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

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Abstract

Chemical composition and biological properties of aqueous and ethanolic extracts of *Inonotus obliquus* (Pers.:Fr.) Pilat from different origins, i.e. of Finland, Russia, and Thailand, were studied. Concerning biological activity, antimicrobial, antiqourum, antioxidative, and antitumor and cytotoxic effects were tested. Oxalic acid was found as the main organic acid, with the highest amount in Russian aqueous extract. Gallic, protocatechuic and *p*-hydroxybenzoic acids were detected in all samples. *I. obliquus* extracts showed high antioxidant and antimicrobial activity. Extracts were tested at subMIC for anti-quorum sensing (AQS) activity in *Pseudomonas aeruginosa* and all of them showed definite AQS activity. The assays were done using twitching and swarming of bacterial cultures, as well as the amount of produced pyocyanin as QS parameters. All the extracts possessed good antitumor effect on four cells lines and did not show hepatotoxicity on porcine liver primary cells designed as PLP2. As the *I. 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.

Keywords: *Inonotus obliquus*; Chemical characterization; Antioxidant properties; Antimicrobial activity; Antiqourum effect; Antitumor activity; Cytotoxic effect.

Introduction

In the present study we searched for comparable bioactive effects in extracts prepared 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. 2012). *In vitro*, the properties of Chaga were found to be non-toxic and mostly antiviral (Ahwad Ali et al. 2003), antioxidative (Nakajima et al. 2007) and antiinflammatory (Kim et al. 2007). Song et al (2007) found opposite results showing that its 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 to the size of the surrounding population. Among QS controlled activities of bacteria, coordination of gene expression will allow different forms of defense against competitors, and of adaptation to changing environments (Ward et al. 2012). Bacteria as *Pseudomonas aeruginosa* use small diffusible signaling molecules that regulate expression of various genes including those for swarming motility, virulence, cell aggregation and production of polysaccharides that protect bacterial biofilms (Danchin et al. 2004, Rasmussen and Givskov, 2006, Fiqua et al. 2001). At least 65 % of all the infectious diseases are associated with bacterial biofilms (Fiqua et al. 2001). Bacteria apply QS based

mechanisms for their protection against antibiotics and are able to rapidly transfer antibiotic resistance throughout their own species as well as to different species. Each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23000 people die each year as a direct result of these infections (CDC, 2013). *Clostridium difficile*, carbapenem-resistant Enterobacteriaceae (CRE) and drug-resistant *Neisseria gonorrhoeae* were considered the most urgent threats, and multidrug-resistant *Acinetobacter*, drug-resistant *Campylobacter,* extended spectrum β-lactamase producing Enterobacteriaceae (ESBLs), multidrug-resistant *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA) and drug-resistant tuberculosis were also among serious threats (CDC, 2013). The rapid emergence of multiple resistances against the present broad spectrum antibiotics used for infectious disease needs immediately 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 (Stojkovic et al. 2014). Based on its immunomodulatory effects, ABM was previously found to show protective activity against bacterial disease (Rumbaugh et al. 2009, Coates et al. 2002, CDC, 2013, Stojkovic et al. 2014). Antimicrobial effects against *Mycobacterium tuberculosis*, *M. bovis* and *Streptococcus pneumoniae* had been related with the immune system (Coates et al. 2002, Stojkovic et al. 2014), but this 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) was a good reason for 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 *P. aeruginosa* than those of ampicillin and streptomycin. The effect was caused by anti-QS compounds present in the extracts of *Agaricus blazei* (Sokovic et al., 2014).

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

Material and methods

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 *I. obliquus* (Russia) was obtained from Life Extension Asia, Singapore, and was (re)identified by one of us (J.G.). Wild *I. 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.

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).

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).

Chemical composition of the extracts

Organic acids. Organic acids were determined by ultra fast liquid chromatography (UFLC, Shimadzu 20A series) coupled with a photodiode array detector (PDA), after dissolving each extract 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.

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.

Evaluation of the antioxidant potential of the extracts

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.

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 colour 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.

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).

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.

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 millilitres of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40ºC 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 50ºC 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 2h of assay/initial absorbance) × 100.

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 37ºC 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 80ºC 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] × 100%, where A and B were the absorbance of the control and the sample solution, respectively.

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 *I. 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.

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 *I. obliquus*, a modified microdilution technique was used (Espaniel-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 *I. 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.

