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Original Article

Immunomodulatory Activity of Bioactive Fraction (PBC) from *Phyllostachys bambusoides*

Sunil Kumar[1*], Gaurav Sharma[1], Amit Kumar[2]

ABSTRACT

Background: The lack of vaccines and limitations of currently available strategies demand a need to develop safe and efficacious immunomodulators. *Phyllostachys bambusoides* is traditionally used for various autoimmune and infectious disorders, a property possibly attributable to presence of flavonoids like orientin and iso-orientin. **Objective:** the objective of this study was, to search a potent immunomodulator which elicit both Th1 and Th2 immune response. **Methods:** The animals were (Balb/c) treated with the bioactive fraction (PBC) from *P. bambusoides* (100 and 200 mg/kg body weight) for 14 days with SRBC (Sheep Red Blood Cells) as an antigen. Haemagglutination antibody (HA) titre, delayed type hypersensitivity (DTH) reaction, phagocytic index, NO production, analysis of cytokines in serum and CD80/CD86 population in spleen. **Results:** PBC significantly enhanced the expression of IgM and IgG titre and DTH response in a dose dependant manner after 24 and 48 h in BALB/c mice with a maximum response at 200 mg/Kg. Besides humoral and cell mediated immunity, it also enhanced phagocytic index, nitric oxide production, which further leads to protection against *Candida albicans* infection. It also, enhanced the expression of CD80 and CD86 in splenic cells. **Conclusion:** Taken together these *in vitro* and *in vivo* data, our results suggest that PBC acts as an effective immunostimulator which specially enhances macrophage function during infection. This further supports the role of PBC in immunopharmacologic applications.
Key words: Macrophages, Immunostimulation, *Phyllostachys bambusoides,* Ha Titre, Cytokine.

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INTRODUCTION

The modulation of the immune response to alleviate the disease has been of great interest for many years. Currently, the plant based immunomodulators are receiving inadequate attention. Many synthetic and natural plant products have been evaluated for their immunomodulatory potential. Plant and animal materials have been used successfully for the treatment of human diseases since ancient times. In Indian system of medicine, a wide range of drugs from natural origin have been advocated for a variety of diseases.[1] Ayurveda is one of the traditional system of medicine practiced in Sri Lanka & India, and can be find back to 6000 BC. Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles. Immunostimulation in a drug-induced immunosuppression model and immunosuppression in an experimental hyper-reactivity model by the same preparation can be said to be true immunomodulation.[2] Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate pathophysiological processes and are hence called immunomodulatory agents.[3]

P. bambusoides represents a remarkable reputation among the indigenous system of medicine and modulates the biologic response of immune cells that enhance the host's ability to resist infections. Mainly it has been used in Chinese medicine for diarrhea, inflammation, stomachache, and restlessness.[4] The various phytochemical studies shown that bamboo is a rich source of flavonoids.[5] Among the various flavonoids present in *P. bambusoides,* orientin seems to be of pivotal importance for its antioxidant property.[6] It is reported that orientin, in combination with other bioactive molecules may have a potential immunomodulatory properties.[7] Also, the plant is already reported for antioxidants,[8] antipyretic, analgesic, anticonvulsant,[9] and anti-inflammatory activity.[10]

On the basis of the importance of leaves for immunological aspects, various fractions were prepared from the whole alcoholic extract and subjected for preliminary study. The most active fraction (PBC) was subjected for a panel of *in vivo* assay to evaluate its immunomoduatory property. The HPLC fingerprinting revealed the presence of the compound of our interest, orientin (Figure 1). It is well known that the agents which are capable of modulating the immune response, treat or prevent immune based disorders.[11] Such agents may increase the antibody responses, or act by modifying the cell mediated immune response. By characterizing the immunological

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correlates, such measurements will aid in the development of prophylactic drugs.

MATERIAL AND METHODS

Reagents

Methanol was purchased from Qualigens, Mumbai, medium RPMI 1640 (Himedia, Bombay, India), fetal calf serum (FCS) (Gibco, USA), trypan blue (Microlabs, Bombay), 96 V wells micro titration plates and micro tissue culture plates (96 U wells) from Tarson, , Concanavalin-A (Con-A), lipopolysaccharide (LPS), dimethylsulphoxide (DMSO), streptomycin, penicillin, levamisole and MTT (3- [(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) from Sigma were used.

