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Evaluation of Widely Consumed Botanicals as Immunological Adjuvants

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Abstract

Background—Many widely used botanical medicines are claimed to be immune enhancers. Clear evidence of augmentation of immune responses *in vivo* is lacking in most cases. To select botanicals for further study based on immune enhancing activity, we study them here mixed with antigen and injected subcutaneously (s.c.). Globo H and GD3 are cell surface carbohydrates expressed on glycolipids or glycoproteins on the cell surface of many cancers. When conjugated to keyhole limpet hemocyanin (KLH), mixed with an immunological adjuvant and administered s.c. the magnitude of the antibody responses against globo H, GD3 and KLH depend largely on the potency of the adjuvant. We describe here the results obtained using this s.c. immunization model with 7 botanicals purported to have immune stimulant effects.

Methods—Groups of 5–10 mice were immunized with globo H–KLH or GD3–KLH mixed with botanical, saline or positive control immunological adjuvant, s.c. 3 times at 1 week intervals. Antibody responses were measured 1 and 2 weeks after the 3rd immunization. The following seven botanicals and fractions were tested: (1) H-48 (Honso USA Co.), (2) *Coriolus vesicolor* raw water extract, purified polysaccharide-K (PSK) or purified polysaccharide-peptide (PSP) (Institute of Chinese Medicine (ICM)), (3) Maitake extract (Yukiguni Maitake Co Ltd. and Tradeworks Group), (4) Echinacea lipophilic, neutral and acidic extracts (Gaia Herbs), (5) Astragalus water, 50% or 95% ethanol extracts (ICM), (6) Turmeric supercritical (SC) or hydro-ethanolic (HE) extracts (New Chapter) or 60% ethanol extract (ICM) and (7) yeast β -glucan (Biotec Pharmacon). Purified saponin extract QS-21 (Antigenics) and semi-synthetic saponin GPI-0100 (Advanced BioTherapies) were used as positive control adjuvants. Sera were analyzed by ELISA against synthetic globo H ceramide or GD3 and KLH.

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Results—Consistent significant adjuvant activity was observed after s.c vaccination with the Coriolus extracts (especially PSK), a 95% ethanol extract of astragalus and yeast β -glucan, and (to a lesser extent) Maitake. Antibodies against KLH in all cases and against globo H in most cases were induced by these botanicals. Little or no adjuvant activity was demonstrated with H48 or Echinacea extracts or the astragalus water extract. Experiments with GD3-KLH as immunogen confirmed the adjuvant activity of the Coriolus, yeast β -glucan and Astragalus extracts. While extraction with ethanol concentrated the active ingredients in astragalus, it had no impact on coriolus where the 90% ethanol precipitate and solute were equally active.

Conclusions—Some, but not all, botanicals purported to be immune stimulants had adjuvant activity in our model. PSK and astragalus were surprisingly active and are being further fractionated to identify the most active adjuvant components.

Keywords

Astragalus; Botanicals; Conjugate vaccine; Cancer vaccine; β -glucan; Immunological adjuvant; PSK; Saponin

INTRODUCTION

Many widely used botanicals are claimed to have immunostimulant effects, but clear evidence that they are able to augment immunological responses against defined antigens is lacking. Screening botanicals for immunomodulatory activity after oral ingestion is difficult due to unknowns such as bioavailability (depending in part on issues such as formulation and concomitant food intake), the amount of active ingredient in the botanical selected, optimal dose and appropriate assay. As a prelude to testing popular botanical medicines as immune modulators after oral ingestion we have chosen to test their immunological activity as immunological adjuvants, mixed with antigens and injected subcutaneously, using resulting antibody titers as read-out. We hypothesized that this would permit us to identify active botanicals and botanical fractions and to identify the active ingredient(s). Our preliminary investigations testing this hypothesis are described here. As the antigenic targets for vaccines against infectious diseases and cancer have become better defined and as our capabilities for synthesizing or extracting these antigens have improved, the need for potent immunological adjuvants or other approaches to augmenting their immunogenicity has become more urgent. A second goal of these studies was to identify new more potent or less toxic adjuvants of value for use in vaccines.

