mm)	Fruit callus zone of inhibition (mm)
1	Control	*Bacillus subtilis*	-	-	-	-	-
		B. licheniformis	-	-	-	-	-
		Azotobacter sp.	-	-	-	-	-
2	2	*B. subtilis*	-	28.7±1.15^{b}	14.0±1.00^{c}	21.3±0.58^{d}	25.0±0.00^{d}
		B. licheniformis	4.3±0.58^{c}	30.3±0.58^{a}	23.3±1.53^{a}	19.0±1.00^{e}	20.7±1.15^{c}
		Azotobacter sp.	-	8.6±0.58^{i}	8.3±1.52^{e}	28.6±0.57^{a}	-
3	6	*B. subtilis*	-	17.7±1.15^{f}	20.7±2.08^{b}	14.0±1.00^{g}	21.3±0.58^{b}
		B. licheniformis	-	26.3±1.52^{c}	11.0±1.73^{d}	19.3±0.58^{e}	20.0±0.00^{d}
		Azotobacter sp.	7.3±2.08^{a}	12.0±1.00^{h}	11.3±0.58^{d}	25.0±0.00^{b}	-
4	10	*B. subtilis*	5.7±0.58^{b}	14.7±1.53^{g}	5.7±0.58^{e}	11.0±0.00^{h}	23.7±1.53^{a}
		B. licheniformis	3.0±0.00^{d}	25.0±0.00^{d}	3.0±0.00^{f}	17.0±1.73^{f}	23.7±1.52^{a}
		Azotobacter sp.	-	20.7±0.58^{e}	-	22.3±1.53^{c}	-

Table 2: Comparison of antifungal activity of different extract concentrations from different explants of *V. rosea.*

S. No.	Extract concentrations (mg/ml)	Tested Microorganism	*In vivo* leaf zone of inhibition (mm)	*In vitro* leaf zone of inhibition (mm)	Leaf callus zone of inhibition (mm)	Nodal callus zone of inhibition (mm)	Fruit callus zone of inhibition (mm)
1	Control	*Aspergillus niger*	-	-	-	-	-
		Alternaria solani	-	-	-	-	-
		Rhizopus oryzae	-	-	-	-	-
2	2	*A. niger*	-	-	30.0±0.00^{b}	9.3±0.58^{d}	7.7±0.58^{b}
		A. solani	-	-	-	18.7±1.15^{a}	8.0±0.00^{a}
		R. oryzae	1.0±0.00^{c}	-	-	2.0±0.00^{f}	-
3	6	*A. niger*	-	-	34.6±0.57^{a}	4.0±1.73^{e}	5.0±0.00^{c}
		A. solani	-	-	-	14.7±0.58^{b}	5.0±0.00^{c}
		R. oryzae	4.3±0.58^{b}	-	-	2.1±0.32^{f}	-
4	10	*A. niger*	-	5.0±1.00^{c}	30.3±1.15^{b}	2.0±1.00^{f}	4.6±0.57^{d}
		A. solani	-	8.0±0.00^{b}	-	10.7±0.58^{c}	4.0±1.00^{e}
		R. oryzae	8.0±1.00^{a}	10.3±0.58^{a}	-	-	-

It is evident from the Table 1 that *in vitro* extracts have strong antibacterial activity as compared to *in vivo* extracts. *In vitro* leaf callus gave higher zone of inhibition against *B. licheniformis* (30.3mm ±0.58^{a}) among all the extracts and followed by *B. subtilis* (28.7mm ±1.15^{b}) in leaf callus. *In vitro* nodal callus also gave 28.6mm ±1.15^{b} zone of inhibition against *Azotobacter sp*.

Antifungal activity

Antifungal activity of different samples including both *in vivo* & *in vitro* grown plant parts of *V. rosea* was observed and their zones of inhibitions were calculated (table 2). MIC values of all different extracts can be seen in the fig. 2. Methanolic extracts of *in vivo* leaf, *in vitro* leaf, *in vitro* leaf callus, *in vitro* nodal callus and *in vitro* fruit callus were tested against three fungal strains i.e. *Aspergillus niger*, *Alternaria solani* and *Rhizopus oryzae*.

