Original Article

Immunomodulatory Polysaccharide from Chlorophytumborivilianum Roots

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Chlorophytum borivilianum Santapau & Fernandes (Liliaceae) is an ayurvedic Rasayana herb with immunostimulating properties. The polysaccharide fraction (CBP) derived from hot water extraction of C. borivilianum (CB), comprising of ~31% inulin-type fructans and ~25% acetylated mannans (of hot water-soluble extract), was evaluated for its effect on natural killer (NK) cell activity (in vitro). Human peripheral blood mononuclear cells (PBMCs), isolated from whole blood on a Ficoll–Hypaque density gradient, were tested in the presence or absence of varying concentrations of each C. borivilianum fraction for modulation of NK cell cytotoxic activity toward K562 cells. Preliminary cytotoxicity evaluation against P388 cells was performed to establish non-cytotoxic concentrations of the different fractions. Testing showed the observed significant stimulation of NK cell activity to be due to the CBP of C. borivilianum. Furthermore, in vivo evaluation carried out on Wistar strain albino rats for humoral response to sheep red blood cells (SRBCs) and immunoglobulin-level determination using enzyme-linked immunosorbent assay (ELISA), exhibited an effectiveness of C. borivilianum aqueous extract in improving immune function. Present results provide useful information for understanding the role of CBP in modulating immune function.

Keywords: ayurvedic Rasayana – NK cell activity – fructan – acetylated mannan – immunoglobulin – immunomodulator

Introduction

Chlorophytum borivilianum Santan F. (Fam. Liliaceae) is widely cultivated throughout India, especially in the states of Madhya Pradesh, Andhra Pradesh and Maharashtra, and exported globally for its medicinal usage (8). Major phytochemical components reported from the roots of C. borivilianum include steroidal saponins, fructans and fructoligosaccharides (FOS), acetylated mannans, phenolic compounds and proteins (9–12). The plant is acclaimed for various health benefits, which include its ability to ameliorate hyperlipidemia, diabetes-induced sexual dysfunction and prevent heat-induced testicular damage. The plant is also considered to be useful as an antioxidant (13–16).

Kapha) (3–5). Evidence collected on various Rasayana herbs signifies their utility as immunomodulators with low toxicity (6,7).

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In our previous study, we reported the improvement of in vivo immunomodulation by the ethanol extract of *C. borivilianum* (17). Extensive phytochemical evaluation of the roots of *C. borivilianum* led to the quantification of nearly 60% w/w of polysaccharides in the hot water-soluble material, which were found to be mainly fructans and acetylated mannan (16). Since these polysaccharides have been cited for their immunomodulatory potential, various fractions of *C. borivilianum* aqueous extract were evaluated for their effect on the modulation or enhancement of human natural killer (NK) cell activity, hemagglutination (HA) titer values against sheep red blood cells (SRBCs) and immunoglobulin G (IgG) levels in rats.

**Methods**

**Plant Material**

Dried roots of *C. borivilianum* were procured from Nandan Agro Farms, Hyderabad, India, and identified at the Department of Pharmaceutical Sciences, Dr. H.S. Gour Vishwavidyalaya, Sagar (India). A voucher specimen of the same has been deposited in the departmental herbarium (MTCB-1608). The powdered roots were subjected to hot water extraction using the methodology reported previously (15) and the procedure on the isolation and fractionation of the aqueous extract of *C. borivilianum* has been detailed in Fig. 1. Cytotoxicity of the fractions was evaluated on the P388 cell line. The aqueous extract of the root powder (CBC), the polysaccharide (CBP) and non-polysaccharide (CBR) fractions were used for evaluating the effect on modulation of NK cell activity in vitro, and HA titer assay and IgG level in vivo.

**Animal Group**

A total of 72 Wistar strain albino rats (either sex) in the weight range of 160–180g were fed a standard pellet diet and water *ad libitum*. The animals were housed at room temperature (24±2°C) on a normal day-night cycle (6.00 a.m. to 6.00 p.m.). The guidelines given by the Committee for the Purpose of Supervision and Control of Experiments on Animals, India, were strictly followed. SRBCs were procured from the Haffkine Biopharmaceutical, Mumbai, India. All the other chemicals and reagents used were of analytical grade.

**Animal Group for HA Titer Value**

Fourteen days prior to experimentation, two sets of six groups, comprising of six animals in each group, received experimental drug administration as per the following schedule.

