IDENTIFICATION OF ERGOSTEROL IN MUSHROOMS

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ABSTRACT

Mushroom has gained popularity as prized table delicacy with high nutritive and medicinal value. Ergosterol is a biological precursor (a provitamin) to vitamin D2. The contents of vitamin D2 and sterols in some wild and cultivated mushrooms were determined. Vitamin D2 was determined using an HPLC method, including saponification and semipreparative normal-phase HPLC purification Lv *et al.* [1]. Currently HPLC methods are regularly practiced for the estimation / determination of ergosterol [2]. The original procedure for extraction of ergosterol was worked out by Seitz *et al.* [3]. Four mushrooms *Calocybe indica*, *Ganoderma lucidum*, *Pleurotus florida* and *Volvariella volvacea* were grown on two synthetic (complete yeast extract agar and Lambert's agar) media and three semi- synthetic (malt extract agar, rice bran decoction and and wheat extract agar) media. The mycelial biomass of each mushroom was subjected to extraction of ergosterol and its identification using high performance liquid chromatography (HPLC). The ergosterol content ranged from 113 to 403µg/g with lowest retention peak was observed in *P. florida* showing 113 µg ergosterol per gram where as *C. indica* showed 243 µg ergosterol per gram and *V. volvacea* shows 159 µg ergosterol per gram and highest retention peak was observed in *G. lucidum* showing 403 µg ergosterol per gram of sample.

Keywords: ergosterol, high performance liquid chromatography (HPLC), *Ganoderma lucidum*

INTRODUCTION

Ganoderma lucidium known as lingzhi in Chinese, reishi in Japanese, yeonghi in Korea is a mushroom in the group commonly known as polypore in the family *Ganoderma taceae* has a worldwide distribution in both tropical and temperate regions [4]. *G. lucidum* has been used widely in the treatment of hepatopathy, chronic hepatitis, nephritis, hypertension, arthritis, insomnia, bronchitis, asthma and gastric ulcer and also as antitumor, immunomodulatory, cardiovascular, antinociceptive (active against pain) effects [5]. The substances extracted from the mushroom can reduce blood pressure, blood cholesterol and blood sugar level as well as inhibition of platelet aggregation. The major compounds in *G. lucidum* which have pharmacological activities are ganoderic acid, triterpenes and polysaccharides. More than 150 triterpenes and more than 50 carcinostatic polysaccharides are isolated and are known to be unique compounds in this mushroom [6]. The extracts of *G. lucidium* contain bacteriolytic enzymes, lysozyme and acid protease and by these it show antimicrobial effect [7].

Ergosterol is an important membrane sterol in almost all eumycotic fungi and has been postulated to be strongly associated with living cytoplasmic fungi in the soil. However, ergosterol is not produced by all fungi and the ergosterol concentrations are known to vary between the same species depending on the physiological state of the fungus. Chiocchio and Matkoviæ [8] studied a modification to an ergosterol extraction technique which has the advantage of having a protocol with few steps of purification. This technique also includes an internal loss marker to evaluate the efficiency of extraction.

Ergosterol is a principal sterol of the cell membrane and is strongly bound to it in fungi [9] and is able to activate expression of a number of defense genes and increase the resistance of plants against the pathogens [10]. Ergosterol is a biological precursor (a provitamin) to vitamin D2. It is turned into viosterol by UV light, and is then converted into ergocalciferol, a form of vitamin D also known as D2 [11]. For this reason, when yeast (such as brewer's yeast) and fungi (such as mushrooms), are exposed to UV light, significant amounts of vitamin D2 are produced. Such vitamin D2 serves as the only available dietary source of vitamin D for those who eat no animal products, although such persons can obtain ample vitamin D through exposure to sunlight.

In HPLC, the analyte is forced through a column of stationary phase (usually a tube packed with small round particle with a certain surface chemistry) by pumping (mobile phase) at high pressure through the column. The sample to be analyzed was introduced in small volume to the stream of mobile phase and was retarded by specific chemical or physical interaction with the stationary phase it transverse the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of column) is called the rentention time and is considered a reasonably unique identifying characteristic [12]. Currently HPLC methods are regularly practiced for the estimation / determination of ergosterol, but a spectrophotometric method was also sensitive for semi micro-determination of ergosterol [2]. The original procedure for extraction of ergosterol was worked out by Seitz *et al*. [3].

MATERIALS AND METHODS

Preparation of mushroom extract

The mushroom mycelium was subjected to analytical assay for the extraction of biomedicinal components.

