ejpmr, 2016,3(10), 250-254.

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article
ISSN 2394-3211
EJPMR

ARISTOLOCHIA BRACTEOLATE: POTENT ENHANCER OF B CELLS AND INHIBITS T CELL POPULATION AGAINST SPECIFIC PROTEIN ANTIGEN

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Article Received on 03/08/2016 **Article Revised on 24/08/2016** **Article Accepted on 14/09/2016**

ABSTRACT
Objective- To investigate its immunopharmacological activity of aqueous leaves extract of *Aristolochia bracteolate* using phosphate buffered saline (PBS, pH 7.2) against hepatitis B vaccine containing surface antigen (HBsAg; 20 µg/ml) and weak antigen i.e. ovalbumin (OVA) pertaining to antibody production determined through Elisa and scrutinize its proliferative response in infected lysed human whole blood with respect to HBsAg and OVA. **Methods-** For these studies, phytochemical (qualitative) analysis were determined and evaluate the presence of primary (protein) and secondary metabolites in aqueous leaves extract. In addition, indirect Elisa was performed using HBsAg and OVA as coating antigen using variable doses (0.156 – 5 mg) of aqueous leaves extract of *Aristolochia bracteolate*. In continuation of these immunological studies, antigen specific immune response were determined in infected human whole blood using HBsAg (20 µg/ml, 50 µl) and OVA (1 mg/ml; 50 µl) using MTT assay. **Results-** The results showed that aqueous extract showed qualitatively the presence of secondary metabolites including protein (40 KDa and 100 KDa) content which is determined through SDS (sodium dodecyl sulphate) PAGE (polyacrylamide gel electrophoresis). In addition, this aqueous leaves extract showed enhancement in anti-HBsAg and anti-OVA IgG titre as compared to standard and control but there is sudden decline in proliferation containing HBsAg and OVA at higher doses. **Conclusion-** Our data suggest that aqueous leaves extract of this medicinal plant may help to raise antibodies against HBsAg and OVA but sudden decline in HBsAg and OVA proliferative response in infected lysed human whole blood. In other words, *Aristolochia bracteolate* could be a potent immune enhancer of B cells and inhibitor of T cells.

KEY WORDS: *Aristolochia bracteolate;* hepatitis B vaccine; ovalbumin; splenocyte; Elisa.

INTRODUCTION
As per the literature, medicinal plants contained number of phytochemicals but the mechanism of action including efficacy or safety of these medicinal plant products for human use is still unknown or needed to be validated scientifically[1, 2]. Recently, infectious diseases are included as hot topic in the area of research and considered to be one of the major human health problem[3]. There are number of remedies based on these medicinal plant products are still reported and showed minimal side effects and relatively high cost of synthetic medicines often makes traditional herbal medicines an affordable option for poor people[1, 2]. Recently, more than 500 medicinal plants are identified and inhibits T cell proliferation[4] which is vital requirement for our immune system against various disorders i.e. autoimmune disease. In view of this, these metabolites extracted from the medicinal plant products that played a crucial role especially in the field of immunology and virology for the treatment and prevention of animal and human diseases.[5, 6].

According to the Ayurvedic system of medicine, long list of medicinal plant products are reported and possessed immunomodulatory activity[7]; anti-inflammatory activity[8]; anti-arthritic activity[9] etc. Now a days, these medicinal plant products are in use for the treatment of various immunocompromised conditions such as HIV, autoimmune disease etc.[7-9] Recently, modulation of immune response whether stimulatory or suppressive to cure various diseases and also promoting the health status by modifying host defences mechanism against different types of pathogens[10, 11]. Therefore a number of medicinal plant products, active fractions in the form of aqueous extract (leaves) have been investigated for immune response modifying activity.

One of the medicinal plants, *Aristolochia bracteolate* (commonly known as worm killer), belongs to the family *Aristolochiaceae*. This medicinal plant is widely distributed in western part of India including Maharashtra[12-14]. Traditionally, whole plant of *Aristolochia bracteolata* has been used for various

inflammatory (arthritis) and cardiovascular (diabetes) diseases and also showed immunopharmacological activities such as antipyretic, antimicrobial etc.[12, 13] In addition, Aristolochic acid is reported in this plant and showed several medicinal properties in various disease conditions[12-14]. In view of this, this study aimed to generate necessary information about phytochemical investigation (related to secondary metabolites including protein content) of this medicinal plant i.e. *Aristolochia bracteolate* and also determined its immunopharmacological activity against different surface antigens i.e. hepatitis B vaccine antigen (HBsAg) and weak antigen (ovalbumin). All these studies were conducted under Institutional biosafety safety committee (IBSC) guidelines and studied its immunopharmacological activity.

MATERIALS AND METHODS

Collection of plants

The study plant *Aristolochia bracteolate* were collected in Baramati region and identification was done by Dr Bharat Shinde, Department of Botany, Vidya Pratishthan, Baramati.

