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Shikimic Acid Production by *Fusarium decemcellulare*, An Endophytic Fungus Isolated from *Flacourtia inermis* Fruits

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Abstract: Microorganisms have produced many of the most potent agents against bacterial and fungal infections, cancer and high cholesterol etc. Studies on secondary metabolites originating from endophytic fungi isolated in Sri Lanka are relatively less studied. Hence we studied the chemistry and bioactivity of an endophytic fungus *Fusarium decemcellulare* isolated from the fruits of *Flacourtia inermis*. A pure culture of fungus was fermented in potato dextrose broth (PDB) for 28 days. The fermentation medium and mycelium was extracted in to ethyl acetate (EtOAc) and methanol (MeOH). These extracts did not show good activity for bioassays against antioxidant, antifungal, α-amylase inhibitory, brine shrimp lethality and phytotoxicity. Chromatographic separation of the EtOAc extract furnished ergosterol while MeOH extract furnished shikimic acid. Isolation of endophytic fungus *Fusarium decemcellulare* from the fruits of *F. inermis* and the production of shikimic acid from *F. decemcellulare* are reported for the first time.

Key words: *Flacourtia inermis*, *Fusarium decemcellulare,* endophytic fungi, fermentation, shikimic acid.

Introduction

Mycological studies have been gained more attention after the discovery of broad spectrum antibiotic penicillin G. Fungi have been the source of many medicines used to safeguard human health. These include the antibiotics penicillin, erythromycin, streptomycin; anti-cancer drugs daunorubicin, doxorubicin and taxol; drugs such as the statins (e.g., lovastatin and mevastatin) to lower cholesterol and indicate the ability of fungi to produce small drug like molecules as secondary metabolites [7]. The fungicidal molecules pyrrolnitrin [3] and the strobilurins [4] isolated from fungi, are used widely in crop protection. Endophytic fungi are the microorganisms that reside inside the plant tissues without causing any external symptoms or any negative effects to the hosts [29]. It is also reported that plants occupied in extreme as well as temperate conditions harbors millions of endophytic organisms. So far only a very small percentage of them are studied in respect to their metabolites and bioactivities [29]. They have been known to produce structurally rare, diverse and unique metabolites which show promising biological activities, which can be used medicinally and agriculturally [13]. In a continuation of our studies on the search for bioactive compounds from Sri Lankan flora, secondary metabolites produced by the endophytic fungus *Fusarium decem-cellulare* (Class: Ascomycota & Order: Hypo-creales) isolated from the fruits of *Flacourtia inermis* Roxb. of the family Flacourtiaceae have been investigated. *F. inermis* is a tree of moderate size, growing in Sri Lanka. Its red-coloured fruits are edible

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and very popular in Sri Lanka. *F. inermis* is a good source of biologically active chemical constituents. Antifungal activities of the acetone extract of *F. inermis* against some human opportunistic pathogens [9], antibacterial activity [10], antiprotozoal activity [11], and antibiotic activity [12] of 2,3-dihydroxybenzoic acid isolated from the fruits of *F. inermis* have been reported. We have recently reported the occurrence of phenolic metabolites including several chlorogenic acid derivatives with high antioxidant activity [17], profiling of phenolics by LC-MS studies [2] and the α-amylase, α-glucosidase and lipase enzyme inhibitors [1] from the fruits of *F. inermis*. In this paper we report the production of shikimic acid from the endophytic fungi *F. decemcellulare* isolated from the fruits of *F. inermis*.

Materials and methods

General

VWR ultrasound cleaner, USC 1700D, was used for extraction. Analytical TLC was performed on Merck Kieselgel $60F_{254}$ aluminum plates. TLC spots were visualized under UV 254 nm and spraying with anisaldehyde reagent followed by heating. Column chromatography (CC) was performed on silica - Merck Art No. 7734 or 9385 and gel chromatography was on Sephadex LH-20 - Fluka Art No. 20100. ^{1}H and ^{13}CNMR spectra were recorded on a Bruker DRX500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer in $CDCl_3$ or CD_3OD solution. Thermo Scientific Multiskan GO Microplate spectrometer was used to the measure the UV absorptions.

