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Research Article

TRYPANOCIDAL ACTIVE COMPOUNDS FROM SCOTTISH ABIES NOBILIS AND PINUS SYLVESTRIS

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ABSTRACT
Three active compounds obtained from the aerial parts of *Abies nobilis* and *Pinus sylvestris* isolated by different chromatographic techniques. Their structures were identified by NMR (^{1}H, ^{13}C, COSY, HMBC) spectroscopic and mass spectrometric data and established as catechin, dehydroabietic acid and Dihydroconiferyl alcohol. The isolated compounds were exhibited activity against blood stream form of parasite *Trypanosoma brucei brucei* (S 427).
KEY WORDS: Pinaceae, *Abies nobilis*, *Pinus sylvestris*, Flavanol, diterpene, cinnamic acid, NMR, Antitrypanocidal activity

INTRODUCTION

The family Pinaceae is the second largest family in geographical range after the Cupressaceae. It contains 220 species and 11 genera, found mainly in the northern but also in southern hemisphere. The Pinaceae family originates in the order Pinales, and consists of 4 subfamilies: the Pinoideae, Piceoideae, Laricoideae and Abietoideae [6].

Humans have at all times used plants in a multitude of applications in a tradition spanning human development. Trypanosomiasis is a major public health problem in various African populations who are exposed to sleeping sickness and tourists are also at risk over 66 million people in 36 countries of sub-Saharan Africa are under the threat of Human African Trypanosomiasis (HAT) and each year an average of 500,000 people are infected with HAT and 50,000 deaths are reported. The reported resistances to the available drugs for different species of the parasite and relapse for unknown reasons suggest that there is an urgent need to search for alternative sources of drugs[1].

Chromatographic techniques were applied for the isolation of catechin (**1**), dehydroabietic acid (**2**) and dihydroconiferyl alcohol (**3**) from the aerial parts of *Abies nobilis* and *Pinus sylvestris.* In this paper, we report the isolation, identification and trypanocidal activities of three compounds from the aerial parts of *Abies nobilis* and *Pinus sylvestris.*

MATERIAL AND METHODS

Plant Aerial parts of *Abies nobilis* were purchased from Albatrees, UK in October 2003 and the aerial parts of *Pinus sylvestris* were collected from Whim bog in Edinburgh, Scotland in April 2006 materials. Herbarium specimens were deposited at the Phytochemistry Laboratory at the University of Strathclyde where they were identified and given the following voucher numbers ABNO1003 (*Abies nobilis*), PISY1003 (*Pinus sylvestris*).

Extraction and isolation of pure compounds

The aerial parts materials were extracted in a Soxhlet apparatus by using different solvent systems starting from non-polar *n*-hexane (60-80°C), semi polar chloroform or ethyl acetate and finally polar solvent as methanol for 2 to 3 days. The extracts were concentrated using a rotary evaporator (BUCHI Labortechnik AG, Switzerland) under reduced pressure at a maximum temperature of 50°C and stored at -20°C before use. A range of chromatographic methods were used; such as Column Chromatography (CC) using silica gel (Kieselgel 60; 0.063-0.020mm, Merck), Sephadex LH-20 (Sigma-Aldrich, UK), Vacuum Liquid Chromatography (VLC) using silica gel (Kieselgel 60H PF_{254}, VWR International Ltd, UK) and Flash Chromatography [Flash Master Personal]^{TM}.

Structural elucidations

NMR spectroscopy was extensively used to elucidate the structures of isolated compounds, IR spectra of samples were recorded in an automatic IR spectrophotometer in the solid state as pressed potassium bromide (KBr) discs, HRESIMS of all isolated compounds was performed in FTMS-Orbitrap (ThermoFinnigan Bremen, Germany) to give the exact mass of the molecular ion that is useful to determine the molecular formula, Specific rotation of compounds with optical activity was measured by an automatic polarimeter Auto pol^{R} V.