Seven widely used botanicals have been selected for initial study based on prevalence of use and reports describing immunomodulatory activity. Multiple samples are tested for some of these seven due to variations in extraction methods and suppliers. The seven botanicals selected for initial study are astragalus[1], coriolus[2,3], echinacea[3,4], H-48 (a formula consisting of ten herbs)[5,6], maitake[4,7], β -glucan of yeast origin[8] and turmeric[4,9]. The preparations tested vary in complexity from multiple botanicals as in H-48 to the more purified coriolus fractions PSK and PSP, and yeast β -glucan. Activity was tested in multiple experiments to limit sampling errors and, in addition, with or without a suboptimal dose of the potent semisynthetic saponin adjuvant GPI-0100.

When the breast cancer carbohydrate antigen globo H[10] or neuroectodermal cancer ganglioside GD3 are conjugated to KLH, mixed with an immunological adjuvant and administered s.c., the magnitude of the antibody responses against globo H and GD3 and against KLH depend largely on the potency of the adjuvant[11]. We test here whether botanicals mixed with the vaccine and injected subcutaneously could function as immunological adjuvants, and if so, whether this could be enhanced by further extraction and

purification. This mix of carbohydrate autoantigen and protein xenoantigen, weak immunogen and strong immunogen, reflects the range of antigens targeted in vaccines against cancer and infectious diseases, and the range of antigens encountered in life. We describe here results of our experiments with these seven botanicals mixed with globo H-KLH and GD3-KLH conjugates and administered s.c. to mice. We find that the coriolus extracts, 95% ethanol extract of astragalus and yeast β -glucan have potent adjuvant activity.

METHODS

Botanicals (see Table 1)

Astragalus was provided by the Institute of Chinese Medicine (ICM), Chinese University of Hong Kong. Three extracts of *Astragalus membranaceus* were prepared using water (Astragalus 1), 50% ethanol (Astragalus 2), and 95% ethanol (Astragalus 3) as solvents.

Coriolus was obtained from the ICM in three different forms: *Coriolus versicolor* raw water extract, purified polysaccharide-K (PSK), and polysaccharide-peptide (PSP). PSK (Krestin) was also obtained from Kureha Corp (Japan). In a final experiment, ICM PSK was fractionated in different concentrations of ethanol and the resulting precipitates (ppt) and soluble (S) fractions tested.

Echinacea was supplied by Gaia Herbs Inc. of Brevard, NC, in three different forms: Lipophilic extract of *Echinacea angustifolia* & *Echinacea purpurea* roots (Echinacea 1), neutral & weak acidic polysaccharides from *Echinacea purpurea* juice extract (Echinacea 2), and strong acidic polysaccharides from *Echinacea purpurea* juice extract (Echinacea 3).

H-48 is a combination of 10 herbal extracts supplied by the Honso Pharmaceutical Co. Ltd.

Maitake Gold 404 and Maitake (*Grifola frondosa*) mushroom extracts were produced by Yukiguni Maitake Co. Ltd, Japan and procured from Tradeworks Group of Brattleboro, VT.

Turmeric was obtained from New Chapter Inc. of Brattleboro, VT. It was extracted from the root of *Curcuma longa* in two forms, a supercritical (SC) extract (Turmeric 1) and a hydroethanolic (HE) extract (Turmeric 2). The ICM also provided a sample of turmeric extracted using 60% ethanol (Turmeric 3).

Yeast Beta glucan (BBG500) was provided by Biotec Pharmacon.

In the case of extractions from raw botanicals, authenticity of botanicals was confirmed by tests described in specific pharmacopeial monographs and compared with an authenticated reference specimen. Voucher specimens of all botanicals studied here have been deposited either at the manufacturers' archive or at the Hong Kong Herbarium.

Vaccine production

Globo H hexasaccharide (molecular weight 1055Da) was synthesized and conjugated to KLH (8×10^6 Da) essentially as previously described[9]. Globo H/KLH molar ratios in the conjugate ranging between 500/1 and 800/1 were used in these studies. The globo H-KLH used here was purchased from Optimer Pharmaceuticals (San Diego, CA) who synthesized it under contract. It was provided as globo H ceramide for use as target in ELISA assays and as globo H-KLH conjugate for vaccine production. GD3 was extracted from bovine buttermilk and purchased from Matreya Inc. (Pleasant Gap, PA), and conjugated to KLH as previously described[13]. The GD3/KLH molar ratio in the conjugate was 950/1. KLH for vaccine production and serological target was purchased from Sigma. GPI-0100 and QS-21 were used as positives controls. GPI-0100 was provided by Galenica Pharmaceuticals, Inc. (now Hawaii Biotech,

Inc., Aiea, HI) and used at a dose of 50–100µg as positive control immunological adjuvant or 10µg when used in combination with other botanicals. QS-21 was provided by Aquila Biopharmaceuticals, Inc. (now Antigenics Inc., New York, NY) and used as positive control at a dose of 10µg. The botanicals were tested at doses between 200µg and 2mg in individual experiments with all tested at a dose of at least 500µg.

