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Epidemiological studies of hepatitis B: Preliminary report on adjuvant potential of *Calotropis gigantea* and *Ficus religiosa* against hepatitis B vaccine containing surface antigen

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ABSTRACT

Background and aims: The frequency of chronic hepatitis B virus infection varies totally in different part of the world but it could be categorized as high, intermediate and low endimicity. In this regard, development of safe and effective vaccines against infectious diseases especially hepatitis B virus that are still required for emerging new pathogens, re-emerging old pathogens and in order to improve the inadequate protection conferred by existing vaccines. In this study, immunological studies were conducted pertaining to investigate the aqueous leaves extract of *Calotropis gigantea* and *Ficus religiosa* for determining its adjuvant effect against hepatitis B vaccine containing surface antigen (HBsAg).
Methods: In this study, our group evaluated the secondary metabolites that are present in the aqueous extract using high performance thin layer chromatography (HPTLC) and estimated its antibody (IgG) titre against HBsAg using variable doses (2.5-10 mg) of aqueous leaves extract of these medicinal plants and also determined splenocyte proliferation assay (*ex vivo* studies) on day 4 where mice (n=5) were immunized subcutaneously on day 0 with HBsAg (20 µg/ml, 100 µl).
Results: The results showed that aqueous leaves extract showed anti-HBsAg titre and also enhanced splenocyte proliferation assay at higher doses (10 mg, 50 µl) as compared to control.
Conclusion: Overall, the results showed that aqueous leaves extract of *Calotropis gigantea* and *Ficus religiosa* showed adjuvant activity against HBsAg.

Keywords: *Calotropis gigantean, Ficus religiosa*, Hepatitis B vaccine, Adjuvant.

Original article

INTRODUCTION

Vaccines are considered to be protective and they saved millions of people all over the world from life-threatening diseases.[1] Most of the microorganisms i.e. pneumococci, rotavirus etc. kills millions of people but it is very difficult to control through old vaccines. However, few of them are in preparation and few of them reached to clinical trials or final

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stages of development.[2,3] For vaccine preparation, major problems are generally reported i.e. instability of microbial genome, absence of adjuvants, lacking of experimentation related to animal model studies, and etc.[4,5] In view of this, it is important to develop new adjuvants (plant based or synthetic) for vaccine antigen that are safe and economically viable. Development or screening of new adjuvants is highly required for vaccine antigen and eliminating various infectious diseases e.g. Hepatitis B virus (HBV).[6,7]

Hepatitis B virus (HBV; *Hepadnaviridae* family) is classified into eight genotypes (i.e. A to H) based on sequence comparison and each genotype showed variation in geographical distribution. Out of three viral particles, HBsAg containing small spherical structures (20 nm diameter) and filaments (variable length, width 22 nm) where as host derived lipids without nucleic acids (viral) are considered to be non-infectious.[6,7] In addition, HBV virion (infectious; double shelled structure; 42 nm in diameter) consisting of lipid envelope containing HBsAg that surrounds inner nucleocapsid composed of hepatitis B core antigen (HBcAg) complexed with virally encoded polymerase and the viral DNA genome.In contrast, detection of HBV disease has been the highest in 2015-16 when 1,200 cases of the total 1.9 lakh samples tested were found to be positive. In comparison, only a 100 were found to be HIV positive (reported in Bowring and Lady Curzon Hospital, Bangalore; 14th June 2016).

In this regard, selection of proposed adjuvant candidates will likely be required to achieve protective immunity against vaccine antigen i.e. HBsAg. Number of immunologists claimed as well as reported that vaccine containing adjuvant (plant based or synthetic) that are capable of generating a broad immune response with high levels of antibodies production (IgG1 and IgG2a) and cytotoxic T lymphocytes that are responsible for eliminating intracellular infections.[6,7] In view of this, there is an urgent need for eliminating this viral disease, lot of vaccines related to HBV that are available. In this study, we used HBV vaccine (Serum institute of India) containing HBsAg (20 µg) adsorbed on aluminium hydroxide (0.5 to 0.8 mg). In view of this, our group focused on those test candidates (i.e. medicinal plantsespecially *Calotropis gigantea* and *Ficus religiosa* will help us how to formulate the proposed adjuvant candidate (aqueous extract) extracted from *Calotropis gigantea* and *Ficus religiosa* in vaccines that are targeted specifically against viral infections. In view of this, these medicinal plants i.e. *Calotropis gigantea* and *Ficus religiosa* were selected because of immunopharmacological applications i.e. anti-inflammatory, antioxidant, proteases etc.[8-11] In the present study, our group evaluated the immunoadjuvant activity of these two medicinal plants against HBsAg.

