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Research Paper

Chemical characterization and biological activity of Chaga (*Inonotus obliquus*), a medicinal "mushroom"

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ABSTRACT

Ethnopharmacological relevance: In Russian traditional medicine, an extract from the mushroom *Inonotus obliquus* (Fr.) Pil´at is used as an anti-tumor medicine and diuretic. It has been reported that *Inonotus obliquus* has therapeutic effects, such as anti-inflammatory, immuno-modulatory and hepatoprotective effects. This study was designed to investigate the chemical composition and biological properties of aqueous and ethanolic extracts of *Inonotus obliquus* from Finland, Russia, and Thailand. Their antioxidative, antimicrobial, and antiquorum properties were tested as well as the cytotoxicity on various tumor cell lines.

Materials and methods: The tested extract was subjected to conventional chemical study to identified organic acids and phenolic compounds. Antioxidative activity was measured by several different assays. Antimicrobial potential of extracts was tested by microdilution method, and antiquorum sensing activity and antibiofilm formation of *Inonotus obliquus* extracts was tested on *Pseudomonas aeruginosa*. Cytotoxicity of the extracts was tested on tumor cells (MCF-7, NCI-H460, HeLa and $HepG_2$) and non-tumor liver cells primary cultures.

Results: Oxalic acid was found as the main organic acid, with the highest amount in the aqueous extract from Russia. Gallic, protocatechuic and *p*-hydroxybenzoic acids were detected in all samples. *Inonotus obliquus* extracts showed high antioxidant and antimicrobial activity. Extracts were tested at subMIC for anti-quorum sensing (AQS) activity in *Pseudomonas aeruginosa* and all extracts showed definite AQS activity. The assays were done using twitching and swarming of bacterial cultures, and the amount of produced pyocyanin as QS parameters. All the extracts demonstrated cytotoxic effect on four tumor cell lines and not on primary porcine liver cells PLP2.

Conclusions: As the *Inonotus obliquus* presence in Chaga conks is limited, further purification is necessary to draw quantitative conclusions. The presence of AQS activity in medicinal mushrooms suggests a broader anti-infectious disease protection than only immunomodulatory effects.

1. Introduction

In the present study we searched for bioactivity in extracts from the well known medicinal mushroom *Inonotus obliquus* (Pers.: Fr.) Pilat, commonly known as Chaga. This "mushroom" is the hyperplastic conk consisting of wood and mycelium that occurs on Birch after infection and invasive growth of the fungus. Extracts of Chaga have been used in China, Korea, Russia and the Baltics for their presumed antibacterial, hepatoprotective, anti-inflammatory, antitumor, and antioxidant activities (Lemieszek et al., 2011). *In vitro*, the properties of Chaga were found to be non-toxic and mostly antiviral (Awadh Ali et al., 2003), antioxidative (Nakajima et al., 2007) and antiinflammatory (Kim et al., 2007). Song et al. (2007) found opposite results showing that *Inonotus obliquus* extract was both toxic and proinflammatory in macrophages *in vitro*. Artificially induced acute colitis in mice could be cured by oral administration of Chaga extract (Mishra et al., 2012), suggesting suppression of inflammatory cytokines. No clear antibiotic effects were reported in the scientific literature for Chaga until now.

Quorum sensing (QS) is a communication system between individuals or individual cells that allows recognizing and reacting

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to the size of the surrounding population. QS allows microorganisms when they reach sufficient numbers to form biofilms that protect from environmental attacks such as from antibiotics and allow escape from their host's immune system. Biofilms are sessile bacterial communities and can form on biotic and abiotic surfaces. Bacteria as *Pseudomonas aeruginosa* use small diffusible signaling molecules that regulate expression of genes including those for virulence, swarming motility, cell aggregation and production of extracellular polysaccharides (Fuqua et al., 2001; Danchin et al., 2004; Rasmussen and Givskov, 2006). Cell motility is the key issue in bacterial pathology; it determines the ability to colonize the environment. Motility is dependent on the flagella involved in swimming in liquid environment, in swarming on semi-solid surfaces, and on type IV pili, that play a role in the adherence to the surface. At least 65% of all the infectious diseases are associated with bacterial biofilms (Fuqua et al., 2001). Each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections (CDC, 2013). Multidrug-resistant *Pseudomonas aeruginosa*, is among the most serious threats (CDC, 2013). It is a well-known biofilm former, and its biofilms are notoriously difficult to eradicate by conventional antibiotic treatment. Biofilm formation is, therefore, being studied extensively in search of novel therapeutic approaches (reviewed in Boyle et al., 2013). Swarming motility raises equally interesting implications. Previous studies have shown that *Pseudomonas aeruginosa* cells in swarming colonies can have distinct phenotypes from planktonic cultures, including gene expression and increased antibiotic resistance (Lai et al., 2009). In addition, the self-produced biosurfactants required for swarming motility (Caiazza et al., 2005) are important for biofilm maintenance and dispersion as well as to kill immune cells (Jensen et al., 2007). Biofilm formation and swarming motility are inversely regulated and the regulation involves the second messenger cyclic diguanylate (c-di-GMP): high levels of c-di-GMP induce biofilm formation and suppress swarming motility (Baraquet et al., 2012). This inverse regulation could be of interest for novel therapeutics against biofilm formation.

The rapid emergence of multiple resistances against the present broad spectrum antibiotics used for infectious disease needs immediate search for alternatives. Non-toxic natural products that can inhibit microbial quorum sensing and thereby stop the appearance of new antibiotic resistant bacterial strains could be such an alternative.

Recently we have described direct antibiotic effects as well as anti-QS properties of extracts of the well known medicinal mushroom *Agaricus blazei* Murill (ABM) against various bacteria (Stojković et al., 2014). Based on its immunomodulatory effects, ABM had previously been found to show protective activity against various bacterial diseases (Coates et al., 2002; Rumbaugh et al., 2009; CDC, 2013; Stojković et al., 2014) of which the effects against *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Streptococcus pneumoniae* had been related with the immune system (Coates et al., 2002, Stojković et al., 2014). The latter was contradicted by the study of Fantuzzi et al. (2011) who described that ABM extract did not promote immunostimulation and protection during experimental *Salmonella enterica* infection in mice. The finding of Fantuzzi et al. (2011) led us to look for possible anti-QS properties of ABM extracts. MIC's and MBC's of these extracts turned out equal to or better for inactivation of *Pseudomonas aeruginosa* than those of ampicillin and streptomycin. The effect was caused by anti-QS compounds present in the extracts of *Agaricus blazei* (Soković et al., 2014).