Plant materials

The fresh leaves of *P. bambusoides* were collected from the herbal garden of University of Horticulture and Forestry, Nauni, Solan, India. A voucher specimen (UHF/12530) has been deposited the Department Forestry products, Nauni University.

Preparation of extract and Isolation

The powdered plant material (1 Kg) was subjected to continuous hot percolation process using Soxhlet apparatus with methanol at room temperature. After exhaustive extraction, the extract was concentrated under vacuum at 50–55 °C. Extract was dissolved in 5% HCl and filtered. Filtrate was treated with chloroform and ethyl acetate respectively in a separating funnel to obtain non-alkaloidal chloroform and ethyl acetate fraction. The chloroform fraction was subjected to silica gel (60–120 mesh) column chromatography. The column was eluted using a gradient of hexane-ethyl acetate (100:0–0:100) to afford 76 fractions. All the fractions were checked on TLC, spots were visualized under UV chamber. Out of 76 fractions, based on TLC pattern 5 broad fraction were prepared and again most bioactive fraction was subjected for repeated column chromatography using same solvent system and 23 fractions were eluted. Out of 23 fractions, four fraction-3-7 (eluted in 15% hexane-ethyl acetate), showed one major spot in TLC. These four fractions were pooled (PBC) and subjected for a panel of *in vivo* assay. The fraction was standardised using HPLC shown in Figure 1.

Animals

Study was conducted on male Balb/c mice (18-22 g, four to six week old). The ethical committee of the CSIR Jammu, approved all protocols. The animals were maintained under standard conditions: temperature (25 ± 2 °C) and photoperiod of 12 h. Commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water were given *ad libitum*. All animals used in experimental work received human care.

Immunization schedule

The blood was withdrawn from Jugular vein of the sheep in Alsever's solution.[12] The SRBCs were washed thrice with pyrogen free saline and a suspension of 5×10^9 cells/mL was prepared for immunization. Mice were divided into four groups, each consisted of six animals. PBC 100 and 200 mg/Kg (in 200 µL of 1 % gum acacia) was administered orally for 14 days, daily. The volume of dose was 200 µL. Control group received 1 % gum acacia. Levamisole (2.5 mg/Kg body weight) was given orally as a positive control. On day 0 all animals were immunized with 200 µL of SRBC (5×10^9) per mouse intraperitoneally (*i.p.*). Additionally immunized groups were challenged on day 7 with SRBC, used for DTH assay.

Anti-SRBC antibody titre and Delayed type hypersensitivity (DTH) reaction in mice

The most active Anti-SRBC circulating antibody (IgM and IgG) titers of mice were determined by haemagglutination titre. Blood samples were collected from individual animals, from retro-orbital plexus on day 7 and 14 for primary & secondary antibody titre respectively. The antibody levels were determined in serum by the haemagglutination technique.[13] For DTH PBC was administered 2 h after immunization and once daily for six days. The thickness of the right hind footpad was considered as the control and was measured with a spheromicrometer (pitch, 0.01 mm). The mice were then challenged by injecting 20 µL of 5×10^9 SRBC/mL into the left hind footpad (intradermally). The thickness of footpad was measured again after 24 and 48 h.[14]

In vitro phagocytic activity of peritoneal macrophages

The effect on phagocytic activity of normal peritoneal macrophages was evaluated by utilizing C. *albicans*. The phagocytic function of peritoneal macrophages was assessed by the method of Lehrer (1981). The resident peritoneal macrophages of mice were harvested by flushing the peritoneal cavity with 5 ml of RPMI. The cells (2×10^6) from the suspension were allowed to adhere to glass cover slips (22×22 mm) for 2 h in CO_2 incubator in absence or presence of different concentrations of PBC. The cover slips were then washed thoroughly with PBS to remove non-adherent cells. During this period, heat-killed (100 °C, 30 min) Candida cells were opsonized for 90 min with 20% mouse serum. Hundred microliters (100 µl) of opsonized candida (2×10^9 cells/ml) cells were then spread over the monolayer and the mixture was again incubated for 15 min. in CO_2 incubator. At the end of this period, the cover slips were washed thoroughly with PBS and stained with Trypan–eosin. The samples were immediately evaluated microscopically to determine the number of yeast cells ingested per cell (phagocytic index). At least 100 adherent cells were evaluated per sample, the phagocytic index were calculated.[15]

