Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(3): 2376-2379
Received: 21-03-2018
Accepted: 25-04-2018

Hoang Van Trung
School of Chemistry, Biology and Invironment, Vinh University, Vietnam

Nguyen Ngoc Tuan
Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Vietnam

Nguyen Tan Thanh
School of Chemistry, Biology and Invironment, Vinh University, Vietnam

Truong Thi Binh Giang
School of Natural Sciences Education, Vinh University, Vietnam

Dinh Thi Truong Giang
School of Natural Sciences Education, Vinh University, Vietnam

Isiaka Ogunwande
Department of Chemistry, Lsagos State University, PMB 0001, Lasu Post Office Ojo, Lagos, Nigeria

Tran Dinh Thang
School of Chemistry, Biology and Invironment, Vinh University, Vietnam

Correspondence
Tran Dinh Thang
School of Chemistry, Biology and Inviroment, Vinh University, Vinh city, Vietnam

Determination of ergosterol and ergosterol peroxide in higher fungi Species by high-performance liquid chromatography

Hoang Van Trung, Nguyen Ngoc Tuan, Nguyen Tan Thanh, Truong Thi Binh Giang, Dinh Thi Truong Giang, Isiaka Ogunwande and Tran Dinh Thang

Abstract
Ergosterol and ergosterol peroxide are clearly the main sterol in mushrooms. Herein, an analytical method for ergosterol and ergosterol peroxide determination in cultivated and wild mushrooms was developed using high-performance liquid chromatography coupled to ultraviolet detection. The chromatographic separation was achieved in a RP 18 (150 x 4,6 mm, 5 mm) column using an isocratic elution with methanol/acetonitrile (85:15, v/v) at a flow rate of 1 mL/min. Different extraction methodologies were tested, using n-hexane, methanol/dichloromethane (75:25, v/v) or chloroform / methanol (20:80, v/v). Ergosterol was the most abundant sterol by a greater extent in all mushrooms. Among wild species, *Fomitopsis dochmius* was the mushroom with the highest content in ergosterol. Results were expressed in fat content, dry weight and fresh weight bases. The assessment of ergosterol and ergosterol peroxide amounts might be very useful due to the bioactive potential that has been attributed to this molecule and its derivatives.

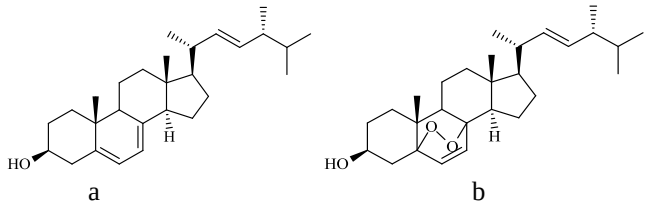
Keywords: ergosterol, higher fungi, ergosterol peroxide, Ganoderma, Daldinia, Fomitopsis

Introduction
For thousands of years medicine and natural products have been closely linked through the use of traditional medicines and natural poisons. Mushrooms have an established history of use in traditional oriental medicine, where most medicinal mushroom preparations are regarded as a tonic, that is, they have beneficial health effects without known negative side-effects and can be moderately used on a regular basis without harm. Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. In particular, and most importantly for modern medicine, they represent an unlimited source of compounds which are modulators of tumor cell growth [1-3]. Furthermore, they may have potential as functional foods and sources of novel molecules [3].

For thousands of years medicine and natural products have been closely linked through the use of traditional medicines and natural poisons. Mushrooms have an established history of use in traditional oriental medicine, where most medicinal mushroom preparations are regarded as a tonic, that is, they have beneficial health effects without known negative side-effects and can be moderately used on a regular basis without harm. Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical Products. In particular, and most importantly for modern medicine, they represent an unlimited source of compounds which are modulators of tumor cell growth [1-3]. Furthermore, they may have potential as functional foods and sources of novel molecules [3].

