

Toxic, but beneficial compounds from endophytic fungi of *Carica papaya*

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Abstract

Fungi remain a promising source of novel biologically active compounds with potentials in drug discovery and development. This study was aimed at investigating the secondary metabolites from endophytic *Fusarium equiseti* and *Epicoccum sorghinum* associated with leaves of *Carica papaya* collected from Agulu, Anambra State, Nigeria. Isolation of the endophytic fungi, taxonomic identification, fermentation, extraction and isolation of fungal secondary metabolites were carried out using standard procedures. Chromatographic separation and spectroscopic analyses of the fungal secondary metabolites yielded three toxigenic compounds - equisetin and its epimer 5'-epiequisetin from *F. equiseti*, and tenuazonic acid from *E. sorghinum*. These compounds are known to possess several beneficial biological properties that can be explored for pharmaceutical, agricultural or industrial purposes.

Keywords: Endophytic fungi, *Carica papaya*, *Fusarium equiseti*, *Epicoccum sorghinum*, secondary metabolites

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Introduction

Carica papaya (Caricaceae) also known as papaya, papaw, or pawpaw is a herbaceous plant indigenous to most tropical regions of the world, including Nigeria (1, 2). Traditionally, the plant is used for the treatment of a wide range of ailments like wounds, ulcers, menstrual irregularities, thermal traumas, dyspepsia, diarrhea, bleeding, haemorrhoids, whooping cough, dysentery, skin diseases and impotence (1-3).

The plant has been reported to show anti-inflammatory (4,5), wound healing (2, 3), anti-fertility (6, 7), anthelmintic (8), anticancer (9, 10), antimicrobial (11-14), anti-hypertensive (15), immunomodulatory (16), antimalarial (17), and diuretic (18, 19) activities.

Fungi are known to be predominantly associated with *C. papaya* diseases and spoilage. These include fungi of the genera *Colletotrichum*, *Fusarium*, *Aspergillus*, *Phoma*, *Cladosporium*, *Phomopsis*, *Penicillium*, *Rhizopus*, etc (20-24). Notwithstanding their ability to cause diseases, some fungi exist asymptotically and symbiotically as endophytes in the tissues of the *C. papaya* without causing disease symptoms. Reports have been made of endophytes associated with *C. papaya* with a view of evaluating the potentials of the plant's endophytic populations in bioprospecting for novel bioactive molecules. Bacterial and fungal endophytes from *C. papaya* capable of producing important extracellular enzymes with biotechnological potentials have been reported (25, 26). Secondary metabolites from a fungus isolated from the leaves of *C. papaya* showed excellent cytotoxic activity against mouse lymphoma cells (27). Wang *et al.* (28) reported the isolation several bioactive compounds from an endophytic *Aspergillus aculeatus* associated with *C. papaya*. Some of the isolated compounds, asperdichrome, RF 3192C, secalonic acids D and F, showed significant cytotoxic activities (28).

In this present study, two endophytic fungi were isolated from healthy leaves of *C. papaya*, and the fungi were identified to be toxigenic species in the genera *Fusarium* and *Epicoccum*. The fungi were chemically investigated for their secondary metabolites.

Materials and Methods

Collection of plant materials

Fresh leaves of *C. papaya* were collected from Agulu, Anambra State, Nigeria. The plant material was authenticated and a voucher specimen (PCG474/A/023) deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Anambra State, Nigeria.

Isolation and identification of endophytic fungi

Isolation of endophytic fungi from the leaves of *C. papaya* was carried out as described by Eze *et al.* (29). The taxonomic identification of the endophytic fungi carried out using DNA amplification and sequencing of the fungal ITS region (30). Two endophytic fungi were isolated and were identified as *Fusarium equiseti* and *Epicoccum sorghinum*. Their DNA sequence data were deposited in the NCBI database (GenBank) with accession numbers KX137847 and MH269246 respectively.

