

## Natural Immunomodulators and their Stimulation of Immune Reaction: True or False?

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**Abstract.** *Natural immunomodulators are getting more and more popular. The popularity, however, often brings over-optimistic claims and mediocre effects. The purpose of the present study was to directly compare eleven most commonly used immunomodulators. Through testing both cellular and humoral branches of immune reactions, we found that most of the immunomodulators tested have limited, if any, effects, with glucan being consistently the most active molecule strongly stimulating every reaction evaluated. These data were also confirmed using a Lewis lung cancer model, where only glucan and resveratrol lowered the number of metastases.*

Natural immunomodulators which offer strong activity with no side-effects have been sought for centuries. The current market is full of both individual immunomodulators and various combinations all promising the golden fleece—inexpensive and active stimulation of immune reactions. On one hand, some, such as  $\beta$ -glucan, have undergone over 10,000 scientific studies published in peer-reviewed journals and are currently the subject of numerous clinical trials. On the other hand, many are simply repeating claims with hardly any substantial scientific background. The resulting mediocre results further lower the satisfaction of the general public and also lower the interest of pharmaceutical companies which is necessary to direct a stimulator toward approval for medicinal use.

Thus far, only a few articles have compared individual immunostimulators (1-3). Based on the limited published comparisons, we decided to compare numerous commercially-available immunostimulators.

To perform this task, we selected the following stimulations, all claiming the effects on immune reactions: *Astragalus*, resveratrol, curcumin, pterostilbene, ellagic acid,

vitamin C, *Chlorella*, Cat's claw, Glucan #300, ginseng, and *Echinacea*. *Astragalus* stimulates IL-2, IL-4 and IFN- $\gamma$  production and cytotoxic lymphocytes (4), vitamin C modulates macrophages (5) and immune parameters (6), *Chlorella* enhances natural killer cells and inflammatory response (7), Cat's claw (extract of *Lopanthus* roots) increases antibody formation (8), Pterostilbene stimulates macrophages and keratinocytes (9), *Echinacea* activates the phagocyte system (10), ellagic acid stimulates apoptosis and inhibits melanoma cell growth (11) curcumin has strong antioxidant activities (12) and cancer growth (13), resveratrol stimulates lymphocytes and NK cells and cytokine production (14), and glucan stimulates macrophages, cytokines, NK cell activity, antibody response, reduces stress and cholesterol levels (for review see 15).

Discovering small natural molecules that regulate the immune system will increase our understanding over how diet and nutrition improve immune functions. The objective of the present study was to compare individual natural molecules with demonstrated immunostimulating properties.

### Materials and Methods

**Animals.** Female, 8-week-old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were performed in accordance with the University of Louisville IACUC protocol. Animals were sacrificed by CO<sub>2</sub> asphyxiation.

**Materials.** Individual immunostimulators were purchased as follows: *Astragalus* (*A. membranaceus*) from Solaway (Park City, UT, USA), resveratrol from Amax (Eugene, OR, USA), curcumin from Natural Remedies (Bangalore, India), pterostilbene from Pteropure (Irvine, CA, USA), ellagic acid (Sigma, St. Louis, MO, USA), vitamin C (ascorbic acid) from Sigma (St. Louis, MO, USA), *Chlorella* from Sun *Chlorella* USA (Torrance, CA, USA) Cat's claw from Piping Rock Health Products (Ronkonkoma, NY, USA), Glucan #300 from Transfer Point (Columbia, SC, USA), ginseng (*Panax ginseng*) from Puritan's Pride (Oakdale, NY, USA), *Echinacea* (*E. purpurea*) from Nature's Way Products (Green Bay, WI, USA). Ovalbumin, sodium citrate, antibiotics, Freund's adjuvant, gelatin, cytochrome *c*, concanavalin A, PMA, RPMI 1640 medium and cyclosporine were purchased from Sigma, fetal calf serum (FCS) from Hyclone Laboratories (Logan, UT, USA).

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**Key Words:** Glucan, resveratrol, vitamin C, *Astragalus*, *Echinacea*, cancer, immunity, natural immunomodulators.