Vaccine administration

Six-week-old female C57Bl/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Groups of 5 mice were immunized s.c. three times at 1 week intervals with globo H-KLH containing 3–5µg of globo H mixed with either various botanicals (see Table 2) in 0.1 ml saline, 10mcg QS-21 or 50–100µg GPI-0100 as positive controls or with a 10µg dose of GPI-0100 plus various botanicals.

Serological Assays

Mice are bled from the retro-orbital sinus under general anesthesia seven days after the third immunization for ELISA, and sera frozen for future testing.

ELISA

Enzyme linked immunosorbent assays are performed as described previously[12,13]. The target antigens are globo H ceramide, GD3 or KLH. To determine the titers of antibodies, ELISA plates are coated with antigen, generally at 0.1mcg/well. Serially diluted sera in 1% HSA in PBS are added to wells of the coated plate and incubated for 1h at room temperature. Goat anti-mouse IgM or IgG conjugated with alkaline phosphatase (Southern Biotechnology, Birmingham, AL) serve as second antibodies. The antibody titer is defined as the highest serum dilution showing an absorbance 0.1 or greater over that of normal sera. A response is considered positive by ELISA if the titer of reactivity increased from undetectable pretreatment to at least 1:40 after vaccination, or if detectable pretreatment, by 8-fold.

Statistical Analysis

For each experimental run, results in serological assays for treated mice were compared to the no-adjuvant or low dose GPI-0100 controls using the Mann-Whitney test. The mean and standard error of the difference between ranks from different experimental runs were then combined using fixed effect meta-analysis. All statistical analyses were conducted using Stata 9.2 (Stata Corp., College Station, TX).

RESULTS

ELISA results after immunizations

Eight separate experiments were conducted with the globo H-KLH vaccine, with all extracts tested in at least two experiments (see Table 1 for botanicals tested, sources and dose range). Antibodies induced against KLH were almost exclusively IgG while those induced against globo H were almost exclusively IgM, as expected. In addition, as expected, antibodies induced against KLH were more sensitive indicators of adjuvant activity than those induced against globo H. Relevant serologic titers from 1 of the 8 experiments using the globo H-KLH vaccine is shown in Table 2 and the results of all 8 individual experiments and a meta-analysis of the pooled data are summarized in Table 3.

Coriolus extracts induced significantly elevated antibody titers against KLH, with PSK having the most activity, both as adjuvant alone and when added to a low dose of GPI-0100. PSK also induced significantly elevated antibody titers against globo H in 3 of 5 experiments. Vaccines containing 95% ethanol extract of Astragalus induced consistent antibodies against KLH

(overall $p < 0.001$) alone and in 1 of 3 experiments against globo H ($p < 0.05$), with the 50% extract showing intermediate activity against KLH ($p = 0.03$ in one experiment). Yeast β -glucan alone or with GPI-0100 resulted in significantly increased antibody titers against KLH in all experiments (overall $p < 0.001$) and against globo H in 2 of 4 experiments (overall $p = 0.002$ and < 0.001 respectively). Turmeric 2 and 3 each induced increased antibody titers against KLH ($p = 0.05$ and 0.002) or globo H ($p = .05$) in one experiment but turmeric of ICM origin also resulted in significantly decreased antibody titer in 1 experiment ($p = 0.04$). None of the other study extracts (Echinacea, Maitake, H-48, or Astragalus water extract) demonstrated consistent adjuvant activity against KLH or globo H in individual experiments, though in the meta-analysis shown in Table 3 Maitake significantly increased antibody titers against KLH. The positive control extract GPI-0100 was strongly positive in all experiments for induction of antibodies against KLH and globo H. Mice were weighed and inspected at 24 and 48 hours and 7 days after vaccination. Loss of vigor or coat grooming or more than 5% of weight were considered evidence of toxicity. Despite doses ranging between 200mcg and 2mg per vaccination, no obvious toxicity was detected with any of these botanicals. The dose administered was limited only by solubility and the volume limitation for subcutaneous vaccinations in mice, 0.2ml.

