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Chemical and biological investigation of the *Agaricus silvaticus* Schaeff ex. Secr

L.Munkhgerel¹, N.Erdenechimeg¹, M.Dumaa¹, G.Zhang², P.Odonmajig¹, D.Regdel¹

¹ Institute of Chemistry and Chemical Technology, MAS, Mongolia

² Chengdu Institute of Biology, CAS, China

Abstract: Extract of *Agaricus silvaticus* Schaeff ex. Secr fungus have been known to have biological activities, including antibacterial, antiviral, anticancer, antioxidant, anticomplementary and immunostimulating effects. In this study we first time identified that *Agaricus silvaticus* Schaeff ex. Secr has interferon like activity. We first time isolated the four known compounds such as, mannitol (**1**), urea (**2**), ergosterol (**3**) and ergosterol peroxide (**4**) from 95% ethanol extracts of *Agaricus silvaticus* Schaeff ex. Secr in Mongolia. Their structures were elucidated on the basis of extensive analysis of spectral data and by comparison with authentic samples.

Keywords: *Agaricus silvaticus* Schaeff ex. Secr., *A. silvaticus*, *A. sylvaticus*, IFN-like activity, luciferase, mannitol, urea, ergosterol (ergosta-5,7,22-trien-3 β -ol), ergosterol peroxide (5,8-epidioxy-5 α ,8 α -ergosta-6,22E-dien-3 β -ol)

Introduction

Mushrooms are considered nutraceuticals or functional foods by many clinicians and researchers, a fact that has also stimulated the search by Brazilian producers for more advanced production techniques along with introduction of new species. According to Urban, there is great genetic variety of native *Agaricus* genus mushrooms cultivated throughout the world [1].

Agaricus is a large and important genus of mushroom containing both edible and poisonous species, with possibly over 300 members worldwide. This genus belongs to Phylum Basidiomycota, Class – Hymenomycetes (newly described Class Agaricomycetes), Order: Agaricales, Family: *Agaricaceae* [2].

Five *Agaricus* sp. proved to have antioxidant activity and mushroom extracts revealed similar electrochemical responses, suggesting similar electroactive chemical

composition and oxidation potentials more positive than those of the standards (ascorbic and gallic acids). *A. silvaticus* was the most efficient species presenting the lowest EC values in the chemical and biochemical assays and the highest antioxidant power in the electrochemical assays [3].

Agaricus silvaticus is able to reduce anemia and improve biochemical parameters in animals with cancer and has no adverse effects on the blood cells of healthy animals. *Agaricus silvaticus* treatment was able to reduce anemia in animals with cancer. Biochemical parameters were nearer the normal levels in animals treated with this mushroom. These results suggest that administration of *Agaricus silvaticus* Schaeff ex. Secr extract has beneficial effects in rats with Walker 256 tumor, especially in the hematopoietic system [4].

Brazilian researchers have reported that the effects of the dietary supplementation with *Agaricus sylvaticus* fungus in relation to

the quality of life in 56 patients with colorectal and gastrointestinal cancer during post-surgery phase. The patients were treated in the randomized study separated as placebo-controlled and *Agaricus sylvaticus* (30mg/kg/day) supplemented groups. After six months of treatment, it was observed an increase of adhesion to physical activity; improved disposition and good mood, reduction of complaints, pains, and alterations of sleep such as insomnia and bad nights of sleep when *A. sylvaticus* and placebo groups were compared [5].

All these studies showed that nutritional supplementation with Agaricales fungi have beneficial effects in patients, suggesting that it may consist in a new therapeutic perspective to cancer treatment.

Experimental

Melting points were determined on an X-6 apparatus (Beijing Fukai Science and Technology Co. Ltd., P. R. China) and are uncorrected. Optical rotations were measured with a Perkin-Elmer 341 automatic polarimeter (Beaconsfield, United Kingdom). Nuclear magnetic resonance (NMR) spectra were recorded on an Avance Bruker 600 spectrometer (600MHz for ^1H NMR and ^{13}C NMR, Bruker Inc., Rhinstetten, Germany) at room temperature. The chemical shifts (δ) are reported in ppm using tetramethylsilane (TMS) as an internal standard, and the coupling constants (J) are given in Hertz (Hz). High-resolution electrospray ionization mass spectra (HRESIMS) and ESIMS spectra were obtained on a Bruker Bio TOF IIIQ mass spectrometer. Column chromatography (CC) was performed on self-packed open column with silica gel from Qingdao Ocean Chemical Engineering Company (QOCEC, P.R. China). Thin layer chromatography (TLC) analyses were carried out on plates precoated with 10–40 μm of silica gel GF254 from QOCEC and detected under a UV lamp at 254 or 365 nm and visualized by spraying 8% phosphomolybdic acid–ethanol solution (w/v), 5% sulfuric acid (w/v) and 10% ninhydrine- n-butanol (w/v) followed by heating, or visualized by iodine [6].