The comparison of different extract concentrations against all tested microorganisms showed that all the *in vitro* extracts showed better clear zones of inhibitions. *In vitro* leaf callus gave higher zone of inhibition against *A. niger* (34.6mm±0.57^{a}) and ranged from 1.0mm-34.6mm in diameter. *In vivo* leaf extract did not show any sensitivity against *A. niger* and *A. solani* except *R. oryzae* which gave very low zone of inhibitions ranging from 1.0mm-8.0mm.

DISCUSSION

Antibacterial activity

It has been reported that Gram-positive bacteria are more sensitive to plant oil and extract (Cosentino *et al*., 1999; Karaman *et al*., 2003). *In vivo* leaf extracts were less sensitive to tested microorganism. The zone of inhibition for different bacterial strains and different extracts ranged from 3.0mm -30.3 mm in diameter. Many of the researchers has been studied the effect of plant extracts on the bacterial growth in different parts of the world (Reddy *et al*., 2001; Erdourul, 2002; Ates and Erdourul, 2003). Muhammad *et al*., (2009) reported the antibacterial potential in crude extracts of different parts of *V. rosea* against clinically significant bacterial strains.

It was previously reported that the pattern of inhibition largely depends upon extraction procedure, plant part, physiological and morphological state of plant, extraction solvent and microorganism tested (Goyal *et al*., 2008; Thongson *et al*., 2004).

Antifungal activity

The best response was shown by *A. niger* followed by *A. solani* and *R. oryzae*. Antifungal activity of forty nine botanical extracts were assayed and the data on effect of plant extracts on the growth of *A. niger* was also presented by Bobbarala *et al*., 2009. Hence it has been supported earlier that the compounds isolated from the medicinal plants possess remarkable toxic activity against bacteria and fungi and possess pharmacological significance (Banso and Adeyemo, 2007). *V. rosea* is an important medicinal plant which might be useful as antimicrobial agents (Jayakumar *et al*., 2010).

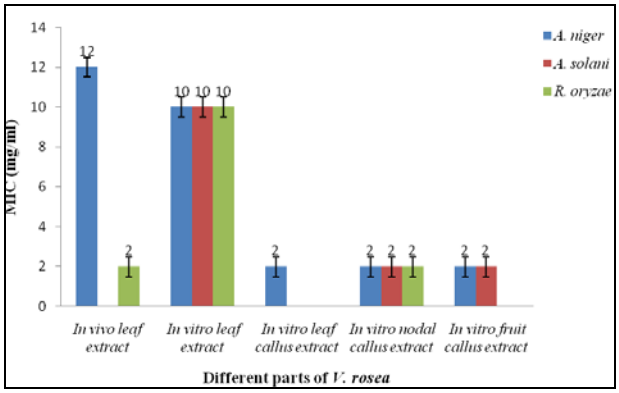

Fig. 2: MIC values of antifungal activity of different parts of *V. rosea.*

The present work also clearly showed that the *in vitro* extract concentrations of all the parts of *V. rosea* exhibited far better results and resistance against both the bacteria and fungi, hence *in vitro* possess greater antimicrobial activity as compared to *in vivo* parts. According to the Roepke *et al*., (2010) and Cowan, (1999), the reason behind this may be that *in vitro* plant parts (leaves & calli) were either too young or immature whereas *in vivo* plant parts (leaves) were mature enough for accumulation of alkolides. The appearance of low levels of catharanthine within older leaves is also reported by Roepke *et al*. (2010). The alkaloid production may be dependent on undifferentiated and immature cells which are mostly present in young leaf and in calluses as compared to the mature and differentiated cells which are present in mature leaves. By this it can be concluded that *in vitro* part (calluses) can be the ideal plant part for the production of antimicrobial compounds.

CONCLUSION

Hence it can be concluded that *in vitro Vinca rosea* propagation can be very useful for the production of antimicrobial compounds, which can prove useful in the medical industry. Further research is needed to isolate many new compounds or alkaloids from *V. rosea,* which are significant medicinally.

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