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Processed Fruiting Bodies of *Lentinus edodes* as a Source of Biologically Active Polysaccharides

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Abstract: Water soluble polysaccharides (WSP) were isolated from *Lentinus edodes* fruiting bodies. The mushrooms were previously subjected to various processing techniques which included blanching, boiling, and fermenting with lactic acid bacteria. Therefore, the impact of processing on the content and biological activities of WSP was established. Non-processed fruiting bodies contained 10.70 ± 0.09 mg/g fw. Boiling caused ~12% decrease in the amount of WSP, while blanched and fermented mushrooms showed ~6% decline. Fourier transform infrared spectroscopy analysis (FTIR) confirmed the presence of β-glycosidic links, whereas due to size exclusion chromatography 216 kDa and 11 kDa molecules were detected. WSP exhibited antioxidant potential in FRAP (ferric ion reducing antioxidant power) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays. Cytotoxic properties were determined on MCF-7 and T47D human breast cell lines using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test. Both biological activities decreased as the result of boiling and fermenting.

Keywords: polysaccharides; *Lentinus edodes*; antioxidant; cytotoxicity; processing; mushrooms; LAB (lactic acid bacteria); fermenting

1. Introduction

Among thousands of mushrooms only about 20 species are cultivated commercially for culinary purposes. Japanese mushroom *Lentinus edodes*, commonly known as Shiitake, is cultivated for both its culinary and medicinal applications [1]. It has been reported that consumption of edible mushrooms provides a significant health improvement as they are low in calories, sodium, fat and cholesterol, but rich in proteins, carbohydrates, fibre, vitamins and minerals [2–4]. Commonly consumed mushroom species exposed to a source of ultraviolet (UV) radiation can generate nutritionally relevant amounts of vitamin D [5]. Providing considerable amount of iron and well absorbed proteins they are often called "the meat of forest" [2]. Mushrooms are rich in immunomodulating compounds which, unlike traditional chemical drugs, do not cause any harmful effect or allergic reactions and put no additional stress on the body [6,7].

Out of all mushroom-derived substances, polysaccharides are known to have the most potent antitumor, antioxidative and immunomodulating properties [6,8,9] but their biological activities differ

greatly depending on the structural and physical features [10]. For example, both in vitro and in vivo antitumor activities of mushroom extracts arise from the presence of β-glucans, especially containing β-1,3 bounds in the polysaccharide chain [2,11]. Studies confirmed that lentinan, the high molecular weight polysaccharide β-1,3-D-glucan with β-1,6-glucopiranoside branches extracted from *Lentinus edodes* fruiting bodies not only has immunomodulating properties but also can suppress the growth of cancer cells and induce them to apoptosis [10,12,13]. However, the details of molecular mechanisms of these processes remain unclear [14,15]. Chen et al. [16] stated that among eight studied species of medicinal mushrooms, *Lentinus edodes* polysaccharides showed the strongest scavenging activity for hydroxyl radicals and were able to inhibit the proliferation of MCF-7 tumor cells. Antibacterial and antifungal properties of Shiitake extracts have also been reported [4,17].

As edible mushrooms are characterized by a short shelf life, 1–3 days at room temperature, they should be consumed directly after harvesting because their nutritional value is then the best then. Additionally, most of mushroom species need to be processed before the consumption. In the literature there are some information about the influence of processing like cooking, baking, drying or freezing on the contents of health promoting compounds [18,19] but little is known about the effect of these processes on mushroom-derived polysaccharides antioxidant and antiproliferative activities [20].

Contemporary consumers are more aware of what they eat and drink, they more often choose home made products or those without any artificial stabilizers. Noticing this tendency the aim of this article was to verify the impact of some processing methods on the content, chemical composition, antioxidant and antiproliferative activity of water soluble polysaccharides (WSP) obtained from *Lentinus edodes*. The processes that were chosen are easy to conduct and commonly used at home, including boiling, blanching and fermenting with lactic acid bacteria (*Lactobacillus plantarum*).

2. Materials and Methods

2.1. Biological Material

Lentinus edodes fruiting bodies were provided by a private producer (AgRoN, Ząbki, Poland). Harvested mushrooms were from the same crop and were transported in five kg plastic trays. Fresh fruiting bodies were stored at 5 °C up to five hours before the analysis.

