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Full Length Research Paper

Fusarium solani, P1, a new endophytic podophyllotoxin-producing fungus from roots of *Podophyllum hexandrum*

Mohammad Nadeem[1]*, Mauji Ram[2], Pravej Alam[2], Malik Mobeen Ahmad[2], Anis Mohammad[2], Fahad Al-Qurainy[1], Salim Khan[1] and Malik Zainul Abdin[2]

[1]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh-11451, Kingdom of Saudi Arabia.
[2]Centre for Transgenic Plant Development, Department of Biotechnology, Faculty of Science, Jamia Hamdard, Hamdard Nagar, New Delhi-110 062, India.

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Podophyllotoxin, a well-known naturally occurring aryl tetralin lignan produced by few plant species is used as precursor for the chemical synthesis of the anticancer drugs like etoposide, teniposide and etopophos phosphate. The availability of this lignan is limited due to the scarce occurrence of its natural sources. Further, synthetic approaches for its production are still commercially unacceptable. This paper reports the synthesis of podophyllotoxin by an endophytic fungus *Fusarium solani* isolated from the roots of *Podophyllum hexandrum*. The presence of podophyllotoxin in fungal biomass was confirmed and quantified by HPLC and mass spectrometry. The fungus is able to produce 29.0 µg/g podophyllotoxin on dry weight basis.

Key words: Endophyte, podophyllotoxin, *Podophyllum hexandrum*, *Fusarium solani*, hypocreaceae.

INTRODUCTION

Podophyllum hexandrum Royle (family: Podophyllaceae, English name-May apple, Hindi name-*Bankakri*, Local name-*Banwaigan and Papri*) is a herbaceous and rhizomatous flowering plant species. It is distributed in restricted areas of the Himalayan region at altitudes ranging from 2000 to 4000 m asl. The plant has some ecolonomic importance due to the production of several unusual lignans, in particularly in the subterrestiral rhizomes. Among these secondary metabolites, podophyllotoxin is arguably the most important one because it is used in the preparation of semi-synthetic drugs namely etoposide, etopophos phosphate and teniposide, well known antitumour agents (Schacter, 1996; Canel et al., 2000). These drugs reportedly arrests mitosis at the metaphase (Stahelin and Wartburg, 1991). *P. hexandrum* contains three times more podophyllotoxin as compared to its American counterpart, *P. peltatum* (Fay and Ziegler, 1985). Limited availability of this plant in the wild is attributed mainly to its long juvenile phase, poor fruit and seed onset, uneven and poor germination leading to low regeneration in nature (Nadeem et al., 2000). The current conservation status of *P. hexandrum* is rare and threatened in the Indian Himalaya primarily due to destruction of its natural habitat and overexploitation (Samant, 1994). Thus, the discovery of effective production of podophyllotoxin without collecting this plant in the wild is a prerogative for future usage and conservation of this threatened plant.

Agricultural production of *Podophyllum* has been unsuccessful since the plant is not amenable to cultivation in the absence of proper climatic conditions (Moraes et al., 2001; Lee and Xiao, 2003). *In vitro* approaches have also not been possible due to the fact that the entire biochemical pathway, including key-enzyme(s) and the genetic blueprint involved in podophyllotoxin biosynthesis, is not known yet. Other biotechnological ways for example, cell/tissue cultures have also not yielded desirable results (Empt et al., 2001; Peterson and Alferman, 2001) and total chemical

*Corresponding author. E-mail: mohammadnadeem911@hotmail.com.

synthesis is not feasible commercially (Damayanti and Lown, 1998; Berkovitz et al., 2000). Hence, alternative approaches such as production of podophyllotoxin through endophytic fungi are currently being vigourously perused.

