



BJP

Bangladesh Journal of Pharmacology

Research Article

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Aquilaria malaccensis Lamk**

Study of an endophytic fungus from *Aquilaria malaccensis* Lamk

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Article Info

Received: 30 March 2010

Accepted: 22 April 2010

Available Online: 5 May 2010

DOI: 10.3329/bjp.v5i1.4687

Cite this article:

Shoeb M, Begum S, Nahar N. Study of an endophytic fungus from *Aquilaria malaccensis* Lamk. Bangladesh J Pharmacol. 2010; 5: 21-24

Abstract

Nine endophytic fungal strains labeled as AL-1, AL-2, AS-1, AS-2, AS-3, AS-4, AR-1, AR-2 and AR-4 were isolated from the medicinal plant *Aquilaria malaccensis* Lamk. Three compounds 1,7-dihydroxy-3-methoxyanthraquinone, propyl p-methoxy phenyl ether and 6-methoxy-7-O-(p-methoxyphenyl)-coumarin were isolated from the ethyl acetate extract of the strain AL-2 by silica gel column chromatography and HPLC. The structures of isolated compounds were elucidated by spectroscopic techniques i.e. UV, IR, 1D and 2D NMR. Extracts of the strain AL-2 were assessed for antibacterial activities against Gram positive and Gram negative bacteria and general toxicity against brine shrimp lethality assay. The ethyl acetate extract showed only mild activity against two Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*).

Introduction

Natural products, derived from microorganisms and plants, have been used for the treatment of various diseases and a large number of modern drugs have been developed from them. The word endophyte means in the plant and refers to all microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no apparent symptoms of disease (Petrini, 1991). Endophytic fungi spend the whole or parts of its life cycle colonizing inter or intracellularly inside the healthy tissues of the host plant. Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal, immunosuppressive and biocontrol compounds. A large number of secondary metabolites have been isolated and characterized from endophytic fungi since the discovery of endophytes in Daren, Germany in 1904 (Strobel et al., 2004). The discovery of penicillin from *Penicillium notatum* inspired scientists to work with fungi. Cryptocandin, isolated from *Cryptosporiopsis quercina*, are currently being considered for use against a number of fungal diseases of the skin and nails

(Strobel et al., 2004; Strobel et al., 1999). Taxol, an anticancer agent, was originally isolated from the bark of the Pacific Yew tree *Taxus brevifolia* (Wani et al., 1971). Later, it was discovered that fungi (*Taxomyces andreanae* and *Pestalotiopsis microspora*) associated with this yew tree also produce taxol (Strobel et al., 1996). As part of our ongoing research on endophytic fungi of Bangladeshi medicinal plants several secondary metabolites were isolated from different endophytes. *Aquilaria malaccensis* Lamk (locally known as Agor) is one of the medicinal plants of Bangladesh. In this paper, we report the isolation of endophytic fungi from *A. malaccensis* and study of the fungal strain AL-2 for chemical and biological properties.

Materials and Methods

Instrument

UV and IR spectra were recorded on Shimadzu UV 160A and Shimadzu IR-470 spectrometer, respectively. The ¹H and ¹³C NMR spectra were recorded on a Bruker 400 MHz spectrometer using tetramethylsilane (TMS)



as the internal reference. High performance liquid chromatography (HPLC) separation was performed on reversed phased C-18 (25 cm x 4.6 mm, 5 μ m, injection volume: 100 μ L) column using Shimadzu Class 10 VP with UV-Visible detector at wavelength 254 nm. Media was prepared under Laminar flow (Thermo Forma. Class 11 A1; Biological safety cabinet). Media was sterilized using Hirayama autoclave (Hirayama MFG Corp.).

Plant collection and sterilization

The medicinal plant *Aquilaria malaccensis* Lamk (locally known as Agor) was collected from Sylhet. Different parts of the plant were cut into small pieces. Each plant part was surface sterilized with 70% ethanol, 3% sodium hypochlorite and sterile water (Schulz et al., 1993). The agar media was autoclaved at 120°C at 1 atmosphere pressure for 30 min.