METHODS

Assembled plant leaves of *Calotropis gigantea* and *Ficus religiosa* were collected from Vidya Pratishthan's garden in the month of March 2016 and studied its immune adjuvant activity against HBsAg using these medicinal plant leaves macerated in liquid nitrogen and dissolved in phosphate buffered saline (PBS) in order to prepare aqueous extract.

For these studies, HPTLC analysis were performed and revealed the presence of secondary metabolites i.e. saponin, terpenoids, flavonoids and phenolics in the phytochemical profile of aqueous leaves extract of *Calotropis gigantea* and *Ficus religiosa*. The retardation factor (Rf) value of secondary metabolites in *Calotropis gigantea* (saponin, 0.31-0.46; terpenoid, 0.92; phenolics <0.72) where as *Ficus religiosa* (saponin, 0.37-0.79; terpenoid, 0.96; phenolics, 1.14).

For immune adjuvant activity, indirect Elisa was performed using standard HBsAg (1:1000 dilution) as coating antigen. Variable doses (2.5-10 mg) of aqueous leaves extract of *Calotropis gigantea* and *Ficus religiosa* were used and anti-HBsAg used as standard for the estimation of IgG antibody titre. Horse anti-serum used as secondary antibody and optical density (OD) measured at 450 nm.[12]

Ex vivo studies were carried out in mice model studies. Mice were immunized on day 0 with HBsAg (20 µg/ml; 100 µl) and collected splenocytes on day 4 for proliferation assay and estimation of Th1 (IFN-gamma and TNF alpha) cytokines from cell culture supernatant. These studies were conducted under ethical guidelines.

Briefly, splenocytes (10^5 cells/well; 100 µl) were taken in 96 well plate for 48 h incubation in carbon dioxide incubator along with variable concentration of aqueous leaves extract (2.5-10 mg; 50 µl). Thereafter, further exposed with HBsAg (20 µg/ml; 10 µl) vaccinein a final volume of 0.2 ml. All these studies were conducted and analyzed at our institute between March to May 2016. After incubation, it was collected the supernatant after centrifuging these splenocytes samples containing HBsAg for estimation of Th1 (IFN-gamma and TNF alpha) cytokines from cell culture supernatant. Finally, freshly added media in 96 well plate and then added MTT (2.5 mg/ml; 10 µl) solution. Incubate 96 well plate for another 3-4 h; centrifuging the plate, discarded the supernatant and formazon crystals settled at the bottom. These crystals were dissolved in dimethyl sulphoxide and the optical density was measured at 570 nm.[13,14]

In addition, measurement of Th1 cytokines (IFN-gamma and TNF alpha) production from splenocytes cell culture supernatant containing HBsAg of *Calotropis gigantea* and *Ficus religiosa* and this experiment was conducted using conventional ELISA kits and reagents, BD Biosciences, according to the manufacturer's instructions.[15]

The difference between control and treated groups of *Calotropis gigantea* and *Ficus religiosa* was determined by one way ANOVA test (using SPSS).

RESULTS

Elisa results confirmed that aqueous leaves extract showed anti-HBsAg titre at higher doses (10 mg) as shown in Figure 1. Anti-HBsAg culture supernatant of splenocytes used as standard for these studies and results showed enhancement of anti-HBsAg titre as compared to control.