The bacterial strain used in the experiment (*Lactobacillus plantarum* IBB76) was obtained from IBB Central Collection of Strains (Warsaw, Poland). This strain was used previously in fermentation of mushrooms [21–23].

2.2. Processing of Mushrooms

Fruiting bodies were divided into four portions. The first one was blanched for five minutes in citric acid solution (0.5% *w*/*v*) at 95 °C. The second portion was boiled in water for fifteen minutes at 100 °C. The third group was blanched as above and fermented with lactic acid bacteria strain, as in the previous study [23]. The last portion (control group) was not processed. All the four portions were lyophilized (Alpha 1–2 LD plus, Christ, Germany) prior the extraction of polysaccharides.

2.3. Extraction of Water Soluble Polysaccharides (WSP)

Lyophilized fruiting bodies were milled into fine powder, extracted with ethanol (80 °C, 1 h) and centrifuged. Insoluble part was rinsed with alcohol and subjected to water extraction (115 °C, 1 h). The obtained water extract was concentred and mixed with 2-propanol to precipitate polysaccharides. Precipitates were then washed three times with alcohol, lyophilized and weighed. The extraction was carried out in triplicate and extraction yields were calculated.

2.4. Chemical Characteristics of Polysaccharides

The extracted WSP were redissolved in water and subjected to three colorimetric assays. Total carbohydrate content was determined with the phenol–sulfuric acid method [24]. Protein

content was determined with Bradford reagent [25] and total phenolics content was quantified with Phenol-Cicalteau reagent [26]. Glucose, bovine albumin and gallic acid (respectively) were used to construct calibration curves.

Fourier transform infrared spectra of the samples were recorded on Nicolet NXR 9650 spectrometer (Thermo, Waltham, MA, USA) equipped with an ATR (attenuated total reflection) module. The samples were scanned within the range of 400–4000 cm^{-1}.

Gel permeation chromatography (GPC) was employed to determine the molecular weight. The analysis was conducted according to the methodology described by Malinowska et al. [27]. Briefly, the samples were dissolved in NaN_3 water solution and applied to the following TSK-GEL columns: G5000PWXL, G3000PWXL andG2500PWXL (Tosoh, Tokyo, Japan). Samples were detected with a Refracto Monitor IV refractive index detector (LDC Analytical, Riviera Beach, FL, USA) and compared with pullulans standards.

2.5. Antioxidant Assays

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity assay was conducted according to Re et al. [28]. The samples (25 µL) were mixed with ABTS + solution (975 µL) and measured at 734 nm after 15 min. Ferric reducing antioxidant power (FRAP) analysis was done according to the methodology of Benzie and Strain [29]. FRAP reagent (1900 µL) was added to the samples (100 µL) and after 90 min of incubation the absorbance was measured at 593 nm. The results of both antioxidant assays were compared with calibration curves made with Trolox and expressed as micromoles of Trolox equivalent (TE) per 1 g of mushroom dry weight.

2.6. Cytotoxic Properties

The MCF-7 adenocarcinoma, cell line was obtained from the American Type Culture Collection (ATCC) cat. no. HTB-22. The T47D ductal, epithelial cancer cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC) no. 85102201. These two human breast cancer cell lines were grown at standard conditions (37 °C, 5% CO_2, 95% humidity) in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 U/mL penicillin (Polfa, Poland) and 100 µg/mL streptomycin (Polfa, Warsaw, Poland). For MCF-7 cells 50 µg/mL of bovine insulin (Sigma) was also added. Cytotoxicity effect of tested WSP was measured with a quantitative colorimetric toxicity MTT assay based on the transformation of yellow, soluble tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to purple-blue insoluble formazan by mitochondrial succinate dehydrogenase of living, metabolically active cells [30,31].

