

Anti-infectious and Anti-tumor Activities of β -glucans

VACLAV VETVICKA and JANA VETVICKOVA

Department of Pathology, University of Louisville, Louisville, KY, U.S.A.

Abstract. *Background/Aim:* The aim of this study was to directly compare the anti-infectious and anti-cancer effects of five commercially available glucans. *Materials and Methods:* We used five different glucans isolated from algae, yeast, bacteria, oat, and mushroom. We compared their effects on the stimulation of phagocytosis of blood cells, on the secretion of IL-2, and on the inhibition of melanoma and breast and lung cancers. In addition, we evaluated the effects of glucan supplementation on two experimental models of infection. *Results:* Most of the tested glucans stimulated phagocytosis and IL-2 secretion, reduced cancer growth, and ameliorated some effects of experimental infections. *Conclusion:* Glucans can produce significant pleiotropic effects, but the activity varies among individual samples.

β -Glucans (hereafter called “glucans”) are well-established natural immunomodulators with documented history of research reaching 60 years back, when a crude yeast extract was discovered to stimulate macrophages by complement activation (1). Glucans were found to be active throughout the animal kingdom and so far, no species has been found to be glucan resistant. Immunostimulating effects of glucans have been demonstrated in shrimp, fish (2), chicken (3), mice, rats, rabbits, goats, sheep, pigs (4), apes (5), and humans [for a review, see Vetvicka 2013 (6)]. Well-established biological effects of glucans include stimulation of immune reactions (7), control of diabetes (8), reduction of chronic fatigue syndrome (9), lowering of cholesterol levels (10, 11), and inhibition of cancer development (12).

With numerous natural sources of glucans, including yeast, mushroom, bacteria, seaweed, and grain, it is not surprising that hundreds of structurally and functionally different glucans exist. However, despite the significant

efforts of many laboratories, a direct link between structure and function remains elusive (13-15).

Currently, more than 30,000 studies on the biological actions of glucans can be found in the peer-reviewed literature. However, there are still problems with results not being fully comparable, as dozens and most probably hundreds of glucans exist, most commercially available. With significant differences in source, purity, physicochemical characteristics, and solubility, it is often difficult to reach a conclusion regarding the general effects of glucans. The only viable solution is to perform direct comparative evaluations; hence, we endeavored to directly compare the anti-infectious and anti-cancer effects of five commercially available glucans.

Materials and Methods

Animals. Female, 8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal work was performed according to the University of Louisville IACUC protocol. Animals were sacrificed by CO₂ asphyxiation followed by cervical dislocation.

Materials. All glucans were either donated or purchased from the manufacturers or distributors as shown in Table I. Concanavalin A (Con A), RPMI 1640 medium, fetal calf serum (FCS), polymyxin B, *Escherichia coli*, and Wright stain were purchased from Sigma (St. Louis, MO, USA). Levine EMB (eosin methylene blue) agar and Tryptic Soy Broth were purchased from Fisher Scientific (Waltham, MA, USA). *Pseudomonas aeruginosa* (ATCC 27853) was purchased from ATT (Manassas, VA, USA).

Cell lines. Murine melanoma B16 cell line (ATCC, Manassas, VA), Lewis lung carcinoma cells (obtained from Dr. G. Ross, University of Louisville, Louisville, KY, USA), and BALB/c mouse-derived mammary tumor cell line Ptas64 (generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Detroit, MI, USA) were maintained in culture at 37°C in a humidified atmosphere supplemented with 5% CO₂ in RPMI 1640 medium supplemented with 10% FCS.

Phagocytosis. Phagocytosis of synthetic polymeric microspheres has been described previously (16). Briefly, 0.1 ml of peripheral blood from mice injected with various doses of glucan or PBS were incubated in vitro with 0.05 ml of 2-hydroxyethyl methacrylate particles (5×10⁸/ml). The tubes were incubated at 37°C for 60 min, with intermittent shaking. Smears were stained with Wright stain. The cells with three or more particles were considered positive. Mice were injected with either glucan or PBS (negative control).

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Correspondence to: Vaclav Vetvicka, University of Louisville, Department of Pathology, 511 S. Floyd, Louisville, KY 40202, U.S.A. Tel: +1 5028521612, e-mail: Vaclav.vetvicka@louisville.edu

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Table I. List of glucans used in this study.