Isolation and identification of the endophytic fungus

Fresh fruits of *F. inermis* were collected from the Central Province of Sri Lanka in July 2014. Endophytic fungus was isolated as described in our previous papers [24, 25, 31]. The reddish coloured endophytic fungus was identified as *Fusarium decemcellular* through molecular means using internal transcribed spacer (ITS) region of rDNA gene. DNA was extracted using Promega, genomic Wizard DNA purification kit A1120. The target ITS regions including 5.8S gene was amplified by polymerase chain reaction (PCR) using universal eukaryotic primers ITS1 and ITS4. Amplified DNA was sequenced and analyzed by BLAST sequences available at NCBI Gene Bank. These experiments were performed by the GeneTech Institute, Sri Lanka. BLAST results 98 % matched with *Fusarium decemcellulare* strain NZD-mf167 (GenBank Accession No. KM 277988.1). *F. decemcellulare* strain (IFS/MQ/EFLF-1) and the photographic evidence of the fruits of *F. inermis* are deposited at the National Institute of Fundamental Studies.

Fermentation, extraction, isolation of compounds and their bioactivity

Pure culture of *F. decemcellulare* was grown on potato dextrose agar nutrient media containing petri dishes for one week and inoculated to forty Erlenmeyer flasks (1L) containing 400 mL of potato dextrose broth (PDB) media. Flasks were allowed to stand at room temperature for 10 days, while shaking every other day to complete a period of 28 days. After completion of the fermentation period, mycelium biomass accumulated in the flasks was separated by suction filtration. The culture broth (filtrate) was successively extracted with EtOAc to get crude EtOAc extract (110 mg). The residual mycelium was sequentially extracted with EtOAc to give other crude EtOAc extract (210 mg) and MeOH to give crude MeOH extract (2.79 g). Solvents were evaporated *in vacuo*. Since TLC analysis indicates the presence of similar compounds in both EtOAc extracts they were combined. Combined EtOAc and MeOH crude extracts were screened for antifungal (against *Cladosporium cladosporioides* with TLC bioautography method) [14], antioxidant (DPPH radical scavenging activity) [1], brine shrimp lethality [19], phytotoxicity (lettuce seed germination bioassay) [23] and α-amylase enzyme inhibitory bioassays [1].

The EtOAc extract (200 mg) was fractionated by normal phase silica CC using *n*-hexane as column equilibrating solvent. After application of the sample, solvents of increasing polarities from 100 % hexane to dichloromethane followed by MeOH in dichloromethane (increasing order of polarity) and finally 100 % MeOH were used for elution. The column fractions were analyzed by using TLC with UV (254 and 365 nm) and by spraying anisaldehyde reagent. Similarly, MeOH extract

was subjected to silica gel CC with CH_2Cl_2 - MeOH, and checked with TLC under UV light (developing solvent: $CHCl_3$: MeOH: H_2O - 7:3:1). The UV active fraction was subjected to Sephadex LH-20 (100 % MeOH and 30 % $CHCl_3$:MeOH) and final purification was carried out by HPLC (C_{18} RP-ODS 25 x 1 cm column, UV detection at 254 nm, with 60 % MeOH-H_2O as eluent with a flow rate of 1 mL/min). The structures of the isolates were identified by the comparison of the ^1H and ^{13}C NMR, data with literature values and confirmed by co-TLC with authentic samples.

Results and discussion

An endophytic fungus with reddish colored, cottony mass and concentric ring shaped white floc with pink pigmentation, was isolated from the fruits of *F. inermis* by following the standard methods. The fermented fungal medium and mycelium were extracted in to EtOAc and MeOH. EtOAc extract and the MeOH extracts were subjected to bioassays against antioxidant, antifungal, α-amylase inhibitory, brine shrimp lethality and phytotoxicity. None of the crude extracts showed good activity for these assays. Chromatographic separation of the EtOAc extract and the column fractions eluted from 100 % dichloromethane gave single spot in TLC with anisaldehyde reagent. This was identified as ergosterol (68 mg) [30]. While silica gel CC separation of the MeOH extract which gave UV active spots on TLC and further purification by HPLC furnished shikimic acid (1, 393 mg) (figure 1). These compounds were identified as ergosterol and shikimic acid (1) by comparison of the ^1H and ^{13}C NMR data with the reported values [30, 32] and TLC comparison with authentic samples. 1H and ^{13}C NMR spectra of shikimic acid (1) are shown in figures 2 and 3.