T47D and MCF-7 cells (100 µL/well) were seeded on 96-well plates at concentrations of 3×10^5 cells/mL and 2×10^5 cells/mL, respectively. After 24 h incubation at standard conditions, when cells in each well reached 75–80% confluence, the growth medium was replaced with WSP dilutions in RPMI 1640 with 2% FBS. The concentrations ranged from 25 µg/mL to 250 µg/mL. Cells were incubated with polysaccharides for 24 h, followed by addition of 25 µL of MTT (Sigma) solution (5 mg/mL in PBS) per well. After 3 h incubation at 37 °C formazan crystals were solubilized with 100 µL of lysis buffer (10% SDS in 0.01 M HCl) per well. Plates were incubated overnight at standard conditions. The absorbance was read at 540 nm with a plate reader (680 XR, Bio-Rad), and the mean value for each concentrations was calculated. The percentage of viable cells were calculated from the absorbance.

2.7. Statistical Analysis

The measurements were done in triplicate and results were expressed as the mean ± standard deviation. The collected data were evaluated using analysis of variance ANOVA with a level of significance set at $p < 0.05$. The LSD test was performed to assess statistically different results. Different letters on graphs and tables (a, b, c, etc.) show significant differences among the data according to LSD test ($p < 0.05$).

3. Results and Discussion

3.1. The Content of Water Soluble Polysaccharides

As shown in Table 1, mushrooms belonging to the control group contained 96.9 ± 0.8 mg/g dw of WSP. Blanching did not cause statistically relevant changes, whereas boiling led to the increase in the amount (112.0 ± 2.0 mg/g dw). The observed increase in the case of boiled fruiting bodies is with agreement with the previous study conducted on *Hypsizygus marmoreus* fungi [32] or button mushroom [22]. The reason for this could be the leaking of easy soluble substances into the brine and changing the ratio between high and low weight molecular weight compounds. Blanched and fermented mushrooms contained the lowest quantity of WSP (86.7 ± 3.1 mg/g dw).

Table 1. The content of the isolated water soluble polysaccharides (WSPs).

Treatment	WSP Content in Dried Fruiting Bodies	WSP Content in Fresh Fruiting Bodies
	(mg/g dw)	(mg/g fw)
Control	96.9 ± 0.8 b	10.70 ± 0.09 c
Blanched	95.3 ± 4.1 b	10.75 ± 0.22 c
Boiled	112.0 ± 2.0 c	9.40 ± 0.20 a
Blanched and Fermented	86.7 ± 3.1 a	10.07 ± 0.21 b

The results were also expressed in mg per g of fresh weight in order to consider the loss of the mass. As can be seen, the highest drop (~12%) was observed in the case of boiled fruiting bodies and slight decrease (~6%) in the case of blanched and fermented samples. Similar results were noticed. Boiling of mushrooms may lead to the changes in their mycelial structure and therefore facilitate the extraction of water soluble polysaccharides [32].

3.2. Chemical Characteristics of Water Soluble Polysaccharides

In the isolated WSPs the level of carbohydrate, protein and phenolics were determined (Table 2). Polysaccharides extracted from non-processed mushrooms contained 72.35 ± 3.77% of carbohydrate, 4.90 ± 0.46% of protein and 0.59 ± 0.02% of phenolic compounds. Previous research demonstrated a similar quantity of carbohydrate in *L. edodes* polysaccharides which ranged from 72% [33] to 78% [34]. Other authors who used different extraction method reported higher quantity of carbohydrate (87%), lower amount of protein (~3.5%) and no phenolic compounds [35]. In accordance with the presented results, previous studies have demonstrated similar content of protein but twice higher amount of phenolics [34]. Mushrooms are highly abundant in phenolics and these compounds have tendencies to bind to polysaccharides with hydrogen or even covalent bounds [36,37]. According to research phenolic antioxidants are released from polysaccharidic matrix by probiotic bacteria and then are absorbed into bloodstream [38].

Table 2. Chemical composition of the isolated WSPs.