In the present work, we report the chemical composition, antioxidant, antimicrobial, anti-QS and antitumor activities of extracts of *Inonotus obliquus* wild samples obtained from Russia, Finland and Thailand.

2. Material and methods

2.1. Mushroom material

Wild *Inonotus obliquus* (Thailand) fruiting bodies were obtained from the Natural Medicinal Mushroom Collection of the Faculty of Biology of Mahasarakham University, Thailand, and had been identified by Prof. Usa Klinhom. Wild *Inonotus obliquus* (Russia) was obtained from Life Extension Asia, Singapore, and was (re) identified by one of us (J.G.). Wild *Inonotus obliquus* (Finland) was bought from COCOVI Import of Kihnio, Finland and (re)identified by one of us (LJLDVG). The three biotypes (IOR14-0018, IOF14-0019, IOT13-0007) have been conserved in the mycotheque of the Mycological laboratory, Institute for biological research Sinisa Stankovic, University of Belgrade.

The samples were lyophilized (LH Leybold, Lyovac GT2, Frenkendorf), reduced to a fine dried powder (20 mesh) and mixed to obtain homogeneity.

2.2. Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Fisher Scientific (Lisbon, Portugal). The standards of organic acids and phenolic compounds, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), phosphate buffered saline (PBS), acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma (St. Louis, MO, USA),. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO) was purchased from Merck KGaA (Darmstadt, Germany). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), DMEM media was from Hyclone (Logan, Utah, USA). Methanol and all other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Extracts preparation

Hot aqueous extraction: The material of *Inonotus obliquus* conks was ground to a fine powder with a grinder. The dry powder (25 g/0.5 l water) was heated for 2 h at 80 °C. The extract was centrifuged at 3000 rpm for 15 min and filtered through Whatman no. 4 filter paper; upon which the filtrate was concentrated in an evaporator at 40 °C (rotary evaporator Buchi R-210, Flawil, Switzerland) to dryness (Hu et al., 2009). *Ethanolic extraction:* The residue from hot aqueous extraction was extracted in 70% ethanol at 70 °C temperature overnight. The extract was then centrifuged at 3000 rpm for 15 min and filtered through the Whatman no. 4 filter paper and dried by a rotary evaporator (Buchi R-210, Flawil, Switzerland) under vacuum at 40 °C (Hu et al., 2009).

2.4. Chemical composition of the extracts

2.4.1. Organic acids

Organic acids were determined by ultra fast liquid chromatography (UFLC, Shimadzu 20A series) coupled with a photodiode array detector (PDA), after dissolving both water and ethanol extracted powders in metaphosphoric acid (4%), at a known concentration (Barros et al., 2013). The organic acids were quantified by the comparison of the area of their peaks recorded at 215 nm with

calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of extract.

2.4.2. Phenolic compounds

Phenolic acids and related compounds were determined using the UFLC mentioned above (Barros et al., 2009), after dissolving the aqueous extract in water and ethanolic extract in 20% aqueous ethanol, at a known concentration. Detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength. The phenolic acids and related compounds were quantified by comparison of the area of their peaks with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of extract.

2.5. Evaluation of the antioxidant potential of the extracts

2.5.1. General

Successive dilutions were made from the stock solution of aqueous and ethanolic extracts in water and ethanol, respectively and further submitted to different *in vitro* assays to evaluate the antioxidant activity of the samples (Reis et al., 2012). The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide/Prussian blue assay) against sample concentrations. Trolox was used as standard.

2.5.2. Folin–Ciocalteu assay

The extract solution (1 mL) was mixed with *Folin–Ciocalteu* reagent (2.5 mL, previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 2 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 °C for color development. Absorbance was then measured at 765 nm (Analytikjena spectrophotometer; Jena, Germany). Gallic acid was used to obtain the standard curve and the reduction of *Folin–Ciocalteu* reagent by the samples was expressed as mg of gallic acid equivalents (GAE) per g of extract.

2.5.3. Reducing power or ferricyanide/Prussian blue assay

The extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, the same with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

2.5.4. DPPH radical-scavenging activity assay

The reaction mixture was made in a 96 wells plate and consisted of 30 μL of a concentration range of the extract and 270 μL methanol containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 1 h in the dark, and the absorption was measured at 515 nm in ELX800 Microplate Reader. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA$=[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample and A_{DPPH} is the absorbance of the DPPH solution.

2.5.5. Inhibition of β-carotene bleaching or β-carotene/linoleate assay

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing 0.2 mL of a concentration range of the extract. The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (Absorbance after 2 h of assay/initial absorbance) × 100.

2.5.6. Thiobarbituric acid reactive substances (TBARS) assay

Porcine (*Sus scrofa*) brains were obtained from official slaughtering animals, dissected, and homogenized with a Polytron in ice cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000*g* for10 min. An aliquot (100 μL) of the supernatant was incubated with 200 μL samples of a concentration range of the extract in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%)$=[(A-B)/A] \times 100\%$, where *A* and *B* were the absorbance of the control and the sample solution, respectively.

2.6. Antibacterial activity of the extracts

Bacteria were routinely grown in Luria–Bertani (LB) medium (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37 °C. The fungi were maintained on potato dextrose agar (PDA), malt agar (MA) and Sabouraud agar (SBA). The cultures were stored at +4 °C and subcultured once a month.

The Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240) and *Listeria monocytogenes* (NCTC 7973), and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Escherichia coli* (ATCC 35210), and *Enterobacter cloacae* (human isolate), were used. The antibacterial assay was carried out by a microdilution method (CLSI, 2009; Tsukatani et al., 2012). The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. Ethanolic and aqueous extracts of *Inonotus obliquus* from Russia, Finland and Thailand were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and immediately added in Tryptic Soy broth (TSB) medium (100 μL) with bacterial inoculum (1.0×10^4 CFU per well). The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MICs obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on reduction of INT ((p-iodonitrotetrazolium violet) [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; Sigma]) color and compared with positive control for each bacterial strain. The MBCs were determined by serial sub-cultivation of 2 μL into microtitre plates containing 100 μL of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank (broth medium plus diluted extracts) and the positive control. Streptomycin (Sigma P 7794) and Ampicillin (Panfarma, Belgrade, Serbia) were used as positive controls (1 mg/mL in sterile physiological saline). Five percent DMSO was used as a negative control.

