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Isolation and identification of vinblastine from the fungus of *Chaetomium globosum* Cr95 isolated from *Catharanthus roseus* plant

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Abstract

Introduction: Endophytic fungi are capable of synthesizing many secondary plant metabolites and as such make available new compounds for pharmaceutical investigation. Thus, the endophytic fungus Cr95 isolate was separated from the stem of *Catharanthus roseus* plant found in Iran and screened for the production of vinblastine.

Materials and methods**:** By considering its morphologic and genotypic properties, it became possible to identify this isolate, followed by grouping in the *Chaetomium globosum* species. Furthermore, the anti-proliferative activities of *C. globosum* Cr95 isolate against *P. oryzae* as a model fungus, were assessed. This endophytic fungus was screened for the production of vinca alkaloids using specific biochemical assays, tryptophan decarboxylase (TDC) encoding gene, and TLC and HPLC analyses.

Results: The endophytic isolate was found to have significant cytotoxic effects and in the biochemical assays, *C. globosum* Cr 95 isolate showed a positive production of vinca alkaloids. TDC gene could be amplified from this isolate. The presence of vinblastine in fungal culture filtrate was confirmed through chromatographic and spectroscopic analyses, and the amount was estimated to be 78 µg/l. The cytotoxic activity of the partially purified fungal vinblastine against conidia of *P. oryzae* was evaluated using tetrazolium salt MTT assay, and maximum susceptibility was found to occur with the IC 50 value of 5 µg/ml.

Discussion and conclusion: Fungal VBL in ethyl acetate extracts was characterized by TLC and HPLC. To the best of the authors' knowledge, this is the first report on vinblastine production from the endophytic fungus, *C. globosum* Cr 95 isolated from the *C. roseus* plant.

Key words: Endophytic Fungi, *Chaetomium globosum*, Vinblastine, *Catharanthus roseus*, TIA Pathway, Anti-proliferative Activities

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Introduction

Microbes produce diverse secondary metabolites. The list of endophytic fungi which naturally synthesize bioactive compounds of pharmaceutical relevance is on the increase. This is a relatively unexplored field and these bioactive metabolites could be classified as: 1) specific or 2) non-specific to the host plant, and have anticancer properties in addition to other therapeutic effects. The total understanding of the molecular, biochemical, and evolutionary principles of endophytic biosynthesis for plant secondary metabolites, has not been impeded by the insufficient research on this issue. Through co-evolution, plants and endophytes can develop similar compounds which have almost identical biological effects on other organisms. However, there are cases in which co-evolution does not produce completely identical compounds. This implies that few endophytes or none were found to possess the desired ability, when bioprospecting the diverse endophytes isolated for synthesizing compounds associated with plants. Endophytic fungi are capable of synthesizing the metabolites associated with plants, in addition to their analogues which also have therapeutic value (1).

Periwinkles *like Apocynaceae* are well-known plants with varied pharmaceutical applications. The formerly included *Catharanthus roseus* (syn. *Vinca rosea*) in genus *Vinca* is most commonly known as a medicinal periwinkle, which is an endemic plant in Madagascar. An earlier scientific name of *Vinca rosea* was given to C. roseus species. As a flowering plant species native to the western Mediterranean Sea, *Vinca major* has got such common names as a big-leaf, large, greater, and blue periwinkle. Different techniques like tissue culture, cell culture, shoot culture, semi-synthesis, and total synthesis are utilized to produce vincristine and vinblastin from *C. roseus* (2). To treat various solid tumors, leukemia, and Hodgkin's disease, vincristine (VCR; Oncovin) and vinblastine (VBL; Velbe) as 2 major vinca alkaloids are most extensively applied in chemotherapy regimens. Via binding to intracellular tubulin, vincristine and vinblastine can inhibit cell proliferation and thus serve as anticancer drugs. In fact, mitotic block and apoptosis occur to tumor cells by vincristine and vinblastine inhibition mechanisms of DNA repair and RNA synthesis as they block the RNA polymerase, which is dependent on DNA (3). This process occurs since they contain a nitrogen moiety of bis (indole) alkaloid derived from tryptophan through Terpenoid Indole Alkaloid (TIA) pathway (4,5). TIA is firstly biosynthesized through the catalysis of TDC reaction when TDC is encoded by a single gene present in *C. roseus* (6,7) The only known sources are *C. roseus* green leaves; yet, the dried leaves have low yields since a massive 300 tons of them can produce 3 kg vincristine and vinblastine after being processed to meet the estimated annual worldwide demand (8). Accordingly, such production methods as cell culture, metabolic engineering, and semi- and total chemical syntheses have been improved through the research on vincristine and vinblastine to satisfy the increasing demand (9). The low vincristine and vinblastine contents of these plants (1) have led to a difficult and expensive search for more sustainable and economic sources for a long time. Natural bioactive molecules can majorly originate from fungi. So far, more than 4000 bioactive metabolites have been described to have a fungal origin. In some cases, the bioactive compounds similar to those of the host plants can be produced by the fungi associated with those plants. The possibility of an inter generic exchange between fungi and plants was raised after gibberellins was discovered to be originated from *Fusarium fujikuroi*