Treatment	Carbohydrate Content	Protein Content	Total Phenolics Content
	(% dw)	(% dw)	(mg Gallic Acid Equivalent per 100 g)
Control	72.35 ± 3.77 a	4.90 ± 0.46 c	0.59 ± 0.02 d
Blanched	79.67 ± 3.45 b	4.03 ± 0.28 b	0.49 ± 0.04 c
Boiled	71.21 ± 3.29 a	5.12 ± 0.25 c	0.45 ± 0.01 b
Blanched and Fermented	93.00 ± 3.59 c	2.30 ± 0.35 a	0.25 ± 0.02 a

The processing of mushrooms caused various changes in chemical composition. Blanching in citric acid solution resulted in higher carbohydrate content (79.67 ± 3.45%) along with lower protein (4.03 ± 0.28%) and phenolics content (0.49 ± 0.04%). Interestingly, boiling for 15 min affected only (negatively) protein quantity when compared with control samples. The most evident changes were observed in the case of blanching and fermenting, where significant decreases of phenolics and protein (0.25 ± 0.02% and 2.30 ± 0.35%, respectively) were noticed. In contrast, the level of

carbohydrate markedly rose reaching 93.00 ± 3.59%. These data is in accordance with previous experiment conducted on *Agaricus bisporus* [22]. The loss of protein and phenolics during lactic acid fermentation of mushrooms can be attributed to enzymes that are produced by bacteria: proteases degrading protein moieties [39] and esterases which release phenolic compounds [40]. Additionally, aqua-thermal processing may cause solubilization or even degradation of low molecular weight phenolics [41].

Fourier transform infrared spectroscopy enables us to detect functional groups which are present in a sample and is often used in the analysis of plant and fungi based polysaccharides [42–44]. FTIR coupled with a ATR accessory a is rapid and non-destructive tool which does not require sample preparation [45]. FTIR analysis may be useful in determination of polymers purity as well as for the detection of the type of glycosidic bond [46]. FTIR analysis of the isolated WSP is presented in Figure 1. The obtained spectra shows bands which are commonly present in polysaccharides mushroom origin. Broad bands at 3000–3500 cm^{-1} and 2800–3000 cm^{-1} are associated with stretching vibrations of O-H, N-H and C-H, respectively. Two signals at ~1645 cm^{-1} (Amide I) and ~1535 cm^{-1} (Amide II) indicate the presence of protein. They are result of C = O stretching vibrations (Amide I) and bending vibrations of N-H groups (Amide II) and were reported previously in *L. edodes* β-glucans [47]. Interestingly, Amide II band is barely visible in WSP isolated from blanched and fermented mushrooms. This is in accordance with the observed sharp drop in protein content shown due to Bradford assay. The signal at ~1520 cm^{-1} can be attributed to C-C stretching of aromatic ring and suggests the presence of phenolic compounds [48]. This signal is the weakest in the case of WSP obtained from blanched and fermented mushrooms and may confirm the significant loss in phenolics. In the region of 1300–1450 cm^{-1} there are signals which can be assigned to bending vibrations of O-H, C-O-H and CH_2 [49], whereas intense peaks between 950–1190 cm^{-1} are due to stretching vibrations of C-O-C, C-O-H, C-C [50]. FTIR analysis allowed to identify the type of glycosidic bonds dominating in the samples. Spectra showed absorbance at ~890 cm^{-1} which is indicative of β-glucans [51,52].