2.7. Antifungal activity of the extracts

Aspergillus fumigatus (human isolate), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate), were used. In order to investigate the antifungal activity of ethanolic and water extracts of three different samples of *Inonotus obliquus*, a modified microdilution technique was used (Espinel-Ingroff, 2001).The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v) and spore suspension was adjusted with sterile saline to a concentration of 1.0×10^5. Ethanolic and aqueous extracts of *Inonotus obliquus* from Russia, Finland and Thailand were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and immediately added in broth Malt medium with inoculum (0.2–6.0 mg/mL). The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 μL of tested compounds dissolved in medium and incubated for 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, and commercial fungicides, bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia), were used as positive controls (1–3000 μg/mL). Five percent DMSO was used as a negative control.

2.8. Antiquorum sensing (AQ) activity of the extracts

2.8.1. Bacterial strains, growth media and culture conditions

Pseudomonas aeruginosa PA01 (ATCC 27853) used in this study is from the collection of the Mycoteca, Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia. Bacteria were routinely grown in Luria–Bertani (LB) medium (1% w/v NaCl, 1% w/v Tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37 °C.

2.8.2. Twitching and flagella motility

After growth in the presence or absence of ethanolic and aqueous extracts of *Inonotus obliquus* (subMIC, 0.5 MIC), streptomycin and ampicillin (subMIC), the cells of *Pseudomonas aeruginosa* PA01 were washed twice with sterile PBS and resuspended in PBS at 1×10^8 cfu/mL (OD of 0.1 at 660 nm). Briefly, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37 °C. Plates were then removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a light microscope (O'Toole and Kolter, 1998a, 1998b).

SubMIC (0.5 MIC; 0.25–0.75 mg/mL) of extracts were mixed into 10 mL of molten MHA (Mueller-Hinton agar) and poured immediately over the surface of a solidified LBA plate as an overlay. The plate was point inoculated with an overnight culture of PAO1 once the overlaid agar had solidified, and was incubated at 37 °C for 3 days. The extent of swimming was determined by measuring the area of the colony (Sandy and Foong-Yee, 2012). The experiment was done in triplicate and repeated two times. The colony diameters were measured three times in different direction and values were presented as a mean values ± SE.

2.8.3. Swarming in PAO1

Fifty microlitres of extracts of *Inonotus obliquus* were mixed into 5 ml of molten Soft Top Agar (STA) and poured immediately over the surface of a solidified LBA plate as an overlay. The plate was point inoculated with an overnight culture of PAO1 once the overlaid agar had solidified and incubated at 37 °C for 3 days. The extent of swarming was determined by measuring the area of the colony using a leaf-surface area meter (Area Meter AM200, ADC Bioscientific Ltd.).

2.8.4. Inhibition of synthesis of Pseudomonas aeruginosa PA01 Pyocyanin

Overnight culture of *Pseudomonas aeruginosa* PA01 was diluted to $OD_{600\ nm}$ 0.2. Then, ethanolic and aqueous extracts of *Inonotus obliquus* were dissolved in 5% of DMSO (0.25–0.75 mg/mL), added to *Pseudomonas aeruginosa* (5.00 mL) and incubated at 37 °C for 24 h. The treated culture was extracted with chloroform (3 mL), followed by mixing the chloroform layer with 0.2 M HCl (1 mL). Absorbance of the extracted organic layer was measured at 520 nm using a Shimadzu UV1601 spectrophotometer (Kyoto, Japan) (Sandy and Foong-Yee, 2012). The experiment was done in triplicate and repeated two times. The values were expressed as ratio (OD_{520}/OD_{600}) × 100.

2.9. Cytotoxicity of the extracts for tumor cell lines and non-tumor liver cells primary cultures

The aqueous and ethanolic extracts were re-dissolved in water at 8 mg/mL to obtain a more efficient dissolution. Successive dilutions were made from the stock solution and tested against five human tumor cell lines: MCF-7 (breast carcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460, HeLa and $HepG_2$) and 2 mM glutamine (at 37 °C, in a humidified air incubator containing 5% CO_2). Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 or 1.0×10^4 cells/well for HeLa and $HepG_2$) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with the diluted sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 μl, pH 7.4) and the absorbance was measured at 540 nm (Monks et al., 1991) in the microplate reader mentioned above. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house. It was designed as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin and 100 μg/mL streptomycin and divided into $1 \times 1\ mm^3$ explants. Some of these explants were placed in 25 cm^2 tissue flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2–3 days using a phase contrast microscope. Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Abreu et al., 2011). Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for Sulforhodamine B colorimetric (SRB) assay was followed. The results were expressed in GI_{50} values (sample concentration that

inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.10. Statistical analysis

For each species, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean values and standard errors, and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 20.0 program.

3. Results and discussion

3.1. Chemical characterization

The yields of ethanolic extracts were 1, 2.4 and 1.3 g for samples from Russia, Finland and Thailand, respectively, while of, aqueous extracts were 2, 5.5 and 0.5 g for the same samples, in all cases starting from 25 g of dry powder.

Oxalic acid was the only organic acid detected in the extracts (6.72–97.59 mg/g extract). Unexpectedly, no betulinic acid was observed, probably caused by the limited solubility of this pentacyclic triterpenoid. The phenolic acids found were gallic, protocatechuic and *p*-hydroxybenzoic acids, and also the related compound cinnamic acid (Table 1). Gallic acid was only found in both, aqueous and ethanolic, extracts from Thailand (0.32 and 0.20 mg/g extract), and protocatechuic acid was detected in all samples except the aqueous extract from Finland (0.07–0.94 mg/g extract). *p*-Hydroxybenzoic acid was found in all samples (0.43–59.20 mg/g extract) and the related compound cinnamic acid was detected in all samples except the aqueous extract from Thailand (0.03–0.40 mg/g extract).

3.2. Antioxidant effects of Inonotus obliquus extracts

Regarding the antioxidant potential (Table 2), it might be concluded that the ethanolic extract of *Inonotus obliquus* from Thailand revealed the lowest EC_{50} values for Ferrocyanide/Prussian blue (0.07 mg/mL), DPPH radical-scavenging activity (0.23 mg/mL), β-carotene bleaching inhibition (0.13 mg/mL) as well as for TBARS inhibition (0.08 mg/mL) assays. *Inonotus obliquus* proved to have high potential for antioxidant purposes, since the obtained EC_{50} values were lower than those reported for other wild edible species (Pereira et al., 2012). In a previous study of antioxidant activity of mycelia of *Inonotus obliquus* (Debnath et al., 2013), the scavenging activity of water, ethanol and methanol extracts on DPPH's were found concentration dependent. The scavenging activities ranged from 12.50 to 200 mg/mL, respectively. All extracts showed higher scavenging activity on DPPH•radical. The IC50's (concentration at which 50% of DPPH•s are scavenged) were 18.96, 16.25 and 24.90 mg/mL for the water, ethanol and methanol extracts, respectively.

