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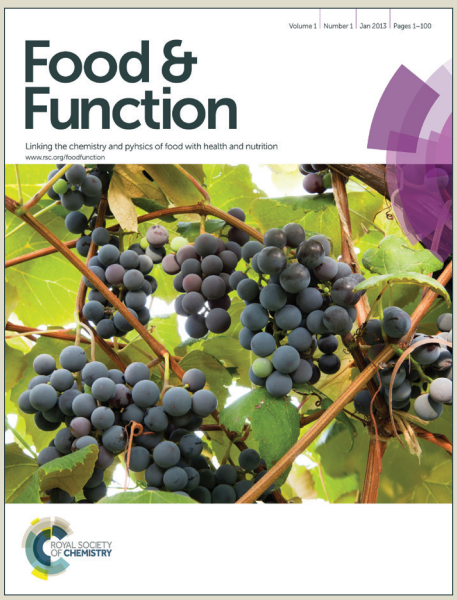

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Bioactive composition, antimicrobial activities and influence of *Agrocybe aegerita* (Brig.) Sing on certain quorum-sensing-regulated functions and biofilm formation by *Pseudomonas aeruginosa*

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Abstract

Agrocybe aegerita (Brig.) Sing is a basidiomycete, white rot fungus. Antioxidant, antimicrobial activity and antiqourum effect on *Pseudomonas aeruginosa* of *A. aegerita* methanolic extract was investigated. The extract showed very good antimicrobial activity against all the tested microorganisms in a dose dependent manner. Effects of the Sub-MIC, MIC and 2MIC of *A. aegerita* methanolic extract regulated virulence factors in quorum sensing (QS) test, as well as biofilm formation on *P. aeruginosa*. Sub-inhibitory and inhibitory concentrations of the extract demonstrated reduction of virulence factors such as pyocyanin production, twitching and swimming motility. The biofilm forming capability of *P. aeruginosa* PAO1 was also reduced in a concentration-dependent manner. Furthermore, the chemical composition of methanolic extract was determined considering phenolic composition. Methanolic extract of *A. aegerita* can be a very good source of bioactive substances. This research is of great importance due to the prevalence of drug-resistant microorganisms.

Key words: *Agrocybe aegerita*, antimicrobial activity, antiquorum effect, *Pseudomonas aeruginosa,* phenolic composition.

1. Introduction

An emerging problem associated with misuse of antibiotic therapy is the worldwide emergence of higher level tolerance of target organisms against available broad spectrum antibiotics [1]. As a result, and considering the rapid spread of multidrug resistance, the development of new antimicrobial or antipathogenic agents that act upon newly adapted microbial targets has become a very pressing priority [2].

Quorum sensing (QS) plays an important role in biofilm formation. It is an intercellular signaling system in which bacteria communicate and regulate gene expression by releasing small compounds called autoinducers in environment [3]. Due to its role in various regulatory processes it can serve as an important target. Knowledge about the biofilm formation and quorum sensing are resulting in identification of new targets for therapeutics against *Pseudomonas aeruginosa* infection [4].

Despite the huge diversity of compounds with antimicrobial properties, resistance of microorganisms to them is increasing dramatically. This fact certainly demands our attention and immediate response. Therefore, the search for new sources of compounds with antimicrobial properties in natural matrices has been intense in the last years [5, 6].

Mushrooms emerged as a good alternative source of new antimicrobials. Some common edible mushrooms like *Ganoderma lucidum* and *Lentinus edodes* have been studied for that purposes [7, 8], but isolated substances or crude extracts derived from lesser-known edible mushrooms are also interesting. Those species are potential sources of diverse biomolecules with nutritional and/or medicinal properties, and could be used for the development of medicines, nutraceuticals and food supplements [9, 10].

Furthermore, wild mushrooms have also emerged as a source of antioxidant compounds, very important to eliminate free radicals and other reactive species produced as a part of the normal process of aerobic metabolism, and that can cause structural damage to cells, being implicated

in several chronic diseases such as various types of cancer, cardiovascular diseases or diabetes [11, 12].

It is estimated that there are about 140000 species of mushrooms on earth and of these only 22000 are known and only a small percentage (5%) has been investigated [6]. Therefore, it is necessary to expand the knowledge and methods available to identify the bioactive components in mushrooms that act on specific target microorganisms.

