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Immunological exploration based studies on *Strychnos nux-vomica* regarding antigen specific immune response

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ABSTRACT: In an effort to determine its cytotoxicity as well as antigen specific immune activity of aqueous leaves extract of *Strychnos nux-vomica* using hepatitis B vaccine containing surface antigen (HBsAg; 20 µg/ml) pertaining to antibody production and scrutinize its proliferative response along with cytokines in lysed human whole blood. For these studies, phytochemical (qualitative) analysis was determined and evaluates the presence of secondary metabolites through high performance thin layer chromatography (HPTLC) and bio-inorganic fingerprinting. In addition, indirect Elisa was performed using HBsAg as coating antigen using variable doses (1-30 mg/ml) of *Strychnos nux-vomica*. In continuation of these immunological studies, antigen specific immune response along with cytotoxicity was determined through MTT assay in infected human whole blood using HBsAg (20 µg/ml, 50 µl). The results showed that *Strychnos nux-vomica* showed qualitatively as well as quantitatively determined the presence of secondary metabolites along with bio-inorganic compounds. In addition, *Strychnos nux-vomica* showed enhancement in anti-HBsAg IgG titre as compared to standard and control but there is sudden decline in proliferation with HBsAg and also showed decline in cytokines (IL-2 and IL-12) level at higher doses as compared to control. Our data suggest that *Strychnos nux-vomica* may help to raise antibodies against HBsAg but sudden decline in HBsAg proliferative response along with cytokines (IL-2 and IL-12) in infected lysed human whole blood and also showed some cytotoxic effect at higher doses. In other words, *Strychnos nux-vomica* could be a potent immune enhancer of B cells and inhibitor of T cells against HBsAg.

Keywords: *Strychnos nux-vomica*; Hepatitis B vaccine; Elisa; Cytotoxicity; Antigen.

1. INTRODUCTION

Medicinal plant products showed numerous properties related to human health care. In other words, these medicinal plant products played a significant effect were reported in case of human especially reported in the prevention of various pathogenic diseases [1, 2]. As per the literature, various medicinal plant products were considered as immunomodulator e.g. immunostimulator (help combat viral infection such as aids and

cancer), immunosuppressor (preventing rejection of transplanted organs) and immunoadjuvants (enhancing humoral or cell mediated immune response) [1-5]. So, these immunomodulators provide some alternative in comparison with conventional chemotherapy for a variety of intracellular (viral) diseases along with autoimmune disorders where selective type of immunosuppressant is used [4-6]. In view of this, most of the medicinal plant products may be in the form of primary and secondary metabolites which constitute a common alternative treatment of immunocompromised patients in many countries around the world.

Out of these, one of the medicinal plant i.e. *Strychnos nux-vomica* (commonly known as Kuchla) belonging to the family *Loganiaceae*, which is commonly found in and grows in Sri Lanka, India (Maharashtra) and Australia. *Strychnos nux-vomica* L. (SN) is widely used in Indian traditional medicine (Ayurveda) for treating a wide range of ailments which include paralysis, diabetes, gonorrhea, anemia, bronchitis, etc. [7-12]. It is also known to possess antioxidant and anti-snake venom activity [13]. The chemical composition of SN is mainly comprised of indole alkaloids. The major alkaloids are strychnine and brucine, which are being employed clinically as efficient stimulants of nervous system [13-25]. In traditional Chinese medicine, *Strychnos nux-vomica* (Fig. 1) has been employed for the treatment of liver cancer. The crude extracts and purified alkaloids from *Strychnos nux-vomica* have been shown to inhibit the growth of various cancer cell lines in vitro. In spite of numerous therapeutic effects of *Strychnos nux-vomica*, its effect on hematologic malignancies is still ill-defined. Due to the lack of awareness about its medicinal properties of *Strychnos nux-vomica*, it is used very less for therapeutic purposes.

