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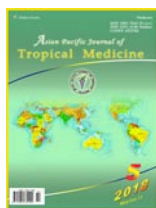
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## Antimicrobial activity of kojic acid from endophytic fungus *Colletotrichum gloeosporioides* isolated from *Sonneratia apetala*, a mangrove plant of the Sundarbans

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### ABSTRACT

**Objective:** To isolate and evaluate the antimicrobial activity of the active principle(s) from the ethyl acetate (EtOAc) extract of endophytic fungus *Colletotrichum gloeosporioides* (*C. gloeosporioides*) isolated from *Sonneratia apetala*. **Methods:** Water agar technique was used to isolate the fungus, and both microscopic and molecular techniques were used for identification of the strain. Potato dextrose broth was used to grow the fungus in large-scale. Reversed-phase preparative HPLC analysis was performed to isolate the major active compound, kojic acid. The EtOAc extract and kojic acid were screened for their antimicrobial activity against two Gram-positive and two Gram-negative bacteria as well as a fungal strain using the resazurin 96-well microtitre plate antimicrobial assay. **Results:** The fungus *C. gloeosporioides* was isolated from the leaves of *Sonneratia apetala*. Initial identification of the fungal isolate was carried out using spore characteristics observed under the microscope. Subsequently, the *ITS1–5.8S–ITS2* sequencing was employed for species-level identification of the fungus *C. gloeosporioides*. Five litres of liquid culture of the fungus produced approximately 610 mg of a mixture of secondary metabolites. Kojic acid (1) was isolated as the main secondary metabolite present in the fungal extract, and the structure was confirmed by 1D, 2D NMR and mass spectrometry. The EtOAc extract and compound 1 exhibited considerable antimicrobial activity against all tested microorganisms. Whilst the minimum inhibitory concentration (MIC) values from the EtOAc extract ranged between  $2.4 \times 10^{-4}$  mg/mL and 2.5 mg/mL, those of kojic acid (1) were between 0.125 mg/mL and 1 mg/mL. The EtOAc extract and kojic acid (1) were most active against *Pseudomonas aeruginosa* (MIC =  $2.4 \times 10^{-4}$  mg/mL) and *Micrococcus luteus* (MIC = 0.125 mg/mL), respectively. **Conclusions:** The results revealed that the endophytic fungus *C. gloeosporioides* could be a good source of commercially important kojic acid, which exhibited antimicrobial properties.

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## 1. Introduction

Mangrove forests are halophytic and salt-tolerant marine tidal forests comprising trees, shrubs, palms, epiphytes, ground ferns and grasses[1]. The mangrove vegetation possesses a unique salt filtration root system that can cope with salt-water immersion, wave action and low-oxygen conditions. Mangrove forests survive through stressful conditions, e.g., harsh environments, high moisture and salinity, intermittent tidal water, and diverse microorganisms and insects, by producing various bioactive compounds, e.g., alkaloids, flavonoids, steroids, tannins and triterpenes[1]. These plants have long been used in traditional medicine, but without proper scientific validation, assessment of toxicity and evaluation of any adverse reactions. Approximately 200 different bioactive secondary metabolites have been reported from the tropical and sub-tropical mangroves[2,3]. Many of these metabolites have antimicrobial properties and protect plants from microbial attacks[3]. In some cases, the endophytic microorganisms growing in symbiosis with mangrove plants could be responsible for the production of some of these bioactive molecules. Consequently, some of these secondary metabolites from endophytes could be useful as leads for new drug discovery and development[4-6]. Endophytes are actually variable life strategies of the symbiosis. The strategies may include facultative saprobic, parasitic, exploitive and mutualistic symbioses. The mangrove endophytes are essentially endosymbiont, often a bacterium or fungus that lives within a plant for at least part of its life cycle without causing any disease. They occupy the upper part of trees as the bases of mangrove trunks and aerating roots are submerged in water permanently or intermittently.