Fermentation, extraction, fractionation and isolation of metabolites

Solid state fermentation was carried out by growing each fungus in 1 L Erlenmeyer flask containing sterile solid rice medium (100 g of rice + 100 mL of distilled water, autoclaved at 121 °C at 15 psi for 1 h) under static conditions at 22 °C for 14 days. After fermentation, the fungal secondary metabolites were extracted with ethyl acetate (EtOAc) and then concentrated under reduced pressure. A weight of 1.5 g of *F. equiseti*'s crude extract (code name: CPL1) was subjected to vacuum liquid chromatography (VLC) on silica gel 60, and stepwise gradient elution of the extract was carried out using dichloromethane:methanol (DCM:MeOH). A dry weight (67.3 mg) of the VLC fraction (DCM 80%:20% MeOH) containing the metabolites of interest was further separated on Sephadex LH-20 with DCM:MeOH (1:1 (v/v)) as the mobile phase. Also, 330 mg of *E. sorghinum*'s crude extract (code name: CP1) was separated on Sephadex LH-20 using DCM:MeOH (1:1 (v/v)) as the mobile phase. The metabolites-containing fractions were further purified using semi-preparative HPLC to isolate the pure compounds. Compounds 1 (4.4 mg) and 2 (1.9 mg) were isolated from *F. equiseti*'s extract; while compound 3 (3.8 mg) was isolated from the extract of *E. sorghinum*. Analytical HPLC was used to evaluate the purity of the isolated compounds.

General procedures

NMR measurements of the isolated compounds were carried out in deuterated chloroform (compounds 1 and 2) and deuterated methanol (compound 3) using a Bruker Avance DMX 600 spectrometer (Bruker BioSpin, Germany). The

NMR spectra were referenced relative to the residual solvent signals. For mass spectral analysis, LC-MS measurements were carried out using a Finnigan LCQ Deca XP LC-MS System (Thermo Electron, Germany); while HR-ESIMS was measured with a UHR-QTOF maXis 4G (Bruker Daltonik, Germany) mass spectrometer. Analytical HPLC analysis was carried out using a Dionex P580 system coupled to a P580A LPG pump and a photodiode array detector (UVD340s, Dionex Softron, Germany). The HPLC instrument consists of a separation column (125 x 4 mm) prefilled with Eurosphere-10 C18 (Knauer, Germany) with MeOH-H₂O mixtures as the gradient solvent system. Semi-preparative HPLC was performed using a Merck-Hitachi HPLC System comprising of a UV detector (L-7400), pump (L-7100), and a Eurosphere column (100 C18, 300 x 8 mm, Knauer, Germany). Gradient MeOH-H₂O mixtures were used as the mobile phase at a flow rate of 5.0 mL/min. Vacuum liquid and open column chromatography were carried out using Silica gel 60 (70–230 mesh, Merck, Germany) and Sephadex LH-20 (Sigma-Aldrich, Germany) respectively. Pre-coated TLC plates (silica gel 60 F254, 20x 20 cm, 0.25 mm thick, Merck, Germany) were used to monitor fractions under UV detection (Camag UV cabinet, Germany) at 254 and 366 nm. The optical rotation of compounds 1 and 2 were measured in MeOH using a P-2000 polarimeter (Jasco, Germany). Distilled solvents were used for column chromatography and spectral-grade solvents were used for spectroscopic measurements.

Results

Chromatographic separation and spectroscopic analyses of the fungal secondary metabolites resulted in the isolation of three compounds (compounds 1 and 2 from *F. equiseti*; and 3 from *E. sorghinum*). HPLC chromatograms of the fungal crude extracts and UV-spectra of the detected compounds (showing their peaks and retention times (RT)) are presented in Figs. 1 and 2. Also, structures of the isolated compounds are shown in Fig. 3.