Endophytic fungi are widespread and perhaps ubiquitous in their occurrence in higher plants (Dighton et al. 2005). Ever since reports by Strobel's group that endophytic fungi isolated from a variety of Yew species produces taxol (Stierle et al., 1995). Since then there has been interest in the biosynthetic capabilities of endophytes for a variety of natural products. Fungal endophytes have begun to be recognized as a rich and diverse source of natural products (Gunatilaka, 2006). Recently, both camptothecin and podophyllotoxin were produced by endophytes from *Notahpophytdes foetida* and *P. hexandrum,* respectively (Puri et al., 2005; 2006). The isolation of two strains (PPE5 and PPE7) of *Phialocephala fortinii* Wang and Wilox from wild collections of *Podophyllum peltatum* has also been reported (Eyeberger, 2006).

In the present study, we report the isolation of an endophytic fungus *Fusarium solani* from *P. hexandrum* growing in the Kumaon Himalaya region of India, which produces podophyllotoxin. This study deals with the standardization of growth parameters of the isolated fungus and the chemo-profiling of its secondary metabolite using chromatographic methods. Spectral data, obtained using HPLC and mass spectrometry of the secondary metabolite produced by fungus was identical to the authentic podophyllotoxin.

MATERIALS AND METHODS

Collection of plant material

Rhizomes of *P. hexandrum* were collected from plants growing in 10 different locations around the Village-Jhuni, in the Distt.–Bageshwar, Uttarakhand, India (2450 to 3260 m asl) during August, 2007. Rhizomes with roots were carefully removed from the soil and transported in perforated plastic containers to the lab. These were washed under running tap water for 30 min, dried under shade and stored at 4°C until usages in further experiments.

Isolation and culture of endophyte

The endophyte was isolated from dried roots of *P. hexandrum* using a modified method described by Arnold et al. (2000). Pieces of roots were thoroughly washed using distilled water, followed by 70% (v/v) ethanol for 1 min and 4% hypochlorite for 7 min to achieve complete surface sterilization. These were subsequently rinsed three times for 2 min in sterile distilled water. The small pieces (1 cm) of roots were placed on aqueous agar in petriplates and incubated at 28 ± 2°C until fungal growth was observed. The tips of fungal hyphae were then removed from the aqueous agar and inoculated on growing medium, that is, potato dextrose agar (PDA, Hi Media, Mumbai, India), containing 0.1% streptomycin sulfate to inhibit the growth of bacteria. Then, the purified endophytic fungal culture was transferred on potato-dextrose-agar and potato- dextrose-broth (Hi Media, Mumbai, India), respectively. The experiments were carried with the same method without sterilization as a negative control to check for surface contaminants. The growing fungal mycelium was serially transferred from culture media several times (more than five subculture) to eliminate the possible contamination of podophyllotoxin carrying from roots. Microscopic slides were prepared, stained using lacto-phenol cotton blue and were examined with a translicent light microscope Photographs were taken using a digital camera attached to the microscope.

Fungal biomass production

The endophyte was grown in a 250 ml flask containing 100 ml potato dextrose broth (pH 5.6) (PDB, Hi Media, Mumbai, India) growth medium (pH 5.6) on an incubated rotary shaker (New Brunswick, USA) at 100 rpm. Agar blocks were used for inoculation from the growing fungus colony on PDA plates. After inoculation, the fungus was allowed to grow for 15 days at 28 ± 2°C in dark. The fungal hyphae were separated from the culture medium by passing through two layers of muslin cloth. The fresh biomass and pH was recorded. Fungal mass was washed with sterilized distilled water to remove the medium. The resulting mass was kept in an oven for drying at 40°C for 24 h. After drying the moisture content and dry weight (biomass) were determined for every sample. These experiments were carried out in triplicate and repeated thrice.