Antiquorum sensing (AQ) activity of the extracts

Bacterial strains, growth media and culture conditions. P. 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.

Twitching and flagella motility

After growth in the presence or absence of ethanolic and aqueous extracts of I. obliquus (subMIC, 0.5 MIC), streptomycin and ampicillin (subMIC), the cells of P. aeruginosa PA01 were washed twice with sterile PBS and resuspended in PBS at 1 x 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,b).

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.

Inhibition of synthesis of Pseudomonas aeruginosa PA01 Pyocyanin

Overnight culture of *P. aeruginosa* PA01 was diluted to $OD_{600\ nm}$ 0.2. Then, ethanolic and aqueous extracts of *I. obliquus* were dissolved in 5% of DMSO (0.25-0.75 mg/mL), added to *P. 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}) x 100.

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 37ºC, 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 48h 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 ºC. 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 1×1 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 37ºC 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 48h 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.

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.

Results and discussion

Chemical characterization, antioxidant and antimicrobial effects I. obliquus extracts

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). The phenolic acids found were gallic, protocatechuic and *p*-hydroxybenzoic acids, as 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).

Regarding the antioxidant potential (**Table 2**), it might be concluded that the ethanolic extract of *I. 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. *I. 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 previous study of antioxidant activity of mycelia of *I. obliquus* (Debnath et al., 2013), the scavenging activity of water, ethanol and methanol extracts on DPPH's were determined, and it was concentration dependent. The scavenging activities were ranged from 12.50–200 mg/mL, respectively. Therefore, the all extracts showed higher scavenging activity on DPPH• radical. The IC50 (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.

The results for antibacterial activity of ethanol and aqueous extracts of *I. obliquus* are presented in **Table 3**. The ethanolic extracts of *I. 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 Thailand. 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 from each one 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 *S. aureus* and *B. cereus*, while the most resistant ones were *L. monocytogenes, E. coli* and *E. cloacae*. All tested extracts exhibited lower antibacterial activity than the control antibiotics, with exception of ethanolic extract from Thailand against *P. 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. A 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 *T. viride,* while the most resistant were *A. niger* and *A. fumigatus.* The antifungal effect of tested extracts on *P. ochrochloron* and *T. viride* was higher than the one observed for ketoconazole (**Table 4**).

Antiquorum sensing activity and cytotoxicity of I. 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 *P. aeruginosa* depends on two cell-associated structures; the flagellum and type IV pili (O'Toole and Kolter, 1998a,b). The flagellum is responsible for swarming motility while the type IV pili are responsible for twitching motility (Henrichsen, 1972). Both types of motility are important in the initial stages of biofilm formation by *P. aeruginosa* (O'Toole and Kolter, 1998a,b). Therefore, we tried to determine if *I. obliquus* extracts can influence either one or both motilities. On swarming plates, the motile strain PAO1 was used as the 100% standard (control) for motility, while the Petri dishes with the same strain plus ethanol and aqueous extracts of *I. obliquus* were compared with the control. The normal colonies of *P. 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 *P. 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 motility. These colonies were incapable of producing such a twitching zone and had almost round, smooth, regular colony edges, the flagella were reduced both in size and in numbers **(Fig. 1A, B**) and the colony diameter was also reduced in comparison with 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 antiquorum effect than EIOF. Colony color was light brown, diameter was reduced (10.5 mm) but the protrusions was only partly reduced (**Fig. 1D**). EIOT extract induced light yellow color of colony and 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 protrsions partly reduced (**Fig. 1F**). Streptomycin changed colony color in white, reduced diameter to 5.0 mm, and completely reduced protrusions (**Fig. 1H**), while Ampicillin changed color in white, and slightly reduced 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 best diameter reduction was shown by both Finland extracts, and the highest protrusion reduction was also shown by one of the Finland extracts, EIOF. Thailand extracts possessed the lowest antiqourum 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 diameter in even lower percentage, protrusions were slightly reduced, still long and wider than AIOF but narrower than the untreated control (**Fig. 1A-I**). I think we should do much more to explain what happens here, especially why this is important for the formation of biofiilms. Maybe there are inhibitors of flagellar growth. I can imagine that we start point inoculation and cover a sector of the plate with a new agarlayer after some hours and again later to see what happens. I think we have to make sure that the readers see the importance of preventing swarming. I am not experienced in bacteriology, but I will think of it. Let's discuss.