3.3. Antimicrobial effects of Inonotus obliquus extracts

The results for antibacterial activity of ethanol and aqueous extracts of *Inonotus obliquus* are presented in Table 3. The ethanolic extracts of *Inonotus obliquus* from Russia, Finland and Thailand exhibited inhibitory effects at 0.30–3.00 mg/mL, 0.75–3.00 mg/mL, 0.50–2.25 mg/mL, respectively. A bactericidal effect was achieved at 1.50–6.00 mg/mL, 1.50–6.00 mg/mL and 0.75–3.00 mg/mL, respectively. The highest antibacterial effect was observed for ethanolic extract obtained from Thai *Inonotus obliquus*. The aqueous extracts showed inhibitory activity at 0.40–3.00 mg/mL, 0.75–3.75 mg/mL and 0.75–3.00 mg/mL for the samples of the mentioned origins, and bactericidal activity at 1.50–6.00 mg/mL, 1.50–7.50 mg/mL and 1.50–6.00 mg/mL. The sample from Russia exhibited the highest antibacterial activity. The most sensitive bacteria to the extracts were *Staphylococcus aureus* and *Bacillus cereus*, while the most resistant ones were *Listeria monocytogenes, Escherichia coli* and *Enterobacter cloacae*. All tested extracts exhibited lower antibacterial activity than the control antibiotics, with exception of ethanolic extract from Thailand against *Pseudomonas aeruginosa* (Table 3).

Regarding the antifungal activity (Table 4), the results were quite similar. All tested extracts showed again considerable antifungal effects on all tested fungi. Inhibitory effect of ethanolic extracts prepared with samples from Russia, Finland and Thailand was 0.40–1.50 mg/mL, 0.75–1.50 mg/mL and 0.40–1.50 mg/mL, respectively. A fungicidal effect was achieved at 0.75–3.00 mg/mL, 1.50–3.00 mg/mL and 0.75–3.00 mg/mL, respectively. Aqueous extracts of the tested samples showed inhibition at 0.20–0.75 mg/mL, 0.20–0.75 mg/mL and 0.20–1.50 mg/mL, respectively. Fungicidal activity of these extracts was observed at 0.75–3.00 mg/mL, 0.40–1.50 mg/mL and 0.40–3.00 mg/mL, respectively. The most susceptible fungus was *Trichoderma viride,* while the most resistant were *Aspergillus niger* and *Aspergillus fumigatus.* The antifungal effect of tested extracts on *Penicillium ochrochloron* and *Trichoderma viride* was higher than the one observed for ketoconazole (Table 4).

3.4. Antiquorum sensing activity of Inonotus obliquus extracts

Many mechanisms of actions have been proposed to interfere with the quorum sensing system such as inhibition of biosynthesis of autoinducer molecules, inactivation or degradation of the autoinducer, interference with the signal receptor, and inhibition of the genetic regulation system (Rasmussen and Givskov, 2006).

In addition to QS, the initiation of biofilm formation by *Pseudomonas aeruginosa* depends on two cell-associated structures; the flagellum and type IV pili (O'Toole and Kolter, 1998a, 1998b). The

Table 1
Organic and phenolic acids in *Inonotus obliquus* aqueous and ethanolic extracts (mean ± SD).

	Russia		Finland		Thailand	
	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract
Organic acids (mg/g extract)						
Oxalic acid	97.59 ± 0.39^{a}	24.15 ± 0.12^{d}	55.62 ± 0.07^{b}	9.50 ± 0.02^{e}	32.86 ± 0.02^{c}	6.72 ± 0.04^{f}
Phenolic acids (mg/g extract)						
Gallic acid	nd	nd	nd	nd	0.32 ± 0.01^{a}	0.20 ± 0.01^{b}
Protocatechuic acid	0.13 ± 0.01^{c}	0.07 ± 0.01^{d}	nd	0.12 ± 0.01^{c}	0.94 ± 0.01^{a}	0.75 ± 0.01^{b}
p-hydroxybenzoic acid	0.43 ± 0.01^{f}	2.77 ± 0.01^{e}	4.85 ± 0.01^{d}	7.41 ± 0.01^{c}	59.20 ± 0.02^{a}	57.48 ± 0.01^{b}
Total phenolic acids	0.56 ± 0.01^{f}	2.84 ± 0.01^{e}	4.85 ± 0.01^{d}	7.53 ± 0.01^{c}	60.47 ± 0.02^{a}	58.43 ± 0.01^{b}
Cinamic acid	0.03 ± 0.01^{d}	0.06 ± 0.01^{c}	0.06 ± 0.01^{c}	0.08 ± 0.01^{b}	nd	0.40 ± 0.01^{a}

nd – not detected. In each row different letters mean significant differences ($p < 0.05$).

Table 2
Antioxidant activity of *Inonotus obliquus* aqueous and ethanolic extracts (mean ± SD).

		Russia		Finland		Thailand	
		Aqueous extract	EtOH extract	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract
Reducing power	Folin–Ciocalteu (mg GAE/g extract)	147.44 ± 0.62^{d}	95.70 ± 0.61^{e}	154.79 ± 1.15^{c}	54.38 ± 0.67^{f}	230.69 ± 1.26^{b}	590.87 ± 1.92^{a}
	Ferricyanide/Prussian blue (EC_{50}; mg/mL)	0.30 ± 0.00^{c}	0.69 ± 0.00^{b}	0.29 ± 0.00^{c}	2.68 ± 0.03^{a}	0.15 ± 0.00^{d}	0.07 ± 0.00^{e}
Radical scavenging activity	DPPH scavenging activity (EC50; mg/mL)	0.48 ± 0.00^{c}	3.21 ± 0.23^{b}	0.53 ± 0.01^{c}	9.22 ± 0.25^{a}	0.23 ± 0.01^{d}	0.23 ± 0.00^{d}
	β-carotene/linoleate (EC_{50}; mg/mL)	0.55 ± 0.04^{b}	0.48 ± 0.03^{c}	0.48 ± 0.02^{c}	0.40 ± 0.01^{d}	0.59 ± 0.03^{a}	0.13 ± 0.00^{e}
Lipid peroxidation inhibition	TBARS (EC_{50}; mg/mL)	0.10 ± 0.00^{c}	0.19 ± 0.00^{b}	0.06 ± 0.00^{f}	0.30 ± 0.01^{a}	0.09 ± 0.00^{d}	0.08 ± 0.00^{e}

Concerning the Folin–Ciocalteu assay, higher values mean higher reducing power; for the other assays, the results are presented in EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance for the Ferricyanide/Prussian blue assay. Trolox was used as standard (EC_{50} values were 0.04 mg/mL for reducing power and DPPH scavenging activity; and 0.02 mg/mL for β-carotene/linoleate and TBARS assays). In each row different letters mean significant statistical differences between samples ($p < 0.05$).