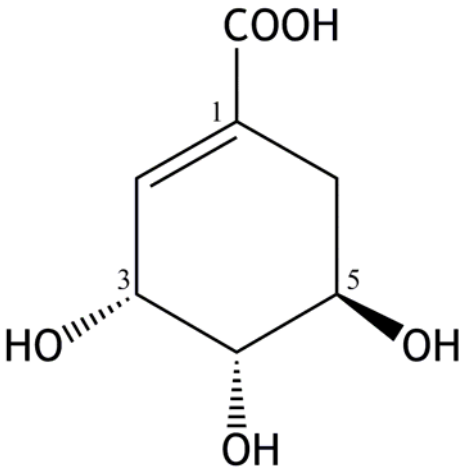

Fig. 1. Structure of shikimic acid (1)

Shikimic acid (1): colorless sticky solid; ^1H NMR (500 MHz, CD_3OD): δ 6.75 (1H, *dt*, *J* = 3.6, 1.7 Hz, H-2), 4.35 (1H, *ddt*, *J* = 4.2, 3.6, 1.7 Hz, H-3), 3.97 (1H, *ddd*, J = 7.6, 5.9, 5.0 Hz, H-5), 3.65 (1H, *dd*, *J* =7.6, 4.2Hz, H-4), 2.70 (1H, *ddt*, *J* = 18.2, 5.0, 1.7 Hz, H_a-6), 2.18 (1H, *ddt*, *J* = 18.2, 5.9, 1.7 Hz H_b-6); ^{13}C NMR (125 MHz,CD_3OD): δ 170.8 (COOH), 137.9 (C-2), 131.7 (C-1), 72.9 (C-3), 68.4 (C-4), 67.4 (C-5), 32.0 (C-6); ESI (-) : *m/z* 173[M-H]$^-$.

Ergosterol is the most abundant fungal sterol [33] which is the major end product of the sterol biosynthetic pathway and is responsible for the membrane fluidity and permeability of fungi especially in yeasts [16]. Natural shikimic acid was initially reported from *Illicium religiosum* in 1885. It is the basic element of aromatic compounds and the benzene ring is formed through shikimate pathway [8]. Shikimic acid is responsible in the biosynthesis of aromatic amino acids (L-phenylalanine, L-tyrosine, L-tryptophan), lignin, flavonoids, tannins, folic acid, vitamins and most of the alkaloids present in plants and microorganisms. Shikimic acid is used to manufacture of the influenza drug Tamiflu (Oseltamir phosphate) which is effective against H5NI, a strain of the influenza virus known as bird flu [5]. Nevertheless shikimic acid is used and being used in medicine and agriculture. There are reports on synthesis of shikimic acid derivatives that are widely used in the preparation of chemotherapeutic drugs, anticoagulants which are capable of reduce blood coagulability and as antithrombiotics [15,21,34]. Agriculturally important herbicide glyphosate (Roundup®) can significantly inhibit the formation of aromatic amino acids which is produced by shikimic acid pathway. It can lead to high productivity of agricultural crops [22].

A series of new cyclic pentapeptides, which are active for both gram-positive and gram-negative bacteria have been reported from *F. decemcellulare* from a Chinese medicinal plant *Mahonia fortune*. Also fusaristatin A displayed

