Research Article

Phytochemical Investigation and Quantitative Comparison of Ergosterol Between *Agaricus bisporus* and *Pleurotus ostreatus* by HPLC and GC-MS Methods

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ABSTRACT

The present study aims to investigate the bioactive components and then the quantitative estimation of ergosterol, provitamine D2, in two types of wild edible Iraqi mushrooms. Qualitative phytochemical analysis of chemical tests revealed that the methanolic extracts of both types contain a number of secondary metabolites like alkaloids, anthraquinone, tannins, saponins, flavonoids, sterols and phlobatannins. Our objective was a comparative study for quantitatively estimation ergosterol in two types of mushrooms. Extraction of ergosterol was carried out by maceration with 95%ethanol for 24hrs. Concentrated then saponified in the presence of alcoholic potassium hydroxide, refluxed at 80-90^{0}C for 20min. After saponification, 20ml of methanol added over the residue to recover the residual ergosterol that taken with hexan for further analysis. Currently high performance liquid chromatography (HPLC) method is regularly practiced for a higher percentage of ergosterol in *Oyster* mushroom than *Agaricus bisporus*. The gas chromatography-mass spectrum (GC-MS) results matched by the National Institute of Standard and Technology (NIST) library confirmed that *Oyster* mushroom contained significantly higher levels of Ergosterol among other active constituents. The validated HPLC and GC-MS methods allowed precise quantitative analysis of ergosterol, provitamine D2, in two types of edible mushrooms. These will be further considered in pharmacological activities and isolation of the individual components would, however, help to find new drugs.

Keywords: *Agaricus bisporus*, Oyster mushroom, Ergosterol, HPLC, GC-MS.

INTRODUCTION

The expansion of the natural product industry influenced significantly by the recognition of the value of traditional medical systems and identification of indigenous medicinal plants[1]. Mushrooms are just a small part of the world of fungi, but they are recently one of the most extensively studied organisms of the present scientific era specially medicinal mushrooms[2]. It is estimated that there are approximately 1.5 million species of mushrooms in the world of which 70,000 species are described[3]. *Agaricus* is a genus of mushrooms containing both edible and poisonous species, with possibly over 300 members worldwide[4]. Among these edible mushrooms *Pleurotus ostreatus* and *Agaricus bisporus* are most accepted in the market, which is recognized not only for its nutritional value but also to possible potential for therapeutic applications. They are used medicinally for many diseases. Most attention has been paid to the investigation of natural drugs from various edible mushrooms[5] . While *Agaricus bisporus* only contains 16 IU of vitamin D as ergocalciferol (vitamin D2), since it also contains high amounts of ergosterol, after temporary exposure to UV light the ergocalciferol contents increase[6]. This is similar to the reaction in humans, where vitamin D3, cholecalciferol (made from 7-Dehydrocholesterol in the skin) was synthesized after exposure to sunlight[7]. Ergosterol (ergosta-5, 7, 22-Turin-3β-ol), is a white crystalline organic solid of the molecular formula $C_{28}H_{44}O$ belonging to the steroid family. It is found only in fungi and is chemically related to cholesterol. Employ as many of the same functions that cholesterol serves in animal cells. Its specificity in higher fungi is thought to be related to the climatic instabilities encountered by these organisms in their typical ecological niches [8] . Ergosterol is converted by ultraviolet irradiation into ergocalciferol, vitamin D_2, a nutritional factor that promotes proper bone development in humans and other mammals. Commercially, ergosterol is produced from yeast and then converted into vitamin D_2, which is used as a food supplement[9] .Vitamin in the form of vitamin D2 or D3 considered biologically inactive until they undergo two enzymatic hydroxylation reactions. The first takes place in liver mediated by 25-hydroxylase, which forms 25-hydroxy vitamin D. The second reaction takes place in kidney mediated by 1a-hydroxylase, which converts 25-hydroxy vitamin D to Calcitriol (1,25 di hydroxyl vitamin D), hydroxy vitamin D is the main circulating form of vitamin D bounded to specific plasma carrier protein (DBP), which also transports vitamin D and Calcitriol.[(6)] There is no doubt that we need Vitamin D in it's both two forms in our daily diet with foods rich in it like: cod liver oil, oily fish, mushrooms, fortified cereals, caviar and dairy products. Also can be used as a supplement, especially in specific conditions like in osteoporosis, hypoparathyroidism, pseudo-hypoparathyroidism, familiar hypophosphatemia[10]. Vitamin D is required to maintain normal blood levels of calcium and phosphate that are in turn needed for the normal mineralization of bone, muscle contraction, nerve conduction, and general

cellular function in all cells of the body[11]. The cell differentiating and immuno-modulatory properties underlie the reason why vitamin D derivatives are now used successfully in the treatment of psoriasis and other skin disorders[12]. According to the Institute of medicine the daily requirement of Vitamin D is illustrated in table 1.

