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(54) Title: A PHARMACEUTICAL COMPOSITION USEFUL AS AN IMMUNOMODULATING AGENT AND A PROCESS FOR THE PREPARATION THEREOF

(57) Abstract: Abstract The present invention is directed towards immunity enhancing activity in Chlorophytum (Safed Musli) due to a novel gel forming aqueous extract of Chlorophytum (Safed Musli) or its partially or completely purified carbohydrate (polysaccharide), and thereby Chlorophytum or its aqueous extract or gel or carbohydrates form a useful and safe health promoting substances that can be consumed alone or as additive in food, food products etc. or as functional food, dietary substances and nutraceutical. Further, the bioactive substance compositions comprising novel carbohydrates particularly but not limited to Chlorophytum or Musli glucomannan (MGM) discovered herein from the plant. Thus the plant, plant aqueous extract, partially purified or completely purified carbohydrate products and its associated processes, properties, uses and applications in any mode form the components of this invention. The novel bioactive polysaccharide/ heteroglycan of the plant invented herein preparations are positive biomodulators for mammalian physiology to jack-up its immune defence in normal as well as diseased and ailing individuals. These compositions if consumed orally or otherwise would improve the well being of mammals suffering from or susceptible to a variety of bodily inefficient functions or disorders in general and immune system related ones in particular. The invention allows alternate designation of Chlorophytum as Indian Konjac.



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**A PHARMACEUTICAL COMPOSITION USEFUL AS AN
IMMUNOMODULATING AGENT AND A PROCESS FOR THE
PREPARATION THEREOF**

5 FIELD OF THE INVENTION

The present invention relates to a pharmaceutical composition useful as an immunomodulating agent and a process for the preparation thereof. In general it is directed toward use and applications of *Chlorophytum* (Safed Musli) as potential immunomodulatory, health promoting, nutraceutical herb. The invention particularly
10 relates to a process for isolating immunomodulatory carbohydrate/polysaccharide including but not limited to *Chlorophytum* Musli Glucomannan (MGM). The present invention further relates to purified compositions of the plant aqueous extracts. In addition, the current invention is directed toward a botanical substance from Safed Musli for immune-response enhancement and curing diseases and ailments related to
15 immunity by prevention of suppression of immunity for any purpose or in any way through the use of the plant in any physical or chemical form.

BACKGROUND OF THE INVENTION

The plant genus *Chlorophytum* belongs to the family Liliaceae. The genus
20 *Chlorophytum* is represented by about 175 valid taxa of rhizomatous herbs distributed predominantly in the tropical and parts of the world and some of them cultivated for ornamental flowers. Its probable centres of origin and diversification lie in tropical and subtropical Africa and Asia where where 85% of the species are found [PC Bordia, A. Joshi and M.M. Simlot 1995 Safed Musli In Advances in Horticulture volume 11
25 (Medicinal and Aromatic Plants) Editors: K.L. Chadha and Rajendra Gupta, pp.430-450, Malhotra Publishing House, New Delhi, India].

Genus-specific features of *Chlorophytum* are fleshy roots/root tubers (fasciculated roots) and attractive green leaves. Leaves are radical, spirally imbricate at the base with acute apex spreading horizontally. The flowers are usually white with ornamental
30 attraction. The plants are monoecious or dioecious herbs, bushes or trees with varied growth habits exhibiting stalks or branches differentiated into two to three different types [PC Bordia, A. Joshi and M.M. Simlot 1995 Safed Musli In Advances in Horticulture volume 11 (Medicinal and Aromatic Plants) Editors: K.L. Chadha and Rajendra Gupta, pp.430-450, Malhotra Publishing House, New Delhi, India].

Typical phytochemical representatives of *Chlorophytum* species believed to be active ingredients so far are the low molecular weight terpenoidal phytochemicals like glycosides/saponins of putatively steroidal/spirostanol skeletal molecules like hecogenin [PC Bordia, A. Joshi and M.M. Simlot 1995 Safed Musli In Advances in Horticulture volume 11 (Medicinal and Aromatic Plants) Editors: K.L. Chadha and Rajendra Gupta, pp.430-450, Malhotra Publishing House, New Delhi, India; S.Mimaki, T. Kanmoto, Y. Sashida, A. Nishino, Y. Satomi and H. Nishino Steroidal saponins from the underground parts of *Chlorophytum comosum* and their inhibitory activity on tumor promoter-induced phospholipids metabolism of HeLa cells, Phytochemistry 41: 1405-1410, 1996; X-C Li, D-Z Wang and C-R Yang, Steroidal saponins from *Chlorophytum malayense*, Phytochemistry, 12: 3893-3898, 1990; S-X Qui, X-C li, Y. Xiong, Y. Dong, H. Chai, N.R. Farnsworth, J.M. Pezzuto and H.H.S. Fong, Isolation and characterization of cytotoxic saponin chloromaloside A from *Chlorophytum malayense*, Planta Medica 66: 587-590, 2000].

Particularly amongst the species used as Safed Musli, *Chlorophytum arundinaceum* and *Chlorophytum birivillianum* are used in Ayurveda and folk medicines as tonic for sexual weakness and aphrodisiac drug. However, there is no specific work on any of its chemical ingredients which may be responsible for its medicinal significance. No chemical substance of the herb is known that may influence spermatogenesis, spermatorrhoea and chronic leucorrhoea. Even effectiveness of these applications, has not been proven scientifically. Sapogenins like stigmasterol, sarsasapogenin, togogenin, neogitogenin and tokorogenin have been shown to be minor constituents (0.0012%, 0.0003%, 0.0003%, 0.0003%, 0.0005%, respectively on dry weight basis have been isolated from *Chlorophytum arundinaceum* (M. Tandon and Y.N. Shukla, Sapogenins from Asparagus adscendens and *Chlorophytum arundinaceum* J. Indian Chemical Society 69: 893, 1992). Also, some other low molecular weight compound slike bibenzyl xylosides and disubstituted tetrahydrofuran have been detected in the tubers of the species (M. Tandon, Y.N. Shukla and R.S. Thakur, 4-hydroxy-8, 11-oxidoheneicosanol and other constituents from *Chlorophytum arundinaceum* roots, Phytochemicstry 31: 2525-2526, 1992; M. Tandon, and Y.N. Shukla, A bibenzyl xyloside from *Chlorophytum arundinaceum*, Phytochemistry 32: 1624-1625, 1993)..

