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ORIGINAL ARTICLE

New biological findings of ethanol and chloroform extracts of fungi *Suillellus rubrosanguineus* and *Tylopilus felleus*

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ABSTRACT

The aim of the research was to determine some basic biological activities of less biomedically studied but commonly known two fungi from the Boletaceae family *Suillellus rubrosanguineus* and *Tylopilus felleus*, which grow in the forests of Middle Europe. The cytotoxicity tests of the ethanol and chloroform extracts were carried out using NIH-3T3 and MCF-7 cell lines. The presence of alkaloids in the extracts was assessed by the reaction with Dragendorff reagent. In all of the extracts the positive reaction with the reagent was observed. In general, the extracts from *Suillellus rubrosanguineus* were more cytotoxic than the extracts from *Tylopilus felleus* and exhibited no selectivity of activities on healthy and cancer cell lines. However, the extracts from *Tylopilus felleus* proved to be selectively cytotoxic for cancer cell line. *Tylopilus* extracts or their isolated bioactive compounds could be considered for further study in pre-clinical experiments.

KEY WORDS: *Suillellus rubrosanguineus*; *Tylopilus felleus*; alkaloids; cytotoxicity; fungi

ABBREVIATIONS

DMEM: Dulbecco´s Modified Eagle´s Medium; **DMSO:** dimethyl sulfoxide; **FBS:** fetal bovine serum; **MTT:** 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; **PBS:** phosphate-buffered saline; **TylEtCap:** ethanol *Tylopilus* extract from the caps and stipes; **TylEtTub:** ethanol *Tylopilus* extract from the tubes; **TylChCap:** chloroform *Tylopilus* extract from the caps and stipes; **TylChTub:** chloroform *Tylopilus* extract from the tubes; **SuiEtCap:** ethanol *Suillellus* extract from the caps and stipes; **SuiEtTub:** ethanol *Suillellus* extract from the tubes; **SuiChTub:** chloroform *Suillellus* extract from the tubes

Introduction

There are only few studies focusing on the two members of the fungi family Boletace: *Suillellus rubrosanguineus* and *Tylopilus felleus* (Figure 1), commonly known as bitter bolete or bitter tylopilus. In the presented study we thus decided to investigate basic biological activities of these two fungus species. *Tylopilus felleus* grows in the whole Northern hemisphere and is common also in Slovakia, where it grows mainly in coniferous forests. *Tylopilus felleus* is typical for its bitter taste and pinkish hymenium. Due to the bitter taste, it is considered not edible (Šutara *et al.,* 2009). Only few studies about biological activities of *Tylopilus felleus* have been done (Grzybek *et al.,* 1994). This mushroom is also known in traditional Chinese medicine (Antonín *et al.,* 2013).

Suillellus rubrosanguineus (syn. Boletus rubrosanguineus, Rubroboletus rubrosanguineus) is a rare species, considered to be poisonous, which grows in middle and east Europe and on the Caucasus. In the Czech Republic it is included in the Red List of fungi in the category of critically endangered (Holec & Beran 2006). In Slovakia this mushroom grows rarely in higher altitudes in coniferous forests under *Picea abies* and *Abies alba.* Its occurrence differs from similar species such as *Boletus legalie, Boletus rhodoxanthus* and *Boletus satanas* (Šutara *et al.,* 2009). *Suillellus rubrosanguineus* is characteristic by its bright carmine colors on stipe and cap.

In this paper we focused on cytotoxicity profile of different extract preparations from *S. rubrosanguineus* and *T. felleus* in healthy and cancer cell lines and their analysis for the presence of alkaloids.

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Materials and methods

Materials

The Dulbecco´s Modified Eagle´s Medium (DMEM), penicillin-streptomycin mixture, phosphate-buffered saline (PBS), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Darmstadt, Germany). Dragendorff reagent, chloroform, ethanol, sulfuric acid, hydrochloric acid, ammonium, ether were obtained from Centralchem (Bratislava, Slovakia).