To confirm that the immune response against a different vaccine would also be enhanced by these botanicals, a final experiment using a GD3-KLH vaccine was conducted (see Table 4). Here the natural glycolipid GD3 ganglioside was substituted for the synthetic carbohydrate globo H conjugated to KLH. Astragalus and especially PSK were again active. PSK was also fractionated in the same general way as astragalus had been, but unlike astragalus where activity was greatly enhanced by extraction with 95% alcohol, alcohol extraction of PSK had no clear impact on the adjuvant potency of the resulting PSK fractions.

DISCUSSION

We have screened 7 separate botanicals all claimed to have immune enhancing activity. Adjuvant activity in our model was found for some, but not all extracts. Our most striking finding was the potency of the four *Coriolous versicolor* extracts. These extracts are known to be rich in β -glucans, the presumed most active ingredient. While all four *Coriolous* extracts had activity over a wide range of doses, PSK supplied by ICM was the most active. In some experiments, this equaled or surpassed reactivity seen with an equal weight of purified β -glucan of yeast origin. The potency of PSK as an adjuvant was confirmed with a second conjugate vaccine as well, GD3-KLH. Since PSK contains less than 25% β -glucan polysaccharides by weight, this suggests that β -(1,4) backbone with β -(1,3) and β -(1,6) glucocytic linkages characteristic of *Coriolous versicolor* β -glucans (as apposed to the β -(1,3) backbone with β -(1,6) linkages characteristic of yeast β -glucans) may have unique potency. This could also be a consequence of the protein core in the protein bound polysaccharides which characterize PSK and PSP[14]. Both PSK and PSP have been described to have wide ranging impact on WBC count, phagocytic functions, T-helper cell activation, T-cell function and cytokine production when tested either *in vitro* or administered *in vivo*[15,16]. Neither have been used as immunological adjuvant mixed with vaccine and there has been no attempt to link particular structures in PSK or PSP with adjuvant activity. Our findings suggest the possibility that β -glucans in *Coriolous* extracts are uniquely potent as immunological adjuvants, a possibility we are pursuing initially by further fractionating PSK and testing the individual fractions. An initial attempt at fractionating PSK demonstrated that ethanol extraction, the method that worked well for astragalus, had no detectable impact on PSK.

We have identified saponins and in particular the saponin fraction QS-21 and the semisynthetic saponin mix GPI-0100 as uniquely potent immunological adjuvants when mixed with conjugate vaccines containing glycolipids or peptides chemically conjugated to keyhole limpet

hemocyanin (KLH)[11,17]. The maximal doses of QS-21 used in mice (20µg) and patients (100µg) were selected for minimal weight loss in mice and acceptable local erythema/induration and systemic flu-like symptoms in patients[18]. The quest continues for immunological adjuvants with more potent adjuvant activity and more limited local and systemic toxicities. While most studies have been focused on *Quillaja saponaria* saponins, there are many additional botanicals expressing other saponins. A possible case in point is *Astragalus membranaceus* which we demonstrate here to have significant adjuvant activity in the 95% ethanol fraction.

A. membranaceus is a well known traditional Chinese medicinal plant used widely for a variety of indications. The main constituents of the *A. membranaceus* root are polysaccharides, saponins and flavonoids. The cyclolanostane-type saponins have been identified as the most active ingredient with significant lymphocyte proliferation and immunostimulatory activities [19]. Using the hemolytic activity of saponins from various botanicals as a surrogate for toxicity, the hemolytic and immunological adjuvant activities of a series of saponin rich botanicals have been compared [20,21]. Saponins were extracted using 70% ethanol, ether and n-butanol. Fifty, 100 and 200mcg had comparable activity, augmenting antibody titers against ovalbumin by approximately 10 fold. Saponins of *A. membranaceus* were identified as having lower hemolytic activity and stronger adjuvant activity than saponins in the other botanicals tested. We demonstrate here that the 95% ethanol extract of *A. membranaceus* saponins (the saponin rich fraction) also augments antibody responses against weak glycolipid autoantigens such as globo H and strong xenoantigens such as KLH. The 95% ethanol extract tested here consists of approximately 40% saponins. At least 15 such separate saponins have been identified in *A. membranaceus* saponins and the chemical structures defined[21]. Four of these (astragalosides I-IV) are commercially available. While purifying the other 11 individual saponins from extracted saponins for use as adjuvants would be difficult, the recent description of the total chemical synthesis of QS-21[22] raises the possibility that these remaining individual *A. membranaceus* saponins could be synthesized. The described low hemolytic activity of these saponins and potent adjuvant activity suggest that this would be fruitful. It is expected that even among this family of saponins some would have greater or lesser toxicity and that this might be distinct from their adjuvant activity.