The activity against pyocyanin production in a flask assay was used to quantify quorum sensing inhibitory activity of the ethanol and aqueous extracts of *I. obliquus*. The effect of extracts on production pyocyanin of *P. aeruginosa* (PAO1) was tested in subMIC concentration (**Figure 2**). The ethanolic and aqueous extracts of *I. 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 in 70.00%, while ampicillin stimulated production of extracts 1.5 times (215.00%). All tested extracts showed reduction of pyocyanin production but only in low amount. The highest reduction of pyocyanin production could be seen for extracts from Finland, especially for ethanolic extract (**Figure 2**).

The results obtained for cytotoxic activity in 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 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).

In summary, our study indicated that aqueous and ethanolic extracts of *I. obliquus* obtained from three different localities possessed clear antioxidant, antimicrobial and anti-quorum

sensing activity, as well as antitumor 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 microscopical 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 *I. 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 kind of bacteria and fungi, and bacterial quorum sensing, but also antitumor effect, offers a new strategy for the using of this mushroom as a medicinal mushroom.

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

	ussia		inland		hailand	
	queous xtract	tOH xtract	queous xtract	tOH xtract	queous xtract	tOH xtract
rganic acids (mg/g extract)						
xalic acid	7.59±0.39a	4.15±0.12d	5.62±0.07b	50±0.02e	2.86±0.02c	72±0.04f
henolic acids (mg/g extract)						
allic acid	d	d	d	d	32±0.01a	20±0.01b
rotocatechuic acid	13±0.01c	07±0.01d	d	12±0.01c	94±0.01a	75±0.01b
-hydroxybenzoic acid	43±0.01f	77±0.01e	85±0.01d	41±0.01c	9.20±0.02a	7.48±0.01b
otal phenolic acids	56±0.01f	84±0.01e	85±0.01d	53±0.01c	0.47±0.02a	3.43±0.01b
inamic acid	03±0.01d	06±0.01c	06±0.01c	08±0.01b	d	40±0.01a

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

Table 2. Antioxidant activity of *I. 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 bacterial (MBC) concentrations of aqueous and ethanolic extracts of *I. 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} 1.50±0.07^{b}	0.30±0.00^{c} 1.50±0.07^{b}	0.75±0.02^{e} 6.00±0.10^{d}	0.75±0.01^{e} 1.50±0.10^{b}	0.75±0.01^{e} 3.00±0.00^{c}	0.75±0.00^{e} 1.50±0.07^{b}	0.040±0.0007^{a} 0.090±0.300^{d}	0.250±0.007^{b} 0.370±0.010^{a}
Bacillus cereus	1.10±0.07^{c} 1.50±0.10^{b}	0.30±0.02^{a} 1.50±0.07^{b}	1.40±0.0.7^{c} 1.80±0.03^{c}	0.75±0.0.2^{b} 1.50±0.10^{b}	0.75±0.02^{b} 3.00±0.10^{d}	0.75±0.00^{b} 1.50±0.01^{b}	0.090±0.030^{a} 0.170±0.010^{a}	0.250±0.030^{a} 0.370±0.010^{a}
Micrococcus flavus	1.50±0.10^{c} 3.00±0.30^{c}	2.25±0.08^{e} 3.00±0.07^{c}	1.80±0.07^{d} 3.75±0.02^{d}	2.25±0.02^{e} 3.00±0.10^{c}	3.00±0.00^{f} 6.00±0.00^{e}	1.10±0.07^{b} 1.50±0.07^{b}	0.170±0.010^{a} 0.340±0.010^{a}	0.250±0.007^{a} 0.370±0.010^{a}
Listeria monocytogenes	3.00±0.07^{e} 6.00±0.10^{e}	3.00±0.00^{e} 6.00±0.10^{e}	3.75±0.08^{f} 7.50±0.07^{d}	3.00±0.10^{e} 6.00±0.00^{c}	1.10±0.03^{c} 3.00±0.07^{b}	2.25±0.05^{d} 3.00±0.10^{b}	0.170±0.010^{a} 0.340±0.010^{a}	0.370±0.010^{b} 0.490±0.010^{a}
Pseudomonas aeruginosa	1.10±0.03^{d} 3.00±0.10^{d}	0.75±0.007^{c} 1.50±0.07^{c}	0.75±0.00^{c} 6.00±0.20^{e}	0.75±0.007^{e} 1.50±0.10^{c}	1.50±0.07^{e} 3.00±0.10^{d}	0.50±0.07^{b} 0.75±0.007^{b}	0.170±0.010^{a} 0.340±0.010^{a}	0.740±0.070^{c} 1.240±0.08^{c}
Salmonella typhimurium	2.25±0.08^{e} 3.00±0.07^{e}	0.75±0.02^{c} 1.50±0.07^{d}	1.10±0.03^{d} 3.00±0.00^{e}	0.75±0.00^{c} 1.50±0.07^{d}	1.10±0.03^{b} 1.50±0.04^{d}	0.50±0.02^{d} 0.75±0.00^{c}	0.170±0.010^{a} 0.340±0.010^{a}	0.370±0.010^{b} 0.490±0.007^{b}
Escherichia coli	1.50±0.07^{e} 6.00±0.20^{e}	2.25±0.08^{d} 3.00±0.10^{c}	3.75±0.08^{f} 7.50±0.07^{f}	2.25±0.08^{d} 3.00±0.00^{c}	3.00±0.00^{e} 6.00±0.20^{d}	1.10±0.03^{b} 1.50±0.07^{b}	0.170±0.010^{a} 0.340±0.01^{a}	0.250±0.007^{a} 0.490±0.030^{a}
Enterobacter cloacae	3.00±0.10^{e} 6.00±0.20^{de}	3.00±0.20^{e} 6.00±0.30^{e}	1.10±0.07^{c} 1.50±0.07^{b}	1.50±0.07^{d} 3.00±0.10^{c}	3.00±0.00^{e} 6.00±0.20^{d}	0.75±0.02^{b} 1.50±0.00^{b}	0.260±0.010^{a} 0.520±0.007^{a}	0.370±0.010^{a} 0.740±0.010^{a}