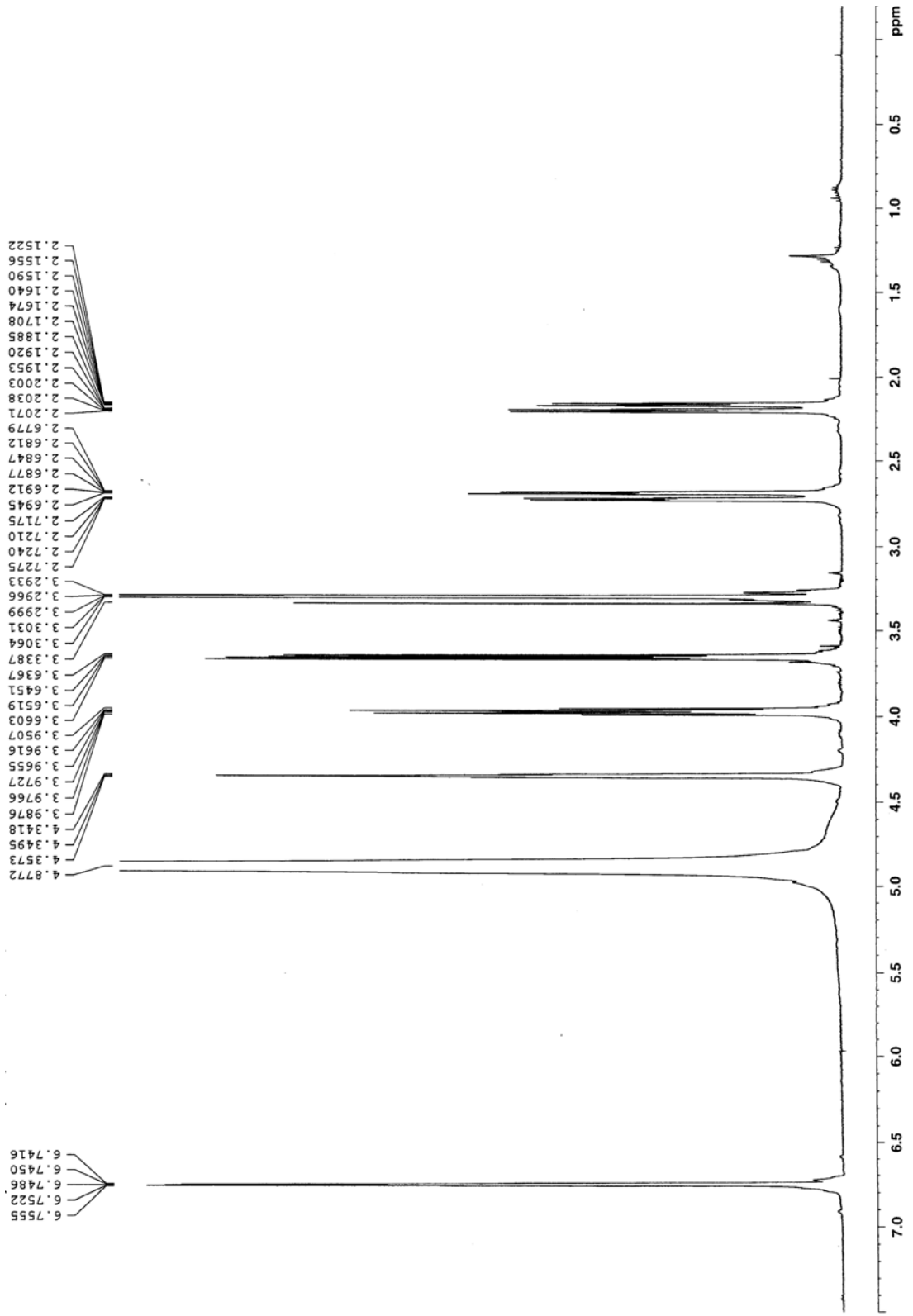

Figure 2. ^{1}H NMR spectrum (500 MHz, CD_3OD) of shikimic acid (1).