Qualitative estimation of secondary metabolites

Aqueous leaves extracts of *Aristolochia bracteolate* were carried out using standard procedure pertaining to phytochemical constituents i.e. alkaloid, flavonoid, phenol, tannin and saponins.

Analysis of protein content through SDS PAGE

Resolving (10 %) and stacking (8%) gels were used for isolation of protein bands of aqueous leaves extracts of *Aristolochia bracteolate*. About 50 µl of aqueous extract of *Aristolochia bracteolate* was loaded into the wells and current of 15 mAh for stacking gel and 25 mAh for separating gel was required to run the gel. After the separation of protein bands through electrophoresis, staining solution was utilized to stain the gel in order to make bands visible. Afterwards the gel was placed in to a destaining solution for 24 hours on shaker and was changed frequently until clear gel was obtained.

ELISA

Indirect Elisa was performed in two different set of experiments using standard HBsAg vaccine (1:1000 dilution) and OVA (100 µg/well, 0.2 ml) as coating antigen. Variable concentration of aqueous leaves extract of *Aristolochia bracteolate* were added and determined its anti-HBsAg and anti-OVA titre. Anti-HBsAg and anti-OVA serum were 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 [11].

Proliferation assay

Infected virally (symptoms such as sneezing, cough etc.) anticoagulant human blood samples were collected from Mangal pathology laboratory, Baramati. In an effort to determine the effect of aqueous leaves extract of *Aristolochia bracteolate* on infected lysed human whole blood (using HBsAg and OVA) and determined its proliferation assay using MTT. For these studies, positive infected blood samples of human (100 µl; 10^5 cells/well) were taken in 96 well tissue culture plate and then treated with variable concentration of aqueous leaves extract (6.25 – 100 mg) of *Aristolochia bracteolate.* Incubate the samples for 24 h at 37°C, 5% carbon dioxide incubator. After incubation, supernatant (100 µl) was discarded and then add fresh complete medium containing fetal bovine serum (FBS). Add MTT solution (5 mg/ml; 10 µl) and then incubate the plate for another 4 h at carbon dioxide incubator. Afterwards, formazan crystals will appear and settled at the bottom and dissolved in dimethyl sulphoxide (DMSO) solution after centrifuging and discarding the supernatant. The optical density (OD) was measured at 570 nm [4, 7].

Statistical analysis

The difference between control, standard and aqueous leaves extract of *Aristolochia bracteolate* is determined by Bonferroni multiple comparison test (One way ANOVA test).

RESULTS

SDS PAGE

The results showed that presence of two prominent bands of around 30 - 40 KDa and 100 KDa as shown in **Fig.1.**

Qualitative based assays

Phytochemical analysis of *Aristolochia bracteolate* revealed the presence of alkaloids, flavonoids, saponin, phenols including tannins.

ELISA

Indirect Elisa assay was performed using HBsAg and OVA as coating antigen as shown in **Fig.2**. The results showed that aqueous leaves extract of *Aristolochia bracteolate* raised antibody production at higher doses as compared to control. In other words, *Aristolochia bracteolate* could be a potent enhancer of B cells against these specific protein antigens.

Proliferation assay

The effect of aqueous leaves extract of *Aristolochia bracteolate* on antigen specific immune response in infected lysed human whole blood as shown in **Fig.3**. The results showed that there is markedly decline in proliferation at higher doses but there is slightly enhancement in proliferation at lower doses as compared to control. Overall, the data indicates that *Aristolochia bracteolate* inhibits T cell proliferation or antigen specific immune response using HBsAg and OVA.

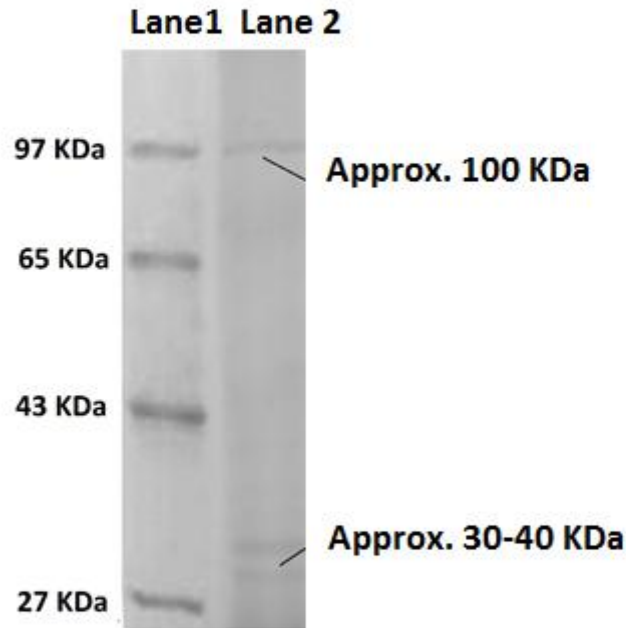

Fig.1. SDS-PAGE analysis of crude protein isolated from aqueous leaves extract of *Aristolochia bracteolate* (LANE1 represents BSA and LANE 2- for aqueous extract)

