

Fucoidans Stimulate Immune Reaction and Suppress Cancer Growth

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Abstract. *Background/Aim:* Fucoidans are gaining popularity as natural immunomodulators. The aim of this study was to compare the immunological activities of both purified samples and commercially available mixtures containing fucoidan. *Materials and Methods:* We evaluated the effects of various samples on phagocytosis, mitogenic response, natural killer (NK) activity, antibody formation and inhibition of breast cancer growth. *Results:* We found significant immunostimulating activity, but the strength of these effects was different among individual samples. *Conclusion:* Fucoidans have strong immunostimulating potential, including inhibition of cancer, with isolated samples offering better activity than commercial mixtures.

Fucoidan is a complex sulfated polysaccharide consisting of sulfated fucose residues and found mostly in brown marine algae, echinoderms (1) and seagrasses (2). Fucoidans exhibit numerous biological activities found both in *in vitro* and *in vivo* experiments. Fucoidan extracts have been found to ameliorate acute colitis (3), have anticancer activity (4) mediate *via* natural killer (NK) cells (5) and affect inflammation, vascular physiology, as well as oxidative stress (6, 7). Some effects differ based on the type of extract, as documented by the differential effects of high and low molecular weight fucoidans on the severity of arthritis in mice. The low molecular weight samples reduced arthritis *via* suppression of Th1-mediated immunity, whereas high molecular weight samples enhanced the inflammatory activation of macrophages (8). Subsequent experiments revealed that high molecular weight fucoidans stimulated spleen cells, whereas low

molecular weight samples had little activity or were toxic (9). The reasons for these differences are still unknown. Using a fish model, however, dietary fucoidan was found to influence blood chemistry and constituents, antioxidation and innate immunity (10). In human models, fucoidan delayed apoptosis and induced proinflammatory cytokines, probably *via* activation of the PI3K/AKT pathway (11). As fucoidan can function as an adjuvant and stimulate antibody response (12), it has been used to improve vaccine efficacy (13).

Cancer development is the most studied effect of fucoidan. The mechanisms are still unknown. However, fucoidan can directly induce cytotoxicity and apoptosis of cancer cells (14) probably explained by an additional study suggesting implication of macrophages and blood leukocytes in relation to fucoidan (15).

In our study, we not only directly compared the biological activities of individual fucoidan samples, but also compared purified samples with commercially available component mixtures. Thus, our study answers the question not only whether fucoidans influence immune reactions, but also if the popular idea of combining biologically active molecules together improves their effectiveness.

Materials and Methods

Materials. Individual samples were purchased from the manufacturers or distributors as shown in Table I. RPMI 1640 medium, sodium citrate, 4-(2-hydroxyethyl)-1-piperazine-ethanol-sulfonic acid (HEPES), antibiotics, Wright stain, Freund's adjuvant, ovalbumin, Concanavalin A (Con A) and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT, USA).

Cells. J774A.1 mouse macrophages and YAC cells were obtained from the ATCC (Manassas, VA, USA). The BALB/c mouse-derived mammary tumor cell line Ptac64 was generously provided by Dr. Wei-Zen Wei of the Michigan Cancer Foundation, Wayne State University, Detroit, MI, USA. The cell lines were maintained in RPMI 1640 medium containing HEPES buffer supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, in plastic disposable tissue culture flasks at 37°C in a 5% CO₂/95% air incubator.

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Table I. Detailed information on samples used in this study.

#	Sample	Composition	Company
1	Absonutrix Fucoidan Max Strength	Fucoidan 500 mg (Brown seaweed)	Manufactured for: Absonutrix 5500 Adams Farm Lane, Suite 206, Greensboro, NC 27407, USA
2	ZRADICAL powder	Sugars 1 g Riboflavin 1.7 mg Niacin B3 20 mg Vitamin B6 2 mg Folic acid 400 mg Vitamin B12 60 mcg Pantothenic acid B5 10 mg Proprietary blend: Fucoidan 50 mg Banana puree, dragonfruit powder, wolfberry juice concentrate, yumberry juice concentrate, milk thistle (<i>Silybum marianum</i>) extract, turmeric, 840 mg	Youngevity 2400 Boswell Road, Chula Vista, CA 91914, USA
3	FUCOIDZ	Vitamin C 250 mg Zinc 5 mg Proprietary blend 450 mg (L-lysine, Astragalus root extract, Eldeberry fruit extract, Olive leaf extract) Fucoidan extract 110 mg	Youngevity 2400 Boswell Road, Chula Vista, CA 91914, USA
4	Lyfetrition Fucoidan	Fucoidan 500 mg (Brown seaweed)	Manufactured for: Absonutrix 5500 Adams Farm Lane, Suite 206, Greensboro, NC 27407, USA
5	PoliNat Brown seaweed ext. 40%	Brown seaweed ext. 40%	Polinat 35240 Ingenio, Las Palmas, Spain
6	PoliNat Brown seaweed ext. 20%	Brown seaweed ext.20%	Polinat 35240 Ingenio, Las Palmas, Spain

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 Institutional Animal Care and Use Committee (IACUC) protocol. Animals were sacrificed by cervical dislocation.