Ergosterol (Fig. 1a) is a principal sterol of the cell membrane to which it is strongly bound in fungi [4] and is able to activate expression of a number of defense genes and increase the resistance of plants against the pathogens [5]. Ergosterol is present in two forms, as free ergosterol and esterified ergosterol, and the relative abundances of free to esterified ergosterol are different among various species [6]. The ergosterol content has been widely used as an estimate of fungal biomass in various environments because a strong correlation has been found between ergosterol content and fungal dry mass [7]. A number of methods have been reported for the determination of ergosterol [8-12]. Most of them are based on its specific UV absorption with a maximum at 282 nm, originating from the double bond at positions 5 and 7 [9]. Previous studies have demonstrated that ergosterol may contribute to potential health benefits and significant pharmacological activities, including reducing pain related to inflammation, reducing the incidence of cardiovascular diseases, and inhibiting cyclooxygenase (COX)

enzyme, antioxidant, antimicrobial, anticomplementary, and antitumor activities [13-22]. The antitumor activity of ergosterol may be due to direct inhibition of angiogenesis induced by solid tumors [17, 21]. Dietary ergosterol is absorbed in the alimentary tract, accumulates in the adrenals and other organs, and can be metabolized *in vivo* to generate newer bioactive products, such as 17R, 24-dihydroxyergosterol, which has been found to be able to inhibit the proliferation of skin cells in culture, as demonstrated in human keratinocytes and melanoma cell lines [23].



a b

Fig 1a: Structure of an ergosterol molecule **b.** Structure of an ergosterol peroxide molecule

Ergosterol peroxide (Fig. 1b) (5α, 8α-epidioxy-ergosta- 6, 22-dien-3β-ol) is a natural steroid that has been found in a variety of fungi, yeast, lichens and sponges [10]. In fungi, ergosterol peroxide was synthesized by the conversion of ergosterol to its epidioxide by the H_2O_2 dependent enzymatic oxidation of ergosterol (Please check this statement properly). This reaction may play a role in the detoxication reaction of reactive oxygen species H_2O_2 in neoplasic cells [12].

Herein, a methodology for sterols extraction and analysis using reversed-phase HPLC and ultraviolet detection (HPLC-UV) was optimised and validated. The developed methodology was afterwards applied to quantify ergosterol and ergosterol peroxide in wild mushrooms (*Daldinia concentrica*, *Fomitopsis dochmius*, *Phellinus igniarius*, *Ganoderma applanatum*, *Fomitopsis carneus*, *Ganoderma lobatum*, *Ganoderma philippii*, *Ganoderma multiplicatum*, *Ganoderma lucidum*, *Trametes gibbosa*). Species were selected according to their relevance and recognised organoleptic properties. As far as we know, most of these species were analysed regarding their ergosterol content for the first time.

2. Materials and Methods

2.1 Mushrooms materials

Mushrooms belonging to different species of the higher fungi (Table 1) were harvested from Pu Mat and Pu Huong National Park, Nghean Province, Vietnam, in May 2012. Voucher specimens TDT 1001 (*Daldinia concentrica*), TDT 1002 (*Fomitopsis dochmius*), TDT 1003 (*Phellinus igniarius*), TDT 1004 (*Ganoderma applanatum*), TDT 1005 (*Fomitopsis carneus*), TDT 1006 (*Ganoderma lobatum*), TDT 1007 (*Ganoderma philippii*), TDT 1008 (*Ganoderma multiplicatum*), TDT 1009 (*Ganoderma lucidum*) and TDT 1010 (*Trametes gibbosa*), respectively, were deposited at the Botany Museum, Vinh University, Vietnam. Mushrooms samples were air-dried prior to extraction. Samples of fruit bodies (10 mushrooms for each species) were homogenized and lyophilized.

2.2 Standards

The standards of sterols (ergosterol and ergosterol peroxide) were isolated from *Ganoderma lucidum*. The structure of sterols were elucidated using a combination of 1D and 2D NMR techniques (^{1}H-, ^{13}C-NMR, HSQC and HMBC), MS, UV and IR.

2.3 Sterols extraction

The assayed methodology was performed following the procedure described by Villares *et al*. [24] with slight alterations. Dried mushroom powder (~2 g; humidity 24%) was extracted with 20 mL of MeOH/MeCN mixture (85:15, v/v) by stirring in an ultrasonic bath at 4 °C for 30 min. Then, the mixture was centrifuged at 3,500 rpm for 10 min. The residue was re-extracted twice, and the extracts were combined. The samples were stored (4 °C in darkness) until HPLC analysis. Prior to HPLC analysis, samples were filtered through nylon syringe membrane (0.45 μm).