*Sonneratia apetala* Buch.-Ham. (*S. apetala*, Bengali name: Kaora), a mangrove plant from the family Sonneratiaceae (alternative name Lythraceae), is one of the dominant mangrove species in the Sundarbans, the largest mangrove forest in the world. However, this plant also grows in mangrove forests in India and Myanmar and has been introduced in some parts of China[7]. Leaves of this plant are used traditionally to treat cardiac complications, dysentery, hepatitis, and sprain and bruises[8,9]. Whilst there are reports on isolation of bioactive compounds from *S. apetala*[9], there is hardly any investigation on the compounds produced by endophytes associated with this plant. Hence, the present investigation was performed to isolate and evaluate the antimicrobial activity of any active principle(s) from the ethyl acetate (EtOAc) extract of endophytic fungus *Colletotrichum gloeosporioides* (*C. gloeosporioides*) isolated from the mangrove plant *S. apetala*.

## 2. Materials and methods

### 2.1. Materials

A Finnigan MAT 95 spectrometer was employed for mass spectroscopic analyses. 1D and 2D NMR spectra were obtained using an Ultrashield Bruker AMX600 NMR spectrometer. Methyl, methylene and methine carbon atoms were differentiated by the DEPT-Q experiment. Homonuclear  $^1\text{H}$ - $^1\text{H}$  scalar couplings were determined from the COSY experiment.  $^1\text{H}$ - $^{13}\text{C}$  one-bond connectivity was detected with the HSQC gradient pulse factor

selection. Two- and three-bond connectivity was identified by the HMBC experiment. Chemical shifts are reported in  $\delta$  (ppm) and coupling constants ( $J$ ) are reported in Hz. Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich (Dorset, UK), and all solvents for both extraction and chromatography were from Fisher Scientific, (Loughborough, UK). NMR solvents were sourced from GOSS Scientific (Crewe, UK).

### 2.2. Isolation of endophytic fungi

Healthy leaves, bark, fruits and roots of *S. apetala* were collected from the Sundarbans, Bangladesh. The samples were stored in tightly sealed polythene bags under humid conditions at room temperature. The plant was identified based on their morphological characters as outlined by Zabala[10], and a voucher specimen (TRN-KU-2017012) was deposited at the Herbarium of Pharmacy Discipline, Khulna University, Khulna, Bangladesh. The isolation of the fungal endophytes commenced within 24 h of collection, using a method described previously[6]. Briefly, plant materials were washed thoroughly in sterile water to get rid of extraneous substances, the samples were then surface sterilized by sequentially immersing in 70% ethanol for 30 s and 5% sodium hypochlorite solution for 1 min, and finally rinsed with sterile distilled water. Small pieces of inner tissues and needles were transferred and impregnated to the petri-dishes containing aqueous agar (1.5% agar-agar in distilled water) supplemented with antibiotic streptomycin (3 mg/100 mL) and incubated at  $(28\pm 2)$  °C until fungal growth was initiated. The tips of the fungal hyphae were removed from the aqueous agar and placed on Potato Dextrose Agar (PDA) medium. After several days of incubation, colony morphology was assessed to determine the purity of each fungal culture. The isolates were cultured several times on PDA to obtain final pure cultures and then transferred into PDA slants.

### 2.3. Microscopic and molecular identification of fungal isolate

Both microscopic and molecular techniques were used to identify fungal endophytes[11]. Fungal DNA was isolated using fungal DNA isolation kit (Cat-26200; NORGEN BIOTEK Corp., 3430 Schmon Parkway, Thorold, ON, Canada). Phylogenetic analyses of the endophytes were performed by the acquisition of ITS1-5.8S-ITS2 ribosomal gene sequencing. The internal transcribed spacer (ITS) region of the fungi were amplified using the forward primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and reverse primer ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3'), using the polymerase chain reaction (PCR)[11]. The PCR amplified products were sent to Cambridge Genomic Services (The University of Cambridge, UK) for sequencing according to their requirements. Consensus sequence of 500 bp of 5.8S rRNA was generated from forward and reverse sequence data using aligner software. Consensus sequences were submitted in the GenBank. Multiple BLASTN searches against the sequence were made at the National Center for Biotechnology Information. Primarily, Clustal Omega was used for Multiple Sequence Alignment and further followed by trimming using trimAI tool for later alignment. Phylogenetic analysis was performed with the neighbour joining method using MEGA 7.0 software.

## 2.4. Extraction of secondary metabolites

The fungal isolate was grown in five 250 mL conical flasks containing potato dextrose broth for 28 d. Culture broth was separated from the mycelium by filtration (Whatman® qualitative filter paper, Grade 1; Sigma-Aldrich, USA) and the culture filtrates were extracted three times with an equal volume of EtOAc in a separating funnel. The EtOAc extract was evaporated under reduced pressure at 40–45 °C using a rotary evaporator to obtain the crude EtOAc extract.

