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ORIGINAL ARTICLE

The anticancer activity of laccase from white rot fungus *Cerrena unicolor* on the example of its action on Caov-3 and NIH:OVCAR-3 ovarian cancer cells

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Abstract

Introduction and Objective. According to epidemiological data, ovarian cancer is diagnosed in 240,000 women per year, which is why it is the seventh most common cancer among women worldwide. In addition, among gynaecological cancers it is characterized by the highest mortality. However, there is still no effective treatment, which is why maintenance therapies have a decisive impact on the quality of life of patients. *Cerrena unicolor* is an example of a medical fungus that produces a laccase enzyme (LAC), as well as synthesizing low-molecular secondary metabolites with a wide spectrum of biological activity. LAC, as demonstrated previously, shows cytotoxic and anti-side activity against cancer cells. The aim of the study was to assess the anti-cancer properties of the extract from the mushroom *Cerrena unicolor* against ovarian cancer cells.

Materials and Method. The study was carried out on two cellular lines of ovarian cancer: NIH:OVCAR-3 and Caov-3, differing in sensitivity to platinum compounds. In the first stage of the study, the values of the concentration of *Cerrena unicolor* extract were determined, at which point growth and proliferation were inhibited without a cytotoxic effect in the tested cell lines. For this purpose, classic MTT and BrdU tests were used. The second stage of the study consisted in examining the impact of the preparation on the proliferative and migration potential of cancer cells, using the ECIS system.

Results. The study showed that the examined laccase showed cytotoxic and antiproliferative effect on the cells of the tested cancer lines.

Conclusions. Following previous and current studies on *Cerrena unicolor*, extracts obtained from this fungus can be safely used in anticancer therapy or chemoprevention with no significant harmful effects on normal cells.

Key words

ovarian cancer, ECIS, anticancer properties, *Cerrena unicolor*

INTRODUCTION

Epidemiological data indicate that ovarian cancer is diagnosed in 240,000 women each year, making it the seventh most common cancer in the female population. Furthermore, it ranks as the eighth leading cause of death among this group, with a five-year survival rate of less than 45% [1, 2]. Additionally, as the third most prevalent gynaecological cancer, it has the highest mortality rate worldwide [3, 4]. Projections from the GLOBOCAN study anticipate a 55% increase in incidence and a 67% rise, respectively, in mortality due to ovarian cancer by 2035 [5]. Standard treatment of ovarian cancer consists of surgery, chemotherapy (CTH) combined with platinum or taxane, and/or radiotherapy (RT). First-line treatment is associated with 80–90% responses to chemotherapy; however, most patients develop resistance to treatment, which translates into disease recurrence and a decrease in the five-year survival rate below 35% [4]. The current chemotherapy regimen involving cisplatin (DDP) has shown favourable response rates and patient survival in the initial stages of therapy. For some patients, an alternative targeted therapy involves the use of anti-VEGF antibodies and PARP inhibitors. However, over half of the patients experience a relapse within two years, which significantly worsens survival rates [6, 7].

An increasing number of natural compounds are being investigated for their anticancer properties. Consequently, there has been a growing interest in exploring the health-

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promoting benefits of mushrooms, which are considered functional foods [8, 9]. According to the latest research, there are 650 known species of mushrooms with documented medicinal properties, and approximately 20 of them are currently used in clinical settings [9]. Bioactive compounds found in mushrooms exhibit significant potential for utilization in medicine. They are categorized into two main groups of compounds: high molecular weight compounds including polysaccharides and proteins, and low molecular weight compounds, such as indoles, terpenoids and phenols, which can penetrate the cell membrane and act as effectors in specific signal transduction pathways [10]. Among the foremost properties are anticancer activity, antibacterial, antiviral, immunomodulatory, antioxidative, anti-inflammatory, antidiabetic, hepatoprotective, antiangiogenic, hypocholesterolemic, homeostasis-modulating, and wound-healing effects [9–11]. Furthermore, research has demonstrated that extracts from medicinal mushrooms possess the capability to impede mitosis and angiogenesis, trigger apoptosis, and hinder the proliferation of cancer cells [9]. The anticancer properties of secondary metabolites of fungi are inhibitors of topoisomerase or kinases responsible for mitosis. Other compounds are able to induce apoptosis and, through their immunomodulatory effects, can stimulate the human immune system to fight and destroy cancer cells [12, 13].

Certain species of medicinal forest mushrooms, belonging to the division *Basidiomycota* and the family *Polyporaceae*, are of particular significance. One such representative is *Cerrena unicolor* [9, 10], a fungus widespread in the environment. It occurs in deciduous and mixed forests, mainly in Europe and North America, but also in other regions of the world such as Georgia, Japan, the Philippines and Hawaii.This fungus is a source of extracellular laccase (LAC) and low-molecular-weight secondary metabolites with a broad spectrum of biological activity (ex-LMS) [11]. Laccase is an enzyme produced by fungi that has been known for many years and many biological properties of laccase have been described. Among other things, it is an effective substance that decomposes lignin [14]. From a medical point of view, an interesting property of laccase is, for example, inhibition of cell proliferation of some cancers [15].