Quantification of podophyllotoxin from fungal biomass

1.0 g powdered fungal biomass was taken in a pestle mortar and ground in 40 ml of 25 mM potassium phosphate buffer, pH 7.0. The homogenate was taken in a 100 ml conical flask at room temperature, while shaking for 30 min. After this, 40 ml ethyl acetate was added to it and the mixture was incubated at room temperature for 5 min. After this, aqueous and organic phases were separated by centrifuging at 5000 rpm for 5 min. The organic phase was collected and evaporated in vacuum. The residue was reconstituted with 1 ml methanol and kept for HPLC analysis. Podophyllotoxin in the sample (20 μL injection volume) was analyzed by HPLC (Waters Corporation, Milford, Massachusettes, Ireland) using reverse phase column (RP18, 5 μm, 4.6*120 mm, LicroSorb, Merck, India) with methanol: water solvent system in the ratio of 70:30 as mobile phase in isocratic mode at constant flow rate of 1 ml/min for 10 min, with the detector set at 290 nm. Podophyllotoxin was quantified with the help of standard podophyllotoxin (Sigma Chemicals, Bangalore, India) curve prepared using HPLC.

Q-TOF MS conditions

The mass spectrometry of the sample was performed on a Synapt mass spectrometer (Waters Q-TOF Synapt). The TOF data were collected between m/z 413 and 423. The Q-TOF Premier TM was operated in +V modes-MS with scan time 1 min and 10 μL injection volume. The compound exhibited identical positive ion mass spectra at m/z 413 [M+] along with some of the diagnostic fragments attributed to the parent compound.

Molecular characterization of Fungus

The total fungal genomic DNA was extracted by CTAB method (Cappiccino and Sherman, 1996). For DNA extraction, fungus was grown as described earlier. About 100 mg of mycelial biomass was taken, washed with sterile Tris-EDTA buffer, followed by the addition of 8 ml of CTAB extraction buffer and 100 μL of

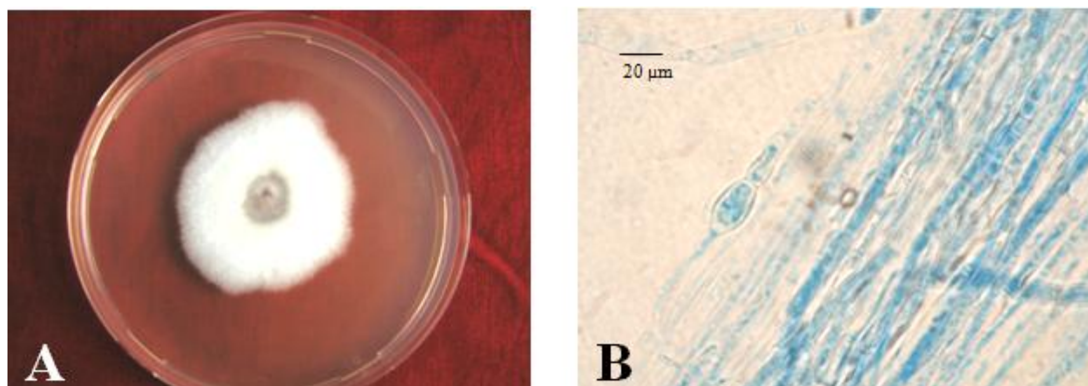

Figure 1. A, Fungal colony of the *Fusarium solani* growing on PDA; B, Microscopic view of the fungal hyphae (100x), Bar scale: 20 μm.

ß-mercaptoethanol. After vortexing, the contents were incubated at 65°C for 30 to 40 min and finally cooled to the room temperature. This was followed by extraction with equal volume of chloroform and centrifugation at 10,000 g for 10 min. Equal volume of isopropanol was added to the supernatant and mixed gently. The DNA pellet obtained was washed with ice cold 70% (v/v) ethanol and vacuum dried. It was then dissolved in 100 µL of TE (pH 8.0).