2.4 Chromatographic Analysis

The analyses were performed by HPLC-UV. The HPLC equipment consisted of an integrated system Aligent 1100 HPLC, Germany. Chromatographic separation was achieved with a RP 18 (150 x 4,6 mm, 5 μm) column operating at 30°C. The mobile phase was acetonitrile/methanol (85:15, v/v), at a flow rate of 1 mL/min, and the injection volume was 20 μL; the detection was performed at 290 nm. Under these conditions the retention time was 16.295 ± 0.2 min (erogsterol) and 4.335 ± 0.2 min (erogsterol peroxide). Ergosterol and ergosterol peroxide were quantified by comparison of the area of its peak with the calibration curve obtained from sterols extraction standard. Cholecalciferol was used as internal standard. The results were expressed in milligrams per gram of fat, milligrams per 100 g of dry weight and milligrams per 100 g of fresh weight.

3. Results and Discussion

In the present study, a mixed solvent of methanol, acetonitrile as the mobile phase and the gradient elution procedure were used to separate the bioactive constituents in 10 mushrooms species. The separation of ergosterol, erogsterol peroxide could be achieved by adjusting the program of the gradient elution and the proportion of methanol and acetonitrile. Using a UV detector, peaks were identified by taking the spectra of each peak during elution. The identification of ergosterol and ergosterol peroxide were achieved by comparing its retention time and UV spectra against the known standard. The external standard method was used for the determination of ergosterol and ergosterol peroxide. The content of ergosterol and ergosterol peroxide were measured by comparing the peak area with the standard. The peak purities might also be examined by the UV detector.

In addition, ergosterol strongly bound to the cell membrane is difficult to extract when the spore is not fully broken. In fact, it is difficult to completely break the sporoderm of a mushroom spore due to its prestressed ovoid structure and very hard bilayer sporoderms, especially on a commercial scale. Thus, the bioactive substances stored in the holes between the inner and the outer walls of spores, especially for ergosterol strongly bound to the cell membrane, are difficult to extract. Thus, a high-performance liquid chromatographic method was established to separate and determine ergosterol and ergosterol peroxide commonly found in fungi. Ergosterol and ergosterol peroxide from the samples were extracted with methanol before subjected to HPLC analysis. Separations were carried out on a reversed-phase RP 18 (150 x 4,6 mm, 5 μm) column using a mobile-phase gradient: a mixture of MeOH: MeCN (85:15, 80:20, 75:25…) were used for 20 min until the end of the chromatographic run. We have succeeded

in fully breaking the cell membrane. We proceed with dried mushroom powder (~2 g; humidity 24%) was extracted with 20 mL of MeOH/MeCN mixture (85:15, v/v) by stirring in an ultrasonic bath at 4°C for 30 min. Then, the mixture was centrifuged at 3,500 rpm for 10 min. The residue was re-extracted twice, and the extracts were combined. The samples were stored (4°C in darkness) until HPLC analysis.

This HPLC method was validated for linearity, the LOD and the LOQ, precision, accuracy, repeatability, and recovery. The calibration curve (A = 2,51607C + 16,16249, R^2 = 0.9999) of the peak-area (A) ratio against the concentration (C) for ergosterol gave a linear response over a wide range of concentrations (50 - 1000 µg/mL). The LOD and the LOQ with a 20 µL injection were 0.46969 and 1.56563µg/mL, corresponding.

The calibration curve (A = 9.18851C + 0.139275, R^2 = 0.9999) of the peak-area (A) ratio against the concentration (C) for ergosterol peroxide gave a linear response over a wide range of concentrations (2 - 10 µg/mL). The LOD and the LOQ with a 20 µL injection were 0.00126 and 0.00342 µg/mL, corresponding.

The method detection limits and quantitative very small range proved highly sensitive equipment that can detect ergosterol and ergosterol peroxide content in the sample analyzed. From the peak area values were calculated and measured concentration C_o (ppm) of ergosterol and ergosterol peroxide and the contents of ergosterol, ergosterol peroxide C (g/l) in the samples (Table 1-2).

