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Full Length Research Paper

Anti-bacterial activity of *in vitro* produced alkaloids in *Catharanthus roseus* L.

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Antibacterial therapeutic failure due to emergence of resistant bacterial strain is a world wide phenomenon. The search for effective antibacterial substances from sources such as plants has become a necessity to overcome emergent of bacterial resistant in clinical practice. *Catharanthus roseus* L. belongs to the family Apocynaceae, is an erector procumbent herb or under shrub containing latex. It possesses known anti-bacterial, anti-fungal, anti-diabetic, anticancer and antiviral activities. In the present study we carried out the screening of this plant for its antibacterial potential adopting the antibacterial assay. The *in vitro* hairy roots and the hairy roots from transgenic of *C. roseus* were used and extracts were subjected to anti-bacterial assay. The alkaloids hairy roots of transgenic of *C. roseus* exhibited anti-bacterial activity against *Staphylococcus epidermis, Staphylococcus saprofiticous, Micrococcus roseous, Micrococcus luteous* and *Bacillus subtilis*. Moreover, the alkaloids hairy roots of transgenic of *C. roseous* were also ineffective against *Bacillus licheniformis*. The alkaloid of hairy root of transgenic plants was more effective than the *In vitro* hairy roots (non-transgenic), which exhibited broad-spectrum anti-bacterial activity against bacteria with the zone of inhibitions measuring between 17-10 mm. The alkaloid of non-transgenic plants exhibited anti-bacterial activity against bacteria with the zone of inhibition ranging between 14-8 mm. The study implicates that bio-active compound(s) of *C. roseus* could potentially be exploited as antibacterial agents.

Key words: *Catharanthus roseus*, hairy roots, transgenic plant, antibacterial assay.

INTRODUCTION

Catharanthus roseus (L.) (Madagascar periwinkle) is a dicotyledonous tropical perennial species (Apocynaceae), which is entirely self-pollinated species with a high heritability (Raza et al., 2009), it produces many kinds of terpenoid indole alkaloids (TIAs), which have many physiological effects on humans and are used in pharmacy. It is especially true that the dimeric terpenoid indole alkaloids, such as 3', 4'-anhydrovinblastine, vincristine and vinblastine, have powerful effects as anticancer drugs, whereas the monomeric compounds (ajmalicine and serpentine) are used in the treatment of cardiac and circulatory diseases (Van der Heijden et al., 2004; Singh et al., 2001).

Discovery of new antimicrobial compounds with diverse chemical structures and novel mechanisms of action is an urgent attention currently. The development of resistant strains of bacteria has increased the need for new antibiotics (Eloff, 1998). Higher plants, through photosynthesis, produce hundreds to thousands of diverse chemical compounds with different biological activities (Hamburger and Hostettmann, 1991). They can work as pollinator attractants and as chemical defenses against insects, herbivores and microorganisms (Harborne et al., 1990). These antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms (Sarac and Ugur, 2007). There are several reports in the literature regarding the antimicrobial activity of plant crude extracts and the bioassay-guided fractionation of those extracts that

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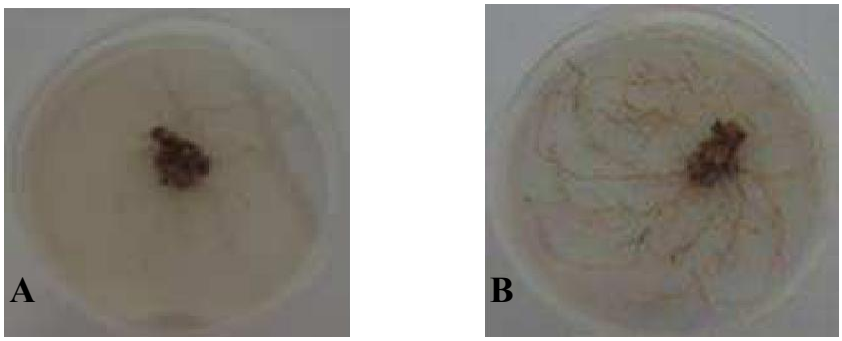

Figure 1. Hairy root regenerated from A) *In vitro* plant B) transgenic calli after inoculation with *A. rhizogenes.*

Table 1. Bacterial strains used in the present study (Vinga et al., 2006).

Strain No.	Bacteria Strain	Gram (+/-)
1	*Staphylococcus epidermidis*	+
2	*Staphylococcus saprophyticous*	+
3	*Micrococcus roseus*	+
4	*Micrococcus loteus*	+
5	*Bacillus subtilis*	+
6	*Bacillus licchniforrmis*	+

yielded active principles (Rabe and Van Staden, 2000; Palombo and Semple, 2001; Portillo et al., 2001; Srinivasan et al., 2001; El-Seedi et al., 2002; Zgoda-Pols et al., 2002; Ramaya et al., 2008, 2009).