**Cells.** Murine tumor cell line YAC-1 was provided by Dr. Julie Djeu of the Moffitt Cancer Research Center, Tampa, FL. The human neutrophil HL-60 cell line was obtained from the ATCC (Manassas, VA, USA). Each cell line was maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics, in plastic disposable flasks at 37°C in a 5% CO<sub>2</sub>/95% air incubator.

**Treatment.** The samples were collected after a 14-day feeding with supplement-containing diet (100 µg/day), except for *in vitro* experiments. All diets (Laboratory Rodent Diet 5001 alone or enhanced with of supplement) were formulated and prepared by Purina (Richmond, IN, USA). Diet ingredients for all groups were identical except for the addition of supplement.

**Phagocytosis.** The technique employing phagocytosis of synthetic polymeric microspheres was described earlier (16). Briefly: peripheral blood cells or peritoneal exudate cells were incubated *in vitro* with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; 5×10<sup>8</sup>/ml). The test tubes were incubated at 37°C for 60 min, with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive. Mice were injected with individual substances or their combinations (or PBS as a control). All experiments were performed in triplicates. At least 200 cells in 60 high-power fields were examined in each experiment.

**IL-2.** Purified spleen cells (2×10<sup>6</sup>/ml in RPMI 1640 medium with 5% FCS) from control or treated animals were added into wells of a 24-well tissue culture plate. After the addition of 5 µg of Concanavalin A into positive control wells; cells were incubated for 72 h in a humidified incubator (37°C, 5% CO<sub>2</sub>). At the end-point of incubation, supernatants were collected, filtered through 0.45-µm filters, and tested for the presence of IL-6 using a Quantikine mouse kits (R&D Systems, Minneapolis, MN, USA).

**Antibody formation.** Formation of antibodies was evaluated using ovalbumin as an antigen. Mice were injected twice (two weeks apart) with 100 µg of albumin and serum was collected 7 days after last injection. Levels of specific antibodies against ovalbumin were detected by ELISA. Combination of ovalbumin and Freund's adjuvant was used as a positive control.

**Lewis lung carcinoma therapy.** Mice were injected i.m. with 5×10<sup>6</sup> Lewis lung carcinoma cells. Cyclophosphamide (150 mg/kg) was used *i.p.* at day 10 after tumor application, Phycarine was used either *i.p.* (250 µg/mouse) or orally (200 µg/mouse) from day 0 to day 14 after tumor application. The control group of mice received daily *i.p.* PBS. Each group held a minimum of 5 mice. At the conclusion of the experiment, mice were euthanized, lungs removed, fixed in 10% formalin and the number of hematogenic metastases in lung tissue was estimated using a binocular lens at 8× magnification.

**Natural killer cell assay.** Spleen cells were isolated from the spleen of mice by standard methods. Cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After elimination of erythrocytes by 10-s incubation in distilled water, and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by the trypan blue exclusion assay. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10<sup>6</sup>/ml; 0.1

ml/well) in V-shaped 96-well microplates were incubated with supplements (2 µg/ml) for 120 min at 37°C and then washed three times with RPMI 1640 medium. After washing, 50 µl of target cell line YAC-1 (three different concentrations of target cells were used so the final effector-target ratio was 10:1, 50:1 and 100:1). After spinning the plates at 250 x g for 5 min, the plates were incubated for 4 hr 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 appropriate control wells 45 min before the end of incubation. The next step was to spin the plates at 250× g for 5 min, followed by transferring 50 µl of supernatant into flat-bottomed, 96-well microplates. After 50 µl 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) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity)=100×[(OD<sub>492</sub> experimental - OD<sub>492</sub> spontaneous) divided (OD<sub>492</sub> maximum - OD<sub>492</sub> spontaneous)] as described in the manufacturer's instructions, where spontaneous release was target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

**Superoxide production.** Firstly, cells were incubated with 1 µg/ml of tested material for 24 h. Next, cells were incubated in a final volume of 200 µl of medium containing 0.1% gelatin and 100 µM cytochrome C. The reaction was initialized by the addition of 5 ng/ml PMA. After gentle mixing, the absorbance was measured after 30 min incubation at 37°C using multiwall spectrophotometer at 550 nm. Results are expressed as nanomoles of cytochrome C reduced/2.5×10<sup>5</sup> cells/30 min, after subtraction of the SOD and spontaneous release controls (17).