Table 1: Contents of ergosterol in 10 fungi species

Sample	C_0 (ppm)	Peak-area	C (g/l)	CV (%)
TDT1001	0.86476	233.38529	0.00086	0.14108
TDT1002	30.60781	7717.28987	0.03061	0.01901
TDT1003	0.77896	212.15442	0.00078	0.65344
TDT1004	7.01148	1780.29818	0.00701	0.00670
TDT1005	1.39210	366.42418	0.00139	0.01580
TDT1006	7.11274	1805.77604	0.00711	0.01575
TDT1007	4.70818	1200.77209	0.00471	0.01848
TDT1008	5.45177	1387.86426	0.00545	0.01137
TDT1009	7.73122	1962.52417	0.00773	0.01281
TDT1010	4.48898	1145.17603	0.00449	0.00735

Repeatability of the method was evaluated by the relative standard deviation (CV, %). This process has been used to determine the amount of ergosterol and ergosterol peroxide in 10 mushrooms species with results shown in Table 1, 2.

Table 2: Contents of ergosterol peroxide in 10 fungi species

Sample	C_0 (ppm)	Peak-area	C (g/l)	CV (%)
TDT1001	0.13714	126.49114	0.00014	4.75490
TDT1002	2.37190	2179.74927	0.00237	1.06244
TDT1003	0.03122	28.78333	0.00003	4.85041
TDT1004	0.03100	28.57558	0.00003	3.67742
TDT1005	0.05916	54.42258	0.00006	2.23123
TDT1006	0.03944	36.53934	0.00004	1.97769
TDT1007	0.03454	32.03350	0.00004	4.86393
TDT1008	0.06534	60.04512	0.00007	2.40382
TDT1009	0.16678	245.43129	0.00017	7.60177
TDT1010	0.02852	26.08780	0.00003	5.18934

This method gave rapid elution, good selectivity and high repeatability for ergosterol and ergosterol peroxide. On the basis of the above results, the contents of ergosterol and ergosterol in 10 mushrooms species were analyzed and the results were also shown in Table 1-2.

Our results suggest that ergosterol and ergosterol peroxide were identified in high quantity in the present TDT1002 samples. The results show that the contents of ergosterol and ergosterol peroxide in the TDT1002 samples have significant differences. This procedure proved to be superior compared with other methods, since the yield of ergosterol yields was higher [6].

4. Conclusion

The contents of ergosterol in TDT1002 were 0.03061g/l. Ergosterol content in TDT1002 is the largest of the 10 samples analyzed fungal. In contrast, only a very small quantity of ergosterol was found in the *Phellinus igniarius*. As shown in Table 2, the contents of ergosterol peroxide in *Fomitopsis dochmius* were 0.00237g/l. Ergosterol peroxide content in *Fomitopsis dochmius* is the largest of the 10 samples analyzed fungal. In the meantime, only a very small quantity of ergosterol peroxide was found in *Phellinus igniarius* and *Ganoderma applanatum*. The results show that the contents of ergosterol and ergosterol peroxide in the 10 mushrooms samples have significant differences.

5. Acknowledgements: This work was financial supported by MOET.

6. References

1. Pujol V, Seux V, Villard J. Research of antifungal substances secreted by higher fungi in culture. Annales Pharmaceutiques Francaise. 1990; 48:17-22.
2. Clericuzio M, Piovano M, Chamy MC, Garbarino JA, Milanesio M, Viterbo D, *et al*. Structural characterization of metabolites from *Pholiota spumosa* (Basidiomycetes). Croatian Chemistry Acta. 2004; 77:605-611.
3. Wasser SP, Weis A. Medicinal properties of substances occurring in higher Basidiomycetes mushrooms: Current perspectives (review). International Journal of Medicinal Mushrooms. 1999; 1:31-62.
4. Czub J, Baginski M. Comparative molecular dynamics study of lipid membranes containing cholesterol and ergosterol. Biophysical Journal. 2006; 90:2368-2382.
5. Lochman J, Mikes V. Activation of different defence-related genes expression by ergosterol. FEBS Journal. 2005; 272:470-470.
6. De Sio F, Laratta B, Giovane A, Quagliuolo L, Castaldo D, Servillo L. Analysis of free and esterified ergosterol in tomato products. Journal of Agriculture and Food Chemistry. 2000; 48:780-784.
7. Pasanen AL, Yli-Pietila K, Pasanen P, Kalliokoski P, Tarhanen J. Ergosterol content in various fungal species and bio contaminated building materials. Applied Environmental Microbiology. 1999; 65:138-142.
8. Sun SJ, Gao YH, Ling X, Lou HX. The combination effects of phenolic compounds and fluconazole on the formation of ergosterol in *Candida albicans* determined by high-performance liquid chromatography/tandem mass spectrometry. Analytical Biochemistry. 2005; 336:39-45.
9. Varga M, Barto´k T, Mesterha´zy A. Determination of ergosterol in Fusarium-infected wheat by liquid chromatography atmospheric pressure photoionization mass spectrometry. Journal of Chromatography A. 2006; 1103: 278-283.
10. Kahlos K. The characterization of some lipid metabolites of Gloeophyllum odoratum grown in vitro. Mycological Research. 1996; 100:23-26.