LAC, primarily produced by white rot fungi, is an enzyme known for its copper ion reduction capabilities. Additionally, the ex-LMS from *C. unicolor* exhibit strong reducing activity and a high capacity for scavenging reactive oxygen species (ROS), making them suitable for use as antioxidants under controlled laboratory conditions [16]. The latest research results have shown that LAC and total ex-LMS produced by *C. unicolor* have cytotoxic and antiproliferative activity against chronic myeloid leukemia cells, acute promyelocytic leukemia (HL-60), human T-cell leukemia (Jurkat), and cervical cancer cells. (SiHa and CaSki), as well as against HT-29 colon cancer cells [9–11]. Cultures of *C. unicolor* represent a highly promising source of other bioactive substances. Therefore, the anticancer activity of this fungus should be explored in the context of other types of cancer [11, 16]. Drug discovery and screening of bioactive compounds are often performed in cell-based test systems to reduce costs and save time. Traditionally microscopy, spectrophotometry, colorimetry, ELISA and flow cytometry techniques are used to examine cell cultures. The above-mentioned methods are considered standard in studies conducted on cell cultures. While these methods may provide insight into the physiological function of each single cell or into pathological changes that could have occurred, they usually require fluorescence, chemiluminescence or radioactive ways of marking, which lead to cell destruction. The marking process causes the loss of important biological information about living cells. The dead cells are easily detached from the electrode surface. The changes in cell adherence to the electrode surface result in the changes in ECIS measurement data; therefore, the cell adherence can be reflected by ECIS data [17]. When performing impedance measurements on intact cells, due to the characteristics of their membrane, the cells act as a parallel connection between resistor and capacitor. Here, resistance represents the opposition to current flow, whereas capacitance (C) describes the separation of electric carriers at the insulating bi-layer of the cell membrane causing polarization of the cell. Capacitance provides an overall measure of electrode coverage. Therefore, the different behaviour of the cells after the seeding, adherence, proliferation and their reaction to substances added to the substrate, as a result produce change in impedance.

In the current study to demonstrate the anticancer potential of a laccase from *Cerrena unicolor*, traditional methods of cytotoxicity and proliferation testing were used, such as MTT and BrdU, as well as the Electric Cell-Substrate Impedance Sensing (ECIS) method. This is an innovative method used for monitoring of live cells is real time analysis of selected electrical parameters (i.e. cell membrane capacity, resistance or impedance). By culturing cells on the electrode surface, the method of monitoring the above-mentioned parameters may directly provide detailed information about the cellular activity by means of measuring the parameters and eliminating the methods of multiple marking, thus facilitating non-invasive examination of cellular properties in real time. Additionally, ECIS measurements provide information about temporal changes in cell-to-cell contacts not available in single cell observations. Depending on the experimental set-up, ECIS measurements show an excellent time resolution ranging from seconds (for example: micromotion) to minutes.

MATERIALS AND METHOD

Microorganism and culture conditions. *C. unicolor* (Bull. ex Fr. Murr.) was accessed from the culture collection of Regensburg University in Bavaria, Germany, and deposited in the fungal collection of the Department of Biochemistry at the Maria Curie-Sklodowska University in Lublin, Poland, under Assigned Strain No. 139 (internal transcribed spacer gene Accession No. DQ056858) [18]. Submerged fungal mycelium fermentation was conducted in an 8-L air-lift bioreactor containing 6 L of a sterilized Lindenberg and Holm medium optimized at 28 °C as in [14]. The aeration of the culture stood at the level of 15 l/min. Ten-day-old homogenized fungal mycelium (10% of total volume) was used as an inoculant for the fermentor. The incubation lasted until the beginning of the idiophase. Determination of the idiophase was performed in accordance with the recommendation of Jennings and Lysek [19].

Preparation of laccase. The fungal cultures of *C. unicolor* were grown for 10 days under idiophasic condition. After this

period, they were filtered through Miracloth (Calbiochem) and washed with MQ water. The culture liquid remaining after fungal separation was centrifuged at 10,000 × g for 15 minutes. Subsequently, the residual culture solution was split into two fractions with an Ultracel mini-cartridge (10 kD cut-off) using the ultrafiltration system (Pellicon 2 Mini holder, Millipore, Bedford (MA), USA). The fraction containing substances with a mass above 10 kDa served as a source of laccase. Concentrated protein fractions underwent anion exchange chromatography on a DEAE Sepharose column (2.5 × 15 cm) using an FPLC system (Bio-Rad, Richmond (VA), USA). The column was pre-equilibrated with a 20 mM Tris–HCl buffer (pH 6.5). The desired protein pool was eluted using a linear NaCl gradient (0.1–0.5 M) at a flow rate of 1 ml/min for 360 minutes and controlled at 280 nm. Fractions showing LAC activity were collected and desalted on a Sephadex G-50 column (5.0 × 20 cm), maintaining the temperature at 4 °C. Lyophilization of the obtained LAC isoforms was performed using the Freezone 12 Freeze Dry System (Labconco, Kansas City (KS), USA). LAC activity was established by measuring the oxidation of 0.025 mM syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) in 50 mM buffer at pH 5.3. In the experiments, 1 mg of lyophilized LAC isoform mixture dissolved in 1 ml of MQ water was used, with an activity of 1.150mkat/L (specific activity of 3.495.4 μkat/mg protein) [20].

Cell lines and culture conditions. In the initial phase, two cultures of ovarian cancer Caov-3 (ATCC® HTB-161™) and NIH:OVCAR-3 (ATCC® HTB-75™) were derived from ATCC and cultured according to the manual. The examined cell lines Caov-3 and NIH:OVCAR-3 were cultured in DMEM medium (Pan-Biotech; Cat. No. P04–03596) and RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. R0883), supplemented with 10% and 20% FBS (Heat-Inactivated Foetal Bovine Serum; Pan-Biotech; Cat. No.: P30–19375), respectively, with the addition of a mixture of antibiotics (100 IU/mL penicillin, 10 mg/mL streptomycin, and 25 g/mL amphotericin B; Pan-Biotech, Aidenbach, Germany; Cat. No. P06–07300). NIH:OVCAR cells were cultured with addition bovine pancreas insulin – 0.01mg/mL (I6634 Sigma-Aldrich). The control group were cells of mouse fibroblast cell line—NCTC clone 929 (L cell, L929, derivative of Strain L) (ATCC® CCL-1™), which was cultured in complete MEM Eagle (Pan-Biotech, Cat. No. P04–08500) supplemented with 10% Heat-Inactivated FBS (Pan-Biotech, Cat. No. P30–19375), in addition with a mixture of antibiotics mentioned earlier. Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in a Galaxy 170R incubator.