Table 1: Recommended Dietary Allowances (RDA) for Vitamin D

Age	Male	Female	Pregnancy	Lactation
0-12 months	400 IU (10mcg)	400 IU (10mcg)		
1-13 years	600 IU (15mcg)	600 IU (15mcg)		
14-18 years	600 IU (15mcg)	600 IU (15mcg)	600 IU (15mcg)	600 IU (15mcg)
19-50 years	600IU (15mcg)	600IU (15mcg)	600IU (15mcg)	600IU (15mcg)
51-70 years	600 IU (15mcg)	600 IU (15mcg)		
>70 years	800 IU (20mcg)	800 IU (20mcg)		

MATERIALS AND METHODS

Collection of Sample

The wild edible mushroom fruit bodies of *Pleurotus ostreatus* (cultivated in Iraq) and *Agaricus bisporus* (wild grown in Iraq). Were collected fresh from the local market, then identified and authenticated by Department of Biology/ College of Science/University of Tikriet.

A voucher sample was kept at the Department of Pharmacognosy/College of Pharmacy/University of Baghdad.

Equipment and chemicals

- All reagents are anhydrous solvents were of annular type and generally used as received from the commercial suppliers.
- Standard Ergosterol is from Bio-Purify comp., China.

Extraction

After collection, Identified samples were collected and cleaned by rubbing, scraping and brushing. The removal of all foreign matters was confirmed. Thereafter they were cut in small pieces of around 2 to 3 cm across using a knife. Then they were wrapped in newspaper and stored in moisture free open places. They were air-dried in shade that took 15 days or more. After drying, they were ground using a metal mortar and pestle into fine powder. 25 g of the dried mushrooms was extracted with 100 ml ethanol overnight at room temperature and was filtered using Whatman No. 2 filter paper. The residue was then extracted with an additional portion of ethanol under the same conditions. The ethanolic extracts were concentrated using a rotary evaporator at 50°C[13].

Phytochemical Screening

Preliminary investigations for the chemical constituents were done using 5% ethanolic potassium hydroxide (KOH) for detection of flavonoids, Mayers and Dragendorffs reagents for detection of alkaloids, and 1% ferric chloride ($FeCl_3$) for detection of tannins, 10% ammonia solution for anthraquinones, Lieberman's test for steroidal glycosides, frothing and emission tests for saponins, 1% aqueous hydrochloric acid for phlobatannins[13].

Separation and Identification of Ergosterol

The ergosterol extraction was carried out through saponification in the presence of alcoholic potassium hydroxide. 20ml of each extract saponified with 25 ml of 25% alcoholic KOH (25 g of KOH, dissolved in 35 ml distilled water) relaxed at 80-90 ºC for 20 to 30min. 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 the 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 chromatographic analysis[14].

HPLC Conditions

P100 isocratic pump (Spectra Physics), UV-100 Detector (Spectra Physics), a data Jet Integrator (Spectra Physics), a flow rate of 1ml / min. The wavelength was set at 290nm for determinate ergosterol. The column used to be LC18 (5µm, 250x 4.6mm). 20 µl of ergosterol standard was first injected with the help of a sterile needle. The mobile phase was Methanol: Acetonitrile, 80:20 v/v (HPLC – grade). Then each sample was injected twice in the HPLC[15].

GC-MS Conditions

Shimadzu GC-MS-QP2010Ultra

Column Oven Temp. : 110.0 °C, Injection Temp. : 250.00 °C, Injection Mode: Split less, Sampling Time: 1.00 min, Flow Control Mode: Pressure, Pressure: 110.0 kPa, Total Flow: 11.4 ml/min, Column Flow: 1.41 ml/min, Linear Velocity: 44.3 cm/Sec, Purge Flow: 3.0 ml/min, Split Ratio: 5.0, High Pressure Injection: OFF, Carrier Gas Saver: OFF, Oven Temp. Program[16]:

Rate, Temperature (°C)	Hold Time (min)
- 110.0	2.00
10.00 200.0	1.00
8.00 280.0	5.00

Pressure Program

Rate	Pressure (kPa)	Hold Time (min)
-	110.0	2.00
10.0	200.0	1.00
8.0	280.	0 5.00

RESULT AND DISCUSSION

Freshly cultivated mushroom contain high moisture content about 87.3%. Variation in water contents among the mushroom samples could be caused by the nature of the mushrooms and the different environmental growth factors. The elimination of water content of the sample to dry state will increase the concentration of nutrient relatively. Thus, drying mushrooms is one method that would extend the shelf life of mushrooms by reducing unnecessary biochemical reaction such as enzymatic browning and lipid oxidation that may lead to quality deterioration[17].