11. Sgarbi DB, da Silva AJ, Carlos IZ, Silva CL, Angluster J, Alviano CS. Isolation of ergosterol peroxide and its reversion to ergosterol in the pathogenic fungus Sporothrix schenckii. Mycopathologia. 1997; 139:9-14.
12. Michael LB, William WR, James DW. Duality of pathways in the oxidation of ergosterol to its peroxide *in vivo*. Journal Chemical Society, Chemical Commununication. 1976; 2:44-45.
13. Zhang YJ, Mills GL, Nair MG. Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom Grifola frondosa. Journal of Agriculture and Food Chemistry. 2002; 50:7581-7585.
14. Zhang YJ, Mills GL, Nair MG. Cyclooxygenase inhibitory and antioxidant compounds from the fruiting body of an edible mushroom, Agrocybe aegerita. Phytomedicine. 2003; 10:386-390.
15. Borchers AT, Keen CL, Gershwin ME. Mushrooms, tumors, and immunity: An update. Experimental Biology and Medicine. 2004; 229:393-406.
16. Yazawa Y, Yokota M, Sugiyama K. Antitumor promoting effect of an active component of polyporus, ergosterol and related compounds on rat urinary bladder carcinogenesis in a short-term test with concanavalin A. Biological and Pharmaceutical Bulletin. 2000; 23:1298-1302.
17. Takaku T, Kimura Y, Okuda H. Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. Journal of Nutrition. 2001; 131:1409-1413.
18. Subbiah MTR, Abplanalp W. Ergosterol (Major sterol of baker's and brewer's yeast extracts) inhibits the growth of human breast cancer cells in vitro and the potential role of its oxidation products. International Journal of Vitamins and Nutrition Research. 2003; 73:19-23.
19. Bok JW, Lermer L, Chilton J, Klingeman HG, Towers GHN. Antitumor sterols from the mycelia of *Cordyceps sinensis*. Phytochemistry. 1999; 51:891-898.
20. Togashi H, Mizushina Y, Takemura M, Sugawara F, Koshino H, Esumi Y, *et al*. 4-Hydroxy-17-methylincisterol, an inhibitor of DNA polymerase-R activity and the growth of human cancer cells in vitro. Biochemical Pharmacology. 1998; 56:583-590.
21. Zaidman BZ, Yassin M, Mahajna J, Wasser SP. Medicinal mushroom modulators of molecular targets as cancer therapeutics. Applied Microbiology and Biotechnology. 2005; 67:453-468.
22. Kim DS, Baek NI, Oh SR, Jung KY, Lee IS, Kim JH, *et al*. Anticomplementary activity of ergosterol peroxide from *Naematoloma fasciculare* and reassignment of NMR data. Archives Pharmaceutical Research. 1997; 20:201-205.
23. Slominski A, Semak I, Zjawiony J, Wortsman J, Gandy MN, Li JH *et al*. Enzymatic metabolism of ergosterol by cytochrome P450scc to biologically active 17R,24-dihydroxyergosterol. Chemistry and Biology. 2005; 12:931- 939.
24. Villares A, García-Lafuente A, Guillamón E, Ramos A. Identification and quantification of ergosterol and phenolic compounds occurring in *Tuber* spp. Truffles, Journal of Food Composition and Analysis. 2012; 26:177-182.