**Statistics.** A test of the null hypothesis that the difference between two responses measured on the same statistical unit has a mean value of zero (Student's *t*-test) was used to statistically analyze the data.

## Results

Stimulation of phagocytosis is usually the first effect of any natural immunomodulator. Using a model of synthetic polymeric 2-hydroxyethylmethacrylate microspheres, we measured the phagocytic activity after two weeks feeding with the tested substances. Our data are summarized in Figure 1 and show that only glucan and *Astragalus* significantly increased the phagocytic activity of blood neutrophils and peritoneal macrophages.

Internalization of foreign material represents just the first step in the complex mechanisms of phagocytosis and elimination of prey. An additional step is connected with a burst of metabolic activity and involves production and secretion of active oxygen species. Therefore, the next part of our study focused on production of superoxide anion. Data shown in Table I demonstrate the activity of several tested

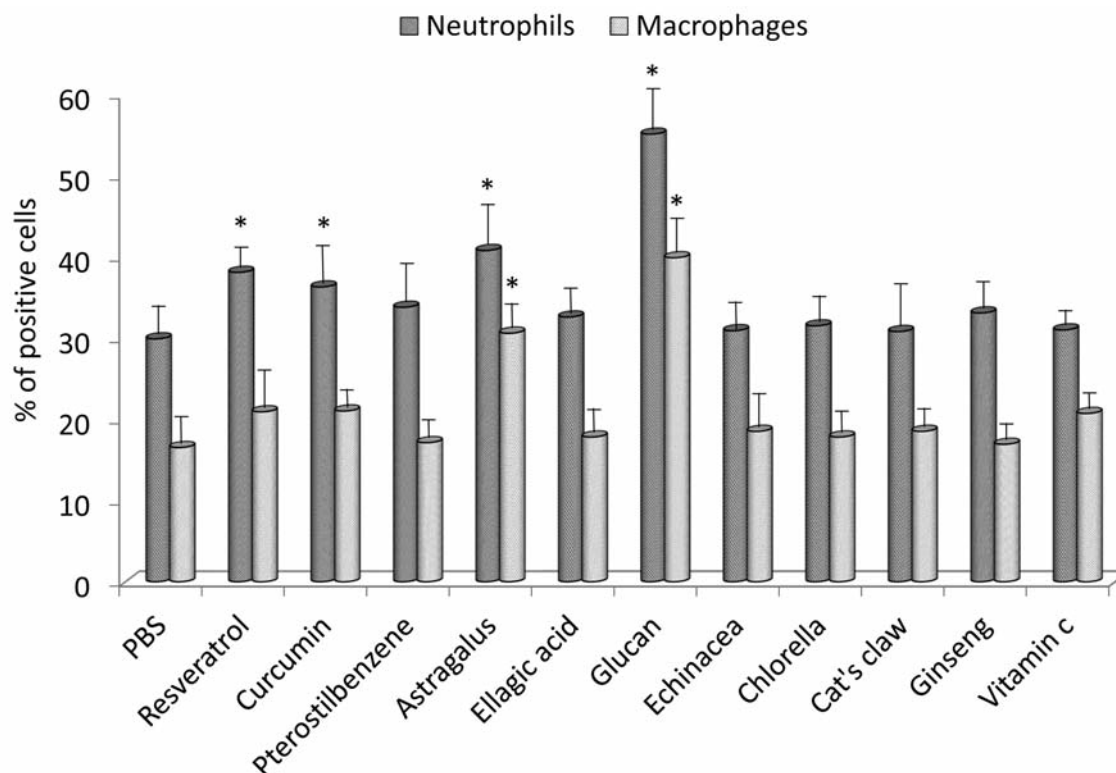


Figure 1. Potentiation of phagocytosis of synthetic microspheres by different immunomodulators. Monocytes or neutrophils with three and more HEMA particles were considered positive. Each value represents the mean $\pm$ SD. \*Represents significant differences between control (PBS) and tested samples at  $p\leq 0.05$  level.

compounds, with curcumin, *Astragalus*, glucan and vitamin C significantly potentiating the production of this anion.

