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ORIGINAL PAPER

Fusarium proliferatum, an endophytic fungus from *Dysoxylum binectariferum* Hook.f, produces rohitukine, a chromane alkaloid possessing anti-cancer activity

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Abstract Rohitukine is a chromane alkaloid possessing anti-inflammatory, anti-cancer and immunomodulatory properties. The compound was first reported from *Amoora rohituka* (Meliaceae) and later from *Dysoxylum binectariferum* (Meliaceae) and *Schumanniophyton problematicum* (Rubiaceae). Flavopiridol, a semi-synthetic derivative of rohitukine is a potent CDK inhibitor and is currently in Phase III clinical trials. In this study, the isolation of an endophytic fungus, *Fusarium proliferatum* (MTCC 9690) from the inner bark tissue of *Dysoxylum binectariferum* Hook.f (Meliaceae) is reported. The endophytic fungus produces rohitukine when cultured in shake flasks containing potato dextrose broth. The yield of rohitukine was 186 μg/100 g dry mycelial weight, substantially lower than that produced by the host tissue. The compound from the fungus was authenticated by comparing the LC–HRMS and LC–HRMS/MS spectra with those of the reference standard and that produced by the host plant. Methanolic extract of the fungus was cytotoxic against HCT-116 and MCF-7 human cancer cell lines (IC_{50} = 10 μg/ml for both cancer cell lines).

Keywords *Dysoxylum binectariferum* · Endophytic fungus · *Fusarium proliferatum* · LC–HRMS/MS · Rohitukine · Flavopiridol

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Introduction

Rohitukine (Fig. 1), a chromane alkaloid (5, 7-dihydroxy-8-(3-hydroxy-1-methyl-4-piperidinyl)-2-methyl-4H-chromen-4-one) was first isolated from *Amoora*

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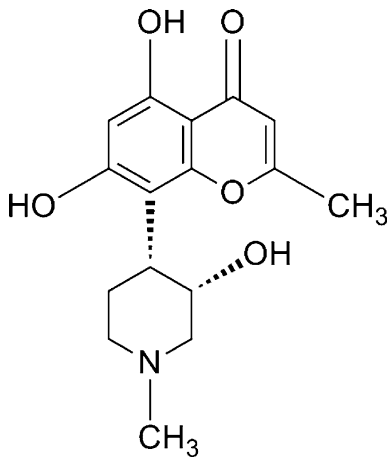

Fig. 1 Chemical structure of rohitukine

rohituka (Meliaceae) (Harmon et al. 1979). Later the alkaloid was also reported from *Dysoxylum binectariferum* (Meliaceae) and *Schumanniophyton problematicum* (Rubiaceae) (Naik et al. 1988; Houghton and Hairong 1987; Houghton and Woldemariam 1993; Yang et al. 2003, 2004). The compound is reported to possess anti-inflammatory, anti-fertility, anti-implantation, anti-cancer and immuno-modulatory properties (Naik et al. 1988; Mohana Kumara et al. 2010; Keshri et al. 2007; Ismail et al. 2009). Several semi-synthetic analogs of rohitukine have been developed of which flavopiridol (also known as HMR 1275 or Alvocidib) is well known (De Souza 1993). Flavopiridol is a potent CDK inhibitor with broad specificity to several kinases including CDK1, CDK2 and CDK4; it arrests the cell cycle at both G1 and G2 phases (Senderowicz and Sausville 2000). It has been shown to be highly effective against breast and lung cancers and chronic lymphocytic leukaemia (Sedlacek et al. 1996; Stadler et al. 2000). Flavopiridol has also been shown to block human immunodeficiency virus Tat trans-activation and viral replication through inhibition of positive transcription elongation factor b (P-TEFb) (Biglione et al. 2007; Perkins et al. 2008). Currently, flavopiridol is under Phase II (Byrd et al. 2006; Karp et al. 2007) and Phase III clinical trials, both as a single agent and in combination with other agents, particularly paclitaxel and cis-platinum against several cancer cell lines (Cragg and Newman 2005; Dancey and Sausville 2003). The biosynthetic pathway of rohitukine in plants is not yet elucidated (Manske and Brossi 1987). Among the reported plant sources of rohitukine, the stem bark of *D. binecteriferum* is one of the richest sources (Mohana Kumara et al. 2010). The tree is sparsely distributed both in the Western Ghat forests of south India and in the Eastern Himalayas (Mohana Kumara et al. 2010).

In this paper, for the first time, the production of rohitukine by an endophytic fungus isolated from the stem bark of *D. binectariferum* is reported. The implication of this finding in the light of the interest in endophytic fungi as a potential alternative source of plant secondary metabolites, in particular, rohitukine is discussed.

