PROSPECTS
IN PHARMACEUTICAL SCIENCES

Prospects in Pharmaceutical Sciences, 22(4), 86-92
https://prospects.wum.edu.pl/

Original article

LYCIUM BARBARUM POLYSACCHARIDE FRACTION – ISOLATION FROM FRUITS AND IMPACT ON THE SECRETION OF INFLAMMATORY MEDIATORS BY HUMAN MONONUCLEAR CELLS AND NEUTROPHILS

Marta Kuśnierek[1] and Monika E. Czerwińska*[2,3]

[1] *Student Scientific Association, Department of Pharmaceutical Biology, Faculty of Pharmacy, Medical University of Warsaw, 02-097 Warsaw, Poland*

[2] *Department of Biochemistry and Pharmacogenomics, Faculty of Pharmacy, Medical University of Warsaw, 02-097 Warsaw, Poland*

[3] *Centre for Preclinical Research, Medical University of Warsaw, 02-097 Warsaw, Poland*

** Correspondence, e-mail: monika.czerwinska@wum.edu.pl*

Received: 19.08.2024 / Accepted: 14.10.2024 / Published: 08.12.2024

ABSTRACT

The barbary wolfberry fruit (*Lycium barbarum* L.) has been used in traditional Chinese medicine for centuries as an anti-aging, anti-fatigue, immune-supporting, and soothing agent for many age-related, mainly metabolic, oxidative, and inflammatory diseases. The purpose of the study was to demonstrate potential properties supporting the work of the immune system of the *Lycium barbarum* polysaccharides (LBP) fraction by studying their effect on the secretion of cytokines or chemokines by human peripheral blood mononuclear cells (PBMC) and neutrophils (PMN). The impact of LBP (5-100 µg/mL) on the secretion of cytokines or chemokines (IL-6, IL-10, TNF-*α*, IL-1*β*, IL-8) by PBMC and PMN was tested by enzyme-linked immunosorbent assay (ELISA). The potential cytotoxicity of LBP was determined by staining with propidium iodide (PI) with flow cytometry. The tested fraction did not show a significant influence on IL-10 and IL-8 secretion by PBMC and PMN, respectively. However, LBP at the highest concentration stimulated PBMC to increase the production of IL-6 and TNF-*α*. The highest efficiency of LBP was shown against the secretion of TNF-*α* and IL-1*β* by PMN.

KEYWORDS: cytokines, chemokines, immunostimulation

1. Introduction

Approximately 97 species belong to the genus *Lycium* (Solanaceae). Among them 31 are edible but only three, including barbary wolfberry (*Lycium barbarum* L.), Chinese barbary (*L. chinense* Mill.), and Russian barbary (*L. ruthenicum* Murray) are particularly widespread in China and used for medicinal purposes. Their fruits and cortex are the most common plant materials used in medicine. Their monographs were included in the European, British, and Polish Pharmacopoeas [1]. An increase in interest in barbary wolfberry fruit (goji berries) as a functional food has been recently noticed in oriental and Western societies [2, 3]. A wide range of studies on the composition and biological functions of *L. barbarum* were provided, which we revised in detail recently [1]. Many pharmacological effects of goji berries are assigned to polysaccharides, carotenoids, and flavonoids [4].

To date, *L. barbarum* phytochemicals are capable of stimulating the immune system, being effective against microorganisms, improving the glycemic and lipidemic profile in humans and animals, and protecting neurons against the effects of damage and aging [3,5-10]. Most reports and reviews concerning *L. barbarum* focus on their polysaccharides (LBP) [4,11-13]. In addition to potential antitumor activity, neuroprotective effects, and immune regulating function, LBP are responsible for health benefits, such as anti-aging and anti-fatigue effects. It is also mentioned that LBP can improve eyesight [13].

Preclinical studies have shown that LBP affects the proliferation of splenocytes, T and B lymphocytes, maturation and activation of macrophages, and dendritic cells in addition to their antigen-presenting function, cytotoxicity, and reduction of apoptosis and necrosis of natural killer cells [14-16]. Moreover, the expression of IL-2 and TNF-*α* in peripheral blood mononuclear cells (PBMC) and the production of T helper lymphocytes from splenic follicles are affected by a polysaccharide-protein complex from *L. barbarum* [17].