The cell viability assay – MTT Assay. Cell viability was assessed by employing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche, Basel, Switzerland). The MTT assay is a colorimetric method for assessing cell metabolic activity, in which the yellow tetrazolium salt is metabolized by succinate dehydrogenase of viable cells to purple, insoluble formazan crystals. Caov-3, NIH:OVCAR-3 and L929 cells were seeded on 96-well microplates (Nunc) at the density of 1×10^5 cells/well and left for 24 h. The following day, the culture medium was removed, and the cells subjected to serial concentrations (0.025, 0.25, 2.5, 10, 25, 50, 100, 150, 200, 250 μg/mL) of LAC, compared to concentrations of cisplatin (1.5, 3, 6, 15, 30 μg/mL), (Calbiochem, Cat. No. 232120) made in a medium enriched with 2% FBS for 24, 48 and 72h. Subsequently, the incubation of cells was performed for 4 h with 20 μL of MTT solution (5 mg/mL). The formazan grains formed by viable cells were solubilized with 200 μL of DMSO, and the colour intensity was measured at the 570 nm wavelength. The experiment was performed in three independent repetitions. The results were calculated using GraphPad Prism.

The cell proliferation assay- BrdU. Cell proliferation was analyzed by bromodeoxyuridine (BrdU) assay (Merck, KGaD, Darmstadt, Germany) according to the manufacturer's instructions. BrdU involves detecting 5-bromo-2-deoxyuridine (a thymidine analogue) using monoclonal antibody (ELISA test). After dsDNA denaturation, anti-BrdU labelled with, e.g. horseradish peroxidase, is used, which eliminates the substrate in the coloured product. Caov-3, NIH:OVCAR-3 and L929 cells were implanted on 96-well microplates (Nunc) at the density of 2×10^5 cells/mL in 100μL/well and left for 24 h. The following day, the culture medium was removed, and the cells were subjected to serial dilutions (0.025, 0.25, 2.5, 10, 25, 50, 100, 150, 200, 250 μg/mL) of LAC, compared to concentrations of cisplatin (1.5, 3, 6, 15, 30 μg/mL), made in a medium enriched with 2% FBS for 24, 48 and 72 h. The plates were read at 450 nm wavelength. The experiment was performed in three independent repetitions. The results were calculated using GraphPad Prism.

ECIS. To measure impedance, the ECIS system's Ztheta instrument (Applied Biophysics Ltd., Troy (NJ), USA) was used. The appliance contained two separate units: outside the incubator, the station controller Zθ was located; while in the incubator space there was a docking station containing two 8-well plates. The standard 8-well ECIS disposable arrays consist of gold film electrodes delineated with an insulating film and mounted on a 20 mil optically clear Lexan® polycarbonate substrate. Polystyrene is used to make the top assembly with eight wells. A typical inverted tissue culture microscope is used to view the cells because of the thin gold layer (about 50 nm). Each well has a surface area for cell attachment and growth of ~0.8 cm^2 and holds a maximum volume of about 600 μL. Gold pads at the edge of the array connect electrodes to the ECIS electronics, via contact with spring-loaded pins within the electrode array station. The electrodes used were 8W10E (Applied Biophysics Ltd., Troy (NJ), USA) and includes eight wells with 10 active electrodes in each well. ECIS electrodes were placed in a holder plate in a humid incubator at 37 °C and 5% CO_2. Proceeding inoculation, the arrays were incubated for 24 h with an appropriate cultural medium in the incubator. After stabilization, the array was removed from the array station and inoculated with cells (Caov-3, NIH:OVCAR-3 and L929) in standard conditions at 37 °C and 5% CO_2 and culture media (paragraph 3). Inoculation of arrays was carried out by 600 μL/well of cell suspension ~1.2×10^5 cells/mL. The LAC was added to inoculated wells after 24 h in concentrations 10, 100 and 250 μg/mL, whereas cisplatin was added in concentrations 1.5, 6 and 30 μg/mL which were selected based on the MTT and BrdU experiments' results.

Following cell manipulation and insertion of the matrix holder in the incubator, real-time measurements were started. In the presented study, default optimal frequencies were used: resistance (R) 4000 Hz, impedance (Z) 32,000 Hz

and capacitance (C) 64,000 Hz. Impedance signals, which represent the alterations in cellular activity in response to the compound, were detected and analyzed using ECIS software (Applied Biophysics Ltd., Troy (NJ), USA). The ECIS system measured the impedance values, which revealed the morphological changes that occurred after the tested substance stimulated the cells.

ECIS wound-healing assay. The preparation was conducted in a manner analogous to the above-mentioned procedure (ECIS), employing a single active electrode (8W1E). Confluent cells (Caov-3, NIH:OVCAR-3 and L929) in standard conditions at 37 °C and 5% CO_2 and culture media (paragraph 3) were wounded by applying an electrical current (2,000 μA, 60 kHz) for 20 s for ovarian cancer cells and (1600μA, 40-kHz,, 30 s) for fibroblasts to induce a disruption in the cell monolayer, followed by a single wash with phosphate-buffered saline to remove cell debris. Subsequently, the fluctuation in impedance as cells migrated back onto the electrode, was observed and analyzed.

Statistical analysis. All analyses were replicated at least three times, and the data analyzed with GraphPad Prism version 7.01 (GraphPad Software, San Diego (CA), USA). Results were shown as mean ± SD. Statistical significance was determined using the one-way ANOVA test and *post-hoc* Dunnett's test of $p < 0.05$.