Compound 1

Compound 1 showed UV maxima at 208.6, 234.9 and 297.0 nm. LC-MS analysis revealed pseudomolecular ions at m/z 374.1 (M+H)⁺ and 372.5 (M-H)⁻ upon positive and negative ionization respectively, thus revealing a molecular weight of 373 g/mol. Analysis of the ¹H-NMR data (CDCl₃, 600 Hz) suggested the molecular formula C₂₂H₃₁NO₄. The optical rotation $[\alpha]_D^{20} [\alpha]_D^{20} = -237.6^\circ$ (c 0.2, MeOH) was recorded. Based on the spectral data, the compound was identified as equisetin (IUPAC name: (3E,5S)-3-(((1S,2R,4aS,6R,8aR)-1,6-dimethyl-2-((E)-prop-1-enyl)-4a,5,6,7,8a-hexahydro-2H-naphthalen-1-yl)-hydroxymethylidene)-5-(hydroxymethyl)-1-methylpyrrolidine-2,4-dione). This compound was originally described from *Fusarium equiseti* (31-34).

Compound 2

Compound 2 also showed UV maxima at 203.2, 234.4 and 295.5 nm. The LC-MS displayed pseudomolecular ions at m/z 374.1 (M+H)⁺ and 372.5 (M-H)⁻ upon positive and negative ionization

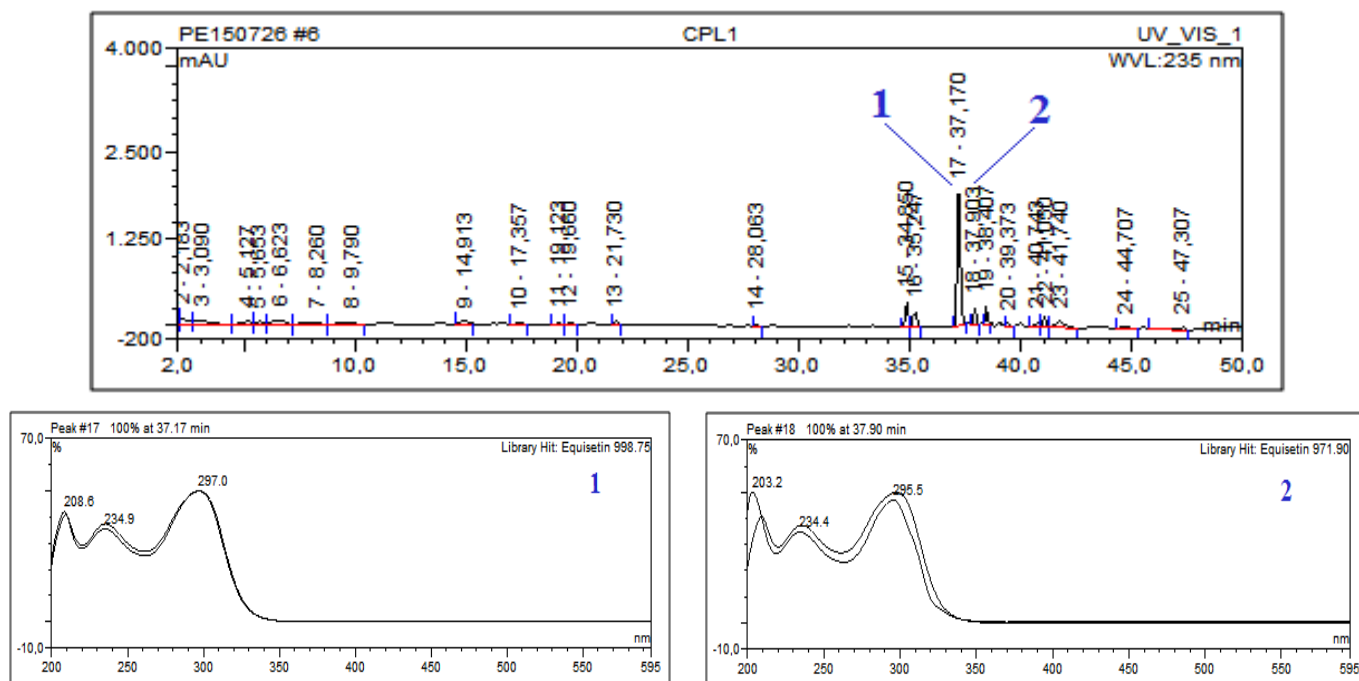


Figure 1. HPLC Chromatogram of *Fusarium equiseti*'s crude extract (CPL1); and UV-spectra of compounds **1** (Peak 17, RT 37.17 min) and **2** (Peak 18, RT 37.90 min).

