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# IDENTIFICATION, CRYSTAL STRUCTURE AND ANTITUMOR ACTIVITY OF FUSARIC ACID FROM THE SUGARCANE FUNGAL PATHOGEN, *FUSARIUM SACCHARI*

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**Abstract**

In this study, we investigated the secondary metabolic profile of the fungus *Fusarium sacchari* chromatographically. Fusaric acid and its analogs, fusaric acid methyl ester and 9,10-dehydrofusaric acid were identified and characterised by spectroscopic methods and reported for the first time as secondary metabolites isolated from the fungal extract of *F. sacchari*. The structure of fusaric acid was fully characterised by x-ray crystallography. Antitumor activity of fusaric acid was tested against larynx cell cancer line (HEP-2), breast cancer cell line (MCF-7), lung cancer cell line (A-549) and colon cancer cell line (HCT-116) and compared with cisplatin as a reference drug. Fusaric acid showed a moderate antitumor activity with $IC_{50}$ 65.9 µM against the HEP-2 cell line.

**Keywords:**

Fusaric acid - crystal structure - HPLC - antitumor activity- *Fusarium sacchari*

## Introduction

*Fusarium sacchari* has been reported to be one of the pathogens of sugarcane which reduces its sugar content by 40-65% and causes Pokkah boeng disease.[1,2] Fusaric acid is a picolinic acid derivative biosynthesised from acetate units and aspartate.[3-5] It is also produced by *Fusarium spp*. and most commonly results from infection of cereal grains.[6] It has been reported to have several effects on humans, and other mammals including cardiovascular and neurological effects.[7] Fusaric acid has also been shown to have antitumor activity against head and neck sqamous cell cancer (HNSCC) by enhancing DNA damage and preventing its synthesis and repair causing apoptosis.[8,9] In our study, we

aimed to isolate and identify the fungal metabolites produced by *Fusarium sacchari* by HPLC and evaluate their antitumor activity on different cancer cell lines.

## Materials and Methods

### Chemistry

Melting points are uncorrected and were recorded by Open Capillary tube method using an Electro-thermal Melting Point apparatus. Infrared spectra (IR) were obtained using a Perkin-Elmer FTIR Spectrum One FT-IR spectrometer for compounds (in the solid or liquid state) mounted directly on the diamond cell (neat). $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded using Varian VNMR 400 MHz and 500 MHz spectrometers. Chemical shifts values (δ) are given in parts per million, and were referenced to trimetrhylsilane (TMS) as the internal reference or an appropriate solvent. Electron ionization (EI) mass spectra were recorded on a VG Analytical Autospec mass spectrometer and Electrospray ionization (ESI) mass spectra were recorded on a Micromass LCT mass spectrometer or a VG Quattro mass spectrometer at the School of Chemistry, Faculty of Science, University of Bristol, UK. Purification of compounds was achieved by HPLC mass-directed purification. Solvents used were: HPLC grade $H_2O$ containing 0.05% formic acid; HPLC grade $CH_3CN$ containing 0.045% formic acid in a gradient flow as follows.

Method 1: 0 min, 33% $CH_3CN$; 15 min, 45% $CH_3CN$; 16 min, 95% $CH_3CN$; 18.50 min, 95% $CH_3CN$; 19 min, 33% $CH_3CN$; 20 min, 33% $CH_3CN$.

Method 2: 0 min, 40% $CH_3CN$; 13 min, 60% $CH_3CN$; 13.50 min, 95% $CH_3CN$; 18.50 min, 95% $CH_3CN$; 19 min, 40% $CH_3CN$; 20 min, 40% $CH_3CN$.

X-ray crystallographic analysis was carried out at 100K on a Burker Microstar rotating anode differactometer using Cu-$K_\alpha$ radiation (λ= 1.54178 Å). Data collections were performed using a CDD area detector from a single crystal mounted on a glass fibre at the X-ray Unit, School of Chemistry, Faculty of Science, University of Bristol, UK.

### Biology

### Strain and Culture Conditions

### Culturing of *F. sacchari* in Solid Media

The *Fusarium sacchari*, originally isolated from field-grown sugarcane plants, was from the First Culture Bank, University of the Punjab (Lahore, Pakistan) supplied by Dr. Asifa Munawar, School of Biological Sciences, University of Bristol. It was propagated on Czapek Dox (CD) Agar (15 g $L^{-1}$) at 28 ˚C for 22 days.