Figure 1. *Strychnos nux-vomica.*

Recently, more than 84 compounds were reported in the form of secondary metabolites i.e. alkaloids, iridoid glycosides, flavonoid glycosides, triterpenoids, steroids and organic acids, among others, have been isolated, purified and identified its structure from *Strychnos nux-vomica* [13-25]. In other words, these compounds in the form of secondary metabolites possess an array of immunobiological activities, including its effects on the nervous system, analgesic and anti-inflammatory actions, anticancer effect, declining in the

growth of various pathogenic microorganisms etc. According to the literature of this plant, *Strychnos nux-vomica* which is already reported as anti-bacterial, anti-hyperglycemic and anti-oxidative activities. Apart from these medicinal properties, uses of this medicinal plant products are also reported e.g. Seeds used in atonic dyspepsia, tincture used in mixtures or its stimulant action on gastro intestinal tract. It stimulates peristalsis; strychnine is used as a gastric tonic in dyspepsia. Brucine is used in pruritus and as a local anodyne in inflammations of the external ear; root is biter, aphrodisiac, tonic. It is effective for skin diseases of scaly nature, leprosy, eczema and scabies [13-25]. It is blood purifier and is therefore helpful in curing skin disorders and allergies. It regulates the histamine secretion and helps building immunity to fight against various allergens. It is also good for inflammation, chronic pains in the muscles and joints; leaves are normally applied as a poultice especially on sloughing wounds and also applied on ulcers as well. These are also applied as well as in the preparation or synthesis of medicated product for hair as well as scalp [13-25]. In view of this literature, leaves of this medicinal plant were investigated its effect on antigen specific immune response.

2. MATERIALS AND METHODS

2.1. Reagents and antibodies

HBsAg vaccine (Serum Institute of India), horse antiserum, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Himedia), capture and detection antibody IL-2 and IL-12 (BD optia Elisa kit).

2.2. Botanical description and demand of plant product

There is a growing demand in pharmaceutical companies especially for fruits/seeds of this species i.e. *Strychnos nux-vomica*. One of the most familiar example is seen where the demand for *Strychnos nux-vomica* fruits in Maharashtra state of India alone as 300 kg per year. Numerous effects were taken by various researchers in order to enhance the production of raw material and used by various pharmaceutical companies. So, there is urgent need in order to enhance its production rate and paid more attention to cultivation of medicinal plants. In this case, seed propagation is one of the most often used and cheapest method, knowledge of seed handling is a prerequisite for growing this species successfully. Till now, there is no information related to the seeds of *Strychnos nux-vomica* possess dormancy. For ex situ conservation purposes related to this plant i.e. seed storage behavior i.e., desiccation sensitivity, temperature of storage along with moisture content of the seeds including longevity of the seeds in storage, should be known.

2.3. HPTLC analysis and bio-inorganic fingerprinting of leaves

The plant was washed with sufficient quantity of distilled water. For the preparation of aqueous extract, leaf sample was macerated to finely powder form and this was used for the immunological studies. The aqueous extraction usually done in phosphate buffered saline and crushed in a mortar pestle and the extract was centrifuged at 10000 rpm at 4°C for 10 minutes. The supernatant was collected and revealed the presence of terpenoids, flavonoids and phenolics. For confirmation, this sample was used for HPTLC analysis and bio-inorganic fingerprinting (using atomic absorption spectrometer analysis) of leaves of *Strychnos nux-vomica*. The solvents including purified reagents along with HPTLC plates (10 x 10 cm) were purchased from

Qualigens and Merck. The stock solution of the leaves of *Strychnos nux-vomica* was prepared for HPTLC studies and dissolved the leaves in phosphate buffered saline or with different solvents. Plate was scanned by using densitometric scanner.

For bio-inorganic fingerprinting, air was generally allowed pertaining to mix with acetylene gas (using gas cylinder, good proportion to ignite the burner of the spectrophotometer). After ignition, calibrate the spectrophotometer using blank (distilled water) and also standard solution (as supplied with the spectrophotometer) as well. Finally, sample aerosol was then aspirated through nebulizer into the flame for analysis. Analysis was done for detecting these elements at their specific wavelength using hollow cathode lamp and its result is displayed on the computer read-out.

2.4. ELISA

Indirect Elisa was performed using standard HBsAg vaccine (1:1000 dilution) as coating antigen. Variable concentrations of *Strychnos nux-vomica* were added and determined its anti-HBsAg titre. Anti-HBsAg serum was used as standard for the estimation of IgG antibody titre. Horse anti-serum used as secondary antibody and absorbance in the form of optical density measured at 450 nm [26].

