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Haemolytic and immunoadjuvant effect of *Butea frondosa* on the immune response to hepatitis B vaccine containing surface antigen in mice

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ABSTRACT

Introduction: Immunological adjuvants derived from various synthetic micro-organisms or from medicinal plant products enhance specific immune responses against vaccine antigens. Immunological studies have already done pertaining to identify compounds from medicinal plant metabolites that are suitable for vaccine formulation. In this study, aqueous leave extracts of *Butea frondosa* were selected to evaluate their haemolytic activity and immunoadjuvant effects.

Methods: For this study, immunoadjuvant activity was investigated *ex vivo* in mice model based studies using splenocyte proliferation assay and also measured IgG titre in cell culture supernatant (using indirect ELISA). Swiss mice were immunized subcutaneously with specific protein antigen i.e. hepatitis B vaccine containing surface antigen (HBsAg, 20 µg/mL; 10 µl) on day 0 and collected the spleen cells on day 4 and proceeded for proliferation assay of variable doses of aqueous leaves extracts of *Butea frondosa* (0.5–30 mg/ml; 50 µl) along with hepatitis B surface antigen (HBsAg) (challenging dose; 20 µg/mL; 10 µl) and also estimated the antibody (IgG) titre in splenocyte cell culture supernatant including determination of its haemolytic activity in human whole blood samples.

Results: The results demonstrated that aqueous leave extracts stimulated HBsAg population at lower doses (0.5 mg/mL) and also enhanced IgG titre as compared with control and HBsAg treated group. In addition, aqueous leaves extracts of *Butea frondosa* showed anti-HBsAg titre at higher doses and also showed slightly haemolytic effect in human whole blood.

Conclusion: These results suggested that aqueous extract of *Butea frondosa* may represent viable candidate for effective vaccine adjuvants due to their higher immune response (with respect to IgG titre and proliferation assay) and lower or non-haemolytic effects.

Implication for health policy/practice/research/medical education:
Butea frondosa aqueous leaves extracts are capable of enhancing both antibody and cell mediated immune responses against hepatitis B surface antigen (HBsAg) in mice. Furthermore, they expressed a lower haemolytic effect and might be safely used as an adjuvant.

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Introduction

Medicinal plants are normally used as raw material for separating the active ingredients which are generally used for the synthesis of compounds (simple or complex). In contrast, these medicinal plant products (stem, root, leaves, flowers, etc.) showed many immunopharmacological properties e.g. anti-arthritic, anti-inflammatory, adjuvant, immunomodulation, anti-tumor, etc (1-5). Lots of research works have already been done related to those medicinal plant products which proved that metabolites (primary or secondary) have their ability to prevent the appearance or burden of some diseases e.g. glycosides from *Picrorhiza kurroa* (6), polysaccharides (7) fraction from *Boswellia serrata*, aqueous extract of *Azadirachta indica*, etc (8). These medicinal plants have their ability to reduce the burden of disease and used as adjuvants for vaccine antigen.

Adjuvants have been used in vaccines for many years pertaining to increase the immunogenicity of vaccine antigen. Only one adjuvant approved for human use i.e.

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alum which increased only humoral response and poorly elicited cell mediated immunity (9). Recently, synthetic formulation or plant derived adjuvants (e.g. oil in water emulsion, water in oil emulsion, liposomes, cytokines, lipid A, bacterial toxins, carbohydrate polymers and saponins) have been categorized into two major subtypes i.e. particulate and non-particulate (9). Thus, immunological efforts have been made pertaining to identify the ideal adjuvant for vaccine antigen (9,10). As per the literature, metabolites (primary or secondary) extracted from various medicinal plants showed some adjuvant effects against s vaccine antigens. For example, saponins showed highly cytotoxic effects and induced both Th1 and Th2 type of immune response (2-4). Thus, researchers focused only on those medicinal plant metabolites with high immune activity with respect to antibody and cell mediated immune response with low haemolytic effect.