Table 3
Minimum inhibitory (MIC) and bactericidal (MBC) concentrations of aqueous and ethanolic extracts of *Inonotus obliquus* (mg/mL).

Bacteria	Russia		Finland		Thailand		Streptomycin	Ampicillin
	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract	Aqueous extract	EtOH		
Staphylococcus aureus	0.40 ± 0.02^{d}	0.30 ± 0.00^{c}	0.75 ± 0.02^{e}	0.75 ± 0.01^{e}	0.75 ± 0.01^{e}	0.75 ± 0.00^{e}	0.040 ± 0.0007^{a}	0.250 ± 0.007^{b}
	1.50 ± 0.07^{b}	1.50 ± 0.07^{b}	6.00 ± 0.10^{d}	1.50 ± 0.10^{b}	3.00 ± 0.00^{c}	1.50 ± 0.07^{b}	0.090 ± 0.300^{d}	0.370 ± 0.010^{a}
Bacillus cereus	1.10 ± 0.07^{c}	0.30 ± 0.02^{a}	$1.40 \pm 0.0.7^{c}$	$0.75 \pm 0.0.2^{b}$	0.75 ± 0.02^{b}	0.75 ± 0.00^{b}	0.090 ± 0.030^{a}	0.250 ± 0.030^{a}
	1.50 ± 0.10^{b}	1.50 ± 0.07^{b}	1.80 ± 0.03^{c}	1.50 ± 0.10^{b}	3.00 ± 0.10^{d}	1.50 ± 0.01^{b}	0.170 ± 0.010^{a}	0.370 ± 0.010^{a}
Micrococcus flavus	1.50 ± 0.10^{c}	2.25 ± 0.08^{e}	1.80 ± 0.07^{d}	2.25 ± 0.02^{e}	3.00 ± 0.00^{f}	1.10 ± 0.07^{b}	0.170 ± 0.010^{a}	0.250 ± 0.007^{a}
	3.00 ± 0.30^{c}	3.00 ± 0.07^{c}	3.75 ± 0.02^{d}	3.00 ± 0.10^{c}	6.00 ± 0.00^{e}	1.50 ± 0.07^{b}	0.340 ± 0.010^{a}	0.370 ± 0.010^{a}
Listeria monocytogenes	3.00 ± 0.07^{e}	3.00 ± 0.00^{e}	3.75 ± 0.08^{f}	3.00 ± 0.10^{e}	1.10 ± 0.03^{c}	2.25 ± 0.05^{d}	0.170 ± 0.010^{a}	0.370 ± 0.010^{b}
	6.00 ± 0.10^{e}	6.00 ± 0.10^{e}	7.50 ± 0.07^{d}	6.00 ± 0.00^{c}	3.00 ± 0.07^{b}	3.00 ± 0.10^{b}	0.340 ± 0.010^{a}	0.490 ± 0.010^{a}
Pseudomonas aeruginosa	1.10 ± 0.03^{d}	0.75 ± 0.007^{c}	0.75 ± 0.00^{c}	0.75 ± 0.007^{e}	1.50 ± 0.07^{e}	0.50 ± 0.07^{b}	0.170 ± 0.010^{a}	0.740 ± 0.070^{c}
	3.00 ± 0.10^{d}	1.50 ± 0.07^{c}	6.00 ± 0.20^{e}	1.50 ± 0.10^{c}	3.00 ± 0.10^{d}	0.75 ± 0.007^{b}	0.340 ± 0.010^{a}	1.240 ± 0.08^{c}
Salmonella typhimurium	2.25 ± 0.08^{e}	0.75 ± 0.02^{c}	1.10 ± 0.03^{d}	0.75 ± 0.00^{c}	1.10 ± 0.03^{b}	0.50 ± 0.02^{d}	0.170 ± 0.010^{a}	0.370 ± 0.010^{b}
	3.00 ± 0.07^{e}	1.50 ± 0.07^{d}	3.00 ± 0.00^{e}	1.50 ± 0.07^{d}	1.50 ± 0.04^{d}	0.75 ± 0.00^{c}	0.340 ± 0.010^{a}	0.490 ± 0.007^{b}
Escherichia coli	1.50 ± 0.07^{e}	2.25 ± 0.08^{d}	3.75 ± 0.08^{f}	2.25 ± 0.08^{d}	3.00 ± 0.00^{e}	1.10 ± 0.03^{b}	0.170 ± 0.010^{a}	0.250 ± 0.007^{a}
	6.00 ± 0.20^{e}	3.00 ± 0.10^{c}	7.50 ± 0.07^{f}	3.00 ± 0.00^{c}	6.00 ± 0.20^{d}	1.50 ± 0.07^{b}	0.340 ± 0.01^{a}	0.490 ± 0.030^{a}
Enterobacter cloacae	3.00 ± 0.10^{e}	3.00 ± 0.20^{e}	1.10 ± 0.07^{c}	1.50 ± 0.07^{d}	3.00 ± 0.00^{e}	0.75 ± 0.02^{b}	0.260 ± 0.010^{a}	0.370 ± 0.010^{a}
	6.00 ± 0.20^{de}	6.00 ± 0.30^{e}	1.50 ± 0.07^{b}	3.00 ± 0.10^{c}	6.00 ± 0.20^{d}	1.50 ± 0.00^{b}	0.520 ± 0.007^{a}	0.740 ± 0.010^{a}

In each row different letters mean significant statistical differences between samples ($p < 0.05$).

Table 4
Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of aqueous and ethanolic extracts of *Inonotus obliquus* (mg/mL).