No phytochemical investigation on Safed Musli (*Chlorophytum borivillianum*) is available so far in the literature. Only general analysis limited to gross gravimetric estimation of non-descript saponins of tubers of some Indian accessions has been done. [PC Bordia, A. Joshi and M.M. Simlot 1995 Safed Musli In Advances in Horticulture volume 11 (Medicinal and Aromatic Plants) Editors: K.L. Chadha and Rajendra Gupta, pp.430-450, Malhotra Publishing House, New Delhi, India] . No *in vivo* or *in vitro* biological activity data for the tubers and active constituents of the species particularly polymeric carbohydrates of the herb are in public domain..

There is a great need to, both, treat or counter growing human population with immuno-response deficiency due to poor body physiology or as an accomplice of disease, infections and their therapies. Synthetic and natural polymeric systems in general and hydrophilic ones in particular are of interest for several pharmacological applications. Mitogenic, comitogenic and immunomodulatory activities of crude polysaccharides of *Salvia officinalis* has been reported (P. Capek and V. Hribalova, Phytochemistry 65: 1983-1992, 2004). As concerns glucomannans, Konjac Glucomannana (KGM) are popular. KGM is extracted from the tubers of *Amorphophallus konjac*. KGM is beneficial to the health of human beings and its ability to cure some diseases is well documented (W. Fang and P. Wu Variation of Konjac glucomannan, KGM from *Amorphophallus konjac* and its refined powder in China, Food Hydrocolloids 18: 167-170, 2004). Polysaccharides are unique in plant specific manner as well as monomeric composition in proportion and sequence are important with respect to biological properties as well as other physical properties (like gelation, colloids) that comprehensively ascribe them wide and specific utilities as materials useful in food, pharmaceutical, cosmetic and related biomedical industries.

There is increasing interest in use of herbals for the maladies of immuno-response deficiency or environmental or otherwise suppression of local and systemic immunity, with scientifically well-demonstrated effectiveness and correlated with chemical(s) or substances contained in the botanical biomass, extract, or partially or completely purified substances there from and products thereof. This issue as related to *Chlorophytum/safed* Musli has been addressed by the listed claims. Following embodiments further explain reiterate the subject matter.

Thus, the invention relates to the use of *Chlorophytum* (Safed Musli) or its one or more polyglyco-component(s) or substances or mixtures of substances obtained therefrom for the prevention or treatment of immunity related problems, risks, disorders, ailments and diseases and secondary consequences thereof.

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OBJECTS OF THE INEVNTION

The main object of the present invention is thus to provide a process for the isolation of bioactive polysaccharides from *Chlorophytum sp* (Safed musli).

Another object of the present invention is to provide a pharmaceutical composition useful as an immunomodulating agent comprising of isolated polysaccharide fractions from the tubers/ fingers (fasciculated roots) of the plant *Chlorophytum borivillianum*.

Still another object of the present invention is to provide *Chlorophytum sp.*(Safed musli) as an immunomodulating agent comprising upto 45% (w/w) isolated polysaccharides, 7 to 10 % (w/w) proteins and 40 to 50% (w/w) of other constituents such as fiber, cellulose, pectin, hemicellulose and free sugars.

Yet another object of the present invention is to provide a pharmaceutical composition having humorral immunomodulatory activity greater than levamisole, a positive control employed in the test systems for immunomodulatory activity.

A further object of the present invention is to provide a herbal non-toxic pharmaceutical composition useful as an immunomodulator.

Yet another object of the present invention is to provide a simple and easy method for the extraction of water soluble and hydro-suspension polysaccharides from safed musli.

SUMMARY OF THE INVENTION

The invention relates to the use of *Chlorophytum* or its aqueous component(s) ['water extractable easily water soluble polysaccharides', 'water extractable hydro-suspension polysaccharides' glucomannan polysaccharides' etc.], or substances or mixtures of substances obtained therefrom for the health benefits with respect to immune-enhancement and immunomodulatory properties associated with the polysaccharides/MGM amassed in the herb. The immune enhancing ability of the *Chlorophytum* or its extracts (to 'water extractable easily water soluble polysaccharides', 'water extractable hydro-suspension polysaccharides' glucomannan MGM polysaccharides' as embodied in the claims or its polymeric carbohydrates

including but not limited to 'water extractable easily water soluble polysaccharides', 'water extractable hydro-suspension polysaccharides' MGM extract is preferably determined by performing *in vivo* assays.

Furthermore, the invention relates to the use of *Chlorophytum* or its aqueous components or substances or mixture of substances obtained therefrom for the production of nutraceutical, dietary supplement, edible additives, pharmaceutical composition for the health benefits like immune strength, antiglycaemic etc. drawn from its novel polysaccharides ['water extractable easily water soluble polysaccharides', 'water extractable hydro-suspension polysaccharides' glucomannan polysaccharides' etc.] and prevention or treatment of diseases caused by immune-suppression (like radiation induced skin cancer).

Furthermore, the invention relates to the use of *Chlorophytum* or its aqueous components or substances or mixtures of substances obtained therefrom for making the immune system potentiating and in the preparation of anti-glycaemic formulations, nutraceuticals, foods, functional foods, dietary supplements and food processing ingredients.

Accordingly, the present invention provides a process for the isolation of bioactive polysaccharides from *Chlorophytum sp* (Safed musli) comprising the steps of :

- [a] homogenizing or otherwise solvation of the *Chlorophytum borivillianum* tissue or biomass in aqueous medium to obtain a supernatant;
- [b] adding 4 volumes of ethanol to the supernatant of step [a] to precipitate the polysaccharides;
- [c] dissolving the precipitated polysaccharide obtained from step [b] in water under stirring followed by filtration to obtain a filtrate and a residue;
- [d] suspending the residue obtained from step [c] in water to obtain water extractable hydro-suspension polysaccharide (Bio-RSIF);
- [e] simultaneously, treating the filtrate of step [c] with known deproteinization agents to precipitate proteins and obtain a deproteinized supernatant;
- [f] subjecting the supernatant obtained from step [e] to ethanol precipitation to obtain a precipitate;

[g] dissolving the precipitate obtained from step [f] in water to get water extractable easily water soluble polysaccharide fraction (Bio-RS1D).