Among medicinal plants, *Butea frondosa* (commonly known as palas; family Fabaceae), grows all over India especially central and western India (11,12). As per the literature, seeds, flowers, leaves, bark etc. have great medicinal value and showed various medicinal properties or uses (11,12). In addition, these medicinal plant parts including leaves have shown anti-inflammatory as well as anti-microbial activities, as well. In this study, our group focused on *Butea frondosa* in order to analyse its haemolytic activity on red blood cells and compared their immunobiological effect to those adjuvants e.g. alum which are commonly used in animal and human vaccination.

Materials and methods

Plant material and phytochemical investigation

Fresh healthy plant leaves of *Butea frondosa* were collected from Vidya Pratishthan's School of Biotechnology garden during the month of January 2016. The plant materials were dried in a shady area and prepared in finely powdered form. For phytochemical investigation, three grams of leaves powder (*Butea frondosa*) were macerated in liquid nitrogen with mortar and pestle and then dissolved in phosphate buffered saline (PBS) (pH 7.2; 30 mL). The aqueous extracts were filtered and used to determine the presence of secondary metabolites. The phytochemical investigation of the aqueous extracts was carried out as per standard methods and evaluated for the presence of phenolic compounds, flavonoids (by alkaline reagent test), saponin (by foam test), terpenoids (by acetic anhydride test) and glycosides. In addition, thin layer chromatography of the aqueous extract was performed using n butanol: acetic acid: water (5:3:2). The observed spots were then visualized using anisaldehyde-sulphuric acid reagent (11).

Immunization

Swiss mice (n = 5) were immunized subcutaneously on day 0 with HBsAg (10 µg) in a final volume of 0.2 mL containing PBS. For this study, PBS was used as negative control.

Splenocyte proliferation assay (ex-vivo)

Splenocyte proliferation was done as per the guidelines of animal ethics committee, CPCSEA. On day 7, Swiss mice were sacrificed by cervical dislocation and the splenocytes were seeded in triplicates at a concentration of 106 cells/mL, 0.1 mL along with variable concentration of aqueous leaves extract of *Butea frondosa* (0.5-30 mg/mL; 50 µl) in flat bottom tissue culture 96 well plate in a final volume of 0.2 mL. hepatitis B surface antigen (HBsAg) (Serum Institute of India; 20 µg/mL; 10 µl) was added to all the wells with or without aqueous extract in a volume of 0.2 mL. After the addition, the samples were incubated for 48 hours at carbon dioxide incubator and then supernatant were collected after centrifugation for IgG titre. After adding fresh medium in 96-well plate, the plate was incubated for another 2 hours. After incubation, MTT solution (2.5 mg/mL; 10 µl) was added. These samples were incubated for another 3 hours at carbon dioxide incubator and then the formazan crystals were observed and dissolved in dimethyl sulphoxide (DMSO). Finally, optical densities (ODs) of each well were evaluated using an ELISA reader at 570 nm (10,11).

ELISA performance

Indirect ELISA was performed using hepatitis B vaccine (HBsAg; 2 µg/mL) as coating antigen. Aqueous leaves extracts of *Butea frondosa* were prepared and the culture supernatants were collected after 48 hours from splenocyte proliferation assay for the estimation of IgG antibody titre. Horse anti-mouse serum was used as secondary antibody, and the OD was measured at 450 nm (8).

Haemolytic activity assay

The haemolytic activity in human whole blood was determined using aqueous extract of *Butea frondosa*. Distilled water was used as positive control for the study. In this study, human whole blood suspension was collected from Mangal Pathology Laboratory, Baramati, and these samples were prepared by diluting the pellet to 0.5% with PBS along with variable concentrations of aqueous leaves extracts of *Butea frondosa*. The samples were incubated for 2 hours at carbon dioxide incubator. After incubation, the samples were centrifuged and the supernatants were collect and estimated the free haemoglobin in the supernatants using spectrophotometer. The OD was measured at 405 nm (13).