## 2.5. Antimicrobial screening

The EtOAc extract of the endophytic fungus, and its major secondary metabolite, kojic acid (1), were screened for their potential antimicrobial activity against two Gram-positive, i.e., *Staphylococcus aureus* (NCTC 12981) and *Micrococcus luteus* (NCTC 7508), and two Gram-negative, i.e., *Escherichia coli* (NCTC 12241) and *Pseudomonas aeruginosa* (NCTC 12903) bacterial strains as well as against a fungal strain, *Candida albicans* (ATCC 90028). The resazurin 96-well microtitre plate based *in vitro* antimicrobial assay was used [12]. A final microbial concentration of  $5 \times 10^5$  cfu/mL was used for this assay. Ciprofloxacin was used as a positive control for bacterial strains, and nystatin for *Candida albicans*. All test samples including the extract, kojic acid (1) and the positive controls were serially diluted from the stock concentration of 1 mg/mL. The average of three values was calculated and that was the minimum inhibitory concentration (MIC) for the test material and bacterial strain.

## 2.6. Large-scale fermentation and extraction of secondary metabolites

As the EtOAc extract of endophytic fungus displayed significant antimicrobial activity, it was subjected to large-scale fermentation. The fungus was grown on PDA medium for 3 d. The mycelium (5 mm) was transferred to 40 conical flasks (250 mL each) containing potato dextrose broth. The flasks were placed at room temperature under continuous shaking for around 28 d at 180 r/min. The mycelia were separated from the medium by filtration and extracted by equal volume of EtOAc. Finally, the extracts were dried using a rotary evaporator.

## 2.7. Fractionation, isolation and structure elucidation of the bioactive compounds

Reversed-phase analytical HPLC analysis of the EtOAc extract was carried out using a Phenomenex C<sub>18</sub> reversed-phase column (250 mm×4.6 mm; particle size 5 µm) on a Dionex Ultimate 3000 analytical HPLC-coupled with a photo-diode-array detector (mobile phase: standard gradient of 30%–100% methanol in water over 30 min, flow rate: 1 mL/min). The crude EtOAc was subjected to preparative HPLC using the same solvent system, but with a flow rate of 10 mL/min and using a HiChrom preparative column (250 mm×20.1 mm; particle size 5 µm) on an Agilent prep-HPLC system to isolate the major compound, kojic acid (1). The purity of the isolated compound was checked by analytical HPLC, and the structure was confirmed by spectroscopic analyses including MS,

and 1D and 2D NMR analyses.

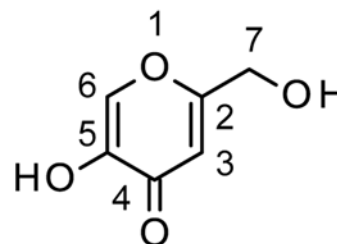
## 3. Results

### 3.1. Microscopic and molecular identification of fungal isolate

Microscopic examination followed by molecular characterization confirmed that the fungal isolate was a species of the genus *Colletotrichum*. The phylogenetic tree revealed that the isolate possessed maximum homology with *C. gloeosporioides* (MF838770.1). The best BLAST matched for endophytic fungus *Colletotrichum* sp. with maximum homology. Because of 5.8S rRNA gene homology the fungal isolated was designated as *Colletotrichum* sp. (*C. gloeosporioides*).

### 3.2. Extraction of secondary metabolites

The secondary metabolites produced by *C. gloeosporioides* on PDA medium was extracted with EtOAc (5 L) to obtain 610 mg of crude mixture of secondary metabolites. Reversed-phase preparative HPLC of the crude mixture afforded kojic acid (1, 58 mg) as the major secondary metabolite present in the EtOAc extract. The structure of kojic acid (1) (Figure 1) was confirmed by 1D and 2D NMR and MS spectroscopic data analyses (Table 1) and compared with published data [13].



**Figure 1.** Structure of kojic acid (1).

[M+1]<sup>+</sup> ion at *m/z* 143.033 5; calculated 143.033 9 for C<sub>6</sub>H<sub>7</sub>O<sub>4</sub>.