Fungi	Russia		Finland		Thailand		Bifonazole	Ketoconazole
	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract		
Aspergillus fumigatus	0.75 ± 0.02^{c}	0.75 ± 0.00^{c}	0.40 ± 0.02^{b}	1.50 ± 0.07^{d}	0.75 ± 0.007^{c}	1.50 ± 0.10^{d}	0.150 ± 0.02^{a}	0.200 ± 0.000^{a}
	1.50 ± 0.07^{c}	1.5 ± 0.00^{c}	1.50 ± 0.10^{c}	3.00 ± 0.20^{d}	1.50 ± 0.10^{e}	3.00 ± 0.00^{d}	0.200 ± 0.02^{a}	0.500 ± 0.020^{b}
Aspergillus versicolor	0.40 ± 0.02^{c}	0.40 ± 0.00^{c}	0.40 ± 0.01^{c}	0.75 ± 0.02^{d}	0.20 ± 0.00^{b}	1.50 ± 0.07^{e}	0.100 ± 0.01^{a}	0.200 ± 0.020^{b}
	0.75 ± 0.00^{cd}	0.75 ± 0.02^{cd}	0.75 ± 0.02^{d}	1.50 ± 0.00^{e}	0.40 ± 0.01^{ab}	3.00 ± 0.20^{f}	0.200 ± 0.02^{a}	0.500 ± 0.030^{bc}
Aspergillus ochraceus	0.20 ± 0.02^{a}	0.75 ± 0.00^{c}	0.40 ± 0.02^{b}	0.75 ± 0.07^{e}	1.50 ± 0.00^{d}	0.75 ± 0.02^{c}	0.150 ± 0.007^{a}	1.500 ± 0.100^{d}
	0.75 ± 0.02^{b}	1.50 ± 0.07^{e}	0.75 ± 0.007^{b}	1.50 ± 0.07^{c}	3.00 ± 0.00^{e}	1.50 ± 0.07^{c}	0.200 ± 0.000^{a}	2.000 ± 0.007^{d}
Aspergillus niger	0.75 ± 0.02^{c}	1.50 ± 0.07^{d}	0.40 ± 0.00^{b}	1.50 ± 0.07^{d}	0.40 ± 0.02^{b}	1.50 ± 0.02^{e}	0.150 ± 0.070^{a}	0.200 ± 0.000^{a}
	3.00 ± 0.10^{c}	3.00 ± 0.20^{c}	0.75 ± 0.00^{b}	3.00 ± 0.20^{c}	0.75 ± 0.01^{b}	3.00 ± 0.00^{e}	0.200 ± 0.010^{a}	0.500 ± 0.000^{b}
Trichoderma viride	0.40 ± 0.00^{b}	0.40 ± 0.02^{b}	0.20 ± 0.01^{a}	0.75 ± 0.02^{c}	0.40 ± 0.02^{b}	0.40 ± 0.00^{b}	0.150 ± 0.000^{a}	1.00 ± 0.100^{d}
	0.75 ± 0.02^{c}	0.75 ± 0.00^{c}	0.40 ± 0.01^{b}	1.50 ± 0.07^{e}	0.75 ± 0.02^{c}	0.75 ± 0.02^{c}	0.200 ± 0.010^{a}	1.00 ± 0.000^{d}
Penicillium funiculosum	0.40 ± 0.01^{b}	0.40 ± 0.00^{b}	0.75 ± 0.07^{c}	0.75 ± 0.01^{c}	0.75 ± 0.00^{e}	0.75 ± 0.02^{c}	0.200 ± 0.010^{a}	0.200 ± 0.020^{a}
	0.75 ± 0.02^{c}	0.75 ± 0.00^{c}	1.50 ± 0.10^{e}	1.50 ± 0.01^{de}	1.50 ± 0.00^{de}	1.50 ± 0.10^{d}	0.250 ± 0.000^{a}	0.500 ± 0.020^{b}
Penicillium ochrochloron	0.40 ± 0.02^{b}	0.75 ± 0.01^{c}	0.40 ± 0.00^{b}	0.75 ± 0.01^{c}	0.75 ± 0.00^{c}	0.75 ± 0.01^{c}	0.200 ± 0.010^{a}	2.500 ± 0.100^{d}
	0.75 ± 0.01^{b}	1.50 ± 0.10^{c}	0.75 ± 0.00^{b}	1.50 ± 0.10^{c}	1.50 ± 0.07^{c}	1.50 ± 0.00^{c}	0.250 ± 0.010^{a}	3.500 ± 0.050^{d}
Penicillium verrucosum	0.40 ± 0.02^{c}	0.75 ± 0.00^{d}	0.40 ± 0.01^{c}	0.75 ± 0.007^{d}	0.75 ± 0.01^{d}	0.75 ± 0.00^{d}	0.100 ± 0.010^{a}	0.200 ± 0.010^{b}
	0.75 ± 0.00^{b}	1.50 ± 0.10^{c}	0.75 ± 0.01^{b}	1.50 ± 0.10^{c}	1.50 ± 0.07^{b}	1.50 ± 0.03^{c}	0.200 ± 0.007^{a}	0.300 ± 0.010^{a}

In each row different letters mean significant statistical differences between samples ($p < 0.05$).

flagella are responsible for swarming motility shown as protusions of the colony border while the type IV pili are responsible for twitching motility (Henrichsen, 1972). Both types of motility are also important in the initial stages of biofilm formation by *Pseudomonas aeruginosa* (O'Toole and Kolter, 1998a, 1998b). Therefore, we tried to determine if *Inonotus obliquus* extracts can influence either one or both