Materials and methods

Isolation of endophytic fungi

The endophytic fungus was isolated from *D. binectariferum* Hook.f (Meliaceae) growing in the central Western Ghats, (Kathagal: 14°29′30″; 74°41′10″), India (Myers et al. 2000). Samples of the tree (flowers and leaves) were taxonomically authenticated by the Botanical Survey of India, Kolkata, India. Voucher specimens (COF\DBT\WG-185-35) were deposited at the Herbarium of the College of Forestry, University of Agricultural Sciences, Sirsi, Karnataka State.

Fungal endophyte was isolated from *D. binectariferum* bark following the method described by Suryanarayanan et al. (1998). The outer bark of the tree at breast height was scraped using a knife and a section of the inner bark (5 cm × 5 cm) was collected into a plastic bag, sealed and transported to the laboratory. The inner bark sample was then cut into small pieces (approximately 0.5 cm^2). The segments were surface sterilized by dipping them in 70% ethanol for 1 min, sodium hypochlorite (2% available chlorine) for 90 s, and sterile distilled water for 5 min. The surface sterilized explants were then placed on an aqueous agar (HiMedia, Mumbai) and maintained at 28 ± 2°C until fungal growth began at the cut ends. A similar procedure, but without surface sterilization, was used as a negative control to check for surface contaminated fungi. As and when the hyphae emerged and grew to cover a reasonable mat area, single hyphal tips were isolated and subcultured on a mycologically rich medium, potato dextrose agar (PDA) and incubated at 28 ± 2°C and

brought to pure culture. The pure culture thus obtained was assigned codes and preserved. The isolates were stored in their vegetative form as slants and in 50% (v/v) glycerol at −80°C. For rohitukine extraction the pure culture isolate was inoculated in 250 ml flask containing 50 ml of pre-sterilized Potato dextrose broth. Flasks were agitated at 200 rpm on a rotary shaker at 28 ± 2°C for 4 days.

Fungal identification using spore morphology and *ITS rDNA* sequencing

Fungal isolate obtained was induced to sporulate by inoculating mycelia on to autoclaved green gram seeds (Shweta et al. 2010). Microscopic slides were prepared, stained using lacto phenol cotton blue and examined under light microscope (Olympus, USA). *ITS* regions of the rRNA gene are often highly variable with respect to nucleotide composition and this characteristic can be used to distinguish both morphologically distinct fungal species and strains of the same fungal species (Guo et al. 2001).

The *ITS* primers make use of conserved regions of 18S, 5.8S and 28S rRNA genes to amplify the non-coding regions between them. ITS1 and ITS4 were used to amplify the different regions of internal transcribed spacer region (White et al. 1990). The fungus was genotyped using the *ITS rDNA* regions. Endophytic fungus isolated from *D. binectariferum* was inoculated into 50 ml potato dextrose broth and incubated for 6 days at 28 ± 2°C. The mycelial mat was harvested, washed with distilled water and air dried for 3 h. Genomic DNA was isolated from the mycelial mass using the method described by Vainio et al. (1998). Amplification of *ITS rDNA* and sequencing was performed using the procedures described in Shweta et al. (2010) and Gurudatt et al. (2010).

Extraction of rohitukine from fungal isolates and HPLC analysis

Rohitukine was extracted from the fungal mycelia following the protocol described by Mohana Kumara et al. (2010). Mycelia and broth was separated by filtration. The mycelia of endophytic fungus was dried to constant moisture content at 60 ^{o} C for 96 h in an hot air oven. The dried mycelia was thoroughly crushed to fine powder in pestle and mortar. About 0.1 g of fine tissue powder sample was taken in 15 ml vial and extracted with 5 ml volume of L.R. grade methanol (99.7%). After fastening the cap, the tissue was extracted in hot water bath at 60°C for 3 h with constant shaking. After cooling to room temperature, the extract was centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.22 µm filter (Tarsons, India) and analyzed for rohitukine. HPLC analysis was performed on a Shimadzu LC20AT instrument. LC separations included reverse phase C18 column (250 × 4.6 mm, 0.5 µm), 254 nm as detection wavelength, acetonitrile and 0.01 M KH_2PO_4 as mobile phase and separated on a gradient mode.

