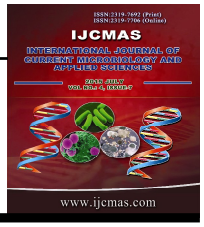
Int.J.Curr.Microbiol.App.Sci (2015) 4(7): 321-329

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 7 (2015) pp. 321-329**
http://www.ijcmas.com



Original Research Article

Cytotoxic Activity of Fungal Endophytes from *Vinca*

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Keywords

Vinca minor, *Vinca erecta*, endophytic fungi, cytotoxicity, bio-active natural products

A B S T R A C T

It has been evaluated that ethyl acetate (EtAc) extracts of endophytes from *Vinca* plants have potential of cytotoxic activity in three cancer cell lines, what indicates the presence of cytotoxic compounds in these extracts. Nine endophytic fungi were isolated from plant tissues of *Vinca minor* and *Vinca erecta* by strict sterile sample preparation and were classified into genera using classical methods. EtAc extracts of five isolates showed significant cytotoxic activity against at least two of three cancer cell lines by MTT assay. The inhibition rates of studied extracts depends on used concentrations and tested cell lines. Even at low concentration the isolates displayed high cytotoxicity that was comparable with one of Cisplatin as positive control. Obtained results indicate the potential of endophytic fungi associated with *Vinca* plants as a source of antitumor agents.

Introduction

Microorganisms that reside asymptomatically and grow intra- and/or intercellularly in the tissues of higher plants known as "endophytes", and have proven to be rich sources of novel organic natural metabolites exhibiting a variety of biological activities (Bacon and White, 2000; Strobel, 2002; Tan and Zou, 2001). Being in symbiotic association with plant, the endophyte produces bioactive metabolites to enhance the growth and competitiveness of the host and to protect it from herbivores and plant pathogens (Guo *et al.*, 2008; Yan *et al.*, 2011). The discovery of endophytes as untapped resource of bio-active natural products means that all the drugs for which plants were earlier being exploited can be synthesized from the endophytic fungi associated with the plants, thus reducing the requirement of any other part of the plant, sparing them from extinction in most places, as well as the drugs could be available at low costs. This also helps in recovering the balance of nature (Guo *et al.*, 2008; Huang *et al.*, 2007; Kusari *et al.*, 2009).

Among the metabolites produced by fungal endophytes special attention is attracted to

compounds with anticancer properties (Gutierrez *et al.*, 2012; Ravindra *et al.*, 2011). Currently 100 anticancer substances classified in 19 different chemical classes with activity against 45 different cancer cell lines have been isolated from 50 fungal species belonging to 6 different groups of endophytic fungi. Of the total number of substances isolated from endophytic fungi, 57% are new or analogues of known compounds (Ravindra et al., 2011). So, for example, Taxol and related compounds, a potent anticancer medicine, taxane prototype, which was first obtained by Wani *et al.* in 1971 (Wani *et al.*, 1971) from the bark of *Taxus brevifolia*, were isolated from a number of endophytic fungi associated with different *Taxus* and related plants (Bashyal *et al.*, 1999; Li *et al.*, 1996; Stierle *et al.*, 1993; Kumaran *et al.*, 2008; Gangadevi and Muthumary, 2009). Another anticancer drug Camptothecin which was initially isolated from the wood of *Camptotheca acuminate*, now can be isolated from the associated endophytic fungus *Fusarium solani* (Lin *et al.*, 2011). Another well known anticancer drug Podophyllotoxin is now being obtained from the endophytic fungus *Phialocephala fortinii* isolated from its host plant *Podophyllum (Sinopodophyllum) peltatum* (Eyberger *et al.*, 2006).

Vinblastine and vincristine, well-known anticancer drugs, firstly isolated from the leaves of field periwinkle *Catharanthus roseus* (syn. *Vinca rosea*), recently the endophytic fungi *Fusarium oxysporum* isolated from *Catharanthus roseus* plant are found to produce both compounds in appreciable amounts (Kumar *et al.*, 2013).