(10,11). A new prospect was arrived at when the probable production of taxol from the endophytic fungi associated with Taxus brevifolia was corroborated by Strobel et al. (1993) through observation. Therefore, to produce the low-volume vincristine and vinblastine as high-valued drugs, researchers were encouraged to make an attempt to isolate endophytic fungi from *C. roseus* after fungal taxol was successfully discovered. The potential reason for finding a fungal source for the mentioned life-saving drugs is reducing their prices and extinction rates of their associated plants in some areas (10-12). Kharwar et al. (2008) (13) isolated a number of endophytic fungi from the *C. roseus* plant found in India, but there are so far no reports of vinca alkaloids from this endophytic fungi.

According to earlier researches, vinca alkaloids (vincristine and vinblastine) were isolated from an unidentified endophytic fungus (14), *F. oxyspo*rum (15,16), *Alternaria* sp. (17), *F. solani* (18), *Talaromyces radicus* (19), *Eutypella* spp. (20) and *Nigrospora sphaerica* (21) from *C. roseus.* This study aimed to isolate endophytic fungi from *C. roseus* by screening for the production of vinca alkaloids using specific biochemical assays, gene-encoding TDC, and TLC and HPLC analyses.

Materials and methods

Identification using morphological characteristics and molecular approaches: The fungus used in this study was isolated from stem cuttings of *C. roseus* plant obtained from the nursery of the Isfahan province, Iran. Morphological studies of the strains were performed with Potato Dextrose Agar (PDA). The cultures were incubated according to the methodologies described by Wang et al. (22). Seven days after inoculation, the colony growth was measured. Morphological studies included colony and conidial morphology of the fungal isolates and was carried out when the mycelium occupied the whole plate or the test tube. Observations and digital images were made with a light microscope and digital camera (Leica or Olympus). For molecular identification, the fungal isolate was grown in test tubes containing Potato Dextrose Agar (PDB) media and allowed to grow at 28˚C for 5-7 days. The mycelium was harvested and extraction of genomic DNA was carried out by using CinnaPure-DNA (Sinaclon, Iran). The ITS region was subjected to PCR with the ITS1 and ITS4 primers (23) and β–tubulin (TUB2) using primers BT2Fw and BT4Rd (22). Each PCR reaction contained 10 × Taq DNA polymerase reaction buffer 5 µl, 2 mM dNTPs 5 µl, 25 mM $MgCl_2$ 5 µl, 10 pmol each of the primers, 2 unit Taq DNA polymerase and 20-100 ng genomic DNA. Amplicons for each locus were generated following the protocols listed in Wang et al (22). The PCR products were sent to the Macrogen sequencing service facilities (Macrogen Inc., Seoul, Korea) for direct sequencing of double strands of DNA. All sequences were assembled by DNA Baser software. The consensus sequence was compared with the database using the nBLAST search tool from NCBI Genbank. The most similar sequences were retrieved from the Genbank; and among them valid and verified data were used to reconstruct the phylogenetic tree

Phylogenetic analyses: Sequences were checked with BioEdit v. 7.0.9.0 (24). The ITS and TUB2 sequences of outgroup (*Achaetomium strumarium* CBS 333.67) and additional isolate was retrieved from GenBank. Sequences were aligned with MUSCLE (25). Manual adjustments were done if necessary after checking the alignments. Using the simple indel-coding implemented by GapCoder, the phylogenetic analyses were carried out based on the phylogenetic information contained in the indels (gaps) (26).

Phylogenetic analyses were performed with PAUP v. 4.0b10 (27) for neighbour-joining (NJ) and maximum-parsimony (MP) analyses as described by Abdollahzadeh et al *(28)*. Bootstrap analysis was carried out with 1000 replicates. A Partition Homogeneity Test (PHT) was used to determine the congruence between the ITS and TUB2 datasets (29). Bayesian Analyses (BA) employing a Markov Chain Monte Carlo (MCMC) method were performed. The general time-reversible model of evolution (30) including estimation of invariable sites and assuming a discrete gamma distribution with six rate categories (GTR+I+Γ) was used. Four MCMC chains were run simultaneously, starting from random trees, for 110 generations. Trees were sampled every 100^{th} generation for a total of 106 trees. The first 105 trees were discarded as the burn-in phase of each analysis. Posterior probabilities (31) were determined from a majority-rule consensus tree generated from the remaining 9000 trees. The analysis was repeated three times starting from different random trees to ensure trees from the same tree space were being sampled during each analysis. New sequences were deposited in GenBank (Table 1)

