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RESEARCH ARTICLE

ISOLATION OF ANTIMICROBIAL COMPOUND BY ENDOPHYTIC BACTERIA FROM *Vinca rosea*

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ABSTRACT

Antimicrobial compound producing endophytic bacteria were isolated from a medicinal plant *Vinca rosea.* One of the isolated endophytes produced potential antimicrobial activity against some selected human pathogenic bacteria and a yeast. Morphological and biochemical characterizations indicated that the isolate, strain Vrb 46 was similar with *Bacillus coagulans.* TLC analysis of concentrated culture filtrate revealed several UV active bands on silica gel plate. Fermented cell free broth was extracted with chloroform-methanol and antimicrobial activity was found in the organic fraction.

INTRODUCTION

Antimicrobial substances are those that are harmful to microorganisms. From the very past, plants were the chief source of such compounds. But after Fleming's discovery of Penicillin, we knew that some microorganisms have ability to produce a particular type of secondary metabolite that could be used as inhibitory agent for other microbes. Till date many potential antimicrobial substances have been isolated from number of microorganisms. But drug resistances of bacteria, out burst of new diseases enforce us to search for new and potential antimicrobial compounds. As we know that the probability of obtaining a novel compound is always higher from a novel source, we selected endophytic bacteria as source of antimicrobial compound.

Microbes that colonize living internal tissues of plant with out causing any immediate overt negative effect are known as endophytic microbes (Bacon *et al.,* 2000). The most common endophyte is fungi though several bacterial report also available. Endophytes have a wide range of antimicrobial producing strains, which are the important potential sources of antimicrobial substances (Ryan *et al.,* 2008). Even the antimicrobial compounds they produce are of relatively low toxicity as the plant itself serves as natural selection system (Strobel *et al.,* 2003). Many bioactive substances that endophyte produce were relatively new to us. Therefore there is a huge potential to screen novel, highly active and low toxicity antimicrobial compounds from endophytic microorganisms. A large number of endophytic *Streptomyces* (Ghadin *et al.,* 2008) and few other eubacteria (Miller *et al.,* 1988) have already been isolated having anti-bacterial or anti-fungal activity.

Endophytic microorganisms have also great contribution in production of antidiabetic (Zhang *et al.,* 1999), anticancerous (Strobel *et al.,* 2003), antiinsecticidal (Demain *et al.,* 2000), antiviral (Guo *et al.,* 2000) and even immunosuppressive compounds (Lee *et al.,* 2005).
Vinca rosea is a commonly found herb in West Bengal and is traditionally used in diabetis. Its alkaloids have hypotensive, seductive and also anti cancerous property. Ethanol extract of leaf of *Vinca rosea* possess wound-healing activity too (Shivananda *et al.,* 2006).

In this experiment we have found several endophytic bacteria possessing antimicrobial compound. Some morphological and biochemical characterization of this organism was done. Bacterial fermented broth was extracted with organic solvent. Activity was found in organic solvent fraction. Present study illustrates the details on the isolation and characterization of an antimicrobial substance producing edophytic strain Vrb 46, from *Vinca rosea*, and isolation of that antimicrobial substance from culture filtrate of that bacterium.

MATERIALS AND METHODS

Plant selection and location

Vinca rosea, a perennial herbaceous plant was collected from different parts of West Medinipore, West Bengal, India. This area represents a tropical climate with huge forest cover**.** Leaves with petioles and stem about 10 cm of the plant was carefully cut out, tagged and stored in plastic bags. The plant materials were kept at 4 °C in and within 5-6 hr taken for bacterial isolation. Healthy plants were selected for endophyte isolation.

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Isolation of endophytes

At first all the plants were washed with running tap water. Stem and leaf (1 cm), and petioles (0.5 cm) of each plant were separated and subjected to surface sterilization treatment to eliminate the contaminating microflora. The procedure includes sequential immersion of each plant parts in 95% (v/v) ethanol for 2 min, sodium hypochlorite (4-6% free chlorine) for 90 sec and 95% (v/v) ethanol for 30 sec. After that those plant parts were washed in autoclaved distilled water gently and were soaked in autoclaved tissue paper (Suryanarayanan *et al.,* 2005). After that using aseptic technique the surface of the stems, and petioles were removed and inner tissues of stem, petioles and leaves were placed onto Nutrient Agar (NA) media. The plates with plant tissues were incubated at 32 °C under illuminating condition. Observation was made for 48 h.