On the territory of Uzbekistan grow four of seven known species of periwinkle - *V. rosea, V. minor, V. major, and V. erecta*. Phytochemical studies conducted at the Institute of Chemistry of Plant Substances of Uzbekistan Academy of Sciences have shown that native species of plants are capable to synthesize many vinca-alkaloids (Baldas *et al.*, 1968). This could suggest the presence of active endophytic cytotoxic microflora in native *Vinca* plants.

The purpose of this work was the screening of cytotoxic activity of fungal endophytes associated with native *V. minor* and *V. erecta.*

Materials and Methods

Study area and material sampling: *V. erecta* and *V. minor* plants were collected in March 2013 on the territory of Tashkent city neighborhoods (Uzbekistan). Plant samples were identified and stored in a herbarium.

Isolation of endophytic fungi: Endophytic fungi were isolated by the method as described previously by Hazalin *et al.* (Hazalin *et al.*, 2009). Roots, stems and leaves were respectively washed in tap water, sterilized in 70% ethanol for 1 min followed by 0.1% $HgCl_2$ for 7 min, rinsed three times in de-ionized water, cut into segments approximately 5 mm in diameter and placed in 90 mm Petri dishes containing Czapek-Dox agarized medium with 50 mg/ml chlortetracycline and 250 mg/ml streptomycin sulfate to inhibit bacterial growth. The plates were incubated for 7-14 days at 28 °C. Different mycelia growing out of the segments were sub-cultured and individually maintained on antibiotics-free Czapek-Dox-agar medium. Colony morphology and growth and spore formation of the isolates were then studied on Potato-Dextrose-agar medium.

Endophytic fungi identification: Isolated strains were identified by classical methods on the basis of morphology using pertinent monographs (Litvinov, 1967). Isolated

strains were deposited at the Institute of Microbiology of the Uzbekistan Academy of Sciences where they were maintained at low temperature (4-5 °C).

Fermentation: To accumulate biomass for further extraction and determination of biological activity, endophytes were grown by submerged fermentation in 500 ml flasks containing 100 ml of Chapek-Dox liquid medium for 5 days at 28 °C.

The extraction of secondary metabolites of endophytic fungi: 5 g of biomass of each isolate was milled in a Potter homogenizer, transferred to a cone flask containing 50 ml of ethyl acetate, and left for extraction at night on a shaker at room temperature. The mixture was filtered through filter paper (Whatman #1) and Na_2SO_4 (40 µg/ml) was added. After the filtration, the extract was striped to dryness on a rotary evaporator and mixed with 1 ml of dimethyl sulfoxide (DMSO). The resulting extract was used as a stock solution and stored at 40 °C.

Cytotoxicity assay: To evaluate the cytotoxic activity of the extracts there were used verified cultures of cancer cells carcinoma of the cervix (HeLa), larynx (HEp-2) and breast (HBL-100) obtained from the Bank of cell cultures of the Institute of Cytology RAS (Saint-Petersburg, Russia), and primary culture of healthy hepatocytes. The growth of cancer cells was determined according to a previously described protocol (Mossman, 1983) by the ability of viable cells to reduce yellow staining of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) with the formation of blue formazan. For MTT assay cells were washed in phosphate buffer and collected by trypsinization, were placed in a 96 well cell plate, incubated, and treated with various concentrations of extracts -100, 10 and 1µg/ml, stock solutions of the extracts were diluted with culture medium to the final concentration of DMSO of 0.1%. After 72 hours incubation, the medium in each well was replaced by MTT solution (5mg/ml in phosphate buffer), cups were incubated for 4 hours under 5% CO_2 and 95% air at 37 °C. Then MTT reagent was removed and the formazan crystals produced by viable cells were dissolved in DMSO and gently shaken. The absorption was determined at 492 nm. Experiments were repeated three times. The percentage growth inhibition was calculated using following formula: % cell inhibition = 100-[(At-Ab)/(Ac-Ab)]x 100, where At - absorption of the sample, Ab - absorption of the blank and Ac - absorption of the control. The effects of the extracts were expressed by IC_{50} values (the concentration of a substance that reduces the absorption of the treated cells by 50% with respect to untreated cells). "Cisplatin" (India) was used as the comparison drug, intact untreated cells was used as the control.