Isolation, purification, and characterization of vinblastine from the endophytic fungus: To isolate vinblastine, the endophytic fungus was screened using a two-stage fermentation procedure. In the first stage, 500 ml Erlenmeyer flasks containing 100 ml of Malt Glucose Yeast Peptone (MGYP) medium, (malt extract (0.3%), glucose (1.0%), yeast extract (0.3%), and peptone (0.5%)) were inoculated with a 7-day-old culture and incubated at 28°C on a rotary shaker (240 rpm) for 4-5 days to be used as a seed culture (stage I). Later, 10 ml of the seed culture was transferred to the 500 ml Erlenmeyer flask containing 100 ml of the production medium called vinca medium-1 (glucose (3%), succinic acid (1%), sodium benzoate (100 mg), peptone (1%), magnesium sulphate (3.6 mg), biotin (1 mg), thiamine (1 mg), pyridoxal (1 mg), calcium pentothenate (1 mg), phosphate buffer (1 ml, pH 6.8), L-tryptophan (0.1%), and geranium oil (0.05%)), which was then incubated at 28°C for 20 days to be used as a shake culture (stage II). After being harvested, it was used for further study. Lyophilization of the culture filtrates and mycelia was followed after their separation through muslin cloth. Then, using ethyl acetate as a solvent, extractions of the lyophilized culture filtrates were done. Using a separating funnel, the organic and aqueous layers were segregated. To obtain a crude extract, the extractions were repeated 3 times. Meanwhile, the solvent was dried and concentrated under a vacuum by using anhydrous sodium sulphate and a rotary evaporator at 40uC, respectively.

Primary screening for vinblastine produced from *C. globosum* Cr 95: By conducting specific biochemical assays on vinca alkaloids (Wagner's and Hager's tests), the screening of ethyl acetate fractions (extracted from the endophytic fungus) for vinblastine was performed (32,33). Wagner's test is a specific test to detect the presence of alkaloids. Wagner's reagent was prepared by dissolving 1.27 g of iodine and 2 g of potassium iodide in 100 ml distilled water. To 100 µl of ethyl acetate fraction of each endophytic fungus, 4-5 drops of wagner's reagent were added and the formation of a reddish-brown precipitate was screened. Vinblastine and methanol were used as the positive and negative controls, respectively. For Hager's Test**,** Briefly, to 100 µl of ethyl acetate fraction, 4-5 drops of Hager's reagent (a saturated solution of picric acid) were added and a yellow precipitate formation was detected (Kodangala et al., 2010). Vinblastine and methanol were utilized as the positive and negative controls, respectively.

Anti-proliferative Assay: The anti-proliferative/cytotoxic bioactivity of the crude extract was examined against the conidial germination of *P. oryzae* as a model. *P. oryzae* conidial suspension (4 × 104 mL^{-1}; 50 µL including 0.02 % of yeast extract) was seeded into each well of a 96-well microtiter plate. To yield the final concentrations of 125, 62.5, 31.25, and 15.62 µg/ mL, 50 µL of the sample extract was added to each well based on a serial dilution. Incubation of the assay plates was done at 28˚C for 16 h. To determine the Minimum Inhibitory Concentration (MIC) for each sample extract, a microscopic observation of 75 conidial germ tubes was carried out in terms of sizes and germination to be compared with the control group. All the experiments were conducted in triplicate.

Genomic screening for TIA-producing fungi based on PCR: TDC gene was employed as a molecular marker for the fungi producing TIA to screen the obtained endophytic fungus. To this goal, the gene-specific primers of TDC (F-5'-ACCTACGACCGTCGAACGC-3' and R-5'-AAACTCGGGACATATACAGG-3') were applied (7), which resulted in a 232-bp amplicon. The PCR products were amplified in a typical reaction mixture of 50 µl genomic DNA (100 ng), TDC primer set (100 µ Meach), dNTPs (150 µM), and Taq DNA polymerase (2.5 U) in 1X Taq buffer by using a PCR thermocycler (Sinaclon, Iran) under an initial denaturation at 94°C for 3 min, 30 denaturation cycles at 94°C for 1 min, annealing at 57.5°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Using agarose gel electrophoresis, analysis of the amplified DNA fragment was performed (19).