The invention further provides a pharmaceutical composition useful as an immunomodulating agent comprising isolated polysaccharide fractions from the plant *Chlorophytum borivillianum* consisting of water extractable easily water soluble polysaccharides and water extractable hydro-suspension polysaccharides either alone or in combination optionally along with pharmaceutically acceptable excipients.

Also provided is use of the plant *Chlorophytum sp.*(Safed musli) as an immunomodulating agent comprising upto 45% (w/w) isolated polysaccharides, 7 to 10 % (w/w) proteins and 40 to 50% (w/w) of other constituents such as fiber, cellulose, pectin, hemicellulose and free sugars.

In an embodiment of the present invention, the extraction may be carried out at any temperature upto boiling point of the extraction medium.

In another embodiment, the ethanol precipitated carbohydrates may be redissolved in normal or alkaline water and deproteinized.

In another embodiment, deproteinization may be carried out by precipitation with TCA, sodium acetate or by other routine way of selective precipitation of proteins.

In another embodiment, the deproteinized supernatant may be subjected directly to ethanol precipitation for sedimenting carbohydrate or after its desalting by dialysis or otherwise.

In another embodiment, the deproteinized polysaccharide preparation may be partially or completely purified through ion-exchange chromatography and/or size exclusion chromatography.

In another embodiment, the yield of bioactive polysaccharide fractions is 8% w/w for 'water extractable easily water soluble polysaccharides' (Bio-RS1D) and 15% w/w for 'water extractable hydro-suspension polysaccharides' (Bio-RS1F).

In another embodiment, the fraction is isolated from *Chlorophytum borivillianum*.

In another embodiment, the plant tissue/biomass is preferably aerial or tubers/ fingers (fasciculated roots) or mixtures thereof.

In another embodiment, the plant tissue/biomass is extracted or used fresh or dry and may be native or peeled or in any other physical form.

In another embodiment, the dose of the composition is 50 to 100mg/Kg body weight administered orally for a period of 1-28days.

- 5 In another embodiment, the amount of RBCs used to induce the humoral immunity was 0.2 ml of 1×10^8 red blood cells.

In another embodiment, the composition is non toxic in rodents as experimentally studied upto a dose of 2000mg/Kg body weight in single oral dose.

- 10 In another embodiment, the humoral immunomodulatory activity of the composition is greater than levamisole, a positive control employed in the test systems for immunomodulatory activity.

- In another embodiment, the cell mediated immunomodulatory activity of the composition is greater than normal and cyclophosphamide treated animals, a control employed in the test systems for delayed type hypersensitivity reaction to measure the
15 cell mediated immunomodulatory activity.

- In another embodiment of the invention, the extraction process of the present invention is desirably carried out using an aqueous solvent, inclusion of other salts or solvents is acceptable so far as it does not jeopardize the extraction of polymeric carbohydrates including but not limited to 'water extractable easily water soluble polysaccharides',
20 'water extractable hydro-suspension polysaccharides' heteroglycans like MGM.

- The invention further relates to methods for the prevention or treatment of diseases or health risks wherein one or more *Chlorophytum* or its aqueous components or substances ['water extractable easily water soluble polysaccharides', 'water extractable
25 hydro-suspension polysaccharides' glucomannan polysaccharides' etc.] or mixtures of substances obtained therefrom are administered to the mammal.

- In a preferred embodiment of the invention, the aqueous extract or components of the herb are selected from a group of polymeric carbohydrates, particularly ['water extractable easily water soluble polysaccharides', 'water extractable hydro-suspension
30 polysaccharides' *Chlorophytum* /Musli glucomannan polysaccharides (MGM)' etc.

In a preferred embodiment of the invention, the *Chlorophytum* parts comprise all botanical parts of the herb.

In a particularly preferred embodiment of the use according to the invention, aqueous extract includes extraction made in water in presence or absence of other solutes or diluents at any temperature to yield the mixture of glycans.

5 In another preferred embodiment, extraction implies to solubilize substances or mixtures of substances in order to recover them in their preferred solvation method from the tissue matrix based on their cellular localization and physico-chemical properties including requirement of additives in the water to aid extraction.

10 In another particularly preferred embodiment, alcohols and/or a mixture thereof can be used for the extraction, purification or precipitation of the substance or the mixture of substances.

The polymeric/oligomeric carbohydrate compounds are like but not limited to 'water extractable easily water soluble polysaccharides', 'water extractable hydro-suspension polysaccharides' MGM demonstrated to exist in the plant may be isolated and purified by persons of ordinary skill in the art employing methods given herein as embodiments
15 of the claims or by other method(s) known in/applicable to the chemical and biochemical art. For example, column chromatography (ion-exchange, size exclusion), selective precipitation, deproteinization, freeze-thawing, dialysis etc. to obtain partially or completely pure compounds from the plant.

20 In another preferred embodiment of the invention, the immunomodulatory activity may be evaluated/ monitored by in vivo or in vitro methods comprising those provided in the examples as well as all others relevant to the issue and in practice.

In another preferred embodiment of the invention the immunomodulatory benefits concern both cellular and humoral.

25 In another preferred embodiment of the invention, the immunomodulatory benefits include primary, secondary, direct or indirect.

In another preferred embodiment, the health promotion potential of the herb or its pure or impure polysaccharide substances extends to those implied in the property of the substances referred herein.

30 It has been observed that aqueous extract of *Chlorophytum* possess immune response enhancing and immunomodulatory activity. Consequently, aqueous extracts may be used to have immunological health gain for normal and suffering person from the plant or its part or its extract or aqueously extracted substance from them. Advantageously,

these aqueous extracts are nutraceuticals that may be safely consumed for preventive, curative or supportive or strengthening for immune system and response.

In addition to immune enhancing, the aqueous extracts of the present invention are preferably isolated from a plant that has a history of safe consumption for long.

- 5 In a particularly preferred embodiment, aqueous extracts are isolated from *Chlorophytum* species.

In another preferred embodiment of the invention, the applications include an oral or topical or other means in practice in food, health, biomedical and cosmetic.