RESULTS AND DISCUSSION

The increasing number of cancer diseases, as well as the high level of resistance to cancer therapies, forces us to look for new therapeutic approaches [21]. Resistance poses a challenge in about 20% of high-grade serous ovarian carcinoma cases. This arises from multiple mechanisms, such as the modulation of cisplatin uptake and efflux, DNA repair processes for treatment-induced damage, and DNA methylation [22]. Such an opportunity is sought among mushrooms, because of their multiple effects on cellular processes. Examples of mushrooms with significant medical properties include: *Agaricus blazei, Agaricus cylvaticus, Ganoderma lucidum, Lentinula edodes, Grofola, frondosa, Herlicumerinaceus* and *Pleurous ostreatus.* All of the above affect IL-6, IFN- γ TNF-α and apoptosis of cancerous cells [23].

One of the most frequently studied biological properties of fungal extracts is their anti-tumour activity, which depends on their immuno-modulatory activities affected by many physical and chemical properties. As can be concluded from the reports of various groups of researchers, medical mushrooms are an interesting source of substances, mixtures of compounds and enzymes potentially effective in anticancer activity, for example, on cervical, ovarian, prostate and leukemia cancer cells [21, 24]. They may possess anticancer properties through the induction of apoptosis, inhibition of proliferation or angiogenesis, epigenetic regulation, or blocking the cell cycle [21, 25].

Herlicum erinaceus, studied by Gravina et al., demonstrated having an anticancer effect. Erinacin A and erinacin S isolated from this mushroom stimulated ROS-dependent apoptosis in gastric cancer cell lines. Pro-apoptotic effects and inhibition of tumour growth by erinacine S have also been shown in colorectal cancer, with the observed changes visible in both *in vivo* and i*n vitro* [26]. Moreover, in the study by Gariboldi et al., agglutinins isolated from *H. erinaceus* indicated antiproliferative effects on breast cancer cells [21].

Another example of the anticancer effect of mushrooms is shown in an ovarian cancer study performed by Neergheen et al. Researchers noted that *Anthrodia camphorata* promotes reduction in the amount of HER-2 oncogene by inhibiting the phosphorylation of PI-3 kinase and AKT kinase. The over-expression of HER-2 in ovarian cancer was observed; moreover, this oncogene contributes to treatment resistance in ovarian cancer. Similar changes have also been detected in cervical squamous cell carcinoma cells [27].

In another study, it was observed that gryfolin isolated from the fungus *Albatrellus confluence* stimulates autophagy in the ovarian cancer cell line (SKOV-3) by blocking the activity of the Akt/mTOR/S6K pathway, which determines tumour growth, survival of cancer cells and cells proliferation [28].

The research hypothesis of the presented study is based on literature reports indicating possible anti-proliferative and apoptotic activity of fungal preparations, and the probability of showing the character and dynamics of these changes by using classical chemical methods as MTT and BrdU for determining cytotoxicity and antiproliferative effects of selected substances, as well as selected electrical parameters, i.e. impedance, resistance and cell membrane capacity by the use of ECIS method. In the first stage of research, an MTT experiment to determine the potential cytotoxic effect of laccase from *Cerrena unicolor* on Caov-3, NIH:OVCAR-3 and a healthy L929 was carried out. As it can be seen in Figures 1–3 B, the largest decrease in vitality, compared to normal cells, was achieved after the LAC administered to the tested cancer cells after 48 h of incubation. However, after 72 h of incubation (Figs. 1–3C), viability also decreased slightly. It should be emphasized that the commercial preparation of cisplatin also showed a similar effect in both types of cancer cells, which should be interpreted as a positive effect of the studied natural preparation. It is of particular interest that the NIH:OVCAR-3 line also showed a decrease in viability after the use of the tested LAC preparation of almost the same order as in the case of the Caov-3 line (Figs 1–2B, 1–2C).

The action of both LAC and cisplatin (CIS) for normal cells L929 is also worth noting. In this case, it can be concluded that the tested preparation from *Cerrena unicolor*, although it caused a decrease in the life of cells L929 after 48 (Fig. 3B) and 72 h (Fig. 3C), compared to cisplatin, only about 40% caused a decrease in normal cell L929 life by up to about 75% (Fig. 3B). After 72 hours, the highest concentrations of LAC caused the strongest cytotoxic effect on Caov-3 cancer cells (Fig. 1C) and NIH:OVCAR-3 (Fig. 2C), but still had the weakest effect on normal cells of the L929 line (Fig. 3C).

A dose dependent effect was also demonstrated. The most visible effect can be noticed in the case of both types of substances (LAC and cisplatin) after 72 hours (Figs. 1–3C). The most severe cytotoxic effect on Caov-3 cells was found in laccase in concentrations 100, 150, 200 and 250 μg/mL, after 72 h of incubation (Fig. 1C). Similarly, after the same time, the above LAC concentrations exerted the strongest cytotoxic effect on NIH:OVCAR-3 cells (Fig. 2C), while the effect of LAC on L929 cells remained at a relatively equal level, reducing viability by up to 60% even at a concentration of 250 μg/mL (Fig. 3C) Matuszewska et al., in a study involving *Cerrena unicolor* and human colorectal cancer cells (HT-29), have shown that a low-molecular fraction

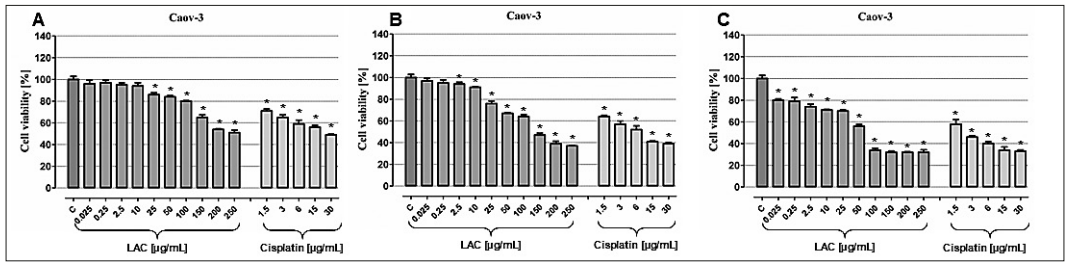

Figure 1. Influence of laccase (LAC) and cisplatin (CIS) after 24 h (A), 48 h (B) 72h (C) on Caov-3 cell line. Cell viability measured by MTT assay