Thin-Layer Chromatography (TLC): Using a mixture of chloroform: methanol (8:2) as a solvent system, a small amount of the crude extract was dissolved in ethyl acetate and subjected to TLC on silica gel-G plates of 0.5 mm thickness. Then, the plates were sprayed with ceric ammonium sulphate as reagent. Brilliant violet and purple colors above the spraying reagent were produced at the spots of vinca alkaloids. The fungal vinblastine was purified using silica-gel column chromatography. The crude extract was loaded on the silica-gel column with mesh size, length and width of 60-120, 40 and 62 cm, respectively. Then, it was eluted with chloroform: methanol gradient of 9:1, 8:2, 7:3, 1:1, and 3:7 associated with 100% chloroform and methanol after pre-equilibrating it with chloroform. After pooling the fractions of the compounds with the same RF values as that of the standard vinblastine, they were subjected to TLC prepared on a silica plate of 0.5-mm thickness (20 x 20 cm) and developed in a solvent system of chloroform: methanol (8:2). Then, following the scraping of the assumed bands of fungal vinblastine, they were eluted with methanol. Checking of the isolated compounds was performed on TLC in the mentioned solvent system in terms of purity (16).

Purification and quantification of vinblastine by HPLC: The fungal vinblastine purity was determined by HPLC using C18 symmetry column (Waters). 40 mg of the sample was poured into 40 ml of acetonitrile and injected into an HPLC column. Then, the gradient elution was carried out at a flow rate of 0.5 ml/min using 5-95% acetonitrile in water together with 0.01% of trifluoroacetic acid. To detect the compound elution from the column, a dual wavelength recorder set was utilized at 220 and 254 nm. The maximum absorption of the purified compound was designated using a Shimadzu PC 101 spectrophotometer. After dissolving the sample in methanol (HPLC grade), the

spectral data were collected over a range of 200-700 nm. Afterwards, a concentration of 2 mg/ml of a standard vinblastine solution in acetonitrile (HPLC grade) was prepared. To prepare a final concentration of 100 mg/ml, 50 ml of the standard solution (2 mg/ml) obtained was added to 950 ml of acetonitrile (HPLC grade). The HPLC was analyzed after injecting 10 ml of the solution. Similarly, after extracting the 20-day culture filtrate, it was purified by the HPLC to quantify vinblastine presence in 1 L of it. Following the fungal vinblastine purification, it was dissolved in 1 ml of acetonitrile (HPLC grade). Then, 10 ml of each purified solution was injected into the HPLC to be analyzed. To estimate the amount of fungal vinblastine in 1 L of the culture filtrate, the data obtained from the area peak vs. the standard concentrations were applied (16,19).

Cytotoxic effects of 'vinblastine' from *C. globosum* Cr 95: First, separation of the prepared fungal extract from *C. globosum* Cr 95 was done with TLC. Then, scraping of the corresponding spot to the crude vinblastine was done and twice its elution with methanol was followed. In this cytotoxic study, the fungal vinblastine (fungal VLB) resulting from the partially purified material was utilized. For the measurement of cell viability, tetrazolium salt MTT was applied to determine the 'fungal VLB' cytotoxicity (µg/ml) against *P. oryzae* conidia (34,35). The methodology of the study was based on a catalyzed reaction of the functional hyphae through hydrogenases, which led to the cleavage of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-iphenyltetrazolium bromide) to MTT-formazan as its purple derivative. MTT-formazan can be quantified via spectrophotometry within 550 nm after being dissolved in isopropanol. 50 µL of the conidial suspension of *P. oryzae* (7.5×104 mL^{-1}) including a 0.02% yeast extract together with a 200-µl aliquot of conidial suspension, was poured into each well of a 96-well plate to yield a final concentration of 15,000 conidia/well. An untreated conidial suspension of fungal VLB extracted from *P. oryzae* was used as the control.

Statistical Analysis: All obtained data were subjected to analysis of variances (ANOVA) and means were compared by least significant differences (LSD) test, using SAS statistical software. The differences between the varied treatments were specified at 5% level (P\0.05).

Results

Host and fungal identities, biogeographies, and biodiversities of endophytic isolate: *C. globosum* Cr 95 isolate was identified using cultural, morphological, and molecular approaches. This isolate was classified according to morphological traits used by Wang et al. (22) (fig 1). A culture of *C. globosum* was deposited in the Fungi Research Institute of Plant Protection Lab at Bu-Ali Sina University (BASU.CR95). For molecular identification, the ITS and TUB2 sequences of fungal isolates were used to reconstruct the phylogenetic tree. The obtained DNA sequences of entophytes isolated species were deposited in the NCBI (Table 1).