**Table 1**

<sup>1</sup>H NMR (600 MHz), <sup>13</sup>C NMR (150 Hz) and HMBC data for kojic acid (1) (spectra obtained in CD<sub>3</sub>OD).

Position	Chemical shifts in δ (ppm)		HMBC	
	<sup>1</sup> H NMR	<sup>13</sup> C NMR	<sup>2</sup> J	<sup>3</sup> J
2	-	170.6	-	-
3	6.52 s, <sup>1</sup> H	110.3	C-2, C-4	C-5, C-7
4	-	177.0	-	-
5	-	147.3	-	-
6	7.97 s, <sup>1</sup> H	141.3	C-5	C-2, C-4
7	4.43 s, <sup>2</sup> H	61.6	C-2	C-3

### 3.2. Antimicrobial activity of the EtOAc extract of *C. gloeosporioides* and kojic acid (1)

The antimicrobial activity of the EtOAc extract of *C. gloeosporioides* and its major compound kojic acid (1) was assessed using the resazurin microtitre assay [12]. The crude EtOAc extract and compound 1 exhibited considerable antimicrobial activity against all

**Table 2**Antimicrobial activity of EtOAc extract of *C. gloeosporioides* and kojic acid (1) (mg/mL).

Test organisms	Ciprofloxacin	Nystatin	EtOAc extract	Kojic acid (1)
<i>Escherichia coli</i> (NCTC 12241)	4.9×10 <sup>-4</sup>	NT	2.500 00	0.500
<i>Micrococcus luteus</i> (NCTC 7508)	9.8×10 <sup>-4</sup>	NT	0.310 00	0.125
<i>Pseudomonas aeruginosa</i> (NCTC 12903)	1.2×10 <sup>-4</sup>	NT	0.000 24	1.000
<i>Staphylococcus aureus</i> (NCTC 12981)	9.8×10 <sup>-4</sup>	NT	0.020 00	0.500
<i>Candida albicans</i> (ATCC 14053)	NT	9.8×10 <sup>-4</sup>	0.039 00	0.500

Ciprofloxacin and nystatin were used as positive controls; NT: not test.

tested microorganisms including four bacterial and one fungal strains (Table 2). Whilst the MIC values from the EtOAc extract ranged between  $2.4 \times 10^{-4}$  and 2.5 mg/mL, those of kojic acid (1) were between 0.125 mg/mL and 1 mg/mL. The EtOAc extract and kojic acid (1) were most active against *Pseudomonas aeruginosa* (MIC =  $2.4 \times 10^{-4}$  mg/mL) and *M. luteus* (MIC = 0.125 mg/mL), respectively.

#### 4. Discussion

*S. apetala* is a mangrove medicinal plant from the Sundarbans. This plant has been used in Bangladeshi folk medicine to treat cardiac complications, dysentery, hepatitis, sprains and bruises [8,9]. Whilst there are reports on isolation of bioactive compounds from *S. apetala* [9], there is hardly any publication on the compounds produced by endophytes associated with this plant. The current study afforded the isolation and systematic identification of the endophytic fungus *C. gloeosporioides* from *S. apetala* for the first time.

Molecular techniques, as employed in the current study, has significantly improved the delimitation of fungal species that are hard to distinguish based on morphology alone, and revealed their phylogenetic relationships [14–16]. *C. gloeosporioides* is a species complex with broad genetic and biological diversity grouped together by similar conidial morphology and ITS sequences [16,17]. Weir and Johnston (Landcare Research, Auckland, New Zealand) presented their research on this species complex and possible approaches to species delimitation through the Genealogical Concordance Phylogenetic Species Recognition [16,17].

This is also the report on the isolation of kojic acid (1) from *C. gloeosporioides* in good quantities. *C. gloeosporioides* appeared to produce kojic acid (1) as a major secondary metabolite, which could easily be extracted by EtOAc and purified successfully using a simple reversed-phase preparative HPLC gradient method. However, kojic acid (1) is a known fungal metabolite, produced mainly by *Aspergillus* and *Penicillium* species, which belong mainly to the *Flavus-Oryzaetamarii* groups [13].