The endophytic fungus was identified by amplifying 18S, ribosomal region of isolated DNA using primers 5’-TCCGTAGGTGAACCTGCGG-3’ and 5’-TCCTCCGCTTATTGATATGC-3’. The amplified products were purified using Qiagen PCR purification kit (Qiagen, USA) and sequencing of PCR product was carried out through automated sequencer at Chromos Biotech, Bangalore (India). The DNA sequence of 541 bases thus obtained was checked for its homology by NCBI BLASTn program (Altschul et al., 1997). The ribosomal gene database (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov) accessed and sequence alignment was used as an underlying basis to identify the fungus. The DNA sequence has been submitted to the GenBank under the accession no GQ451337. The fungal strain P1 has been deposited and identified at Fungal Identification services of ARI, Pune, India. The fungus was also submitted for Indian Patent (accession no. MTCC 5500) at Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, Punjab, India.

RESULTS AND DISCUSSION

P. hexandrum was selected as a source plant for isolation of endophyte. The emerging fungal hyphae from cultured root segments on day 20 were transferred to PDA plates. As a result, three pure isolates of endophytic fungi (named P1, P2 and P3) were obtained. These strains were screened for the production of podophyllotoxin with the help of HPLC against standard compound. Among these strains, only one (P1) was found positive and shown the presence of podophyllotoxin. Therefore, P1 was finally selected for further studies.

The fungus P1 was characterized on the basis of morphological characteristics. It showed white cottony growth when mycelia were young and later turned to light pink (Figure 1A). In microscopic view, endophytic hyphae were septate and hyaline (Figure 1B). The alignment of rDNA sequence obtained from P1 showed 99% homology with the corresponding gene sequences of *Fusarium solani*. Thus, it can be concluded that the DNA identification supports close affinities to *Fusarium solani*. Shake flask experiment was performed to study the growth kinetics and production of podophyllotoxin. The fungal growth period was observed up to 15 to check the biomass production and podopyllotoxin yield (Tables 1 and 2). The fungus started very slow growth reaching to maximum biomass yield on 10th day of inoculation.

Whereas the maximum yield of podophyllotoxins was recovered from biomaas collected on 8th day growth. HPLC analysis of the extract from fungal biomass has shown the presence of podophyllotoxin (Figures 2 and 3). In case of mass analysis, the authentic podophyllotoxin yielded an *m/z* (M+) peak at 413 (Figures 4 and 5), which corresponded to fungal podophyllotoxin. Maximum production of podophyllotoxin was observed on day 8 (29 µg/g dry weight of mycelia, Table 2). No podophyllotoxin was observed in un-inoculated culture broth and processed culture broth at zero h. This eliminated the possibility that podophyllotoxin had been carried from the original plant material source of the fungus via the mycelium (Kour et al., 2008).

The discovery of fungal endophytes that produce podophyllotoxin has significant biological and commercial implications. Commercially, the fungal culture can be scaled up to provide adequate production for new drug development and patient’s treatments needs. This would reduce the requirement to harvest wild populations of the source plant, conserving this specie in nature. Although, the actual source organism (fungus or plant) of paclitaxel and camptothecin remains in question due to this kind of research, the low production of podophyllotoxin in culture by endophyte from *P. hexandrum* does not lend strong support to the endophyte as the source of the high content of this metabolite in the leaf and rhizome. The

Table 1. Growth pattern of *Fusarium solani* on PDB medium.

Fungal growth days	Fresh weight (g) ± SD	Dry weight (g) ± SD	Moisture content % ± SD
0	0	0	0
2	8 ± 1.0	0.6 ± 0.1	92.5 ± 2.5
4	9.5 ± 0.5	0.72 ± 0.02	92.4 ± 1.2
6	14 ± 1.8	0.85 ± 0.05	93.9 ± 1.0
8	16.8 ± 0.8	1.03 ± 0.03	93.8 ± 0.2
10	17.5 ± 0.5	1.5 ± 0.2	91.4 ± .83
12	17.33 ± 1.04	1.56 ± 0.05	93.36 ± 1.26
15	17.67 ± 1.8	1.53 ± 0.15	93.7 ± 1.57

SD = standard deviation.