Mushroom material

The whole mushrooms of *Agaricus silvaticus* *Schaeff ex. Secr* were collected from Bayantes soum, Zavkhan province, Mongolia, in 2009. The mushroom sample was identified by researcher B.Burenbaatar and N.Kherlenchimeg of Institute of Botany, Mongolian Academy of Sciences. A voucher specimen was deposited at the Herbarium of Institute of Botany and Institute of Chemistry and Chemical Technology, MAS.

Extraction and Isolation

The air dried *Agaricus silvaticus* *Schaeff ex. Secr* (900g) was extracted with 95% ethanol at room temperature for 3 times (1 time*7 days), to give ethanol extract, filtered and concentrated to yield the ethanol extract **As3** (71g). The crude ethanol extract was suspended methanol with methanol soluble **As3A** (61g) and methanol insoluble **As3B** (18mg) to give into two sub- fractions. The methanol soluble extract (**As3A**) was suspended in water and subjected to column chromatography with resin D101 and eluted H_2O , 10%, 30%, 50%, 70% and 95% ethanol to give into 6 sub-fractions.

The ethanol soluble fraction was recrystallized to yield compound **1** (**AaI** 55mg). A5 (5.12g) sub fraction was subjected to column chromatography with silica gel (200-300 mesh) and eluted using mixture of petroleum: acetone: acetic acid (5:1:0.1), chloroform:methanol (20:1 to 0:1) solvents, to afford As3A5A and As3A5B into 2 sub-fractions. As3A5B 63-69 were recrystallized to yield compound **2** (**As3A5BT** 8mg) from fraction 130-140.

A6 (5.8g) was subjected to column chromatography with silica gel (200-300 mesh) and eluted using a stepwise gradient of petroleum and acetone (20:1 to 0:1), of chloroform and methanol (10:1 to 0:1) to afford 560 sub-fractions. Fractions 141 and 229 were chromatographed on silica gel column using petroleum: acetone (20:1) solvents, giving compounds **3** (**As3A6Da** **20mg**). Fractions 273-343 were rechromatographed on silica gel column (sil 60 mesh) with Pe: Ace (gradient) as the solvent system, giving compounds **4** (**As3A6Ea** 8mg).

Interferon-like activity The IFN-like activity was determined by Luciferase Reporter Assay System according to the manufacturer's instruction (Promega), using Thermo Scientific Varioskan® Flash. The extracts library were dissolved in DMSO to a final concentration of 100 µg/mL and stored at -20 °C until use. Working solution was prepared in RPMI 1640 medium at a final concentration 100 µg/mL in the plate.

Cell line: The human hepatocellular carcinoma HepG2 cell line was obtained from ATCC, and was maintained in RPMI-1640 (GIBCO, Thermo) medium supplemented with 10% (v/v) calf serum and antibiotics (100 U/ml penicillin and 0.1 g/L streptomycin) at 37 °C in the presence of 5% CO₂. The HepG2-ISRE-Luc2 firefly Luciferase reporter cells was generated by transfecting the HepG2 cells with pISRE-Luc plasmid.

Luciferase Assay for screening: Screen was performed at 96 well format, for each plate, 16 wells were used for negative and positive control, the remaining 80 wells contained test samples which were diluted to 100µg/ml. HepG2-ISRE-Luc2 cells were plated at 5 × 10⁴ cells/well in 96-well plates. After incubation for 24 h at 37 °C in 5% CO₂, cells were stimulated with test samples and 200IU IFN-α as positive control for 24 h. Cells were lysed in Reporter Lysis Buffer and luciferase activity was measured by Luciferase Reporter Assay System according to the manufacturer's instruction (Promega), using Thermo Scientific Varioskan® Flash [7].

Results and Discussion

As seen from biological studies, **A2, A3, A4** groups of *A.silvaticus* extract have showed relatively high interferon identical activities than **A5** and **A6**, while water extract (**A1**) and methanol non-dissolved part (**As3B**) did not show those activities, respectively (Fig.1).

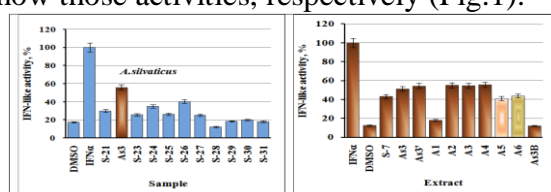


Fig.1. IFN-like activity extracts of *Agaricus silvaticus* Schaeff ex. Secr

Activities of **A2, A3, A4** groups of *Agaricus silvaticus* Schaeff ex. Secr extract were similar to alfa-interferon, which was used as a control, so it was considered that those extracts have some compounds identical to interferon in high amount.