The next part of our study focused on production of IL-2. IL-2 levels that were measured after a 72 h *in vitro* incubation of spleen cells isolated from control and stimulant-treated animals. Since the secretion of IL-2 by non-stimulated cells (PBS) was always zero, all tested material showed significant stimulation of IL-2 production (Figure 2). The most active materials were resveratrol, *Astragalus*, ellagic acid and glucan.

Next, for evaluation of the effects on NK cells, human YAC-1 cells were incubated with mouse spleen cells isolated from mice stimulated by tested samples (Figure 3). Resveratrol, curcumin, *Astragalus*, glucan, *Echinacea* and ginseng showed significant stimulation. Data shown in this Figure represent a 50:1 effector-target ratio. Identical results were obtained when using 10:1 and 100:1 ratios, respectively.

Despite the fact that most natural immunomodulators stimulate cellular immunity, recent studies also showed significant effects on humoral branch. As an experimental model, we used immunization with ovalbumin as an antigen. Mice were injected twice (two weeks apart) with 100  $\mu$ g of albumin and serum was collected 7 days after the final

Table I. Superoxide anion production.

Sample	nm per $2.5\times 10^5$ HL-60 cells
Resveratrol	0.29 $\pm$ 0.07
Curcumin	0.82 $\pm$ 0.11
Pterostilbenzene	0.20 $\pm$ 0.04
Astragalus	0.44 $\pm$ 0.09
Ellagic acid	0.12 $\pm$ 0.04
Glucan	1.55 $\pm$ 0.27
Echinacea	0.15 $\pm$ 0.03
Chlorella	0.10 $\pm$ 0.03
Cat's claw	0.21 $\pm$ 0.10
Ginseng	0.15 $\pm$ 0.08
Vitamin C	0.49 $\pm$ 0.12
PBS	0.13 $\pm$ 0.04

injection. All tested samples stimulated antibody formation, with glucan stimulation being by far the strongest (Figure 4).

In the next step, we focused on the role of tested substances in cancer inhibition. Using a model of Lewis lung carcinoma cells, we showed earlier that cyclophosphamide administered in the used dose caused 70% inhibition of the