It seems that the relevant potential of goji berries is in their influence on the immune system and their antioxidant properties. Therefore, our study was performed on the

blood morphotic elements such as PBMC and human neutrophils (PMN) that are capable of secretion of inflammatory mediators such as pro-inflammatory (IL-1β, TNF-α, IL-6) and anti-inflammatory (IL-10) cytokines, or chemokines (IL-8). These proteins are engaged in the regulation of hemopoiesis, fibrinolysis, coagulation, granulation, and immune response necessary for fighting infection [18].

The study aimed to demonstrate the potential properties of the *L. barbarum* polysaccharide fraction supporting the functioning of the immune system by testing their effect on the secretion of cytokines or chemokines by PBMC (IL-6, IL-10, TNF-α) and PMN (IL-8, IL-1β, TNF-α).

2. Materials and methods

2.1. Plant material

The dried fruits of *L. barbarum* (Smakosz, Brzeziny, Poland) were purchased in the herbal store. The batch number was 270717.

2.2. Reagents

Ethanol (96%), acetone, isoamyl alcohol, and dichloromethane were purchased from POCh S.A. (Gliwice, Poland). The Savage reagent was prepared by mixing isoamyl alcohol and dichloromethane (49:1, *v*/*v*). Water purification system Millipore Simfilter Simplicity UV (Molsheim, France) was used to obtain ultra-pure water. Citric acid, sodium citrate tribasic dihydrate, and glucose for citrate dextrose solution (ACD) were purchased from Chempur (Piekary Śląskie, Poland). Dextran from *Leuconostoc mesenteroides*, propidium iodide (PI), dexamethasone (Dex), and Triton X-100 were purchased from Sigma-Aldrich (Saint Louis, MO, USA). RPMI 1640 medium and amphotericin B were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Pancoll Human was from PAN-Biotech (Aidenbach, Germany). Phosphate buffered saline (PBS) and fetal bovine serum (FBS) as well as penicillin-streptomycin were purchased from Biowest (Nauillé, France). Lipopolysaccharide (LPS) from *Escherichia coli* and formic acid were obtained from Merck (Darmstadt, Germany). Sets of immunosorbent-assay for human IL-6, IL-10, IL-8, TNF-α, and IL-1β were purchased from BD Biosciences (Erembodegem, Belgium).

2.3. Extraction and isolation of LBP

A 50 g-portion of dried *L. barbarum* fruits was ground using an electric grinder and macerated twice with 96% (*v*/*v*) ethanol (1:10, *m*/*v*) for 24 hours. After filtering through cotton wool, the remaining raw material was extracted twice with 96% ethanol (1:10, *m*/*v*) in an ultrasonic bath for 30 minutes and filtered again. The supernatants were discarded and the raw material was poured with hot distilled water at a temperature of 80 ºC (500 mL) and then shaken for 3 hours (Incubator Shaker Series, 100 rpm, 80ºC). Shaking was repeated once. After filtration, the residues of the raw material were extracted twice with distilled water (500 mL) in an ultrasonic bath for 30 minutes at 80ºC and filtered. The supernatants after aqueous extractions were combined and excess water was evaporated using a vacuum evaporator (Rotavapor R-3, Buchi, Flawil, Switzerland). Next, the proteins were precipitated using Savage reagent (1:1, *v*/*v*). The dichloromethane layer was centrifuged at 2000 rpm for 10 min. (MPW-350R, MPW Med. Instruments, Poland) to completely separate the remaining aqueous phase. The aqueous phases were combined to obtain approximately 150 mL of solution. To precipitate polysaccharides, the extract was concentrated to 40 mL using a vacuum evaporator, then poured with ice-cold 96% ethanol (1000 mL) and stored in a refrigerator (4 ºC) for 12 hours. The ethanol was poured off from the precipitated polysaccharides. This step was followed by washing polysaccharides twice with acetone (50 mL) [19]. After evaporating the acetone, the obtained fraction of LBP was dried by freeze-drying (Telstar Cryodos 50, Telstar International, S.L., Terrassa, Spain). The percentage value of the yield was calculated following the equation:

$$\boldsymbol{Yield}\ (\% \cdot \boldsymbol{w/w}) = \frac{\boldsymbol{M_{LBP}}}{\boldsymbol{M_s}} * \mathbf{100}$$