- **Group I**: Served as control and was administered vehicle only.
- **Group II**: 100 mg of *C. borivilianum* aqueous extract per kilogram of body weight (CBC).
- **Group III**: 50 mg of *C. borivilianum* polysaccharide fraction per kilogram of body weight (CBP50).
- **Group IV**: 100 mg of *C. borivilianum* polysaccharide fraction per kilogram of body weight (CBP100).
- **Group V**: 50 mg of *C. borivilianum* non-polysaccharide fraction per kilogram of body weight (CBR 50).
- **Group VI**: 100 mg of *C. borivilianum* non-polysaccharide fraction per kilogram of body weight (CBR 100).

**HA Titer Determination**

On day 0, paralleling the above mentioned treatment, animals were immunized by injecting 0.5 ml of 5.0×10⁹ SRBC/ml via the intraperitoneal route (i.p.). On day 7 the animals were challenged by injecting the same volume of SRBCs. Blood samples were collected by retro-orbital puncture on day 14 for antibody titer. Hemagglutination antibody titer was determined by using the micro-titration technique described by Damre et al. (18). For experimentation, 40 μl of 0.1% (w/v) bovine serum albumin (BSA) solution in normal saline was pipetted into the wells of micro-titration plates. To this solution, 40 μl of serum of either the treated or control animal was added, which was later serially diluted 2-fold. Further 20 μl of a 0.1% suspension of SRBCs in BSA-saline was added to each well; the plate was initially incubated at 37°C for

![Figure 1. Fractionation of crude aqueous extract and various fractions evaluated for biological activity.](image-url)
Two-fold serial dilutions of chlorambucil and curcumin were made starting at concentrations of 600 and 100 μg/ml, respectively. Solvent and media controls were also included in the assay. The samples were assayed at various concentrations of CBC, CBP and CBR starting at 10 mg/ml. Testing was performed in duplicate. For this, 50 μl of sample was added to each well along with 50 μl of P388 cell suspension. The cells and samples were incubated at 37°C for 24 h in a humidified 5% CO₂ incubator (Sanyo, Japan). Measurement of cell proliferation (ATP) was performed as per ATPLite kit protocol (Perkin Elmer, Netherlands) using the Micro Beta 1450 plate reader (Perkin Elmer, USA).

**NK Cell Activity**

Samples (CBC, CBP and CBR) were prepared by dissolving the material at an initial concentration of 10 mg/ml in de-ionized Milli Q water (Millipore, UK) and appropriately diluted in water to obtain the final concentrations as required. The samples were vortexed and sonicated for 10 min to ensure complete solubility.

*In vitro* NK cell activity was assayed by flow cytometry using a Becton Dickinson FACS Calibur instrument. The methodology reported by Standen et al. (20) was used with little modification. In brief, peripheral blood mononuclear cells (PBMCs) were prepared from fresh, whole, lithium-heparinized blood using Ficoll–Hypaque (Amersham Biosciences). Human PBMCs were suspended in advanced Roswell Park Memorial Institute (RPMI)-1640 medium (2% fetal BS; 1% l-glutamine; 2% penicillin-streptomycin) and aliquots of cells were pre-incubated with each extract dilution for 2 h at 37°C in 5% CO₂. The PBMCs (NK cells/effectors) were then incubated for 2 h (37°C, 5% CO₂) with target cells (K562; ATCC) pre-labeled with Vybrant DiO cell labeling solution (Molecular Probes, Invitrogen, V-22886), a green fluorescent dye that allows differentiation of target from effector cells. The effector:target cell ratio used was 30:1. Propidium iodide (Molecular Probes, P-3566), a red fluorescent DNA dye, was added following incubation to label target cells, which were rendered permeable by NK cell activity. A target cell control (no effectors) was run for each sample to monitor spontaneous target cell death. Protein-bound polysaccharide (PSK), which has been validated for its selective activation of NK cells in a mouse experimental tumor model, was used as a positive control (21). A solvent control (water) was also run and each sample was tested in duplicate. The percentage of dead target cells was determined by flow cytometry using CellQuest Pro software. The percentage of specific cytotoxicity was determined by subtracting the percentage of dead cells in the target control tube from the percentage of dead target cells in each test sample.
Statistics
Statistical analysis and sample size was determined using Instat v 2.01. Confidence was set at 95%. All the groups were compared to control using one-way Analysis of Variance (ANOVA) followed by Dunnet’s test. Significance was set at $P < 0.05$.

Results
Effectiveness of *C. borivilianum* on various aspects of the immune system was clearly demonstrated in the present study. CBC, CBP and CBR were evaluated for their effect on NK cell activity, humoral and cellular immune response.