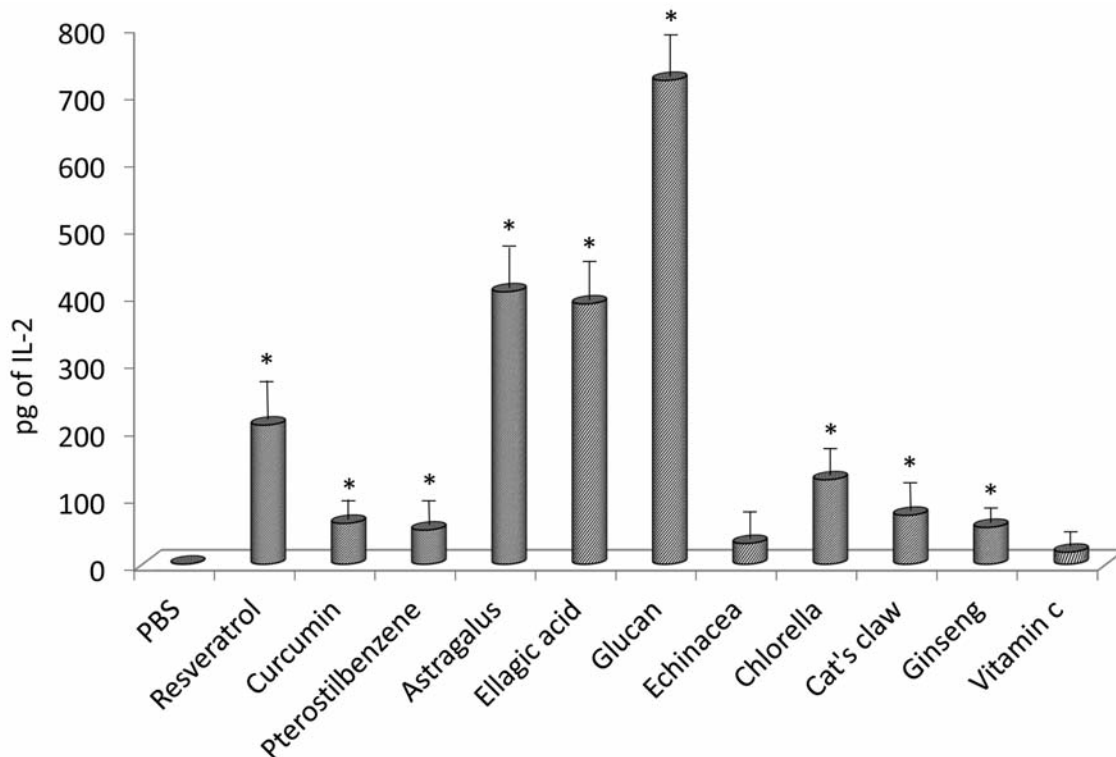


Figure 2. Stimulation of IL-2 production by different immunomodulators. Each value represents the mean $\pm$ SD. \*Represents significant differences between control (PBS) and tested samples at  $p \leq 0.05$  level.

number of lung metastases in comparison to the control group (18). Our data, summarized in Figure 5, show that only resveratrol and glucan significantly lowered the number of lung metastases, with resveratrol showing 24% inhibition and glucan showing 47% inhibition.

## Discussion

Inexpensive and effective natural immunomodulators represent the holy grail of current alternative medicine. Certain immunomodulators have been extensively studied for decades with an impressive number of peer-reviewed scientific articles (such as glucan), with some giving confusing results based on isolation sources (such as *Echinacea*, where results widely differ based on part of plant used for isolation (19)). When we consider differences between individual batches, based on the natural source of material, it is understandable why big pharmaceutical companies still are not convinced. Some of the natural immunomodulators have already reached clinical trials and with dozens of clinical trials under way, their use in regular clinical practice is only a question of time. However, despite clear and well-established biological effects of these immunomodulators, the search for even better effects continues.

Immunomodulators usually offer non-specific and often systemic effects and the mechanisms of their effects are often unknown. The present study focused on the comparison of eleven most commonly used natural immunomodulators. With only a limited number of studies directly comparing the immunological effects of various immunostimulating compounds (2, 3, 20), we decided to monitor their effects on the most important reactions covering both branches of the immune response, *i.e.*, both cellular and humoral immunity.

All immunomodulators act primarily on innate immunity and particularly on cellular branch. Therefore, the first reaction tested was phagocytosis with the use of peripheral blood neutrophils and peritoneal macrophages and synthetic polymeric particles as a model. Only four immunomodulators stimulated phagocytosis (glucan, curcumin, *Astragalus* and resveratrol), with only glucan and *Astragalus* being able to stimulate both neutrophils and macrophages. For glucan, resveratrol and *Astragalus*, these results were expected (21, 22), results of (10) suggesting the effects of *Echinacea* were not confirmed.

Ligand-receptor interactions during individual steps of phagocytosis resulted in a substantial outburst of metabolic activity. Particularly during early stages, the cells exhibit a large



