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Chemical constituents and potential cytotoxicity of Hygrophorus agathosmus

E. Penevaa, B. Trushevaa, M. Gyoshevab, P. Georgievab, S. Popova, V. Bankovaa

[a]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
[b]*Institute of Botany, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*
E mail-bankova@orgchm.bas.bg

ABSTRACT - The macromycete Hygrophorus agathosmus was found to be toxic to Artemia salina nauplii and the active chloroform fraction of the total extract was chemically studied. Several hydrocarbons, including paraffins, prystan, phytan, and squalene were identified. The sterol composition of this mushroom was analyzed for the first time, the main sterol being ergosterol. 5α, 8α-Epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol was isolated for the first time from H. agathosmus. The presence of the latter compound, which might be regarded as an artifact obtained by oxidation of the corresponding Δ 5,7 sterol, could provide at least partial explanation of the observed toxicity of the chloroform fraction.

KEYWORDS - *Hygrophorus agathosmus*, *A. salina* toxicity, constituents

INTRODUCTION

The macromycete *Hygrophorus agathosmus* Fr.:Fr. (family Tricholomataceae, order Agaricales, class Basidiomycetes) is widespread in Europe, North Africa, Asia and North America. It forms mycorrhizae with coniferous trees, most often with spruce *Picea abies* L. (Pinaceae). This mushroom is edible but the strong odor is a limitation for using it as foodstuff (1). Till now, the chemical composition of *H. agathosmus* has barely been studied: Rapior et all. (2) analyzed its volatiles and identified a number of aromatic compounds (benzaldehyde, phenylacetonitril, etc.) and tridecanal. In other members of the genus *Hygrophorus*, biologically active (fungicidal and antibacterial) compounds, 4-oxo-2-alkenoic fatty acids (3) and 5-(hydroxyalkyl)-2-cyclopentenone derivatives (hygrophorones) have been found (4), for this reason, a phytochemical study of *H. agathosmus* appeared of interest. In this paper, we report on the study of the toxicity of *H. agathosmus* extracts to *Artemia salina* nauplii and the identification of some chemical constituents.

MATERIALS AND METHODS

Biological material

Hygrophorus agathosmus was collected in November 2003 in Rila Mountain, in the locality of Borovets, 1200 m a.s.l. The material was identified by Dr. Melania Gyosheva. Voucher is kept in the Mycological Collection of the Institute of Botany, Bulgarian Academy of Sciences, voucher specimen #SOMF 25989.

Extraction

The fresh fruit bodies of *H. agathosmus* (1300 g) were cut into small pieces and consecutively extracted with ethanol (2L), ethanol-chloroform (1:1) (2L) and chloroform (2L). The three extracts were combined, water was added and the chloroform layers were removed and evaporated *in vacuo* to yield 5.0 g dry residue. The water-ethanol layers were extracted twice with *n*-butanol and the butanol layer was evaporated *in vacuo,* (0.84 g dry residue).

Isolation and identification of compounds

The chloroform extract was subjected to column chromatography on silica gel with an *n*-hexane - acetone gradient (30:1 – 5:1) to produce eight fractions (**I** – **VIII**).

The first fraction of this column, **I** (215 mg) was subjected to GC-MS analysis with a gas chromatograph (Hewlett Packard 5890 Series II Plus) linked to mass spectrometer system (Hewlett Packard 5972) equipped with a 23 m long, 0.25 mm id, 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 100^{o}C to 310^{o} C at a rate of 5^{o}C.min^{-1}. Helium was used as a carrier gas, flow rate 0.7 ml.min^{-1}. Split ratio 1:80, injector temperature 280^{o}C, ionization voltage 70eV.

Fraction III. after further purification by preparative TLC (silica gel G, n-hexane – acetone 10:1) yielded 111 mg sterol mixture, which was analyzed by GC-MS. A gas chromatograph (Hewlett Packard 5890) linked to mass spectrometer (Hewlett Packard 5972) with a capillary column SPB-50 (30 m * 0.32 mm, 0.25 μm film thickness) was used. The carrier gas was helium and a temperature program of 270 °C – 290 °C at 4 °C.min-1 and a 20-min hold was used. The ion source was set at 250 °C and the ionization voltage was 70 eV.

From fraction **VI.** by preparative TLC (silica gel, *n*-hexane – methyl ethyl ketone 10:1), 5α,8α-epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol (15.2 mg) was isolated and identified by comparisons of its EIMS, ^{1}H- and ^{13}C-NMR spectra with literature data (5).

GC-MS identification.