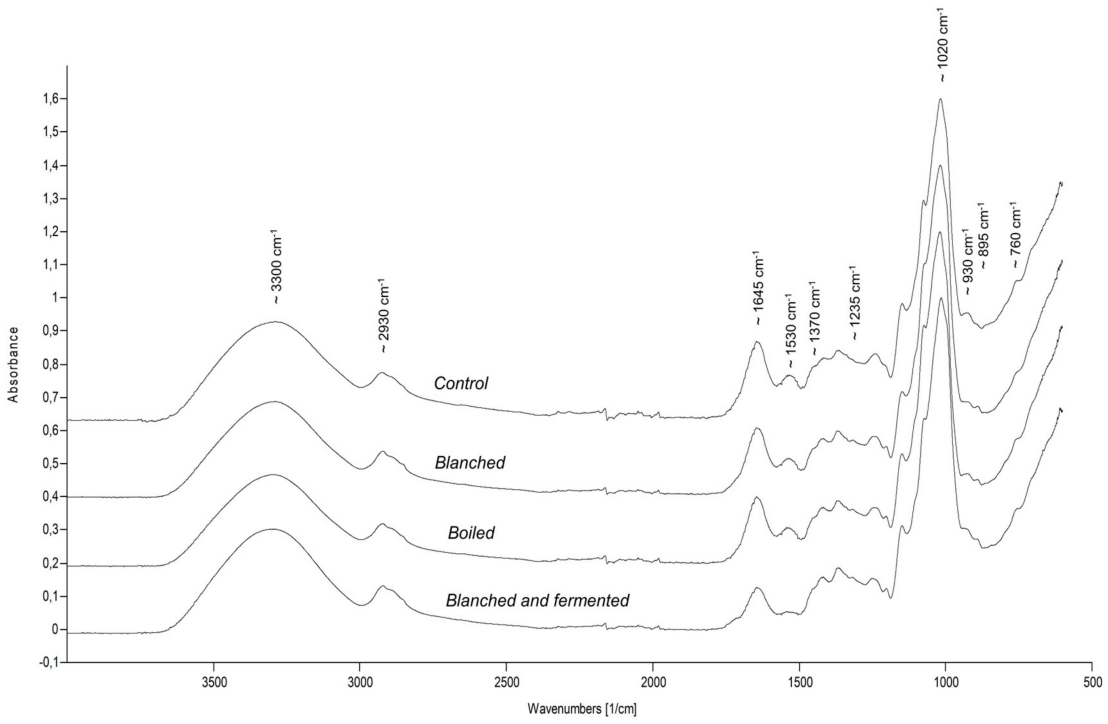

Figure 1. FTIR spectra of WSP.

In order to establish molecular weight of the extracted polysaccharides, size exclusion chromatography was used (Figure 2). All the tested WSP gave intense signals at ~18.8 mL. These sharp

peaks correspond to molecules of 216.3 kDa. This finding was also reported by Chen et al. [53] who isolated proteoglycan from *L. edodes*, having the molecular weight of 220 kDa and capable of scavenging free radicals. It is also consistent with the previous research in which 200 kDa polysaccharide (showing immunomodulating activities) was obtained from *L. edodes* water extract [54]. However, this study was unable to demonstrate the presence of larger molecules exceeding 600 kDa as was earlier reported by other authors [55,56]. Chromatogram of the control sample also contains the peak at 22.6 mL. It can be attributed to the smaller compound having the molecular weight of 11 kDa. This peak is removed due to the processing and a possible explanation for this might be that this compound was solubilized and extracted during blanching or boiling.