Bioassays

Alamar Blue Assay (resazurin–reduction test) was carried out to assess the trypanocidal activities of isolated compounds against blood stream form of parasite *Trypanosoma brucei brucei* (S 427).

Test organism

Trypanosoma brucei brucei (S 427) was obtained from Prof. Mike Barratt, Glasgow University.

Preparation of sample and plate

The samples were dissolved in DMSO in such a way that the concentration was 10 mg/ml. 4µl of test solutions were added into column 2 of the plate.

A 196µl of HMI-9 medium was introduced in all wells of the microplate. Serial dilution was carried out from well 2 to 11 which followed half fold dilution in the consecutive well from left to right. A solution of suramin (Sigma) was prepared in filtered sterilized water and had a concentration of 100µM. A 2µl of this solution was added in the wells of 12th column. 100µl suspension of blood stream form of *T. b. brucei* S427 (2-3X10^{4} trypanosomes/ml) was added to each well.

The plate was then placed in an incubator at 37°C under 5% CO_2 humidified air. After a 48h incubation period, 20µl of the REDOX indicator Alamar blue was added to each well and then incubated again for a period of 24hrs. Fluorescence was determined in Wallac Victor at excitation and emission wavelengths of 530 and 590nm, respectively. The protocol was modified according to the previous paper [5].

RESULTS AND DISCUSSION

The biological activities of the isolated compounds were assessed against *T.b.brucei* and the test results were

summarized in Fig **1**: catechin (**1**), dehydroabietic acid (**2**) and dihydroconiferyl alcohol (**3**) showed activity against *T. b. brucei* (S427) with an MIC 21.55µM, 83.27µM, 68.60 µM and, respectively when compared to 0.90µM suramin.

The MIC of the isolated compounds was determined at concentrations ranging from 0.19-100µg/ml using the Alamar Blue Assay and the results are presented in Figure **1**. This is the first report of compound **1**, **2** and **3** having anti-trypanosomal activity.

The analysis of the ethyl acetate part of the methanolic extract, n-hexane extract of *Abies nobilis* and the chloroform extract of *Pinus sylvestris* using column and flash chromatography separations, led to the isolation of three active natural products such as catechin, dehydroabietic acid and dihydroconiferyl alcohol. The structures were elucidated by mainly NMR spectroscopic and MS spectrometry. The activities of isolated compounds were assessed by Alamar Blue Assay.

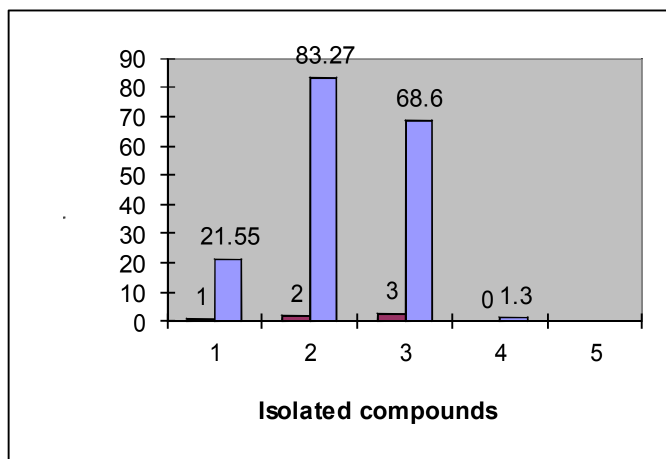

Figure 1: MICs in µM of isolated compounds against *T. b. brucei*

Isolated compounds; catechin 1, dehydroabietic acid 2, dihydroconiferyl alcohol 3 and standard drug (suramin) as a positive control, MIC (Minimum Inhibitory Concentration).