MATERIALS AND METHODS

Seed culture

Seeds of *C. roseus* were obtained from Research Center Mahallat and Shahre Rey. Seeds surface were sterilized under aseptic conditions of laminar flow hood, using 5% NaOCl (hypochlorite) for 30 min and were washed by sterile water for 3 - 4 times. Sterilized seeds were then cultured on solid MS medium (Murashige and Skoog, 1962). Hypocotyl explants obtained from 15-day-old per winkle seedlings, were cut into 0.7 – 1 cm long explants and cultured on MS agar medium supplemented with treatment 6-Benzyladenine (31.1 µM) and Naphthalen acetic acid (5.4 µM) (Zargar et al., 2010) Each treatment consisted of 4 explants per dish with 20 replicates. All cultures were placed on dark conditions at 25 - 30°C for 8 - 12 weeks.

Agrobacterium rhizogenes culture

Agrobacterium rhizogenes RI000 was used for inoculation of the callus explants. The bacteria for growth and multiplication were cultured into Luria broth (LB) medium. The bacteria were inoculated into half strength liquid MS basal medium before callus explants were placed into the tube. After 30 min of incubation, callus explants were placed onto solid MS basal medium and co-cultivated.

Hairy roots culture medium

For hairy roots production, callus explants (after inoculate), were transferred onto solid MS basal medium (free hormone) with 400 mgl^{-1} cefotaxime and placed in dark condition (Figure 1).

Alkaloids extraction

About 3-5 g of fresh weight of hairy roots was homogenized (1 min in 60-100 ml ethanol) and was set in water bath in bathroom 60°C for 10 min. The filtrated, alkaloids were extracted and purified according the modified method of Renaudin (1984) as described by Miura et al. (1987). For purification, ethanolic extracts were dried by vacuum evaporator at 40°C and stored in 4°C for furth er examination. Alkaloids isolation stages were: (1). Acidic phase was isolated by sulfuric acid (5%) and diethelic ether (50/50; v/v) in a decanter. (2). Acidic phase was separated and made basic (pH 10) with 10 N NaOH and was concentrated by vacuum evaporator at 40°C. The resulting alkaloids extracts extracted with 60 - 100 ml chloroform in decanter. (3). Chloroformic phase were were dissolved in 1 ml ethanol and was than used for alkaloids assay.

Preparation of bacteria for the experiment

Bacterial species used in the current study were obtained from Bacteriology Laboratory, Islamic Azad University, Qom Branch. These were *Staphylococcus epidermidis, Staphylococcus saprphyticus M. roseus, Micrococcus loteus, B. subtilis* and *B. licheniformis* (Table 1).

The bacterial isolates were homogenized with 3 ml of nutrient broth. A loopful of broth containing the bacteria was inoculated on blood agar. It was then incubated at 37°C for 24 h. Purity and viability of the organisms were checked by plating, Gram staining,

Table 2. Anti-bacterial activity of alkaloids from *C. roseus*.

Strain No.	Microorganism	The zone of inhibitory growth (mm) of plant part	
		In vitro plant	Hairy root transgenic
1	*Staphylococcus epidermidis*	10±0.6	17±0.1
2	*Staphylococcus saprophyticus*	14±0.5	15±0.4
3	*Micrococcus roseus*	14±0.5	15±0.3
4	*Micrococcus loteus*	10±0.1	10±0.2
5	*Bacillus subtilis*	8±0.4	14±0.2
6	*Bacillus licheniformis*	0	0