Qualitative phytochemical analysis of mushroom extracts showed the presence of alkaloids, anthraquinone, tannins, saponins, flavonoids, terpenoids, phenolics, as well as steroids. The results have shown that wild edible mushrooms are rich in health-promoting phytochemical compounds than cultivated. Both *Pleurotus ostreatus* and *Agaricus bisporus* mushroom may be used as potential sources of phytochemicals and thus can be used for designing drugs that can prove to be of keen interest in the treatment and prevention of diseases like cancer, tumor, heart diseases, etc[18]. The identification of phytochemical compounds in the HPLC chromatograms of ergosterol standard together with a methanolic extract for each type is based on their retention times, and estimated the concentration according to the area under the peak. These are shown in the following figures (1-3) and table 2 respectively.

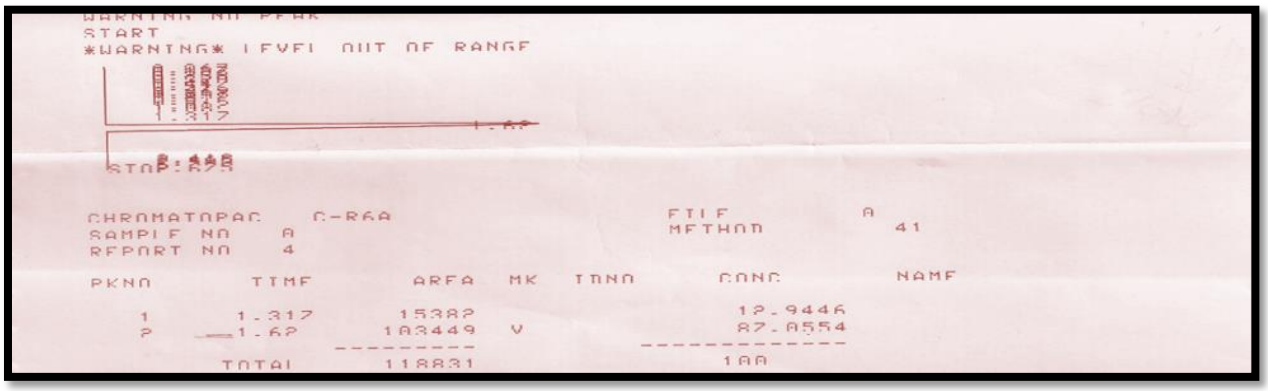

Figure 1: Ergosterol standard chromatogram.

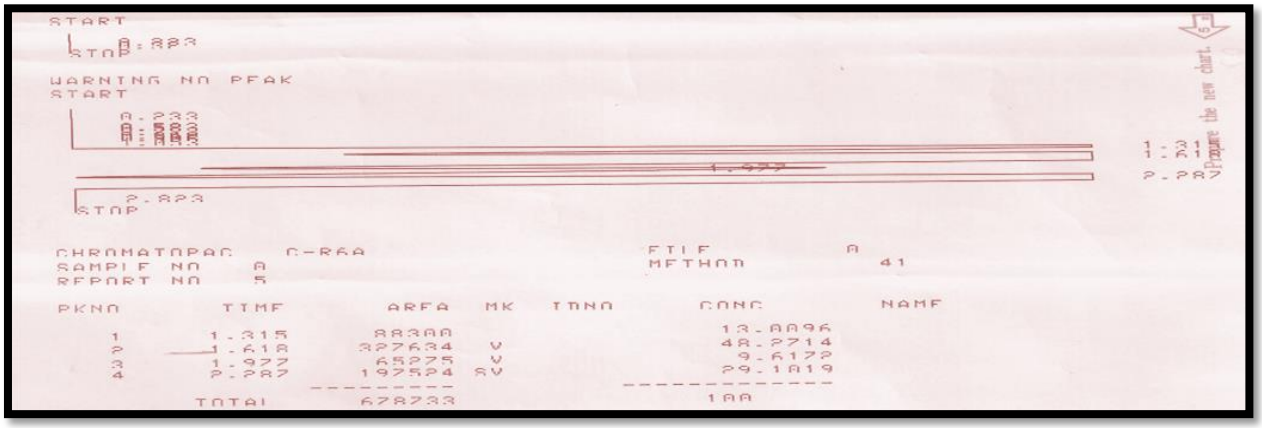

Figure 2: Methanolic extract of *Pleurotus ostreatus* mushroom.