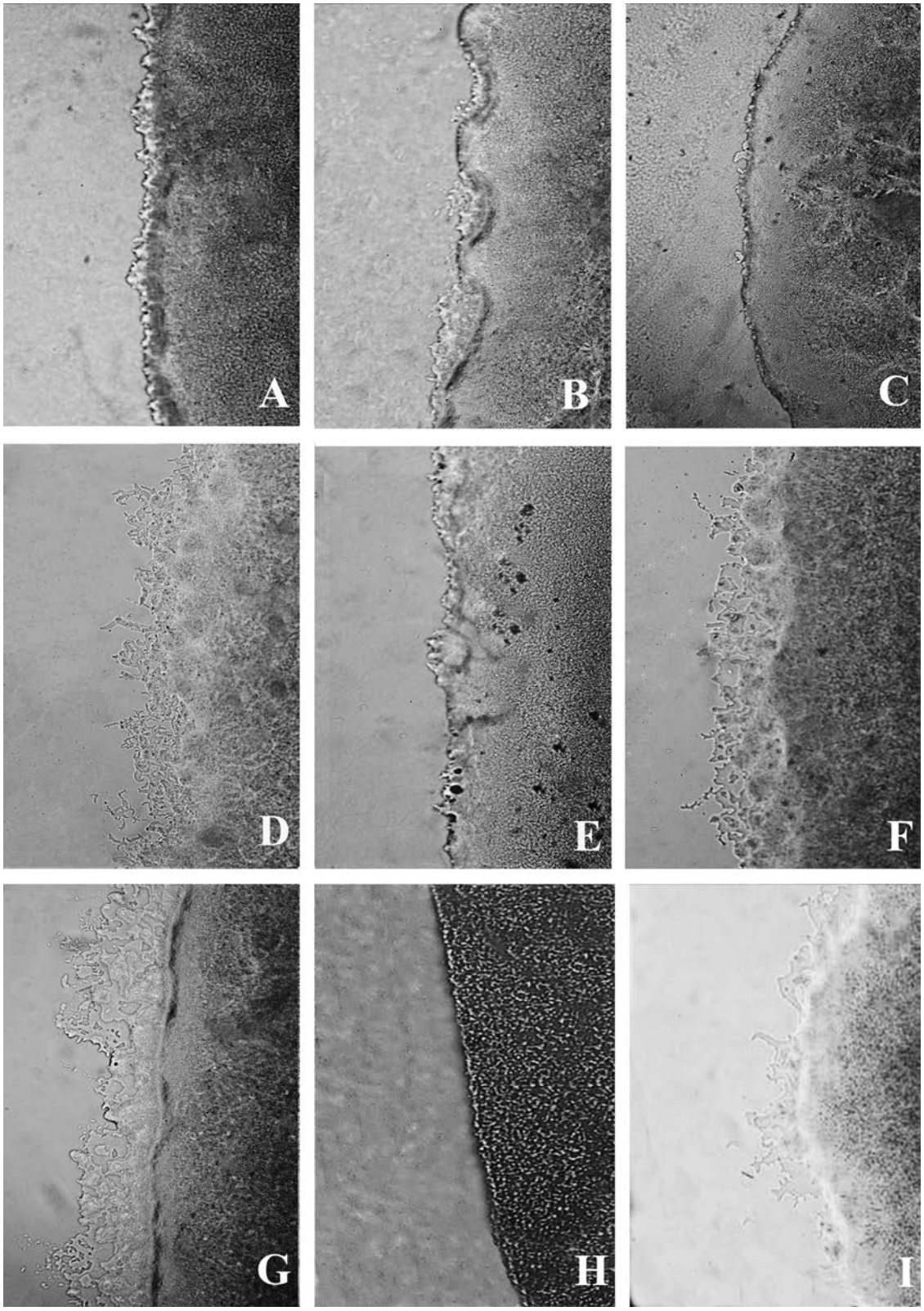

Fig. 1. Light microscopy of colony edges of *Pseudomonas aeruginosa* in twitching motility, grown in the presence or absence of *Inonotus obliquus* (EIO and AIO) extracts at a concentration of 0.5 MIC. The colonies from the bacteria grown with *Inonotus obliquus* (Russia) ethanolic extract (A) and aqueous extract (B) were rounded, had a smooth domed shape and lacked a hazy zone surrounding the colony. Colony with ethanolic extract of *Inonotus obliquus* (Finland) with reduced protrusions (C), and with aqueous extract with regular long protrusions (D). Colonies with *Inonotus obliquus* (Thailand) ethanolic extract with reduced protrusions (E) and with aqueous extract which reduced the protrusions (F). *Pseudomonas aeruginosa* produced a flat, widely spread, irregularly shaped colony with regular protrusions in the absence of extracts, control (G); *Pseudomonas aeruginosa* colony in the presence of streptomycin without protrusions (H) and with ampicillin with almost regularly formed protrusions (I); magnification: (A–E) × 100.

motilities. Our strain did not show swarming but it formed biofilm. On swimming plates, the motile strain PAO1 was used as the 100% standard (control) for motility; the Petri dishes with the same strain with ethanolic and aqueous extracts of *Inonotus obliquus* were compared to the control. The normal colonies of *Pseudomonas aeruginosa*, i.e. in the absence of extracts, were flat with a rough appearance displaying irregular colony edges (Fig. 1G) and a hazy zone surrounding the colony. The cells grew in a very thin layer. After 2 days of incubation at ambient temperature, colony expansion occurred very rapidly due to twitching motility; the control *Pseudomonas aeruginosa* isolates produced swimming zones (Table 5) of 12.6 ± 1.0 mm. Statistically they all had the same colony size; the color of all treated samples was however different and went from white to light brown. Microscopically it was seen that bacteria grown with EIOR and AIOR extracts had reduced twitching and swimming motility. These colonies were incapable of producing a twitching zone and had almost round, smooth, regular colony edges, the protrusions were reduced both in size and in numbers (Fig. 1A, B) and the colony diameter was also reduced compared to the control (10.7 mm). EIOR caused flatter protrusions than AIOR and the colony had a different color. EIOF extract influenced the colony color (orange) and diameter (10.3 mm), while also the protrusions were flatter. AIOF extract showed slightly lower swimming effect than EIOF. Colony color was light brown, diameter was reduced (10.5 mm) but the protrusions were only partly reduced (Fig. 1D). EIOT extract induced light yellow color of the colony and its diameter was reduced to 11.3 mm, and protrusions were flatter (Fig. 1E). AIOT extract showed lower AQ effect; colony color was white yellow, diameter was 11.6 mm and protrusions were partly reduced (Fig. 1F). Streptomycin changed colony color into white, reduced its diameter to 5.0 mm, and completely reduced protrusions (Fig. 1H), while Ampicillin changed color to white, and slightly reduced its diameter to 12.0 mm and did not affect the formation of protrusions at all (Fig. 1I). It can be seen that extracts from Russia in general showed the highest reduction of protrusions. The strongest diameter reduction was shown by both Finland extracts, and the highest protrusion reduction was shown by one of the Finland extracts, EIOF. Thailand extracts possessed the lowest swimming activity of the three different extract origins investigated; colony diameter was reduced in low percentage, protrusions were reduced but still wide and merged by EIOT, while AIOT reduced the diameter in even lower percentage; protrusions were slightly reduced, still long and wider than AIOF but narrower than in the untreated control (Fig. 1A–I).

Table 5
Twitching and motility activity of *Inonotus obliquus* aqueous and ethanolic extracts at MIC 0.5 mg/mL (mean ± SD).