Isolation of endophytic fungi

The surface sterilized different part of plant materials i.e. leaves, stems and roots were inoculated on autoclaved potato-carrot agar media on a sterilized petri dish (90 mm in diameter) and after 21 days, twenty one fungal strains were isolated for identification. By dereplication of common fungi, nine strains labeled as AL-1, AL-2, AS-1, AS-2, AS-3, AS-4, AR-1, AR-2 and AR-4 were found to have optimum growth for further cultivation. The fungal strain AL-2 was selected for chemical and biological studies.

Culture extraction

The fungal strain AL-2 was fermented in a large scale (400 petri dishes of 90 mm diameter) in the potato-carrot agar semi solid medium at ~22-25°C. After 21 days the fungi were collected in a round bottom flask and the content was then subjected to freeze-drying. The dried powdered material was extracted with ethyl acetate (3 x 1,500 mL for 24 hours, at room temperature). The ethyl acetate extract was evaporated to dryness and 250 mg extract was obtained. A small part of the extract (~20 mg) was saved for anti-bacterial and brine shrimp lethality assays. The rest of the extract was used to isolate pure compounds.

Antibacterial activity assay

The antibacterial activity of ethyl acetate extract of the strain AL-2 was performed against two Gram positive bacteria i.e. *Bacillus cereus*, *Staphylococcus aureus* and five Gram negative bacteria i.e. *Escherichia coli* 0157, *Salmonella typhi*, *Shigella boydii*, *Pseudomonas aeruginosa* and *Vibrio cholerae* by disk diffusion assay (Atta-ur-Rahman et al., 1999).

The specific concentration of the extract was 12 mg/mL. Sterile filter paper disc of 6 mm in diameter were loaded with 200 μ g/ disc using micropipette and was

dried under laminar airflow hood. Standard antibiotic, *Kanamycin* (30 μ g/ disc) was used as a positive control. The loaded discs were placed in petri dish (90 mm in diameter) containing sterile nutrient agar medium seeded with test microorganisms using sterile transfer loop for antibacterial screening. The plates were kept at 4°C for 12 hours. Then the plates were kept in an incubator at 37°C for 12-18 hours. The antibacterial activities of the test agent were determined by measuring the diameter of the zone of inhibition in mm. Zone of inhibition ranging from 7-9 mm was considered insignificant and zone of inhibition 10-12 mm, 13-15 mm and above 15 mm were considered as mild, moderate and significant activity, respectively.

Brine shrimp lethality Assay

The ethyl acetate extract of the strain AL-2 was tested against brine shrimp lethality assay for general toxicity using different concentration of the extracts and following the protocol proposed by Meyer (Meyer et al., 1982). By counting the number of brine shrimps died after 24 hours and after 48 hours the LD50 value for the parent ethyl acetate extract was calculated manually.

Chemical studies of the extract

The extract (200 mg) was dissolved in chloroform and silica gel (400 mg) was added to it. A column was packed with silica gel using hexane as column equilibrating solvent. After application of the sample, solvents of increasing polarities from 100% hexane to dichloromethane (DCM) and finally 50% methanol with dichloromethane were used for elution. The fractions were collected in test tubes and on the basis of their R_f values on TLC, sixteen fractions were obtained. Among them one fraction gave single spot in TLC and collected as pure compound I (4.0 mg) after washing with *n*-hexane. One of the fractions S-16 (20 mg) gave three spots with tailing in TLC (6% methanol in dichloromethane) and was purified by HPLC using mobile phase methanol and water (40:60). Finally, two compounds II (3.5 mg) and III (3.5 mg) were isolated by HPLC from S-16.

Properties of the isolated compounds

Compound I: Yellow colored and gummy (4.0 mg), UV (in DCM): λ_{max} 262, 274 and 415 nm. IR (KBr pellet): ν_{max} 2900, 2850, 1720, 1450 and 1250 cm^{-1} . 1H NMR (CDCl₃, TMS as standard): 7.8 (d, J=7.1 Hz, 2H), 7.7 (d, J=7.1 Hz, 2H), 7.5 (s), 7.1 (s), 7.0 (s), 3.9 (s), 12.1 (bs) ppm.