**Preparation of Czapek Dox (CD) Liquid Medium**

Medium for culturing the fungus *F. sacchari* in this work was prepared by combing 30 g sucrose with 50 mL of Czapek stock solution A (60 g $NaNO_3$, 10 g KCl, 10 g $MgSO_4.H_2O$, 0.2 g $FeSO_4.7H_2O$ in 1000 mL distilled water) and 50 mL Czapek stock solution B (20 g $K_2HPO_4$ in 1000 mL distilled water) levelled to 1 liter with distilled water. The medium solution was made into 100 mL portions in 500 mL baffled Erlenmeyer flasks, covered with foam bung coated with aluminum foil and sterilized by autoclaving.

**Culturing of *F. sacchari* in Liquid Media**

*Fusarium sacchari* was cultured in Czapek Dox broth (CDB) (100 mL per 500 mL baffled flask) at 28 ˚C, shaking at 250 rpm in dark on an orbital gyratory shaker for an incubation period of 12 days. Spores and mycelia from *Fusarium sacchari* (22 days old) were inoculated into all broth flasks at 28 ˚C.

For purification of compounds the crude extract was made into a solution of 50 mg $mL^{-1}$ in HPLC grade methanol and 200 µL aliquots were injected in each run of mass-directed HPLC preparative purification at a rate 4 mL $min^{-1}$.

**Antitumor Activity against HEP-2, A-549, MCF-7 and HCT-116 Cell Lines**

Antitumor activity was performed in the National Cancer Institute - Cairo University.

1- Cells were plated in 96-multiwell plates ($10^4$ cells/ well) for 24 h, before treatment with the compounds to allow the attachment of cells to the wall of the plate.

2- Different concentrations of the compounds under test (0, 1, 2.5, 10 µg/ml) were added to the cell monolayer.

3- Triplicate wells were prepared for each individual dose.

4- Monolayer cells were incubated with the compounds for 48 h at 37 ˚C and in 5% $CO_2$ atmosphere.

5- After 48 h, Cells were fixed, washed and stained with Sulforhodamine B (SRB) stain.

6- Excess stain was washed with acetic acid and the attached stain was recovered with Tris EDTA buffer.

7- Color intensity was measured in an ELISA reader.

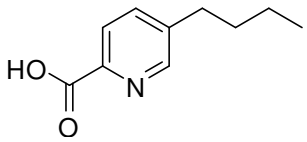
The relation between the surviving fraction and drug concentration is plotted to obtain the survival curve of the tumor cell line after the specified compound was added. $IC_{50}$ and standard errors (S.E) for the $IC_{50}$ values were calculated by Graph Pad Prism 5 software.

**Results**

**Fusaric acid (5-*n*-Butylpicolinic acid[10,11]) (Figure 1)**

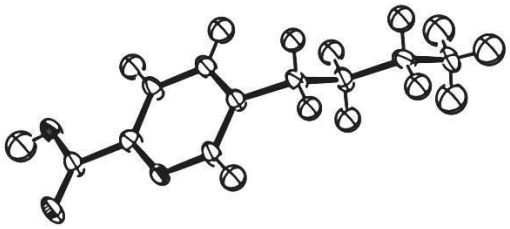
mp 99-100 °C, was isolated as white crystals by preparative HPLC using method 1, R*t* = 4.0 min; UV/vis $\lambda_{max}$ ($H_2O$/MeOH) 270.2 nm; IR $\upsilon_{max}$ (neat) 3436, 2956, 2932, 2861, 1721, 1591 $cm^{-1}$; $^1H$ NMR (400 MHz $CDCl_3$) δ 0.96 ppm (3H, t, *J* = 6 Hz, H-10), 1.38 (2H, tt, *J* = 4, 8 Hz, H-9), 1.65 (2H, tt, *J* = 4, 8 Hz, H-8), 2.73 (2H, t, *J* = 6 Hz, H-7), 7.75 (1H, dd, *J* = 1.6, 8 Hz, H-4), 8.15 (1H, d, *J* = 6.4 Hz, H-3), 8.46 (1H, d, *J* = 2 Hz, H-6), $^{13}C$ NMR (100 MHz $CDCl_3$) δ 13.8 ppm (C-10), 22.2 (C-9), 32.8 (C-8), 32.9 (C-7), 123.5 (C-3), 138.0 (C-4), 143.4 (C-5), 143.9 (C-2), 148.2 (C-6), 164.3 (C-1); MS (EI) m/z (%): 180 (77)$[M]H^+$.