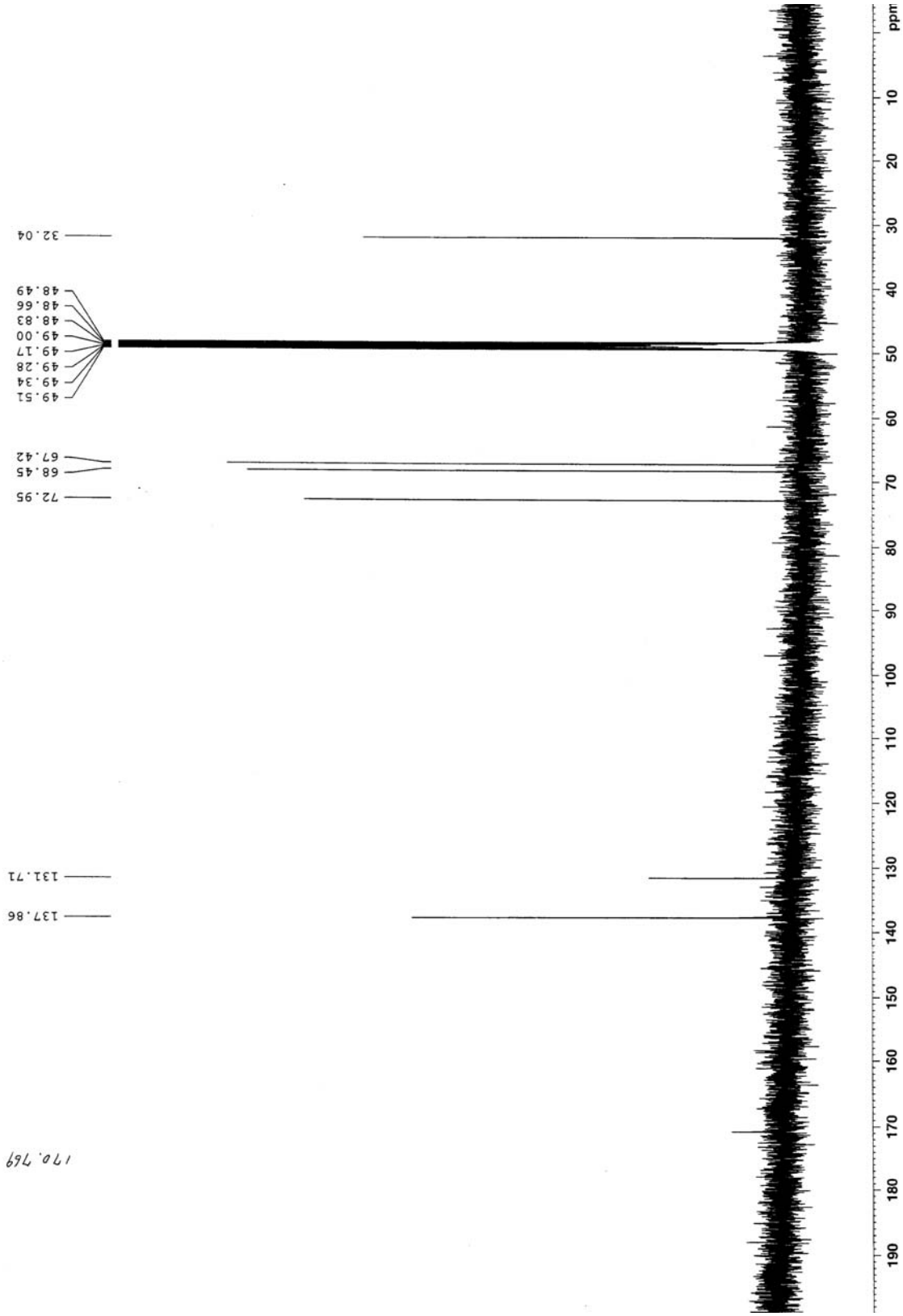

Figure 3. ^{13}C NMR spectrum (125 MHz, CD_3OD) of shikimic acid (1).

allelopathic effect on *Glomerella acutata* [20]. *F. decemcellulare* has been previously reported to cause pathogenicity, and inflorescence wilt, vascular necrosis, and flower necrosis in agriculturally important fruit crops in many countries [26].

Shikimic acid plays an important role in medicine and agriculture as described above. Therefore natural shikimic acid producing plant sources are diminishing quickly [8]. The current supply of influenza drug Tamiflu is able to cover about 2 % of the world population. Therefore alternative sources are welcome in this regard. This is the first report of the isolation of shikimic acid from an endophytic fungus *Fusarium decemcellulare* isolated from the fruits of *F. inermis*, although it has been previously isolated from limited fungal sources such as *Trichoderma ovalisporum* [6] and *Penicillium griseofulvum* [28]. Therefore, *Fusarium decemcellulare* represents additional fungal source of shikimic acid. These fungal strains could be a source for metabolic engineering to produce a higher amount of shikimic acid. Indeed fermentative production of shikimic acid by a metabolic engineered *Escherichia coli* has been reported [18]. It is also reported that optimization of culture conditions of a bacterium *Citrobacter* sp. resulted in an increased production of shikimic acid [27].

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Conflict of interests

The authors declare that they have no conflict of interest.

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