Highly active **A2, A3, A4** group of extracts did not show any spot and picks by TLC and HPLC, where there are no necessities to additional study or change of methodology; therefore, phytochemistry study was performed in **A5** and **A6** extracts separating them by column chromatography.

The four isolated compounds, mannitol (**1**), urea (**2**), ergosterol (**3**) and ergosterol peroxide (**4**) were identified in comparison with EI-MS, ¹H-NMR and ¹³C-NMR spectral data of published earlier.

Compound **1** (AaI) was isolated as an amorphous powder and its elemental composition was determined to C₆H₁₄O₆ by ESI-MS. AaI which was isolated from the ethanol extract was believed to be a monosaccharide based on ¹HNMR measurement and its polarity. ¹H NMR peaks were observed between δ 3.59, 3.71 to 3.79 and only three carbons at δ 70.8, 69.3, and 63.2 were evident in the ¹³C NMR spectrum (Fig.2). Based on an extensive literature search, this compound was believed to be mannitol. In addition, the ¹H and ¹³C NMR spectra of compound AaI were in good agreement with those of mannitol. Therefore, compound AaI was identified as mannitol [8].

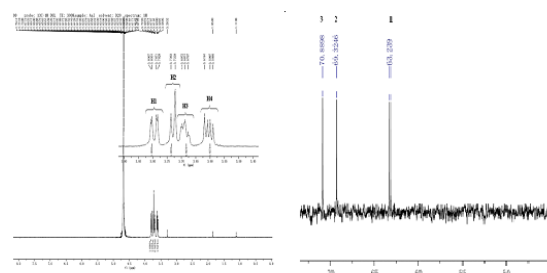
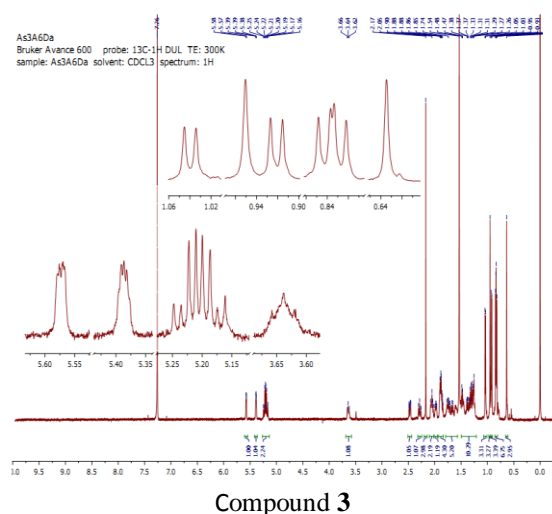


Fig.2. ¹H and ¹³C NMR spectral data of mannitol (**1**)

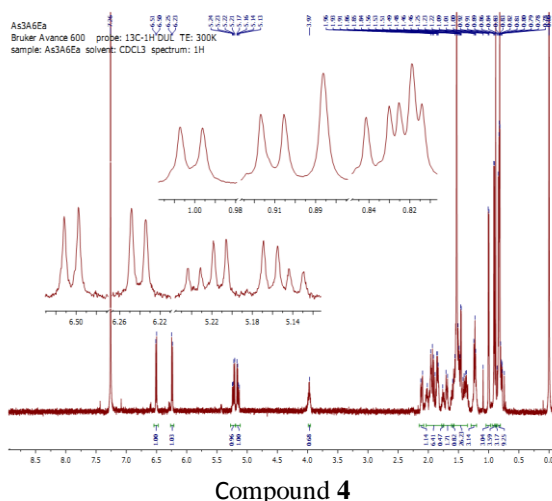
Urea (**2**) and ergosterol (**3**) structures were elucidated on the basis of extensive analysis of spectral data and by comparison with authentic samples [9].

Compound (**4**) was isolated as a colorless crystals and its elemental composition was determined to be C₂₈H₄₄O₃ ESI-MS. Ergosterol peroxide (5,8-epidioxy-5α,8α-

ergosta-6,22E-dien-3 β -ol) seemed to have an ergosterol (ergosta-5,7,22-trien-3 β -ol) type steroid structure by comparing its NMR spectral data with those of ergosterol (**3**).



Compound 3



Compound 4

Fig.3. ^1H NMR spectral data of ergosterol (**3**) and ergosterol peroxide (**4**)

The difference in molecular formula between ergosterol (**3**) and compound **4** is two oxygens, implying that compound **4** is a peroxidated derivative of ergosterol (**3**). From the ^1H and ^{13}C NMR spectral data, compound **4** was identified as ergosterol peroxide (Table 1). The ^{13}C NMR spectrum showed carbon signals of two double bonds at δ 130.68 (C-7), δ 132.26 (C-23), δ 135.16 (C-22), δ 135.39 (C-6). Other characteristic signals in ^1H NMR spectrum were δ 5.183 (m, 2H, H-22, H-23), δ 3.961 (m, 1H, -OH), δ 0.998 (d, J = 6, 3H, H-21), δ 0.908 (d, J = 7, 3H, H-28), δ 0.881 (s, 3H, H-19), δ 0.831 (d, J = 8, 3H, H-27), δ 0.818 (d, J = 8.5, 3H, H-26), and δ 0.815 (s, 3H, H-18) [9, 10].