Edible mushroom *Agrocybe aegerita* (a synonym of *Agrocybe cylindracea*; black poplar mushroom) is an agaric fungus that colonizes deciduous wood and bark mulch, preferably stumps of poplar trees. This mushroom is found in North America, Europe and Asia, and it seems to prefer warm or mild climates. It is a popular edible mushroom in southern Europe, especially in Italy (so called Pioppino mushroom) where it is also commercially cultured [13]. *A. aegerita* is a popular and highly nutritional edible mushroom, which has been used as a traditional Chinese herbal medicine. It has an abundant amount of proteins, which accounts for 25–30% of dry fruiting bodies [14].

In the present study, a methanolic extract obtained from a wild sample of *A. aegerita,* collected in Serbia, was explored for its antimicrobial activity against plant, animal and human pathogens, as well as food spoilage agents, possible inhibition of quorum sensing activity on *P. aeruginosa,* and antioxidant potential. Furthermore, being an edible species, the mushroom was fully characterized regarding nutritional properties, hydrophilic and lipophilic compounds.

2. Material and methods

2.1. Standards and reagents

Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Dimethylsulfoxide (DMSO), (Merck

KGaA, Germany) was used as a solvent. 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, USA).

2.2. Mushroom species

Agrocybe aegerita was collected from the wood logs of poplar trees at Jabučki rit (Northern Serbia) during April 2012 and authenticated by Dr Jasmina Glamočlija (Institute for Biological Research "Siniša Stanković"). Voucher specimen has been deposited at the Fungal Collection Unit of the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, under number Aa-001-2012. Fresh fruiting bodies were randomly divided to smaller samples and immediately dried by lyophilization (LH Leybold, Lyovac GT2, Frenkendorf). When reaching constant mass, specimens were milled to a fine powder, mixed to obtain an homogenate sample, and kept at 4ºC untill further analysis.

2.3. Preparation of the extract

The methanolic extract was obtained by stirring the dry fruiting body of *A. aegerita* (8 g) with 100 mL of methanol for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then re-extracted with 20 mL of methanol for 1 h. The combined methanolic extracts were evaporated at 40ºC (rotary evaporator Büchi R-210) to dryness. The extracts were re-dissolved in *a)* methanol for the antioxidant activity assays (20 mg/mL), *b)* 5% solution of DMSO in distilled water for the antimicrobial activity and antiqourum assays (100 mg/mL).

2.4. Evaluation of the antimicrobial activity

2.4.1. Antibacterial activity

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 [15, 16]. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. Mushroom methanolic extract was dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and 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 an INT ((p-iodonitrotetrazolium violet) [2-(4-iodophenyl)-3-(4-nitrphenyl)-5-phenyltetrazolium chloride; Sigma]) color and compared with positive control for each bacterial strains. 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.

2.4.2. Antifungal activity

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 for this test. In order to investigate the antifungal activity of mushroom extract, a modified microdilution technique was used [17, 18]. 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 1.0×10^5l. Extract was dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (10 mg/mL) and added in broth Malt medium with inoculum (0.005-3 mg/mL for extracts). The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 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.

2.5. Antiqourum (AQ) sensing activity

2.5.1. Bacterial strains, growth media and culture conditions

P. aeruginosa PA01 (ATCC 27853) used in this study is from the lab collection. 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.

2.5.2. Biofilm formation

Biofilm was grown as described previously [19]. The effect of different concentrations of extract (ranging from 0.5 to 0.125 of MIC) on biofilm forming ability was tested on polystyrene flat-bottomed microtitre 96 well plates as described by Spoering and Lewis, [20] with some modifications. Briefly, 100 µL of overnight culture of *P. aerugonosa* was added to each well of the plates in the presence of 100 µL subinhibitory concentrations (subMIC) of extract (0.5, 0.25 and 0.125 MIC; 0.30 mg/mL, 0.15 mg/mL, 0.07 mg/mL) or 100 mL medium (control). After incubation for 24 h at 37° C, each well was washed twice with sterile PBS (pH 7.4), dried, stained for 10 min with 0.1 % crystal violet in order to determine the biofilm mass. After drying, 200 µL of 95% ethanol (v/v) was added to solubilize the dye that had stained the biofilm cells. The excess stain was washed off with dH_2O. After 10 min, the content of the wells was homogenized and the absorbance at λ= 625 nm was read on a Sunrise™ - Tecan ELISA reader.