Extraction of Ergosterol

The ergosterol extraction was carried out through saponification in the presence of alcoholic potassium hydroxide. The protocol was standardized with some modification in the method given by Brevik and Owades [13]. Mycelial biomass harvested from the broth was dried by pressing against filter paper to remove moisture and was further used for extraction of ergosterol. Dried sample each having 20-30% moisture was sponified with 25 ml of 25% alcoholic KOH (25 g of KOH, dissolved in 35 ml distilled water bath at 80-90 ºC for 2 to 3 hours. After saponification, samples were allowed to cool and the supernatant thus obtained was transferred to separatory funnel and 20 ml of methanol added over the residue to recover the residual ergosterol. Two aliquots were joined in the separatory funnel and 30-40 ml hexane (HPLC grade) was poured from top into the funnel. The contents were shaken and allowed to stand till the layers separate out. Hexane layer was collected, dried by evaporation in rotavapor and stored at 4 ºC till the HPLC analysis.

Identification of ergosterol

The aliquots prepared were used for the identification of bioactive molecules using high performance liquid chromatography (HPLC) equipment DGU-20A5 at Punjab Agricultural University, Ludhiana. The operation condition of HPLC consisted a high rate flow of methanol and acetonitrile (80:20) at 1ml min > 1 through C=18 reverse phase column. Detection was carried out by a photodiode array detector at 280 nm as most of the compound present in the sample can be detected in this range. The column was first washed with water at least six times and then with solvent. The column clearance was done to remove the impurities and for base line stabilization.

After baseline stabilization, 20 µl of standard was first injected with the help of a sterile needle. The standard was allowed to run for 30 minutes. The photodiode array detector detects the retention time at which various compounds elute from the column and chromatogram was obtained. After 30 minutes the equipment automatically stops for the running of standard. The column was again cleaned by giving 2-3 injections of solvent. Samples were then run as above and after each injection of sample, column was thoroughly cleaned by washing it with solvent

The column was first calibrated with the standard. Standard of ergosterol was obtained from sigma aldrich (E6510) and stored at 4 ºC. The standard was dissolved in methanol with composition of 200 µg/ml and 1 mg/ml.

Quantity of egrosterol present in each sample could be quantified by using the formula given below:

$$\text{Quantity of ergosterol (µg/g)} = \frac{\eta g \text{ of std injected}}{\text{area of peck of std}} \times \frac{\text{area of sample}}{\mu l \text{ of sample injected}} \times \frac{\text{vol of sample}}{\text{wt of sample}}$$

RESULTS

Extraction and Identification of Ergosterol

Biomass of *Calocybe indica, Ganoderma lucidum, Pleurotus florida* and *Volvariella volvacea* was harvested after 15 days of inoculation on malt extract broth, complete yeast extract broth and rice bran decoction broth, respectively. The dried mycelial cake was used for the extraction of ergosterol. Samples were subjected to sponification using methanol and hexane. Final hexane layer containing ergosterol was dried to its minimum in rotavapor to the volume 1.6-2.5 ml. From each sample, 20 µl of solution was injected into C-18 reverse column (3.4.2). After 40 min of HPLC run, retention time graph was obtained for each sample (Plates I-III). The highest retention peak was observed in *G. lucidum* showing 403 µg ergosterol per gram of sample whereas lowest retention peak was observed in *P. florida* showing 113 µg ergosterol per gram (Table 1, Fig. 2)

Table 1. Ergosterol content from mycelium of mushroom cultures

Culture	Retention peak of sample	Area under the peak	Ergosterol content(µg/g)
Calocybe indica (C3)	32.862	426432	243
Ganoderma lucidum (GL-I)	32.993	1059612	403
Pleurotus florida (PF)	33.161	190640	113
Volvariella volvacea (VV12)	33.623	279254	159
CD@ 5%		16	

Retenton time of standard – 32.639, Volume of sample injected -20µl, Weight of sample-1g.