Specimens used in the study

Tylopilus felleus: Slovakia, Záhorská nížina, Holubičky, in coniferous forest, grass under *Pinus* trees; 19th of June, 2016; Leg. Drahomír Ďuriška et Det. Ondrej Ďuriška. *Suillellus rubrosanguineus:* Slovakia, Veľká Fatra, Valča; in mixed forest, under *Picea abies* trees; 3rd of July, 2016; Leg. et Det. Ondrej Ďuriška. Dr. Ondrej Ďuriška (co-author) made the photos of both collected fungi (Figure 1).

Extract preparations

Dried mushrooms samples were powdered in a blender before the extraction process. Mushroom samples were extracted by 96% ethanol or by concentrated chloroform for two weeks at occasional shaking. After evaporation of the solvents under reduced pressure, dry extracts were dissolved in DMSO and stored at –20 °C and used as mother liquor for all experiments. Separate extracts were prepared from stipes and caps (TylEtCap, TylChCap, SuiEtCap) and from hymenium (tubes) (TylEtTub, TylChTub, SuiEtTub, SuiChTub).

Cell cultures

NIH-3T3 (mouse embryonic fibroblast cells) were obtained as a gift from dr. Diana Vavrincová (Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University in Bratislava, Slovakia). MCF-7 (human breast adenocarcinoma cells) were donated by dr. Peter Gál (Department of Pharmacology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Slovakia). Cells were grown at 37 °C in humidified atmosphere with 5% CO_2 in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were subcultured twice a week.

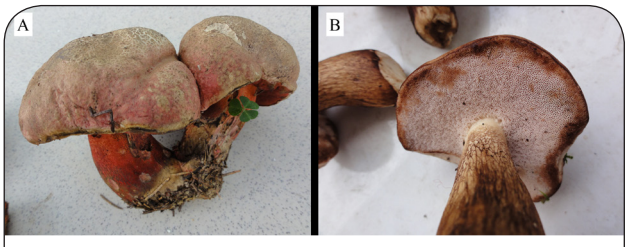

Figure 1. Photographs of collected fungi *Suillellus rubrosanguineus* (**A**) and *Tylopilus felleus* (**B**) made by dr. Ondrej Ďuriška (co-author).

In vitro analysis of cytotoxicity and cell proliferation

The effects of the compounds tested on the activity of mitochondrial dehydrogenases and proliferative functions of both cell lines were assessed using the reduction of tetrazolium salt MTT. The cells were seeded (10 000 cells/100 µL/well) in the 96-well-plate in complete medium. After 24 h, different concentrations of extracts, dissolved in complete medium, were added. The appropriate blanks were included into the experiment as well. The compound quercetin was used as a reference and it was dissolved in DMSO and then in complete medium. The final concentration of DMSO never exceeded 0.1%. Following 24-h incubation, a MTT solution was added to the wells (final concentration 0.4 mg/mL) except blank and after 4 h of incubation, the medium was removed and 100% DMSO was added to lyse the cells. The absorbance was measured (λ=570 nm) in Infinite M200 spectrofluorometer (Tecan, Switzerland). The amount of generated formazan (correlating to the number of viable and metabolically active cells) was calculated as a percentage of control cells and was set to 100%.

Qualitative determination of alkaloids

The presence of alkaloids in the extracts was identified with alkaloid-specific reagent Dragendorff, which creates red-orange precipitate with alkaloids. Dry chloroform extracts of fungi were dissolved in chlorform and were shaken out with 0.5 M sulfuric acid. The Dragendorff reagent was added into the water phase. Dry ethanol extracts of fungi were dissolved into the ether and a small amount of 15% ammonia was added. The solutions were shaken out with 0.1 M hydrochloric acid. The alkaloids were determined by adding a few drops of Dragendorff reagent. Extracts from leaves of *Atropa bella-donna* and from bark of *Cinchona succirubra* were employed as reference standards in tests of alkaloid detecting solutions.