In vitro NO production assay

A volume of 10 mL of ice cold RPMI was injected into fresh Balb/c mice. After proper lavage, the medium was taken out using same syringe and centrifuged at 1800×g for 10 min at 4°C. The pellet was re-suspended in RPMI medium and macrophages (3×10^6) were seeded in 96-well culture plate with different concentration of PBC and incubated for 18 h. After incubation 100 µL of supernatant was transferred to another plate in the same fashion and 100 µL of Griss reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid) was added. The plate was read at 540 nm.[16]

Determination of IFN-γ and IL-4 by ELISA and Phenotypic analysis of (CD80 and CD86) co-stimulatory molecules by flow cytometry

Serum was collected 4 h after final oral administration of the PBC. The IFN-γ and IL-4 were measured with an enzyme linked immunosorbent assay (ELISA kit, R&D Systems Quantikine) according to the instructions of the manufacturer.[17]

For phenotypic analysis, splenic cells from PBC-treated BALB/c were suspended in RPMI media after removing the RBCs by RBC lysis buffer. Cells (1×10^7 cells/mL) were washed and incubated for 1 h at 37 °C. To measure the expression of co-stimulatory molecules, the cells were stained with FITC-labeled anti-CD80 and anti-CD86 mAbs. The data were analyzed for ten thousand cells with FACScan flow cytometer.[18]

Statistics

Data were expressed as mean±SEM, and statistical analysis was carried out using Bonferroni correction multiple comparison test (One way ANNOVA). All in vitro experiments were carried out in triplicates and represented as mean ± SEM. P values < 0.05 were being taken as statistically significant.

RESULTS

HPLC fingerprinting of PBC

The HPLC chromatogram of PBC is shown in Figure 1. It showed 8 peaks with varying retention times (2.40, 6.27, 9.60, 11.92, 13.63, 14.20, 16.29, 19.63,) including the presence of iso-orientin (11.92); orientin (13.63).

Effect of PBC on anti-SRBC antibody titre and DTH response

Bioacive fraction PBC (100–200 mg/Kg, p.o.) produced a dose-related increase in the primary and secondary antibody synthesis and also showed the significant cell-mediated immunity in terms of the footpad thickness, expressed as DTH reaction given in Table 1.

Effect of PBC on phagocytic activity

A highly significant increase in phagocytic index was obtained at 50 µg (Figure 2). PBC caused a dose dependent increase in phagocytic index as compared with control group.

Effect of PBC on nitrite content

The effect of PBC on production of NO was observed in peritoneal macrophages isolated from Balb/c mice. PBC showed a dose dependent increase in nitrite content as compared to the control group (Figure 3).

Effect of PBC on IFN-γ and IL-4 and on the expression of CD80 and CD86 in mice

PBC significantly up-regulated Th1 (IFN-γ) and Th2 (IL-4) cytokines compared to the control group (Figure 4a); a similar trend was observed in expression of CD80 and CD86 at 100 and 200 mg/Kg (Figure 4b).

DISCUSSION

The integrity of immune response along with other factors, depends on the involvement of molecular communication through release of chemical mediators and its interaction with T & B cells. There are three types of cells which modulate the immune response and these include T & B lymphocytes and macrophages.[19] Now a day, the demand for drugs having potential for enhancing both specific and non-specific immune response is increasing continuously. Therefore the focus of the present study was to investigate in depth, the role of macrophages stimulated by PBC in host defense mechanism.