2.5. Immunopharmacological activity

Determination its immunopharmacological activity on the basis of its bioassays for measuring its cytotoxic (MTT assay in human whole blood) and antigen specific immune activity (lymphocyte proliferation assay using HBsAg and estimating cytokines i.e. IL-2 and IL-12 as well through Elisa activity) of *Strychnos nux-vomica*.

2.5.1. Cytotoxicity and anti-inflammatory activity

Lysed human whole blood (10^6 cells/ml; 100 µl) were taken in 96 well plate in absence or presence of HBsAg (20 µg/ml; 10 µl) vaccine for 24 h incubation in carbon dioxide incubator along with variable concentration of *Strychnos nux-vomica* in a final volume of 0.2 ml. Centrifuging the plate, collect the supernatant for the estimation of cytokines. After collection, add MTT (5 mg/ml; 10 µl) solution in 96 well plates. Incubate the plate for another 2-3 h and observed formazan crystals settled at the bottom. These crystals were dissolved in dimethyl sulphoxide and the optical density was measured at 570 nm [27, 28] using microplate reader (Bio-Rad Laboratories India).

2.5.2. Estimation of cytokines

Th1 (IL-2 and IL-12) were measured with an enzyme linked immunosorbent assay (BD optia kit) according to the instructions of the manufacturer [29]. In this kit, add ELISA diluent (50 µl) into each well (96 well Elisa plate; high protein binding) and also add standard or sample (100 µl). So, incubate these samples for 2 h at room temperature. Thereafter, aspirate and wash the sample including standard five times. Add working detector (100 µl) to each well and then incubate these samples for one hour at room temperature. Finally, aspirate and wash these samples (seven times) and then add TMB (100 µl) substrate reagent to each

well. Incubate these samples at room temperature and then add stop solution (50 µl) to each well. Read these samples at 450 nm within 30 minutes.

2.6. Statistical analysis

The difference between control, standard and *Strychnos nux-vomica* is determined by Bonferroni multiple comparison test (One way ANOVA test).

3. RESULTS

3.1. ELISA

The results of these studies may indicate that *Strychnos nux-vomica* showed higher antibody production at higher doses as compared to control (Fig. 2).

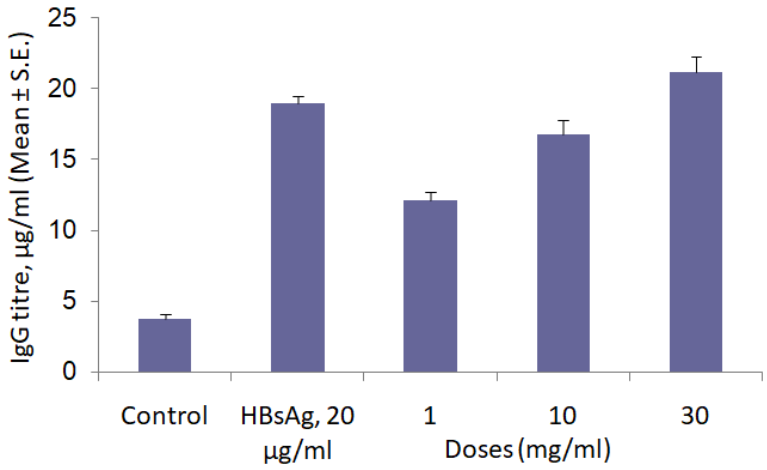

Figure 2. ELISA assay. Indirect Elisa was performed using standard HBsAg as coating antigen. Variable concentration of *Strychnos nux-vomica* was used for the estimation of anti-HBsAg antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm. The difference between the control and standard is determined by one way ANOVA test. *P<0.05; **P < 0.01 and ***P < 0.001.

3.2. HPTLC analysis and Bio-inorganic fingerprinting

The mobile phase for *Strychnos nux-vomica* was ethyl acetate: n-butanol in the ratio of 3:2. So, resolution for *Strychnos nux-vomica* was obtained at 220 nm. The results showed that maximum concentration with an area under the curve of 200 and its peaks also obtained at 1.21. In terpenoids, *Strychnos nux-vomica* showed its presence in retention factor (RF) value i.e. 0.93. In addition, bio-inorganic fingerprinting of *Strychnos nux-vomica* is done by using atomic absorption spectroscopy (AAS). *Strychnos nux-vomica* plant leaves are analyzed for trace metal contents- copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), calcium (Ca) and zinc (Zn). Sample preparation was done in both water and HCl. Data is recorded in ppm. The aqueous extract showed the presence of Cu (0.26), Fe (0.34), Mn (4.42), Mg (0.11), Ca (4.99) and Zn (0.644). Further studies are taken, HPLC analysis of the aqueous extract it was observed that it's totally endotoxin free fraction.