10 DETAILED DESCRIPTION OF THE INVENTION

Plant Material: Plants of *Chlorophytum borivillianum* were raised at the experimental farm of Central Institute of Medicinal and Aromatic Plants, Lucknow (India) following standard agronomic practices. At the crop maturity, the tubers (fasciculated roots) were harvested by digging out and were surface-peeled off and were shade dried to complete
15 dryness and stored in a cool dry place until used.

Isolation of Polysaccharides: The dried tubers were fine powdered and extracted with 20 volumes (20 ml per g dry mass) of water at room temperature for 12 h with constant stirring and filtered. The filtrate was saved and the residue was re-extracted with 20
20 volumes of water and filtered, as above. The two filtrates were pooled and added with ethanol to a saturation of 80% and the contents were thoroughly mixed and allowed to stand for 6 h at room temperature. The polysaccharide precipitated was recovered. The polysaccharide was dissolved in water and filtered. The filtrate constituted water extractable easily water solubilizable polysaccharide fraction (*Pro-Bio-RS1D*) of the
25 crude polysaccharide whilst the residue after filtration was suspended in water to give water extractable hydro-suspension polysaccharide fraction of the crude polysaccharide (*Bio-RS1F*). The former being easily water soluble was further purified by treatment with 6% (final concentration) trichloro acetic acid and precipitates were separated out by centrifugation and discarded. The clear solution (supernatant) obtained after
30 centrifugation was subjected to ethanol precipitation to recover the water extractable easily water solubilizable polysaccharide (*Bio-RS1D*).

Characterization of the Polysaccharide: The water soluble polysaccharide fraction (Bio-RS-1D) was subjected to molecular sieve exclusion chromatography through Sephacryl HR-200 column. For this, an aliquot (0.5 ml) of the polysaccharide was loaded on to a molecular weight calibrated column (38.5X 1.4 cm) of Sephacryl HR-200 (void volume 17.5 ml) and the column was eluted with distilled water and fractions (2.0 ml) collected. An aliquot (300 microlitre) of each fraction was made up to 80% ethanol saturation by the addition of absolute ethanol and precipitates were collected, measured gravimetrically, redissolved in 300 microlitre of water and subjected to anthrone test of carbohydrates. The elution volume of the polysaccharide gave an estimate of its molecular weight more than 200, 000 daltons.

The polysaccharides identity was discerned through its compositional analysis in terms of the nature of the constituent repeating unit of monosaccharide(s). For this, the polysaccharide was subjected to hydrolysis with TFA (tri-fluoro acetic acid) and thin layer chromatography (TLC) of the hydrolyzed as well as unhydrolyzed polysaccharide. For this, the solution of the polysaccharide was subjected to hydrolysis with 2M TFA in a boiling water bath for 5 hours in a sealed glass tube. After hydrolysis the mixture was neutralized by the addition of sodium bicarbonate till effervescence stopped. 2µl of neutralized solution as such or appropriately diluted (upto 10 times) was subjected to TLC on precoated TLC plates (E. Merck-kieselgel without fluorescent indicator) along with standard monosaccharides (1.0 mg per ml, 2 µl each). The solvent system used for TLC was ethyl acetate: isopropyl alcohol: water: pyridine in the ratio of 26:14:8:2 (v/v). Detection of sugars was done by spraying the spray reagent comprising of diphenylamine (2% w/v), aniline (2% v/v), phosphoric acid (10% v/v) in ethanol. The plate was heated at 110 °C for 10 minutes and spots of authentic standards and sample hydrolysate were visualized and compared. The comparative analysis of migration of the hydrolyzed products from the polysaccharide and the standard monosaccharides revealed that the hydrolyzed products were glucose and mannose, thus, discerning that the polysaccharide is glucomannan. As polysaccharides from different tissues differ in size and extent/degree of polymerization, they are usually characteristic for each plant in absolute terms. Therefore, the Safed Musli glucomannan was designated as Musli Glucomannan (MGM). The polysaccharide isolation may also

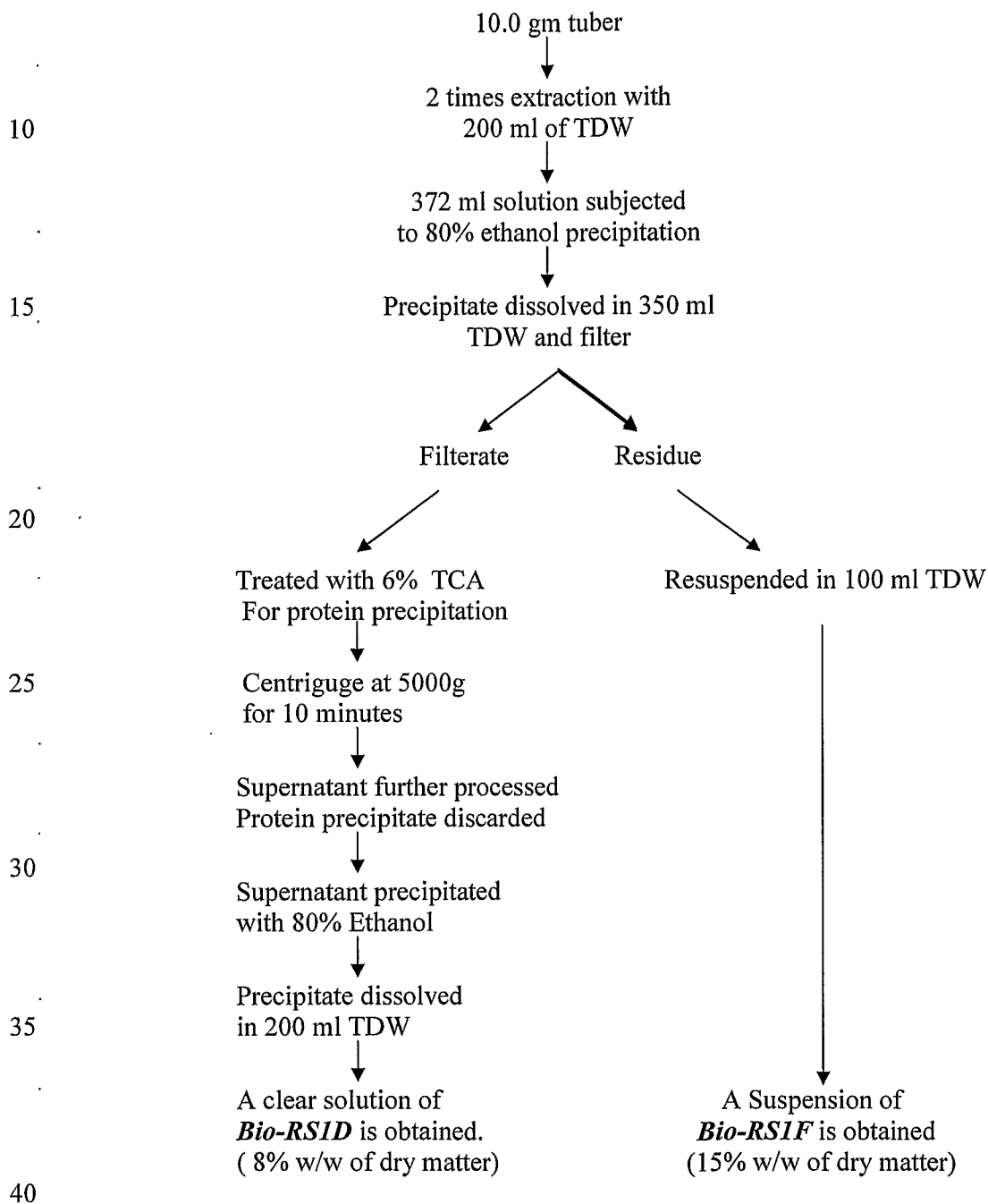
start with hot water extraction or prior defatting of the tissue with chloroform-methanol (1:1 ratio).