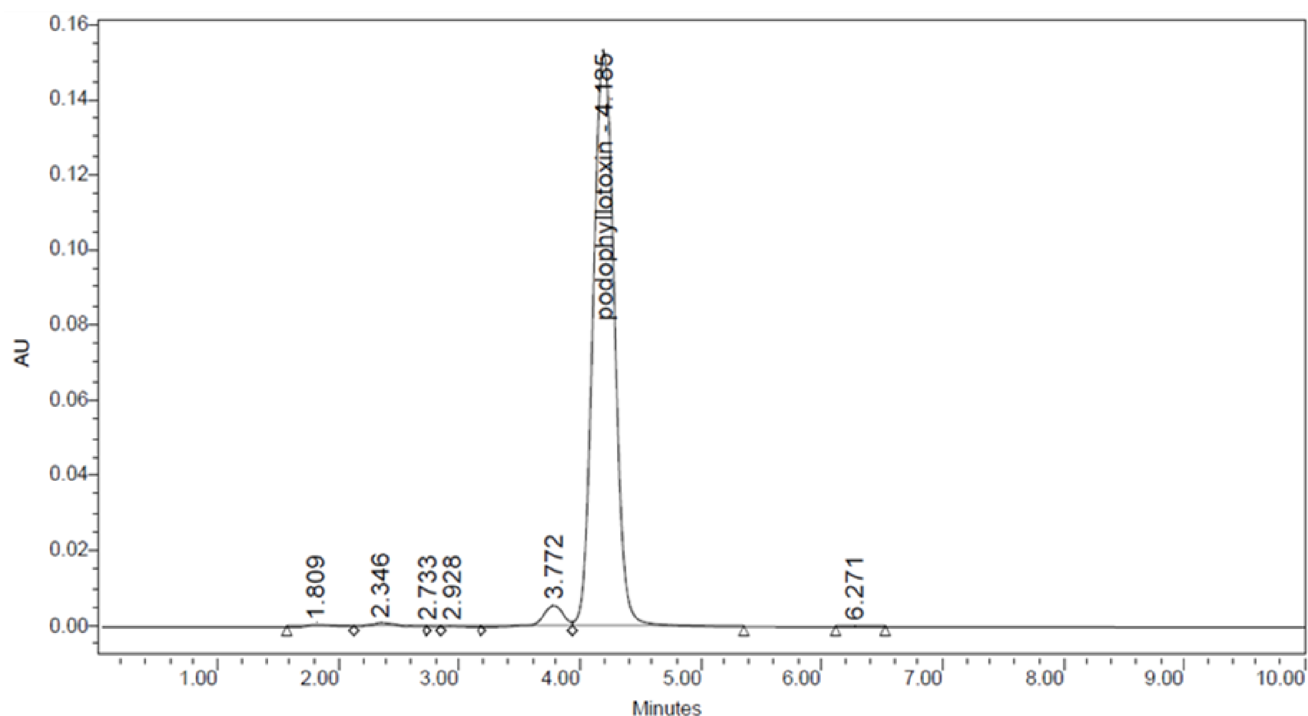

Figure 2. HPLC-Chromatogram of standard podophyllotoxin.

role of the fungus in the production of podophyllotoxin in *P. hexandrum* and regulation of that production needs further investigation (Eyeberger, 2006). In addition to optimization the production, studies are required to improve the production by the endophyte.

The production of podophyllotoxin by *F. solani* supports the theory that during the co-evolution of endophytes and their host plants, endophytes adapted themselves to their special microenvironments by genetic variation, including uptake of some plant DNA into their own genomes (Germaine et al., 2004). This could have led to the ability of certain endophytes to biosynthesize some phytochemicals originally produced by the host plant (Stierle et al., 1993). It is possible to isolate hundreds of endophytic species from a single plant, and among them, at least one generally shows host-specificity (Tan and Zou, 2001). Previous workers have reported the production of the anti-leukemic and anti-tumor drug taxol from the endophytes of *Taxus* spp. like *Taxomyces andreanae* and *Pestalotiopsis microspora* (Kour et al., 2008; Strobel et al., 1996a, b). The results of our study indicate that *Fusarium solani*, a non-pathogenic fungus of animals can be a promising source for large-scale production of podophyllotoxin. The molecular studies of *F. solani* and optimization of fermentation conditions are in progress in our laboratory to scale up the

