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Original article

Antimycobacterial quinoline alkaloid from the root wood of teclea amaniensis engl.

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ABSTRACT

The phytochemical analysis on the root wood of Teclea amaniensis afforded a quinoline alkaloid veprisine. Its chemical structure was deduced using Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) analyses. Veprisine was screened for antimycobacterial activity against two mycobacterial strains namely; Mycobacterium madagascariense (MM) DSM 44641 and Mycobacterium indicus pranii (MIP) DSM 45239. It exhibited moderate to higher antimycobacterial activity against test organisms with the MIC values of 657.9 µM and 2.63 x 103 µM against MM and MIP respectively. In the same assay isoniazid (INH), a first line anti-TB drug lacked efficacy, even at higher concentration. Consequently, veprisine was further screened to determine its ability to potentiate the activity of isoniazid against the two resistant mycobacteria strains. The assay was done by screening the combination of 1/2 to 1/16 MIC values of veprisine and isoniazid (adopted MIC values of INH against M. tuberculosis Mtb H37Rv strain). Veprisine potentiated the activity of INH against MM and MIP with fractional minimum inhibitory concentration (FMIC) values of 328 µM and 164 µM respectively. The FMIC values are equivalent to 1/2 and 1/16 MIC values of veprisine against MM and MIP respectively. When INH and veprisine were tested alone within this range, they lacked efficacy

against the test organisms. These results show that veprisine is bioactive against Mycobacterium species and it has the ability to potentiate the activity of isoniazid against resistant strains.

1. Introduction

Teclea Engl., (Rutaceae) is a small genus of shrubs, lianas and small trees widespread in the tropical and subtropical regions. Plant species of this genus have various ethnomedical applications which include treatment of chest complaints, venereal diseases, as an analgesic and antipyretic (Al-Rehaily et al., 2003; Waffo et al., 2007). Previous reports indicate that Teclea species are rich in acridone and furanoquinoline alkaloids (Al-Rehaily et al., 2003; Waffo et al., 2007; Magadula et al., 2008).

Teclea amaniensis Engl., is a small tree endemic to the Amani Nature Reserve, Tanga region in Tanzania. Little has been done to investigate its phytochemical constituents of this species. The report by Magadula and co-workers (2008) revealed the presence of furanoquinoline and acridone alkaloids which is a typical chemotaxonomical characteristic of the genus Teclea. Although there is only one report on its chemical constituents, nothing is known about pharmacological properties of its extracts and compounds. In our continued interest to search for potential antimycobacterial natural products, this article reports the isolation and in vitro antimycobacterial activity of veprisine, a quinoline alkaloid isolated from the root wood of Teclea amaniensis.

2. Materials and methods

2.1. General procedures

The 1-D and 2-D NMR Spectral data of the isolated compounds were obtained using Bruker Avance NMR machine operating at a spectrometer frequency of 300 MHz for 1H-NMR and 75 MHz for 13C-NMR. EI-MS spectra were recorded on a Finnigan MAT SSQ 7000 Single Quadrupole Instrument. Melting points were measured on a Stuart Scientific (SMP1) melting point apparatus.

2.2. Chemicals and growth media

All solvents were purchased from Carlo Erba (France), Middlebrook 7H9 broth base was obtained from HIMEDIA (India), Glycerol (AR) obtained from Lab Equip Ltd (Tanzania), iodonitrotetrazolium (INT) chloride, Ciprofloxacin and Isoniazid (R&D) were purchased from Sigma (UK). Ninety six wells micro-titre plates supplied by KAS Medics (Tanzania). Silica gel Kiesegel 60 PF254 obtained from Merck South Africa Pty. Pre-coated Aluminium backed silica gel 60 F254 (0.2 mm thickness) TLC plates were obtained from Merck UK.

2.3. Plant materials

The roots of Teclea amaniensis were collected from Amani Forest reserve in Muheza, Tanga. The plant species was identified by a plant taxonomist MR Haji Selemani from the Department of Botany, University of Dar es Salaam. A voucher specimen HS1367 was thereafter deposited at the Institute of Traditional Medicine Herbarium of Muhimbili University of Health and Allied Sciences for future references. The root woods were chopped into small pieces and allowed to air dried for 7 days before being pulverized using electric miller. The powdered root woods were kept in an air tight container ready for extraction processes.