Table 4. Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of aqueous and ethanolic extracts of *I. 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} 1.50±0.07^{c}	0.75±0.00^{c} 1.5±0.00^{c}	0.40±0.02^{b} 1.50±0.10^{c}	1.50±0.07^{d} 3.00±0.20^{d}	0.75±0.007^{c} 1.50±0.10^{e}	1.50±0.10^{d} 3.00±0.00^{d}	0.150±0.02^{a} 0.200±0.02^{a}	0.200±0.000^{a} 0.500±0.020^{b}
Aspergillus versicolor	0.40±0.02^{c} 0.75±0.00^{cd}	0.40±0.00^{c} 0.75±0.02^{cd}	0.40±0.01^{c} 0.75±0.02^{d}	0.75±0.02^{d} 1.50±0.00^{e}	0.20±0.00^{b} 0.40±0.01^{ab}	1.50±0.07^{e} 3.00±0.20^{f}	0.100±0.01^{a} 0.200±0.02^{a}	0.200±0.020^{b} 0.500±0.030^{bc}
Aspergillus ochraceus	0.20±0.02^{a} 0.75±0.02^{b}	0.75±0.00^{c} 1.50±0.07^{e}	0.40±0.02^{b} 0.75±0.007^{b}	0.75±0.07^{e} 1.50±0.07^{c}	1.50±0.00^{d} 3.00±0.00^{e}	0.75±0.02^{c} 1.50±0.07^{c}	0.150±0.007^{a} 0.200±0.000^{a}	1.500±0.100^{d} 2.000±0.007^{d}
Aspergillus niger	0.75±0.02^{c} 3.00±0.10^{c}	1.50±0.07^{d} 3.00±0.20^{c}	0.40±0.00^{b} 0.75±0.00^{b}	1.50±0.07^{d} 3.00±0.20^{c}	0.40±0.02^{b} 0.75±0.01^{b}	1.50±0.02^{e} 3.00±0.00^{e}	0.150±0.070^{a} 0.200±0.010^{a}	0.200±0.000^{a} 0.500±0.000^{b}
Trichoderma viride	0.40±0.00^{b} 0.75±0.02^{c}	0.40±0.02^{b} 0.75±0.00^{c}	0.20±0.01^{a} 0.40±0.01^{b}	0.75±0.02^{c} 1.50±0.07^{e}	0.40±0.02^{b} 0.75±0.02^{c}	0.40±0.00^{b} 0.75±0.02^{c}	0.150±0.000^{a} 0.200±0.010^{a}	1.00±0.100^{d} 1.00±0.000^{d}
Penicillium funiculosum	0.40±0.01^{b} 0.75±0.02^{c}	0.40±0.00^{b} 0.75±0.00^{c}	0.75±0.07^{c} 1.50±0.10^{e}	0.75±0.01^{c} 1.50±0.01^{de}	0.75±0.00^{e} 1.50±0.00^{de}	0.75±0.02^{c} 1.50±0.10^{d}	0.200±0.010^{a} 0.250±0.000^{a}	0.200±0.020^{a} 0.500±0.020^{b}
Penicillium ochrochloron	0.40±0.02^{b} 0.75±0.01^{b}	0.75±0.01^{c} 1.50±0.10^{c}	0.40±0.00^{b} 0.75±0.00^{b}	0.75±0.01^{c} 1.50±0.10^{c}	0.75±0.00^{c} 1.50±0.07^{c}	0.75±0.01^{c} 1.50±0.00^{c}	0.200±0.010^{a} 0.250±0.010^{a}	2.500±0.100^{d} 3.500±0.050^{d}
Penicillium verrucosum	0.40±0.02^{c} 0.75±0.00^{b}	0.75±0.00^{d} 1.50±0.10^{c}	0.40±0.01^{c} 0.75±0.01^{b}	0.75±0.007^{d} 1.50±0.10^{c}	0.75±0.01^{d} 1.50±0.07^{b}	0.75±0.00^{d} 1.50±0.03^{c}	0.100±0.010^{a} 0.200±0.007^{a}	0.200±0.010^{b} 0.300±0.010^{a}