Table 1. Inhibition zones against the test pathogens produced by the culture extracts of different endophyte isolates.

Endophyte	Inhibition zone (mm) against different pathogens (avg ±SD)[a]				
	Bacillus cereus	*Klebsiella pneumoniae*	*Vibrio cholerae*	*Escherichia coli*	*Candida albicans*
Vrb 45	0	0	17±0.5	14.5±0.3	0
Vrb 46	18.5±0.6	14.5±0.3	15±0.6	16±0.1	17.5±0.3
Vrp 44	0	14.5±0.3	13.5±0.6	15±0.3	17±0.6
Vrl42	0	17.5±0.5	16.5±0.6	16±0.3	0

[a] Average (avg) of inhibition zone diameter ± Standard Deviation.

Table 2. Differential characteristics of Vrb 46.

Characteristics studied	Result
Gram staining	+
Shape	rod (slender)
Cellular arrangement	single, few in long chain
Pigmentation	white
Endospore	+
Location of spore	terminal
Motility	+
Capsule	–
Starch hydrolysis	–
Casein hydrolysis	–
Citrate utilization	–
Gelatin hydrolysis	–
Urea hydrolysis	–
Catalase	–
Methyl red	–
VP test	–
Aerobic growth	+
Anaerobic growth	–
Utilization of carbohydrates (gas production)	
i. Glucose	–
ii. Fructose	–
iii. Arabinose	–
iv. Galactose	–
v. Sucrose	–
vi. Maltose	–
vii. Lactose	–
viii. Mannitol	–
ix. Inositol	–
x. Glycerol	+

Test microorganisms

Pathogenic strains of *Bacillus cereus*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Escherichiae coli* and *Candida albicans* were used in this study. The pathogens were obtained from Department of Microbiology, Vidyasagar University, West Bengal, India. The pathogens were grown in nutrient broth and maintained at -20 °C with 50 % autoclaved glycerol (v/v).

Primary antimicrobial screenings

Isolated endophytic bacteria from *Vinca rosea* were cultured in 30 ml Nutrient broth (NB) medium at 32 °C for 5 days in a rotary shaker (150 rpm). After 5 days culture medium was centrifuged at 8000 rpm for 8 min. and filtrate was screened for antimicrobial activity by agar-diffusion technique on Muller Hinton agar (All media and other ingradients are purchased from HiMedia Laboratories, India.) media that was previously seeded with test pathogens. Sterile broth was set as control. Formation of any inhibition zone was recorded.

Morphological and biochemical characterization

One of the isolated endophytioc bacteria was characterized with its shape, colony character, Gram reaction, endospore formation, and some biochemical reactions. Biochemical tests like methyl red test, VP test, enzymatic hydrolysis tests, sugar utilization test etc. were carried out according to Cappuccino and Sherman (2004).

The characters here considered following Bergey's Mannual of Systematic Bacteriology (Sneath *et al.,* 1986).