Phagocytosis. The technique employing phagocytosis of synthetic polymeric microspheres was described in Vetvicka *et al.* (16, 17). Briefly, peripheral blood cells were incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; 5×10^8 /ml). The test tubes were incubated at 37°C for 60 min, with intermittent shaking. Smears were stained with Wright stain. Cells with three or more HEMA particles were considered positive. Mice were injected intraperitoneally with individual samples or phosphate-buffered saline (PBS; control). All experiments were performed in triplicate. At least 200 cells in 60 high-power fields were examined in each experiment. In *in vitro* experiments, cells were incubated with 0.01 ml of HEMA particles/well. Incubation and staining was identical to *in vivo* experiments.

Mitogenic response assay. For the mitogenic response assay, splenic lymphocytes (5×10^5 per well) were co-cultured with tested samples (10 µg) and either 10 µg of Con A or 1 µg of LPS in 200 µl of

RMPI 1640 plus 10% FCS for 72 h. Proliferation was evaluated using the Biotrak cell proliferation ELISA system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

***In vitro* cytotoxicity assay.** Spleen cells were isolated from mice by standard methods. Cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After erythrocyte elimination by 10-second incubation in distilled water and five washes in cold PBS, cells were re-suspended in PBS and counted. Viability was determined by Trypan blue exclusion. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6 /ml; 0.1 ml/well) in V-shaped 96-well microplates were incubated with individual samples (2 µg/ml) for 30 min at 37°C and then washed three times with RPMI 1640 medium. After washing, 50 µl of target cell line YAC-1 (two different concentrations of target cells were used so the final effector-target ratio was 32:1 and 64:1, respectively). After spinning the plates at $250 \times g$ for 5 min, the plates were incubated for 4 h at 37°C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, 10 µl of lysis solution was added into the appropriate control wells 45 min before the end of incubation. The next step was to spin

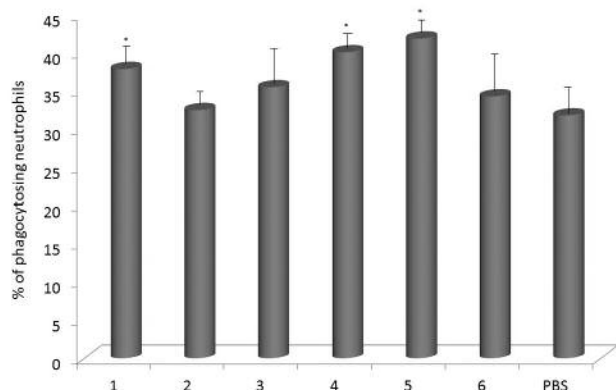


Figure 1. Effects on phagocytosis by peripheral blood monocytes by orally administered individual samples against phosphate-buffered saline (PBS). Each value represents the mean \pm SD. *Represents significant differences between control (PBS) and tested samples at $p\leq 0.05$ level. All experiments were performed in triplicate.

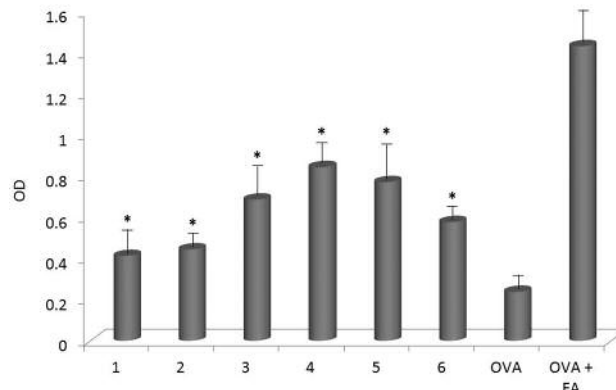


Figure 2. Effects on formation of antibodies against ovalbumin (OVA) by orally administered individual samples. Animals were injected twice (two weeks apart) with antigen. The serum was collected 7 days after last injection. Level of specific antibodies against ovalbumin was detected by ELISA. As positive control, Freund's adjuvant (FA) was used. *Represents significant differences between control (OVA alone) and samples at $p\leq 0.05$ level.