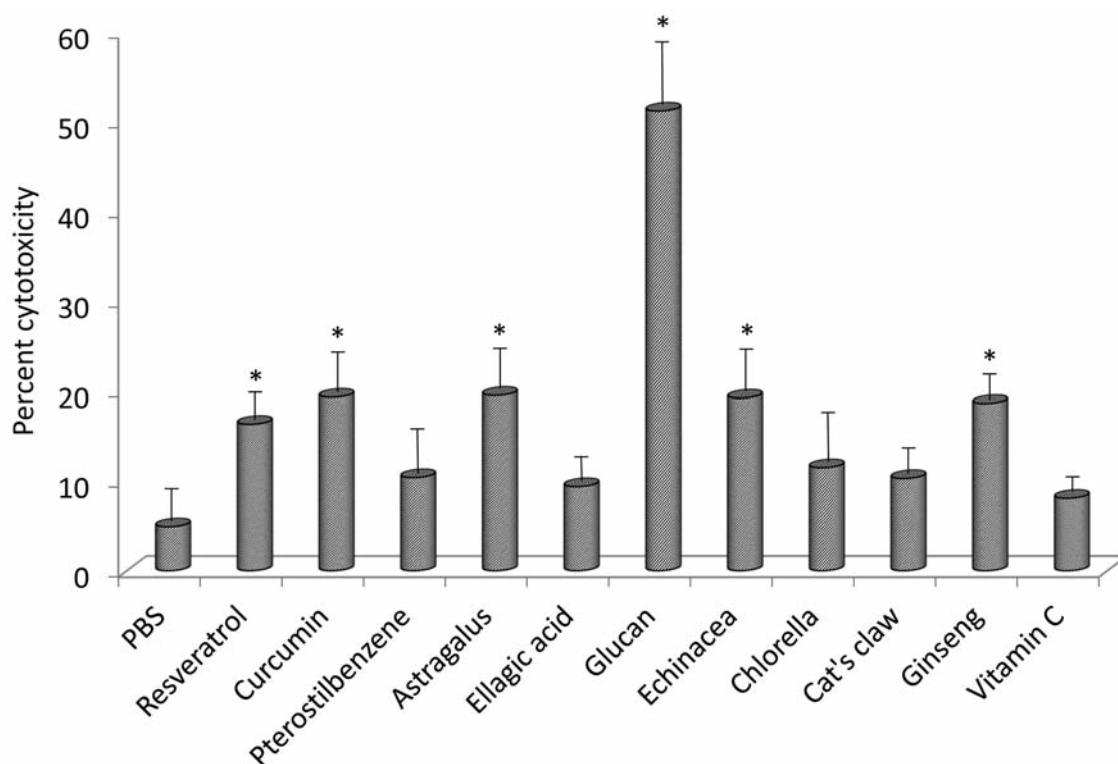


Figure 3. Effects of tested samples on NK cell cytotoxicity of YAC-1 cells. Different ratios of NK/target cells were tested for cytotoxicity in the presence or absence of  $\beta$ -glucans for 30 min at 37°C. The data points shown are mean values from three experiments using only 1:50 ratio. The differences were significant at  $p \leq 0.05$  level.

increase in oxygen consumption, hexosemonophosphate shunt activity, and production of active oxygen molecules during oxidative burst, which is necessary for the destruction of invading microorganisms. Numerous bioactive molecules were found to stimulate oxidative burst (23). However, five of the tested compounds failed (ellagic acid, *Echinacea*, chlorella, cat's claw and ginseng), the highest effects of glucan #300 were in agreement with previously published data (24).

Next, we studied the effects of various compounds on NK cell activation. Numerous modulators showed stimulation of cytotoxicity, namely resveratrol, curcumin, *Astragalus*, *Echinacea* and ginseng, but again, glucan was much more effective.

In addition to the direct effects on various types of immunocytes, the action of natural immunostimulators also causes synthesis and/or secretion of several cytokines, including IL-1, IL-2, IFN- $\gamma$  and TNF- $\alpha$ . Until now, only one glucan has been found not to stimulate any cytokine production (25). We focused on stimulation of IL-2 secretion by splenocytes. With a minimal production by unstimulated cells, it is not surprising that most modulators stimulated IL-2 secretion. The three most active of these were glucan, *Astragalus* and ellagic acid, which in the case of glucan and

*Astragalus* was in agreement with previous studies (4, 1). With *Echinacea*, we were not able to confirm older studies (26), which is most probably due to the significant differences among individual batches of *Echinacea*.