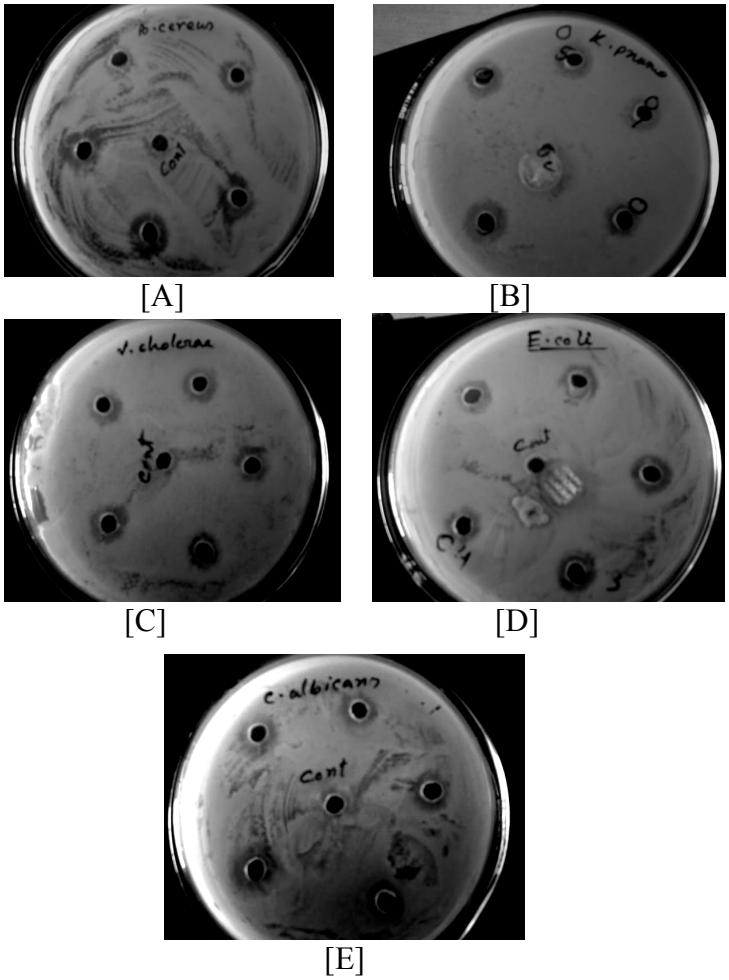


Fig 1. Cell free supernatant showing antimicrobial activity against [A] *B. cereus*, [B] *K. pneumoniae*, [C] *V. cholerae*, [D] *E. coli*, [E] *C. albicans*. All the peripheral wells contain Vrb 46 culture supernatant and central well contain sterile broth

Fermentation and Thin Layer Chromatography

Selected bacterium (Vrb 46) was grown in NB medium for 24 hr and used as inoculum. 2 % inoculum was added for antimicrobial compound production in 250 ml Erlenmayer flask containing 80 ml sterile Nutrient Broth Yeast extract (NBY) media (peptone 0.5 %, beef extract 0.3 %, yeast extract 0.1 %) for five days in a rotary shaker (150 rpm) at 32 °C.

After incubation the cells are separated by centrifugation at 10,000 rpm for 10 min and the fermented supernatant was concentrated to 2 ml by using rotary evaporator (EYELA - Japan) at 40 °C. The antimicrobial activity was again assessed and inhibition zones produced were recorded. The concentrated material was Thin Layer Chromatogrammed (TLC) on silica gel plate.

Different solvents mixtures were tested for better separation including: n-butanol, methanol, ethanol, acetone, chloroform, glacial acetic acid, water. Bands were observed on TLC plates by illuminating the plate with UV Transiluminator.

Extraction and fractionation

The cell free fermentation broth of this endophytic bacterium was finally extracted with same volume of chloroform-methanol (4:0.7) mixture. After fractionation, *in vacuo* evaporation of organic layer was done, and residual material was dissolved in 1 ml chloroform. Antimicrobial activity of this chloroform dissolved substances was detected on MHA plates by agar diffusion technique where pure chloroform and distilled water were treated as control. The aqueous fraction was also verified for antimicrobial activity.

RESULTS

After 48 hr of incubation, bacterial colony was found emerging from the ends of plant parts on nutrient agar media. Isolated bacteria showed different colony characters. After Gram staining their morphological variations were noted. All the morphologically distinct isolates were preserved in 50 % glycerol stock at -20 ˚C.

In primary screening four endophytic bacteria (Vrl 42, Vrp 44, Vrb 45, Vrb 46) were found producing antimicrobial compound against the various test pathogens among all the isolated endophytes of *Vinca rosea* (Table 1).

One bacterium (Vrb 46) isolated from surface sterilized stem of *Vinca rosea* showed antimicrobial activity against all the test pathogens (Fig 1). This rod shaped, Gram positive, motile, endospore forming bacterium was unable to degrade starch, casein, or gelatin (Table 2). This organism showed most of the characters similar with *Bacillus coagulans*. A living culture of this bacterium was maintained with 50 % autoclaved glycerol at -20 °C in our laboratory.

Best separation of the compounds in fermented broth was observed with chloroform – methanol (4:0.7). Six distinct bands were observed after exposing the TLC plates on UV light (Fig 2). After extraction and fractionation, a huge milkfish precipitation was observed at the interfaces of water and organic solvent (Chloroform-methanol). The organic solvent phase was separated carefully by using a separatory funnel. The evaporated fractionated residue did not dissolved well in distilled water. However it was dissolved in 1 ml Chloroform. *In vitro* test showed that the antimicrobial activity retains in only the organic fraction. Inhibition zone produced against *Klebsiella pneumoniae*, *Vibrio cholerae* is shown in Fig 3.