respectively, thus revealing a molecular weight of 373 g/mol. Interpretation of the $^1\text{H-NMR}$ data (CDCl_3 , 600 Hz) suggested the molecular formula $\text{C}_{22}\text{H}_{31}\text{NO}_4$. The optical rotation $[\alpha]_D^{20} = -196.4^\circ$ (c 0.2, MeOH) was recorded. The compound was thus identified as 5'-epi-equisetin, an epimer of equisetin (IUPAC name: (3E,5R)-3-(((1S,2R,4aS,6R,8aR)-1,6-dimethyl-2-((E)-prop-1-enyl)-4a,5,6,7,8,8a-hexahydro-2H-naphthalen-1-yl)-hydroxy-methylene)-5-(hydroxymethyl)-1-methyl-

pyrrolidine-2,4-dione). Results of the spectroscopic analysis of compound **2** are confirmed by the report of Phillips *et al.* (33).

Compound 3

Compound **3** displayed UV maxima at 218.0 and 277.3 nm. HR-ESIMS showed pseudomolecular ions at m/z 196.3 (M^-) upon negative ionization, revealing a molecular weight 197 g/mol Interpretation of the $^1\text{H-NMR}$ data (MeOD, 600 Hz)

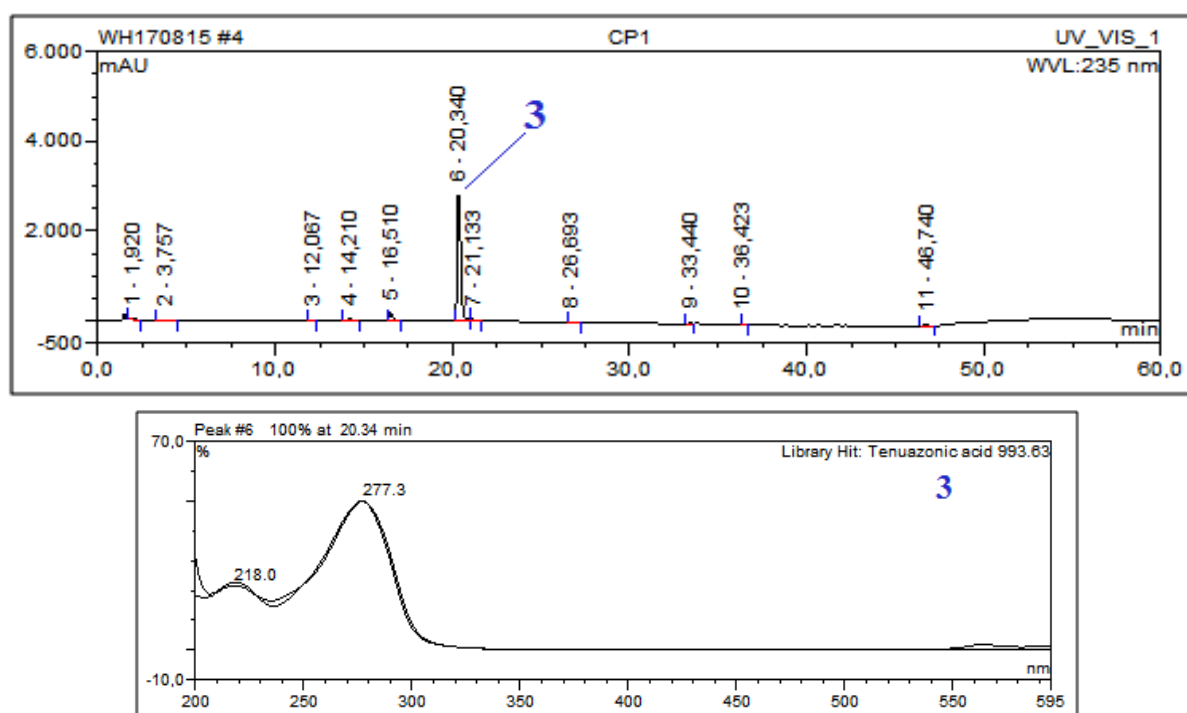


Figure 2. HPLC Chromatogram of *Epicoccum sorghinum*'s crude extract (CP1); and UV-spectrum of compound **3** (Peak 6, RT 20.34 min).