Compound II: Light yellow colored and solid (3.5 mg), UV (in DCM): λ_{max} 228, 278 nm. IR (KBr pellet): ν_{max} 1512, 1457, 1075 cm^{-1} . 1H NMR (CDCl₃, TMS as standard): 7.5 (d, J= 8.4 Hz, 2H), 6.6 (d, J= 8.4 Hz, 2H), 4.8 (d, J=6.8 Hz, 2H), 4.6 (d, J=6.9 Hz, 2H), 3.7 (s), 3.7 (t, J=7.2 Hz), 1.3 (m), 1.0 (t, J=7.2 Hz) ppm.

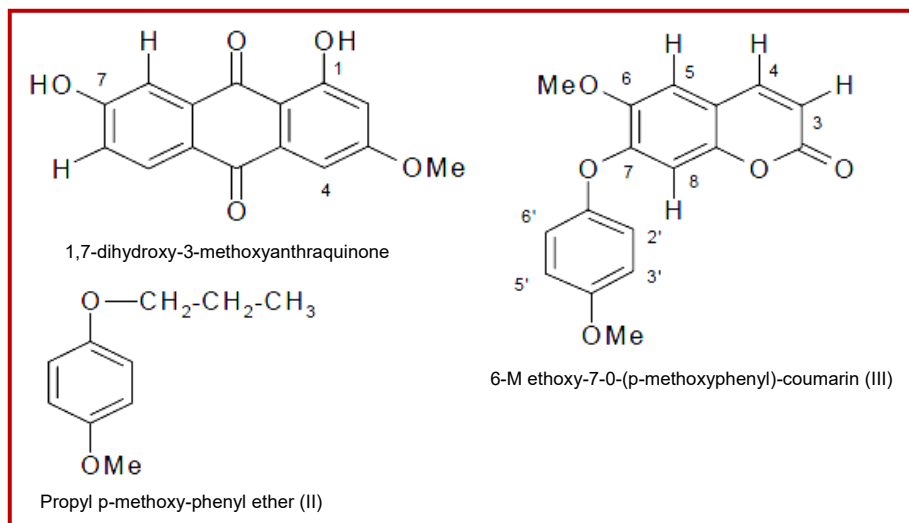


Figure 1: isolated from the ethyl acetate extract of the strain AL-2 by silica gel column chromatography and HPLC

Compound III: Off-white and solid (3.5 mg), UV (DCM): λ_{max} 230, 260, 295, 345 nm. IR (KBr pellet): ν_{max} 1720, 1512, 1490 cm^{-1} . ^1H NMR (CDCl_3 , TMS as standard): 7.6 (d, $J=9.0$ Hz), 7.3 (d, $J=7.4$ Hz), 7.1 (s), 7.0 (d, $J=7.4$), 6.6 (s) 6.1 (d, $J=9.0$), 3.8 (s), 3.6 (s) ppm.

Results and Discussion

Nine endophytic fungal strains labeled as AL-1, AL-2, AS-1, AS-2, AS-3, AS-4, AR-1, AR-2 and AR-4 were isolated from *A. malaccensis*. Three compounds I, II and III were isolated from the ethyl acetate extract of the strain AL-2 by silica gel column chromatography. The NMR spectrum of I gave five signals in the aromatic

region. Two doublets at 7.8 ($J=7.1$ Hz) and 7.7 ppm ($J=7.1$ Hz) indicated two aromatic protons in o-position coupling each other. Other three singlets in the aromatic region at 7.5, 7.1, 7.0 ppm indicated that they do not have any neighboring hydrogen. One sharp signal at 3.9 ppm was assigned for oxygenated methyl proton. The chemical shift at 12.11 ppm was due to the chelation of H-bonding of hydroxyl group and carbonyl-oxygen of aromatic system (Aly et al., 2008). The ^1H NMR data of I was similar to macrosporin, a substituted anthraquinone, reported from an endophytic fungus of *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*. The compound I showed UV absorbance at 262, 274 and 415 nm. The UV absorption fitted very well with reported values (264, 274 and 416 nm) of anthraquinone isolated from other sources (Aly et al., 2008; Debbab et al., 2009). From ^1H NMR and UV absorbance, the structure of I was confirmed as 1,7-dihydroxy-3-methoxyanthraquinone (Figure 1). The IR spectrum of II showed absorbances at 1512, 1457 and 1075 cm^{-1} due to the presence of aromatic moiety and ether group. In ^1H NMR spectrum of II two most downfield signals at 7.5 ($J=8.4$ Hz, 2H) and 6.6 ppm ($J=8.4$ Hz, 2H) indicated the presence of four aromatic protons. Two sets of doublet at 4.8 ppm ($J=6.8$ Hz, 2H) and 4.6 ppm ($J=6.9$ Hz, 2H) were accounted for two protons of oxygenated methylene group. A sharp singlet (3H) at 3.7 ppm was for an oxymethyl group. Triplet at 3.7 was due to two protons attached of oxygenated methylene group. Chemical shift at 1.0 and 1.3 ppm were due to methyl proton and methylene proton, respectively. In the ^2D - ^1H COSY spectrum strong crossed peak between 6.6 and 7.5 ppm indicated that they coupled with each other.