Cytotoxicity Evaluation
As previously reported, CBC was found to be non-toxic up to a dose of 2 g per kilogram of body weight (16). In the present study, an evaluation of the cytotoxicity and determination of IC$_{50}$ was carried out on the sensitive mouse leukemic P388 cell line. Curcumin and chlorambucil were used as positive controls for the determination of cytotoxicity. The results confirmed the non-toxic nature of the *C. borivilianum* extract under investigation. CBC and CBP exhibited IC$_{50}$ values of 1219.67 and 722.31 μg/ml, respectively (Fig. 2).

HA Titer Value
The values for HA titer in the case of the vehicle-treated control group animals were established at 147.6 ± 11.9. Significantly higher HA titer values of 169.2 ± 6.1, 174.3 ± 3.1 and 168.6 ± 2.6 were observed for CBC and CBP at 100 μg/ml and CBP at 50 μg/ml, respectively. However, CBR only showed mild increases in HA titer of ~8.6% and 8.1% at 100 and 50 μg/ml, respectively. The results for HA titer values observed are detailed in Fig. 3.

IgG Level
ELISA-based measurements were used for the secondary antibody responses in immunized animals. Administration of CBC resulted in a significant increase ($P < 0.01$) in IgG level (1.93 ± 0.02), followed by CBP100 (1.85 ± 0.01) and CBP50 (1.81 ± 0.04). CBR50 and CBR100 gave similar IgG levels of 1.82 ± 0.12 and 1.82 ± 0.11 units, respectively. In the case of control animals (vehicle only), the value was found to be 1.74 ± 0.013 units (Fig. 4).

NK Cell Activity
In comparison to the control, a significantly higher effect of CBP ($P < 0.01$) in augmenting the NK cell activity was observed. Although CBC stimulated NK cells significantly ($P < 0.05$), most of the effect appeared to be contributed by CBP alone. Therefore, polysaccharides of *C. borivilianum* appeared to be solely responsible for NK cell augmentation. In the case of CBR, no effect was observable, and the result obtained was equivalent to that of the solvent control group. At a final concentration of 5 μg/ml the CBP fraction resulted in an ~2-fold increase in NK cell activity (98 ± 2.5%), whereas at 25 μg/ml, the % increment was 58.4 ± 0.3%. This indicates that the isolated polysaccharide fraction is more effective at the lower concentration tested. In the case of CBC, some semblance of a dose-dependent effect was observed, wherein a 72.9 ± 4.4% and 86.6 ± 0.7% increase in NK cell activity was observed with CBC at 5 and 50 μg/ml, respectively. There was no enhancement in relative NK cell activity for CBR at 5 and 25 μg/ml.

![Figure 2.](image-url) **Figure 2.** IC$_{50}$ values of *C. borivilianum* aqueous extract and fractions against P388 cells.

![Figure 3.](image-url) **Figure 3.** Effect of *C. borivilianum* aqueous extract and fractions on HA titer values in Wistar rats.
The differences in relative activity compared to control groups have been shown in Fig. 5.

**Discussion**

Ayurvedic Rasayan herbs have been acclaimed for their ability to enhance the functionality of the immune system. Appropriate modulation of biological homeostasis in order to boost the ability to fight infection, counteract diseases, prevent cancer, etc., is the primary focus of Rasayan therapy (22). *C. borivilianum* is renowned for its ability to boost immune function (17). Interactions among immune cells and those of immune cells with the other tissues in the body are highly diversified and mostly unexplored. The ability to counteract infections or to fight against cancer mainly involves a three-tiered functionality: humoral immunity, cellular immunity and the regulators of immune system, such as cytokines (23). Previously we had reported the effectiveness of ethanol fraction of the *C. borivilianum* extract on improving non-specific immunity (17); in the present study we have been able to determine the possible role of CBP on immune modulation.

A burgeoning area of research is the development or discovery of immunomodulatory agents that are free from toxic side effects and can be used for a long duration, thus resulting in continuous immuno-activation (24). Previous research carried out on *Rasayan* herbs has validated that they activate immune functionality without causing an imbalance in overall physiology; this aspect has been excellently reviewed by Vayalil et al. (6). Similar to many immunomodulatory substances, Rasayanas are not directly cytotoxic to tumor cells; rather they produce significant inhibitory effect on ascites tumor development and solid tumor growth in mice during the treatment period (25). Rasayanas have been previously considered to act via *in vivo* augmentation of NK cell activity as well as antibody-dependent cellular toxicity. In the present study, an enhanced NK cell activity of CBP as well as CBC against K562 myeloid leukemic cells clearly validates the immunopotentiating ability of fructans and mannans isolated from *C. borivilianum* (26).