Result and Discussion

Periwinkles *Apocynaceae* comprises well known plants with many uses as pharmaceutical source. The most known periwinkle *Catharanthus roseus* (syn. *Vinca rosea*) is used for production of vincristine and vinblastin by different techniques such as tissue culture, cell culture, shoot culture, semi synthesis as well as total synthesis (Kumar *et al.,* 2012). A number of endophytic fungi have been isolated by Kharwar *et al.* (Kharwar *et al.,* 2008) from the *Catharanthus roseus* plant found in India. As reported by Zahng *et al.* and Tung *et al.* (Zhang *et al.,* 2000; Tung *et al.,* 2002) vincristine is produced by *Fusarium oxysporum*, an endophyte of *Catharanthus roseus*. Guo and Kunming isolated vinblastine from *Alternaria sp.* associated with the same plant found in China and

showed the production based upon TLC and HPLC (Guo and Kunming, 1998). But not much is known about cytotoxic ability of endophytes associated with *V. minor* and *V. erecta*.

Since cytotoxic screening of samples is the preliminary methods to identify active compounds, we have investigated the cytotoxic activity of the extracts isolated from endophytes of these two native plants.

From the roots, stems and leaves of *V. erecta* and *V. minor* we isolated nine strains of endophytic fungi identified as belonging to the genera *Acremonium, Alternaria, Aspergillus, Cladosporium, Penicillium,* and *Sclerotium.* To study the cytotoxic activity there were used extracts at 10 μg/ml concentration. Testing of extracts were carried out on verified cell lines of carcinoma of the cervix (HeLa), larynx (HEp-2) and breast (HBL-100) and primary culture of hepatocytes with Cisplatin as positive control (Table 1).

The obtained data showed that endophytic fungi isolated from *V. minor* and *V. erecta* have a wide range of antitumor activities. All nine tested fungi displayed antitumor activity against at least one of three cancer cell lines. At the same time in comparison with Cisplatin all tested isolates, except isolate VML85, showed significantly lesser cytotoxicity against primary hepatocytes. But the cytotoxic abilities of endophytic fungi were different depending on tested cancer cell lines as well as concentration of extracts.

Therefore, the growth inhibition activity of five most active isolates' extracts in three cancer cell lines and primary culture of hepatocytes were studied at concentrations 1, 10 and 100μg/ml. The results of cytotoxicity tests of extracts are shown in Figures 1 - 4.

Thus, the highest proportion of active isolates was observed on the tests against HBL-100 cells. Inhibitory activity relatively close to one of Cisplatin was found in all three tested concentrations of extracts (Fig. 1). The highest inhibitory activity (91% in compare with 93% for Cisplatin) was displayed by isolate VML85 from *Vinca minor* leaf at concentration 100μg/ml, while at lowest concentration isolates VER90, VER93, VEL98 from *Vinca erecta* displayed almost the same activity as Cisplatin (49%, 48%, 45% accordingly, against 49% for Cisplatin).

At screening test with HEp-2 cells pronounced cytotoxic effects of the cells growth caused by extracts of VMS86, VER90 and VER93 (Fig. 2). At highest tested concentration (100μg/ml) inhibitory activity of VER93 was 90% against 93,5% cytotoxicity of Cisplatin, while at lesser concentration (1μg/ml) this extract caused even more inhibition than Cisplatin (37,5% and 31%, accordingly).