A nBLAST search for CR 95 isolate indicated 100% similarity to *C. globosum* strain CBS 160.62 (KT214565.1 and KT214742.1). The phylogenetic analyses were conducted by combining the 2 unlinked regions of the genes including ITS/TUB2. The internal nodes were observed to highly support the phylogenies resulted from the stable and reproducible ITS/TUB2. Production of the trees with similar topologies was represented by the individual congruent datasets since no significant divergence (P=0.59) was seen through the partition homogeneity test in PAUP 4.0 b10 (27). Therefore ITS and

TUB2 datasets were combined for analysis. The combined ITS and TUB2 sequences for 11 in-group and 1 out-group taxa contained 1254 characters including alignment gaps, of which 334 characters were excluded, 674 were constant, 391 were variable and parsimony-uninformative, and 189 were parsimony-informative. A heuristic search of the remaining 408 parsimony-informative characters resulted in a single most parsimonious tree of 575 steps (CI = 0.83, HI = 0.16, RI = 0.79). The Bayesian and NJ analyses produced phylogenetic trees with the same topology as the MP tree. The NJ tree is shown in Fig. 2 with BI/MP/NJ posterior probabilities and bootstrap support values at the nodes.*C. globosum* Cr 95 was identified with a combination of morphologic and genotypic characters. The *C. globosum* Cr 95 was placed in a similar clade of *C. globosum* CBS 160.62 with a high bootstrap value (fig 2). Overall, *C. globosum* isolate was identified by sequence analysis of ITS and TUB2 as well as morphological identification.

Primary screening for the endophytic fungi producing vinblastine: The 2 biochemical tests (Wager's and Hager's tests), which were specific for the detection of vinca alkaloids, were used for vinblastine detection in ethyl acetate extracts of *C. globosum* Cr 95 isolate (Table 2). In the analysis, the isolate was found to be positive for vinblastine production. The reddish brown and yellow precipitate formations in Wager's and Hager's tests, respectively, confirmed the presence of alkaloidal compounds in the samples.

Anti-proliferative/cytotoxic bioactivities of the *C. globosum* Cr 95 culture extracts: The crude extract of *C. globosum* Cr 95 isolate was then evaluated for anti-proliferative activity against *Pyricularia oryzae*. *P. oryzae* was used as a fungal model target for the primary screening of antitumor and antifungal activities (36,37). Thus, to assess the anti-proliferative and growth inhibition activities of the fungal metabolites, their conidial germination and germ tube development from *P. oryzae* were adapted. Four different concentrations (15-125 µg/ml) of the sample were tested. It was found that crude extract of *C. globosum* Cr 95 significantly inhibited conidial germination of *P. oryzae* (Table 2).

Table 1- Details of strains included in phylogenetic analyses and endophytic fungus isolate . Isolates and GenBank accession numbers in italic were newly generated in this study.

Species	Isolate	GenBank Accession No[1]	
		ITS	TUB
Chaetomium globosum	CBS 160.62	KT214565.1	KT214742.1
	CBS 105.40	KT214566.1	KT214743.1
	CR 95	*MF319195*	*MF319194*
Chaetomium pseudoglobosum	CBS 574.71	KT214573.1	KT214750.1
Chaetomium umbonatum	CBS 293.83	KT214575.1	KT214752.1
Chaetomium novozelandicum	CBS 124555	KT214576.1	KT214753.1
	CBS 124556	KT214577.1	KT214754.1
Chaetomium afropilosum	CBS 145.38	KT214574.1	KT214751.1
Chaetomium unguicola	CBS 128446	KT214567.1	KT214744.1
Chaetomium tenue	CBS 139.38	KT214568.1	KT214745.1
	CBS 138.38	KT214569.1	KT214746.1
Achaetomium strumarium	CBS 333.67	AY681204.1	AY681238.1

[1]ITS: internal transcribed spacer regions and intervening 5.8S ribosomal RNA; TUB: beta-tubulin

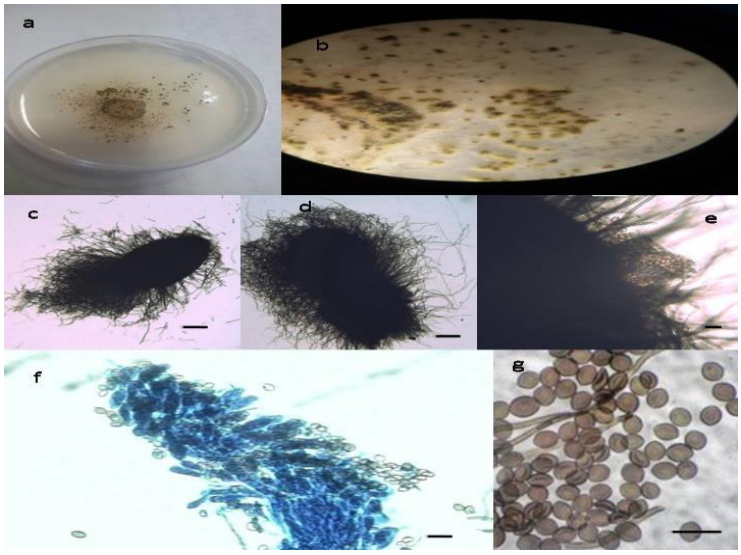

Fig. 1- a. Ascomata and masses of ascospores on OA, top view; b. ascomata on OA,; c , d and e. structure of ascomatal wall in surface view; f. asci; e and g. ascospores. Scale bars: c,d = 200 µm; e-g = 10 µm.