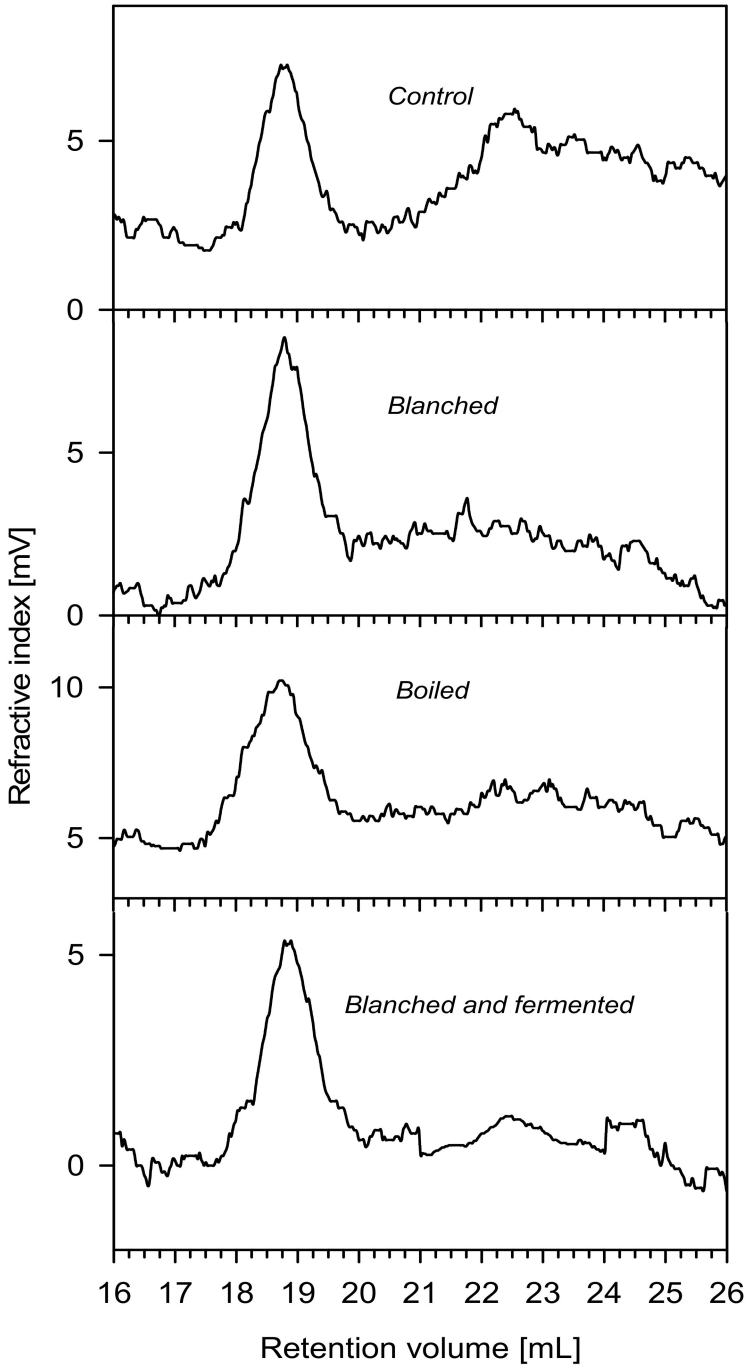

Figure 2. Size exclusion chromatography of WSP.

3.3. Antioxidant Potential

Mushrooms are rich in low molecular weight antioxidants, mainly phenolic acids. Such compounds are mainly present in free form and can be easily extracted with both water and organic solvents. However, some antioxidants bind to larger molecules and become less or non-extractable [57]. These compounds are often bound to mushroom derived polysaccharides and affect their antioxidant capacities. All the isolated WSP exhibited antioxidant activity in both assays (Figure 3). The highest antioxidant parameters were noticed in the case of the control samples (23.53 ± 0.30 µmoles of TE/g dw for ABTS and 12.29 ± 0.87 µmoles of TE/g dw for FRAP). Antioxidant activity of *L. edodes* polysaccharides was reported before and this study confirms earlier findings [16,35,58]. Blanching caused a significant drop in FRAP test (7.59 ± 0.75 µmoles of TE/g dw), while no relevant change in ABTS parameter was noticed. Boiling resulted in the decrease of ABTS activity (20.83 ± 1.24 µmoles of TE/g dw), whereas no further change in the case of FRAP test was observed. The most profound changes caused blanching and fermenting (7.78 ± 1.50 µmoles of TE/g dw for ABTS and 3.56 ± 0.33 µmoles of TE/g dw for FRAP). The results clearly showed that aqua-thermal processing may have negative impact on antioxidant parameters of polysaccharides. Previous findings showed that antioxidant activity of polysaccharides is related to total phenolics and protein content [59–61]. In this study, a high correlation was observed between ABTS values and total phenolics (R = 0.93) as well as protein content (R = 0.85). In the case of the FRAP parameter, the correlation coefficients were similar to ABTS (R = 0.96 and R = 0.82, respectively). Therefore, the reason for the drop in antioxidant capacity is very likely caused by the removal of phenolics and protein. Further studies should be carried out to isolate and identify antioxidant compounds which are bound to polysaccharidic matrix by covalent bonds [57].