The HRMS analysis of the purified compound from *C. gloeosporioides* revealed the pseudomolecular ions peak  $[M+1]^+$  at  $m/z$  143.033 5, calculated 143.033 9 for  $C_6H_7O_4$ , confirming the molecular formula of the compound  $C_6H_6O_4$  consistent with the structure of kojic acid (1,5-hydroxy-2-hydroxymethyl- $\gamma$ -pyrone). The <sup>1</sup>H NMR spectrum of 1 displayed only three signals: two olefinic methines ( $\delta$  6.52 and 7.97) and an oxymethylene ( $\delta$  4.43), assignable to H-3, H-6 and H<sub>2</sub>-7 of kojic acid. The <sup>13</sup>C NMR spectrum revealed signals for six <sup>13</sup>C atoms: one carbonyl carbon ( $\delta$

177.0), two olefinic oxygen-linked quaternary carbons ( $\delta$  170.6 and 147.3), two olefinic methine carbons ( $\delta$  141.3 and 110.3) and an oxymethylene carbon at  $\delta$  61.6. All these <sup>13</sup>C NMR signals together with the <sup>1</sup>H NMR signals established the structure of kojic acid (1). Further confirmation was obtained through <sup>1</sup>H-<sup>13</sup>C HSQC (<sup>1</sup>J) and HMBC (<sup>2</sup>J and <sup>3</sup>J) correlations. Thus, the structure of the major secondary metabolite from the EtOAc extract of *C. gloeosporioides* was determined conclusively as that of kojic acid (1). All spectroscopic data are in agreement with the published data [13]. From biosynthetic consideration, kojic acid (1) is a putative intermediate in the shikimic acid pathway [13], which involves the condensation of phosphoenol pyruvate (C3 unit) with a tetrose (derythrose-4-phosphate) to produce 3-deoxy-D-arabinoheptulosonic acid, which could be converted to shikimic acid or could undergo intramolecular condensation and subsequent dehydration and oxidation to kojic acid (1) [13]. Since shikimic acid is a key intermediate in the biosynthesis of quinones, compound 1 could also be considered as a putative precursor in the biogenesis of the quinone-based natural products [13].

Kojic acid (1) showed antimicrobial activity against tested microorganisms and was most effective against *Micrococcus luteus* (MIC = 0.125 mg/mL) and the least against *Pseudomonas aeruginosa* (MIC = 1 mg/mL). However, it is noteworthy that the crude EtOAc extract showed remarkable activity against *Pseudomonas aeruginosa* (MIC =  $2.4 \times 10^{-4}$  mg/mL), which was just half the potency of the positive control ciprofloxacin MIC =  $1.2 \times 10^{-4}$  mg/mL. The significant loss of activity in the isolated compound (1) against *Pseudomonas aeruginosa*, compared to that of its parent extract, indicated that there might be other active compounds present in the extract, which could not be detected by the HPLC-PDA because of lack of presence of any detectable chromophores in those molecules. Previous bioactivity studies on kojic acid (1) revealed its antioxidant [18], tyrosinase inhibitory [19], anti-pigmentation (skin whitening) [20,21], nematocidal [22], antifungal [23] and antileishmanial [24] properties. Kojic acid (1) is a chelation agent produced by several species of fungi, especially *Aspergillus oryzae*, which has the Japanese common name koji. This compound (1) is a commercially important ingredient for various topical cosmetic products, especially for skin-lightening [21]. The approved and permitted maximum concentration of kojic acid (1) in cosmetic products, e.g., powders, serums, creams, cleansers, and soaps, is 1%. Some products, like soaps and cleansers, are meant to be washed off immediately, but others, like creams and serums, are formulated to be left on and absorbed into the skin [21–24]. Products containing kojic acid (1) are frequently used on the face and hands, but can be applied on all non-sensitive areas of the body. It possesses



antifungal properties and is incorporated to some antifungal products to enhance their efficacy. Moreover, kojic acid (1) could be useful in treating fungal infections of the skin including yeast infections, candidiasis and ringworm or athlete's foot[21-24]. Regular use of soap containing kojic acid (1) may help prevent both topical bacterial and fungal infections on the body. The antimicrobial property, as observed in the current study, has reinforced the scientific basis for its effectiveness as an antimicrobial agent. Furthermore, the results revealed that the endophytic fungus *C. gloeosporioides* could be a good source of commercially important kojic acid (1).

### Conflict of interest statement

The authors declare that they have no conflict of interest.

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