2.4. Extraction and isolation of veprisine from root extracts

800g of pulverized root wood were soaked in methanol (AR) for 24 hours. The obtained filtrate/extract was concentrated in vacuo using a rotary evaporator to yield 26 g of crude extracts. Eleven grams of methanol extracts were adsorbed in silica gel and loaded on a silica gel column chromatography eluting with Pet-ether/dichloromethane (1:9). A total of 22 fractions each with 150 ml of eluates were collected. Thereafter the mobile phase was changed to dichloromethane/acetone (9.5:0.5) yielding 20 more fractions each with 150 ml of eluates. After TLC analysis, fractions 1 – 6 were discarded, while fractions 7 - 8 and 9 – 21 were combined. The

remaining fractions 22 – 40 were combined and concentrated in vacuo and kept for future use. A combined fractions 9-21 was adsorbed in silica gel and loaded on column chromatography eluting with chloroform/Pet-ether/acetone (7.5:2:0.5). A total 27 sub-fractions were collected and left to stand overnight. From sub-fractions 8-19 cream colored crystals formed and were collected and washed with acetone 100% to afford a pure compound weighing 382 mg. Furthermore, combined fractions 7 – 8 were loaded on a silica gel 60FP column and eluting with chloroform/pet ether (9:1) mobile phase. After TLC analysis, sub-fractions 8 – 14 seemed to have the same compound as a single spot. These were left to stand overnight to afford cream crystals weighing 36.2 mg. The two separately collected crystals were spotted on TLC and developed using Chloroform/pet-ether/acetone (9:0.5:0.5), and they all had the same retention factor (RF) values. The TLC was sprayed with Dragendorff reagent to give deep orange colored spots against yellow background. The combined crystals were later identified as a quinoline alkaloid, veprisine.

2.5. Antimycobacterial screening of veprisine

2.5.1. Test organisms

The mycobacteria strains, namely Mycobacterium madagascariense (MM) DSM 44641 and Mycobacterium indicus pranii (MIP) DSM 45239 were supplied by the Germany Resource Centre for Biological Materials, Braunschweig, Germany. The two fast growing mycobacteria strains were used as markers for determination of potential anti-TB efficacy of veprisine.

2.5.2. Sub-culturing of Mycobacterium species

The strains were sub-cultured in Middlebrook 7H9 broth base supplemented with glycerol. The medium was prepared by suspending 1.18 g of Middlebrook 7H9 broth base in 230 ml of distilled water in a Scotch bottle (500 ml) followed by addition of 1 ml of glycerol (AR). The mixture was heated to dissolve the broth base completely, thereafter autoclaved at 121oC for 15 minutes. The mixture was left to cool to 31 and 35oC under lamina flow, before separately being inoculated with Mycobacterium madagascariense (MM) and Mycobacterium indicus pranii (MIP) respectively. Thereafter MM was incubated at 31oC while MIP was incubated at 37oC. The optimal growth of bacteria cultures were observed between 5 to 7 days, and were ready for antimycobacterial screening.

2.5.3. Determination of minimum inhibitory concentration (mic)

The MIC values of steroids against two Mycobacterium strains were determined by two fold microdilution method as described by Ellof (1998) and Erasto et al., (2011).

2.5.4. Potentiation of antimycobacterial activity of isoniazid

Isoniazid (INH) has generally been found to be inactive against M. madagascariense and M. indicus pranii even at higher concentration. This offers the opportunity to investigate the ability of veprisine to potentiate the efficacy of INH against MM and MIP. Adopting the method of Ellof [4], with modification, the fractional minimum inhibitory concentration (FMIC) of veprisine was determined by screening 1/2 to 1/16 MIC values of alkaloids against MM and MIP, blended with 1/2 to 1/16 of the documented MIC value of isoniazid against Mycobacterium tuberculosis (Mtb H37Rv). This implied that, the first wells had 1/2 MIC values of veprisine and INH which was then diluted two folds to the last well which had 1/16 MIC values of test samples. The control wells in this assay were as follow; two rows with veprisine (1/2 to 1/16 MIC values), mycobacteria inoculums and broth only, two rows with INH, mycobacteria innoculum and broth only and positive control wells which had ciprofloxacin, mycobacteria innoculum and broth. The FMIC values of veprisine were determined by addition of 40 µl (0.2 mg/ml) iodonitrotetrazolium (INT) chloride salt into each well and plates incubated at 31oC (MM) and 37oC (MIP) for 1 hour. The FMIC values were read at the concentration where a marked no change in color formation as a result of INT metabolism by active mycobacteria was observed.

