ANALYTICAL AND BIOLOGICAL CHARACTERIZATION OF THE LIPOPHILIC EXTRACT PROFILE OF CHAGA (*INONOTUS OBLIQUSS*)

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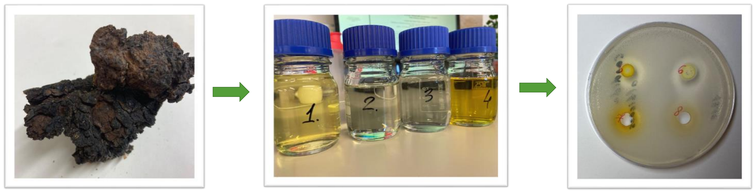
Abstract

The lipophilic compounds of chaga were isolated applying conventional (Soxhlet), non-conventional (ultra-sound assisted, accelerated solvent) and environmentally friendly (supercritical fluid) extraction methods utilizing both polar and non-polar solvents. The yield and profile of lipids, phytosterols, and triterpenoids of chaga were discussed and potential application in terms of antimicrobial and anticancer activity against opportunistic pathogens demonstrated.



Introduction

Chaga (*Inonotus obliquus*) (Fr.) Pilát is a black perennial fungus of the *Hymenochaetaceae* family that parasites on adult birch trunks. *Inonotus obliquus* produces a wide range of bioactive metabolites, including triterpenoids, phytosterols, polysaccharides, polyphenols. These compounds have antioxidant, antitumor, and antiviral activity and the ability to increase immunity to pathogenic microbial infections. Since the 12th century, chaga has traditionally been used in Russia, China, Poland, Baltic States to treat and prevent gastrointestinal disorders, cardiovascular diseases, cancer, tuberculosis, diabetes and skin problems

The aim of the study is to obtain the maximum high yield of extracts with beneficial biology activity. This would allow the development of industrially based components based on green raw material that can participate in the correction and prevention of pathological disorders.

Experimental

To evaluate the profile of lipophilic fractions and validate the potential application of the compounds present in chaga fungus the I. obliquus fungi was collected.

Extraction of lipophilic compounds was done using different extraction techniques (Soxhlet, ultrasound, supercritical fluid, accelerated solvent extraction) with different polarity solvents. The obtained lipophilic extracts were derivatised with BSTFA and analyzed by gas chromatography-mass spectrometry. Chaga's chloroform extract was fractionated by different polarity solvents and hexane/ethyl acetate gradient. The effect of the obtained fractions on pathogenic microorganisms (*Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia colli, Candida albicans*) and A-549 (human lung adenocarcinoma cells), MCF7 (human breast adenocarcinoma cells), B16-F10 (murine melanoma cells), CaCo-2 (human colorectal adenocarcinoma cells) cancer cell lines was tested. The antimicrobial properties of the obtained extracts were characterized by agar diffusion test and anticancer activity by MTT (colorimetric assay for assessing cell metabolic activity) test.

Results

Black alder (*Alnus glutinosa*) chaga fungi (*Inonotus obliquus*) was extracted with 10 different polarity solvents and different methods. **Figure 1** show that the extraction yield increases with increasing solvent polarity – the chaga contains more polar compounds. The extraction method does not significantly affect the extraction result, but a small increase with all solvents is observed in Soxhlet extraction. Although it takes more time. Extraction of the sample with a mixture of CO_2/ethanol (85:15) in the supercritical state gives the same extraction result as the use of hexane with different extraction methods.

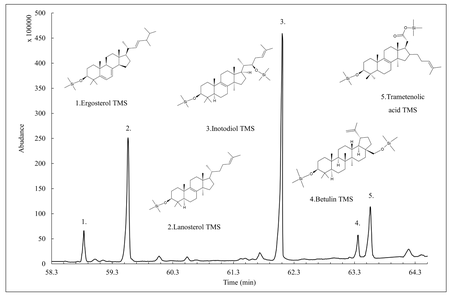


Fig. 2. Profile of phytosterols and triterpenoids of chaga GC-MS chromatogram (solvent - ethyl acetate; extraction - ASE)

Chromatograms with a mixture of phytosterols and triterpenoids were obtained by analysing chaga-derived (TMS) extracts: ergosterol, lanosterol, inotodiol, trametenolic acid and betulin. **Figure 2** shows the chromatogram of alder chaga extract, where the inotodiol fraction predominates. It is specific and located only in the sclerosis of *Inonotus obliquus*.

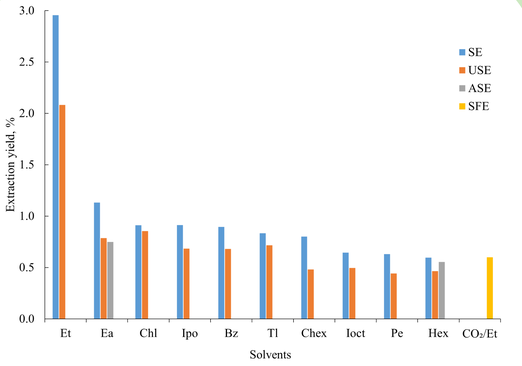


Fig. 1. Extraction result with different polarity solvents (SE – Soxhlet extraction; USE – ultra-sonic extraction; ASE – accelerated solvent extraction; Et – ethanol; Ea – ethyl acetate; Chl – chloroform; Ipo – isopropanol; Bz – benzene; Tl – toluene; Chex – cyclohexane; Ioct – isooctane; Pe – petrol ether; Hex – hexane)