the plates at $250 \times g$ for 5 min, followed by transferring 50 μ l of supernatant into flat-bottomed, 96-well microplates. After 50 μ l of reconstituted substrate was added into each well, plates were covered and incubated for 30 min at room temperature at dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC, USA) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity) = $100 \times [(\text{OD}_{492} \text{ experimental} - \text{OD}_{492} \text{ spontaneous}) / (\text{OD}_{492} \text{ maximum} - \text{OD}_{492} \text{ spontaneous})]$ as described in manufacturer's instructions, where spontaneous release was target cells incubated with medium alone and maximum release was obtained from target cells lysed with the solution provided in the kit.

Macrophage inflammatory assay. One hundred thousand of J774A.1 mouse macrophages were incubated in RPMI 1640 medium supplemented with 10% FCS in 12-well tissue culture plates. The cells were incubated with either 5 μ g/ml of LPS or with LPS plus tested samples for 18 h. The supernatant was collected, filtered and stored at -80°C . Interleukin (IL)-10 and tumor necrosis factor (TNF)- α levels were determined using an ELISA and Quantikine mouse IL-10 or TNF- α kit (R&D Systems, Minneapolis, MN, USA), respectively.

Antibody formation. Formation of antibodies was evaluated using ovalbumin (OVA) as an antigen. Mice were injected twice (two weeks apart) with 0.1 mg of OVA and the serum was collected 7 days after last injection. Experimental groups received daily intraperitoneal injections of tested material. ELISA detected the level of specific antibodies against OVA. As a positive control, a combination of OVA and Freund's adjuvant was used.

Breast cancer model. Mice were injected directly into the mammary fat pads with 1×10^6 /mouse of Ptas64 cells in PBS. The experimental treatment begun after palpable tumors were found (usually 14 days after injection of cells) and after mice were assigned to experimental

groups. Experimental treatment was achieved by intraperitoneal injections of tested samples diluted in PBS (once/day for 14 days). After treatment, the mice were sacrificed, tumors removed and weighed (18).

Results

The list of individual samples of purified fucoidans and various commercially available mixtures is given in Table I. The purpose was to directly compare the effects of laboratory and commercial samples. In each case, the doses of fucoidan used in experiments were calculated from the information given by the manufacturer.

As fucoidans can influence phagocytosis of blood leukocytes, the study of such a process seemed imperative. As summarized in Figure 1, only samples #1, #4 and #5 showed significant stimulation of phagocytosis of HEMA microspheres. A different situation was found when the antibody response was tested. Using OVA as an antigen, all samples significantly increased specific antibody response compared to antigen alone. However, none of the samples reached the levels of antigen and adjuvant (Figure 2).

Subsequently, proliferation of T and B lymphocytes after mitogenic stimulation was measured. The dose of 10 μ g of tested samples shown in Figure 3 was based on the best results from the 0.1 to 100 μ g range (data not shown). In Con A-stimulated splenocytes, only sample #4 showed significant effects; the rest of samples had no effect. In case of LPS-stimulated proliferation, samples #4 and #5 were active.

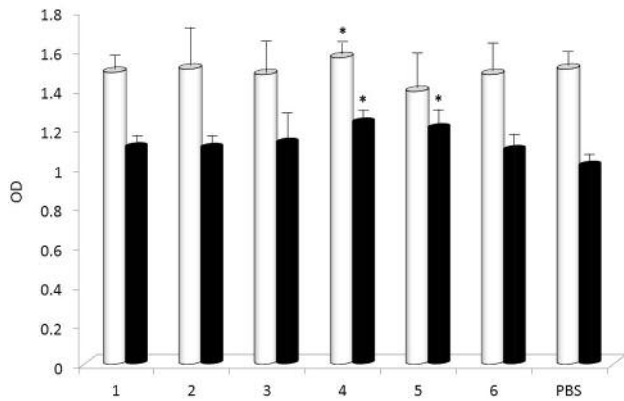


Figure 3. Administered samples affect concanavalin A (Con A)-induced proliferation of T lymphocytes (white column) and lipopolysaccharide (LPS)-induced proliferation of B lymphocytes (black column). Each value represents the mean±SD. *Represents significant differences between control and experimental samples at $p \leq 0.05$ level. OD, Optical density.

In an activated macrophage assay, we found significant stimulation of both IL-10 and TNF- α cytokines by all samples except #2 (Figure 4A and B). In several samples, the stimulation was the same or even strongly exceeded that of LPS.

