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COMPARATIVE EVALUATION OF AQUEOUS EXTRACT AND PROTEASE FROM *ADHATODA VASICA* FOR DETERMINING ITS IMMUNOSUPPRESSIVE ACTIVITY

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Abstract:
In order to compared its immunopharmacological activity of protease (against Casein) and aqueous leaves extract of Adhatoda vasica (using phosphate buffered saline, PBS, pH 7.2) against hepatitis B Vaccine containing surface antigen (HBsAg; 20 µg/ml) and determined antibody (IgG) production through indirect Elisa and also examined its proliferative response including nitric oxide production. For these studies, qualitative assays were determined and evaluate the presence of secondary metabolites in aqueous leaves extract whereas concentration of protein is also determined for protease separation. The results showed that aqueous leaves extract and protease at higher doses showed enhancement in anti-HBsAg IgG titre as compared to standard and control but there is sudden decline in proliferation including NO production containing HBsAg at higher doses. In short, protease and aqueous leaves extract of Adhatoda vasica could be a potent immune enhancer of B cells and inhibitor of T cells.

Key words: *Adhatoda vasica; aqueous extract; protease; nitric oxide; proliferation; Elisa*

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INTRODUCTION:

India has a long history of using various medicinal plant products for treating various inflammatory and cardiovascular diseases. Most of the Indian plants have been investigated for its beneficial use in various types of diseases and reported as well as published in numerous scientific journals [1, 2]. The medicinal value of various plant products that contained chemical substances in the form of primary and secondary metabolites that produce a define physiological action on human body [3]. The use of these medicinal plants as a traditional medicine is well known especially in rural areas of many developing countries. Traditionally, medicinal plant products in the form of medicine are cheaper as well as effective an showed less side effects as compared to synthetic drugs [4]. Therefore, screening of these medicinal plants for pharmacological activities that are important for finding potential new compounds for therapeutic use.

In recent years, researchers showed some interest related to proteases for the treatment of various infectious diseases. Some potent protease based drug have been reported and isolated from medicinal plant products and showed anti-diabetic, anti-microbial effect etc. [5-7] further research is still required for the lead molecule as well as the search for new bioactive compounds from various medicinal plant products. For the last ten years, people were aware about the importance and useful aspects of medicinal plants which have shown to exhibit potent immunosuppressive effect in the treatment of various viral diseases by using various models [8-10]. To achieve this objective, one of the medicinal plants i.e. *Adhatoda vasica* (commonly known as Adulsa) [11-14] belongs to the family *Acanthaceae* that are used generally for various diseases especially in case of asthma and bronchitis patients. In addition, this medicinal plant also showed various immunopharmacological applications e.g. hepatoprotective, antioxidant, analgesic etc. As per the literature, comparative difference between aqueous extract and isolated protease from the leaves of *Adhatoda vasica* is not studied still so far related to its immunosuppressive activity especially in human whole blood

MATERIALS AND METHODS:

Collection of plants

The study plant *Adhatoda vasica* was collected in Baramati region and identification was done by Late Dr Sharadini Dahanukar (founder of Nakshatra udyan, Vidya Pratishthan's).

Aqueous extract preparation and qualitative estimation

Two grams (2 g) of powdered plant material of *Adhatoda vasica* were macerated (using liquid nitrogen) and then dissolved in phosphate buffered saline (PBS, 50 mL) using mortar and pestle. During filtration, supernatant was collected for the estimation of secondary metabolites in aqueous leaves extract. Further, these studies were carried out using standard procedure pertaining to phytochemical constituents i.e. terpenoid, flavonoid, phenolics and saponins.

Extraction of protease

Leaves samples are dried at room temperature (3-4 days). Then the leaves sample were grinded and make it in powder form. This powder is used for extraction of protease sample using solvent (100 % methanol) extraction process. The extract was loaded on silica gel column and eluted sample were collected and confirmed using qualitative assay (i.e. Casein agar plate test and spot was visualized on TLC plate). The protease (1.2 mg/ml) content of the fraction where estimated spectroscopically (UV visible spectrophotometer) and used for further studies.

Enzyme linked immunosorbent assay (ELISA)

Indirect Elisa was performed using hepatitis B vaccine containing surface antigen (HBsAg) as coating antigen. Variable doses (0.312– 1 mg) of aqueous leaves extract and protease of *Adhatoda vasica* were used. For these studies, horse anti-serum used as secondary antibody and the absorbance values in the form of optical density (OD) and these readings are measured at 450 nm [15].

Proliferation assay

Human whole blood samples (10^6 cells/ml, 100 µl) were cultured along with variable doses of protease and aqueous leaves extract of *Adhatoda vasica* in RPMI 1640 medium containing 10% heat-inactivated Fetal calf serum (FCS) along with penicillin (100 IU/ml) and streptomycin (100 µg/ml) in 96 well tissue culture plate. Incubate the plate for 24 h at carbon dioxide incubator. After incubation, MTT solution was added and again incubate it for another 4 h. Centrifuge the plate, collect supernatant (nitric oxide, NO production) and observed formazon crystals settled at the bottom. Dissolve the formazon crystals in dimethyl sulphoxide (DMSO) solution and then analyzed its optical density (OD) at 570 nm [16, 17].