3. Results

3.1. Structure elucidation of veprisine

The phytochemical analysis on the roots of Teclea amaniensis afforded 418.2 mg of a quinoline alkaloid, veprisine (1). The chemical structure was deduced through 1-D and 2-D NMR analysis and with comparison with

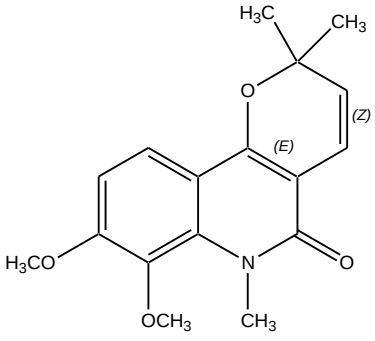
the data existing in literatures. The 1H NMR spectrum of compound 1 showed the presence of two ortho-coupled aromatic proton peaks at δH 7.71 (1H, d, J = 9.0 Hz) and 6.87 (1H, d, J = 9.0 Hz). It further showed two proton peaks which are trans to each other resonating at δH 6.79 (1H, d, J = 13.2 Hz) and δ 5.52 (1H, d, J = 13.2 Hz); two methoxy groups at δH 4.01 and 3.53 (each 3H, s); N-methyl group signal at δH 3.96 (3H, s) and a germinal dimethyl group resonating at δH 1.49 (6H, s). See Table 1 (Ayafor et al., 1980; Ramesh et al., 1984; Ngadjui et al., 1988).

The 13C NMR spectrum showed sixteen peaks out of which eight were quaternary (δC 162.5, 155.5, 155.0, 136.8, 134.6, 112.1, 103.9, 78.6); four methine carbon peaks (δC 125.5, 119.0, 118.0, 107.2); two methoxy carbon peaks (δC 61.6, 56.2); one N-methyl carbon peak at δC 33.5 and two germinal methyl carbons resonating at the same chemical shift of δC 28.1 as shown in Table 1. The spectroscopic data above compared well with the reported by Ngadjui and co-workers (1988). Compound 1 was therefore confirmed as veprisine (Fig. 1).

Veprisine (1): Cream crystals (418.2 mg); mp 176 - 179°C; EI-MS: m/z 301 [100%, M+] calculated for C17H19NO4; 1H NMR (CDCl3, 300 MHz;): δ 7.71 [1H, d, J = 9.0 Hz], 6.87 [1H, d, J = 9.0 Hz], 6.79 [1H, d, J = 13.2 Hz], 5.52 [1H, d, J = 13.2 Hz], 4.01 [3H, s], 3.96 [3H, s], 3.53 [3H, s], 1.49 [6H, s]; 13C NMR (CDCl3, 75 MHz;) δ 162.5 (C-1), 155.5 (C-8), 155.0 (C-7), 136.8 (C-3), 134.6 (C-9), 125.5 (C-3′), 119.0 (C-5), 118.0 (C-4′), 112.1 (C-2), 107.2 (C-6), 103.9 (C-4), 78.6 (C-2′), 61.6 (C-8OCH3), 56.2 (C-7OCH3), 33.5 (NCH3), 28.1(2) (C-2′CH3).

Table 1
1H and 13C NMR data for veprisine (1).

Position	δC, type	δH (J in Hz)	COSY	HMBC
C-1	162.5, C			
C-2	112.1, C			
C-3	136.8, C			
C-4	103.9, C			
C-5	119.0, CH	7.71, 1H, d, J = 9.0	H-6	C-4, C-6
C-6	107.2, CH	6.87, 1H, d, J = 9.0	H-5	C-5, C-7
C-7	155.0, C			
C-8	155.5, C			
C-9	134.6, C			
C-2′	78.6, C			
C-3′	125.5, CH	5.52, 1H, d, J = 13.2	H-4′	C-2′, C-3′
C-4′	118.0, CH	6.79, 1H, d, J = 13.2	H-3′	C-1, C-2, C-3′
C-7 OCH3	56.2, OCH3	4.01, 3H, s		C-7, C-8
C-8 OCH3	61.6, OCH3	3.53, 3H, s		C-7, C-8, C-9
NCH3	33.5, CH3	3.96, 3H, s		C-1, C-9
C-2′CH3(2)	28.1, CH3(2)	1.49, 6H, s		C-2′, C-3′



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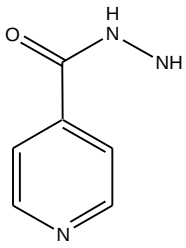

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Fig. 1. Chemical structure of Veprisine (1) and Isoniazid (2).