Table 1. ^1H NMR spectral data of ergosterol (**3**) and ergosterol peroxide (**4**)

^1H (Shin Yusoo <i>et al</i>)	A6Ea	^1H NMR (S.Kim <i>et al</i>)	A6Da
3.96, m	3.97, m, 1H, -OH	3.633, m	3.64, m, (H-3)
6.24, d, J=8.	6.24, d, H-6	5.577, 5.389, J=2.358	5.577, 5.389
6.51, d, J=8.6	6.50, d, H-7	5.181, 5.122, J=2.358	5.181, 5.122
0.82, s	0.83, s, 3H, H-18	0.633, s	0.63, s, (H-18)
0.88, s	0.89, s, 3H, H-19	0.949, s	0.95, s, (H-19)
1.00, d, J=6.6	1.00, d, 3H, H-21		
5.16, d, J=7.6	5.16, dd,		
5.20, d, J=6.8	5.22, d,		
0.82, d, J=6.7	0.82, d, 3H, H-26	1.038, d, J=6.6	1.04, d, (H-26)
0.84, d, J=6.7	0.84, d, 3H, H-27	0.841, d, J=6.7	0.838 d, (H-27)
0.91, d, J=6.9	0.91, d, 3H, H-28	0.919, d, J=6.8	

The all four compounds are first found in *Agaricus silvaticus* Schaeff ex. Secr in Mongolia. The chemical structure of isolated four compounds is shown in Fig. 4. Ergosterol, a sterol, is the biological precursor to Vitamin D2. It is turned into viosterol by ultraviolet light, and is then converted into ergocalciferol, which is a form of Vitamin D. Ergosterol is a component of fungal cell membranes, serving the same function that cholesterol serves in animal cells. Ergosterol is not found in mammalian cell membranes [11].

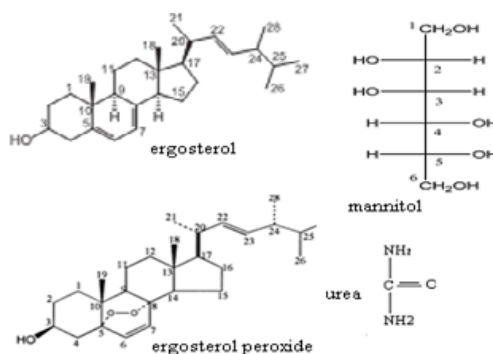


Fig.4. Structures of the compounds from *A.silvaticus*

Ergosterol is also used as an indicator of fungal biomass in soil. Though ergosterol does degrade over time, if kept below freezing in a dark environment, this degradation can be slowed or even stopped completely. Research has shown ergosterol may have antitumor properties [12].

Ergosterol peroxide have been previously isolated and identified from *Inonotus obliquus*, common wood-rotting fungi in Filand, Lanum lyratum and

Naematoloma fasciculare which is toxic mushroom widely distributed in the world. It was reported that ergosterol peroxide from *Lobliquus* not only inhibited the growth of cancer cells but also killed them in the antitumor tests. Recent study also revealed its inhibitory effects on induced inflammation and tumor promotion in mouse skin [9].

Based on the summarized results described above, it could be concluded that ergosterol peroxide (4), which is peroxidated at C-5 and C-8 of ergosterol (3), have strong antitumor activity, cytotoxic activity and anticomplementary activity [10].

Ergosterol peroxide (EPO) is a major antitumor sterol produced by edible or medicinal mushrooms. These findings suggest that bacterial metabolites of EPO play a significant role in its cytotoxic activity against human colorectal cancer cells [13].

Conclusions

- In this study we first time identified that *Agaricus silvaticus Schaeff ex. Secr* has an interferon like activity.
- Four known compounds such as mannitol (1), urea (2), ergosterol (3) and ergosterol peroxide (4) from fruit bodies of *Agaricus silvaticus Schaeff ex. Secr* from Mongolia has been isolated successfully. Their structures were elucidated on the basis of extensive analysis of spectral data and by comparison with authentic samples.
- The extraction of chemical compound which has interferon like activity from the natural raw materials is very important as nowadays the cancer and the hepatitis disease increasing highly. Therefore, the findings we demonstrated here is useful for producing the pharmaceuticals to depress the activity of hepatitis B and C viruses.

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