2.5.3. Discs-diffusion method for determination of AQ activity against P. aeruginosa

Filter paper discs were impregnated with tested extract solutions (subMIC; 0.30 mg/disc, 0.15 mg/disc, 0.07 mg/disc), streptomycin and ampicillin (subMIC; 0.5 MIC, 0.25 MIC and 0.125 MIC; 0.1, 0.05, 0.025 mg/disc for streptomycin and 0.4, 0.2 and 0.1 mg/disc for ampicilin) to determine whether they have antiqourum activity against bacteria and impair bacterial growth. To do this, filter paper (filter paper 4 mm; Whatman) were used. Discs were soaked in the indicated solutions, then dried at room temperature (3 h, protected from light), and aseptically placed onto the plates prior to bacterial inoculation. After incubation, it was recorded whether the inhibition or antiqourum zones were obtained [21].

2.5.4. Twitching and flagella motility

After growth in the presence or absence of extract, 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 [22]. Fifty microlitres of tested extract (sub MIC; 0.5 MIC, 0.25 MIC and 0.125 MIC) was mixed into 10 mL of molten MH medium 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 swimming was determined by measuring the area of the colony [23].

2.5.5. Anti-QS against P. aeruginosa PA01 Pyocyanin

Overnight culture of *P. aeruginosa* PA01 was diluted to OD_{600} nm 0.2. Then, tested extract (250 µL disolved as 0.5 MIC, MIC and 2 MIC) was added to *P. aeruginosa* (4.75 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 using the UV-visible spectrophotometer (UV1601, Shidmazu, Kyoto, Japan) at 520 nm [24].

2.6. Chemical characterization of the methanolic extract in terms of bioactive compounds

The analysis was performed by HPLC-DAD-ESI/MS methodology using 280 nm and 370 nm as preferred wavelengths, according to a procedure previously described by the authors. The phenolic compounds were characterized according to their UV and mass spectra, retention times, and by comparison with authentic standards when available. For the quantitative analysis of phenolic compounds, a calibration curve was obtained by injection of known

concentrations (5-80 µg/mL) of different standard compounds: *p*-hydroxybenzoic acid (y=16420x+12914; R^2=0.9999); *p*-coumaric acid (y=51195x+1×^{106}x; R^2=0.992); cinnamic acid (y=86366x+88451; R^2=0.999). The results were expressed as µg per g of extract.

3. Results and Discussion

3.1. Antimicrobial activity

It was determined that the yield of the methanolic extraction from *A. aegerita* fruiting body is very good, and is 1.40 g out of 5.00 g of fruiting body.

The methanolic extract of *A. aegerita* was tested against four species of Gram-negative bacteria, four species of Gram-positive bacteria, and eight species of fungi. Extract exhibited excellent activity at tested concentrations on all tested microorganisms. Among the tested bacteria, *S. aureus* and *P. aeruginosa* were the most susceptible to the activity of the extract, with MIC (0.59 mg/mL) and MBC (1.18 mg/mL). Commercial antibiotics, streptomycin and ampicillin, showed inhibitory activity in the range of 0.05-0.30 mg/mL, and bactericidal activity in the range of 0.10-0.50 mg/mL. *L. monocytogenes* was the most resistant bacteria with MIC (4.74 mg/mL) and MBC (9.49 mg/mL) (**Table 1**).

Regarding micromycetes, *A. versicolor* was strongly inhibited by the extract, with MIC value of only 0.025 mg/mL, and MBC 0.95 mg/mL. The most resistant microfungi was *A. fumigatus* with inhibitory concentration at 2.38 mg/mL and fungicidal concentration 18.96 mg/mL (**Table 1**). Antifungal drugs ketoconazole and bifonazole exhibited high activity in the range of 0.10-3.50 mg/mL (**Table 1**). It is important to notice that the extract showed inhibitory activity against all the tested bacteria and micromycetes.

Numerous mushroom extracts have been reported to have antimicrobial activity against pathogenic microorganisms, including extracts from *Agaricus bisporus* (the most cultivated

mushroom in the world), *Armillaria mellea*, *Boletus edulis*, *Cantharellus cibarius*, just to mention a few [9]. It should be highlighted that as far as our literature survey could ascertain, scarce information is available on the *in vitro* antimicrobial activity of *A. aegerita* extracts. There are literature data (from 1992) concerning the antifungal activity of the extract from the submerged culture, and since then methodology has significantly changed concerning the isolation of the extracts and assays for antimicrobial activity evaluation [25]. Also, data concerning the compound agrocybolacton, derived from the culture representatives of the genus *Agrocybe*, showed moderate antibacterial activity against Gram-positive bacteria *B. subtilis* and *M. smegmatis* [26]. Agrocybin, a peptide isolated from *A. dura* and *A. cylindracea* exhibited activity against Gram-negative bacteria [27] and also antifungal activity [28]. Since there are no literature data providing information about antimicrobial activity of crude extracts of *A. aegerita*, these results are of great importance.