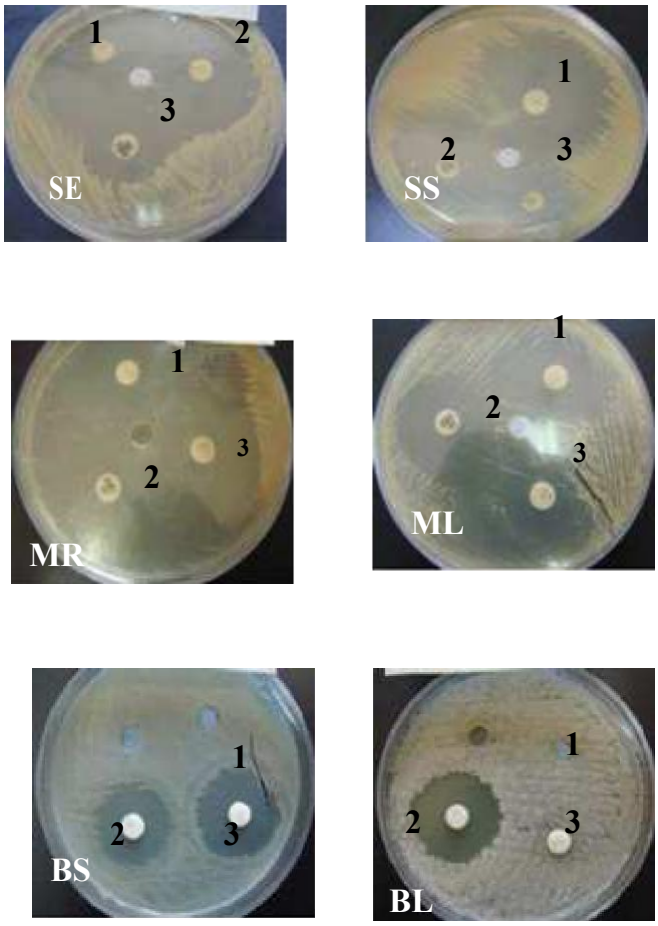

Figure 2. Antimicrobial activity of hairy root transgenic *C. roseus* alkaloids against some bacteria. SE- *Staphylococcus epidermis,* SS- *S. saprphyticus,* MR- *Micrococcus roseus,* ML- *M. loteus,* BS- *Bacillus subtilis,* BL- *B. licheniformis,* 1- Alkaloid, 2, 3- positive controls (gentamycin, erythromycin).

and conducting primary and secondary biochemical tests (Acheampong et al., 1988). The test bacteria were suspended into sterile universal bottles containing nutrient broth separately and incubated at 37°C for 18 h. Normal saline was added g radually to adjust the culture turbidity to that of tube No. 0.5 MCFarland turbidity standard, which corresponds to approximately (1.5×10^8 CFU/ml).

Determination of antibacterial activity

The antibacterial activity was tested using agar well diffusion and broth dilution methods (Lino and Deogracious, 2006; Sahm and Washington 1990).

Agar well diffusion

Briefly, 1 ml of the test culture (1.5×10^8 CFU/ml) was inoculated into a sterile plate with 20 ml Muller Hinton molten agar (50°C) and the plate was shaken for even spread and proper mixing of the organisms on the medium and was then allowed to solidify. Four wells of approximately 8 mm in diameter were made on the surface of the agar plates using a sterile borer. One well of the four wells were filled with 0.35 ml of the plant extracts. The second and third wells were filled 13.13 µg equivalent of gentamycin and erythromycin in 0.35 ml of distilled water and served as and a positive control, respectively. Distilled water (0.35 ml) was applied as negative control. The plates were then incubated at 37°C for 24 h and zone of inhibition was measured with a pair of calipers and ruler millimeter and results were tabulated.

Statistical analysis

The experiment was repeated for 3 times for each treatment used and data were analyzed by analysis of variance test (ANOVA) followed by least significant difference test (LSD).

RESULTS AND DISCUSSION

Results of the present study indicate that antibacterial activity of the alkaloids varied significantly depending upon the part used, namely *in vitro* hairy roots and hairy roots transgenic calli. Further, data obtained demonstrates that the antibacterial activity of plant parts depends largely upon type and amount of alkaloids used and the bacterial strains tested, results of this experiments shows (Table 2), the alkaloids prepared from hairy roots transgenic exhibited better antibacterial activities than those alkaloids prepared from *In vitro* of hairy roots. Almost both of parts of the plant (*in vitro* hairy root and the hairy root transgenic calli) showed significant antibacterial activity. The hairy root transgenic alkaloids exhibited maximum inhibition, followed by *in vitro* hairy root alkaloids (Figure 2).