Glucan name	Source	Solubility	Manufacturer
Glucan #300	Yeast	Insoluble	Transfer Point, Little Mountain, SC, USA
Super Pure Beta 1,3 Glucan	Algae	Insoluble	The Synergy Company, Moab, UT, USA
Glucan Elite	Bacterial	Semisoluble	Pro Formulations MD, Orange, CA, USA
Beta Glucan	Oat	Semisoluble	Bioimersion, Bellevue, WA, USA
Maitake Gold	Mushroom	Soluble	NutraGenesis, Brattleboro, VT, USA

All experiments were performed in triplicate. At least 300 cells were examined in each experiment.

IL-2 secretion. Purified spleen cells (2×10^6 /ml in RPMI 1640 medium with 5% FCS) obtained from mice injected with 100 µg glucan or PBS were added into wells of a 24-well tissue culture plate. Cells were incubated for 48 h in a humidified incubator (37°C, 5% CO₂/95% air). Addition of 1 µg of Concanavalin A (Sigma) was used as a positive control. At the endpoint of incubation, supernatants were collected, filtered through 0.45 µm filters, and tested for the presence of IL-2 using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN, USA).

Tumor inhibition in vivo. Mouse mammary, lung, and melanoma cancer models have been described previously in full detail (12).

Urinary tract infection. Urinary tract infection was induced via direct inoculation of 10 µl of *E. coli* into the bladder using a syringe equipped with soft polyethylene tubing as previously described (17, 18). Tested glucans were used orally at 100 µg/mouse doses for 14 days. Mice were sacrificed at various intervals (6 h, 24 h, and 72 h) after infection and bladder and right kidney were aseptically collected from each. After weighing, organs were homogenized in sterile PBS. Obtained material was centrifuged to discard the tissue fragments. Appropriate dilutions were plated on Petri dishes with Levine EMB agar (Fisher Scientific (Waltham, MA, USA) and incubated at 37°C. Colony-forming units (CFU) were evaluated 24 h later.

P. aeruginosa. *P. aeruginosa* was incubated at 37°C overnight on 5% sheep blood agar plates. Colonies were grown overnight in Tryptic Soy Broth. Bacteria were centrifuged at $4,000 \times g$ washed with PBS, and suspended to a density of 1×10^8 CFU/ml. The density for both instillation and bronchoalveolar lavage fluid (BALF) collection were determined by serial culture dilutions. Pneumonia was induced by intranasal instillation of 10^6 CFU of *P. aeruginosa*. Three days later, BALF was collected and bacterial counts and neutrophil counts were performed (19).

Statistical analysis. Statistical significance was evaluated by paired *t*-test and by unpaired *t*-test using a GraphPad Prism 502 software (GraphPad Software, San Diego, CA, USA).

Results

Commercial samples, particularly when used from broken capsules, might contain small amounts of fillers such as gelatin or microcrystalline cellulose. These fillers are

biologically inert and the risk of influencing tested biological reactions is only theoretical. In addition, they might slightly lower the dose of the tested material, as the amount of the tested material in the capsule is slightly lower than the amount on the bottle. However, our previous experiments showed that direct comparison of one particular glucan obtained either from a commercial capsule or directly from pure glucan powder yielded identical results (12). Another possible way to achieve defective results is possible contamination with lipopolysaccharide. We prepared lipopolysaccharide-free samples and compared their effects on phagocytosis but found no differences from untreated samples (data not shown). For our experiments, we have chosen five commercially available glucans (Table I). Glucan #1 is one of the most studied glucans (20), glucan #2 is an insoluble glucan isolated from algae, glucan #3 is Glucan Elite, an Agrobacterium-derived glucan (21), glucan #4 is a semisoluble oat-derived glucan, and glucan #5 is Maitake Gold isolated from mushrooms (22).

The glucans were originally used as nonspecific immunomodulators of cellular immunity. We decided to test their ability to stimulate phagocytosis of peripheral blood neutrophils. The glucans #1, #2, #3, and #5 had significant effects on phagocytic activity, with glucan #1 showing the highest effects (Figure 1).