Statistical analysis

The difference between control and treated groups of *Butea frondosa* was determined by one-way analysis of variance (ANOVA) test (Bonferroni multiple comparison test). Values were expressed as mean ± standard error of mean (SE).

Results

Splenocyte proliferation assay

The effects of variable doses of aqueous leaves extracts of *Butea frondosa* on splenocyte proliferation assay in mice model are shown in Figure 1. The results showed that there was enhancement in proliferation with respect to

HBsAg using aqueous leaves extracts at lower doses. HBsAg was used as standard and there was enhancement in proliferation as compared to control.

ELISA results

The effects of variable doses of aqueous leaves extracts on IgG antibody titre are as shown in Figure 2. The aqueous leaves extract showed IgG antibody titre at higher doses whereas cell culture supernatant of spleen cells collected after 48 hours showed enhancement of IgG antibody titre at lower doses as compared to control.

Haemolytic activity

The effects of variable doses of aqueous leaves extracts of *Butea frondosa* on haemolytic activity are shown in Figure 3. The aqueous leaves extract did not show any haemolytic activity at higher doses whereas distilled water was used as positive control and showed high haemolytic activity as compared to control.

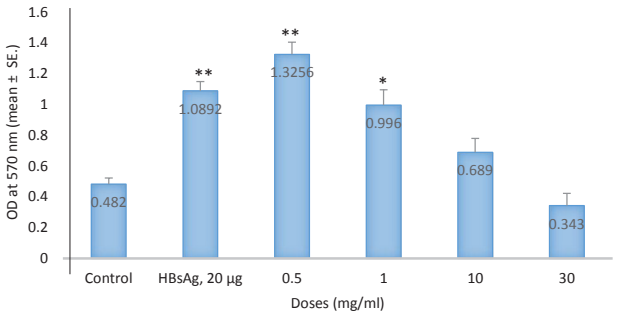

Figure 1. Splenocyte proliferation assay (*ex vivo*). Splenocytes of Swiss mice were prepared on day 4 after immunization with HBsAg, 10 µg on day 0 and cultured with HBsAg or together with variable concentration of aqueous leaves extract (0.5–30 mg/ml; 50 µl) for 48 hours. Splenocyte proliferation was measured using MTT assay as described in materials and methods section. The values are presented as the mean ± SE (n = 6). Significant differences with control groups (samples blank) were designated as $*P < 0.05$ and $**P < 0.01$.

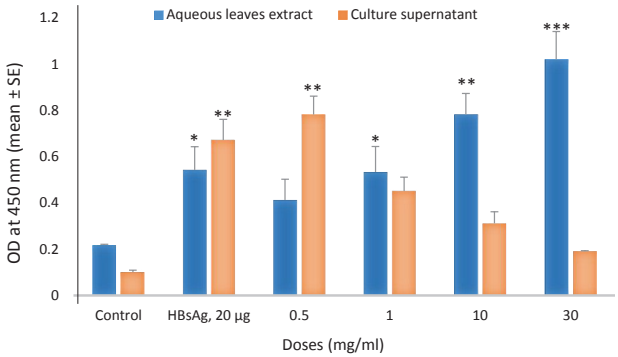

Figure 2. Indirect ELISA (IgG titre). Indirect Elisa was performed using standard HBsAg (1:1000 dilution) vaccine as coating antigen. For IgG titre, aqueous leaves extract of Butea frondosa were prepared and also collect the splenocyte cell culture supernatant were used for the estimation of anti-HBsAg antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm. The values are presented as the mean ± SE (n = 6). Significant differences with control groups (samples blank) were designated as $*P < 0.05$ and $**P < 0.01$.