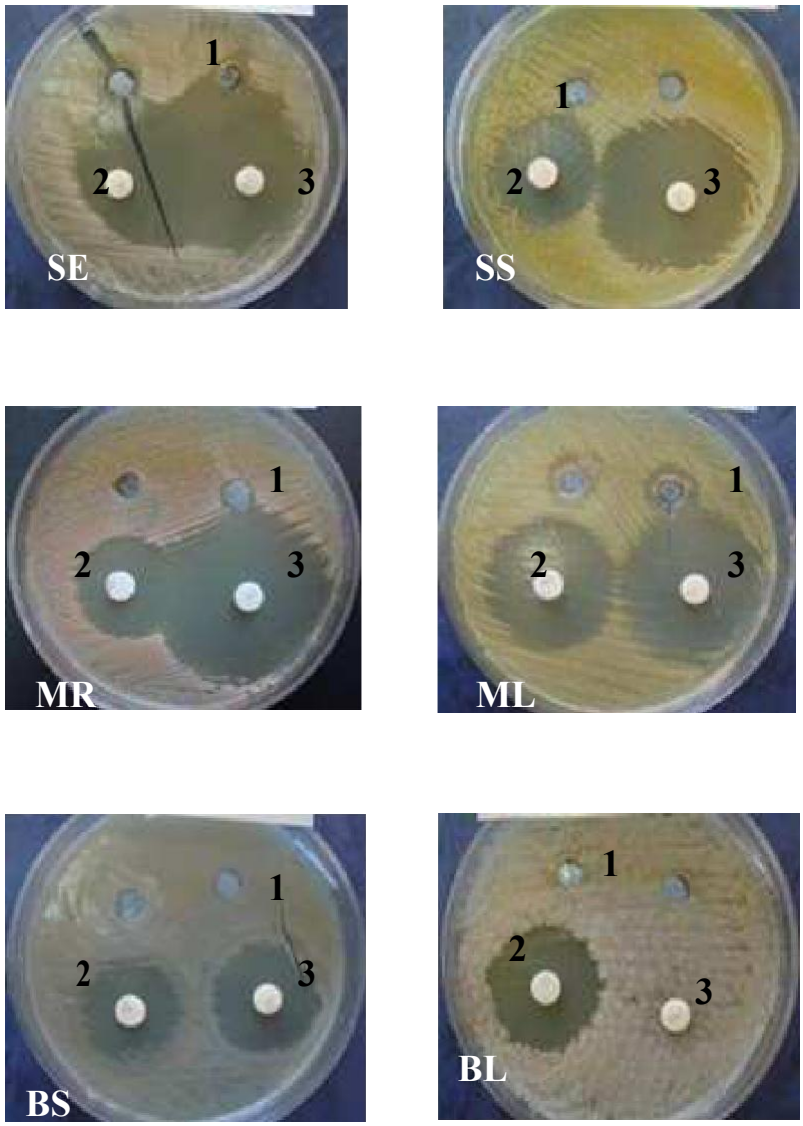

Figure 3. Antimicrobial activity of *In vitro* hairy root *C.roseus* alkaloids against some bacteria. SE- *Staphylococci epidermis,* SS- *S. saprophyticus,* MR- *Micrococci roseus,* ML- *M. loteus,* BS- *Bacillus subtilis,* BL- *B. licniformis,* 1- Alkaloid, 2, 3- positive controls (gentamycin, erythromycin).

Both kinds of alkaloids from the *In vitro* hairy root and the hairy roots transgenic calli of *C. roseus* were effective against *S. epidermis, S. saprphyticus, M. roseus, M. loteus* and *Bacillus subtillis*. The alkaloids of *In vitro* hairy root exhibited a zone of inhibition measuring between 14 to 8 mm (Figure 3). The alkaloids hairy root transgenic calli exhibited powerful antibacterial activity with a maxi-mum inhibition zone 17, 15, 15, 10 and 14 mm against *Staphylococci epidermis, S. saprophyticous* and *Micrococci roseus, M. loteus* and *Bacillus subtillis,* respectively. Antibacterial activities were dose-dependent. However, the efficacies of plant alkaloids were less than the standard, gentamycine and erthromycine antibiotics.

Herbal medicines are a valuable and readily available resource for primary health care and complementary health care systems. Unfortunately, many species of plants containing substances of medicinal value have yet to be discovered; though large numbers of plants are constantly being screened for their antimicrobial effects. It has been suggested that phytochemical alkaloids from plants holds promise to be used in allopathic medicine as they are potential sources of antiviral, anti-tumoral and antimicrobial agents (Nair et al., 2005). The present study

reveals the antibacterial potential of crude alkaloids of different parts of *C. roseus*. Almost all parts of the plant exhibited significant antibacterial activity. Nevertheless, hairy roots transgenic calli alkaloids demonstrated maximum antibacterial activity. Therefore it is obvious that alkaloids prepared using organic solvents were more active against bacterial species. Similar observations have been reported by Thongson et al., (2004). In a study with *C. roseus* it has been pointed out that the pattern of inhibition largely depends upon extraction procedure, plant part, physiological and morphological state of plant and microorganism tested.

This implicates that if a lead molecule is identified from such studies, plant tissue culture techniques can be harnessed for the production of plant secondary metabolites (Verpoorte, 1998). Therefore, a study with large number of clinical pathogens with phytochemicals is expected to provide a hint to fish-out an effective lead molecule.

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