Antibody response is often an overlooked area of action by natural immunostimulators. However, some of the recent studies showed that at least some of the natural molecules strongly stimulate the antibody reaction either alone (18) or during vaccination (27). We found five molecules showing strong effects.

As most natural immunomodulators claim to help fight cancer, we evaluated effects of oral administration on the reduction of lung metastases. Our data showed that only resveratrol, curcumin, and glucan significantly lowered the number of metastases. The question of vitamin C and cancer remains controversial, and most studies showing positive effects use much higher doses *via* different routes of administration (28). Minimal effects of *Echinacea*, ginseng and *Astragalus* on tumor progression are in agreement with data as reviewed by Block and Mead (29).

Our study provides a uniform, although limited, survey of the immunomodulatory properties of eleven commonly used natural immunomodulators. Data presented in the present

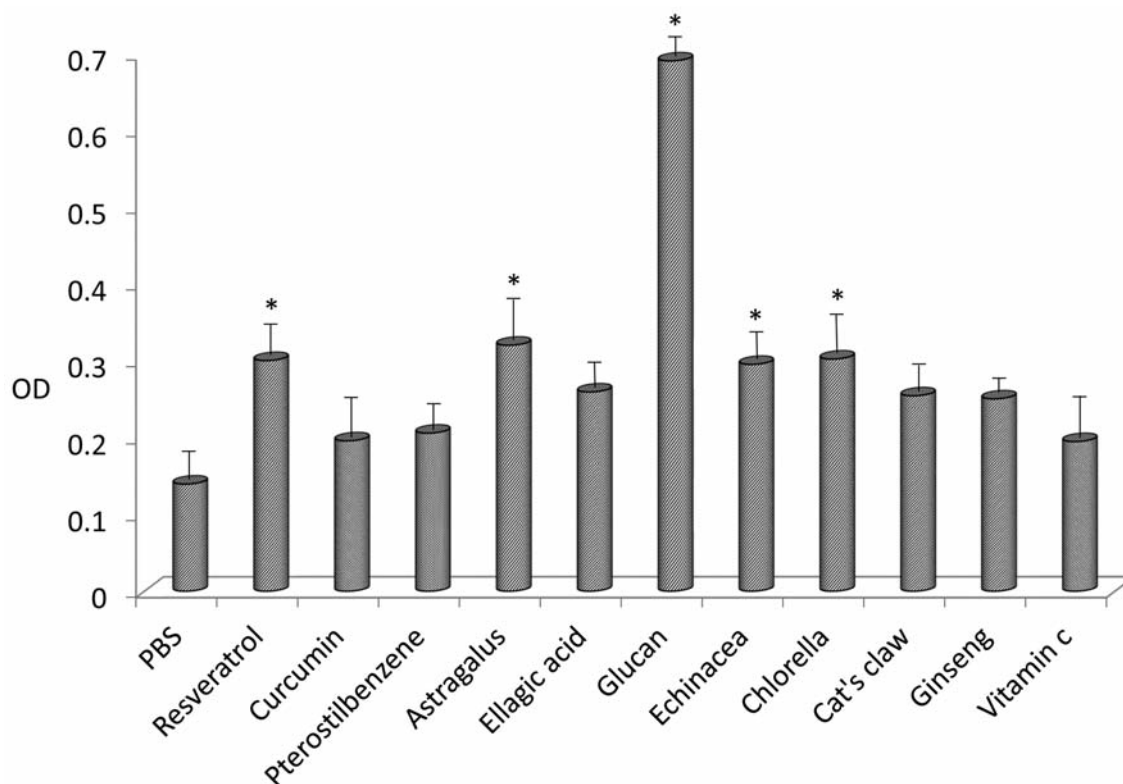


Figure 4. Effects of dietary supplementation on formation of antibodies against ovalbumin. Mice were injected twice (two weeks apart) with antigen and the serum was collected 7 days after the last injection. Level of specific antibodies against ovalbumin was detected by ELISA. \*Represents significant differences between control (ovalbumin alone) and samples at  $p \leq 0.05$  level. All experiments were performed in triplicate.

study demonstrated significant differences among individual types of immunostimulating agents. Where some of the products showed rather limited or even marginal immunostimulatory effects, some, such as cat's claw, vitamin C and pterostilbenzene had almost no activity at all. The only product with consistently high activities was glucan.

