Tenuazonic acid: a promising antitubercular principle from *Alternaria alternata*

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Abstract

Bioactivity guided isolation of dichloromethane extract of *Alternaria alternata* identified tenuazonic acid (1) as potentially active against *Mycobacterium tuberculosis* H37Rv, MIC at 250 µg/mL concentration. This active metabolite 1, was also evaluated for osmotic hemolysis using the erythrocyte as a model system. It was observed that this fungal metabolite showing antitubercular activity exhibited concentration dependent toxicity to human erythrocytes.

Introduction

Fungi play an important role among a variety of species produced by nature.¹ Fungi produce and metabolize a broad range of simple to very complex organic compounds.¹ These fungal metabolites are generally termed as mycotoxins due to their toxic effects and risk to humans.² Ironically, in the recent past these fungal metabolites have become an interesting source of molecules due their possible uses as therapeutics.¹ *Alternaria* is a widespread fungal genus known for the high pathogenicity of some of its members and several species are known as plant pathogens.^{3,4} *Alternaria alternata* is an opportunistic pathogenic fungi on numerous hosts causing leaf spots, rots and blights on many plant parts. A number of metabolites are already reported from this fungus.⁵

In the present study, we have investigated antitubercular activity of the endophytic fungal strain *Alternaria alternata*, which was isolated from the leaves of the *Indigofera enneaphylla*. On systematic chromatographic isolation, we found tenuazonic acid (1, TA) as bioactive compound responsible for antitubercular activity.

Materials and Methods

General

All the laboratory chemicals were purchased from Thomas Baker, India. The Nuclear Magnetic Resonance (NMR) solvents were procured from Sigma Aldrich USA. Thin layer chromatography (TLC) was done on Merck aluminium sheet thin layer chromatography (TLC, UV254nm) plates. NMR spectra were measured on a Bruker Avance 300MHz instrument with TMS as an internal standard. Electron Impact ionization mass (EI mass) spectra were recorded on Perkin-Elmer Turbo Mass GC-MS system after dissolving the compounds in methanol.

Isolation of endophytic fungus from medicinal plant

The healthy medicinal plant (*Indigofera enneaphylla* L.) leaf samples were obtained from Herbal Garden, Centre for Advanced Studies in Botany (CAS in Botany), Guindy Campus, University of Madras, Chennai, India. *Alternaria alternata* SVJM015 was isolated from surface-treated leaves of *I. enneaphylla* and the fungus was identified using standard monographs. The identified strain (*A. alternata* SVJM015) was deposited to Madras University Botany Laboratory (MUBL), CAS in Botany, University of Madras, Chennai - 600 025.

The collected leaf samples were surface sterilized for the isolation of endophytic fungi. The healthy leaf samples were first washed in running tap water and processed as follows. The samples were sliced into small segments (approximately 0.5 cm²) to screen the presence of endophytic fungal isolates. Leaf segments were surface sterilized with 70% ethanol for 5 sec., immersed in 4% NaOCl for 90 sec., rinsed in sterile distilled water and then dried on sterile filter paper. The surface sterilized plant segments were inoculated on to a petriplates containing potato dextrose agar (PDA) medium amended with streptomycin (30 µg/mL). The petriplates were sealed with parafilm™ and incubated at 25±1°C in a growth chamber with 12 h light followed by 12 h of dark regime and checked from the second day for fungal growth.⁶ The hyphae growth was observed from the tissues on PDA plates, immediately it's were transferred in to fresh PDA plates for further studies.

Molecular characterization of endophytic fungal isolate (*A. alternata* SVJM015)

The fungal DNA was amplified using the Internal Transcribe Sequencer (ITS) primers such as ITS1 (F 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (R 5'-TCC TCC GCT TAT TGA

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TAT GC-3') for the molecular identification.⁷ Amplification was carried out with 20-µL reaction mixture containing 1X PCR buffer, 2.5 µM MgCl₂, 0.6 µM of each dNTPs, 0.25 µM of each primers, 1.25 U Taq polymerase and 4 ng gDNA. The reagents were obtained from QIAGEN (QIAGEN, Chatsworth, CA, USA). PCR amplification was performed in a T-Gradient thermal cycler (Biometra, Gottingen, Germany). The amplification starts with initial denaturation at 95°C for 3 min., followed by 34 cycles with denaturation at 94°C for 15 sec., annealing at 55°C for 45 sec., extension at 72°C for 55 sec. and the final extension at 72°C for 7 min. Negative control (no template DNA) was included in this study. The amplified PCR products were purified with QIAquick PCR purification kit (QIAGEN). The purified PCR products were sequenced; the sequence data was BLAST and submitted with NCBI database (National Centre for Biotechnology Information, USA) for GenBank accession number.

Fungal culturing and extraction

The endophytic isolate, *A. alternata* SVJM015 was selected for the screening of bioactive compounds production and grown in 4 L Hopkins flasks containing 2 L of M1D medium supplemented with soytone (1 g/L) and incubated at 25°C±1 for 21 days in still culture.⁸ After incubation, the fungal culture was passed through a four layered of cheese-cloth.