Another correlation was also observed between 1.0 ppm with 1.3 ppm. Combining IR, ^1H -, ^2D - ^1H COSY

Table I		
Zone of inhibition of ethyl acetate extract of AL-2		
Name of organisms	Zone of inhibition ^a	
	Kanamycin (30 pg/disc)	Ethyl acetate extract (200 pg/disc)
Gram-positive bacteria		
<i>Bacillus cereus</i>	36	12
<i>Staphylococcus aureus</i>	40	10
Gram-negative bacteria		
<i>Escherichia coli</i> 0157	37	0
<i>Shigella boydii</i>	39	0
<i>Pseudomonas aeruginosa</i>	42	0
<i>Salmonella typhi</i>	40	0
<i>Vibrio cholerae</i>	35	9

^a7-9 mm: insignificant, 10-12 mm: mild activity, 13-15 mm: moderate activity, above 15 mm: significant

spectral data, II was determined as propyl p-methoxy phenyl ether.

The UV spectrum (in methanol) of III showed absorption bands at 230, 260, 295 and 345 nm which suggested the presence of coumarin skeleton (Kwak et al, 2001). In the IR spectrum, absorbances were observed at 1720, 1512 and 1490 cm^{-1} indicating the presence of coumarin skeleton. In the ^1H NMR spectrum, two doublets at 7.6 ($J=9.0$ Hz) and 6.1 ($J=9.0$ Hz) ppm were due to two aromatic protons. Two singlets at 7.1 and 6.6 ppm were also for two aromatic protons without having any neighboring hydrogen. The ^1H NMR spectrum also showed two doublets at 7.3 ppm ($J=7.4$ Hz) and 7.0 ppm ($J=7.4$ Hz). Strong correlation between chemical shift at 7.3 ppm and chemical shift at 7.0 ppm in the ^1H - ^1H COSY spectrum indicated that they were p-substituted aromatic protons. Two sharp singlets at 3.8 (3H) and 3.6 (3H) were due to two oxymethyl groups attached to aromatic moiety. The ^1H NMR signals of III was indicative of a coumarin nucleus (Kwak et al., 2001). Combining spectroscopic data i.e. UV, IR, ^1H NMR and 1H-1H COSY, the structure of III was elucidated as 6-methoxy-7-O-(p-methoxyphenyl)-coumarin.

The antibacterial activity of ethyl acetate extract of AL-2 was performed against two Gram positive bacteria i.e. *Bacillus cereus*, *Staphylococcus aureus* and five Gram negative bacteria i.e. *Escherichia coli* 0157, *Salmonella typhi*, *Shigella boydii*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. The extract showed mild activity against two Gram positive bacteria i.e. *Bacillus cereus* and *Staphylococcus aureus* (Table I). The ethyl acetate extract of AL-2 was also tested for general toxicity against brine shrimp lethality assay and was found low activities. Further study is necessary to determine the relationship between the endophyte and host plants.

Conflict of Interest

Authors declare no conflict of interest

Acknowledgement

The authors are grateful to International Foundation for

Sciences, Sweden and International Program in the Chemical Sciences, Uppsala, Sweden for financial supports. Shahanara is grateful to BOSE Center for Advanced Studies and Natural Sciences, University of Dhaka for research fellowship.

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