Determination of Biological Activity of the Musli Polysaccharide: The biological activity of the polysaccharide was assayed in terms of *in vivo* immunomodulatory (humoral and cell mediated) activity in normal mice (*Mus musculus*). For humoral immunomodulatory activity, normal healthy outbred mice, maintained under standard conditions ($22 \pm 3^{\circ}\text{C}$, 12:12 hr light : dark cycle, pellet diet and soaked Bengal gram), 5 animals in each group were taken for the experiment. The animals were fed with the test compound at the dose rate of 100mg/Kg body weight for a period of 1-28 days. Freshly collected and washed rabbit RBC's was used as an antigen which was used to immunize the animal on day 7 followed by the booster dose on day 14th. Blood was collected from the orbital plexus after 21 days and assessed for haemagglutinating (HA) titre. The haemagglutination was carried upon using a serial two fold dilution of the serum in 96 well 'U' bottom microtitre plates. The highest dilution showing visible agglutination was taken as the antibody titre.

For cell mediated activity in normal rat (*Rattus norvegicus*) through delayed type hypersensitivity model (DTH): Normal healthy outbred Sprague Dawley rats, maintained under standard conditions ($22 \pm 3^{\circ}\text{C}$, 12:12 hr light: dark cycle, pellet diet and soaked Bengal gram), 5 animals in each group were taken for the experiment. The animals were fed with the test compound at the dose rate of 50-100mg/Kg body weight for a period of 15 days. The animals were primed with washed RBC's as antigen (0.2 ml containing 1×10^8 cells subcutaneously) five days after the start of the drug treatment followed by a booster dose on 12th day with the same amount of antigen subcutaneously. The animals were challenged with the same antigen in the plantar surface of the hind limb paw 48 hrs after the last antigen dose. Readings were taken plethysmometrically 24 hrs after.

Toxicological response evaluation (Single dose response) was carried out wherein normal Healthy mice (*Mus musculus*) were fed with a single oral dose of 2000mg/Kg body weight and observed for any abnormal symptoms. At this dose, the animals were normal and did not show any evident toxicity proving the safety for a single dose of

2000mg/Kg body weight. For toxicological response evaluation (repeated dose response), normal Healthy mice (*Mus musculus*) were fed with a repeated oral dose of 100mg/Kg body weight for 28 days and observed for any abnormal symptoms. At this dose, all the animals were normal and did not exhibit any evident toxicity, proving the safety for repeated oral dose of 100mg/Kg body weight.



Flow chart for the process of isolation of bioactive polysachharides from the tubers of *Chlorophytum*. The two forms of the polysachharide, Bio-RS1D and Bio-RS1F represent water extractable easily water soluble form and water extractable hydrosuspension form, respectively. TDW, triple distilled water; TCA, trichloroacetaic acid.

According to the invention, the term "*Chlorophytum* carbohydrate substances(s)" relates to all the parts of the whole plants and covers the polymeric substances including oligomeric glycans. These can be used individually or together in combination and same or similar or analogous constituents of different *Chlorophytum* species can be used individually or together. The *Chlorophytum* or its substances embodied in the claims here can be used after pre-treatment or without pre-treatment.

"Substances or mixtures of substances" which can be obtained from any part of *Chlorophytum* can be used as such as contained in the herb or in pure or impure form. These active agents can be used individually or in combination as active agents or mixture of active agents. Mixtures of active substances can be obtained from the same *Chlorophytum* plant part or from different of the same species or a different species.

"Substances or mixtures of substances" according to the invention can consist of the same or of different representatives of the glycan class of chemical compounds. These chemical compounds can be used in natural form, i.e. unmodified by the obtaining process or modified by the obtaining process. In this context, derivatives relate to all the compounds that can be derived from the compounds mentioned and can be achieved by chemical modification, attachment etc. For example, such derivatives can be formed by substitution, addition, esterification, saponification, condensation reactions, etc.

Within the meaning of the present invention, "prevention" relates to the broad use of *Chlorophytum* or substances or mixtures of substances obtained therefrom as a prophylaxis for the operational maintenance of immune-response apparatus in an individual without any clinically diagnosed disorder or prevention of the development

of immuno-deficiency due to any physiological abnormality or immune system functionality risking exposure to environment, infection or disease and treatment.

The formulations and products based on *Chlorophytum* and its substances as embodied in the claims may be optionally employed in a specific or combination
5 forms/ways/modes/carrier mediated/concentration pharmaceutically acceptable or physiologically compatible.

EXAMPLES

*The following examples are given by way of illustration of the present invention and
10 therefore should not be construed to limit the scope of the present invention.*

Terms and Definitions:

To facilitate understanding of the invention, a number of terms and abbreviations as used herein are defined below:

15. "Purified" means partially purified and/or completely purified. Therefore, a "purified" substance may be either partially purified or completely purified.