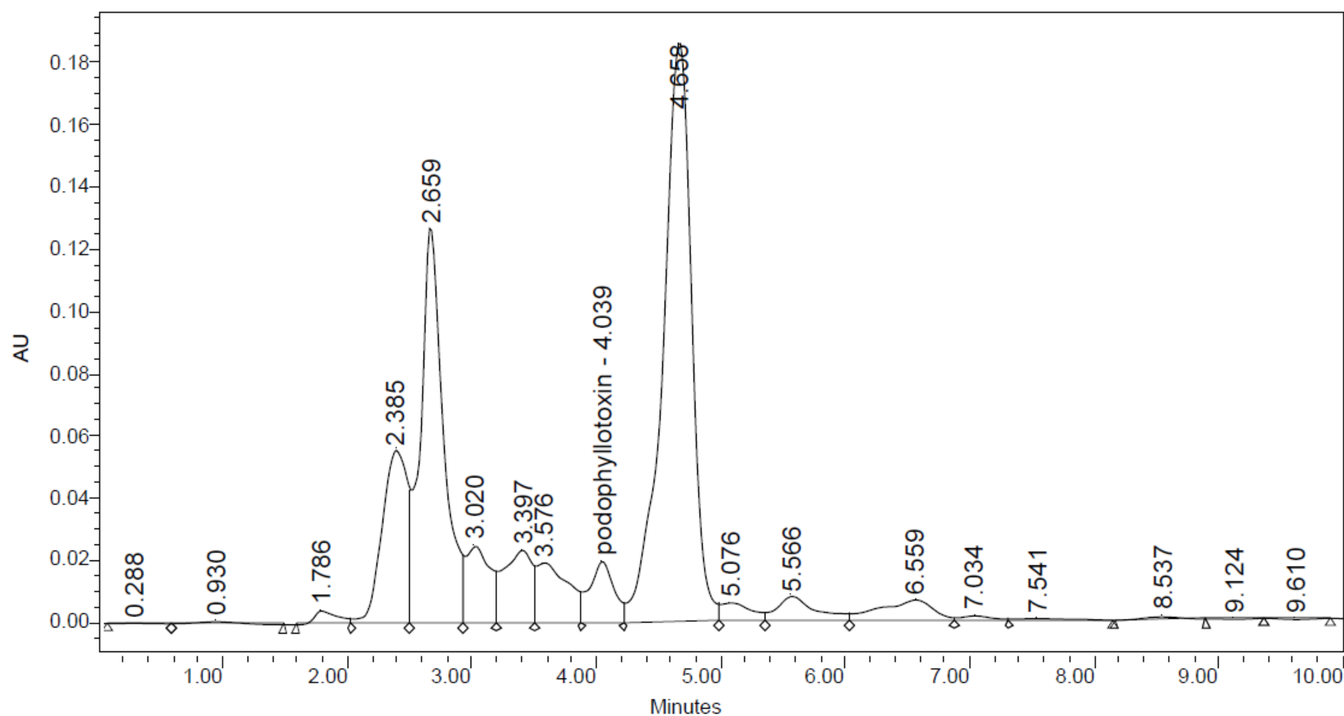

Figure 3. HPLC-Chromatogram of sample from isolated fungi (Isolate P1).