The similar growth inhibition was observed when testing the extracts against HeLa cells. VER93 extract in concentration 1μg/ml had higher cytotoxicity effect than Cisplatin (39% and 32%, respectively), while inhibitory activity of VMR85 extract in 100 μg/ml concentration was 82 % in compare with 97% for Cisplatin.

It should be mentioned that tested extracts, except VMR85, in 1 and 10μg/ml concentrations showed in 2.5 times lesser inhibitory activity against hepatocytes than Cisplatin. Cytotoxicity effect similar to one of Cisplatin was found in 100μg/ml concentration (Fig. 4).

Table.1 Cytotoxic activity of EtAc extracts of Vinca endophytes against HeLa, HEp-2 and HBL-100 cell lines

#	Isolate*	Hepatocytes	HeLa	HEp-2	HBL-100
1	VMR83	47,1±1,80	17,5±0,20	0±0,02	33,0±0,09
2	VML84	40,0±0,90	20,5±0,15	27,0±0,79	47,0±1,60
3	**VMS86**	**30,0±0,60**	19,5±2,10	**61,0±6,80**	**43,0±4,20**
4	VML96	20,0±1,50	6,0±0,30	5,0±0,04	27,0±0,97
5	**VML85**	72,0±4,10	6,5±0,60	26,0±1,90	**47,0±3,80**
6	**VER90**	**28,6±0,90**	**44,0±3,60**	**45,5±3,10**	**54,0±3,07**
7	VEL89	32,0±0,70	6,0±0,01	20,0±0,90	50,0±5,30
8	**VER93**	**30,0±3,20**	**48,0±4,60**	**39,0±1,20**	**53,5±4,70**
9	**VEL98**	47,0±0,02	28,5±2,20	30,5±0,30	**64,0±5,09**
Cisplatin		76,0±9,10	59,0±6,90	84,0±9,60	76,0±4,90

*VMR:*Vinca minor* root, VMS:*Vinca minor* stem, VML: *Vinca minor* leaf, VER: *Vinca erecta* root, VEL:*Vinca erecta* leaf, VES:*Vinca erecta* stem.Extracts concentration: 10 µg/ml; cell inhibition, %; control - untreated cells;significant difference vs. control, P< 0.05.

Fig.1 Cytotoxic activity of EtAc extracts of Vinca endophytes against HBL-100 cell line