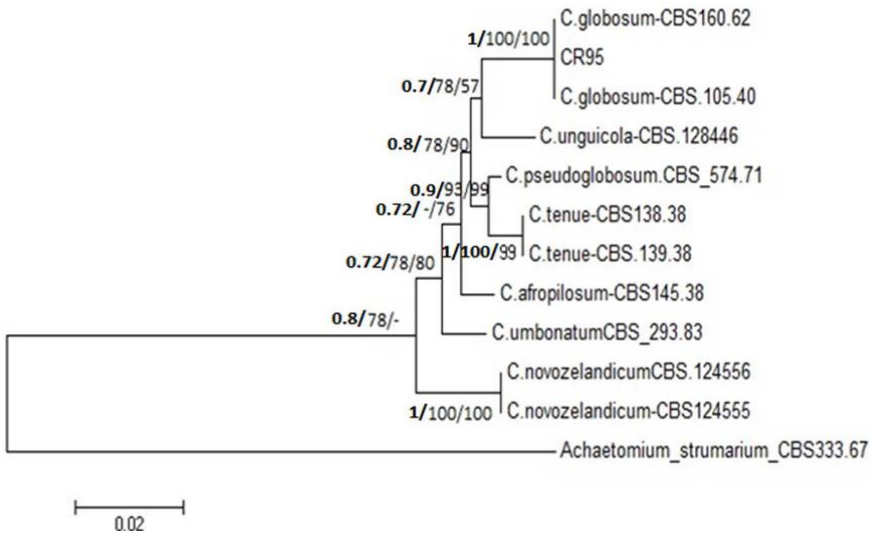

Fig. 2- The NJ consensus tree obtained using the GTR+I+Γ substitution model on the combined ITS and EF1-α sequence data. BI/MP/NJ posterior probabilities and bootstrap support values are given at the nodes. The tree is rooted to *Achaetomium strumarium* CBS 333.67. c: *Chaetomium*.

Table 2- Endophytic fungus isolate, according to plant tissue, cytotoxic activity and biochemical assays

isolate	Plant host	Idification	Plant segment	[a]Cytotoxic activity IC50 (lg mL^{-1})				[b]Primary Screening Test	
				filtrate extracts				Wagner's	Hager's
				125.0	62.5	31.2	15.6		
CR 95	*C. roseus*	*Chaetomium globosum*	stem	+++	+++	++	+	++	++

Data (significant at P B 0.05) were obtained from three replicates. Data are reported as IC50 values, the observations were averages of 4–6 assays

a;* The *P. oryzae* conidial germination was completely inhibited; +++ Strong growth inhibition of germ tube (≤1/3 of control); ++ moderate Growth inhibition of germ tube (1/3–2/3 of control); + low Growth inhibition of germ tube (≥2/3 but less than control); – not Inhibited (as control).

b; The observations were averages of 4–6 assays

+++ indicate very good precipitation, ++ moderate precipitation; + low precipitation; – not precipitation.

Genomic screening for the *C. globosum* Cr 95 producing TIA based on PCR: To determine the presence of TDC genes, *C. globosum* Cr 95 isolate was screened through PCR amplification, and fungus genomic DNA and TDC gene-specific primer applications. As a molecular marker, TDC can help in the identification of the fungi producing TIA. This isolate amplified the production of TDC gene (Fig 3). The amplification patterns of *C. globosum* Cr 95 based on TDC gene could be compared with that of standard *C. roseus* serving as the positive control. Hence, Cr 95 strain was found to be the potential candidate for the production of vinca alkaloids by harboring TDC genes in its genomes.

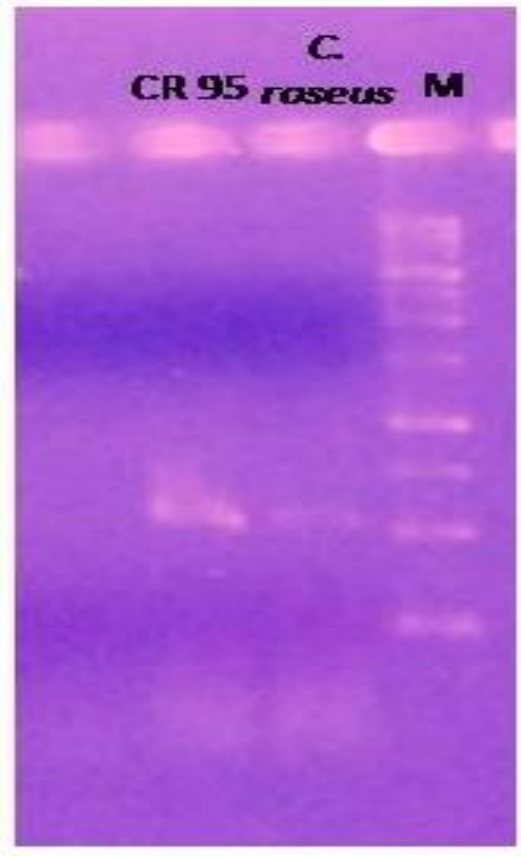

Fig. 3- Genomic screening for TIA-producing fungi based on PCR. Lane M: 1kb ladder; Lanes CR: the PCR amplification products of the TDC gene in CR 95 endophytic fungus isolate. Arrowheads show the amplified products in CR and C. roseus (which served as the positive control).