It has been claimed by proponents of botanical medicine that crude or simple extracts of botanicals or mixtures of botanicals have unique potencies that can not be replicated or exceeded by any of the individual chemical constituents. Such a claim is a testable hypothesis, at least with regard to the saponins in the 95% ethanol extract of astragalus and β-glucans in PSK. This is especially relevant in the case of immunological adjuvants where efficacy frequently varies with dose but dose administered is limited by toxicity. The clear superiority of QS-21 over cruder fractions or unfractionated *Quillaia saponaria* saponins was largely a consequence of the superior immunogenicity/toxicity ratio. It may be that saponins are not the only immunologically active components in the 95% ethanol fraction or that a mixture of these saponins will prove superior to any one, but again this should be testable. The same applies to the unexpected potency of the β-glucans in PSK. In addition, if the goal is optimizing the adjuvant activity of botanicals, identification of the most active components is a necessary first step to developing relevant markers for improved methods of extraction and for confirming relevant batch to batch consistency. The studies described here are our first steps in these directions.

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The abbreviations used are

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; PBS, phosphate buffered saline.

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Table 1

Botanicals Tested with Globo H-KLH vaccines, Source, Dose, and Active Ingredients

Botanical Extract	Source	Dose	Probable Active Ingredients
Coriolus	<i>Coriolus versicolor</i> Water Raw extract, ICM	500µg	
PSK	<i>Coriolus versicolor</i> Protein bound polysaccharides, ICM	0.25–2mg	
PSK	50% Ethanol precipitate, ICM	2 mg	β-glucan protein linked β-1,4 backbone β-1,3 and β-1,6 side chains
PSK	80% Ethanol precipitate, ICM	2 mg	
PSK	80% Ethanol soluble, ICM	2 mg	
PSP	<i>Coriolus versicolor</i> Protein bound polysaccharides, ICM	250µg	
PSK	<i>Coriolus versicolor</i> Protein bound polysaccharides, (Krestin) Kureha	250µg	
Maitake	Maitake Gold 404 Extract, Tradeworks Group, Inc. (Yukiguni)	250µg	β-glucan β-1,6 backbone β-1,3 side chains
H-48	All Inclusive Great Tonifying Formula, Honso Pharmaceutical Co, Ltd.	250µg, 1mg	
Echinacea 1	<i>Echinacea angustifolia</i> & <i>Echinacea purpurea</i> root 05090601, Gaia	250µg	Polysaccharides
Echinacea 2	<i>Echinacea purpurea</i> (Neutral & weak acidic polysaccharides) 05090602, Gaia	250µg	
Echinacea 3	<i>Echinacea purpurea</i> (Strong acidic polysaccharides) 05090603, Gaia	250µg	
Turmeric 1	Turmeric Supercritical (SC) Extract <i>Curcuma longa</i> D502401, New Chapter	100µg-2mg	Curcuminoids
Turmeric 2	Turmeric Hydro-ethanolic (HE) Extract <i>Curcuma longa</i> D502402, New Chapter	100µg-2mg	
Turmeric 3	Turmeric Hydro-ethanolic (HE) Extract <i>Curcuma longa</i> , ICM	0.5–2mg	
Astragalus 1	<i>Astragalus membranaceus</i> – Water Extract 05 1105 01, ICM	500µg	Saponins
Astragalus 2	<i>Astragalus membranaceus</i> – Ethanol 50% Extract 05 1105 02, ICM	500µg	
Astragalus 3	<i>Astragalus membranaceus</i> – Ethanol 95% Extract 05 1105 03, ICM	0.5–2mg	
Yeast β-glucan	Yeast - Biotec Pharmacon	100µg 0.25–2mg	β-glucan β-1,3 backbone β-1,6 side chains
Positive Controls			
QS-21	<i>Quillai Saponaria</i> – fraction 21, Antigenics Inc.	10–20µg	
GPI-0100	<i>Quillai Saponaria</i> – semisynthetic, Hawaii Biotech	100µg	

Table 2
ELISA Antibody Titers after Vaccination with Globo H-KLH plus or minus Botanicals