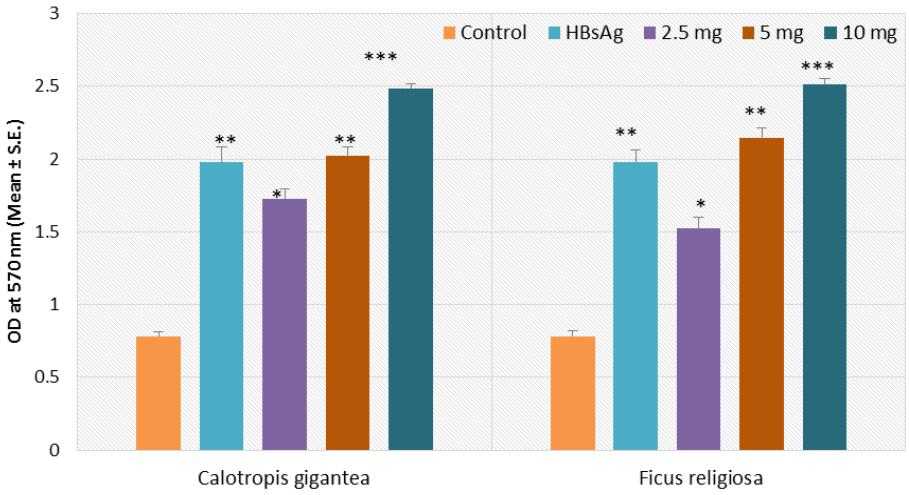


Figure 1: ELISA assay

Indirect Elisa was performed using standard HBsAg (1:1000 dilution) as coating antigen. Aqueous leaves extract of Calotropis gigantea and Ficus religiosa, anti-HBsAg samples 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 difference between the control and standard is determined by one way ANOVA test.

The effect of aqueous leaves extract on splenocyte proliferation assay as shown in Figure 2. The results showed that aqueous leaves extract showed anti-HBsAg proliferation at higher doses (10 mg; 50 µl). Anti-HBsAg cell culture supernatant was used as standard for these studies and results showed enhancement of proliferation as compared to control.

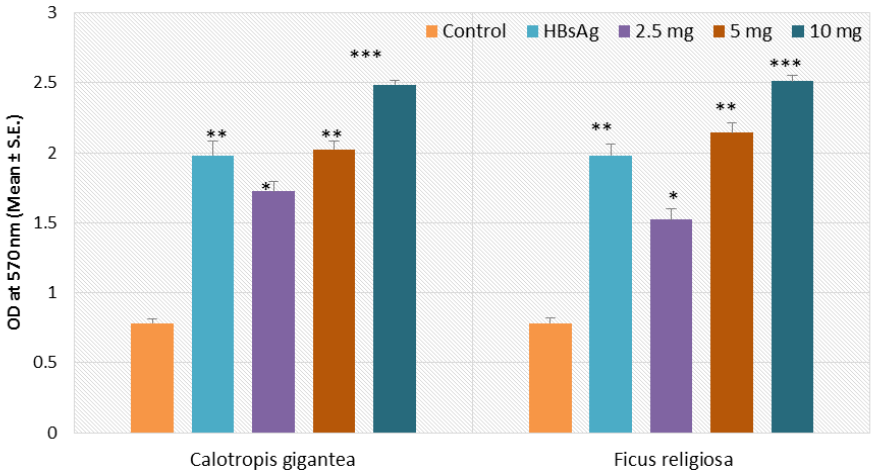
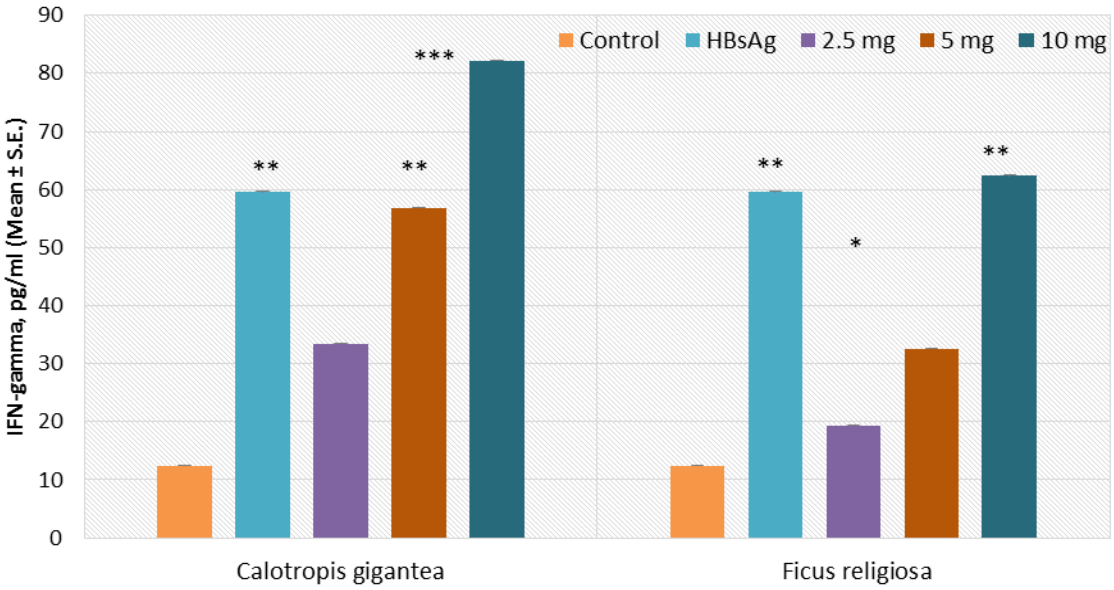