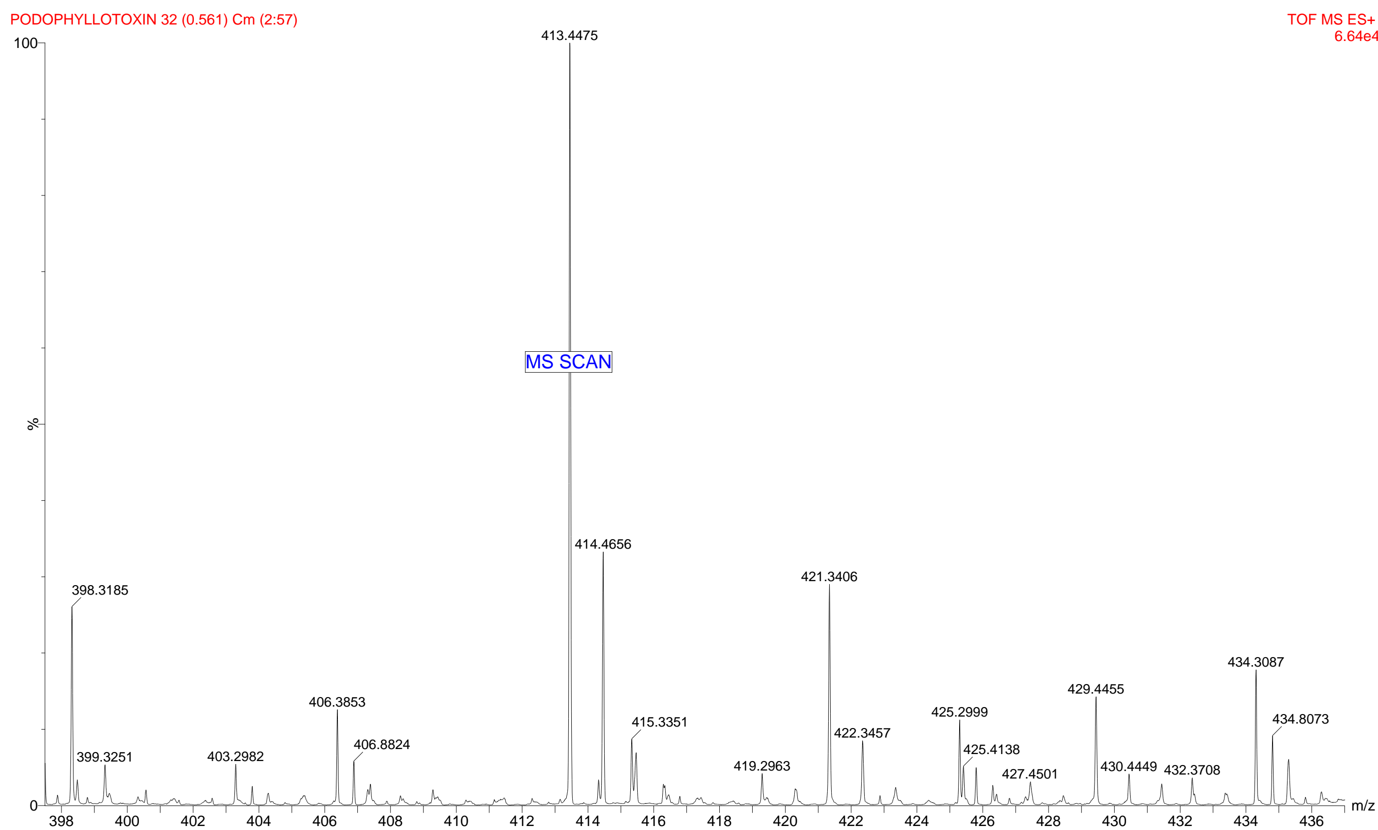

Figure 4. ESIMS of standard podophyllotoxin.