3.3. MTT assay

In cytotoxicity based assay, MTT assay is generally used for determining dose response curve of *Strychnos nux-vomica* against HBsAg (Fig. 3). From these studies, it showed inhibitory effect at higher doses in human whole blood after exposure with variable concentration of *Strychnos nux-vomica*. This activity is due to the presence of secondary metabolites (flavonoids, terpenoids and phenolics) which is determined qualitatively.

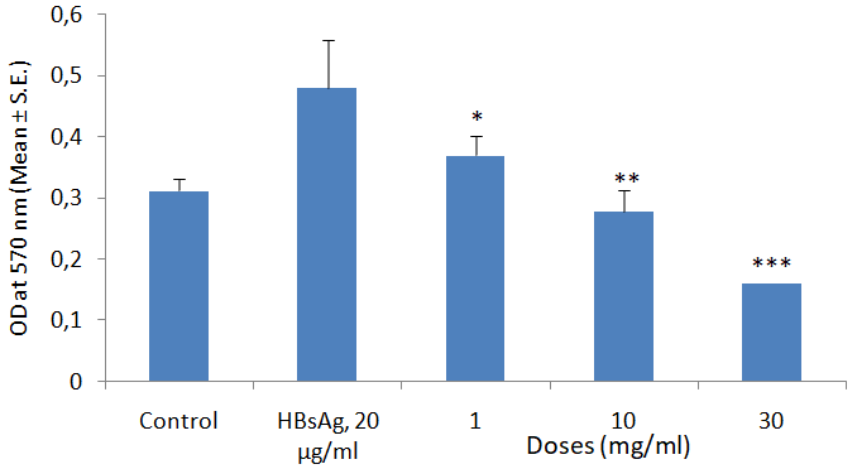

Figure 3. Inhibition of antigen specific immune response using HBsAg in human whole blood which is determined through MTT assay. Values are expressed as Mean ± S.E. Statistical analysis is performed between control vs standard; standard vs variable concentration. *P<0.05; **P<0.01 and ***P<0.001

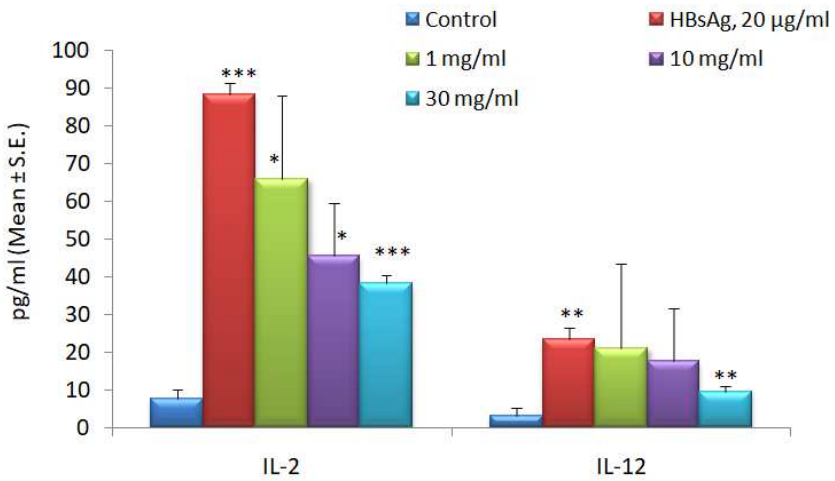

Figure 4. Estimation of cytokines from cell culture supernatant in lysed human whole blood containing HBsAg. Values are expressed as Mean ± S.E. Statistical analysis is performed between control vs standard; standard vs variable concentration. *P<0.05; **P<0.01 and ***P<0.001

3.4. Estimation of cytokines

In anti-inflammatory studies, following assays were used i.e. lymphocyte proliferation assay using HBsAg and also measuring cytokines from cell culture supernatant. The result of these studies related to

Strychnos nux-vomica which inhibits T cell immunity with respect to HBsAg (specific antigen) can be shown by the reduction of lymphocyte proliferation response. In other words, *Strychnos nux-vomica* may significantly decreases the activation potential of T cells observed in human whole blood. For confirmation, Elisa test were performed for determining cytokines from human whole blood cell culture supernatant in presence of *Strychnos nux-vomica* extracted treated with HBsAg. The results may clear cut indication that *Strychnos nux-vomica* showed significant effect on IL-2 and IL-12 cytokines, thereby confirming its inhibitory effect on the cell-mediated immune response (Fig. 4).