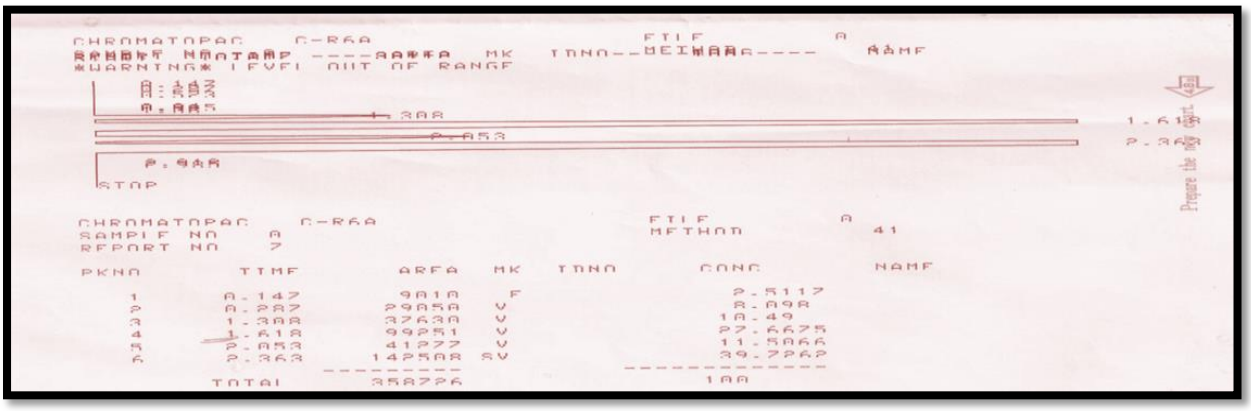

Figure 3: Methanolic extract of *Agaricus bisporus* mushroom

Table 2: Ergosterol content from different types of mushrooms

Sample	Retention time	Area under the peak	Ergosterol content(microgram)
Ergosterol Standard	1.62	103449	87.0554
Pleurotus Ostreatus	1.618	327634	48.2714
Agaricus bisporus	1.618	99251	27.6675

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.[15]Ergosterol content from different types of methanolic extracts of mushrooms was measured by HPLC analysis with UV detector as shown in Figures (2,3) and compared with ergosterol standard in Fig. (1). Ergosterol is easily quantitated because of its ultraviolet (UV) absorption peaks at 282 and 293 NM and its UV spectrum, which differs from those of other plant sterols. The conjugated double bonds in ergosterol at carbons5 - 6 and 7 - 8, whereas other plant sterols absorb weakly at wavelengths greater than 240nm. Prior to the quantification, a saponification is performed to release esterified ergosterol from cytosolic lipid particles. These hydrolysis allow total ergosterol quantification (free ergosterol from fungi walls and cellular/ cytoplasmic ergosterol stemming from ergosterylesters). The total ergosterol amount is usually considered as the most sensitive marker of fungal biomass.

The average yields of ergosterol from extractions of *Oyster* and *Agaracus* mushroom were 48.2 and 27.66% (w/w), respectively. HPLC chromatographies condition in this study gave agood resolution for the analysis of ergosterol in which the retention time was1.62min. As shown in Table (2).*Oyster* mushroom extract contained significantly higher levels of Ergosterol. We determined ergosterol in arange (0.27-0.43) µg/g depending on the sample.So our method to quantify ergosterol showed to be efficient and able to be carried out in only a few steps. It should be noted that there were no overlapped peaks in the HPLC procedure. Several chromatographic methods have been proposed to assess ergosterol including recently both gas chromatography – mass spectrometry and liquid chromatography –atmospheric pressure chemical ionization mass spectrometry. Most of them are based on UV absorption of ergosterol but we used reverse phase High Pressure Liquid Chromatography [HPLC] which becomes the reference method. Interpretation of mass spectrum GC-MS of methanolic extract as shown in (Fig.4) was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were summurised in table (3), in which about 39 compounds were identified.