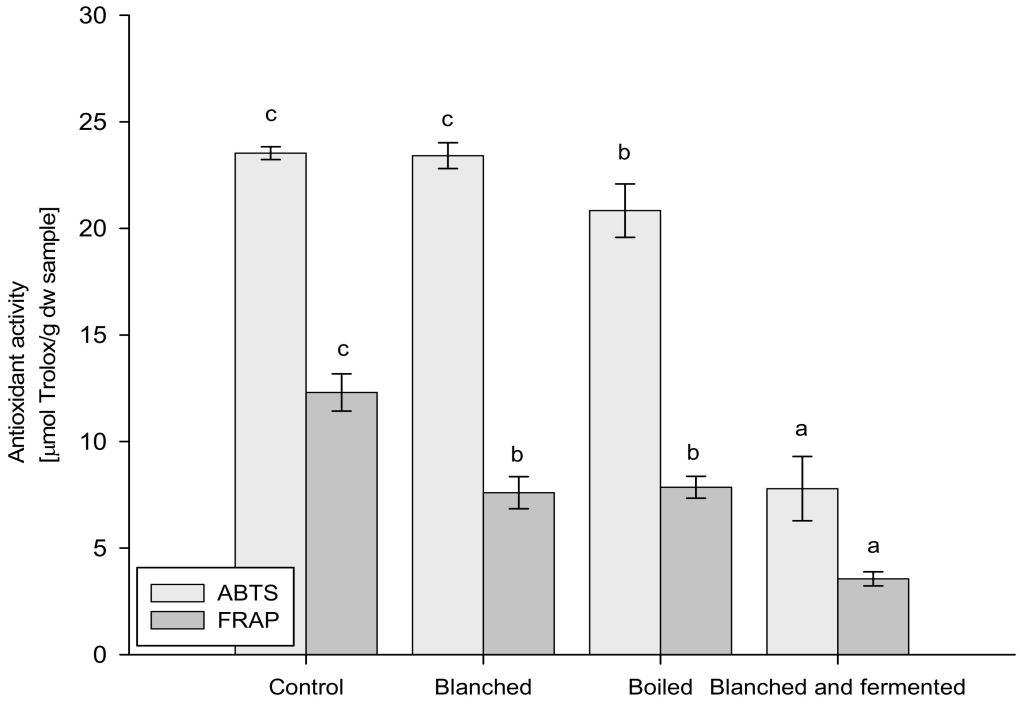

Figure 3. Antioxidant capacity of WSP.

3.4. Cytotoxic Properties of WSP

It has been reported that many mushroom polysaccharides show direct cytotoxicity to cancer cells [62]. To determine the response of cells to water soluble mushroom polysaccharides (WSP) we used MTT assay that measures the reduction in cell viability when exposed to cytotoxic substances. The control T47D and MCF-7 cells viability was assessed as 100%. Our results showed a dose dependent cytotoxic effect of crude polysaccharide extract from *Lentinus edodes* on MCF-7 cells

(Figure 4a). The control, a not processed WSP sample, caused a 73% and 41.7 ± 4.7% decrease of MCF-7 cell viability after treatment with 50 and 250 µg/mL concentration respectively. The processing of *Lentinus edodes* fruiting bodies particularly blanching and blanching with further fermentation had suppressed cytotoxic activity of applied WSP. After incubation with the highest polysaccharides concentration MCF-7 cells viability reached 74.0 ± 5.7% and 82.5 ± 3.5%, respectively. Boiling in water changed cytotoxic properties of polysaccharides in the least degree. MCF-7 cells viability after treating with (250 µg/mL) WSP from boiled mushrooms was 57.9 ± 5.8% [63]. Israilides et.al. achieved a 50% reduction in viability of MCF-7 cells, however the time of incubation with 73 ± 14 µg/mL of water extract from *Lentinus edodes* took 48 h. Moreover, it is known that whole mushroom extracts contain a lot of substances other than polysaccharides with very well documented direct cytotoxic activities achieved 50% reduction in viability of MCF-7 cells, however, the time of incubation with 73 ± 14 µg/mL of water extract from *Lentinus edodes* took 48 h. It is known that whole mushroom extracts contain a lot of substances other than polysaccharides with very well documented direct cytotoxic activities [64].