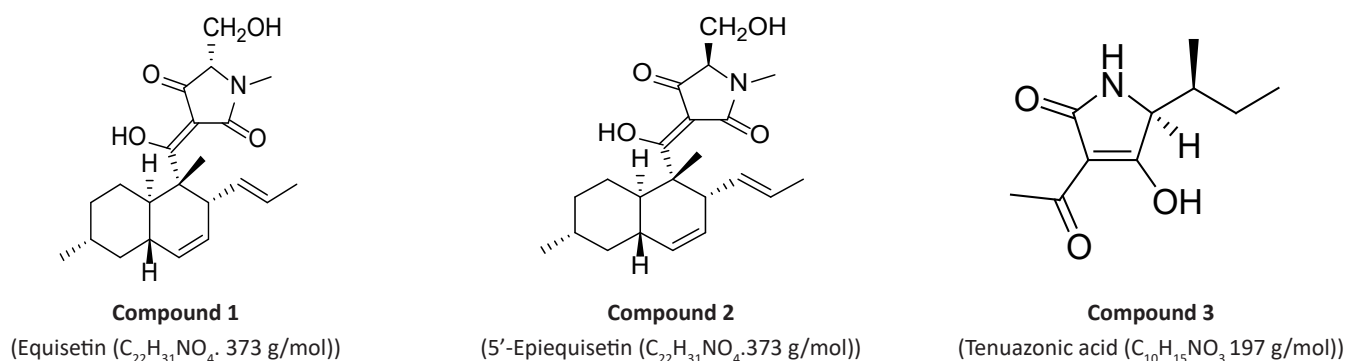


Figure 3. Structures of isolated compounds.

suggested the molecular formula C₁₀H₁₅NO₃. Compound 3 was thus elucidated as a tetramic acid derivative - tenuazonic acid (IUPAC name: (5*S*)-3-acetyl-5-((2*S*)-butan-2-yl)-4-hydroxy-2,5-dihydro-1*H*-pyrrol-2-one). Results of the spectroscopic analysis of the isolated compound are confirmed by the report of Davis *et al.* (35).

Discussion

Fusarium species are important plant pathogens that are widely distributed throughout the world. They can exist as opportunistic colonizers of plants and agricultural commodities, or as saprophytes on debris and plant materials (32,36). Several species of *Fusarium* cause a broad range of plant diseases, including vascular wilt, seedling blight, fruit, root or stem rot, and cereal ear rot. Some *Fusarium* strains can synthesize several mycotoxins like the trichothecenes, zearalenones, and fumonisins. Moniliformin, beauvericin, and fusaproliferin have also been synthesized by the fungal strains (36). Among the endophytes from medicinal plants, many studies have revealed *Fusarium* sp. as the most prevalent species and a potent source of biologically active compounds (37, 38). Many studies have been carried out on metabolites production from several *Fusarium* species especially *F. oxysporum* and *F. solani* (39-42).

Fusarium equiseti (Nectriaceae) (teleomorph: *Gibberella intricans*) is a toxigenic species and a soil inhabitant known to cause disease in several plant species. The fungus can infect seeds, fruits, roots and tubers of several crop plants from diverse climatic regions (43, 44). *F. equiseti* is capable of expressing a vast range of phytotoxic and cytotoxic metabolites (45, 46).