M_{LBP} – the weight of extracted LBP
M_S – the weight of plant material used for the extraction

2.4. Isolation of leukocytes - PBMC and PMN

Leukocytes were isolated from buffy coats obtained commercially from healthy volunteers below 35 years old (n=7) from the Warsaw Blood Donation Centre (Poland).

The PBMC were isolated according to the method originally described by Böyum [21]. Neutrophils were isolated by dextran sedimentation and centrifugation in density gradient with buffer for leukocyte isolation (Pancoll Human, 1.077 g/mL) [22]. The details of isolation procedures were provided in the previous report [20].

Following isolation, the cells were suspended in an appropriate medium, such as RPMI 1640, and were maintained at 4°C before use. The cells were seeded in 96-well plates for cytokines secretion assays (2×10^6 cells/well in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, and 10 mM HEPES). The cells were treated with LBP at concentrations of 5, 50, and 100 µg/mL and 10 µL LPS (100 ng/mL), which was applied after 30 min. of incubation of cells with LBP. The incubation was continued for a further 20 h in standard conditions in a humidified atmosphere with 5% CO_2 at 37 ºC. Dexamethasone (Dex) was used as a positive control when the secretion of cytokines was determined.

Leukocyte cytotoxicity was assessed by flow cytometry [23,24]. The cells were treated with LBP at 5, 50, and 100 µg/mL concentrations for 20 h. Next, they were suspended in 500 µL of PI solution (0.5 µg/mL) and analyzed by flow cytometry FACS Calibur (BD Biosciences, San Jose, CA, USA), and data from 10,000 events were recorded. Cells that displayed high permeability to PI were expressed as a percentage of PI (+) cells. Triton X was used as a positive control in the cytotoxicity assay.

The experiments were performed in triplicate for each concentration.

2.5. Enzyme-linked immunosorbent assays for cytokine detection

After incubation of leukocytes with LBP the supernatant was collected to evaluate cytokine concentration with ELISA. The results were shown as a percentage of LPS-stimulated control (100%).

2.6. Statistics for analysis of results

The Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, United States) software was used to analyze the results. The mean, standard deviation (SD), standard error of the mean (SEM), coefficient of variation (CV), and statistical significance for the differences between the results from the tested extract and the control were calculated.

The statistical significance of the differences between means was evaluated by one-way ANOVA. The non-parametric methods such as Kruskal-Wallis or U-Mann-Whitney's tests were used if necessary and obtained *p-values* below 0.05 were considered statistically significant.

3. Results and discussion

In our study, we tested the fraction of polysaccharides isolated from fruits of *L. barbarum* in the models of morphotic cells of the blood engaged in the innate immune. Polysaccharides are the most abundant biological macromolecules of plant tissues, which occur in animals and microorganisms. These structures are polymeric carbohydrates, which consist of more than ten monomers linked by glycosidic bonds. Two classes such as homopolysaccharides which contain just one type of monomer, and heteropolysaccharides containing more than one type of monomer, can be distinguished. Several polysaccharides are utilized for pharmaceutical purposes such as drug delivery, including encapsulation, gene therapy, wound healing applications, tissue engineering, and the preparation of contact lenses for drug delivery [25]. Moreover, polysaccharides are known for their interaction and influence on the immune response. For this reason, they are considered a potential source of health benefits for humans. Apart from starch, cellulose, and pectin occurring in plant tissues playing a structural or storage role, polysaccharides from yeast and mushrooms such as β-glucans have been found to affect several types of immune cells, including macrophages, natural killer cells, and neutrophils engaged in the first defensive line of innate immunity [26]. Many polysaccharides, worth noting dietary polysaccharides, have been shown to stimulate the immune system through different pathways. However, there is still a gap in research on their structure-activity relationship [27]. Fractions of LBP include some acidic heteropolysaccharides, polypeptides, or proteins. Their monosaccharide and amino acid residues constitute glycoconjugates. The structure of LBP is based on glycan backbone, branching sites, and side chains. The molecular weight of LBPs ranges from 10 to 2300 kDa [13].