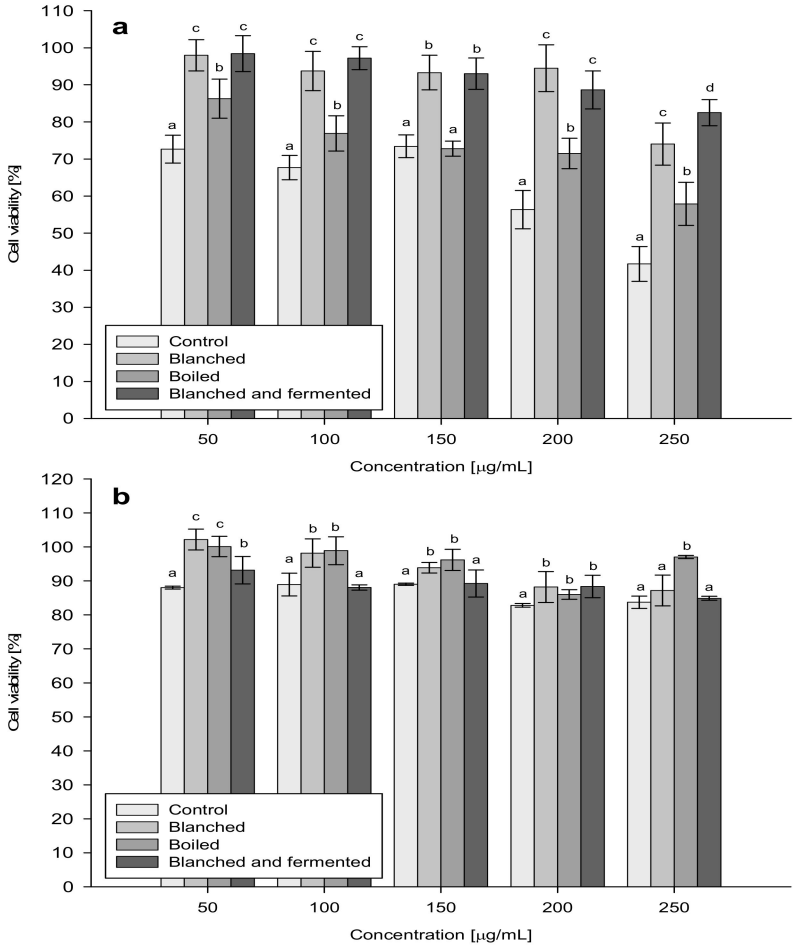

Figure 4. Cytotoxic effect of water soluble polysaccharides (WSP) from blanched, boiled, blanched and fermented *Lentinus edodes* on MCF-7 (**a**) and T47D (**b**) cell lines.

In the cell line T47D (Figure 4b), we have assessed no significant sensitivity to polysaccharides fractions from processed and control (*Lentinus edodes* fruiting bodies) mushrooms. Viability of T47D cells treated with maximal WSP concentration was consecutively 87.2 ± 4.5% for blanched, 84.9 ± 0.6% for blanched with further fermentation and 83.7 ± 1.8% for control sample. Interestingly boiling in water have led to complete loss of WSP antiproliferative activity (cells viability was 97.0 ± 0.5%).

These observations are consistent with the results of previous studies [23] carried out on *Pleurotus ostreatus* fungus where it was stated that hydro-thermal processing of mushrooms cause

the loss of direct cytotoxic activity of mushroom derived polysaccharides. Similarly other authors reported that different ways of mushrooms conservation treatment and cooking affect the composition of nutritional values and their antioxidant properties [18]. To sum up, from both studied breast cancer cell lines MCF-7 and T47D, the first one seemed to be more sensitive to water soluble polysaccharides. The strongest antiproliferative activity to MCF-7 cells was stated for water soluble polysaccharides from non-processed *Lentinus edodes* fruiting bodies.

4. Conclusions

Mushrooms fruiting bodies are rarely eaten raw and certain processing needs to be applied prior the consumption. The main purpose of the present paper was to determine the effect of three processing methods on the content and biological activity of water soluble polysaccharides. The study has shown that boiling and fermenting with lactic acid bacteria may lead to small decrease in the amount of polysaccharides. Moreover, all the techniques, namely blanching, boiling and fermenting cause the decrease in both antioxidant and antiproliferative capacities. However, these processes do not diminish biological activity completely and polysaccharides still retain their properties to some extent. A natural progression of this work would be to verify the impact of different processing methods e.g., baking, frying or microwaves. In addition, further studies should be conducted to understand mechanisms beyond antiproliferative activities of polysaccharides and residues which are attached to them.

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