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

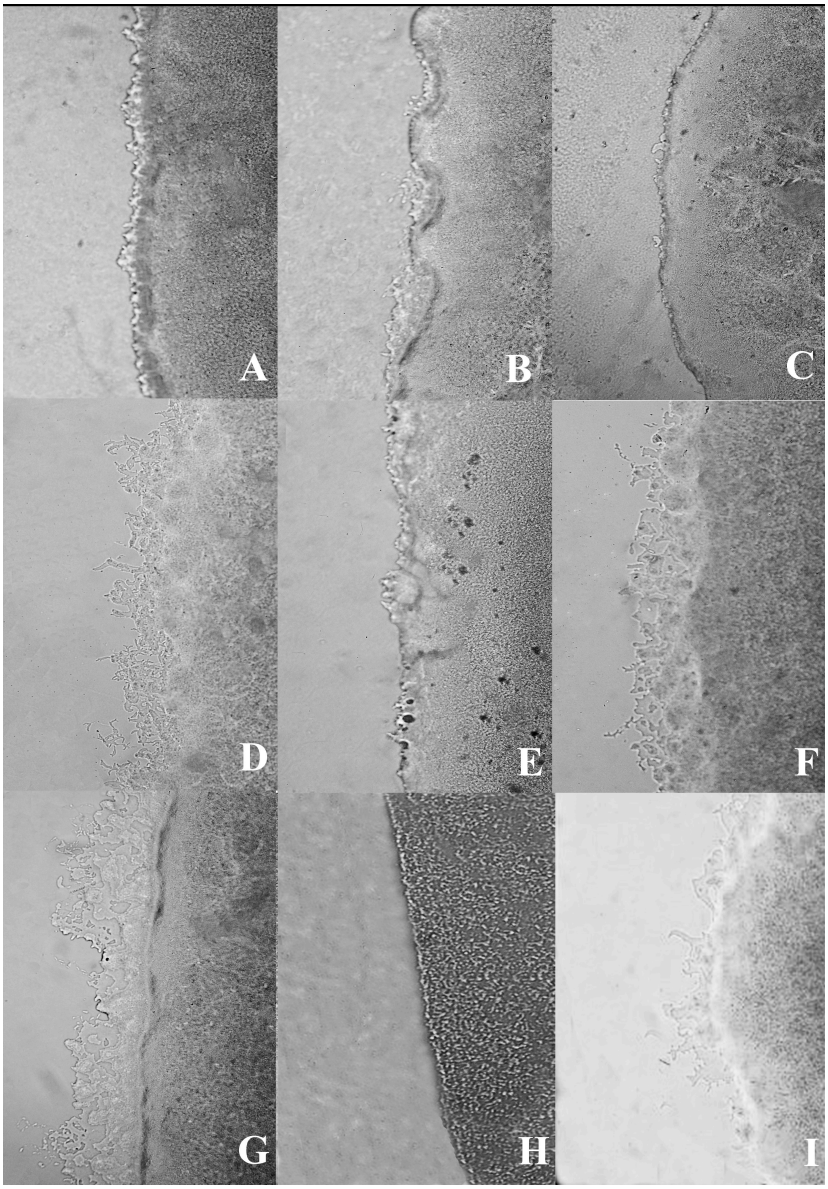
Agents	Colony diameter (mm ± SD)	Colony color	Colony edge on microscope
Aqueous Russia	10.7±0.76	white brown	reduced flagella
EtOH Russia	10.7±1.51	light brown	reduced flagella
Aqueous Finland	10.5±0.65	light brown	partly reduced flagella
EtOH Finland	10.3±0.63	orange	reduced flagella
Aqueous Thailand	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