Augmentation of NK cell activity is considered an important parameter for improved immunological function. NK cells are a subset of lymphocytes that are important in the body’s defense against viral infections and malignancy, participating in innate immunity and early defenses (26,27). They are defined functionally by their ability to mediate spontaneous cytotoxicity, lysing a broad range of target cells without prior sensitization and without restriction by major histocompatibility complex (MHC) antigens (28,29). Impaired NK cell activity is associated with increased sensitivity to infection (30). NK cells are a major force in counteracting, and fighting, against cancer.

As previously mentioned, Rasayan herbs are generally regarded as non-toxic even at high doses. A toxicity assay using the ATPLite kit clearly suggested that the extracts and fractions from *C. borivilianum* were non-cytotoxic against P388 cells, a sensitive mouse cell line. In our previous studies on the plant, *in vivo* toxicity was evaluated and the extracts were found to be non-toxic even at a dose of 2g per kilogram of body weight. Lack of

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**Figure 4.** Effect of treatment with *C. borivilianum* aqueous extract and fractions on Immunoglobulin (IgG) levels in Wistar rats after 28 days.

**Figure 5.** Effect of *C. borivilianum* aqueous extract and fractions on *in vitro* NK cell activity against K562 cells.
cytotoxicity is an important attribute of Rasayan drugs and this was validated in the case of *C. borivilianum* as well.

The crude *C. borivilianum* extract as well as the polysaccharide fraction were able to enhance the antibody titer, while the non-polysaccharide fraction was effective in enhancing a cell-mediated response. Augmentation of the humoral response was evidenced by increased antibody production in response to SRBC challenge in the post-immunization drug treatment. The enhanced responsiveness is indicative of up-regulation of macrophages, dendritic cells and B-lymphocyte subsets involved in antibody synthesis. T-lymphocyte activation is also considered an important attribute of polysaccharides, which is directly correlated with immuno-stimulation. This cascade of functionalities provides evidence for an enhancement of humoral as well cellular immune responsiveness (31). A diagrammatic representation of the overall assessment of *C. borivilianum* is shown in Fig. 6.

*Rasayana* herbs or their extracts have been found to be effective in enhancing the production of cytokines such as interleukin (IL)-2, interferon (IFN)-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) in albino rats (32). Further studies in this direction are warranted on the extracts/fractions of *C. borivilianum*.

In recent years, there have been reports on the presence of specific polysaccharides, such as fructans, acetylated mannans, xylans and glucans, in *Rasayana* herbs. These polysaccharides from medicinal plants exhibit biological activities of importance for improving human health. Perhaps the most important activity would be on the immune system, which may lead to the production of nutritional supplements in cancer treatment (33,34). As validated in the case of *C. borivilianum*, the use of these polysaccharides will stimulate the immune system and may also contribute to lowering the dose of existing immune therapeutics. These polysaccharides can be used in relatively large doses with no side effects; accompanying these facets are the juxtaposed benevolent attributes such as their effects on different viral infections, diabetes and aging-related problems (35,36).

The present study provides further insight into the potential immunomodulator herb *C. borivilianum* and provides some *in vitro* evidence. It can be further inferred from this study that the polysaccharide fraction is the most effective in augmenting the NK cell activity as well as humoral immunity. The remaining fraction of extract, which is rich in phenolics and saponins (13), was useful in improving the HA titer, but had no effect on NK cell activity. Thus, it can be concluded that the *C. borivilianum* extract acts via a cascade of mechanisms, modulating the immune system to improve and restore a healthy state. The polysaccharides from *C. borivilianum* could be considered for cancer treatment via post-chemotherapy revival of the immune system. This study further validates and provides molecular evidence for the potential benefits of *Rasayana* herbs of the ayurvedic system of medicine.

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References

1. Thakur M, Chauhan NS, Bhargava S, Dixit VK. A comparative study on aphrodisiac activity of some Ayurvedic herbs in male albino rats. *Arch Sex Behav* 2009;38:1009–15.

2. Patwardhan B, Warude N, Pushpangadan P, Bhatt N. Ayurveda and traditional Chinese medicine: a comparative overview. *eCAM* 2005;2:465–73.