In the next set of experiments, we focused on NK cells. All samples (except #2) significantly increased the activity of NK cells (Figure 5). Only one of the three tested effector-target ratios is shown. The effects, however, of the 10:1 and 100:1 ratios were identical (data not shown).

The protective effects of fucoidans were supported by findings that feeding with no-commercial samples affected relative portions of early phases of apoptosis in spleen cells (Figure 6). Samples #1 and #5 showed significant effects, while the remaining samples had no effect.

Finally, we evaluated the effects of tested samples on cancer development. We used mice challenged with Ptas64 mammary tumors. These experiments were also repeated with LPS-free samples with identical results (data not shown). Our data, summarized in Figure 7, show significant lowering of tumor growth by samples #1, #3, #4 and #5.

Discussion

Fucoidans are mostly isolated from seaweed. As each seaweed species comprises a unique fucoidan, the literature evaluating their biological effects often offers contradictory results. When directly compared, the immunological effects differ among the individual samples (19). The study's objective was to compare immunomodulatory activity of

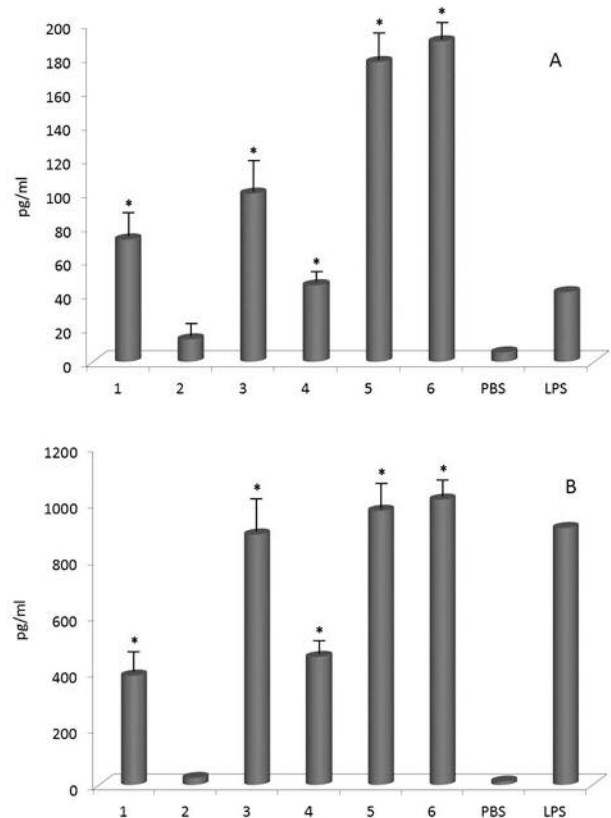


Figure 4. Administered samples affect interleukin (IL)-10 (A) and tumor necrosis factor (TNF)- α (B) production by activated macrophages against phosphate-buffered saline (PBS) and lipopolysaccharide (LPS). Each value represents the mean±SD. *Represents significant differences between control and experimental samples at $p \leq 0.05$ level.

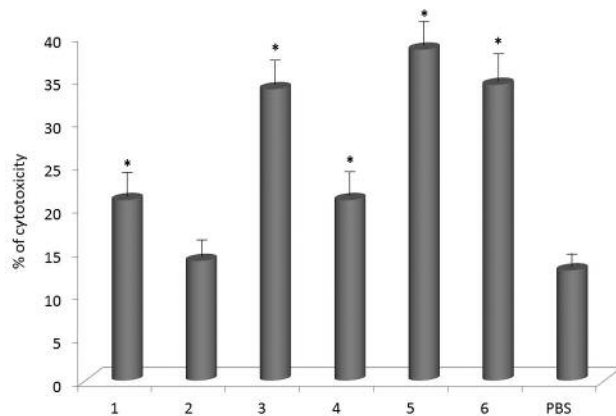


Figure 5. Administered samples affect NK cell activity against phosphate-buffered saline (PBS). A ratio of 50:1 was used. Each value represents the mean±SD. *Represents significant differences between control and experimental samples at $p \leq 0.05$ level.