The *Fusarium* toxin equisetin, an N-methylserine-derived acyl tetramic acid, was first isolated in 1974 from the white mold *F. equiseti* (46). It is known to possess an impressive biological activity profile including antibiotic and HIV inhibitory activities, as well as cytotoxic and mammalian DNA binding properties (34). Equisetin exists as two epimers - equisetin (EQ) and epiequisetin (epi-EQ) (47). EQ and its C-5' epimer epi-EQ have been reported to be majorly produced by cultures of *F. equiseti* (34, 46-48). However, it has also been isolated from other *Fusarium* species such as *F. pallidoroseum* (47) and *F. heterosporum* (31).

EQ demonstrates antibiotic and cytotoxic activities (48-50). EQ inhibits mitochondrial ATPase activity (31,51). It inhibits HIV-1 integrase (31, 34, 52, 53). EQ has been reported to show strong antibiotic activity against some Gram-positive bacteria and mycobacterium (46). Epi-EQ, the phytotoxic isomer of EQ, is also reported to inhibit HIV-1 integrase (53). Wheeler *et al.* (47) reported the phytotoxicity of the EQ and epi-EQ to certain plants during seed germination or seedling growth.

Compounds with anticancer properties also isolated from *F. equiseti* include Diacetoxyscirpenol (54, 55) and Fusarochromanone and its derivatives (56, 57). Other compounds from *F. equiseti* are 4-acetylnivalenol, nivalenol, scirpentriol zearalenone, beauvericin, fusarochromanone, equisetine and butenolide (58, 59).

Epicoccum sorghinum (Didymellaceae), formerly known as *Phoma sorghina* (60), is a facultative plant pathogen considered to be majorly associated with the sorghum grain-mold disease complex (61). The fungus is also the cause of leaf spot disease of plants such as *Eichhornia crassipes* (62), *Colocasia esculenta* (63), *Nicotiana tabacum* (64), *Oxalis debilis* (65), and *Phytolacca Americana* (66). *E. sorghinum* is known to produce the mycotoxin tenuazonic acid (TA) (61, 66-68). Several other phytotoxins such as epoxydon, desoxyepoxydon, phyllostine, diphenyl ether, and 6-methyl salicylate ether have been expressed by the fungus (66). In contrast to its pathogenic activity on sorghum grains, *E. sorghinum* is reported to be the most prevalent endophytic fungal species associated with the leaves of sorghum field plants (69, 70). It has also been found as endophytes of *Annona senegalensis* (71), *Rhodomyrtus tomentosa* (61), and *Tithonia diversifolia* (66).

TA a tetramic acid derivative, is a mycotoxin and phytotoxin majorly produced by *Alternaria alternata* (*A. tenuis*), as well as by other phytopathogenic *Alternaria* species (36, 68, 72, 73). The compound is also expressed by other species of fungi such as *E. sorghinum* and *Pyricularia oryzae* (36, 68). TA inhibits protein biosynthesis, and it's the most toxic of the *Alternaria* toxins (68, 74). TA is a non-specific phytotoxin as it exhibits significant phytotoxic effects on both monocotyledonous and dicotyledonous plants (75-77). TA is also toxic to mice, chick embryos and chickens (36). The compound has been implicated as a possible cause or contributing factor to 'Onyalai', a human

hematological disorder occurring in Africa after consumption of sorghum (78). TA has shown antiviral, antitumor, antibiotic, and phytotoxic activities (35, 36, 75, 79). The compound possesses broad spectrum and quick killing properties, and as such can be employed as a bioherbicide (72, 77).

Apart from expressing these biologically important compounds, these two toxigenic endophytic fungi isolated from *C. papaya* are reported to have industrial applications. *F. equiseti* and *E. sorghinum* have been shown to have potentials for use as biocontrol agents of weeds and plant pathogens (62,80-84). *F. equiseti* is also employed as a plant growth promoting fungus (82,83). Also, an *F. equiseti*-derived protease showed excellent stain removal property and was compatible with several commercial laundry detergent formulations (85).

Conclusion

F. equiseti and *E. sorghinum* isolated from *C. papaya* produced toxic compounds known to have beneficial potentials for pharmaceutical, agricultural or industrial purposes.

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Conflict of interest statement

The authors declare no conflict of interest.

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