**Figure 1**: **Structure of fusaric acid.**




Crystals were obtained by slow evaporation of an aqueous methanolic solution. White needles, size/mm = 0.16 × 0.03 × 0.03, formula $C_{10}H_{13}N_1O_2$, *M* = 179.21, monoclinic, space group *C*2, *a* = 16.076(2) Å, *b* = 9.5828(12) Å, *c* = 19.507(3) Å, *α* = 90.00˚, *β* = 99.337(6)˚, *γ* = 90.00˚, *V* = 2965.4(7) $Å^3$, *Z* = 12, μ.$mm^2$ = 0.68, $\theta_{min/max}$ = 4.6, 62.7˚, *F*(000) = 1152, reflections: total/independent = 2609/2550, $R_{int}$ = 0.000, Final *R*1 and *wR*2 = 0.078,0.229, $\Delta p_{max,min}/eÅ^3$ = 0.43,-0.55. The Cambridge Crystallographic Data Centre (CCDC) Deposition Number: 981036.

**Figure 2**: **The molecular structure of fusaric acid showing displacement ellipisoids at the 50% probability level using Ortep3.**

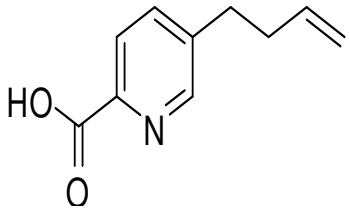


**9,10-Dehydrofusaric acid (5-(But-3-enyl)picolinic acid[10,11]) (Figure 3)**

mp 118-120 ˚C, was isolated as white solid by preparative HPLC using method 1, R*t* = 3.4 min; UV/vis $\lambda_{max}$ ($H_2O$/MeOH) 272 nm; IR $\upsilon_{max}$ (neat) 3436, 2956, 2932, 2861, 1721, 1591 $cm^{-1}$; $^1H$ NMR (500 MHz $CDCl_3$) δ 2.43 ppm (2H, dd, *J* = 6.5, 8.5 Hz, H-8), 2.85 (1H, t, *J* = 10 Hz, H-7), 5.03-5.06 (2H, m, H-10), 5.79-5.84 (2H, m, H-9),

7.77 (1H, d, *J* = 10 Hz, H-4), 8.15 (1H, d, *J* = 8.5 Hz, H-3), 8.46 (1H, s, H-6), $^{13}$C NMR (125 MHz $CDCl_3$) δ 32.5 ppm (C-8), 34.8 (C-7), 116.6 (C-10), 138.3 (C-9), 123.6 (C-3), 136.5 (C-4), 143.2 (C-2), 145.5 (C-5), 153.0 (C-6), 164.0 (C-1); MS (EI) *m/z* (%): 91 (100), 178 (60) $[M]H^+$.

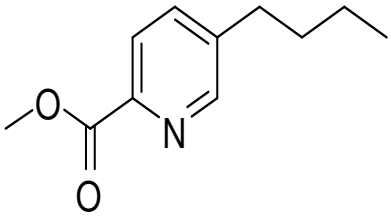
**Figure 3: Structure of 9, 10-dehydrofusaric acid.**




**Fusaric acid methyl ester (Methyl 5-butylpicolinate[12]) (Figure 4)**

Isolated as yellow oil by preparative HPLC using method 2, R*t* = 15.3 min; UV/vis $\lambda_{max}$ ($H_2O$/MeOH) 230, 269 nm; IR $\upsilon_{max}$ (neat) 2956, 2932, 2861, 1721, 1591 $cm^{-1}$; $^1$H NMR (400 MHz $CDCl_3$) δ 0.89 ppm (3H, t, *J* = 8 Hz, H-10), 1.32 (2H, tt, *J* = 6.8, 8 Hz, H-9), 1.58 (2H, p, *J* = 6.8 Hz, H-8), 2.64 (2H, t, *J* = 8 Hz, H-7), 3.95 (3H, s, H-11) 7.59 (1H, d, *J* = 8 Hz, H-4), 8.00 (1H, d, *J* = 8 Hz, H-3), 8.52 (1H, s, H-6), $^{13}$C NMR (100 MHz $CDCl_3$) δ 13.8 ppm (C-10), 22.3 (C-9), 32.8 (C-8), 33.0 (C-7), 52.8 (C-11), 125.0 (C-3), 136.7 (C-4), 142.3 (C-5), 145.6 (C-2), 150.1 (C-6), 165.9 (C-1); MS (ESI) *m/z* (%): 194 (62) $[M]H^+$, 216 (100) $[M]Na^+$.