LC–HRMS/MS analysis

The compound was identified by LC–HRMS and LC–HRMS^2 (LTQ-Orbitrap spectrometer, Thermo Fisher Scientific, Bremen, Germany). Retention times and exact masses were consistent with the reference standards (Mohana Kumara et al. 2010). Separation was performed on a Surveyor-LC HPLC system (Thermo Fisher Scientific, Bremen, Germany) with a quaternary, low-pressure mixing pump with vacuum degassing, an autosampler with temperature-controlled tray (T = 10°C), and a column oven (30°C). Injection volume was 5 µl. For mass spectrometric detection, nitrogen was used as sheath gas (6 arbitrary units). The separations were performed by using a Synergie Fusion RP column (5 µm, 150 × 2 mm) (Phenomenex, aschaffenburg, Germany) with a H_2O (+10 mM ammonia acetate) (A)/methanol (+0.1% HCOOH) (B) gradient (flow rate 0.25 ml/min). Samples were analysed by using a gradient program as follows: 90% A isocratic for 3 min, linear gradient to 100% B within 23 min. After 100% B isocratic for 8 min, the system was returned to its initial condition (90% A) and equilibrated for 7 min. The spectrometer was operated in positive mode (mass range: 150–1000) with mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz. Automatic gain control was applied to provide high-accuracy mass measurements within 2 ppm deviation using one internal lock mass; *m/z* 391.284290; bis-(2-ethyl-hexyl) phthalate. The compound was monitored at their exact mass and the product ion spectra were produced by collision energy of 35.

Cytotoxicity studies

Cell culture and maintenance

Breast cancer cell line MCF-7 and colon cancer HCT-116 were obtained from DTP NCI. The cells were maintained in RPMI medium supplemented with 10% fetal bovine serum.

Analysis of chromatin condensation

Chromatin condensation analysis was done by subjecting cells for Hoechst 33342 staining. The cells were grown on 96 well plates, after indicated treatment with extract as well as standard. Cells were stained with 0.5 μg/ml of Hoechst 33342 for 10 min and observed under UV filter sets using Nikon Epi-fluorescent microscope TE2000E. Number of cells with apoptotic-condensed nuclei were scored and expressed as per cent cell mortality for each treatment at least by two investigators.

Results and discussion

Endophytic fungus was isolated from inner stem bark of *D. binectariferum* and brought to broth culture. Molecular analysis of the fungus based on *ITS rDNA* showed a 99% match with *Fusarium proliferatum* (GenBank accession number GQ24905) and *Gibberella fujikuroi* (Gen Bank accession number EU214560). However, since the fungus produced only conidia and not ascospores, the isolate was inferred to be *Fusarium proliferatum* (Leslie et al. 2006).

The DNA sequence of the strain has been deposited in the NCBI GenBank (accession number HM245296). Representative sample of the fungal culture has been deposited at the MTCC (Microbial Type Culture Collection and GenBank), Chandigarh, India (accession number MTCC 9690).

Both the fungal extract and the broth in which the fungus was cultured were subjected to LC–HRMS, LC–MS/MS and LC–HRMS/MS analysis and compared with those of the reference standard and the plant material; however since the broth did not yield any signal, further analysis was restricted to only the fungal extract. High resolution LC–MS/MS analysis of the fungal extract showed a peak eluting at retention time of 16.98 min, with a mass of *m/z* 306.133 $[M+H]^+$ and subsequent MS analysis of the parent ion resulted in the following molecular ions; *m/z* 288.1226 $[M–H_2O]^+$, *m/z* 245.0804 $[M–C_2H_5ON]^+$, *m/z* 221.1122 $[M–C_4H_4O_2]^+$, *m/z* 114.0911 $[M–C_{10}H_7O_4]^+$ (Fig. 2a–c). Masses of the fragments calculated based on the chemical formulae matched well with the observed masses of the fragments in LC–HRMS of standard rohitukine with mass deviations of less than around 1.32 ppm (calcd *m/z* 288.124, obsd 288.1226, calcd *m/z* 254.0814, obsd *m/z* 245.0804, calcd *m/z* 221.105 obsd *m/z* 221.1122, calcd *m/z* 114.092, obsd *m/z* 114.0911), suggesting unequivocal structural match based on fragmentation pathway (Fig. 2d). Finally, LC–MS spectrum of the rohitukine peak in the mycelial extract matched well with the MS spectrum of rohitukine in stem bark sample of the host, which in turn matched with the MS spectrum of the standard rohitukine. These results clearly indicated that the fungus produces rohitukine in culture under in vitro conditions independent of the host association.

For quantification of rohitukine in the host and fungal extract using LC–MS/MS analysis, the molecular ion with the highest intensity in the MS spectrum (*m/z* 245.08057) was chosen. LC–MS/MS analysis of stem bark sample of *D. binectariferum* showed 1.49 mg of rohitukine in 100 mg of dried tissue, which is approximately 1.49% rohitukine by dry weight. In the fungus, it was 186 μg/100 g dry mycelial weight. The amount of rohitukine declined with sub-culture of the fungus; for instance from the first generation sub culture with 186 μg/100 g the rohitukine decreased to 120 μg/100 g in the second generation and 50 μg/100 g in the third generation.