3.2. Biofilm formation

The effect of *A. aegerita* extract on biofilm formation of *P. aeruginosa* was tested with 0.5, 0.25 and 0.125 of determined MIC. **Table 2** shows that the extract tested reduced biofilm formation better than streptomycin and ampicillin, especially at 0.5 MIC. The extract reduced biofilm formation in 84.24%, while streptomycin and ampicillin reduced biofilm in 50.60% and 30.84%, respectively.

The quorum-sensing inhibition zones were determined by disc diffusion method. It can be seen that extract showed antiquorum sensing (AQ) activity at all concentrations in range of 7.70-10.30 mm. Ampicillin possessed AQ activity at higher concentration (7.60 mm), while streptomycin showed the best AQ activity presenting the zones in range of 15.50-22.06 mm (**Table 2).**

The flask incubation assay was used to quantify quorum sensing inhibitory activity of the extract that produced quorum-sensing inhibition zones. The tested extract of *A. aegerita* demonstrated concentration-dependent pyocyanin inhibitory activity and showed reduction of pigment in all tested concentration, sub MIC, MIC and 2 MIC. The best reduction pyocyainin ability was noticed at 2 MIC. In general, all the tested concentrations (sub MIC, MIC and 2 MIC) showed higher reduction ability of pigment. A decrease level in green pigment content was demonstrated in the extract at all the tested concentrations. The extract showed higher reduction of pigment than ampicillin and streptomycin (**Figure 1**). Promising anti-quorum sensing compounds have been demonstrated to disrupt bacterial biofilms and make the bacteria more susceptible to antibiotics, and these compounds also provide the ability to reduce bacterial virulence factors as well as promote clearance of bacteria in infectious animal models. 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 [29].

In addition to QS, the initiation of biofilm formation by *P. aeruginosa* depends on two cell-associated structures; the flagellum and type IV pili [22, 30]. The flagellum is responsible for swimming motility while the type IV pili are responsible for twitching motility [31]. Both types of motility are important in the initial stages of biofilm formation by *P. aeruginosa* [22, 30]. Therefore, we tried to determine if our extract influence on either one or both motilities. On swimming plates, the motile strain PAO1 was used as the 100% standard (control) for motility while the Petri dishes with the same strain plus *A. aegerita* extract were compared with control. Extract reduced the twitching motility of *P. aeruginosa*. The normal colonies of *P. aeruginosa,* i.e. in the absence of the extract, were flat with a rough appearance displaying irregular colony edges (**Fig. 2B**) and a hazy zone surrounding the colony. The cells were 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 to 100% (**Table 3)** and it was 14.0 mm. Bacteria that were grown with the *A. aegerita* extract solution 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, and the colony diameter swimming zones was also reduced (15.33 mm) (**Fig. 2A**, **Table 3**). Streptomycin reduced the flagellas completely (**Fig. 2C**), while Ampicillin did not affect the formation of flagella at all (**Fig. 2D**).

In summary, our study indicated that *A. aegerita* extract possessed antimicrobial, antibiofilm and anti-quorum sensing activity. Inhibition of bacterial quorum sensing offers new strategy for the treatment of bacterial infections. Anti-quorum sensing property of this plant species may play an important role in antibacterial activity and offers an additional strategy for fighting bacterial infection.

3.3. Chemical composition regarding bioactive compounds

Chemical composition of *A. aegerita* regarding bioactive compounds of its methanolic extract are presented in the **Table 4**. Two phenolic acids namely, *p*-hydroxybenzoic and *p*- coumaric acids were identified, as well as cinnamic acid as phenolic related compound. Phenolic acids account for almost a third of dietary phenols and there is an increasing awareness and interest in their antioxidant behavior and potential health benefits [32]. Antimicrobial activity of phenolic acids is well documented in the literature [33]. *A. aegerita* was also reported to contain several bioactive metabolites, such as indole derivatives with free radical-scavenging ability [34], polysaccharides with hypoglycemic activity [35] and agrocybin, a peptide with anti-fungal activity [36]. Previously, Gao et al. [37] reported that the protein components from

A. aegerita showed tumor rejection activity. Also two antitumor lectins, AAL and AAL-2, were identified from the protein components of *A. aegerita* [38, 39].