In the present study, immunomodulatory effect of PBC were established in BALB/c mice employing a panel of *in vitro, in vivo* and *ex vivo* assay models. Orientin is the principle bioactive compound present in PBC thus it may be suggested that orientin might be responsible for immunomodulatory potential shown in the present study. These results are in agreement with the previously published report.[7] The antibody response was observed by HA titre using SRBC as an antigen and found increased number of IgM and IgG antibodies. The augmentation of IgM and IgG production in response to SRBC is due to increased responsiveness of macrophages because macrophages are closely associated with production of antibodies. Further, T cells contribute to delayed type of hypersensitivity reaction via participating in the expression of cell mediated immunity.[20] PBC enhances the delay type hypersensitivity reaction compared to control group, as reflected by increased in footpad thickness and it revealed that macrophages in-filtered at inflammatory sites.[21,22]

The effect of PBC on the non-specific defence mechanism were determined. Since Macrophages and granulocytes play an important role in non-specific defence therefore *in vitro* phagocytic function was performed. In the present result we assessed the phagocytic function of macrophages by ingestion of candida particles in PBC treated macrophages. Results revealed that PBC enhances the non-specific immune response. Moreover, Th1 and Th2 cytokines have an important role in both adaptive and innate immunity.[23] They are the major components involved in regulation of the immune mechanism to antigens and infectious agents. IFN- γ, a type of Th-1 cytokines are capable of inducing the generation of macrophages and T cells, while Th-2 cytokine up-

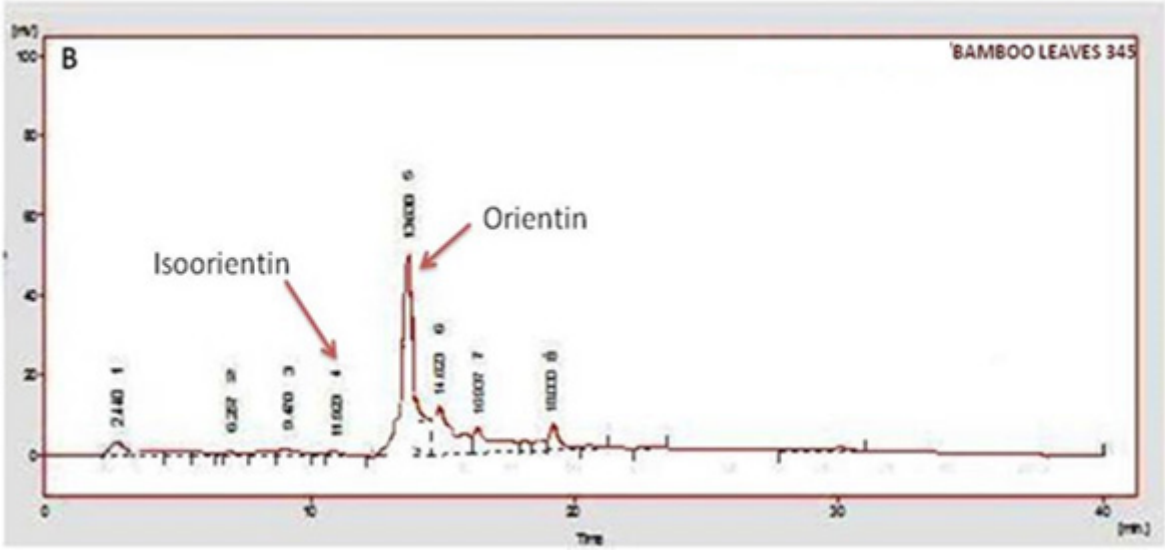

Figure 1: HPLC fingerprinting of PBC showing presence of orientin (13.63) and isoorientin (11.92) at different Rt. The separation was carried out Eclips XBD C18 column, 4.6 × 150 mm, 5 µm particle size, and the temperature was maintained at 25 °C.

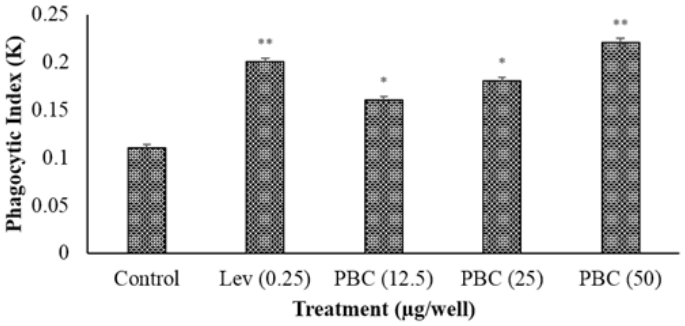

Figure 2: Phagocytic index was calculated in murine macrophage induced by different concentration of PBC PBC (12.5, 25 and 50 µg/well). Data are mean ± S.E. of five animals. *p<0.05 and **p<0.01 compared with control group.