Agents	Colony diameter (mm ± SD)	Colony color	Colony edge on microscope
Aqueous Russia	10.7 ± 0.76	White brown	Reduced flagella
EtOH Russia	10.7 ± 1.51	Light brown	Reduced flagella
Aqueous Finland	10.5 ± 0.65	Light brown	Partly reduced flagella
EtOH Finland	10.3 ± 0.63	Orange	Reduced flagella
Aqueous Thailand	11.6 ± 0.06	White yellow	Slightly reduced flagella
EtOH Thailand	11.3 ± 0.36	Light yellow	Reduced flagella
Ampicillin	12.00 ± 1.00	White	Regular flagella
Streptomycin	5.0 ± 0.03	White	No flagella
Control P.a. 10^9	12.6 ± 1.00	Green	Regular flagella

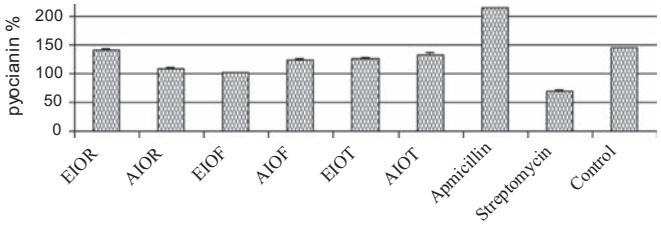

Fig. 2. Effects of ethanolic and aqueous extracts at 0.5 MIC of *Inonotus obliquus* on production of pyocyanin by *Pseudomonas aeruginosa* (PAO1). Aqueous extract from Russia (AIOR), Finland (AIOF) and Thailand (AIOT), ethanolic extract from Russia (EIOR), Finland (EIOF) and Thailand (EIOT).

Biofilm formation is an essential trait for *Pseudomonas aeruginosa*, both in environmental and clinical settings (Kolter and Greenberg, 2006), and is known to be inversely regulated to swarming motility (Boyle et al., 2013). An inverse relationship has previously been shown between swarming motility and biofilm formation in a cohort of 237 clinical isolates (Murray et al., 2010). Our strain shows a similar trend where good biofilm formers tend to be poor swarmers, whereas poor biofilm formers can be good swarmers. Our strain did not show swarming activity. It seems that it this represents the natural diversity of *Pseudomonas aeruginosa.* An experiment with swarming and biofilm formation provides a unique example of parallel evolution and suggests an evolutionary trade-off between motility and biofilm formation.

The activity against pyocyanin production in a flask assay was used to quantify quorum sensing inhibitory activity of the ethanol and aqueous extracts of *Inonotus obliquus*. The effect of extracts on production pyocyanin of *Pseudomonas aeruginosa* (PAO1) was tested in subMIC concentration (Fig. 2). The ethanolic and aqueous extracts of *Inonotus obliquus* demonstrated concentration-dependent pyocyanin inhibitory activity. The pyocyanin assays revealed that subMIC amounts of extracts produced less (102–141%) pyocyanin than PAO1 (145.00%). Streptomycin reduced the production of pyocyanin to 70.00%, while ampicillin stimulated the production of extracts 1.5 times (215.00%). All tested extracts showed reduction of pyocyanin production but only in low amount. The highest

Table 6
Antitumor cell line activity of *Inonotus obliquus* aqueous and ethanolic extracts L (mean ± SD).

	Russia		Finland		Thailand		
	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract	Aqueous extract	EtOH extract	Elipticine
MCF-7 (breast carcinoma) (GI_{50}, μg/mL)	212.10 ± 10.12^{b}	182.61 ± 2.45^{c}	212.03 ± 19.72^{b}	239.43 ± 4.74^{a}	92.65 ± 2.74^{e}	174.32 ± 12.92^{d}	1.21 ± 0.02
NCI-H460 (non-small cell lung cancer) (GI_{50}, μg/mL)	91.20 ± 5.35^{d}	80.93 ± 13.04^{e}	177.94 ± 6.64^{b}	> 400	267.27 ± 6.37^{a}	144.33 ± 4.61^{c}	1.03 ± 0.09
HeLa (cervical carcinoma) (GI_{50}, μg/mL)	> 400	245.66 ± 9.34^{b}	224.91 ± 24.42^{c}	217.36 ± 11.95^{d}	318.19 ± 18.19^{a}	225.99 ± 1.95^{c}	0.91 ± 0.11
HepG2 (hepatocellular carcinoma) (GI_{50}, μg/mL)	336.48 ± 27.96^{a}	228.23 ± 22.78^{d}	281.12 ± 4.15^{b}	247.60 ± 18.66^{c}	217.79 ± 4.46^{e}	94.24 ± 3.61^{f}	1.10 ± 0.09
Hepatotoxicity PLP2 (GI_{50}, μg/mL)	> 400	> 400	> 400	> 400	> 400	> 400	2.29 ± 0.18

GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

reduction of pyocyanin production could be seen for extracts from Finland, especially for the ethanolic extract (Fig. 2).

3.5. Cytotoxicity and antitumor effects of Inonotus obliquus extracts

The results obtained for cytotoxic activity on human tumor cell lines are presented in Table 6. The lowest GI_{50} values were obtained for both Russian extracts in all cell lines except NCI-H460, which proved to be more sensitive to the extracts (80.93–91.20 μg/mL). All tested extracts were particularly active on breast carcinoma cells (MCF-7; 92.65–239.43 μg/mL), non-small cell lung cell line (NCI-H460; 91.20–267.27 μg/mL), with exception of the ethanolic extract from Finland, cervical cell line (HeLa; 217.36–318.19 μg/mL) with exception of aqueous extract from Russia, and hepatocellular carcinoma cells (HepG2; 94.24–336.48 μg/mL). At the mentioned concentrations, the different fractions did not show toxicity against non-tumor liver primary cells (PLP2; $GI_{50} > 400$ μg/mL).

4. Conclusion

In summary, our study indicated that aqueous and ethanolic extracts of *Inonotus obliquus* obtained from three different localities possessed clear antioxidant, antimicrobial and anti-quorum sensing activity, as well as anti tumor cell line effects without toxicity for non-tumor liver cells. It should be noted that extracts were prepared from Chaga conks, hyperplastic structures from Birch cortex that consist only partly of fungal material. One of us (LJLDVG) estimated the amount of mycelium in the conks on 10% only, based on micrscopical observation. This indicates that further purification of the extracts is needed to draw definite conclusions on the possible anti-QS activity of Chaga and of *Inonotus obliquus* extracts. Anti-quorum sensing property of this "mushroom" derived material may play an important role in antibacterial activity and could offer an additional strategy for fighting bacterial infection. Inhibition of different species of bacteria and fungi, and of bacterial quorum sensing, but also the antitumor effect that we showed, offers a new strategy for the using of this mushroom as a medicinal mushroom.

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