Nowadays, mushroom research is intense and hundreds of species have demonstrated a broad spectrum of activities, including antimicrobial and antioxidant. It is evident that there are very good effects of extracts, but there is a gap in the identification of the individual compounds responsible for antimicrobial properties. Only a few low-molecular weight compounds and some peptides and proteins have been described. After elucidation of the mechanism of mushroom extracts activity or pure compounds activity, mushroom metabolites or other related compounds could be used to develop nutraceuticals or microorganisms effective drugs. The reason that some of the mushroom extracts/preparations are not available yet as medicines may be the difficulty relating to massive production. Since *A. aegerita* is part of the mushroom cultivation, and the fungal material is primarily available, in the light of new data of the antimicrobial activity of the extract, attention should definitely be dedicated to it.

4. Conclusion

According to chemical profile and bioactivities presented herein, *A. aegerita* might be explored regarding antimicrobial purposes. Further research and gaining knowledge about the mechanisms of action of the different mushroom compounds might lead to the discovery of new active principles involved in the bioactivity.

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Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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Table 1. Antibacterial and antifungal activity of *Agrocybe aegerita* methanolic extract (mg/mL) (mean ± SD).

	Bacteria	*S. aureus*	*B. cereus*	*L.monocytogenes*	*M.flavus*	*P.aeruginosa*	*E.coli*	*S.typhimurium*	*En. cloacae*
A. aegerita	MIC	0.59±0.01	1.18±0.01	4.74±0.01	2.38±0.02	0.59±0.02	2.38±0.02	2.38±0.01	1.18±0.03
	MBC	1.18±0.02	2.38±0.03	9.49±0.03	4.74±0.03	1.18±0.03	4.74±0.03	4.74±0.01	2.38±0.01
Streptomycin	MIC	0.25±0.03	0.05±0.02	0.15±0.03	0.125±0.02	0.05±0.03	0.05±0.01	0.05±0.02	0.05±0.03
	MBC	0.5±0.01	0.10±0.03	0.30±0.01	0.25±0.01	0.10±0.03	0.10±0.01	0.10±0.02	0.10±0.02
Ampicillin	MIC	0.10±0.03	0.10±0.01	0.15±0.03	0.10±0.03	0.10±0.01	0.30±0.02	0.15±0.01	0.15±0.01
	MBC	0.15±0.01	0.15±0.02	0.30±0.03	0.15±0.02	0.20±0.03	0.50±0.03	0.20±0.03	0.20±0.01
	Fungi	*A.fumigatus*	*A.versicolor*	*A.ochraceus*	*A.niger*	*T.viride*	*P.funiculosum*	*P.ochrochloron*	*P.cyclopium*
A. aegerita	MIC	2.38±0.01	0.025±0.01	1.19±0.01	2.38±0.02	2.38±0.02	2.38±0.02	1.19±0.01	2.38±0.00
	MFC	18.96±0.02	0.95±0.03	4.74±0.03	9.49±0.03	2.38±0.03	4.74±0.03	4.74±0.01	4.47±0.01
Ketoconazole	MIC	0.20±0.03	0.20±0.02	0.15±0.03	0.20±0.02	0.20±0.03	2.50±0.01	0.20±0.02	0.25±0.03
	MFC	0.50±0.01	0.50±0.03	0.20±0.01	0.50±0.01	0.30±0.03	3.50±0.01	0.50±0.02	0.50±0.02
Bifonazole	MIC	0.15±0.03	0.10±0.01	0.15±0.03	0.15±0.03	0.10±0.01	0.20±0.02	0.20±0.01	0.25±0.00
	MFC	0.20±0.01	0.20±0.02	0.20±0.03	0.20±0.02	0.20±0.03	0.25±0.03	0.25±0.03	0.50±0.03

Table 2. Effects of *Agrocybe aegerita* methanolic extract on biofilm formation of *P. aeruginosa* (PAO1) and disc-diffusion method for detection of antiquorum (AQ) concentrations

Biofilm formation* (%)				AQ** (mm)		
Agents	0.5MIC	0.25MIC	0.125MIC	0.125 MIC	0.25 MIC	0.5 MIC
A. aegerita	15.76±0.03	44.97±0.30	80.63±0.46	7.70±0.58	8.00±0.00	10.30±0.58
Ampicillin	69.16±0.65	56.46±0.46	92.16±0.37	-	-	7.6±0.6
Streptomycin	49.40±0.46	70.97±0.36	88.36±0.42	-	15.0±2.1	22.6±2.3

*Biofilm formation values were calculated as: (mean A_{620} treated well)/(mean A_{620} control well)x100.
Values are expressed as means ± SD. In each column, different letters mean significant differences between samples ($p<0.05$).
** - No effect of AQ

Table 3. Twitching activity of *Agrocybe aegerita* methanolic extract.