Next, we focused on the evaluation of the effects of glucans on the secretion of IL-2 by splenocytes. The basal levels of IL-2 without any stimulation are almost zero. All glucans showed significant stimulation of IL-2 secretion, which was comparable to the effects of Con A (Figure 2).

After establishing the basic effects on some immune reactions, we focused on their effects on cancer development. We used two different experimental models of tumor growth. Using a breast cancer model, we evaluated the changes in tumor weight. Our data (Figure 3) showed that the tested glucans again differed in their activity towards breast cancer inhibition and only glucans #1, #2, and #5 significantly suppressed the growth of breast cancer. Next, we used a well-described Lewis lung carcinoma assay. Our data (Figure 4) confirmed the breast cancer experiments, with additional suppression being found in using glucan #3. Again, glucan #1 showed the highest effects. Similar results were found in case of melanoma inhibition (Figure 5).

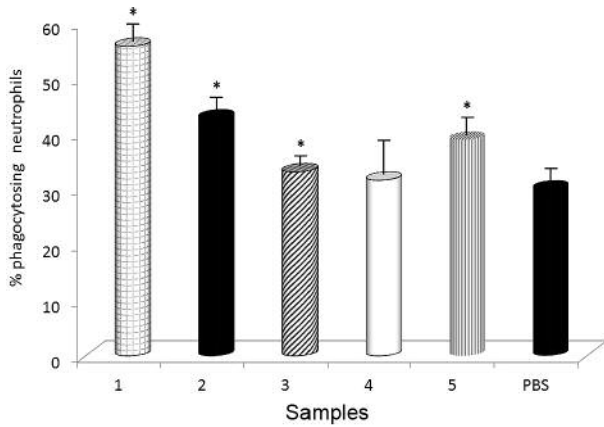


Figure 1. Effects of glucan treatment on the phagocytosis of microparticles by peripheral blood neutrophils. The experiments were repeated three times, results are given as mean \pm SD. *Significant at $p < 0.05$.

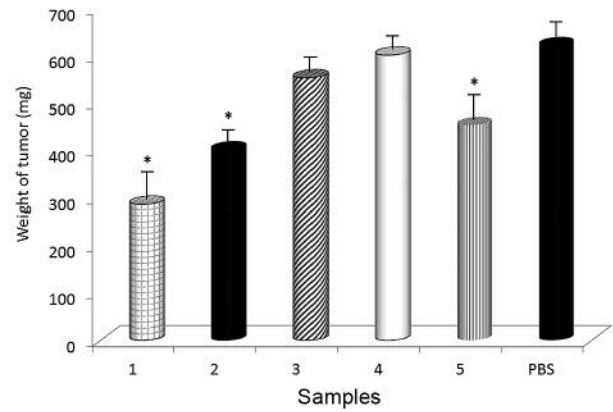


Figure 3. Effects of glucan supplementation on the weight of primary breast cancer tumors. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

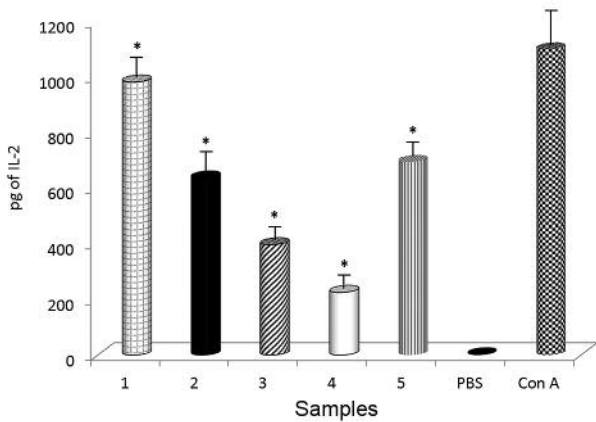


Figure 2. Effects of the tested glucans on the secretion of IL-2 by spleen cells. As the control (PBS) production of IL-2 is not detectable, all columns represent significant differences between control (PBS) and samples ($p < 0.05$). All experiments were performed in triplicates.