**Figure 4: Structure of fusaric acid methyl ester.**




***In-vitro* Antitumor Activity of Fusaric Acid against Cancer Cell Lines**

**Table 1: $IC_{50}$ µM values of the tested compounds on HEP-2, A-549, MCF-7 and HCT-116 cell lines.**

| Compound | **$IC_{50}$ µM ± SE** | | | |
|---|---|---|---|---|
| | **HEP-2** | **MCF-7** | **A-549** | **HCT-116** |
| **Fusaric acid** | 65.9 ± 0.13 | 82.7 ± 0.11 | 97.8 ± 0.14 | 101.1 ± 0.16 |
| **Cisplatin** | 14.3 ± 0.16 | 12.3 ± 0.09 | 15.0 ± 0.20 | 11.3 ± 0.13 |

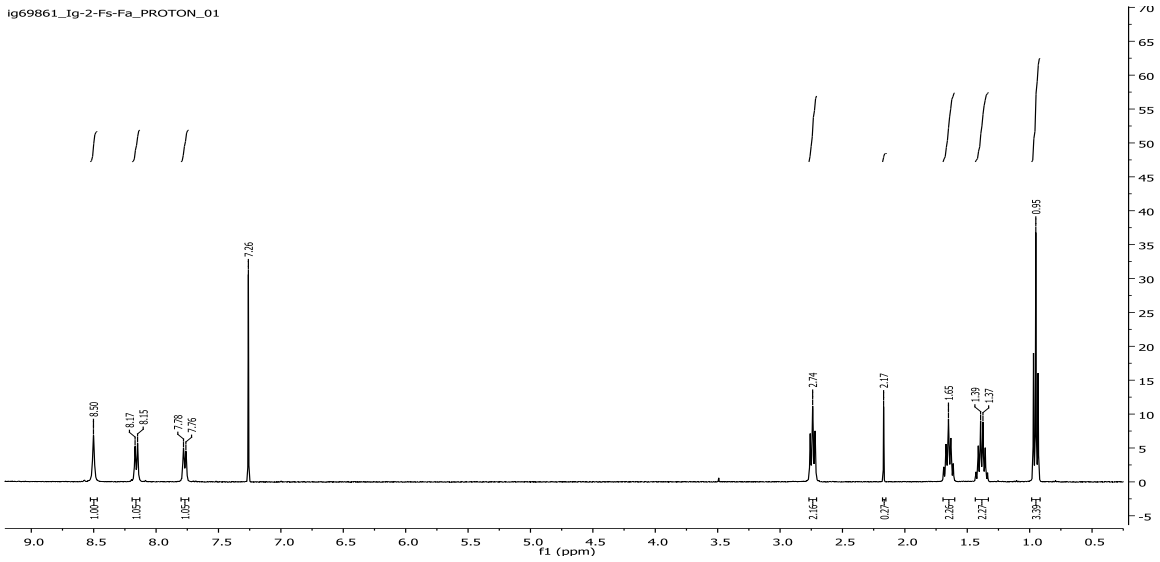
**Discussion**

**Isolation and Identification of Metabolites**

*Fusarium sacchari* strain was supplied by Dr. Asifa Munawar, University of Bristol and obtained from First Fungal Culture Bank, University of the Punjab, Lahore, Pakistan (accession number 144). The fungal extract on Czapek Dox (CD) medium was analyzed by LC-MS.

The $^{1}$H-NMR spectrum of fusaric acid revealed a butane chain (**figure 5**) and the $^{13}$C-NMR spectrum revealed four aliphatic carbons, one carboxyl and five aromatic carbons. $^{13}$C NMR chemical shift analysis showed that the carboxyl must be in the 2 position and the aromatic proton coupling pattern indicated that the butane chain must be in the 5 position. The EI$^{+}$ MS showed an ion *m/z* 180 Da [M]H$^{+}$. The structure of fusaric acid was confirmed by the X-ray single crystal. It's the first time to report the sole x-ray structure of fusaric acid.