4. DISCUSSION

Medicinal plants are widely useful and also claimed to be very effective in the treatment of various diseases especially using various traditional systems of medicine. Various S*trychnos* species are already reported traditionally e.g. *S. potatorum*, used in the treatment of cardiovascular disease i.e. diabetes and is found to be every effective. Considering these facts, this study deals with the evaluation of cytoxicity and anti-inflammatory activity in *Strychnos nux-vomica* [13-18]. Till date, numerous chemical compounds especially strychnine and brucine are already reported and isolated from different plant parts of *Strychnos nux-vomica* and considered them as major active principle which is responsible for its therapeutic potential and toxicity [13-25].

Availability of current treatment are there in order to control various types of immunopathological disorders only using various types of immunosuppressive drugs. In this regard, various approaches should be taken pertaining to induce or suppress antigen specific immunological tolerance that has the ability to induce or control or suppress cellular as well as humoral immune responses at the appropriate level. These type of responses are generally desirable in order to maintain and regulating the proper immune system. However, further immunopharmacological research should be designed pertaining to determine its cytotoxicity and antigen specific immune activity of *Strychnos nux-vomica* in human whole blood. The results showed that *Strychnos nux-vomica* showed decline its activity in presence or absence of HBsAg antigen in lysed human whole blood, thereby supporting its cytotoxic as well as declining in antigen specific immune properties. Both these activities could be possible only due to the presence of secondary metabolites that are reported in *Strychnos nux-vomica*.

The effect of *Strychnos nux-vomica* at various concentrations on lysed human whole blood and was evaluated its cytotoxicity against HBsAg using MTT assay. The optical density of proliferation of lymphocytes at different concentrations and showing that *Strychnos nux-vomica* inhibited HBsAg stimulations at higher concentrations. In other words, *Strychnos nux-vomica* indicates its potential as an effective immunosuppressive compound. In addition, inhibition of HBsAg specific immune response is mainly due to decline in T lymphocytes production in case of human lysed whole blood. According to the literature, T lymphocytes may play a central role seen in case of antigen (e.g. HBsAg) specific immune response against various pathogens [29]. Finally, our data show that *Strychnos nux-vomica* decline in the proliferation of T cells in human lysed whole blood with respect to HBsAg stimulation. Overall, the data reveals that *Strychnos nux-vomica* decrease HBsAg proliferation rate at higher doses.

Modulation of cytokine secretion in the form of stimulation or suppression showed some novel approaches for the treatment of various diseases [29]. One of the observation is seen in case of *Strychnos nux-vomica* showed some modulation i.e. inhibition in cytokine expression. IL-12 is a cytokine released by DCs which can induce differentiation of T cells to Th1 and cellular immunity. Our results showed that *Strychnos nux-vomica* decreased production of IL-12 comparing with control however this change was highly significant. In addition, *Strychnos nux-vomica* decreased production of IL-2 cytokine but this change was also significant. This inhibitory activity of *Strychnos nux-vomica* is controlled by various mediators i.e. cytokines such as IL-2 and IL-12 cytokines. These cytokines are probably the most valuable mediator of anti-microbial and anti-tumoral defense and these cytokines have the ability to destroy vital cellular structures like lipids and proteins and to inhibit growth and proliferation of cells and pathogens [29]. These studies may be confirmed that *Strychnos nux-vomica* significantly decreased IL-2 and IL-12 cytokine production.

5. CONCLUSION

Aqueous leaves extracts of *Strychnos nux-vomica* showed some cytotoxic as well as inhibition in antigen specific immune activity in lysed human whole blood using HBsAg. This medicinal plant might be of some value especially in case of T cell mediated disorders i.e. autoimmune disease. Further type of immunopharmacological should be done pertaining to determine the exact phytoconstituents or secondary metabolites that are responsible for inhibiting T cell proliferation.

Conflict of Interest: The author declares no conflict of interest.

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