Research Journal of
Phytochemistry

ISSN 1819-3471

Academic
Journals Inc.

www.academicjournals.com

Research Journal of Phytochemistry 5 (4): 216-221, 2011
ISSN 1819-3471 / DOI: 10.3923/rjphyto.2011.216.221

Chemical Composition of Vietnamese Black Lingzhi *Amauroderma subresinosum* Murr

[1]D.N. Quang, [1]T.T. Nga and [2]L.X. Tham
[1]Faculty of Chemistry, Hanoi National University of Education, Hanoi, Vietnam
[2]Department of Science and Technology, Lam dong, Vietnam

Corresponding Author: D.N. Quang, Faculty of Chemistry, Hanoi National University of Education, Hanoi, Vietnam

ABSTRACT

Fatty acid composition of Vietnamese black lingzhi, *Amauroderma subresinosum* was investigated by GC (Gas chromatography) with authentic samples. Fourteen fatty acids were identified, in which the concentration of unsaturated fatty acid, 11-octadecaenoic is the highest (around 51.01% of total fatty acids). Furthermore, two fatty acids, lignoceric acid and 11-octadecaenoic together with three esters of cholesta-7,22-dien-3β-ol with three fatty acids 14- methylpentadecanoic, 8,11-octadecaenoic and 8-octadecenoic with the ratio 1/1/1 were isolated and structural elucidated. These results support a new evidence for classification of Ganodermataceae family, in which *A. subresinosum* is placed in the distinct genus *Amauroderma*, isolated from *Ganoderma*, since it does not produce any triterpenoid, typically elaborated from *Ganoderma* species, particularly *G. lucidum*.

Key words: *Amauroderma subresinosum*, sterol, fatty acid, fungus, back lingzhi

INTRODUCTION

The fungal family Ganodermataceae is represented by about 322 species (Foroutan and Vaidya, 2007), which mostly occur in subtropical and tropical regions. The fruiting bodies of *Ganoderma* species have been widely used in traditional Chinese, Japanese, Korean and Vietnamese medicine to treat a variety of conditions from ancient time (Kleinwachter *et al*., 2001). In addition, *Ganoderma* species could also be used as feed supplement in animal diet since it produced various compounds such as steroids, cardiac glycosides, saponins, resins and amino acids (Ogbe *et al*., 2009). Interesting biological activities have been observed in these mushrooms; *Ganoderma lucidum* showed cytotoxic (Min *et al*., 2000) and antiviral activities (El-Mekkawy *et al*., 1998), *Ganoderma colossum* showed anti-inflammatory, cytotoxic (Kleinwachter *et al*., 2001) and antimicrobial activities (Ofodile *et al*., 2005), *Ganoderma applanatum* (Lee *et al*., 2006) revealed antibacterial and aldose reductase inhibitory activities, *Ganoderma concinna* induced apoptosis in human promyelocytic leukemia HL-60 cells (Gonzalez *et al*., 2002) and *Ganoderma pfeifferi* showed antimicrobial activity (Mothana *et al*., 2000). These basidiomycetes are known to be prolific producers of lanostane type triterpenoids and over 130 such compounds have been recognized from the genus *Ganoderma*. However, chemical investigation on Vietnamese *Ganoderma* is very poor. So far, only isolation of colossolactones from *G. colossum* was reported (Kleinwachter *et al*., 2001). In the course of our investigation on Vietnamese fungi, we study the chemical constituents of *A. subresinosum* in order to get more

evidence for its classification as well as to look for novel bioactive substances. This study reports the fatty acid composition as well as the isolation of two fatty acids and ester of sterol from fruit bodies of *A. subresinosum*.

MATERIALS AND METHODS

Material: Fruit bodies of *A. subresinosum* was collected in Cattien National Park in November 2008 and identified by L.X. Tham. Voucher specimens were deposited at faculty of Chemistry, Hanoi University of Education (ND0801).

Methods

General: NMR spectra were recorded on Varian Brucker Avance 500 MHZ, using $CDCl_3$ as solvent. Chemical shifts are given with TMS used as internal standard (^{1}H-NMR) and δ 77.03 (ppm) from $CDCl_3$ as a standard (^{13}C-NMR). Mass spectra were recorded on a JOEL JMS AX-500 spectrometer. Fatty acid content was analyzed by using a Gas chromatography (Finnigan Trace GC) with an ultra-column BPX70. The temperature programming of GC analysis was performed from 50°C isothermal for 3 min, then 50-250°C at 4°C min^{-1} and finally isothermal at 250°C for 10 min. TLC was performed on silica gel plates (Kieselgel 60 F254, Merck) and reversed phase C_{18} silica gel plates (Merck).

Extraction and isolation: Dried *A. subresinosum* (230 g) was extracted with methanol. The methanolic extract was concentrated to give a residue (6.8 g) which was further divided into four fractions. Fraction 2 (278 mg) was purified by reversed phase RP-18 column, using MeOH as solvent to afford compound 1 (30 mg). Compounds 2 (40 mg) and 3 (52 mg) were obtained from fraction 3 (1068 mg) by silica gel column, eluting with $CHCl_3$.