The novel LBP extraction technologies include ultrasound-, enzyme-, microwave-supported extraction, and supercritical fluid extraction. The water extraction of polysaccharides from *L. barbarum* seems the best, except for a microwave-assisted extraction method, for this class of compounds. The extraction yield usually ranges between 7.46-7.63% [13]. The extraction of LBP from dried fruits of *L. barbarum* in our study resulted in obtaining 4.58 g of water-soluble polysaccharide fraction. The yield was then recalculated to 9.16%. It can be concluded that the yield was even higher than the values available in the literature.

To begin with, the potential cytotoxicity of LBP was studied in *ex vivo* models of PBMC and PMN (**Fig. 1**). Both LBP and Dex as a standard anti-inflammatory drug did not affect the viability of both types of cells.

In the next step, we assessed the inflammatory functions of PBMC treated with LBP and LPS (**Fig. 2**). The tested fraction caused a statistically significant increase in IL-6 secretion in a concentration-dependent manner (**Fig. 2A**). At 100 µg/mL, it increased IL-6 secretion by PBMC to 145.4 ± 13.72% compared to the (+) LPS control (100.00 ± 5.49%). The fraction in the lowest LBP concentration did not cause a significant change in IL-6 release relative to the (+) LPS control.

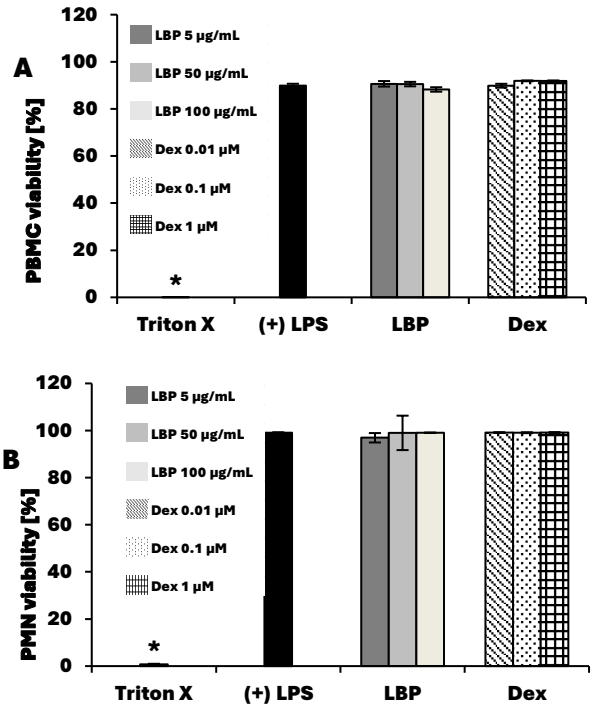

Fig. 1. Cell viability of PBMC (A) and PMN (B) treated with *Lycium barbarum* polysaccharides (LBP) and dexamethasone (Dex); Triton X - positive control. * p < 0.001 vs. (+) LPS

In the case of IL-10, no significant effect of LBP on its secretion by PBMC was observed (**Fig. 2B**). The percentage of secretion of this cytokine ranged between 77.47 ± 7.49% for LBP at a concentration of 5 µg/mL and 112.09 ± 10.54% for LBP at a concentration of 100 µg/mL, while PBMC incubated with Dex (positive control) and LPS-stimulated cells released from 101.01 ± 7.34% to 111.55 ± 7.00% (Dex) and 100.00 ± 8.34% (LPS) of IL-10, respectively. The most important function of IL-10 includes inhibiting excessive inflammatory reactions and pro-inflammatory cytokine production that could lead to tissue damage, therefore the lack of a significant decrease in the concentration of this cytokine under the influence of LBP is a beneficial phenomenon [28].