Statistical analysis

Data were evaluated as means ± SEM from the three independent measurements. Comparisons among groups were made using the Student t-test with equal variance.

Results

Because of few data about cytotoxicity of two Boletaceae species, we provided MTT cytotoxicity tests on healthy mouse fibroblasts NIH-3T3 and on human cancer cell line MCF-7. We used the concentration range 6.25–100 µg/mL for *Suillellus rubrosanguineus* extracts and *Tylopilus felleus* extracts as well. The well-studied flavonoid quercetin was used as a reference with the concentrations from 6.25 to 50 µg/mL. The generation of formazan correlates directly with the number of viable cells with active mitochondrial reductases. The results are shown in Figure 2 (A, B, C).

Almost all higher concentrations of the four *Tylopilus* extracts caused significant decrease of the MTT reduction by cancer cells MCF-7 ($p<0.05$; $p<0.01$) (Figure 2 A). On

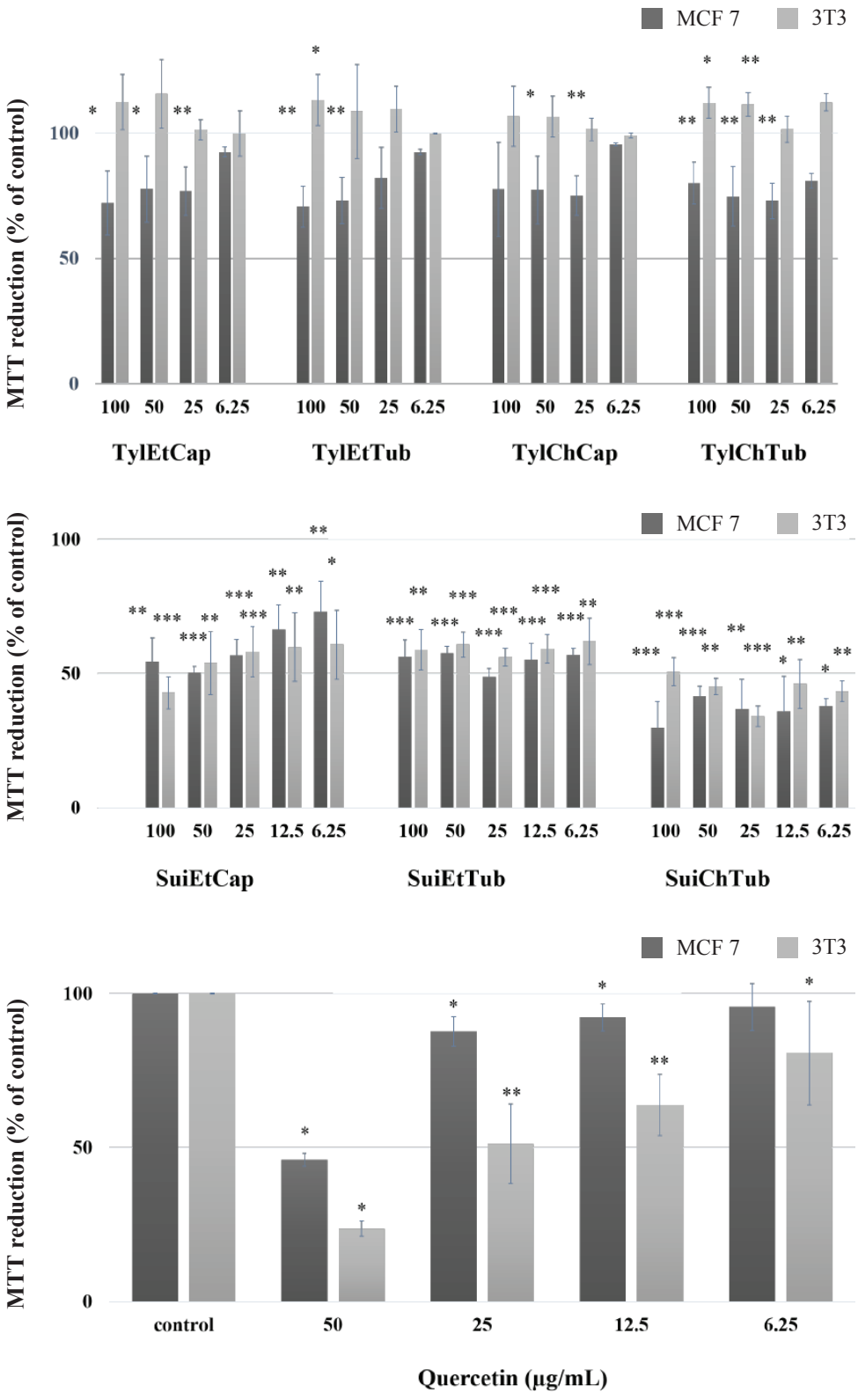

Figure 2. Comparison of viability/proliferation (assessed by mitochondrial reduction of MTT) of MCF-7 cells (dark gray) and 3T3 cells (light gray) after 24-h treatment with *Tylopilus* extracts (**A**), *Suillellus* extracts (**B**) and well-studied flavonoid quercetin used as a reference for set up the model (**C**) expressed as the percentage of control. *p<0.05, **p<0.01, ***p<0.001 *vs*. control. TylEtCap – ethanol *Tylopilus* extract from the caps and stipes; TylEtTub – ethanol *Tylopilus* extract from the tubes; TylChCap – chloroform *Tylopilus* extract from the caps and stipes; TylChTub – chloroform *Tylopilus* extract from the tubes; SuiEtCap – ethanol *Suillellus* extract from the caps and stipes; SuiEtTub – ethanol *Suillellus* extract from the tubes; SuiChTub – chloroform *Suillellus* extract from the tubes.