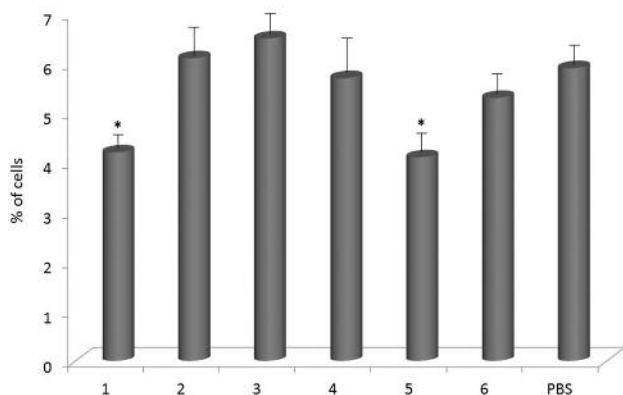


Figure 6. Administered samples affect relative portions of early phases of apoptosis in spleen cells against phosphate-buffered saline (PBS). Each value represents the mean \pm SD. *Represents significant differences between control and experimental samples at $p\leq 0.05$ level.

several fucoidan samples. We compared isolated fucoidans with commercial samples that contained either fucoidan alone or in combination with other molecules. In each case, we weighted the content of the capsule and calculated the dose of fucoidan, therefore eliminating possible errors due to the inert material in the capsule.

Fucoidans have a variety of biological functions (20), including mobilization of progenitor cells (21), stimulation of dendritic cells and radioprotection of bone marrow cells (22). A wide range of findings led to studies on proviral loads in humans with T-lymphotropic virus type-1. The results showed inhibition of cell-to-cell transmission and a 42% proviral load (23). Our previous study showed strong *in vivo* activity of fucoidan, which was comparable to that of mushroom- or yeast-derived glucans (24).

As fucoidans are known for their stimulation of phagocytosis (15), we employed synthetic microspheres known for minimum spontaneous adhesion to the cell surface and showed stimulation of internalization. A different situation was found, however, in evaluating the effects of fucoidans on antibody response. All samples were active, suggesting that these molecules can influence the cellular and humoral branch of immunity differently. Recent clinical trials examining the effects on antibody production in subjects over 60 years of age showed that both the antibody titers and NK cell activity were affected (25). Our study clearly demonstrated the effect on phagocytosis, whereas antibody and cytokine secretion confirmed previously published data further supporting the broad spectrum of fucoidans' reactivity.

Fucoidan-containing food supplements have been traditionally used in cancer patients in China, Korea and Japan. Some studies showed effects on apoptosis (14), which we confirmed. Direct mechanisms of anticancer effects are

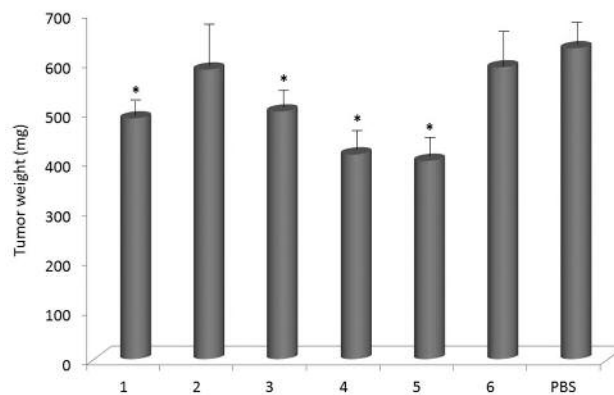


Figure 7. Administered samples on the therapy of Balb/c mice with Ptas64 mammary carcinoma against phosphate-buffered saline (PBS). Data from three independent experiments are shown. For each experiment, groups of three mice were tested for a response to therapy as indicated by the weight of tumors after two weeks of supplementation. Each value represents the mean \pm SD. *Represents significant differences between control and experimental samples at $p\leq 0.05$ level.

not known; however, activation of NK cells (also found in our study) and indirect stimulation of immune system are likely to occur. As fucoidans are similarly effective after intraperitoneal, intravenous and oral application, there is a potential for clinical practice. A comprehensive review of anticancer effects of fucoidans in preclinical development has been previously published by Kwak (26).

Our study used both isolated fucoidans and commercial samples, sometimes in a complex with other potentially active substances. From the presented results, it is clear that fucoidans have significant biological activities. Only some of our samples influenced all reactions and rarely all samples affected one tested reaction. The most active samples were pure fucoidans (samples #1, #4 and #5), followed by a Fucoidz combo (sample #3). Addition of more molecules to the same fucoidan as #3 lowered the effects obtained (sample #2). In addition, samples #5 and #6 showed dose-dependent action. From our results, it is not possible to conclude if addition of other biological substances lowers the effects of tested fucoidans. At the same time, our results do not provide evidence for any improvement as well. Additional experiments focusing on purified and substantially characterized fucoidans, as well as possible mechanisms of action, are currently under way.

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