The LBP fraction showed significant stimulation of TNF-α secretion by PBMC (**Fig. 2C**) and PMN (**Fig. 3**). In the case of PMN the increase was in a concentration-dependent manner (p < 0.001). The LBP at the highest concentration caused a significant increase in TNF-α secretion by PBMC (192.00 ± 8.53%) and PMN (175.48 ± 9.19%). The percentage of TNF-α secretion by cells stimulated with LPS was 95.30 ± 10.46% and 100.00 ± 6.18% for PBMC and PMN (**Fig. 3C**), respectively.

The study of the influence of the LBP fraction on the secretion of IL-1β (**Fig. 3A**) showed that the LBP fraction at concentrations of 50 and 100 µg/mL caused significant stimulation of PMN to produce this cytokine (197.47 ± 13.43% and 245.09 ± 25.10%, respectively), compared to the (+) LPS control, for which the percentage of IL-1β secretion was 100.00 ± 3.40%. It is worth noting that PBMC and PMN were treated simultaneously with LPS and LBP solutions. In the case of both TNF-α and IL-1β secretion, addition or synergism of the effects of LPS and LBP could occur. Therefore, it is necessary to perform further experiments, including trials in which cells will be treated with LBP without the addition of LPS.

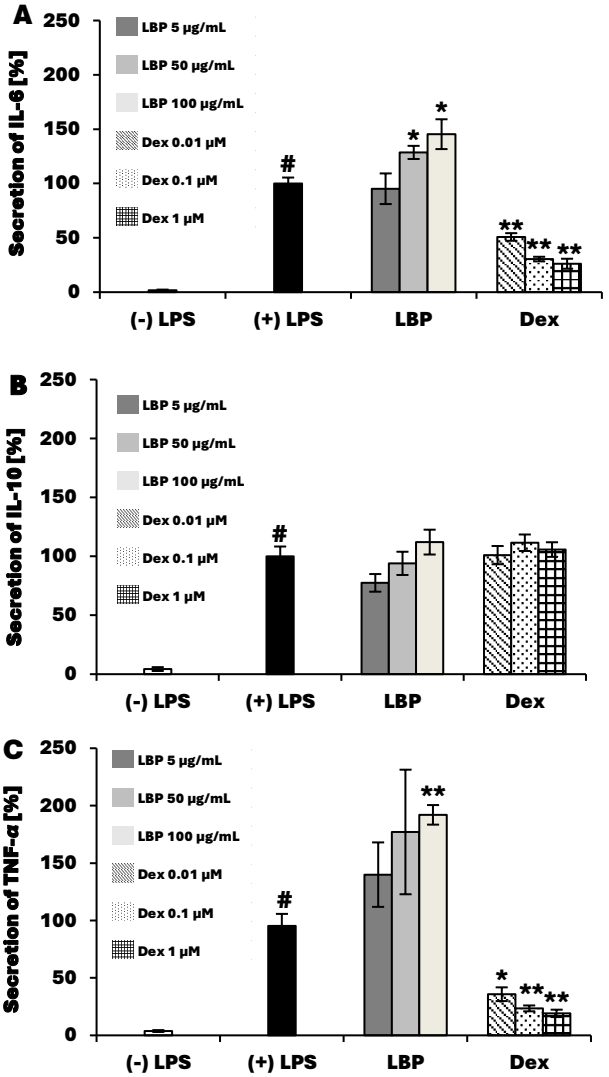

Fig. 2. Effect of *Lycium barbarum* polysaccharides (LBP) on the secretion of IL-6 (A), IL-10 (B), and TNF-*α* (C) in PBMC; dexamethasone (Dex) – positive control. # $p < 0.05$ vs. (-) LPS; * $p < 0.05$ vs. (+) LPS, ** $p < 0.001$ vs. (+) LPS