The GC/MS investigation was based on the interpretation of the mass spectral fragmentation followed by comparisons of the spectra obtained with those of authentic samples. Computer searches in a HP Mass Spectral Library NIST98 (Hewlett Packard, Palo Alto, California, USA) were also applied in most cases. When the spectra of some isomers were very similar and these compounds could not be identified unambiguously, comparisons of the GC retention times, obtained under the same conditions, were used. When there were no suitable authentic samples and spectra for comparison, no identifications were made. Only the unambiguously identified compounds were reported. The ion current generated depend on the characteristics of the compound and for this reason are not a true quantitation. The results obtained by GC/MS might be used for characteristics of the biodiversity in the investigated organisms, as well as for comparisons between different groups of metabolites in them.

Cytotoxicity assay.

Artemia salina (nauplii) lethality (6) was determined using caffeic acid phenethyl ester (CAPE) as active reference compound. The nauplii were hatched using simulated sea water (10g marine salt, (Pomorie, Bulgaria) and 100 mg $NaHCO_3$ in 500 ml water) at 27-28ºC, under aeration and illumination. Concentrations of 1000, 100, 10 and 1 ppm for the test substances were used, 10 *A. salina* nauplii per concentration, plus 10 nauplii control group. The experiment was performed in triplicate for every concentration and control group. The results are presented as mean ±SD. Differences between the mean values were tested by Student's t-test ($t<0.05$).

RESULTS AND DISCUSSION

Chloroform and butanol fractions of the fruiting bodies's extract were tested for their toxicity against *A. salina* nauplii. The results are represented in Table 1.

Table 1 - Cytotoxicity assay of fractions of H. agathosmus extract

Sample	$EC_{50}\pm SD$ (mg.ml^{-1})[a]
Chloroform fraction	10±6
Butanol fraction	>1000
CAPE[b]	0.45±0.05

[a] *Mean of three measurements ;* [b] *Positive control*

The chloroform fraction demonstrated significant toxic effect (EC_{50}=10±6), which is an indication of potential cytotoxicity, while the toxicity of the butanol fraction was much lower (EC_{50} >1000). For this reason, the chemical composition of the chloroform fraction was further studied. The extract was subjected to column chromatography and several fractions were obtained. The first fraction was analyzed by GC-MS and the results are given in Table 2.

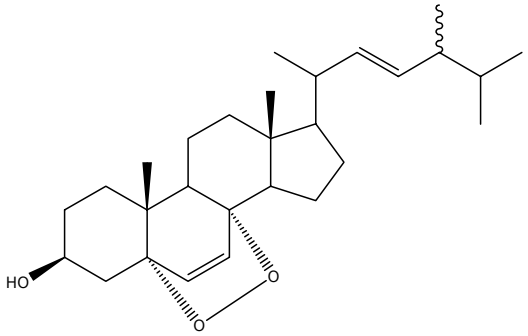
Table 2- Composition of fraction I. from the chloroform fraction(GC-MS)

Compound	% of total ion current[a]
Tetradecane	3.2
Pentadecane	4.0
Haexadecane	3.8
Heptadecane	2.8
Pristan	1.6
Diisopropyl naphthalene	8.2
Octadecane	2.3
Phytan	1.8
Nonadecane	2.2
Methyl hexadecanoate	1.2
Ethyl hexadecanoate	1.4
Eicosane	1.2
Henicosane	1.6
Ethyl linoleate	27.9
Docosane	0.9
1-Undecyl decahydronaphtalene	9.9
Pentacosane	5.3
Squalene	5.5

[a] *The ion currents generated depend on the characteristics of the compound and for this reason are not a true quantitation.*

Besides the paraffins and cycloparaffins, fatty acid esters and several isoprenoid hydrocarbons (prystan,

phytan, squalene) were identified. The ethyl esters of fatty acids might be artifacts as a result of transesterification during extraction procedures. The concentrations of the straight chain hydrocarbons were very similar for the groups with odd and even number of carbon atoms, which is an indication for the presence of bacteria in the investigated mushroom. Another fraction of the column chromatography, after additional purification by preparative TLC, yielded the sterol mixture which was analyzed also by GC-MS. The main sterol was 22E-ergosta-5, 7, 22-triene-3β-ol (ergosterol) (82%), typical fungal sterol, accompanied by two other ergostane derivatives: (22E)-ergosta-5,7,9(11),22-tetraene-3β-ol (11.5%) and ergosta-7-ene-3β-ol (6.5%). This is the first report on sterol composition of *H. agathosmus* and it seems similar to the ones reported for other *Hygrophorus* species studied (7).



1.

From fraction **VI**, after preparative TLC a crystalline compound was isolated and identified as 5α,8α-epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol by comparing its spectral data (MS, ^{1}H- and ^{13}C-NMR) with literature data (5). It was isolated for the first time from this species. This type of compounds have recently been demonstrated to have inhibitory action against HTLV-1 virus and cytotoxic activity against human breast cancer cell line (MCF_7WT) (5). The presence of this compound, which might be regarded as an artifact obtained by oxidation of the corresponding $\Delta^{5,7}$ sterol, could provide at least partial explanation of the observed toxicity from the chloroform extract.

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