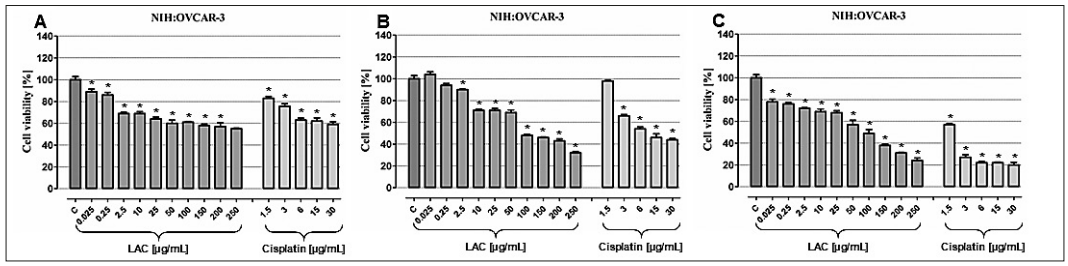

Figure 2. Influence of laccase (LAC) and cisplatin (CIS) after 24 h (A), 48 h (B) 72h (C) on NIH:OVCAR-3 cell line. Cell viability measured by MTT assay

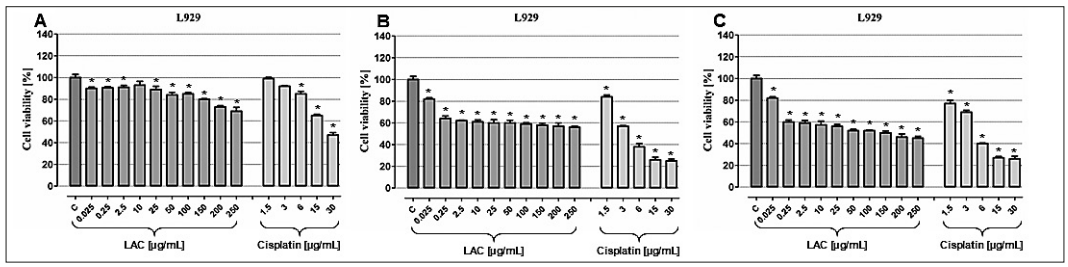

Figure 3. Influence of laccase (LAC) and cisplatin (CIS) after 24 h (A), 48 h (B) 72h (C) on L929 cell line. Cell viability measured by MTT assay

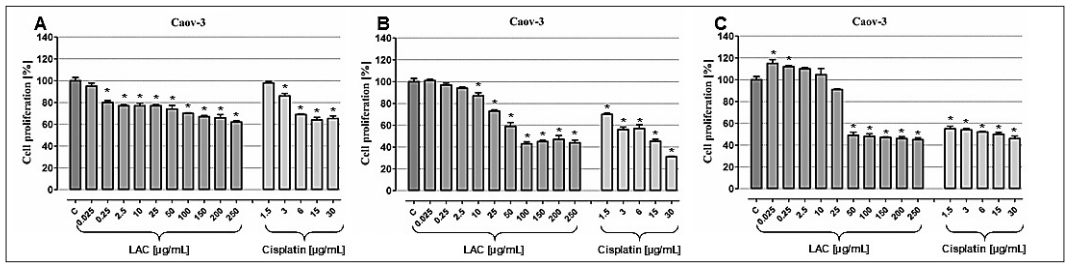

Figure 4. BrdU assay on the influence of laccase (LAC) and cisplatin (CIS) after 24 h (A), 48 h (B) 72h (C) on Caov-3 cell line

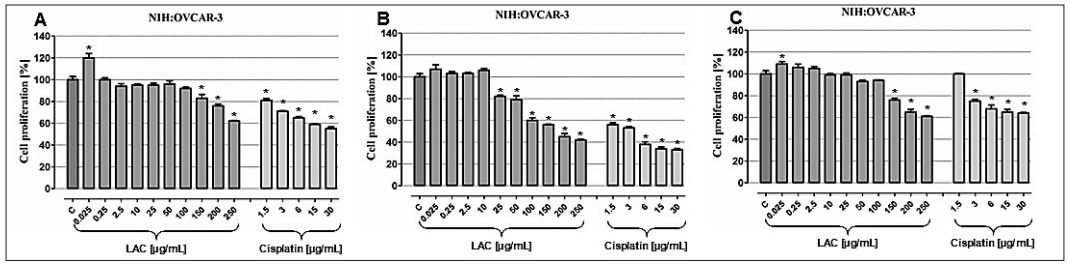

Figure 5. BrdU assay on the influence of laccase (LAC) and cisplatin (CIS) after 24 h (A), 48 h (B) 72h (C) on NIH:OVCAR-3 cell line

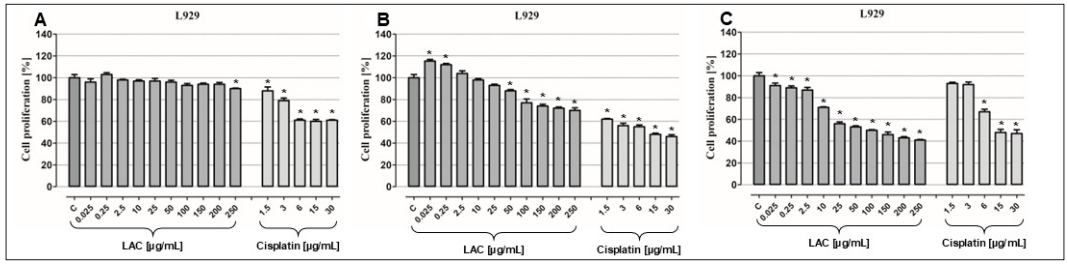

Figure 6. BrdU assay on the influence of laccase (LAC) and cisplatin (CIS) after after 24 h (A), 48 h (B) 72h (C) on L929 cell line