Hydrolysis of 3: The mixture 3 (10 mg) in MeOH (2 mL) was added KOH (5 mg) and the mixture was stirred at room temperature for 3 h. Then, the reaction mixture was concentrated and water (2 mL) was added and extracted with n-hexane (2 mL) for 2 times. The n-hexane extract was further purified by silica gel column, using n-hexane: EtOAc = 4:1 (v/v) as eluent to yield compound 4 (2.5 mg). The water phase was neutralized and methylated as the same method as mentioned below and analyzed by GC to determine the fatty acid content.

Methylation of crude extract: Crude extract of *A. subresinosum* (50 mg) was added 5 mL H_2SO_4 in methanol in a flask and the mixture was stirred at 80°C for 4 h. The reaction mixture was concentrated, then added 2 mL of water and finally extracted with n-hexane (2 mL). The n-hexane extract was directly injected to GC for fatty acid analysis.

- **Compound 1: ^{1}H NMR ($CDCl_3$):** δ 0.88 (3H, t, J = 6.5 Hz, H-24), 1.28 (m, from H-5 to H-22), 1.30 (2H, m, H-23), 1.66 (4H, m, H-3 and H-4), 2.34 (2H, t, J = 7.5 Hz, H-2)
- **Compound 2: ^{1}H NMR ($CDCl_3$):** δ 0.88 (3H, t, J = 6.4 Hz, H-18), 1.40 (m, from H-4 to H-9 and H-14 to H-17), 2.34 (2H, m, H-13), 2.78 (2H, m, H-10), 5.30 (2H, m, H-11 and H-12).
- **Compound 2: ^{13}C NMR ($CDCl_3$):** δ 14.0 (C-18), 29.3 (C-3), 29.4 (C-14 to C-17), 29.6 (C-4 to C-9), 31.9 (C-10, C-13), 34.0 (C-2), 129.7 (C-12), 130.0 (C-11), 179.8 (C-1)
- **Compound 4: EI-MS: m/z 398. ^{1}H NMR ($CDCl_3$):** δ 0.54 (6H, s, H-18 and H-19), 0.82 (3H, d, J = 6.9 Hz, H-27), 0.84 (3H, d, J = 6.9 Hz, H-26), 0.91 (3H, d, J = 6.9 Hz, H-28), 1.02

(3H, d, J = 6.6 Hz, H-21), 3.60 (1H, m, H-3), 5.15 (1H, m, H-7), 5.17 (1H, m, H-23), 5.20 (1H, m, H-22). ^{13}C NMR ($CDCl_3$): δ 11.8 (C-18), 12.1 (C-19), 17.9 (C-28), 19.6 (C-26), 20.0 (C-27), 21.1 (C-21), 22.7 (C-15), 23.0 (C-11), 27.6 (C-6), 28.1 (C-2), 28.3 (C-16), 33.1 (C-25), 34.5 (C-10), 37.0 (C-1), 39.4 (C-4, C-12), 40.0 (C-20), 42.8 (C-24), 42.9 (C-5), 43.3 (C-13), 49.4 (C-9), 55.8 (C-14), 56.8 (C-17), 71.1 (C-3), 117.5 (C-7), 131.9 (C-23), 135.6 (C-22), 139.6 (C-8)

RESULTS AND DISCUSSION

Table 1 shows that fatty acid composition of *A. subresinosum*. Accordingly, fourteen major fatty acids were found. The concentration of unsaturated fatty acid, 11-octadecaenoic is highest (51.077%), followed by 9,12- octadecadienoic (22.979%) which is dominant in Rumen liquor (Achenef *et al*., 2009) and hexadecanoic (19.05%). Consequently, total unsaturated fatty acids in *A. subresinosum* are about 80% of total fatty acid. Analysis of fatty acid composition of several *Ganoderma* sp. also has revealed that they possess mainly polyunsaturated fatty acids (PUFAs), especially 18-carbon unsaturated fatty acids (Martinez *et al*., 1991). Previous experimental suggest that one of the food components that reduces serum cholesterol and high blood pressure is PUFAs content (Bavelaar and Beynen, 2004; Abd El-Baky *et al*., 2004). PUFAs also are anticancer (Ndem *et al*., 2008) and antioxidant agents (Ogbuewu *et al*., 2010).

In addition, repeated column chromatographs resulted in the isolation of three compounds. Compound 1 was obtained as an oil. Analysis of its ^{1}H NMR spectrum suggests that it contains one methyl group (δ_H 0.88 ppm) and no proton signal at low field, thus, it should be a saturated fatty acid. By comparing its NMR data with those of previous publication, compound 1 is characterized as lignoceric acid (Kang *et al*., 2011; Onwuliri *et al*., 2004). Compound 2 was also obtained as white powder. Interpretation of its ^{1}H and ^{13}C NMR revealed that it is an unsaturated fatty acid with one double bond at $C_{11\text{-}12}$ in its molecule. Thus, compound 2 was found to be 11-octadecaenoic as shown in Fig. 1.