Figure 2: Splenocyte proliferation assay

Spleen cells (106 cells/ml; 100 µl) were treated with variable doses of aqueous leaves extract in presence HBsAg (as already described in materials and methods section). Values are expressed in Mean ± S.E.The difference between the control and standard is determined by one way ANOVA test.

In this study, splenocyte cell culture supernatant containing HBsAg were collected for estimation of Th1 cytokines as shown in Figure 3. The results showed that aqueous leaves extract showed enhancement in Th1 cytokines at lower doses (0.625 mg) as compared to control and standard (HBsAg).

A) IFN- gamma



B) TNF alpha

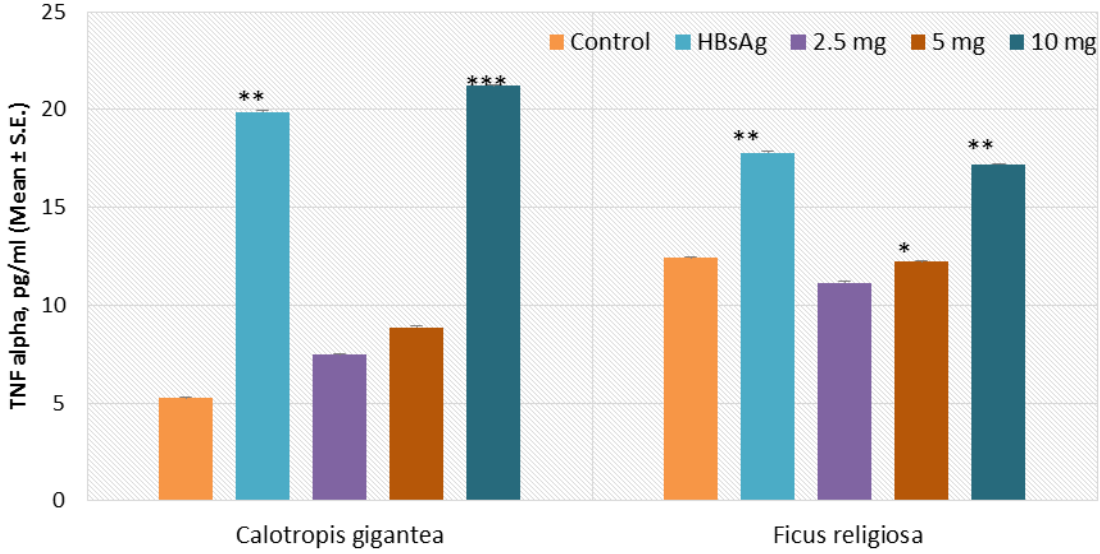


Figure 3: ELISA assay (Th1 cytokines)

Spleen cells (106 cells/ml; 100 µl) were treated with variable doses of aqueous leaves extract in presence HBsAg and cell culture supernatant was collected for the estimation of cytokines (IFN-gamma and TNF alpha). Values are expressed in Mean ± S.E.The difference between the control and standard is determined by one way ANOVA test.