DISCUSSION

The endophyte isolates indicates that the plant *Vinca rosea* is enriched with various bacterial populations. Among the isolates four bacteria were found to be antimicrobial compound producer, so we have tried to isolate new active compounds. Endophytes are reported as novel source of bioactive compounds (Strobel and Daisy, 2003). Among all the isolates Vrb 46 showed antagonistic activity against all pathogens, so it was considered as good source of antimicrobial compound. It is active against both the tested Gram positive and Gram negative bacteria. However according to morphological and

biochemical properties the strain showed similar properties with *Bacillus coagulans* (Sneath, 1986).

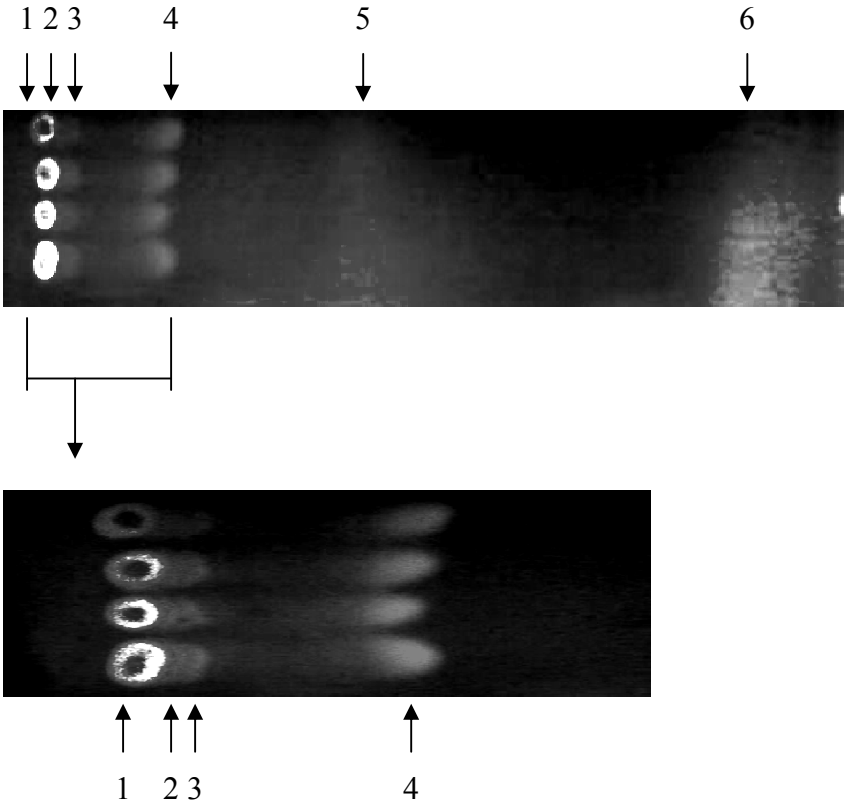
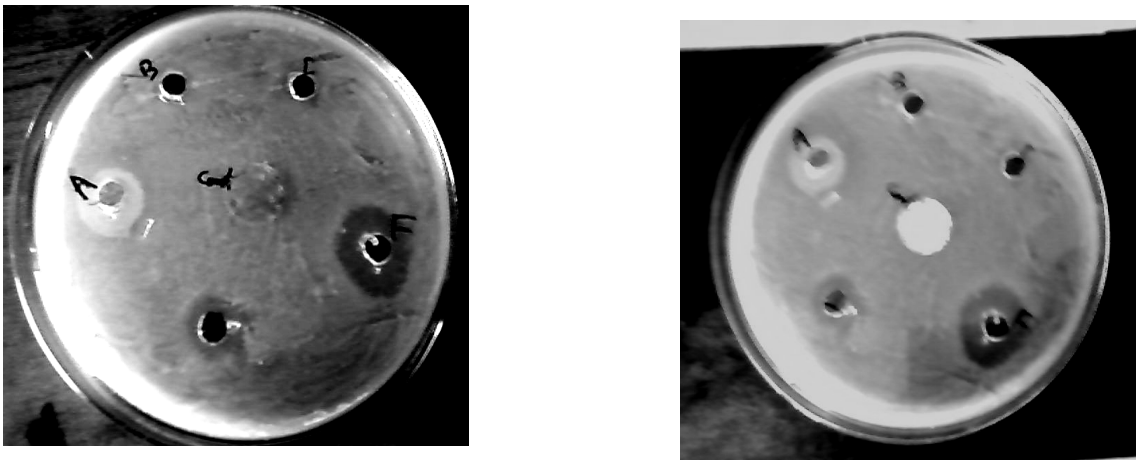


Fig 2. Bands of different compounds (total six) on UV exposed TLC plates at solvent combination, chloroform- methanol; (4: 0.7).