"Extract" means crude extract, purified extract, and purified composition obtained by purification of the extract.

"Immuno-modulatory activity means" means the ability of the substance to alter
20 immune status of the subject.

"Titer" means the maximum dilution of the serum containing induced antibodies showing haemagglutination with the RBC's.

"Serial Dilution" is related to microplate format dilutions in series with dilution of serum by factors like 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048 etc.

25 "Haemagglutination" means visible agglutination in the form of serrated shield or button in a U-bottom microtiter plate.

"Antigen" means the rabbit RBC's used for induction of immunity in mice.

"Serum" means the supernant from the clotted blood.

"Plant or part" means either the the whole plant or any part of the the plant such as an
30 aerial part, seed, leaf, stem or roots or flower and any combination thereof.

Example 1

25 grams of the dried tubers were ground to fine powder. This powder was then defatted with 20 volumes of chloroform: methanol in the ratio of 1:1 (v/v). Defatted residue was further extracted with 500 ml of hot water (at 100 °C) for 3 hours and the contents were filtered/sedimented and filtrate or supernatant was collected. The supernatant was added with 4 volumes of ethanol to precipitate the polysaccharides. The precipitated polysaccharide preparation was filtered/centrifuged out and then dissolved in 200 ml of water with stirring (water soluble polysaccharide rich extract). The solution was quick frozen by liquid nitrogen and allowed to thaw slowly at around 4 to 10°C overnight. The precipitates thus formed were collected by centrifugation at 5000 rpm for 10 min. at 4-10°C. The precipitates were subjected to freeze drying to obtain a fluffy material or flakes (9.583 grams). The polysaccharide fraction accounted for 38.3% of the tuber dry weight basis. The substance or preparation finally obtained was freeze dried flake form of crude polysaccharide (designated as source *Bio-RS1*).

Example 2

25 gram of dried tubers were ground to a fine powder. This powder was then subjected to hot water (20 vol.) extraction at 100°C for 3 hours. The resultant solution was filtered and the filtrate was collected and residue re-extracted above. The filtrates were pooled (950 ml) and concentrated under vacuum to 400 ml. 50 ml of the concentrate was saved and 350 ml of the concentrate was subjected to ethanol precipitation by addition of four volumes of ethanol. The contents were filtered and precipitate collected by filtration. The precipitates were dissolved in 200 ml water at 50°C and the solution quick frozen with liquid nitrogen and allowed to thaw at 4 to 10 °C slowly. The resultant precipitates were filtered out and dissolved in 100 ml of water (solution or gel form of the crude polysaccharides). The solution was diluted four times to yield the preparation with water extractable total polysaccharide at the concentration of 1 mg per ml. It was designated as *Bio-RS1B* and subjected to immunomodulatory activity (Example 7).

Example 3

10 gram of dried tuber was fine powdered and then extracted in 20 volumes of water twice at room temperature with stirring. 372 ml solution was obtained by filtration and subjected to 80% ethanol precipitation. The precipitate thus obtained was dissolved in 5 350 ml of water and subjected to filtration. The filtrate constituted water extractable easily solubilizable polysaccharide fraction of the total crude polysaccharide and was retained as *Pro-Bio-RSID*. The filtration residue was suspended in 100 ml of water. The suspension was referred as water extractable hydro-suspension polysaccharides (15mg/ml) fraction of crude polysaccharides and were designated as *Bio-RSIF* and 10 subjected to immunomodulatory activity evaluation (Example 6). The filtrate (*Pro-Bio-RSID*) was treated with 6% TCA (final concentration) to precipitate out proteins. Protein precipitates were removed by centrifugation (5000g for 10 minutes) and the supernatant (350ml) was subjected to ethanol precipitation to recover polysaccharides (partially purified water extractable easily water soluble fraction). The precipitates were 15 collected by filtration and dissolved in 200 of ml water. This clear deproteinized polysaccharide solution (a concentration of 4mg/ml) was designated as *Bio-RSID* and employed to evaluate immunomodulatory activity. The preparation was also subjected to chemical compositional analysis (as in Example 5) and further purification through gel filtration chromatography (as in example 4).

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Example 4

Subsequent to partial purification of polysaccharides by selective precipitation through alcohol and freeze-thaw method and deproteinization, *Chlorophytum/Musli* glucomannan (MGM) fraction of soluble polysaccharides was purified by molecular 25 sieve exclusion chromatography through Sephacryl HR-200 column. For this, 0.5 ml aliquot of soluble polysaccharide was loaded on to a 38.5 x 1.4 cm column of Sephacryl HR-200 (void volume 17.5 ml) and the column was eluted with triple distilled water. 2.0 ml fractions were collected and an aliquot (300 µl) raised to 80 % ethanol, precipitates collected and redissolved in 300 µl of water and subjected to 30 carbohydrate assay (anthrone method). Single peak of carbohydrates was eluted from the preparation chromatographed as presented in Fig. 1. The purified polysaccharide

was subjected to compositional analysis through hydrolysis with TFA followed by thin layer chromatography (TLC) as in example 6.

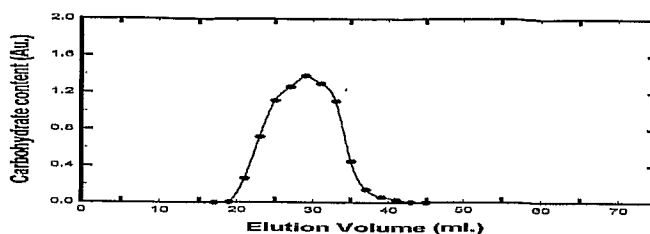


Fig. 1 Molecular exclusion chromatography of water extractable easily water soluble polysaccharide preparation of *Chlorophytum borivillianum* (Safed musli) through Sephacryl HR-200 column.