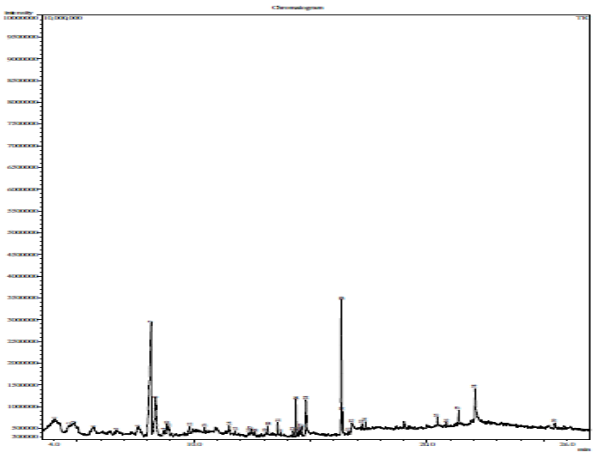

Figure 4: Methanolic extract of *Pleurotus ostreatus* chromatogram.

Table 3: Identification of 39 components by GC-MS

Peak	Time	Area%	Height%	Name
1	4.01	13.18	2.12	Glycerin
2	4.67	1.01	0.47	Hexane, 2,4,4-trimethyl-
3	4.88	2.98	1.02	2-Pyrrolidinone
4	5.67	3.39	1.18	1,2,4,5-Tetrazine, hexahydro-1,2,4,5-tetramethyl-
5	6.67	1.78	0.78	4-Pyridinecarboxylic acid
6	7.61	2.95	1.17	2-Furanmethanamine, tetrahydro-N-[(tetrahydro-2-furanyl)methyl]-
7	8.17	26.52	18.68	DL-Proline, 5-Oxo-, methyl ester
8	8.36	8.88	6.11	Niacinamide
9	8.73	0.80	0.76	DL-Phenylalanine, methyl ester
10	8.85	0.77	1.22	2-Pyrrolidinecarboxylic acid-5-Oxo-, ethyl ester
11	8.92	0.24	0.60	S-[1-Phenyl-2-[2,2-dimethylpropyl]aminoethyl] thiosulfate
12	9.82	0.78	1.16	2-Cyclohexen-1-one, 2-hydroxy-4,4,6,6-tetramethyl-
13	10.46	0.65	1.01	1H-Pyrrole, 1-(4-methylphenyl)-
14	11.51	0.85	1.29	2-Acetyl- 1,5-dimethyl-8-oxabicyclo [3.2.1] octane
15	11.77	0.50	0.72	Glycyl-L-glutamic acid
16	12.36	0.32	0.45	Tetradecanoic acid
17	12.46	1.00	0.97	2,11-Dodecadiene, 4-acetoxy-
18	12.61	0.39	0.61	2,7-Diazaspiro [4.4] nonane, 2-ethyl-
19	13.09	0.18	0.43	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
20	13.17	0.72	1.46	Pentadecanoic acid, methyl ester
21	13.59	1.05	2.07	Pentadecanoic acid
22	13.71	0.26	0.39	Phthalic acid, isobutyl octadecyl ester
23	14.25	0.52	0.98	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
24	14.37	2.82	6.12	Hexadecanoic acid, methyl ester
25	14.43	0.29	0.53	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
26	14.56	0.35	0.56	Methyl 3-O-benzyl-alpha-d-glucofuranoside 5,6-carbonate
27	14.62	0.56	1.22	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester
28	14.81	3.75	5.51	L-(+) -Ascorbic acid 2,6-dihexadecanoate
29	16.32	9.77	22.18	9,12-Octadecadienoic acid (Z, Z) -, methyl ester
30	16.38	1.24	3.31	9-Octadecenoic acid, methyl ester, (E)-
31	16.65	0.26	0.58	Octadecanoic acid, methyl ester
32	16.77	2.27	1.54	9,12-Octadecadienoic acid (Z, Z)-
33	17.21	0.52	1.04	Dodecanamide
34	17.37	0.72	1.34	5-Adamantan-1-yl-2H-pyrazol-3-ol
35	20.45	0.68	1.38	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester
36	20.83	0.42	0.70	Bis(2-ethylhexyl) phthalate
37	21.36	1.03	2.24	Acetic acid, 4,4a,6b,8a,11,11,12b,14a-octamethyl-3-oxodocosahydropicen-2-yl ester
38	22.06	4.74	5.19	9,12-Octadecadienoic acid (Z, Z) -, 2,3-dihydroxypropyl ester
39	25.48	0.88	0.95	Anthiaergosatn-5, 7,9,22-tetraen, 3-acetoxy-
		100.00	100.00	

CONCLUSION

The presence of various bioactive compounds justifies the use of the medicinal mushrooms for various ailments by traditional practitioners. The isolation of these compounds was supportive to identify new drugs to treat various diseases.

The present work highlights the possible use of medicinal mushrooms as the main source of vitamin D supplement, especially in specific conditions like in osteoporosis and hypoparathyroidism.

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