Vinblastine isolation and identification: Through genomic analyses based on PCR, it was found that *Chaetomium globosum* Cr 95 produces TIA. The presence of vinblastine in Cr 95 strain cultures after 21 days was verified, using the obtained chromatographic and spectroscopic data. The crude extract is the brown residue which was yielded after solvent removal and extraction of the culture filtrates with ethyl acetate (38). Upon TLC, brilliant spots with a purple color were produced by the crude extract when using silica gel G and a solvent system of chloroform: methanol (8:2) sprayed with ceric ammonium sulphate reagent. Identical RF values of the spots (0.69 for vinblastine) obtained through the crude extract TLC by using the solvent system of chloroform: methanol (8:2) and standard vinblastine were found after their comparison. After spraying with ceric ammonium sulphate reagent, identical colors were seen for vinblastine spots in the crude extract and the standard ones. Fractioning of the crude extract on the silica gel column with chloroform: methanol was done to purify vinblastine from the culture filtrates ((100% chloroform (9:1, 8:2, 7:3, 1:1, 4:6, and 3:7) and 100% methanol). The partially purified vinblastine was shown to have the same chromatographic properties as the standard vinblastine. Significantly pure compounds were obtained from the partially purified vinblastine subjected to preparative TLC. The dark purple color of the single spot obtained for vinblastine from the preparative TLC later turned to a very dark purple color with an RF value of 0.69 after spraying it with ceric ammonium sulphate reagent on the TLC (Figure 4) (39,40).

Quantification of vinblastine production by HPLC: A single symmetrical peak with Rt 37.5 min was achieved for the purified compounds through HPLC analysis, thus showing their homogeneity on Symmetry C18 column of vinblastine (Figure 5).

High and rather low intensities at 220 and 254 nm were observed for the eluting compound absorbance, respectively. A peak absorption at 220 nm was shown via the UV absorption analysis. The maximum absorbance of the standard vinblastine is shown in Table 3 for comparison. To estimate the fungal vinblastine quantity, the data of area peak vs. vinblastine concentration was obtained for the standard sample. 7^ mg of vinblastine was obtained by isolating these vinca alkaloids from 1 L of the culture filtrate from *C. globosum* Cr 95 (41).

Cytotoxic effects of 'fungal VLB' on conidial *P. oryzae*: Vinblastine from *C. globosum* Cr 95 was used for its cytotoxic activities. The cytotoxic activity of 'fungal VLB' against conidial *P. oryzae* was determined through tetrazolium salt MTT assay. Upon treatment with fungal VLB with an IC50 value of 5 µg/ml, *P. oryzae* conidial suspension had a dose-dependent inhibition. However, no inhibition was found to occur in the untreated fungal VLB of *P. oryzae* conidial suspension (Figure 6).

Fig. 4- TLC of partially purified fungal vinblastine from culture filtrates along with standard vinblastine on silica gel using chloroform: methanol (8:2) solvent system. VLB: Standard vinblastine .Detection: Ceric ammonium sulphate reagent.

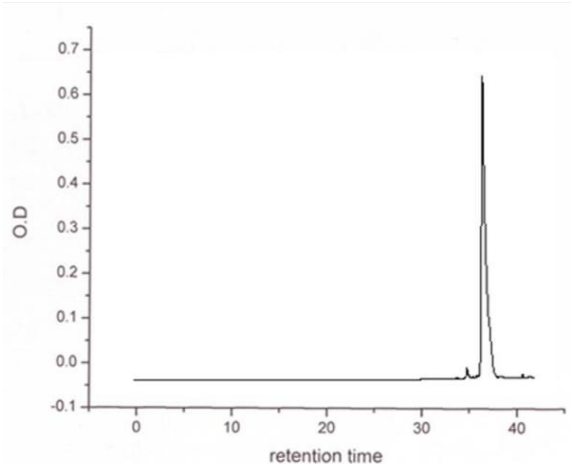

Fig. 5- HPLC profile of pure fungal vinblastine with retention time of 37.5 min.