Several conclusions can be drawn; most of the commercial immunostimulating compounds have only very limited, if any, effects on the immune system including cancer. In addition, doses recommended on the label might not be sufficient, but no solid data on dosages exist with exception of glucan (1). And, glucan consistently showed the highest effects throughout the entire study. In view of the known synergy between glucan and vitamin C (30) and glucan and resveratrol (and vitamin C) (24), it may be possible to consider the combination of glucan with some of the herbal molecules tested in this study. However, more research on possible positive or negative effects of such combinations is necessary.

### Conflicts of Interest

No conflicts of interest exist for the Authors.

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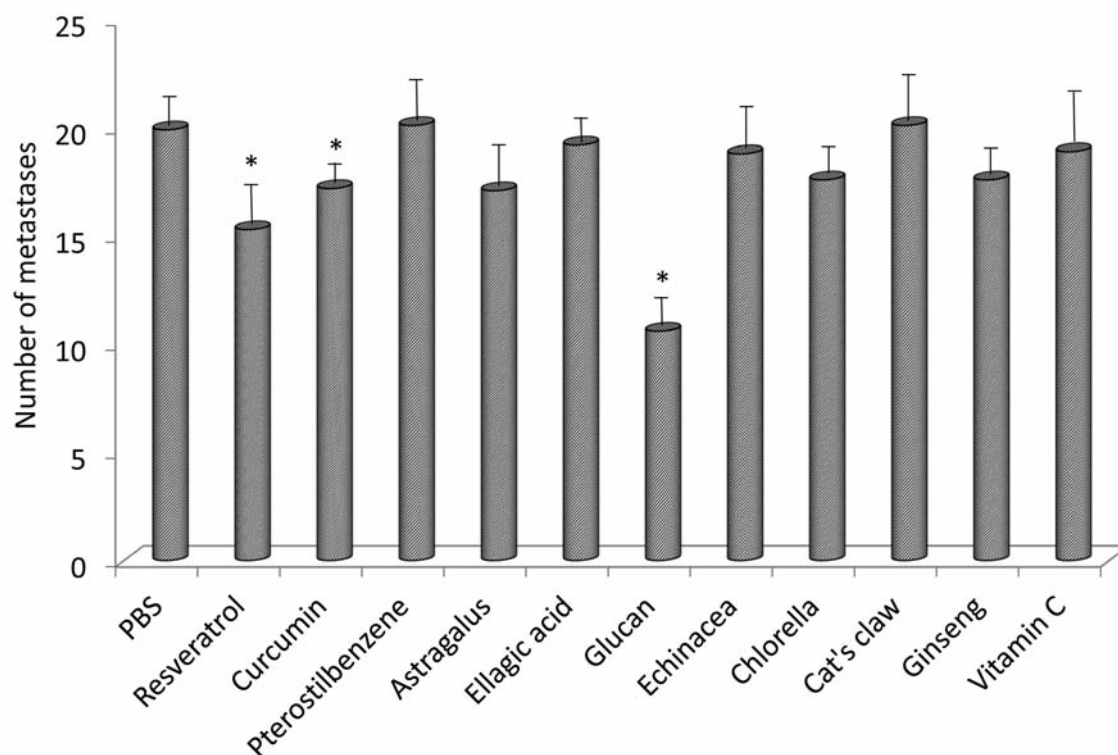


Figure 5. Effect of tested substances on lung cancer growth in cyclophosphamide (CY)-treated mice. CY (30 mg/kg) was injected into mice on day 8 of the inoculation of  $1 \times 10^5$  tumor cells. Other experimental groups obtained the daily injections of individual substances over 5 days starting 48 h after injection of CY. Individual substances were used at an 100  $\mu$ g/dose. Each value represents the mean  $\pm$  SD. \*Represents significant differences between the control and samples at  $p \leq 0.05$  level. All experiments were performed in triplicate.

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