All data presented as mean value ± SD p: * P ≤ 0.05 one-way ANOVA, Dunnett's test

isolated from the fungus induced apoptosis in cancer cells [29]. Previous studies by this centre indicated the antioxidant and anticancer properties of a low molecular weight isolate of the fungus *C. unicolor* [10]. In the cited study, it was noted that subfractions obtained from a low-molecular weight isolate inhibited the migration of HT-29 cancer cells, while healthy colon cells responded by stimulating cell migration, preventing metastases [29]. Pięt et al. conducted a study on the supplementation of *C. unicolor* and *Pycnoporus sanguineus* in colorectal cancer cells (HT-29). It was observed that supplementation of extracts from both mushrooms shows an increase in the cytotoxic effect, an elevated apoptosis rate, as well as physiological and morphological changes in cancer cells [8]. The properties of *C. unicolor* were also analyzed in a study on chronic lymphocytic leukemia (CLL) conducted by Matuszewska et al., in which *C. unicolor* produced a highly active extracellular Laccase (ex-LAC) [9, 30]. The study used cells from the following lines: HL-60, Jurkat, RPMI 8226, K562 and primary CLL cells isolated from the blood of nine patients. Cytotoxic effects were observed on the following cell lines: HL-60, Jurkat, RPMI 8226 and K562, and on primary leukemic cells. In the case of Jurkat and RPMI 8226 cells, induction of apoptosis was additionally demonstrated even at low concentrations of ex-LAC [9].

As can be seen, there are many literature reports on the impact of various ingredients obtained from mushrooms, including even the enzyme laccase, which affect the proliferation of cancer cells. It should be noted, however, that in the case of the fungus *Cerrena unicolor*, its potential remains to be tested. In the current research in the BrdU assay, the LAC antiproliferative potential was tested to compare it with the commonly used cisplatin for cells of both types of ovarian cancer and normal fibroblasts (Figs. 4–6). In the case of LAC treatment for 24 hours (Figs. 4–6 A), it should be noted that in the case of both Caov-3 and NIH:OVCAR-3 cells, the antiproliferative effect was present, although it was insignificant. Only the highest concentrations of LAC, especially in the case of NIH:OVCAR-3 cells, only the concentration of 250 μg/mL equalled the effect of cisplatin (Fig. 5). After 24 hours of incubation, virtually no antiproliferative effect of LAC was observed on normal L929 cells, and a decrease of up to 60% under the influence of cisplatin (Fig. 6A). A particularly clearly visible antiproliferative effect of both types of substance: natural product- LAC and chemical substance: cisplatin, can be seen after 48 hours (Figs. 4–6B), with the point that Caov-3 cells proved to be much more sensitive for both preparations. Similar to the MTT study, the most effective doses of the natural preparation were from 50–250 μg/mL, the higher the concentration, the stronger the antiproliferative effect. After 72 h of incubation, interesting results were obtained indicating that the LAC preparation at concentrations of 50–250 μg/mL had a comparable effect on Caov-3 and L929 cells, causing a decrease in the proliferation of both mentioned cell types to approximately 40%, compared to the control (Figs. 4,6C). Moreover, a very interesting pro-proliferative effect of laccase was observed at lower concentrations (0.025–2.5 μg/mL) on cells of both types of ovarian cancer (Figs. 4–5C).

What seems important is that cisplatin did not have this effect, nor did the LAC preparation have this effect on normal L929 cells (Fig. 6C).

Mizerska-Dudka et al. [18] found that laccase dose-dependently inhibited the proliferation of SiHa and CaSki cancer cells and normal fibroblasts (HSF), which corresponds with the results of the current study. In the latter study, very interesting results were obtained on the effect of laccase from *C. unicolor* on cells of two types of ovarian cancer, compared to the effect of cisplatin on these cells. As established earlier [31], a cell line NIH:OVCAR-3 from malignant as cites of a patient with progressive adenocarcinoma of the ovary, is resistant to cisplatin (5 x 10~7 M), with a survival rate of 77%. The authors of the current study also noted a lower response to cisplatin than in Caov-3 ovarian cancer cells, with a strong cytotoxic effect on normal fibroblast cells. Under the same conditions, the tested medicinal mushroom laccase preparation caused comparable cytotoxic and antiproliferation effects on cells of both types of ovarian cancer, with a much weaker effect on healthy cells. Every cell type has its characteristic adhesion and growth curve that can be manipulated by, e.g. varying seeding density or other stimuli.

One of the major difficulties in studying these processes is differentiation between adhesion, spreading and proliferation, and describing in detail the use of a combination of resistance and capacitance in order to distinguish between those parameters [32]. The results obtained by the authors coincide with observations by Wegener on resistance and capacitance, and complement each other. Variation in cell behaviour after cell attachment, spreading, proliferation, micromotion, demise, and/or responses to external substances, result in changes in impedance [33]. Electrical impedance is defined as the opposition to an electrical current within a circuit. In systems utilizing direct current, the impedance is simply the resistance, but in systems utilizing alternating currents, the changing electric and magnetic fields create additional and varying opposition to the applied current [34]. ECIS is a system using alternating current. In the presented study, the effect of the chosen LAC concentrations – 10, 100 and 250 μg/mL and commercial cisplatin – 1.5, 6, 30 μg/mL on NIH:OVCAR-3, was examined for 160 h by the use of ECIS system (Fig. 7). The obtained results clearly show that the tested preparation has significant anticancer potential, which can be concluded from the comparison of changes in electrical parameters in the culture of the tested cells, compared to the use of the traditional chemotherapeutic cisplatin. Using the ECIS device, changes were found in the impedance, resistance and capacitance values, both in the case of LAC preparation and cisplatin. The effect of the biological preparation was much milder compared to cisplatin. Changes in impedance (Fig. 7 A) and resistance (Fig. 7 C) values decreased gently in the case of both concentrations of 10 and 100, while concentration of 250 μg/mL quite significantly reduced the impedance from 1,100 ohms to just over 400 ohms. In this case, the dose effect was clearly visible. At the same time, cisplatin caused a drastic decrease in the mentioned parameters (Fig. 7B, D). The decrease in capacity (Fig. 7E, F) recorded between 40–130 h with increasing impedance and resistance (Fig. 7A-D), should be interpreted as cell proliferation, and in this respect, both values complement each other and should be analyzed in parallel as a standard.

