

ANTIMICROBIAL METABOLITE FROM THE ENDOPHYTIC FUNGI *Aspergillus flavus* ISOLATED FROM *Sonneratia alba*, A MANGROVE PLANT OF TIMOR-INDONESIA

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ABSTRACT

The endophytic fungi *Aspergillus flavus* was isolated from *Sonneratia alba*, a mangrove plant growing in Timor Island which is a hot and dry region with high intensity of sunlight throughout the year. Chemical investigations of the ethyl acetate extract of the endophytic fungi *Aspergillus flavus* led to the isolation of kojic acid (**1**) by simple chromatographic separation. The chemical structure of **1** was determined from the comprehensive analyses of the NMR and mass spectral data. Kojic acid (**1**) showed comparable antibacterial activity as positive control tetracycline with a diameter of zone inhibition of 10.3 mm and 8.8 mm against *Staphylococcus aureus* and *Escherichia coli*, respectively. Besides its antibacterial properties, kojic acid has been widely used in the cosmetics and pharmaceutical industry especially for UV B protection. High yield of kojic acid production by this endophytic fungi *A. flavus* revealed the influence of production a specific compound as a result of abiotic and biotic ecological stress.

Keywords: Mangrove, *Sonneratia alba*, Timor, Endophytic Fungi, Kojic Acid, Antibacterial.

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INTRODUCTION

Endophytic fungi have attracted great attention as a new source for valuable compounds as it requires only a small quantity of the plant materials and thus it can prevent the diminishing of slow-growing, valuable and rare plants.¹⁻³ Fungal endophytes have been also known to produce the same and highly valuable chemical metabolites as their host plants such as paclitaxel, vincristine and vinblastine.⁴ Among the fungal endophytes from plants, mangrove derived endophytic fungi have attracted special interest due to its unique habitat located in the zone of transition between marine and terrestrial ecosystems. As tolerance to biotic and abiotic stress had been shown to correlate with endophytic fungal natural products, it is likely that endophytic fungi from mangrove plants produce a wide variety of bioactive compounds.⁵ Several bioactive compounds had been reported from Indonesian plants⁶⁻⁸ that might be correlated with the presence of their endophytes.⁹ As part of our ongoing objective finding the pharmacologically active metabolites from endophytic fungi isolated from plants growing in Timor Island, Indonesia,¹ we isolated the endophytic fungi *Aspergillus flavus* from leaves of mangrove plant *Sonneratia alba*. Chemical investigation of extract from large scale cultivation on solid rice media led to the isolation of antibacterial compound kojic acid (**1**).

EXPERIMENTAL

Isolation of Endophytic Fungi

The fresh and healthy leaves of *S. alba* was collected from Mangrove forest, Kupang, East Nusa Tenggara Province, Indonesia. The leaf was washed with sterilized distilled water and subsequently dipped into 70% ethanol for 1-2 min, followed by washing it twice in sterilized distilled water. The sterile mangrove

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leaf was then cut into small parts (approximately 1cm x 1cm) and placed on a Petri dish having potato dextrose agar (PDA). Hypha growing from the mangrove leaf was transferred to a new dish with the same PDA medium and subcultured again until the pure colony was obtained. The pure culture was periodically observed for its purity.^{1,3}

Cultivation and Extraction of Secondary Metabolites

Cultivation of the endophytic fungi on large scale was performed in two Erlenmeyer flasks (1 L). A small portion of a pure culture grown on PDA was transferred to the sterile solid rice media (100 g of rice in 110 mL of distilled). The fungal strain was then cultivated on rice medium under static conditions for 14-30 days at room temperature. After reaching their stationary phase, 250 mL of ethyl acetate was put into the culture to stop the fungal growth. The mixture of cultures and solvents was left for one night and then the cultures were filtered. The ethyl acetate was evaporated under a vacuum with a rotary evaporator. The ethyl acetate crude extract of *Aspergillus flavus* (19 g) was fractionated using column chromatography under vacuum (VLC) with a combination of solvent from non-polar (mixtures of *n*-hexane and ethyl acetates) to polar gradients (a mixture of dichloromethane and methanol). Fraction 8 eluted with CH₂Cl₂-MeOH (7:3 and 1:1) yielded pure kojic acid (1.2 gram).