Compound **1** was isolated from the ethyl acetate part of the methanolic extract of *Abies nobilis.* The HRESI-MS showed an $[M+H]^+$ at *m/z* 291.0220 for the molecular formula of the compound as $C_{15}H_{14}O_6$. The ^1H and ^{13}C NMR data [400MHz, CD_3OD, Table 1] of compound **1** revealed to be catechin and confirmed this indication by the *meta*-coupled aromatic signals at δ 5.84 and δ 5.91 for ring-A, the ABX spin aromatic system (δ 6.75, 6.70 and 6.82) for ring-B and the characteristic signals at δ 2.52, 2.84, 3.96 and 4.55 for the flavanol ring C. In the ^{13}C NMR spectrum, aromatic ring system, two oxygen- bearing methines and one methylene were observed among 15 signals, suggesting the flavanol-type compound. The sample yield was low and the optical rotation was not recorded. Hence, the absolute stereochemistry could not be confirmed. However, the large coupling constant between H-2 and H-3 indicated a *trans* diaxially relative stereochemistry. Therefore, The structure of compound **1** was identified as catechin (**1**) as compared with the literature data (Benavides *et al.,* 2006).

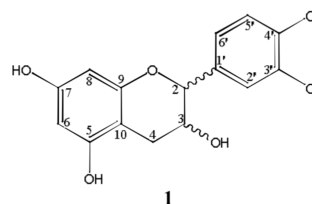

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Table 1: ^1H and ^{13}C NMR data for compound 1 in CD_3OD

Position	^1H	^{13}C	Position	^1H	^{13}C
2	4.55 (d, *J*= 8.0Hz)	81.7	8	5.91(d, *J*= 2.2Hz)	96.1
3	3.96 (dt, *J*= 5.2, 8.0Hz)	67.9	9		157.0
4	2.52 (dd, *J*=16.2, 8.0Hz) 2.84 (dd, *J*= 16.2,5.2Hz	27.4	10 1' 2'	 6.82 (d, *J*= 1.7Hz)	100.5 131.0 115.0
5		157.3	3' 4'		 145.4
6	5.84 (d, *J*= 2.2Hz)	97.0	5'	6.75 (d, *J*= 8.5Hz)	115.5
7		157.0	6'	6.70 (dd, *J*=1.7Hz, 8.5Hz)	120.2

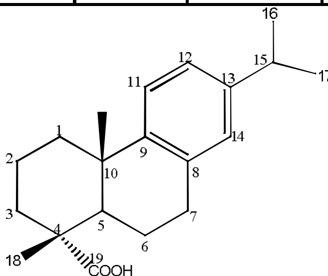

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Table 2: ^{1}H and ^{13}C NMR data of compound 2 in $CDCl_3$

Position	^{1}H	^{13}C	^{3}J HMBC
1	1.49, 2.34m	37.9	
2	1.69, 1.90m	18.6	
3	1.68, 1.83m	36.7	C-19, Me-18
4		47.4	
5	2.23 dd, *J*= 1.8, 12.3Hz	44.6	
6	1.55, 1.87m	21.8	C-8
7	2.87, 2.93m	30.0	C-9, C-14
8		134.7	
9		146.8	
10		36.9	
11	7.16 d, *J*= 8.0Hz	124.2	
12	6.99 dd, *J*= 1.8, 8.0Hz	123.9	
13		145.8	
14	6.88 d, *J*= 1.8Hz	127.0	
15	2.88septet	33.5	C-12, C-14
16	1.21 d, *J*= 7.0Hz	24.0	C13, C17
17	1.21 d, *J*= 7.0Hz	24.0	
18	1.27 (s)	16.3	C3, C5, C19
19		184.5	
20	1.20 (s)	25.2	C1, C5, C9