Nitric oxide production

For nitric oxide (NO) estimation using cell culture supernatant (50 µl) from lysed human whole blood along with variable doses of protease and aqueous leaves extract of *Adhatoda vasica* was mixed with Griess reagent (50 µl containing 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2- 2.5% phosphoric acid) in 96 well flat bottom plate. Incubated the plates at room temperature for 10 minutes, and absorbance at 540 nm was measured. The cell culture medium (PBS, phosphate buffered saline containing 10 % fetal bovine serum) was used as a blank. The nitrite quantity (µM) was determined from a sodium nitrite standard curve [17].

RESULTS:

ELISA

As shown in **Fig.1**, the results showed that aqueous leaves extract and protease of *Adhatoda vasica* at a dose range of 1 mg showed enhancement in IgG titre as compared to control. Out of two, aqueous extract showed higher IgG titre as compared to protease.

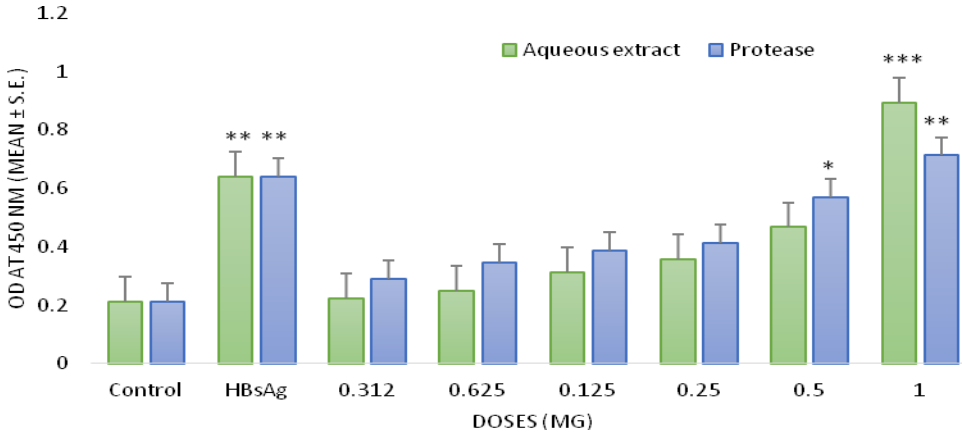

Fig 1: Indirect Elisa. For this experiment HBsAg vaccine used as coating antigen. Aqueous leaves extract and protease samples (0.312 – 1 mg) were used for the estimation of antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm. The difference between the control vs standard and variable doses of aqueous extract and protease is determined by one way ANOVA test. *P<0.05, **P<0.01 and ***P<0.001.

Proliferation assay

The effect of protease and aqueous leaves extract of *Adhatoda vasica* on proliferation (HBsAg) assay as shown in **Fig.2**. The results showed that there is decline in proliferation assay at higher doses and maximum inhibition is reported in case of protease as compared to aqueous leaves extract.

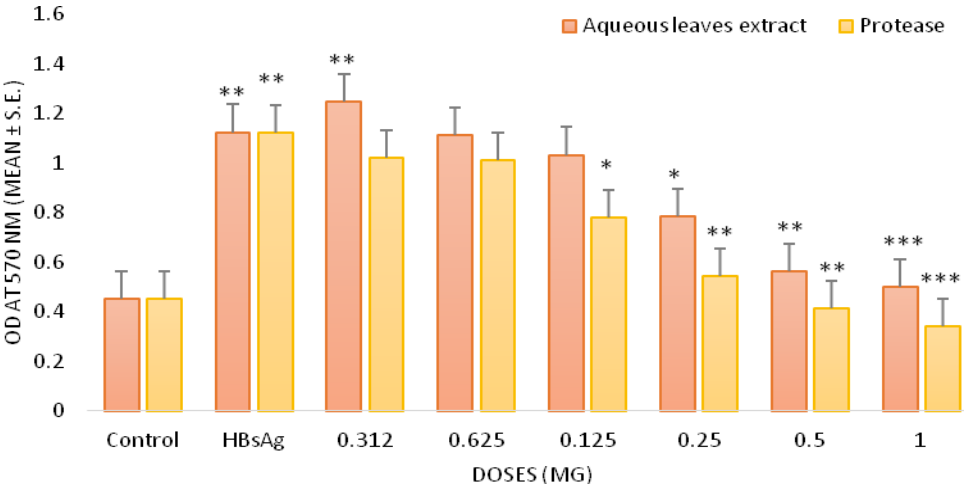

Fig 2: Proliferation assay. Lysed human whole blood were treated with variable concentration (0.312 – 1 mg) of protease and aqueous leaves extract samples. After incubation, the optical density was measured at 570 nm. The difference between the control vs standard and variable doses of protease and aqueous extract is determined by one way ANOVA test. *P<0.05, **P<0.01 and ***P<0.001.