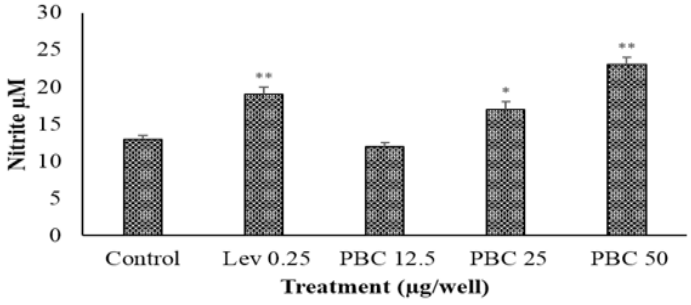

Figure 3: Peritoneal macrophages (3×10^6 cells/mL) were cultured with different concentration of PBC (12.5, 25 and 50 µg/well) for 18 h. Results are expressed in µM. Data are mean ± S.E. of six animals. *p<0.05 and **p<0.01 compared with control group.

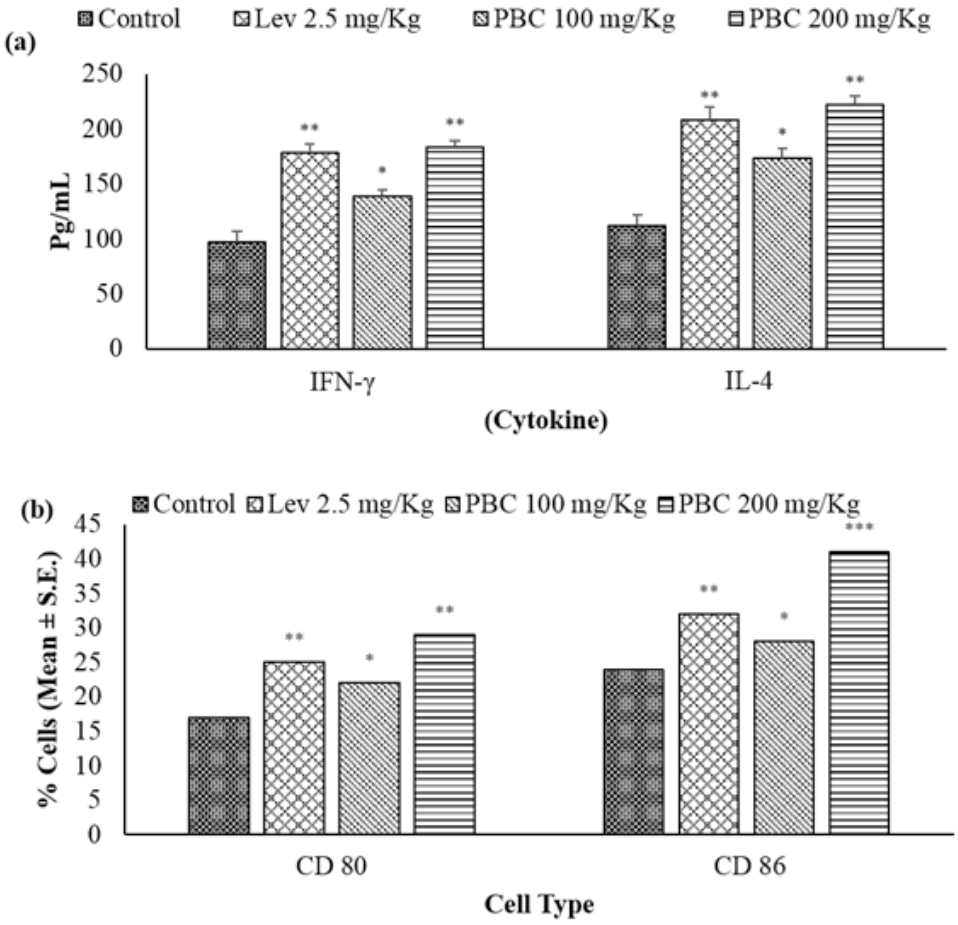

Figure 4: (a) Influence of PBC on the cytokines (IFN-γ and IL-4) expression on mouse serum collected on day 14 after immunization. **(b)** The expression of CD80/CD86 molecules in splenic macrophages. Values are means ± SE; *p<0.05, **p<0.01, and ***P<0.001 (control vs. PBC-treated groups; using one-way ANOVA).