Table 3- UV absorption and identity of peaks in the chromatogram

Peak maxima (nm) in literature (Palem et al, 2015; kumar et al, 2013a, 2013b)	Identity	Peak maxima (nm)	Retention Time (min)
220, 252, 254	vinblastine	220, 254	37.5

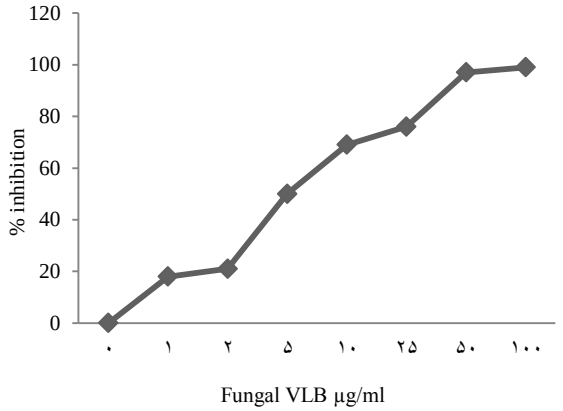

Fig. 6- Cytotoxic activity of 'fungal VBL' in conidial of *P. oryzae.* IC 50= 5µg/ml., no inhibition was found to occur in the untreated fungal VLB of *P. oryzae* conidial suspension

Discussion and conclusion

To the best of the authors' knowledge, this is the first report on vinblastine production from the endophytic fungus, *C. globosum* Cr 95 isolated from the *C. roseus* plant (13,15,19,21,42).

In this study, endophytic fungus was isolated from the stem of *C. roseus* plant

and grouped in *C. globosum* species based on the partial sequencing of ITS and TUB2. The findings further indicated that the endophytic fungus isolate exhibited significant anti-proliferative inhibition capacities (Table 2). Previously, it was shown that ethyl acetate (EtAc) extracts of endophytes from *C. roseus* have potential cytotoxic activities in cancer cell lines. In another study, the anti-proliferative activities of the endophytic fungi extracted from *C. roseus* were assayed in HeLa cells using MTT assay (14,15,19).

Since this study aimed at finding a viable alternative source of vinblastine, *C. globosum* Cr 95 isolate was screened for specific biochemical assays and TDC gene presence. In the biochemical assays, *C. globosum* Cr 95 isolate showed a positive production of vinca alkaloids. The key enzyme for the biosynthetic pathway of TIA in the early steps is TDC. Strictosidine as a precursor of the pharmaceutically important alkaloids of vincristin and vinblastine is yielded by Tryptamine (TDC product) and secologanin condensation (43). A TIA consists of an indole moiety provided by tryptamine and a terpenoid component derived from the iridoid glucoside secologanin. A single gene in *C. roseus* is involved in TDC encoding and *C. globosum* Cr 95 isolate represent this gene amplification. Palem et al. (19) screened 22 endophytic isolates from *C. roseus* for the presence of the gene-encoding TDC. The mentioned gene could only be amplified from *T. radicus* isolate to produce vinblastine and vincristin. The ability of a fungus to produce host/plant-specific compounds is believed to result from its symbiotic association with a plant, from which some biosynthetic genes are horizontally transferred to the fungus. In fact, the biosynthetic genes of some plants for taxol and camptothecin are found in endophytes as candidates for production of these compounds (10,44,45). TDC gene and vinblastine presence in *C. globosum* Cr 95 extracts producing vinblastine at 7^ mg/L were corroborated by this study. TLC and HPLC were used to characterize fungal VBL in ethyl acetate extracts. Also, the cytotoxic effects of fungal VBL against *P. oryzae* were determined, which indicated its maximum susceptibility. This study revealed that a reliable approach for the pre-screening of vinblastine-producing fungi is achieved through PCR amplification of the genes involved in TIA biosynthesis. Since the fungus can synthesize vinblastine, the synthesis of other vinca alkaloids (including vindoline, ajmalacine, secologonin, catharanthine etc) is highly possible. Once the basis for the existence of endophytic microbes in higher plants is understood, it is possible to design a drug discovery program based on these organisms, using some specific examples. The search for vinblastine and vincristine (two rare and expensive anti-proliferative products) is made easier by the systematic investigation of the ability of endophytic microorganisms present in certain plants, to synthesize other pharmacological products. Moreover, it is possible that the fungus could produce other compounds with better anticancer properties as compared to the present drugs.

Abbreviations

CI: Consistency Index, ECA: ethyl acetate, EFα-1 and tef: elongation factor, HI: Homoplasy Index, ITS: internal transcription spacer, MP: maximum-parsimony, MTT: 3-(4, 5- dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide, PHT: partition homogeneity test, NCBI: national center of biotechnology information, N: neighbour-joining, PDB (Potato Dextrose Agar), RI: Retention Index, Rf :retention factor , VCR: vincristine, VBL: vinblastine

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