Table 6. Antitumor activity of *I. 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 tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

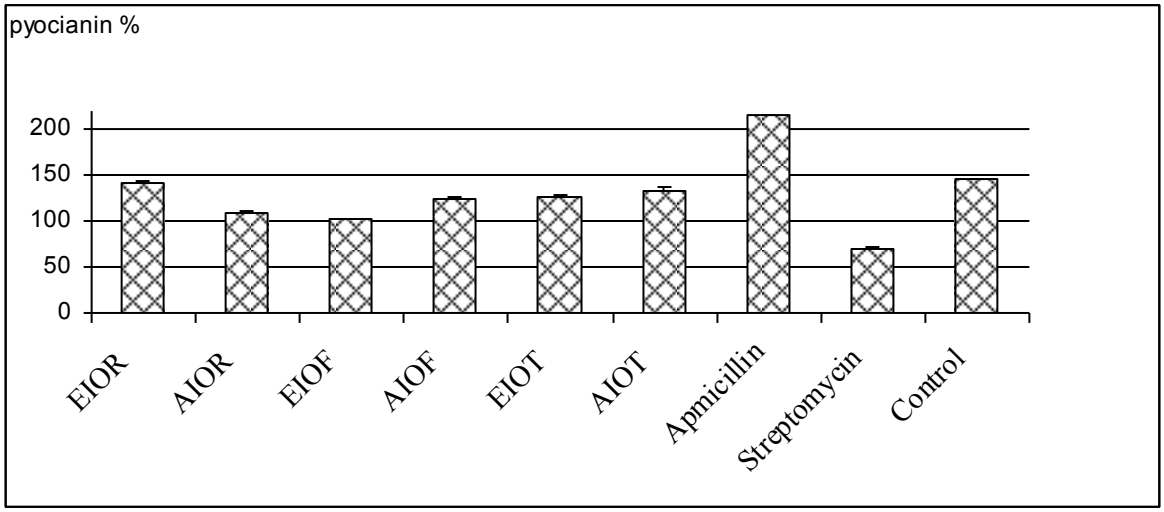
Figure 1. Light microscopy of colony edges of *P. aeruginosa* in twitching motility plates, grown in the presence or absence of ethanolic and aqueous extracts of *I. obliquus*



Light microscopy of colony edges of *P. aeruginosa* in twitching motility, grown in the presence or absence of *I. obliquus* (EIO and AIO) extracts in concentration of 0.5 MIC. The colonies from the bacteria grown with 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 the presence of Finland ethanolic extract with reduced flagella (**C**), and with presence of aqueous extract with regular long flagella (**D**). Colonies with the presence of Thailand ethanolic extract with reduced flagella (E) and with aqueous extract which partly reduced flagella (**W**). *P. aeruginosa* produced a flat, widely spread, irregularly

shaped colony with regular flagella in the absence of extracts, control (**G**); *P. aeruginosa* colony with presence of streptomycin without flagella (**H**) and ampicillin with almost regularly formed flagella (**I**); Magnification: (A–E) ×100.

Figure 2. Effects of ethanolic and aqueous extracts at 0.5 MIC of *I. obliquus* on production of pyocyanin by *P. aeruginosa* (PAO1).



Aqueous extract from Russia (AIOR), Finland (AIOF) and Thailand (AIOT), ethanolic extract from Russia (EIOR), Finland (EIOF) and Thailand (EIOT).