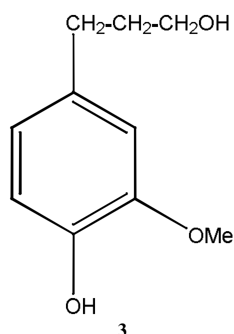

Table 3: ^{1}H (400MHz) and ^{13}C NMR data of compound 3 in $CDCl_3$

Position	^{1}H	^{13}C
1		133.8
2	6.70 d, *J*= 1.8Hz	111.1
3		147.2
4		143.7
5	6.82 d, *J*= 7.9Hz	114.3
6	6.68 dd, *J*= 7.9, 1.8Hz	121.0
7	2.63 t, *J*= 7.9Hz	31.9
8	1.86 tt, *J*= 7.9, 6.2Hz	34.6
9	3.62 t, *J*= 6.2Hz	62.4
3- OMe	3.82	55.9

Compound **2** was isolated from VLC fraction of n-hexane extract of *Abies nobilis.* The HRESI-MS indicated a molecular ion at *m/z* 300.2087 which was analyzed for the molecular formula $C_{20}H_{28}O_2$. IR (KBr) absorption at 1464, 2649, 2927cm^{-1} were indicative of the presence of C=C, O-H and C-H groups stretching respectively.

The ^{1}H NMR spectrum [400MHz, $CDCl_3$, Table 2] showed aromatic, aliphatic protons and 4 methyls; two appearing as equivalent doublets at δ 1.21 ppm (each d, *J*= 7.0 Hz, Me-16, Me-17) and two other methyl groups attached to quaternary carbons and appearing as singlets at δ 1.27ppm (Me-18) and δ 1.20 ppm (Me-20). In the aromatic region of the spectrum it showed a doublet at δ 7.16 (*J*= 8.0 Hz, H-11), doublet at δ 6.88 (*J*=1.2Hz, H-14) and doublet of doublets at δ 6.99 (*J*= 1.8Hz, 8.0Hz, H-12) indicating an ABX substitution pattern. A septet proton at δ 2.88ppm (H-15) showed COSY correlation to two methyl groups (Me-16 and Me-17) whereas these methyls also showed COSY correlation to each other indicating the presence of an isopropyl group. In ^{13}C NMR data [100MHz, $CDCl_3$, Table 2] showed 20 carbon atoms including one at δ184.5ppm due to a carbonyl function. This number of carbon atoms corresponds to diterpene moiety which contains a carboxylic acid group and 4 methyls. Two methyls at positions 16 and 17 showed HMBC correlations to the aromatic ring and another methyl (δ 1.27ppm) gave a correlation to the carboxylic carbon. Some methylens (δ 1.55, 1.87, 2.87 and 2.93) exhibited HMBC correlation to the aromatic ring whereas some other methylens (δ 1.68 and 1.83) showed correlation to the carboxylic acid and one methyl (δ 1.27). The optical rotation of compound **2** was found to be $[\alpha]_D$ = +57 which is comparable with the value found in the literature (Kayoko *et al.,* 2008). Further comparisons of the NMR data to those of the literature identified that the compound is dehydroabietic acid (**2**). This is the first report of this compound being found from this plant.The experimental data were in agreement with the published data (Gigante *et al.,* 1995).

Compound **3** By using VLC and further purification over sephadex LH20 column chromatography and the flash chromatography, JE7 was isolated from the chloroform extract of *Pinus sylvestris.* Compound **3** solved for $C_{10}H_{14}O_3$ according to HRESI-MS at *m/z* 182.0943. ^{1}H NMR spectrum [400 MHz, $CDCl_3$, Table 3] showed signals for both aromatic and aliphatic protons. The aliphatic protons (H-7, H-8 and H-9) resonated from δ 1.86 to 3.62 ppm and methoxy group at δ 3.82 as a singlet peak was observed. From the HMBC NMR data: H-5 of the aromatic ring and the methoxy proton showed ^{3}J correlation to C-3 indicating the methoxylation at this position. In the ^{13}C NMR data (Table 3): 10 signals were observed and a methoxy group at 55.9ppm was present. By using ^{1}H NMR and ^{13}C NMR spectroscopy and comparing with the previous data (Ma *et al.,* 2000), compound **3** was identified as dihydroconiferyl alcohol (**3**).

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