The crude methanol extract of *F. proliferatum* was found to be cytotoxic against HCT-116 and MCF-7 human cancer cell lines (IC_{50} = 10 μg/ml for both cancer cell lines; compared to an IC_{50} of 1 μg/ml using the positive control, camptothecin). The fungal extract showed a better anti-cancer activity than the one reported by our earlier study for pure rohitukine. It is likely that the presence of other compounds in the crude extract might be synergizing the anti-cancer activity of rohitukine.

In recent years considerable interest has been evinced in endophytic fungi as a possible alternative source of plant secondary metabolites (Strobel and Daisy 2003; Aly et al. 2010; Zhao et al. 2011). Novel

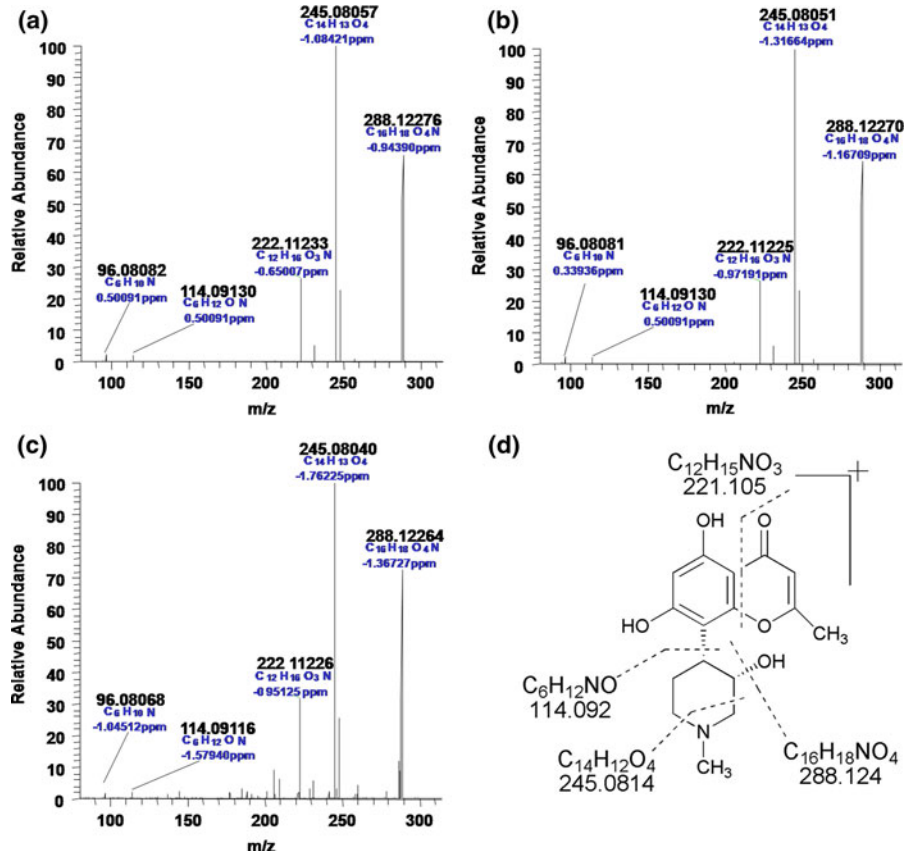

Fig. 2 LC–HRMS spectra of **a** reference rohitukine, **b** *F. proliferatum* (MTCC-9690) mycelia extract, **c** stem bark extract of host plant *D. binectariferum*, and **d** ion fragmentation of rohitukine

plant secondary metabolites including taxol, camptothecin, hypercin and a host of other metabolites have been shown to be produced by endophytic fungi isolated from plants producing the metabolites (Aly et al. 2011; Shweta et al. 2010; Stierle et al. 1993; Puri et al. 2005; Amna et al. 2006; Kusari et al. 2008, 2009). This study reports for the first time the production of rohitukine by the endophytic fungus, *F. proliferatum*. The fungus retains the ability to produce the chromane alkaloid for 2 or 3 serial subculture generations before showing signs of attenuation, a feature now reported for several other endophytic fungi (Gurudatt et al. 2010; Kusari et al. 2009; Priti et al. 2009). While the present study raises the hope of exploiting the endophytic fungi as an alternative source of rohitukine, clearly, more studies are required to optimize the growth as well as conditions that would predispose the fungus in producing the metabolite without perceptible attenuation. The latter would require understanding of the primary biosynthetic pathway of rohitukine as well as in identifying the elicitors of rohitukine.

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