regulation id directly correlated with phagocytic potential, since it leads to generation of superoxide and NO production.[24] In PBC treated groups, Th-1 (IFN- γ) was found to be up along with an increase in the level of Th-2 (IL-4) cytokine. It was also observed that PBC augmented the release of nitric oxide (*in vitro*). The results presented here show that PBC may stimulate the immune function of macrophages through up-regulation of IFN- γ, IL-4 and NO production.

Further, co-stimulatory signals play important role in the delivery of the required immune response through the cell-cell cross talk. CD80 and CD86 have been found essential in maintaining the state of immune response and in maintaining the immune memory respectively.[25] The PBC treated mice showed a significantly enhanced expressions of CD80 and CD86 in splenic macrophages, supporting its potential to activate the antigen presenting cells. PBC up-regulates the co-stimulatory bio-

Table 1: Effect of PBC on HA titre and DTH response

Treatment	Dose (mg/Kg)	Primary antibody titre	Secondary antibody titre	DTH response after 24 h	DTH response after 48 h
Control	-	6.2 ± 0.4	5.8 ± 0.4	1.74 ± 0.06	1.62 ± 0.04
Levamisole	2.5	8.4 ± 0.6*	7.8 ± 0.4*	2.32 ± 0.08**	2.14 ± 0.04*
PBC	100	7.8 ± 0.4*	7.2 ± 0.4*	2.16 ± 0.08*	2.02 ± 0.02*
PBC	200	9.6 ± 0.4**	8.8 ± 0.6**	2.54 ± 0.04**	2.22 ± 0.04**

Data are mean ± S.E. of six animals. *P<0.05 and **P<0.01 compared with control group.

molecules by secreting various cytokines and chemokine which further helps in the generation of effective immune response. In conclusion it may be suggested that immunomodulatory effect of PBC are at least in part due to presence of orientin. We further emphasise that orientin might be effective in nursing the diseases where the underlying defect is a phagocytic dysfunction as it enhances the immune response by improving the function of phagocytic cells. However more studies are required to reinforce the fact.

CONCLUSION

In conclusion, PBC from *P. bambusoides* displayed immunomodulating potential by increasing haemagglutination antibody titre, DTH response, phagocytic activity and also enhanced cytokine production & CD80/CD86 expression. Taken together, these results suggest that it could be a drug of choice, effective in treating the diseases where the underlying defect is a T-cell and B-cell deficiency or phagocytic dysfunction.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest, financial or otherwise.

ABBREVIATION USED

MTT: 3-(4,5-di-methylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide; **CTL:** Cytotoxic T lymphocytes; **LC:** Liquid chromatographyIL-2: Interleukin-2; **IL-4:** Interleukin-4; **IFN-γ:** Interferon-γ; **DTH:** Delay Type Hypersensitivity.

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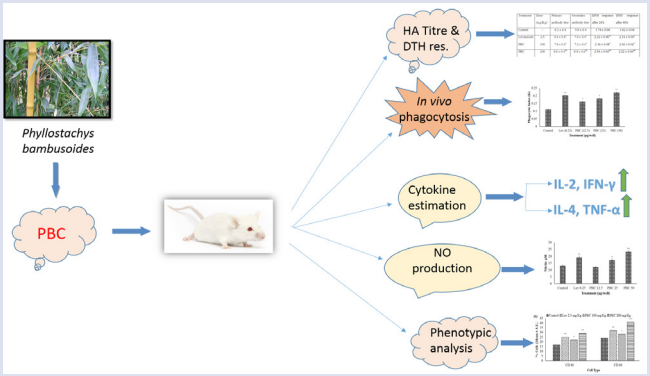
GRAPHICAL ABSTRACT



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