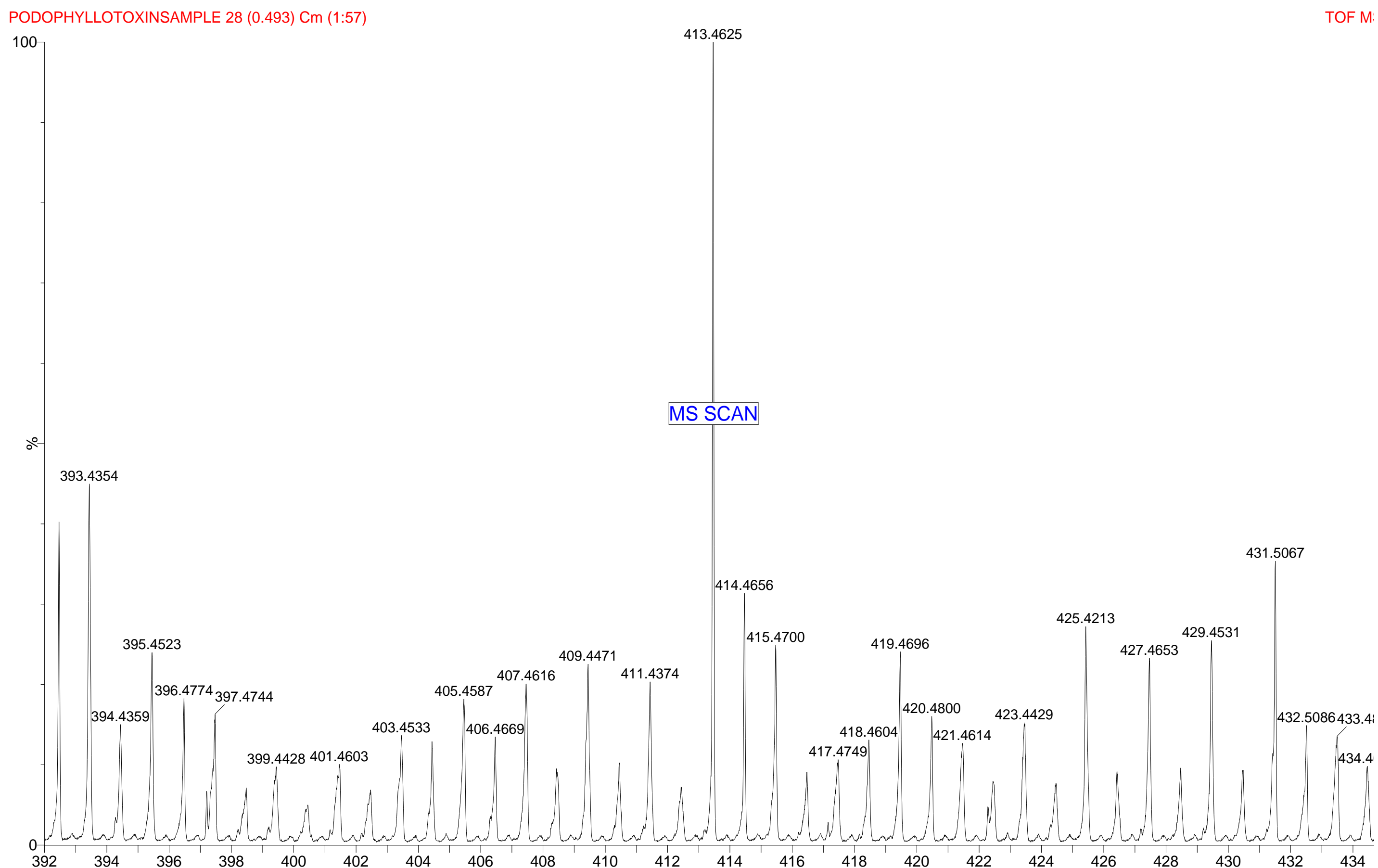

Figure 5. ESIMS of podophyllotoxin isolated from the fungi (P1).

Table 2. Podophyllotoxin content during growth phase of the fungi.

Fungal growth days	Podophyllotoxin in µg/g ± SD
0	0
2	3.2 ± 0.2
4	10.6 ± 0.6
6	18.3 ± 0.3
8	29.0 ± 1.0
10	29.0 ± 1.5
12	29.03 ± 1.3
15	29.16 ± 0.57

SD = standard deviation.

podophyllotoxin production.

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