17.36 ± 0.88	2.66 ± 0.10	7.23 ± 0.18	6.11 ± 1.60	0.39 ± 0.06

Agents	Colony diameter (mm ± SD)	Colony color	Colony edge
A. aegerita	15.33±4.51	green with white edges	reduced flagella
Streptomycin	5.00±0.06	white	flat
Ampicillin	12.00±1.00	white	regular flagella
Control P.a. 10^9	14.00±1.00	green	regular flagella

Table 4. Chemical composition of *A. aegerita* methanolic extract

Phenolic acids	mg/100 g dw
p-Hydroxybenzoic acid	0.61±0.04
p-Coumaric acid	0.19±0.01
Total phenolic acids	0.80±0.04
Cinnamic acid	1.04±0.02

Figure 1. Reduction of pyocyanin pigment by *A. aegerita* extract.

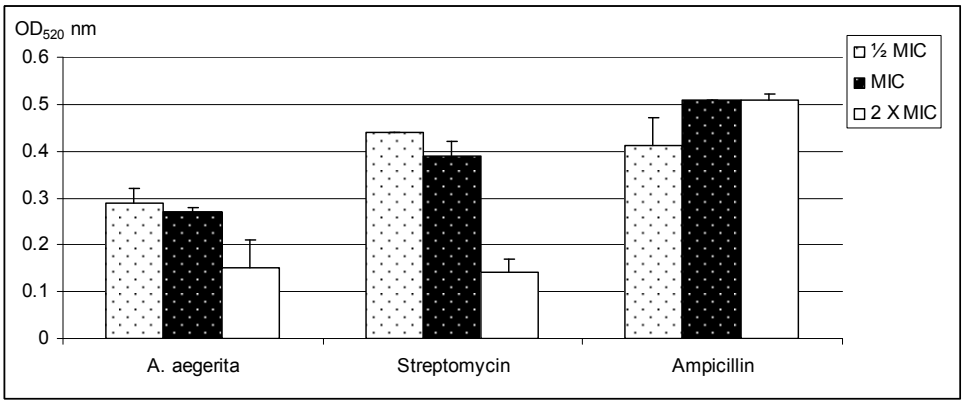
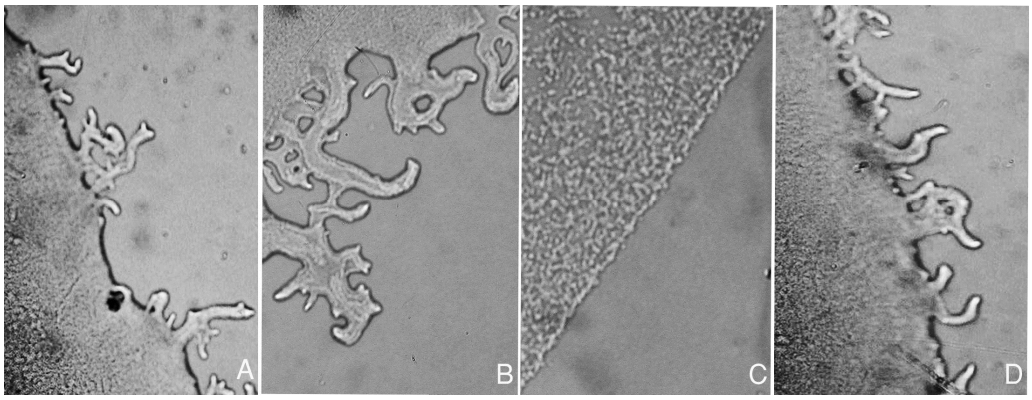


Figure 2. Light microscopy of colony edges of *P. aeruginosa* in twitching motility plates, grown in the presence or absence of *A. aegerita* extract.



The colonies from the bacteria grown with *A. aegerita* extract (**A**) were with small changes in flagella shape and numbers. *P. aeruginosa* produced a flat, widely spread, irregularly shaped colony in the absence of extracts (**B**). *P. aeruginosa* colony with presence of Streptomycin without flagella (**C**) and Ampicillin with almost regularly formed flagella (**D**). Magnification: (**A-D**) x100.