[I]

Fig 3. Chloroform dissolved material after fractionation of fermented broth showing antimicrobial activity against [I] *V. cholerae,* [II] *K. pneumoniae*.

A : Precipated between solvent interface; B : Aquas fraction; C : Pure Chloroform; D : Distilled water; E : Organic fraction; F : Vrb 46 culture filtrate supernatant

The evaporated fractionated residue was yellowish oily substance and did not dissolved well in distilled water indicating hydrophobic nature of the compound. There are many report of lipo-peptide antimicrobial compound (Bie *et al.,* 2005), our isolated compound also bears similar properties. Moreover compared with other chemicals lipo-peptides are also safer for our environment (Sun *et al.,* 2006). Such compounds are highly applied in present medicine and food industries. Further investigation is

underway on the chemical properties to ascertain if it may have the potential to be used in biological controls.

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REFERENCES

Bacon, C. W. and White, J. F. 2000. *Microbial Endophytes*. Marcel Dekker Inc. N.Y. New York.

Bie, X. M., Lu, Z. X., Lu, F. X. and Zeng, X. X. 2005. Screening the main factors affecting extraction of the antimicrobial substance from *Bacillus* sp. fmbJ using Plackett-Burman method. *W. J. Microbiol. Biotechnol.* 21:925-928.

Cappuccino, J. G. and Sherman, N. 2004. *Microbiology: a laboratory manual.* (Bernjamin Cummings: UK)

Demain A. L., 2000. *Microbial natural products: A past with a future. In: Biodiversity: new leads for pharmaceutical and agrochemical industries,* (Wringley SK, Hayes MA, Thomas R, Chrystal EJT, and Nicholson N, ed). The Royal Society of Chemistry, Cambridge, United Kingdom, pp 3-16.

Ghadin, N., Zin, N. M., Sabaratnam, V., Badya, N., Basri, D. F., Hing, H. L. and Sidik, N. M. 2008. Isolation and characterization of a novel endophytic *Streptomyces* SUK06 with antimicrobial activity from Malaysian plant. *Asian J. Plant Sci* 7:2:189-194.

Guo, B., Dai, J., Huang, N. Y., Leong, C., Ong, W. and Carte, B. K. 2000. Cytonic acids A, and B: Novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonema* sp. *J. Nat. Prod.* 63:602-604.

Lee J., Lobkovsky, E., Pliam, N. B., Strobel, G. A., and Clardy, J., 1995. Subglutinols A and B: immunosuppressive compounds from the endophytic fungus *Fusarium subglutinans*. *J. Org. Chem.* 61:7076-7077.

Miller, R. V., Miller, C. M., Garton-Kinney, D., Redgraane, B., Sears, J., Condron, M., Teplow, D., and Strobel, G. A., 1998. Isolation of unique antimycotic Ecomycin from *Pseudomonas viridiflava*. *J. App. Microbiol.* 84:937-944.

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N., 2008. Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.* 278:1-9.

Shivananda, N. 2006. Influence of ethanol extract of *Vinca rosea* on wound healing in diabetic rats. *J. Biol. Sci.* 6:3:40-44.

Sneath, H. A. P. 1986. *Bergey's Manual of Systematic Bacteriology*, Vol. 2 ed by Sneath, H.A.P., Mair, N. S., Sharpe, M, E., and Holt, J. G., The Williams & Wilkins, Baltimore, pp 1104-1138.

Strobel, G. A., and Daisy, B., 2003. Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiol. Mol. Bio. Rev.* 67:491-502.

Suryanarayanan, T. S., Wiltlinger, K. S., and Faeth, H. S. 2005. Endophytic fungi associatied with cacti Arizona. *The British Microbiol. Soc.* 109: 635-639.

Sun, L., Lu, Z., Bie, X., Lu, F., and Yong, S., 2006. Isolation and characterization of co – producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2 from *Scutellaria baicalensis* Georgi. *W. J. Microbiol. Biotechnol.* 22:1259-1266.

Zhang, B., Salituro, D., Li, Z., Szalkowsski, D., Zhang, I., Dez, M., Pelaez, F., Ruby, C., Mao, X., Grifilin, P., and Moller, D. E., 1999. Discovery of small molecule insulinmimetic with antidiabetic activity in mice. *Science*, 284:974-981.

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