Compound 3 was obtained a white powder. From TLC (both silica gel and reversed phase) analysis, only one spot was detected. However, analysis of its ^{1}H NMR suggests that it has two moieties, fatty acid and sterol parts attached together by an ester linkage. Thus, EI-MS was recorded and several peaks at m/z 600-700 were observed. Then, compound 3 was treated with

Table 1: Fatty acid compositions (percentage of total fatty acids) of *A. subresinosum*

Name of acids	Molecular formula	Rt (min)	Percentage (% total fatty acids)
Butanoic	$C_4H_8O_2$	5.51	0.070
Decanoic	$C_{10}H_{20}O_2$	6.85	0.114
Tetradecanoic	$C_{14}H_{28}O_2$	12.08	0.348
Pentadecanoic	$C_{15}H_{30}O_2$	14.15	1.091
Hexadecanoic	$C_{16}H_{32}O_2$	16.93	19.052
9-hexadecaenoic	$C_{16}H_{30}O_2$	17.81	0.256
Heptadecanoic	$C_{17}H_{34}O_2$	19.83	0.323
11-octadecaenoic	$C_{18}H_{34}O_2$	25.37	51.077
9,12-octadecadienoic	$C_{18}H_{32}O_2$	27.56	22.979
6,9,12-octadecatrienoic	$C_{18}H_{30}O_2$	30.22	0.595
Nonadecanoic	$C_{19}H_{38}O_2$	31.99	0.676
5,8,11,14-eicosatetraenoic	$C_{20}H_{32}O_2$	33.76	0.340
5,8,11,14,17-eicosapentaenoic	$C_{20}H_{30}O_2$	41.12	1.454
7,10,13,16,19-docosapentaenoic	$C_{22}H_{34}O_2$	52.85	1.625

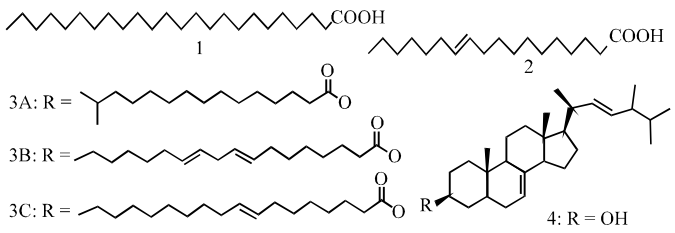

Fig. 1: Structures of compounds 1-4

KOH and extracted with n-hexane. Compound 4 was obtained from n-hexane extract. Its EI-MS shows the molecular peak at m/z 398. The ^{1}H and ^{13}C-NMR spectral data of 4 showed the presence of four tertiary methyls, one tertiary alcohol (δ_C 71.1), three olefinic protons (δ_H 5.15, 5.17 and 5.20). On the basis of the above spectral data, 4 was deduced to be a sterol (Reich *et al*., 1969). The structure of 4 was then determined by 2D NMR spectra. The position of the first double bond was found at C_{7-8} by the HMBC correlation between H-6, H-9 and H-14 to C-7 and C-8. There are HMBC correlations between H-21, H-24, H-28 to C-22 and C-23, thus, the second double bond was decided at C_{22-23}. Therefore, compound 4 was characterized as cholesta-7,22-dien-3β-ol.

The fatty acid moiety was later analyzed by GC analysis. The result shows that it is a mixture of three fatty acids, 14- methylpentadecanoic, 8,11-octadecaenoic and 8-octadecenoic with the ratio 1/1/1. Consequently, compound 3 is a mixture of three esters (3A-C) of cholesta-7,22-dien-3β-ol with three fatty acids 14- methylpentadecanoic, 8,11-octadecaenoic and 8-octadecenoic with the ratio 1/1/1.

Even, *Ganoderma* sp. has already been characterized by a combination of morphological, chemotaxonomy and sequences of rDNA 26S. But, the morphological concepts for identification of *Ganoderma* sp. in tropical area is not well-established (Zakaria *et al*., 2009). Therefore, these above results play a very important role in classification and support a new and further taxonomy of the Ganodermataceae family. *A. subresinosum* is now isolated from *Ganoderma* genus and created a distinct genus *Amauroderma*, since it does not produce any lanostane triterpenoid, typically elaborated from *Ganoderma* species, particularly *G. lucidum* (Tham, 2005). This classification is in good agreement with that by examining its basidiospore structure as well as complete sequencing of D1, D2 regions (26S) of *A. subresinosum* (Tham, 1998).

CONCLUSION

Chemical composition of black lingzhi *A. subresinosum* collected from Cattien National Park has been investigated. Fourteen fatty acid are found in its crude extract. Fruit bodies of *A. subresinosum* mainly produce unsaturated fatty acids which cover about 80% of total fatty acids. In addition, from its MeOH extract, two fatty acids, lignoceric acid and 11-octadecaenoic together with three esters of cholesta-7,22-dien-3β-ol with three fatty acids 14- methylpentadecanoic, 8,11-octadecaenoic and 8-octadecenoic with the ratio 1/1/1 were purified and structural elucidated.

ACKNOWLEDGMENTS

Financial supports of Ministry of Education and Training (project No. B2010-17-239) and International Foundation of Science, IFS (Sweden) (project No. F/4896-1) are greatly acknowledged.

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