Discussion

Immunomodulation means to modulate the immune system either stimulatory or suppressive showed some adjuvant effects against specific (e.g. protein) as well as non-specific (e.g. sheep red blood corpuscles; sub-immunogenic dose) antigens (14). For many years, immunopharmacologists have focused on various medicinal plant products in order to carry out their various immunobiological activities e.g. anti-viral, anti-bacteria, anti-inflammatory with respect to antibody and cell mediated immune response (2-5). According to the literature, lots of research works has already been done with respect to medicinal plants that are responsible for curing immunological disorders. In addition, these medicinal plants also played a vital role in the promotion of health by strengthening host defence mechanisms against various diseases (10,11). In order to achieve this objective, our group focused on its haemolytic as well as immunoadjuvant activity of aqueous leaves extract of *Butea frondosa*.

In Asian countries especially India, people believe on various medicinal plants including *Butea frondosa* that have been used in traditional system of medicine for several thousand years and these plants constituted to be an important and basic resource for ethnobotanical research in many ways. In the present study, our group focused on immunoadjuvant activity of *Butea frondosa* using HBsAg as specific protein antigen pertaining to determined its T cell-mediated immunity (i.e. complex interactions between the lymphoid and phagocytic cells) that played an important role in eliminating intracellular infections and was responsible for enhancing the lymphocyte proliferative response (10,11). In this study, HBsAg was used as standard for the study and enhanced the lymphocyte proliferation as compared to control. As shown in Figure 1. Our data showed that aqueous leaves extract of *Butea frondosa* at lower doses enhanced proliferative response as compared to HBsAg and control. Overall, the data showed

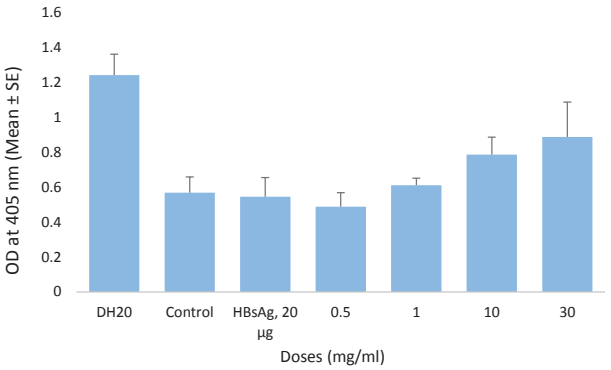

Figure 3. Haemolytic activity. Human whole blood samples were prepared by diluting the pellet in PBS along with variable concentration of aqueous leaves extract of *Butea frondosa*. Haemolytic percentages of PBS and distilled water were included as minimal and maximal haemolytic controls. All values represent the mean ± SE (n = 6). The optical density (OD) was measured at 405 nm.

that the aqueous leaves extract could significantly enhance the T cell response i.e. cell mediated immunity.

The results also showed that aqueous leaves extracts caused enhancement in antibody titre at higher doses whereas splenocyte cell culture supernatant showed some enhancement at lower doses as compared to control. HBsAg containing alum was used as standard for the study and enhanced the production of IgG titre as compared to control. Overall this study claimed that aqueous leaves extract of *Butea frondosa* were effective on antibody and cell mediated immune response with respect to IgG titre and lymphocyte proliferative response using HBsAg containing alum. In addition, aqueous leaves extracts showed slightly haemolytic effect as compared to control. In short, ex vivo experimental results indicated the adjuvant activity of *Butea frondosa* against specific antigen i.e. HBsAg.

Conclusion

Butea frondosa, aqueous leaves extract are capable of enhancing both antibody and cell mediated immune responses against HBsAg in mice. Furthermore, they expressed a lower haemolytic effect and could be safely used as an adjuvant.

Authors' contributions

This work was carried out in collaboration between two authors. AG designed the study, wrote the protocol and interpreted the data. AG and SRC anchored the field study, gathered the initial data and performed preliminary data analysis. AG and SRC managed the literature searches and produced the initial draft. Both the authors read and approved the final manuscript.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

These studies were conducted under ethical guidelines with registration no. 1814/PO/ERE/S/15/CPCSEA.

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