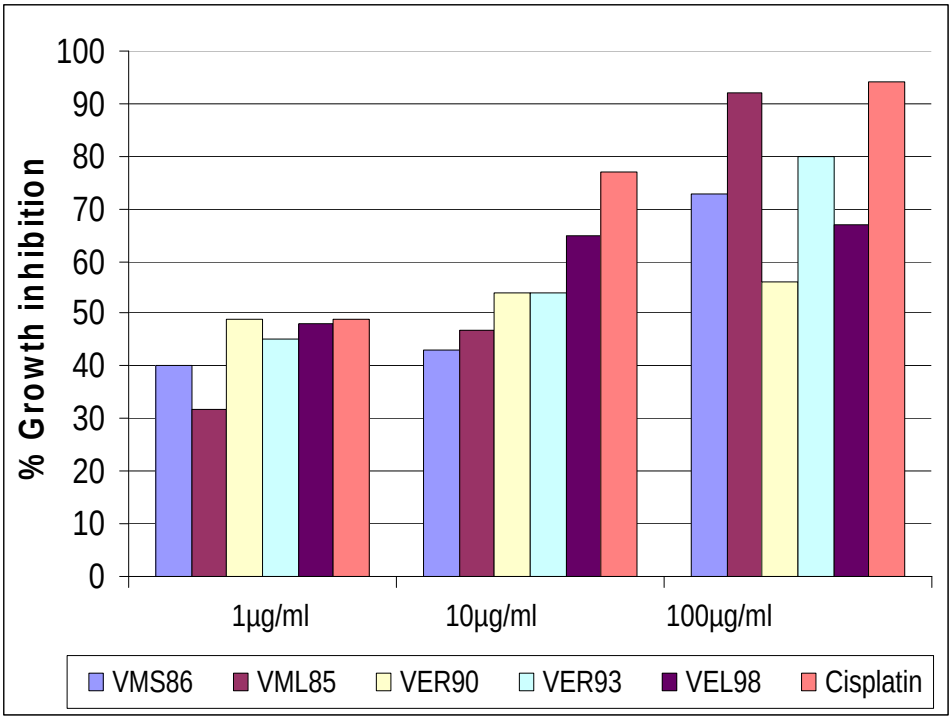


Fig.2 Cytotoxic activity of EtAc extracts of *Vinca* endophytes against Hep-2 cell line

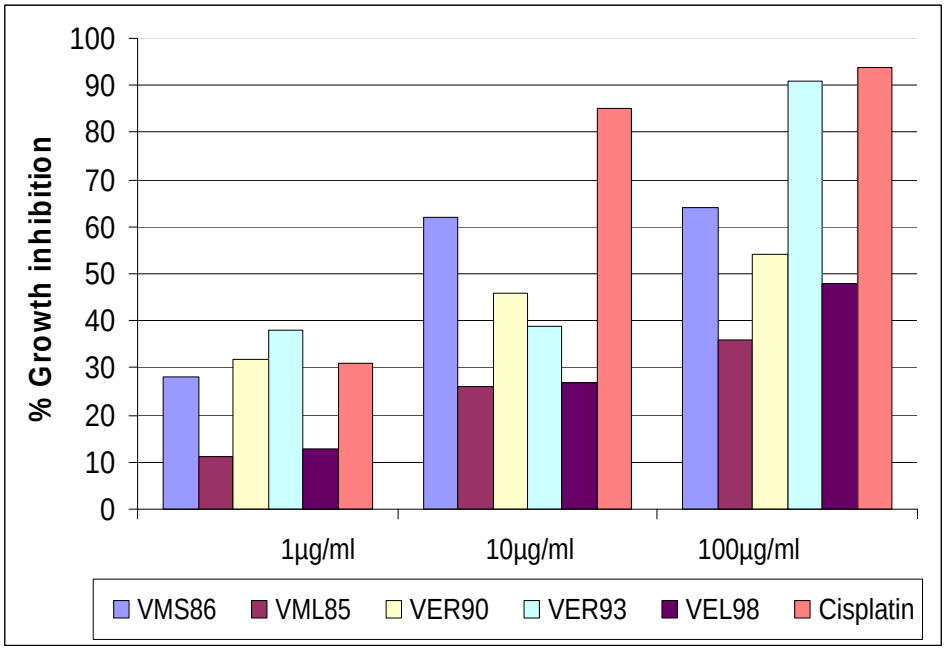


Fig.3 Cytotoxic activity of EtAc extracts of *Vinca* endophytes against HeLa cell line