Concentrations of compounds of the same extract vary depending on the solvent and the extraction method. Inotodiol, the major triterpenoid, which has been shown anti-cancer activity on cancer cells was compared by different extraction (**Figure 3**). Ultrasonic extraction results showed the highest results for inotodiol using chloroform and Soxhlet extraction – with cyclohexane. Accelerated solvent extraction is low in triterpenoid amount, especially with hexane. The optimization of SFE by varying temperature, pressure, time, flow rate, co-solvent and particle size of raw material can provide high amount of lipophilic extract. Among other things, SFE is a green chemistry extraction method that is environmentally friendly

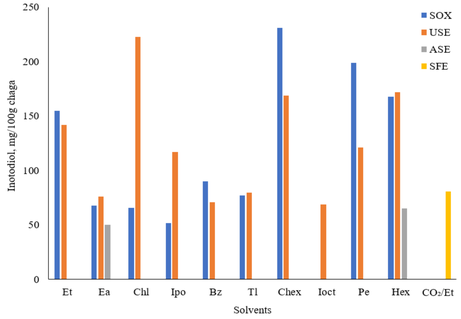


Fig. 3. Extraction result of inotodiol with different methods

Escherichia coli, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are resistant to chaga triterpenoids. Chaga lipid extracts act as good inhibitors of pathogenic *Candida albicans* yeasts (**Figure 4**). The antifungal activity of aqueous extracts of chaga samples is lower.

After the first fractionation, the ethyl acetate (**3F**) fraction, which dissolves almost all sterols and triterpenoids, has the highest antimicrobial activity among other fractions against yeast. To determine which compounds inhibited the growth of *C. albicans*, the ethyl acetate fraction was re-fractionated. The mixture of triterpenoids - inotodiol and betulin (**2SF**) - has antimicrobial activity against pathogenic yeasts.

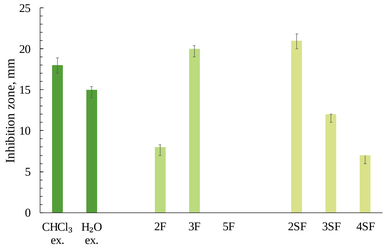


Figure 4. Antimicrobial activity of chaga extracts against *Candida albicans* yeast

The crude chloroform extract was found to be cytotoxic to all cancer cell lines used, but its activity was lower than that of the other fractions (**Table 1**). Cell viability assessed by the MTT assay showed that MCF-7 cancer cells are most sensitive to chaga phytosterols and triterpeoids. The concentrated mixture - lanosterol, inotodiol and betulin in the SF2 subfraction - has strong anti-cancer activity against the MCF-7 cancer cell line. But SF3, which mainly contains stigmasterol and tramethenolic acid is stronger against B16-F10 and CaCo-2.

Table 15

Inhibitory effects on the growth of the cancer cell lines

Ekstrakts	IC_{50} (µg/mL)			
	A-549	MCF-7	B16-F10	CaCo-2
$CHCl_3$ ekstr.	57,16 ± 1,04	42,3 ± 1,3	72,3 ± 1,2	88,9 ± 1,2
F3	**35,97 ± 1,04**	43,9 ± 1,1	42,1 ± 1,3	49,06 ± 1,02
SF2	48,41 ± 1,02	**31,8 ± 1,5**	74,59 ± 1,06	63,53 ± 1,07
SF3	51,10 ± 1,00	37,9 ± 1,3	**35,9 ± 1,5**	**45,62 ± 1,06**

Since chaga drinks have been used in folk medicine for the prevention and treatment of cancer, it is reasonable to assume that an aqueous extract has cytotoxic activity against cancer cell lines. The aqueous extract has a significantly lower anticancer activity (40 times) compared to the lipid fraction of chaga, especially with B16-F10 cells (**Figure 5**). Concentration of the extract up to 400 µg/mL has a beneficial effect on the growth of B16-F10 cells.

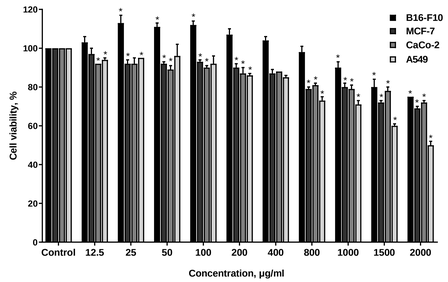


Figure 5. The effect of an aqueous extract of chaga on different cancer cell lines depending on the concentration.

Concluisons

Chaga is a rich source of unique lipids. The polarity of the solvent affects the lipid extraction result of the chaga. To obtain the maximum lipid yield, it is necessary to use extraction with chloroform by ultrasonic extraction. **Extraction using CO_2 in the supercritical state in the presence of a cosolvent is considered perspective**.

Chaga lipids and aqueous extracts have **antifungal properties**. All lipophilic chaga extracts show **cytotoxicity against various cancer cell lines**. Fragmentation of chaga lipids may allow the identification of specific compounds that determine it.

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