the contrary, higher concentrations of the chloroform and ethanol extracts of the tubes caused significant increase of the viability of NIH-3T3 ($p<0.05$; $p<0.01$) and extracts from the caps and stipes caused mild enhancement of the viability and/ or proliferation of the NIH-3T3 cells.

In general, the extracts from *Suillellus rubrosanguineus* are significantly more cytotoxic than the extracts from *Tylopilus felleus.* All three *Suillellus* extracts in the concentrations used significantly decreased the metabolic activity or/and proliferation of both used cell lines ($p<0.05$; $p<0.01$; $p<0.001$) (Figure 2 B). The chloroform extract from the tubes of *Suillellus rubrosanguineus* is more toxic than the two ethanol extracts tested. There is no significant difference in the effects of the three extracts of *Suillellus rubrosanguineus* on NIH-3T3 compared to MCF-7.

The flavonoid quercetin used as a reference (Figure 2 C) showed higher cytotoxicity to healthy NIH-3T3 than to cancer cell line MCF-7. Our results for quercetin cytotoxicity are in accordance with previous *in vitro* studies (Chou *et al.,* 2010; Danihelova *et al.,* 2013). These results serve to ensure that this method is set correctly.

In our study we detected the presence of alkaloids by alkaloid-specific Dragendorff reagent, which creates red-orange precipitates with them (Table 1). All extracts from both fungi reacted positively with the Dragendorff reagent, the ethanol extracts showed stronger reactions than the chloroform extracts. The alkaloids are localized in the tissues of caps as well as tubes of fungi.

Table 1. Response of the tested extracts to specific alkaloid indicator.

Extracts	Reaction with Dragendorff reagent
TylEtCap	***
TylEtTub	***
TylChCap	**
TylChTub	**
SuiEtCap	***
SuiEtTub	***
SuiChTub	**

From the extracts the alkaloid cations were released and they could make positive reaction with Dragendorff reagent as a red precipitate.
*** intense reaction; ** moderate reaction; * weak reaction; – no reaction (range inspired by Furr & Mahlberg, 1981).