In the same time interval of 0–160 h, as in the case of NIH:OVCAR cells, changes in electrical parameters were monitored in the culture of Caov-3 ovarian cancer cells exposed to LAC at concentrations of 10, 100 and 250 μg/mL, and cisplatin at concentrations of 1.5, 6 and 30 μg/mL. From the obtained values of impedance changes, it can be

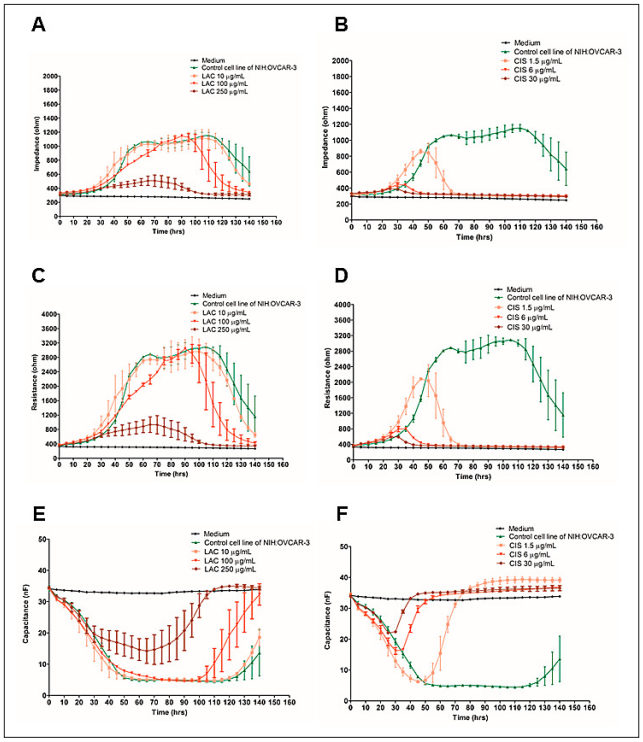

Figure 7. Impedance, resistance, and capacitance monitoring of cell lines NIH:OVCAR-3 during treatment with LAC in concentrations 10, 100, and 250 µg/mL (A,C,E), and cisplatin in concentrations 1.5, 6 and 30 µg/mL (B, D, F).
Data presented as mean value ± SEM

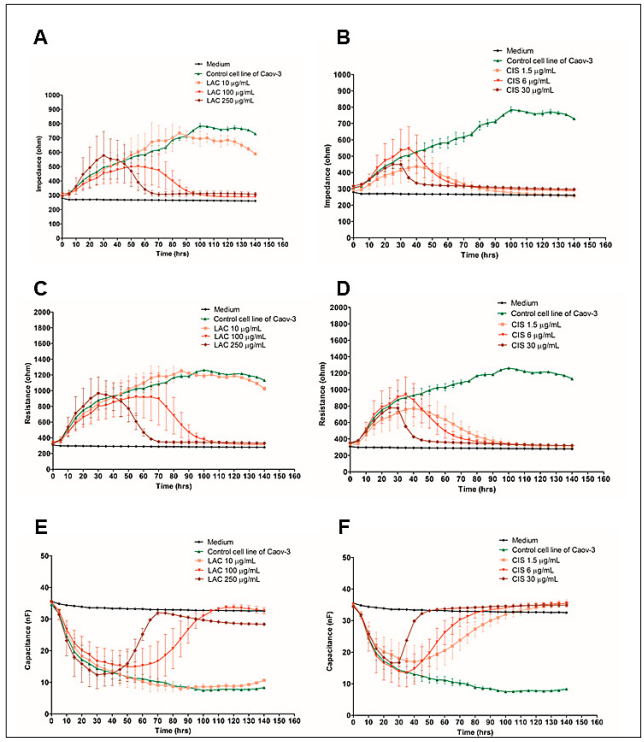

Figure 8. Impedance, resistance, and capacitance monitoring of cell lines Caov-3 during treatment with LAC in concentrations 10, 100, and 250 µg/mL (A,C,E), and cisplatin in concentrations 1.5, 6 and 30 µg/mL (B, D, F).
Data presented as mean value ± SEM