Globo H-KLH Vaccine Plus:	Globo H IgM	KLH IgG
No adjuvant	200, 400(2) *, 800(6), 1600	400(2), 800(2), 1600, 3200(5)
Turmeric 2 500µg	800(2), 1600(2), 6400	6400(3), 12800, 51200
Turmeric 2 2 mg	1600(4), 3200	12800, 25600, 51200(3) **
PSK 500µg	800(3), 1600, 3200(2)	25600(3), 51200, 204800
PSK 2 mg	800, 1600(2), 3200(2)	51200, 102400(2), 204800(2)
Astragalus 3 500µg	800(2), 1600, 6400, 12800	6400, 12800(2), 25600, 51200
Astragalus 3 2mg	800(2), 3200, 6400, 25600	3200, 12800, 25600, 51200(2)
Yeast β-glucan 500µg	1600(2), 3200, 12800, 25600	12800, 102400(3), 204800
Yeast β-glucan 2 mg	1600, 3200, 6400, 12800, 25600	51200, 102400, 204800, 409600(2)
QS-21 10µg	6400(2), 12800(2), 25600	409600, 819200(2), 1638400(2)

* Reciprocal antibody titer. Number in parentheses is the number of mice with that titer, if more than 1.

** Groups in bold are significantly ($p < 0.05$) higher than the no adjuvant control group.

Table 3
Summary of ELISA Results of Individual Experiments and of Meta-analysis

Botanical Extract	Proportion of positive experiments* compared to respective no adjuvant negative controls, and meta-analysis p value					
	No GPI-0100			Plus GPI-0100 (10 µg)		
	Globo H	KLH		Globo H	KLH	
Coriolus	0/1					
PSK	3/5	0.9	1/1	0.002	0/1	0.7
PSP	0/1	<0.001	4/5	<0.001	0/1	0.3
Maitake	1/4	0.6	1/1	0.002	0/1	0.5
H-48	0/1	0.6	1/4	0.03	0/1	0.15
Echinacea 1	0/1	0.6	0/1	0.7	0/1	0.7
Echinacea 2	0/1	0.09	0/1	0.4	0/1	0.5
Echinacea 3	0/1	0.02***	0/1	0.8	0/1	0.02
Turmeric 1	0/1	0.4	0/1	0.9	0/1	0.1
Turmeric 2	0/1	0.3	0/1	0.07	0/1	0.2
Turmeric 3	1/2	0.06	1/2	0.4	0/1	0.9
Astragalus 1	0/1	0.04***	1/1	0.002	0/1	0.5
Astragalus 2	0/1	0.9	0/1	0.4	0/1	0.08
Astragalus 3	1/3	0.7	1/1	0.03	0/1	0.02***
Yeast β-glucan	2/4	0.002	4/4	<0.001	0/1	0.4
GPI-0100 (100 µg)	5/5	<0.0001	5/5	<0.001	0/1	0.9
QS-21 (10 µg)	1/1	<0.002	1/1	<0.002	0/1	0.9
					1/2	0.012
					4/4	<0.001

* Positive experiment = p value for higher titers induced by indicated botanical compared to no adjuvant or low dose GPI-0100 controls <0.05.

** Compared to mice receiving no adjuvant

*** Botanical induced antibody titers that were significantly lower than the controls in these individual experiments.

Table 4

ELISA Antibody Titers after Vaccination with GD3-KLH plus or minus Botanicals

GD3-KLH Vaccine		GD3	KLH
Plus		IgM [*]	IgG
No adjuvant		0(3), 20(2)	0(2), 6400(3)
PSK	2mg	0(2), 40, 80(2)	6400, 25600, 102400(2), 409600
PSK 50% Ethanol ppt ***	2mg	40(2), 80(3) ^{**}	6400, 25600, 102400(2), 409600
PSK 80% Ethanol ppt	2mg	0(2), 20(2), 160	6400(2), 25600, 102400(2)
PSK 80% Ethanol sol	2mg	0(4), 20	1600, 6400, 25600(2), 102400
Astragalus 95% Ethanol sol	2mg	0(5)	6400, 25600(3), 102400
GPI-0100	50µg	40, 160, 320(2), 640	25600, 102400, 409600, 1638400(2)

* Reciprocal antibody titer. Number in parentheses is the number of mice with that titer, if more than 1.

** Groups in bold are significantly (p<0.05) higher than the no adjuvant control group.

*** Extraction in the indicated percent Ethanol was performed and the precipitate (ppt) and solute (sol) tested.