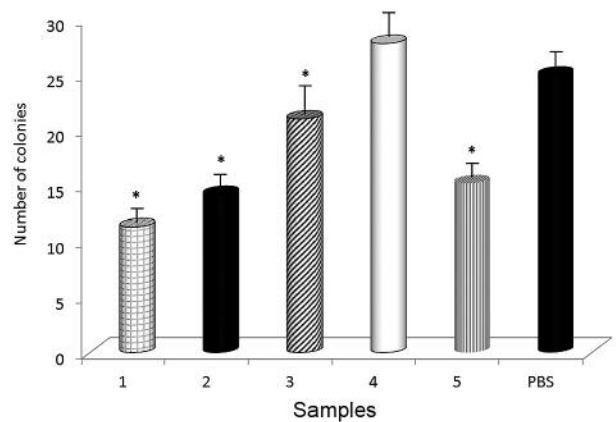


Figure 4. Effects of glucan supplementation on the number of lung colonies. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

Next, we focused on the antibacterial effects. Pneumonia was induced by intranasal application of 10^6 CFU of *P. aeruginosa*. Three days later, BALF was collected and bacterial counts and neutrophil counts were performed. In the pneumonia conditions, the BALF bacterial counts were significantly lower in animals treated with glucans #1, #2, #3, and #5, with glucans #1 and #3 being the most active (Figure 6). When we measured the changes in the numbers of neutrophils in BALF, all glucans suppressed their numbers (Figure 7).

Using a model of mouse urinary tract infection, we observed the appearance of *E. coli* as CFU in bladder after 14 days of oral treatment. Figure 8 shows that four of the tested glucans significantly decreased the amount of *E. coli*

in the bladder of tested animals in both tested intervals (24 h and 72 h). Furthermore, we found that all glucans significantly reduced the number of bacteria in the kidney samples after 3 days post infection. The results taken 7 days after infection were similar but, in this case, glucan #4 showed only a small decrease of CFU (Figure 9).

Discussion

The immunomodifying effects of glucans are well established. From the pioneering work of Nicolas DiLuzio, who demonstrated significant stimulation of macrophages, improved defense reactions, and tumor resistance (23),

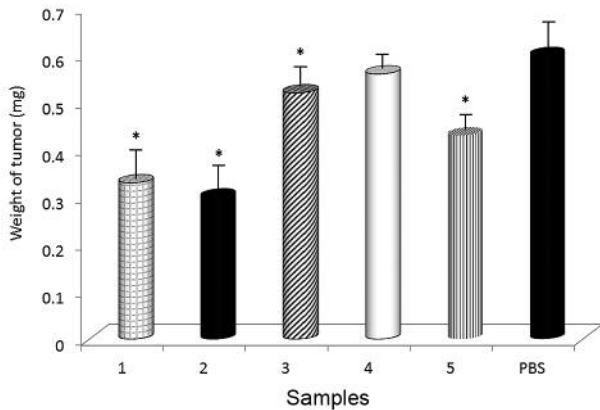


Figure 5. Effects of glucan supplementation on the weight of melanoma tumors. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

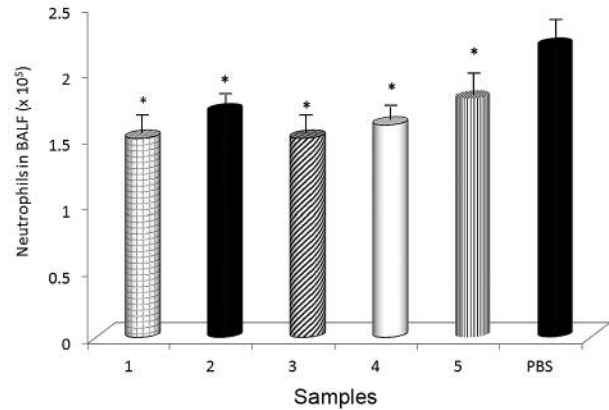


Figure 7. Effects of glucan supplementation on neutrophil counts in BALF. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