Identification of the Isolated Compound

The isolated compound **1** was identified its structure with the NMR and MS spectral data. NMR spectral data were performed on 500 MHz for ¹H and 125 MHz for ¹³C with deuterated methanol (MeOD) as a solvent. FID (Free Induction Decay) data were processed using Mestre Nova program.

Antibacterial Activity

The antimicrobial activity of the isolated compound was performed by the paper disk method against *Staphylococcus aureus* and *Escherichia coli*. Kojic acid was prepared in the concentration of 1 mg/mL using distilled water that has been sterilized. 10 µL of kojic acid solution was pipetted into 0.66 cm sterile paper disk on the surface of the medium containing bacteria test strain. Paper disks were also inoculated with aquades (10 µL) as a negative control. Paper disks that had already impregnated with tetracyclin (30 µg) were used as a positive control. The plates were put in an incubator at 37 °C for approximately 24 hours. Inhibition zones were measured and recorded. Screening of antibacterial activity was repeated twice.

RESULTS AND DISCUSSION

The endophytic fungi *A. flavus* was cultivated in solid rice media in order to produce a large number of secondary metabolites. The ethyl acetate extract from the grown fungi was checked for its chemical profile with HPLC (Fig.-1) and was further chromatographed under vacuum to afford several fractions. The chemical profile of each fraction from VLC was then analyzed with HPLC. Fraction 8 and 9 eluted with 30% and 50% methanol in dichloromethane afforded pure compound (1.2 gram) based on the result from HPLC analysis (Fig.-2).

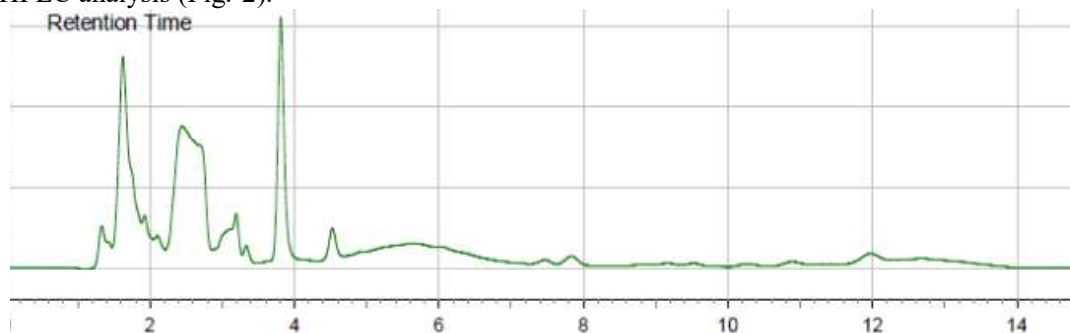


Fig.-1: Chromatogram Ethyl acetate Extract of *A. flavus*

The structure of pure compounds from fractions 8 and 9 were then elucidated based on the analysis from high-resolution ESI MS together with 1D and 2D NMR data. The molecular formula of compound **1** was determined as C₆H₆O₄ by HRESIMS (*m/z* 143.0335 [M+H]⁺ calculated for 143.02661 [M+H]⁺), indicating

4 degrees of unsaturation. The ^1H NMR spectra had two singlets of aromatic signals at δ_{H} 6.50 and 7.96 together with an oxy-methylene signal at δ_{H} 4.41 (Fig.-4).