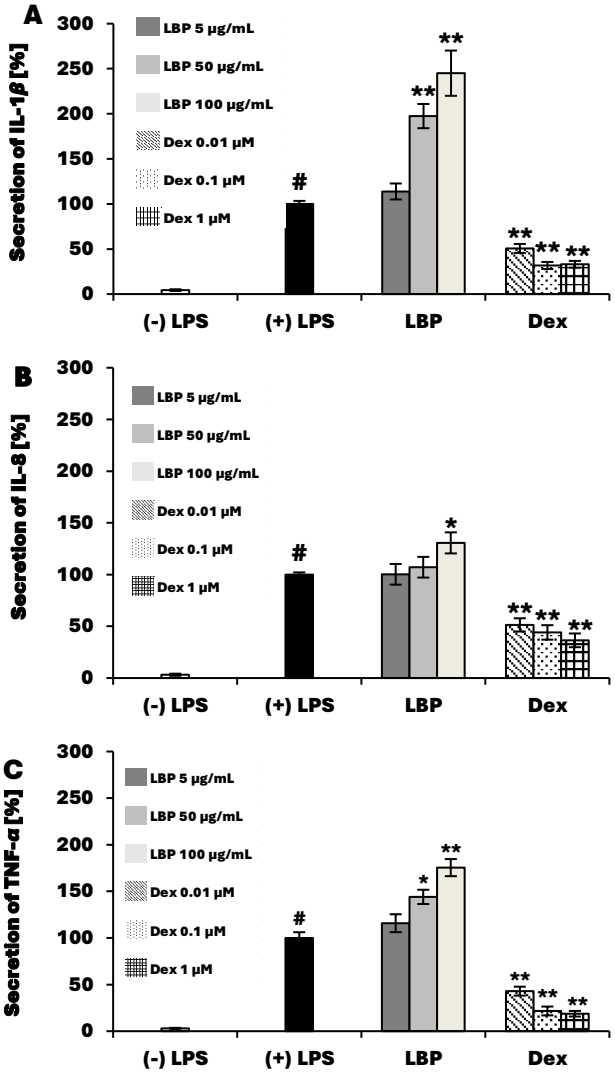

Fig. 3. Effect of *Lycium barbarum* polysaccharides (LBP) on the secretion of IL-1*β* (A), IL-8 (B), and TNF-*α* (C) in PMN; dexamethasone (Dex) – positive control. # $p < 0.05$ vs. (-) LPS; * $p < 0.05$ vs. (+) LPS, ** $p < 0.001$ vs. (+) LPS

The secretion of IL-8 secretion by PMN (**Fig. 3B**) incubated with the LBP fraction showed a tendency to increase in a concentration-dependent ($p < 0.05$) manner ranging from 100.29 ± 10.00% (5 µg/mL) to 130.67 ± 10.18% (100 µg/mL). Nevertheless, the differences between the obtained results were not statistically significant compared to the LPS-stimulated control (100.00 ± 2.12%), except for LBP at 100 µg/mL.

Innate immunity is the first line of defense against pathogens and cooperates with specific immunity mechanisms. Thanks to a fast reaction, it often enables completely stopping the invasion of microorganisms or the spreading of infection. Granulocytes and monocytes appear most quickly at the site of inflammation. Neutrophils constitute approximately 30% of bone marrow cells. After reaching maturity, they enter the bloodstream, where nearly half of the neutrophils are found in the circulating blood and the rest - in the marginal pool [29,30]. They respond to chemotactic stimuli and demonstrate the ability to move in a targeted manner. Monocytes are also formed and mature in the bone marrow, after which they enter the peripheral blood. They constitute approximately 4-9% of leukocytes. After entering the tissues through the vascular endothelium, they transform into macrophages. The most important functions of these cells include phagocytosis and the production of many regulatory proteins, including cytokines [31,32].