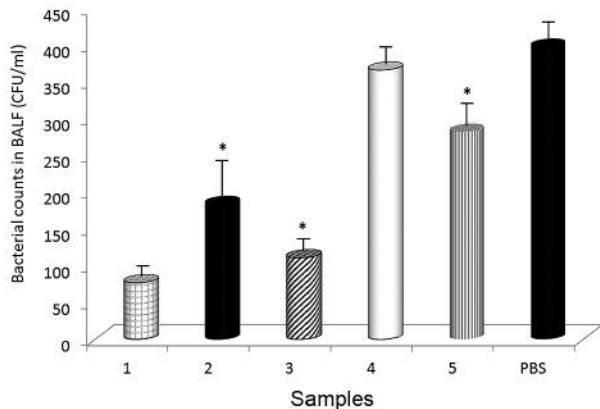


Figure 6. Effects of glucan supplementation on bacteria counts in BALF. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

decades of intensive research performed all around the world have confirmed that glucans represent the most active natural immunomodulator. Glucan effects are manifested *via* binding to several specific receptors, such as CR3 and dectin-1, and subsequent glucan-related signaling pathways include the NF κ B-receptor tyrosine kinase axis (24). Their effects include not only improvements of immune reactions against cancer and infection (7, 25), but also protection against mitochondrial toxicity (26) or improvements in diabetic retinopathy (27). Recently, glucans have been evaluated for their use in vaccine preparation, either as a part of the targeting delivery system or as a nanoparticle-based delivery platform (28).

Despite the fact that glucans are the most studied natural immunomodulators and have been an approved as drugs in Japan for decades (29), they are not without problems. Several

studies have examined the effects of glucans, however, direct comparison of their effects is difficult. The use of synthetic glucan-based oligosaccharides was expected to overcome these problems, but their use is limited due to the difficult and expensive preparation (30). To better understand the biological effects of glucans and to further increase the chance that glucans are used in Western medicine, it is important to directly compare glucans using identical methods. Our previous studies compared over 60 individual glucans (12, 31, 32), but the ever increasing multiplication of new commercial glucans led us to compare some of the new glucans with glucan #300, which has been previously shown to be superior.

Glucans were originally considered to be nonspecific stimulators of cellular immunity; therefore, phagocytosis was and still is the test of choice, because the universal dogma stating that glucans not stimulating phagocytosis will not affect immunity is still valid. The effects of glucans on phagocytosis are mediated *via* interaction with glucan receptors (6). Here, we used synthetic microbeads based on 2-hydroxyethyl methacrylate with demonstrated low spontaneous adhesion to the cell surface and, therefore, low false positivity (16). Our results demonstrated that glucans really stimulate phagocytosis of professional phagocytes, but that there are significant differences in the activities among the tested glucans.

In order to show that glucans affect additional facets of the immune system, we evaluated the potential stimulation of IL-2 secretion. The relationship between glucans and cytokines is complicated. On one hand, high systemic stimulation of secretion of many cytokines is not desirable; on the other hand, there is only one glucan described in literature as having no effects on cytokine secretion (33). We confirmed that all glucans significantly increased IL-2 secretion by spleen cells. However, it is important to note that without any stimulation, splenocytes do not secrete IL-2.

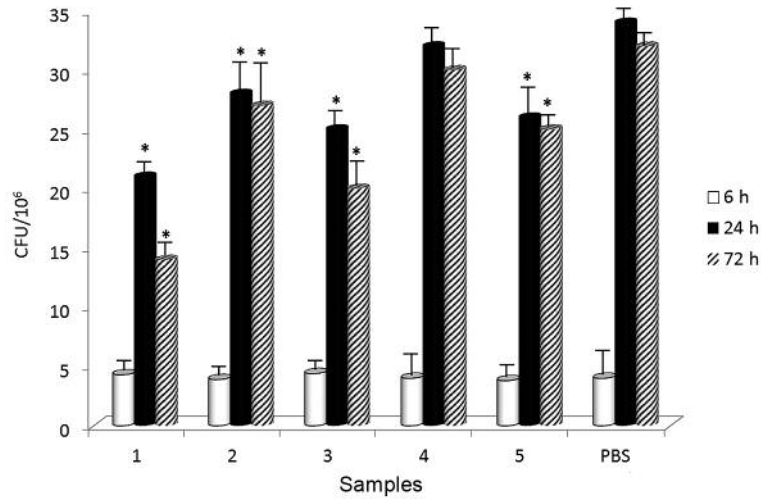


Figure 8. Effects of glucan supplementation on CFU in the bladder at different time intervals. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