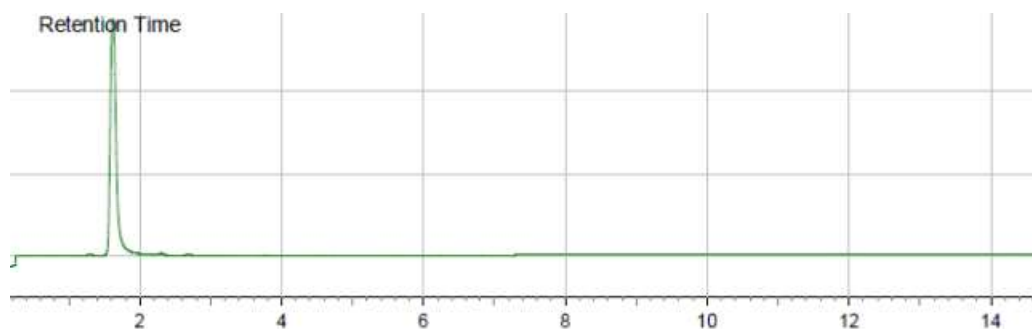


Fig.-2: Chromatogram of the isolated Kojic Acid (1)

The ^{13}C NMR data displayed 6 carbon signals (Table-1) including one carbonyl carbon resonating at δ_{C} 177.1 ppm, two oxygenated olefinic quaternary carbons resonating at δ_{C} 168.9 and 145.9 and two olefinic methine carbons at δ_{C} 139.3 and 109.3 together with a methylene carbon attached to oxygen at δ_{C} 61.2 (Fig.-5). The presence of quaternary and methylene carbon was also confirmed from the DEPT experiment. DEPT spectra revealed only one signal of a methylene carbon and two olefinic methine carbons. The NMR data of compound **1** matched very well with those previously reported for kojic acid.¹⁰⁻¹⁴ UV spectra of compound **1** having λ_{max} at 219 and 270 nm (in methanol) confirmed the structure of compound **1** as kojic acid (Fig.-3).¹⁴ Kojic acid was then evaluated for its antimicrobial activity against *S. aureus* and *E. coli* with tetracycline as a positive control. Kojic acid (10 μg) was able to inhibit the growth of *S. aureus* and *E. coli* with the diameter of zone inhibition at 10.3 mm and 8.8 mm, respectively. This result was in agreement with the earlier study on the antibacterial activity of kojic acid produced by the endophytic fungi *Colletotrichum gloeosporioides* associated with the mangrove tree *Sonneratia apetala*.¹⁰

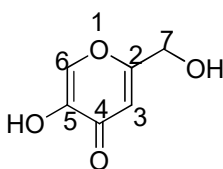


Fig.-3: Chemical Structures of Kojic Acid (1)

Table-1: ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data for Kojic Acid in CD_3OD

No	Kojic acid (1)		Reference ^{10,12-14}	
	^1H NMR	^{13}C NMR	^1H NMR	^{13}C NMR
1				
2	-	170.41		170.2
3	6.49 s, 1H	110.74	6.50 s, 1H	110.6
4	-	176.86		176.7
5	-	147.37		147.2
6	7.95 s, 1H	141	7.95 s, 1H	140.8
7	4.40 s, 2H	61.18	4.41 s, 2H	61.1

This finding revealed the importance of ecological factors in directing the production characteristic metabolites from endophytic fungi.² Kojic acid was known as a UV protector and skin whitening agent in the various formulation of cream, lotion and soap.¹⁵ The endophytic fungi of *A. flavus* used in this study was isolated from the mangrove plant *S. alba* that grows in the dry region area with high intensities of

sunlight throughout the year. The production of high kojic acid in this endophytic fungus might serve as multi protector for the plant from the damage caused by high intensities of UV radiation from the sun light^{15,16} and from the invasion of pathogenic bacteria. The amount of kojic acid produced of the fungus collected from tropical dryland used in this study (1.2 gram) was much higher compared with the earlier reports about 631 mg by the endophytic fungi *Colletotrichum gloeosporioides* from the leaves of mangrove tree *S. apetala*¹⁰ collected in Sundarbans (Bangladesh) and about 351.7 mg by *Aspergillus flavus* isolated from worker bees in Portici, Italy.¹² This result confirmed the influence of ecological factors in directing the production of specific compound as a response toward the environmental abiotic and biotic stress.