Previous studies have shown that aqueous extracts of *L. barbarum* fruit or isolated aqueous or water-methanol fractions of LBP and their combinations with proteins stimulate the secretion of cytokines IL-1*β*, IL-6, IL-8, IL-10, TNF-*α* by macrophages RAW264.7 cell line and mouse spleen cells [5,33]. The secretion of IL-2 and IL-12 has been affected by LBP only in higher concentrations but LBP did not influence IL-13 [5]. Thus, the results of our study confirmed the previous reports. It is worth noting that in the study of Zhang et al. (2011) LBP itself at a concentration range of 10-100 µg/mL induced the secretion of cytokines even more significantly than LPS [5]. On the other hand, in the study of Feng et al. (2020) LBP regulated inflammatory response in mouse macrophages RAW264.7 oppositely [34]. Both LBP at 400 and 800 µg/mL and LBP with LPS decreased IL-6 and TNF-*α* compared to LPS alone. As the authors stated LBP significantly promoted the release of NO, TNF-*α*, and IL-6 from

macrophages in a dose-dependent manner, but the extent of the stimulating effect was much lesser than that of LPS. In addition, LBP was also observed to have a significant inhibitory effect on LPS-induced NO, TNF-*α*, and IL-6 overproduction [34]. A less relevant impact of the tested fraction was observed in the case of the secretion of IL-8 and IL-10 in the model cells used in our study. It is supposed that the production of these cytokines is more strongly influenced by fractions of LBP protein conjugates than by crude polysaccharides. Moreover, previous studies indicate that polysaccharide fractions from goji fruits have a greater impact on the functions of macrophages than T and B lymphocytes [5], which justifies the selection of cellular models to assess the activity of LBP fractions. According to our knowledge, the influence of LBP on cytokine secretion by human neutrophils *ex vivo* was investigated for the first time. However, some *in vivo* studies indicate that LBP inhibited serum levels of inflammatory factors including IL-2, IL-6, TNF-*α*, interferon (IFN)-*α*, monocyte chemoattractant protein (MCP)-1, and intercellular adhesion molecule (ICAM)-1 compared to diabetic rats [35] or alleviated CCl4-induced liver fibrosis in Wistar rats through inhibiting the TLRs/NF-κB signaling pathway expression [36]. For this reason, further studies, including the LBP metabolism in the gut are necessary to provide the unequivocal conclusion.

The immunomodulatory activity of plant substances is often due to the content of polysaccharides or their derivatives. Examples are *Aloe arborescens* (Mill.) leaf components or its extract [37]. The aqueous extracts of *Aloe arborescens* are commonly used as medicinal products supporting the functioning of the immune system (e.g. Biostimina®, Bioaron C®) [38]. Recent studies on a model of *Sparus aurata* fibroblast cell line SAF-1, gingival fibroblasts and human peripheral blood mononuclear cells show that the activity of aqueous extracts from *Aloe arborescens* leaves and acemannan (partially acetylated mannose derivatives) involves, among others, stimulation of the release of pro-inflammatory cytokines (IL-1*β*, IL-6, IL -10 and TNF-*α*) [37,39-41]. As in the case of LBP, the extracts or *Aloe* polysaccharides may also show the opposite anti-immune effects [42].

4. Conclusions

The polysaccharide fraction from *L. barbarum* fruit can stimulate innate immunity, initiated by pathogens such as bacteria, viruses, and fungi, which are primarily fought by inflammatory mediators secreted by neutrophils and monocytes. Therefore, the fruits of *L. barbarum* and their constituents may be a potential dietary plant material preventing many immunological diseases, but further research confirming this hypothesis and justifying its use in medicine is still necessary. The observed significant increase in the secretion of cytokines, especially TNF-*α* and IL-1*β*, might result from the additive or synergistic effect of LBP and lipopolysaccharide. Identification of sugar components and molecular weight of the tested LBP may constitute the basis for continuing research on fruits of *L. barbarum*.

Author Contributions: Conceptualization, M.E.C.; methodology, M.E.C.; validation, M.K. and M.E.C.; investigation, M.K.; resources, M.E.C.; data curation, M.K.; writing—original draft preparation, M.E.C.; writing—review and editing, M.K. and M.E.C.; visualization, M.K.; supervision, M.E.C.; project administration, M.E.C.; funding acquisition, M.E.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Informed Consent Statement: Patient consent was omitted due to the commercial obtaining of resources.

Conflicts of Interest: The authors declare no conflict of interest.

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