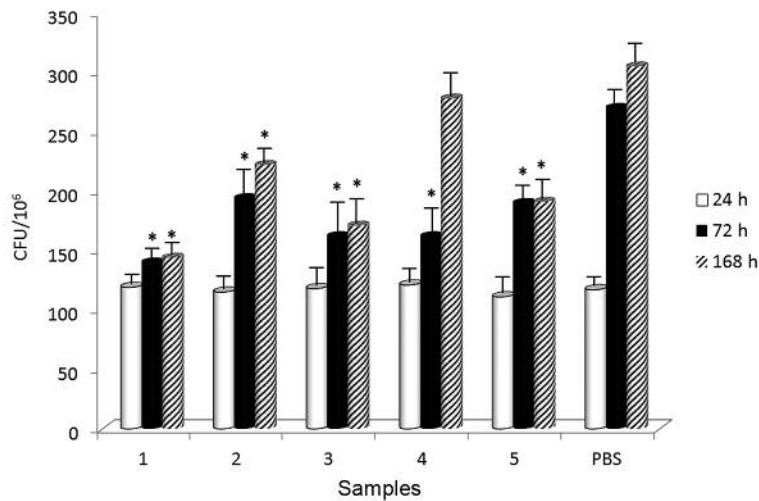


Figure 9. Effects of glucan supplementation on CFU in kidney at different time intervals. Results represent mean from three experiments \pm SD. *Represents significant differences from control, $p < 0.05$.

Glucans are well-established natural immunomodulators with significant anti-cancer properties (34). Glucans can either directly inhibit cancer cell growth or stimulate cells such as NK cells or neutrophils to kill cancer cells upon binding to glucan receptors. In addition, some novel roles of glucan in cancer treatment have recently been described, ranging from glucans being a delivery platform for anti-cancer drugs (35) to regulating trained immunity, where innate cells take on memory phenotypes. To better compare the effects of glucan supplementation on cancer development, we used three different models of breast cancer, lung cancer, and melanoma. Our data showed that

most of glucans inhibit cancer development by reducing the primary tumor weight and the number of lung cancer colonies. Glucan #4, however, showed only small, nonsignificant inhibition. Based on our previous data, we hypothesized that these effects are mediated by a combination of stimulation of effector cells, such as NK cells, and general stimulation of immune system (20).

Similar to cancer, glucans have also established as anti-infectious agents (36, 37). In our comparative study, we used two different models of infection. First, we focused on urinary tract infection, as it represents a common clinical problem. Repeated infections are, at least partly, caused by

depressed immune system. This infection includes both infection of the kidney and infection of the bladder and is most commonly caused by *E. coli*.

Using a model of mouse urinary tract infection, we counted the CFU of *E. coli* in the bladder after 14 days of oral treatment and found that four of the tested glucans significantly decreased the amount of *E. coli* at both intervals (24 h and 72 h). When we measured the number of bacteria in the kidney, we found that all glucans significantly reduced their numbers after 3 days post infection. All glucans were active, but significant differences among glucans mirrored those found in previous tests.

The second experimental infection model was a mouse model of pneumonia. We chose this model because bacterial-caused pneumonia is a leading cause of acute respiratory distress syndrome and significantly increases the mortality of patients in intensive care units (38). Food supplementation with glucan resulted in significant depression of high neutrophil counts caused by infection. In addition, four out of the five tested glucans reduced bacterial counts in BALF. This model of *P. aeruginosa* infection is highly relevant to clinical situation (39) and our results suggest the potential use of glucans as a supportive treatment in pneumonia patients.

Conclusion

In conclusion, this study is a follow-up of our previous comparative studies testing over 60 different glucans. From the current and previous studies, we can clearly confirm that glucans have significant immuno-stimulating, anti-infectious, and anti-cancer effects. Furthermore, we clearly showed that there are huge differences in activities among the individual glucans and that the source of glucan is not the most important factor responsible for glucan effects. Current literature on glucan often yields contradictory results. Based on our results, we believe that the most probable explanation are the differences in biological activities among individual glucans. However, glucan #300 remains the most active glucan.

Conflicts of Interest

The Authors declare that there are no conflicts of interests related to this study.

Authors' Contributions

VV designed the project, supervised the experiments, analyzed data and wrote the manuscript. JV performed the experiments, prepared the Figures and co-wrote the manuscript.

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