Example 5

An aliquot (1.0 ml) of the clear solution of Bio-RS1D was taken and subjected to hydrolysis with 2M TFA on a boiling water bath for 5 hours in a sealed tube. The resultant mixture was neutralized with sodium bicarbonate till effervescence stopped. 2 μ l of neutralized solution as such or appropriately diluted (upto 10 times) was subjected to TLC on precoated TLC plates (E. Merck-kiesel without fluorescent indicator) along with standard monosaccharides (1.0 mg per ml, 2 μ l each). The solvent system used for TLC was ethyl acetate: isopropyl alcohol: water: pyridine in the ratio of 26:14:8:2 (v/v). Detection of sugars was done by spraying the spray reagent comprising of diphenylamine (2% w/v), aniline (2% v/v), phosphoric acid (10% v/v) in ethanol. The plate was heated at 110 °C for 10 minutes and spots of authentic standards and sample hydrolysate were visualized and compared.

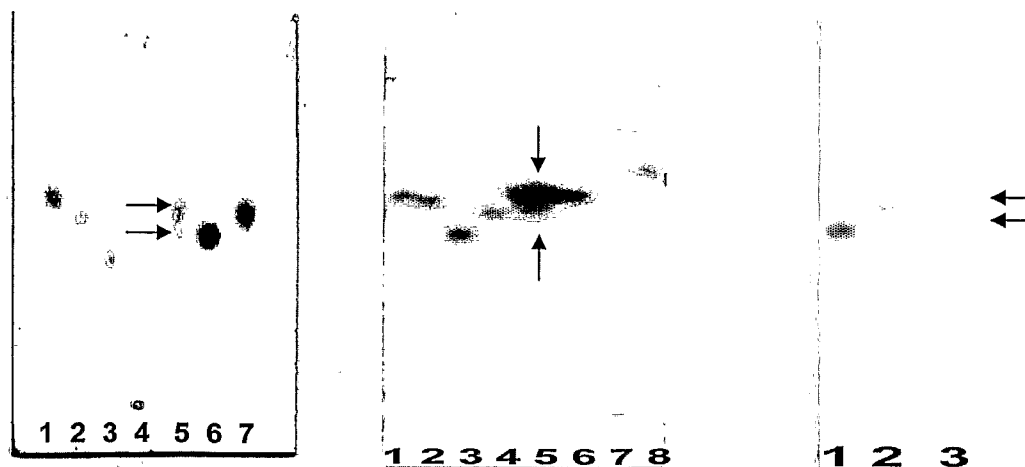


Figure 2. Compositional analysis of *Chlorophytum* polysaccharides. Left Plate- 1, arabinose, 2, fructose, 3, galactose, 4, unhydrolyzed 'water extractable easily water soluble polysaccharide (*Pro-Bio-RSID*), 5, hydrolyzed *Pro-Bio-RSID*, 6, glucose, 7, mannose; Middle Plate- 1, arabinose, 2, fructose, 3, galactose, 4, glucose, 5, hydrolyzed purified *Bio-RSID* (MGM), 6, mannose, 7, rhamnose, 8, xylose; Right Plate- 1, glucose, 2, mannose, 3, gel filtration chromatographed *Bio-RS-1D* (MGM).

Thus, two monomeric monosaccharide sugars (arrow indicated), glucose and mannose constitute the bioactive polymeric heteroglycan MGM of *Chlorophytum*/Safed Musli.

The bioactive polysaccharide substance is resourced in the plant as one of the major cellular component comprising as an example (without any limitation) 38 % (total) and 8% MGM and that compositional analysis method is TLC with authentic co-migration comparison with the development system of ethyl acetate: isopropyl alcohol: water: pyridine in the ratio of 26:14:8:2 (v/v)

Example 6

Immunostimulant (humoral) activity in normal mice (*Mus musculus*) : Normal healthy outbred mice, maintained under standard conditions ($22 \pm 3^{\circ}\text{C}$, 12:12 hr light : dark cycle, pellet diet and soaked Bengal gram), 5 animals in each group were taken for the experiment. The animals were fed with the test compound at the dose rate of 100mg/Kg body weight for a period of 1-28 days. Freshly collected and washed rabbit RBC's was used as an antigen which was used to immunize the animal on day 7 followed by the booster dose on day 14th. Blood was collected from the orbital plexus after 21 days and assessed for haemagglutinating (HA) titre. The haemagglutination was carried upon using a

serial two fold dilution of the serum in 96 well 'U' bottom microtitre plates. The highest dilution showing visible agglutination was taken as the antibody titre.

Results are represented in the following table (Table 1).

Table 1: Haemagglutination titre of the animals treated.

Animal No.	Control Normal	Control Levamisole	Bio RS- 1F	Bio RS- 1D
1.	256	4096	64	64
2.	128	1024	8092	16384
3.	32	2048	16384	32768
4.	64	32	1024	64
5.	4	32	64	64
Mean	96.8	1446.4	5146.6	9868.8

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The data demonstrate that the polysaccharides of Chlorophytum tubers (fingers/fasciculated roots) possess humoral immunity stimulating activity that is 3.55 times and 8 times higher than positive control (levamisole) on mean and median value comparisons, respectively. Similarly, the chlorophytum/Musli glucomannan (MGM) fraction of the polysaccharides possess significant humoral immunity stimulating activity that is 6.8 fold and 16 fold higher than positive control (levamisole) on mean and median value comparisons, respectively.

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Example 7:

15 Immunostimulant (cell mediated) activity in normal rat (*Rattus norvegicus*) through delayed type hypersensitivity model (DTH): Normal healthy outbred Sprague Dawley rats, maintained under standard conditions ($22 \pm 3^{\circ}\text{C}$, 12:12 hr light: dark cycle, pellet diet and soaked Bengal gram), 5 animals in each group were taken for the experiment. The animals were fed with the test compound at the dose rate of 50-100mg/Kg body weight for a period of 15 days. The animals were primed with washed RBC's as antigen (0.2 ml containing 1×10^8 cells subcutaneously) five days after the start of the drug treatment followed by a booster dose on 12th day with the same amount of antigen subcutaneously. The animals were challenged with the same antigen in the plantar

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surface of the hind limb paw 48 hrs after the last antigen dose. Readings were taken plethysmometrically 24 hrs after. Results are represented in the following table.

Table 2.: Mean delayed type hypersensitivity reaction (%age of inflammation in the paws) of the animals treated.

RS Bio 1B	Normal	(-ve) control
24.47	7.79	3.92

The data (table 2) illustrate that the differential precipitation purified polysaccharides of *Chlorophytum* tubers impart significant cellular immune response enhancement (24.47% in comparosin to 7.79 % and 3.92 % in positive and negative controls.