Then, the culture filtrate was extracted with two equal volumes of methylene chloride and the organic phase was evaporated to dryness under reduced pressure at 35°C. The residue obtained was further processed for purification.

Bioactivity guided isolation of fungal metabolites

The crude mass obtained (315 mg) from methylene chloride was charged on silica gel (100-200 mesh, 2x40 cm glass) column and eluted successively with hexane (200 mL), chloroform-hexane (20% x 500 mL) and subsequently increasing polarity by adding chloroform (40%, 60% and so on). At 60% chloroform-hexane compound A (~4 mg, 2) was obtained as oil. At 80% chloroform-hexane another compound B (~42 mg, 1) was obtained as oil.

In vitro antimycobacterial assay by BACTEC radiometric susceptibility assay

The antimycobacterial assay was done radiometrically as per previously reported method.^{9,10} Rifampicin (Sigma Chemicals, USA), a standard drug for tuberculosis was used as positive control.

Determination of erythrocytes osmotic fragility

The *in vitro* erythrocyte osmotic fragility test was done as per previously described procedure.^{11,12,13} Curcumin was used as erythrocyte membrane stabilizer (positive control), while hydrogen peroxide was used as erythrocyte fragility inducer (Figure 1).

Results and Discussion

On chromatographic purification of methylene chloride soluble fraction of *A. alternata* resulted in identifying TA (1, 5S,6S-3-acetyl-5-sec-butyl-4-hydroxypyrrolidone-2,4-dione) as active constituent against *Mycobacterium tuberculosis*. TA obtained as oil, showed molecular ion peak at 197 [M⁺] and 198 [M+1]⁺ consistent to molecular formulae C₁₀H₁₅NO₃. In ¹³C NMR, compound 1 showed 3 methyls, one methylene, two methines, and four quaternary carbons. Compound 1 was ascertained as tenuazonic acid on the basis of ¹H NMR, ¹³C NMR, distortionless enhancement of polarization transfer (DEPT)-90, DEPT-135. The ¹H and ¹³C NMR spectral data of TA are well in agreement with the earlier published data¹¹ (Table 1). Another compound 2 was also isolated as oil, but due to insufficient quantity, its complete structure could not be ascertained. Antitubercular compounds from this fungus have not been reported previously. In this study, antitubercular activity of TA was found to be twice as active as the dichloromethane extract. The antitubercular activity of dichloromethane extract was at 500 µg/mL against *Mycobacterium tuberculosis* H₃₇Rv strain through BACTEC assay. The minimum

inhibitory concentration (MIC) of test compound was noted on the basis of GI (growth index) value. The antitubercular activity of TA was found to be 250 µg/mL against the same strain of *M. tuberculosis* (Table 2).

Antifungal, antibacterial, cytotoxic and antiviral properties of TA are previously reported. TA has exhibited antibiotic activity in inhibiting *Paenibacillus larvae*, which is the causal agent of American foulbrood, a honeybees' disease.¹⁴ Recently, some of the analogues of TA have been reported as antifungal

and herbicidal agents¹⁵ We confirm here antitubercular activity of TA which is not reported earlier.

In conclusion, on bioactivity guided isolation tenuazonic acid has been reported as antitubercular constituent in *A. alternata*. This compound exhibited toxicity to human erythrocytes in a concentration dependent manner i.e. higher concentration of the compound increased the fragility whereas, lower concentration did not. This may provide some structural clues for future development of new antitubercular agents.

Figure 1. Osmotic fragility curve of erythrocyte treated with compounds 1 and 2.

Table 1. Spectral data of Tenuazonic acid (1).

Assignments	¹³ C (CDCl ₃ , 75MHz) δ values (ppm)	Compound 1 ¹ H (CDCl ₃ , 300MHz) δ values (ppm)	Mass (EI Mass)
N1	--	7.79 (s, 1H, exchangeable, NH)	197 [M] ⁺ , 198
C2	175.63 (QC)	---	[M+1] ⁺ , 195 [M-
C3	102.45 (QC)	---	2] ⁺ , 168 [M-29] ⁺ .
C4	184.20 (QC)	11.55 (exchangeable, OH)	
C5	67.23	3.79 (CH)	
C6	36.83	2.51 (CH)	
C7	23.46	1.23 (CH ₂)	
C8	11.51	0.998 (CH ₃)	
C9	15.67	1.02 (CH ₃)	
C10	195.71 (QC)	---	
C11	19.34	2.46 (CH ₃)	

Assignments are based on ¹H, ¹³C, DEPT 135 & DEPT 90 experiments. QC= quaternary carbon, OH= hydroxyl group, NH= amine group.

Table 2. Antimycobacterial activity of compounds and extract against *M. tuberculosis* H37Rv.

S. no.	Compound no.	Minimum Inhibitory Concentration (MIC) µg/mL
1.	Dichloromethane extract	500
2.	1	250
3.	2	Inactive
4.	Rifampicin	2

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