3. Mahdihassan S. The rational interpretation of the Indian doctrine of humorology. *Am J Chin Med* 1982;10:40–3.

4. Joshi H, Parle M. Brahmi rasayana improves learning and memory in mice. *eCAM* 2006;3:79–85.

5. Shinde VM, Dhalwal K, Mahadik KR, Joshi KS, Patwardhan BK. RAPD analysis for determination of components in herbal medicine. *eCAM*. 2007;6:Suppl 1:21–3.

6. Vayalil PK, Kuttan G, Kuttan R. Rasayanas: Evidence for the concept of prevention of diseases. *Am J Chin Med* 2002;30:155–71.

7. Chopra A. Ayurvedic medicine and arthritis. *Rheum Dis Clin North Am* 2000;26:133–44.

8. Thakur M, Dixit VK. Effect of *Chlorophytum borivilianum* root on lipid metabolism in hyperlipaemic Studies on the immunomodulatory activity of flavonoidal fraction of *Tephrosia purpurea*. *Fitoterapia* 2003;74:257–61.

9. Kahn J, Harmon CC, Buckner GP, Richardson GJ, Russell MW, Michalek SM. Protective salivary immunoglobulin A responses against *Streptococcus mutans* infection after intranasal immunization with *S* *mutans* antigen I/II coupled to the B subunit of cholera toxin. *Infect Immun* 1993;61:1964–71.

10. Stadden MD, Connellan PA, Leach D. Natural killer cell activity and lymphocyte activation: Investigating the effects of a selection of essential oils and components in vitro. *Int J Aromatherapy* 2006;16:133–9.

11. Pedrinaci S, Algarra I, Garrido F. Protein bound polysaccharide (PSK) induces cytotoxic activity in the NKL human natural killer cell line. *Int J Clin Lab Res* 1999;29:135–40.

12. Ali M. Rasayana therapy in classical literature of Ayurveda: a review. *Bull Indian Hist Med* 1998;28:95–110.

13. Kubena KS, McMurray DN. Nutrition and immune system: A review of nutrient-nutrient interactions. *J Am Diet Assoc* 1996;96:1156–64.

14. Su PF, Staniforth V, Li CJ, Wang CY, Chiao MT, Wang SY, et al. Immunomodulatory effects of phytocompounds characterized by in vivo transgenic human GM-CSF promoter activity in skin tissues. *J Biomed Sci* 2008;15:813–22.

15. Vayalil PK, Kuttan G, Kuttan R. Effect of Rasayanas on normal and tumor bearing animals. *J Exp Clin Cancer Res* 1994;13:67–70.

16. Pedersen BK. Natural killer cells in relation to disease and treatment. *Allergy* 1985;40:547–57.

17. Brittenden J, Heys SD, Ross J, Eremin O. Natural killer cells and cancer. *Cancer* 1996;77:1226–43.

18. Herberman RB. Natural killer (NK) cells and their possible roles in resistance against disease. *Clin Immunol Rev* 1981;1:1–65.

19. Walker W, Aste-Amegaza M, Kastelein RA, Trinchieri G, Hunter CA. IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12-induced IFN-gamma. *J Immunol* 1999;162:5894–901.

20. Andersson BL, Farrar WE, Golden-Kreutz D, Katz LA, MacCallum R, Courtney ME. Stress and immune responses after surgical treatment for regional breast cancer. *J Natl Cancer Inst* 1998;90:30–6.

21. Fulzele SV, Burchandani PM, Kanoje VM, Joshi SB, Dorle AK. Immunostimulant activity of Ashtamangal ghrita in rats. *Ind J Pharmacol* 2002;34:194–7.

22. Rekha PS, Kuttan G, Kuttan R. Immunopotentiating activity of *Brahma Rasayana*. *Amala Res Bull* 1998;18:85–90.

23. Paulsen BS. Biologically active polysaccharides as possible lead compounds. *Phytochem Rev* 2002;1:379–87.

24. Paulsen BS. Plant polysaccharides with immunostimulatory activity. *Curr Org Chem* 2001;5:939–50.

25. Hsu JW, Huang HC, Chen ST, Wong CH, Juan HF. *Ganoderma lucidum* polysaccharides induce macrophage like differentiation in human leukemia THP-1 cells via caspase and p53 activation. *eCAM* 2009; doi:10.1093/ecam/nep107.

26. Han Y, Son SJ, Akhalaia M, Platonov A, Son HJ, Lee KH, et al. Immunomodulatory activity of fructo-oligosaccharides and essential oils by ginsan. *eCAM* 2005;2:529–36.

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