Discussion

So far, only a few studies concerning biological activities of the two fungi of Boletaceae family *Suillellus rubrosanguineus* and *Tylopilus felleus,* which grow in the forests of Middle Europe, were done.

For this reason we focused on this topic. Our research includes basal cytotoxicity test on healthy and cancer cell lines and qualitative determination of the presence of alkaloids.

In general, the high selectivity of the plant extracts or synthetic compounds for cancer cell lines is preferred. Well-known cytostatics paclitaxel, doxorubicin, tamoxifen have inhibition concentrations IC_{50} more times lower for cancer cell lines than for normal cells (Hasanpourghadi *et al.,* 2017). Prsumably, no studies about the cytotoxicity on the healthy or cancer cell lines of the extracts from the mentioned two fungi have been done. Only some cytotoxicity studies of the β-glucan tylopilan isolated from *Tylopilus felleus* were provided. Tylopilan showed antitumor activity on 180-TG Crocker cells in the concentration range 300–37.5 μg/mL (Grzybek *et al.,* 1994). In general, β-glucans are natural polysaccharides present in plants, fungi, yeast, bacteria and algae. Several studies indicate that β-glucans could activate cells of the immune system against pathogens or against cancer cells, as well as exert direct cytostatic, antibacterial and antiviral activities and regenerative effects (Browder *et al.,* 1990; Markova *et al.,* 2003; Baldwin *et al.,* 2015).

Our results demonstrate that only *Tylopilus* extracts exhibit selective cytotoxicity to cancer cells over healthy non-tumorigenic cells in pharmacological concentrations. Similar results have been seen by Jafaar *et al.* (2014) with β-glucan, which at higher concentrations acted toxic to breast cancer cells but promoting proliferation to healthy cells. Lentinan, β-glucan isolated form the mushroom *Lentinus edodes*, selectively inhibited proliferation of breast cancer cells and showed good safety profile in normal cells (Xu *et al.,* 2017). This shows the worth of continuing in the following cytotoxicity tests. Precisely because our experiments proved only mild toxicity of extracts to cancer cells, further tests could be done with higher concentratons of extracts, or with extract isolates to achieve higher toxicity to cancer cells while preserving the preferred selectivity.

The extracts from *Suillellus rubrosanguineus* have proven to be significantly more cytotoxic than extracts from *Tylopilus felleus* to both cell lines in the concentration range used. These results could contribute to the knowledge about known poisonousness of the mushroom *S. rubrosanguineus*.The mushroom *Tylopilus felleus* is generally considered to be non-edible due to its bitter taste.

The presence of alkaloids in the fungi could contribute to the whole image of toxicity. Alkaloids are naturally occurring organic nitrogen-containing bases of plants as well as fungi. They have diverse biological effects on humans and animals in very low concentrations and toxic effects with the higher concentrations (Nugroho *et al.,* 2015; Bun *et al.,* 2008).

Probably no data are known about quantitative or qualitative characterization of alkaloids in fungi *Suillellus rubrosanguineus* and *Tylopilus felleus.* Some alkaloids have been identified in some species of the Boletaceae family (Mahmood *et al.,* 2010).

In the present study, we provided some novel additional data concerning the biological profile of less biomedically studied but commonly known two fungi species of the Boletaceae family *Suillellus rubrosanguineus* and *Tylopilus felleus.* The results show beneficial specificity

of cytotoxicity of the four different *Tylopilus* extract preparations. They are cytotoxic for human breast cancer cells MCF-7 and they caused slightly enhanced proliferation/ metabolic activity in mouse fibroblasts NIH- 3T3 in pharmacological concentrations. The *Suillellus* extracts showed greater cytotoxicity than *Tylopilus* extracts, however, with a comparable extent in both cell lines. The presence of alkaloids was found in all extracts, which could contribute to their cytotoxicity. The extracts of *Tylopilus felleus* showed biological activities, which could open a perspective for future detailed study focusing, for instance, on their potential use in adjuvant therapy of cancer.

Acknowledgments

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