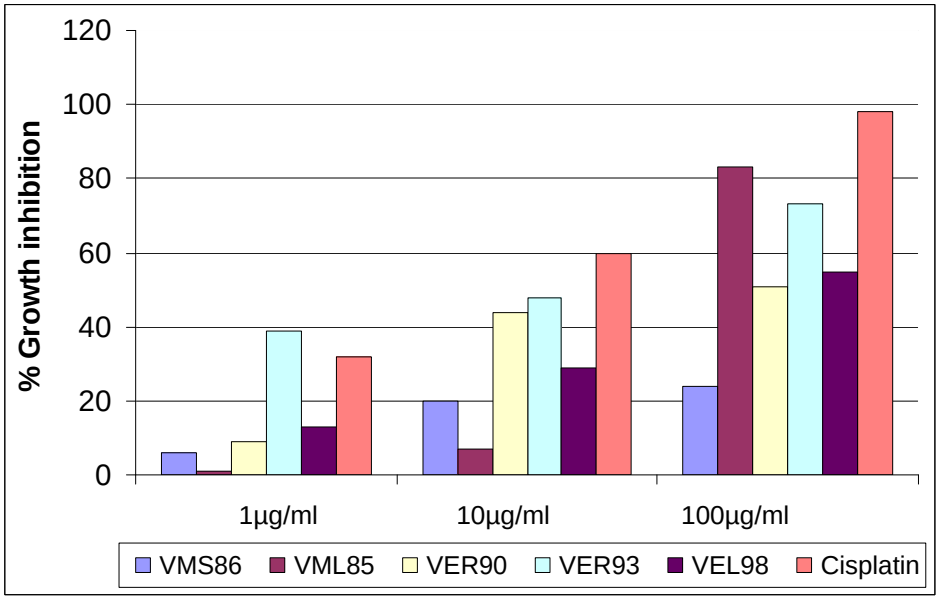
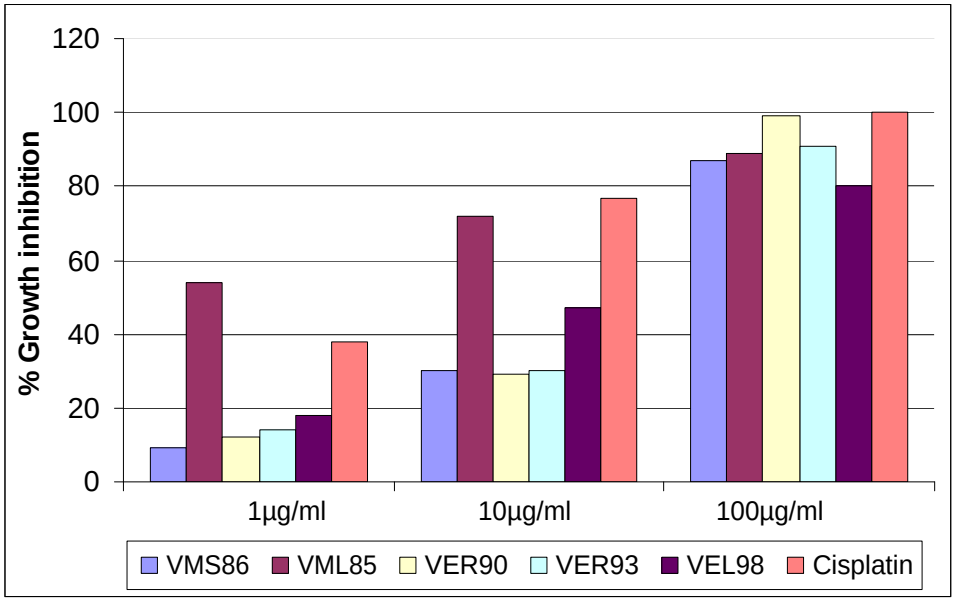


Fig.4 Cytotoxic activity of EtAc extracts of *Vinca* endophytes against hepatocytes



Overall, this study evaluate that EtAc extracts of endophytes from *Vinca* have potential of cytotoxic activity in three cancer cell lines, indicating the presence of cytotoxic compounds in these extracts. Preliminary research on the discovery of *Vinca* alkaloids in the extracts by thin-layer chromatography showed that five extracts of periwinkle direct (*V. erecta*) and small periwinkle (*V. minor*) endophytes contain compounds relevant to vincristine (unpublished data).

The study provides only basic data, and further studies are necessary for isolation and identification of biologically active compounds from these endophytes.

Thus, obtained data indicate that the periwinkle plants growing on the territory of Uzbekistan are inhabited by endophytes producing metabolites with high cytotoxic activity. Of nine isolated endophytic fungi, EtAc extracts of five endophytes, even at low concentration, possesses pronounced cytotoxicity effect what suggests the promising research basis to create anti-cancer drugs.

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