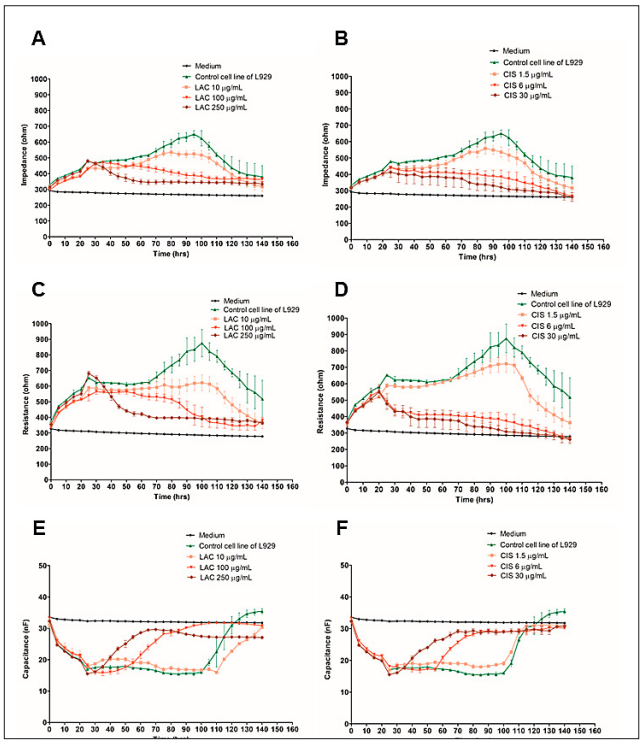

Figure 9. Impedance, resistance, and capacitance monitoring of cell lines L929 during treatment with LAC in concentrations 10, 100, and 250 µg/mL (A,C,E), and cisplatin in concentrations 1.5, 6 and 30 µg/mL (B, D,F).
Data presented as mean value ± SEM

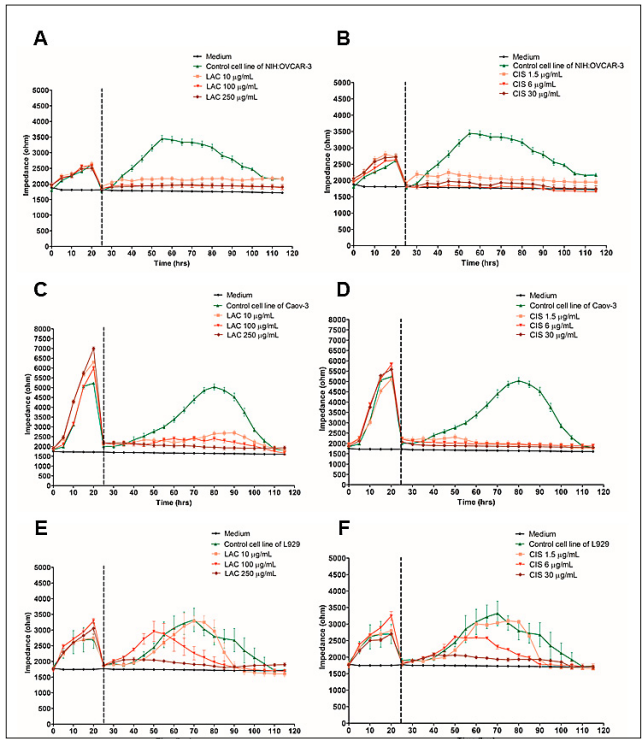

Figure 10. Effect of LAC and cisplatin on NIH:OVCAR (A, B), Caov-3 (C,D) and L929 (E,F) cells line in scratch assay in 3 concentrations of LAC 10, 100, and 250 µg/mL (A,C,E), and cisplatin 1.5, 6 and 30 µg/mL (B, D, F).
Data presented as mean value ± SEM. The dashed line represents the moment the scratch was made

seen that the largest decrease in the parameter compared to control cultures was obtained after the use of LAC at a concentration of 250 µg/mL, and cisplatin at a concentration of 30 µg/mL(Fig. 8A, B). In the case of the LAC preparation at the mentioned concentration, the strongest decrease was observed after 60 h of the experiment, while cisplatin caused the strongest effect after 35 h of the experiment. The same type of changes was recorded in the case of resistance tested in the

mentioned research system (Fig. 8C, D). Characterization of the electrical parameters in the cultures is complemented by increases in capacitance [nF], corresponding with decreases in impedance and resistance (Fig. 8E, F).

The LAC preparation and cisplatin had a comparable effect on normal L929 fibroblast cells, causing the greatest decrease in impedance after 60 h of culture with the LAC preparation, and after 30 h with cisplatin (Fig. 9 A, B). The corresponding changes in resistance were recorded in the same time intervals (Fig. 9C, D).

The wound-healing test showed that normal cells L929, after scratching, regain confluence within 60 h at the lowest concentration of laccase 10 µg/mL. Subsequently, at higher concentration at this time was slightly longer for 100 µg/mL, and in a case of the highest concentration cells, did not regain the confluence (Fig. 10E, F).

In the case of NIH:OVCAR and Caov-3 cancer cells, the graphs are different because from the very beginning the cells show lower viability (Fig. 10A-D), both in the case of the laccase and cisplatin preparations. The measurement of selected electrical parameters, i.e. impedance, resistance, and capacitance of the cell membrane in real time, may be an important complement to results obtained by traditional methods of analyzing cell cultures.

Assuming that electric changes precede biochemical changes, the electric system of monitoring the above-mentioned parameters is a method allowing the analysis of cellular activity during growth in cell culture.

In another study, it was observed that gryfolin isolated from the fungus *Albatrellus confluence* stimulates autophagy in the ovarian cancer cell line (SKOV-3) by blocking the activity of the Akt/mTOR/S6K pathway, which determines tumour growth, survival of cancer cells and cells proliferation. In the current study, very interesting results were obtained on the effect of laccase from *C. unicolor* on cells of two types of ovarian cancer, compared to the effect of cisplatin on these cells, as established earlier.

CONCLUSIONS

Based on the presented results, it can be concluded that the enzyme from *Cerrena unicolor* – laccase is active towards ovarian cancer cells of the Caov-3 and NIH:OVCAR-3 lines, demonstrating cytotoxic and antiproliferative effects on the mentioned cells. Moreover, it was found that Caov-3 line cells showed greater sensitivity to the tested laccase preparation than NIH:OVCAR-3 cells. The effect of laccase on normal cells of the L929 line was much weaker than the effect of cisplatin, which can be interpreted as potentially promising in future *in vivo* studies. The results of the conducted research presents the possibility of progression in the treatment of ovarian cancer. The multitasking nature of substances isolated from medical mushrooms, including *Cerrena unicolor*, suggests considering the implementation of mushrooms into cancer therapy as a supportive treatment.

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