3.2. Antimycobacterial screening of veprisine

Veprisine was screened for antimycobacterial activity using a two folds microdilution method. In this assay, two mycobacteria strains namely; Mycobacterium madagascariense (MM) and M. indicus pranii (MIP) were used to determine the efficacy of veprisine. The alkaloid exhibited moderate to higher antimycobacterial activity against test organisms. It had the MIC values of 657.9 µM and 2.63 x 103 µM against MM and MIP respectively (Table 2). As reported in various literatures, MM and MIP are resistant to INH even at higher concentration, this was similarly observed in this assay (Kadza et al., 1992; Saini et al., 2009; Erasto et al., 2011; Erasto, 2012). In the same experiment, ciprofloxacin exhibited higher antimycobacterial activity with an MIC value of 0.05 µg/ml against all test organisms.

The drug combination experiment between INH (1/2 to 1/16 MIC value of INH against Mtb) with micro-molar concentration of veprisine (ranging from 1/2 to 1/16 MIC value), was designed to investigate whether the alkaloid can potentiate the activity of isoniazid against the two resistant strains. In this assay, the MIC value of INH used was that recorded against Mycobacterium tuberculosis (Mtb H37Rv) which is 8.75µM (~0.12 µg/ml) [12]. Therefore the concentration of INH in the drug combination ranged from 4.375 µM (1/2 MIC) to 0.547 µM (1/16 MIC) against that of veprisine which was 328.9 µM to 41.13 µM for MM and 1315 µM (1/2 MIC) to 164.4 µM (1/16 MIC) for MIP respectively. Veprisine potentiated the activity of INH against MM and MIP with the fractional minimum inhibition concentration (FMIC) of 328 µM and 164 µM respectively (Table 2). These figures are equivalent to 1/2 MIC and 1/16 MIC values of veprisine against MM and MIP respectively. Within the same range, veprisine and isoniazid tested alone lacked efficacy against test organisms. This confirmed further that synergism between the two bioactive molecules exists.

Table 2
Minimum Inhibition Concentration (MIC) and Fractional Minimum Inhibition Concentration (FMIC) of Veprisine (1).

	MIC (µM)		**FMIC (µM)**	
Veprisine and combination of veprisine with INH*	MMa	MIPb	MM	MIP
Veprisine (1)	657.9	2.63 x 103	-	-
Veprisine + INH** (½ to 1/16 MIC values)	-	-	328 (4.375)**	164 (0.547)**
Veprisine (1/2 to 1/16 MIC)	-	-	NA	NA
INH (1/2 to 1/16 MIC)	-	-	NA	NA
Ciprofloxacin (µg/ml)	0.05	0.05	0.05	0.05

INH* = Isoniazid, aMycobacterium madagascariense; bMycobacterium indicus pranii
** The figures in parenthesis are fractional minimum inhibition concentration values of isoniazid (INH) in the drug combination assay.

These results indicate that some quinoline alkaloids such as veprisine may be useful in enhancing INH activity against resistant mycobacteria strains. The FMIC values for veprisine were quite low compared to their respective MIC values. Further interesting observations were that, the efficacy of veprisine against MIP when screened in combination with INH was twice higher than that of against MM, whilst in the normal antimycobacterial activity, the alkaloid was more active against MM than MIP (Table 2). Similarly, INH had FMIC value of 4.375 µM against MM and 0.547 µM against MIP. These intriguing observations require further investigation to determine the nature of interaction between veprisine and INH. It is important to explore the mode of action through which veprisine potentiates the efficacy of INH against resistant mycobacteria strains. Two of the possible areas to examine include 1) Does veprisine promote inhibition of mycolic acid cell wall synthesis through other mechanisms which may not involve KatG protein, 2) Veprisine promote inhibition of Mycothione reductase redox cycle and active efflux pumps. Once the mechanism of action is deduced, similar experiments can be conducted to evaluate the ability of veprisine to potentiate activity of INH against drug resistant M. tuberculosis strains.

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