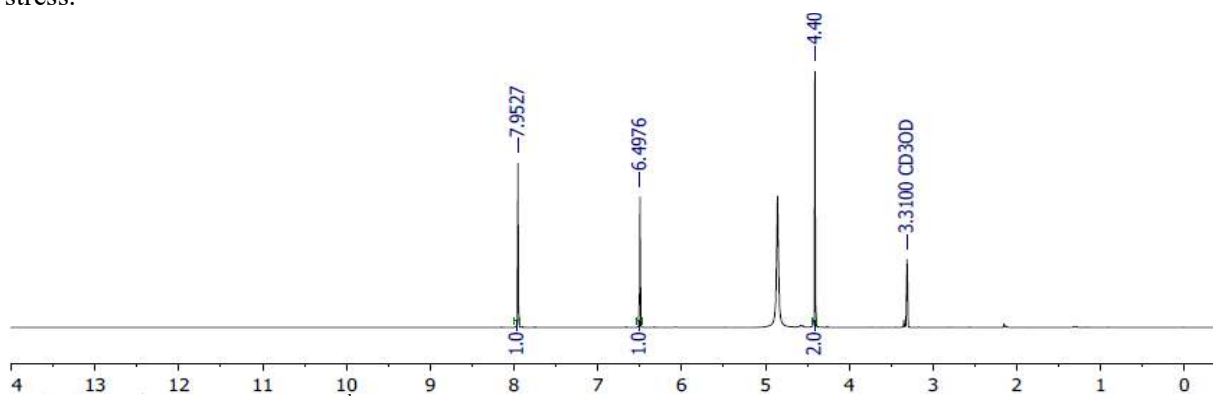


Fig.-4: ¹H NMR (500 MHz) Spectra for Kojic Acid (1) in CD₃OD

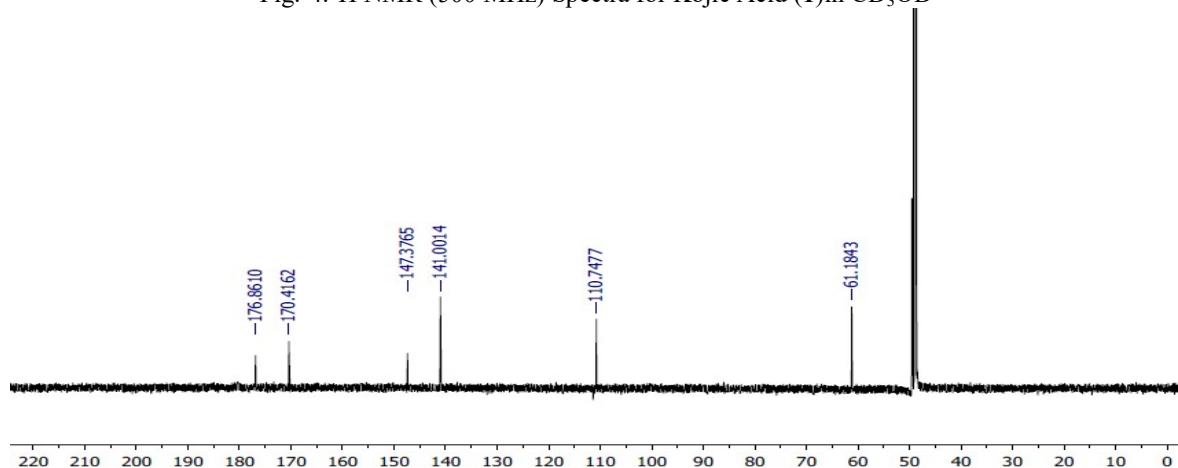


Fig.-5: ¹³C NMR (125 MHz) Spectra for Kojic Acid (1) in CD₃OD

CONCLUSION

An antibacterial metabolite, kojic acid was isolated in high yield from the Endophytic fungi *Aspergillus flavus* collected from the leaves of mangrove plant *Sonneratia alba* in Timor Island, Indonesia. Besides its antibacterial properties, kojic acid has been widely used in the cosmetics and pharmaceutical industry especially for UV B protection. The result obtained in this study confirmed the importance of the ecological factor in directing the specific compound production by the endophytic fungi.

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