10 Example 8

Toxicological response evaluation (Single dose response): Normal Healthy mice (*Mus musculus*) were fed with a single oral dose of 2000mg/Kg body weight and observed for any abnormal symptoms. At this dose, the animals were normal and did not show any evident toxicity proving the safety for a single dose of 2000mg/Kg body weight.

15 Example 10

Toxicological response evaluation (repeated dose response): Normal Healthy mice (*Mus musculus*) were fed with a repeated oral dose of 100mg/Kg body weight for 28 days and observed for any abnormal symptoms. At this dose, all the animals were normal and did not exhibit any evident toxicity, proving the safety for repeated oral dose of 100mg/Kg body weight.

ADVANTAGES:

The advantages of the invention are follwing:

1. It provides a novel and mainframe therapeutic/health benefit use of the plant *Chlorophytum* (Safed Musli), different from that/those of peripheral and non-descript nature (like aphrodisiac).
2. The invention of bioactive non-nutrient polysaccharide underlines the multififerous uses of the congener plant/plant part as a functional food for weight

- loss, lower glycemic food ingredient with added advantage of immunity against disease development/combating.
3. It provides Chlorophytum as an alternative to Konjac for food, nutraceutical and medicinal purposes.
 - 5 4. It provides the multiferous uses of the plant as a value additive ingredient in foods, food recipies, cuisines, restaurants etc.
 5. It can be used a thickening agent or otherwise calorie discounted additive agent in foods, snacks, chocolates, soups, jams, icecreams, jellies etc. with attendant health gains and avoiding calorie gains.
 - 10 6. The pharmaceutical compositions with crude or enriched polysaccharides can be used as immune strengthening/disease resistance safe herbal prescriptions.

We claim:

1. Use of tubers/ fingers (fasciculated roots) of *Chlorophytum sp.*(Safed musli) either individually or in combination as an immunomodulating agent comprising upto 45% (w/w) isolated polysaccharides, 7 to 10 % (w/w) proteins and 40 to 50% (w/w) of other constituents such as fiber, cellulose, pectin, hemicellulose and free sugars.
2. The use as claimed in claim 1, wherein the plant used is *Chlorophytum borivillianum*.
3. The use as claimed in claim 1, wherein the isolated polysaccharide is a herteroglycan comprising of a polymer of glucose and mannose as constituent units with a molecular weight of more than 200 KDa.
4. The use as claimed in claim 1, wherein the isolated polysaccharide fraction is water extractable easily water soluble polysaccharides.
5. The use as claimed in claim 1, wherein the isolated polysaccharide fraction is water extractable hydro-suspension polysaccharides.
6. A pharmaceutical composition useful as an immunomodulating agent comprising isolated polysaccharide fractions from the plant *Chlorophytum borivillianum* consisting of water extractable easily water soluble polysaccharides and water extractable hydro-suspension polysaccharides either alone or in combination optionally along with pharmaceutically acceptable excipients.
7. A composition as claimed in claim 6, wherein the fraction is isolated from *Chlorophytum borivillianum*.
8. A composition as claimed in claim 6, wherein the plant tissue/biomass used is preferably aerial or tubers/ fingers (fasciculated roots) or mixtures thereof.
9. A composition as claimed in claim 6, wherein the plant tissue/biomass is extracted or used fresh or dry and may be native or peeled or in any other physical form.
10. A composition as claimed in claim 6, wherein the dose is 50 to 100mg/Kg body weight administered orally for a period of 1-28days.
11. A compsiton as claimed in claim 6, wherein the amount of RBCs used to induce the humorral immunity was 0.2 ml of 1×10^8 red blood cells

12. A composition as claimed in claim 6, being non toxic in rodents as experimentally studied upto a dose of 2000mg/Kg body weight in single oral dose.
13. A composition as claimed in claim 6, wherein its humoral immunomodulatory activity is greater than levamisole, a positive control employed in the test systems for immunomodulatory activity.
14. A composition as claimed in claim 6, wherein the cell mediated immunomodulatory activity is greater than normal and cyclophosphamide treated animals, a control employed in the test systems for delayed type hypersensitivity reaction to measure the cell mediated immunomodulatory activity
15. A process for the isolation of bioactive polysaccharides from *Chlorophytum sp* (Safed musli) comprising the steps of :
 - [a] homogenizing or otherwise solvation of the *Chlorophytum borivillianum* tissue or biomass in aqueous medium to obtain a supernatant;
 - [b] adding 4 volumes of ethanol to the supernatant of step [a] to precipitate the polysaccharides;
 - [c] dissolving the precipitated polysaccharide obtained from step [b] in water under stirring followed by filtration to obtain a filtrate and a residue;
 - [d] suspending the residue obtained from step [c] in water to obtain water extractable hydro-suspension polysaccharide (Bio-RS1F);
 - [e] simultaneously, treating the filtrate of step [c] with known deproteinization agents to precipitate proteins and obtain a deproteinized supernatant;
 - [f] subjecting the supernatant obtained from step [e] to ethanol precipitation to obtain a precipitate;
 - [g] dissolving the precipitate obtained from step [f] in water to get water extractable easily water soluble polysaccharide fraction (Bio-RS1D).
16. A process as claimed in claim 15, wherein extraction may be carried out at any temperature upto boiling point of the extraction medium.
17. A process as claimed in claim 15, wherein the ethanol precipitated carbohydrates may be redissolved in normal or alkaline water and deproteinized.

18. A process as claimed in claim 15, wherein deproteinization may be carried out by precipitation with TCA, sodium acetate or by other routine way of selective precipitation of proteins.
19. A process as claimed in claim 15, wherein the deproteinized supernatant may be subjected directly to ethanol precipitation for sedimenting carbohydrate or after its desalting by dialysis or otherwise.
20. A process as claimed in claim 15, wherein the deproteinized polysaccharide preparation may be partially or completely purified through ion-exchange chromatography and/or size exclusion chromatography.
21. A process as claimed in claim 15, wherein the yield of bioactive polysaccharide fractions is 8% w/w for 'water extractable easily water soluble polysaccharides' (Bio-RS1D) and 15% w/w for 'water extractable hydro-suspension polysaccharides' (Bio-RS1F).