DISCUSSION

Hepatitis B is highly endemic disease and is reported in various developing regions (with large population) i.e. South East Asia, China, sub-Saharan Africa, and etc. In these areas, more than 80 % of the population showed serological evidence of hepatitis B viral infection. Most of these infections are generally caused during infancy or childhood. Since most of these infections in children are asymptomatic but evidence is very less related to hepatitis B viral infection, but the rates of chronic liver disease or liver cancer are highly reported in adults. In this regard, our group focused on those medicinal plants that tried to reduce the burden of this disease i.e. hepatitis B. Lot of commercial vaccines that are available in the market but still trying to increase the immunogenicity of this vaccine antigen. In other words, medicinal plant products were considered and proved as alternative sources of vaccine adjuvants against specific protein antigens e.g. HBsAg. Lot of research work is done related to plant based adjuvants for vaccine production. Most of the commercial vaccines containing adjuvant i.e. alum that are available and administered subcutaneously, intraperitoneally, intradermal, and etc. which have the capability to enhance antibody response. Alum is one of the approved adjuvant for human use that elicits only humeral antibody (Th2) response and poorly elicited cell mediated (Th1) immunity. In an effort to screen those molecules or test adjuvant candidates extracted from medicinal plants that enhanced antibody profile (IgG) and cell mediated (T cell derived cytokines i.e. IFN-gamma and TNF alpha) immune response against vaccine antigen (HBsAg).[6,7] For these studies, indirect Elisa was performed using HBsAgas coating antigen andtestedvariable doses of aqueous leaves extract of *Calotropis gigantea* and *Ficus religiosa* and showed anti-HBsAg titre. For further confirmation of its immunoadjuvant activity tested in mice model studies using *ex vivo* studies and performed splenocyte proliferation assay.

In animal model studies, mice were immunized with HBsAg intraperitoneally on day 0 and collect the splenocytes on day 4 and were further exposed to HBsAg in the presence of aqueous leaves extract of these two medicinal plants and showeddose dependent change (enhancement at lower doses) in the proliferation as compared to HBsAg and control. In addition, HBsAg was recognized strongly by aqueous leaves extract containing primary as well as secondary metabolites and antibody-secreting cells from vaccines. Studying this interaction will allow us to further elucidate the effectiveness of antigen adjuvant combinations. For its isolation, extraction as well as purification of adjuvants from various medicinal plants were considered to be safe and totally free from animal or human pathogens, and also provided some beneficial effects in terms of cost, distribution and production.

As per the literature which reveals that Th1 cytokines (IFN-gamma and TNF alpha) are the indicators of cell mediated immunity.[6,7] In this study, we evaluated immunoadjuvant activity of aqueous extract and can be determined through cytokines

from splenocyte cell culture supernatant whether these aqueous extract showed stimulatory or suppressive effect. The results showed that aqueous extract showed enhancement in Th1 cytokines at lower doses as compared to HBsAg and control.Overall, the results showed that aqueous leaves extract of *Calotropis gigantea* and *Ficus religiosa* showed vaccine adjuvant potential, but it's urgently needed some preservatives to store for long time in clinical trial studies.

The potencyrate of routine infant hepatitis B immunization is significantly reduced or inhibiting the prevalence of chronic hepatitis B viral infection has been reported in number of countries. However, there are still many challenges or approaches to achieve the goal of universal childhood immunization against these infectious diseases especially hepatitis B, such as imperfect immunization delivery infrastructure, low coverage and lack of financial sustainability. Therefore, to continue to promote access to hepatitis B vaccines worldwide, great efforts are needed to support countries to ensure sustained funding for immunization programs. Additionally, because the current vaccine must be given by injection, the development of an effective and safe oral HBV vaccine would be advantageous.

CONCLUSION

Medicinal plants i.e. *Calotropis gigantea* and *Ficus religiosa* were selected because of number of immune pharmacological studies as well as their integral role in cellular and humoral immunity. These findings about these proposed adjuvants can also be used to formulate with other vaccine antigens.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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