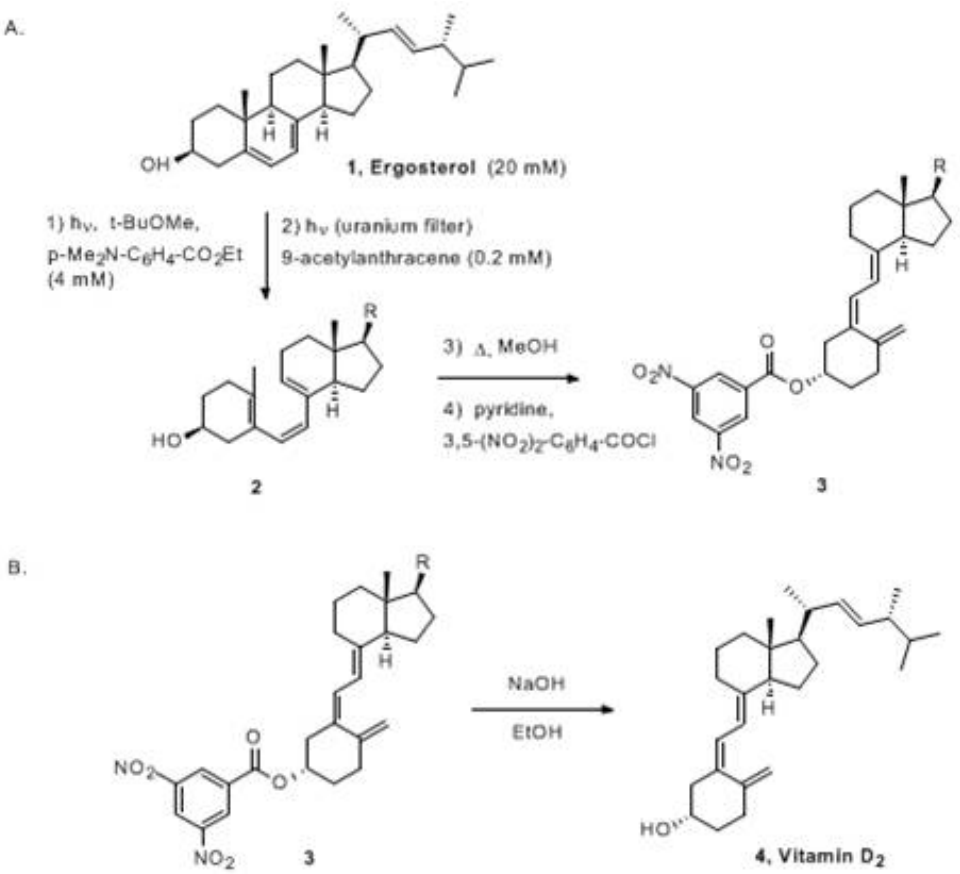

Figure 1. Formation of Vitamin D_2 from Ergosterol
[9,10-Secoergosta-5,7,10 (19),22-tetraen-3-ol, (3â)- from Ergosta-5,7,22-trien-3-ol, (3â)-]

DISCUSSION

HPLC method for determining ergosterol was established for *G. lucidum* and various cultured tree species [14]. Samples from 17 different varieties and 14 different tree species were quantified by HPLC. The RP-HPLC was conducted on Diamonsil C18 column with acetonitrile as the mobile phase at 40 degrees C. The flow rate was 1.0 ml/min; and the detection wavelength was 282 nm. The fluctuations of ergosterol content was 0.093% - 0.243% among different varieties and 0.080%-0.227% among different tree species. Ergosterol was the most abundant sterol found in mushrooms, and its contents were higher in cultivated mushrooms (602.1–678.6) than in wild mushrooms (296–489 mg/100 g dry weight) [15].

The contents of vitamin D2 and sterols in some wild and cultivated mushrooms were determined. Vitamin D2 was determined using an HPLC method, including saponification and semi-preparative normal-phase HPLC purification before analytical reversed-phase quantification with an internal standard. Lv *et al.* [1] studied two species of *Ganoderma, G. sinense and G. lucidum*, used as Lingzhi in China. However, the content of triterpenoids and polysaccharides, main active compounds, were different, though the extracts of both *G. lucidum* and *G. sinense* have antitumoral proliferation effect. It was suspected that other compounds also contributed to their antitumoral activity. Sterols and fatty acids also have bioactivity. To elucidate the active components of Lingzhi, ergosterol, a specific component of fungal cell membrane, was rich in *G. lucidum* and *G. sinense*. But its content in *G. lucidum* (median content 705.0 ìg g^{-1}, range 189.1-1453.3 ìg g^{-1}, n = 19) was much higher than that in *G. sinense* (median content 80.1 ìg g^{-1}, range 16.0-409.8 ìg g^{-1}, n = 13). The mycochemical profiles and the antioxidant activities of the lipophilic extracts of the white and brown button mushrooms. Showed that only free ergosterol were present in both mushrooms at 2.04-4.82 mg/g dry matter (DM).

Ergosterol concentration was higher in early growth stages but decreased as the mushrooms grew, and it distributed evenly between the caps and stems during early developmental stages but accumulated more in the caps after maturation. The photochemiluminescence (PCL) values of the two mushrooms were 5.49-10.48 nmol trolox equivalent/mg DM, and the EC50 values of 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay ranged 20.19-41.49 mg DM/ìg DPPH. The ergosterol content positively correlated with the antioxidant activities (r^2>0.89) recorded by Shao *et al* [16].

CONCULSION

G. lucidum had the highest ergosterol content (403 ì g/g). The observation made during the present study indicated that the mushrooms have medicinal properties like ergosterol, β-glucan which act against pathogenic bacteria.

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