NO Production

For these studies, the maximum inhibition is reported in case of protease followed by aqueous leaves extract of *Adhatoda vasica*. As shown in **Fig.3**, both of them showed inhibition at higher doses.

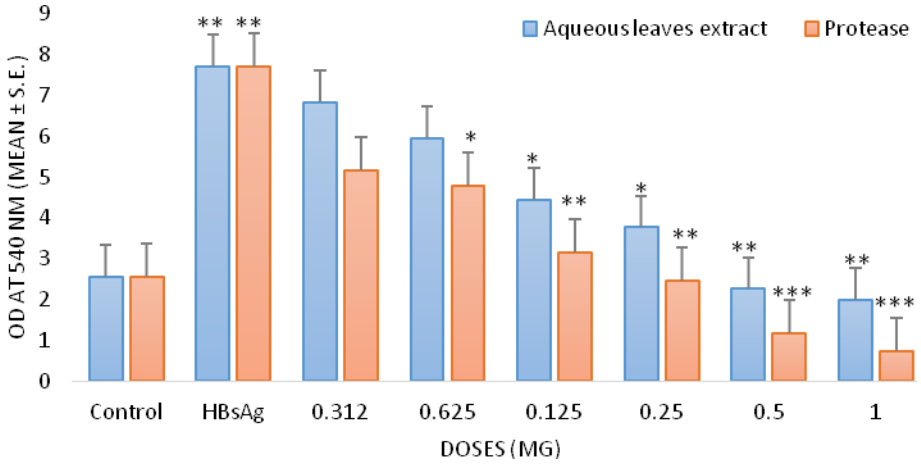

Fig 3: NO Production. Lysed human whole blood were treated with variable concentration (0.312 – 1 mg) of protease and aqueous leaves extract samples. After incubation, supernatant was collected for the estimation of NO through Griess reagent methods (described in materials and methods section). The optical density was measured at 540 nm. The difference between the control vs standard and variable doses of protease and aqueous extract is determined by one way ANOVA test. *P<0.05, **P<0.01 and ***P<0.001.

DISCUSSION:

Although a number of research papers including review articles that are already published or in process related to identification and screening of various bioactive compounds extracted from aqueous leaves extract. The activity of these aqueous extract is not because of single compound or molecule but rather a combination of compounds interacting in an additive or synergistic manner [1, 4, 10]. For these studies, we compared the activity of aqueous extract and protease from the leaves of *Adhatoda vasica* and determined its immunological activity on human whole blood against HBsAg. In this regard, our preliminary results showed that aqueous leaves extract at higher doses showed enhancement in antibody production whereas protease showed maximum inhibition in proliferation rate including NO production as compared to control.

Drug screening is essential criteria for the discovery of various immunomodulatory compounds using specific as well as non specific antigen. Diverse *in vitro* as well as *in vivo* immunoassays are already exist i.e. cell based assay e.g. MTT assay (cell viability). These assays are already standardized and time consuming and therefore, other new methods are also used for drug screening [6, 9, 11]. In this study, we compared its proliferative activity of protease and aqueous leaves extract containing HBsAg and the results showed that protease at higher doses showed maximum inhibition as compared to aqueous leaves extract. Out of these, protease showed highly immunosuppressive activity as compared to aqueous leaves extract of *Adhatoda vasica*.

One of the assays i.e. ELISA is frequently used to detect antibody (IgG) against HBsAg using variable doses of protease and aqueous leaves extract. The results showed that aqueous leaves extract showed more antibody production as compared to protease. In other words, both the samples were exposed to HBsAg, there is dose dependent change in antibody production which is confirmed through ELISA. In addition, this study was further supported pertaining to analyse NO production from cell culture supernatant of treated samples of aqueous extract and protease containing HBsAg. In short, this study showed that protease showed maximum inhibition in HBsAg proliferation and NO production but showed less antibody production as compared to aqueous leaves extract. Overall, the data represents immunosuppressive activity of protease and aqueous leaves extract against HBsAg.

CONCLUSION:

These studies suggest that protease and aqueous leaves extract of *Adhatoda vasica* showed significantly inhibited proliferation rate, NO production but enhancement in antibody production. Further investigations of protease and aqueous leaves extract should be done through *in vivo* assessment for immunopharmacological studies in mice models with identification of the

major active candidates responsible for immunosuppressive activities. In addition, protease may act as way towards vaccine development against infectious diseases and the current data support that the protease inhibiting antigen specific immune response in human whole blood samples.

AUTHORS' CONTRIBUTIONS:

This work was carried out in collaboration between Dr Gupta, Dr Chitte and Dr Chaphalkar. All the authors designed the study, wrote the protocol and interpreted the data. Dr Gupta anchored the field study, gathered the initial data and performed preliminary data analysis whereas Dr Chitte for protein and protease estimation. All the authors managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS:

Authors have declared that no conflicts of interest exist.

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