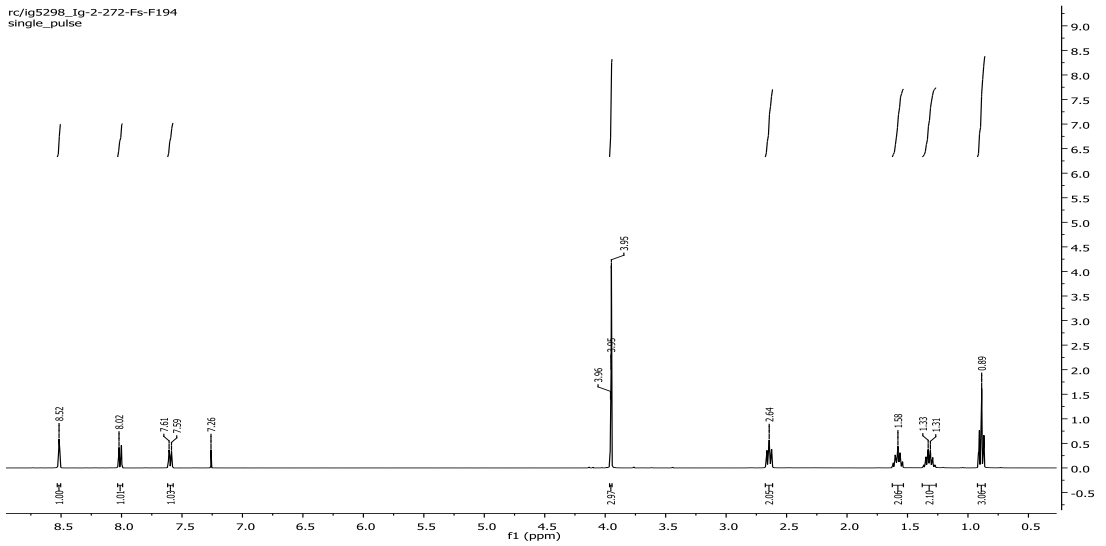
**Figure 5: $^{1}$H NMR spectrum of fusaric acid.**




The $^{1}$H-NMR spectrum of 9,10-dehydrofusaric acid revealed a buteneyl chain which shows differences in their chemical shifts (deshielded) from fusaric acid that revealed one terminal double bond, 2.43, 2.85, 5.03 and 5.79 ppm. $^{13}$C-NMR spectrum revealed four aliphatic carbons, one carboxyl and five aromatic carbons. $^{13}$C-NMR chemical shift analysis showed that the four aliphatic carbons are more deshielded than the ones in fusaric acid, 32.5, 34.8, 116.6 and 138.3 ppm. The EI$^{+}$ MS showed an ion *m/z* 178 Da [M]H$^{+}$ which confirms the presence one degree of unsaturation.

$^{1}$H-NMR of fusaric acid methyl ester was the same as the one of fusaric acid with addition of singlet peak at 3.95 ppm which corresponds to methyl protons (**figure 6**). $^{13}$C-NMR revealed an extra carbon peak corresponds to the carbon of $CH_3$ at 52.8 ppm. The ESI$^{+}$ MS showed an ion *m/z* 194 Da [M]H$^{+}$ and 216 Da [M]Na$^{+}$.

**Figure 6: $^1$H NMR spectrum of fusaric acid methyl ester.**




***In-vitro* Antitumor Activity of Fusaric Acid against Cancer Cell Lines**

Fusaric acid was tested for its antitumor activity and its $IC_{50}$ on (HEP-2) larynx cancer cell line = 65.9 µM, on (MCF-7) breast cancer cell line = 82.7 µM, on (A-549) lung cancer cell line = 97.8 µM and on (HCT-116) colon cancer cell line = 101.12 µM. Fusaric acid has been shown to have an antitumor activity against in an order as follows, HEP-2>MCF-7>A-549>HCT-116. It was also found that fusaric acid is less potent than cisplatin against all the cancer cell line tested.

**Conclusion**

Fusaric acid, 9,10-dehydrofusaric acid and fusaric acid methyl esters were isolated as metabolite toxins from the fungal extract of *Fusarium sacchari*. Fusaric acid has been shown to have higher antitumor activity on larynx (HEP-2) cell line than the other cell lines tested.

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