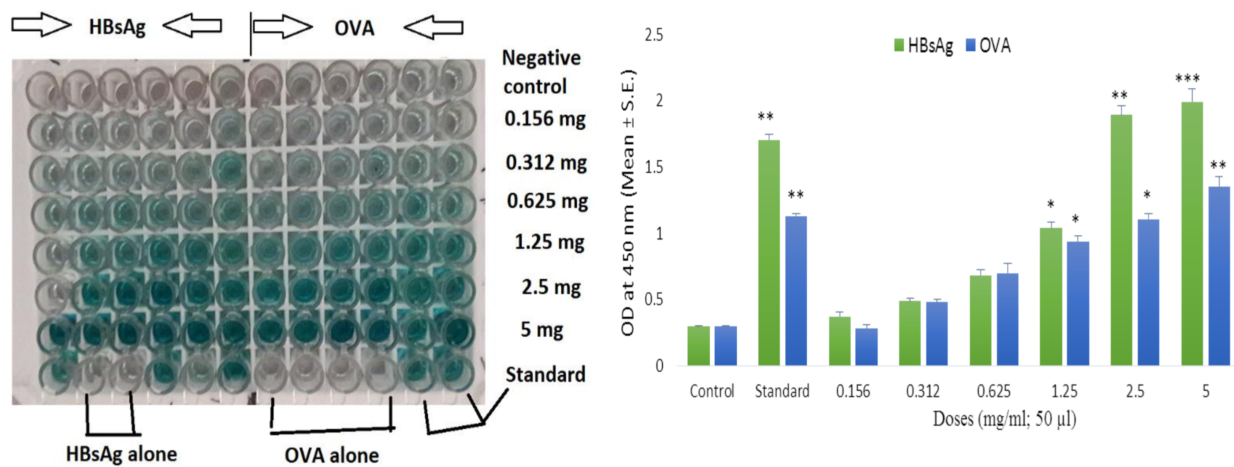

Fig.2. ELISA assay. Indirect Elisa was performed using standard HBsAg vaccine (1:1000 dilution) and Ovalbumin (OVA, 100 µg/well) as coating antigen. Aqueous extract of *Aristolochia bracteolate*, anti-HBsAg serum and anti-OVA serum were used as standard for the estimation of anti-HBsAg and anti-OVA antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm. The difference between the control and standard including aqueous leaves extract is determined by one way ANOVA test.

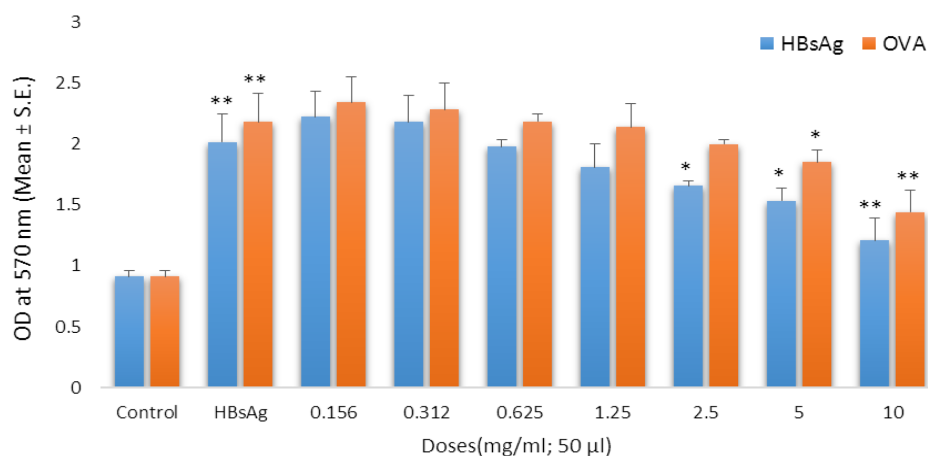

Fig.3. Proliferation assay in human whole blood. Cells were cultured for 48 h along with variable doses of aqueous leaves extract of *Aristolochia bracteolate*. After incubation, proliferation was measured by MTT assay. The results are presented as Mean ± S.E. The difference between control, standard and aqueous leaves extract is determined through one way ANOVA test. *P* values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

DISCUSSION

Use of these medicinal plant products as a source of medicine has been reported in Ayurveda and Unani system of medicine. These medicinal plants should be considered as an important component for human health care. Recently, pharmaceutical companies have produced lot of antibiotics or drugs in the last 20 years against various microorganisms. The problem of microbial resistance is still growing and outlook for the use of antimicrobial drugs from medicinal plant products is still in progress[15]. In the present study, aqueous leaves extracts of *Aristolochia bracteolate* was investigated for its antimicrobial activity in infected lysed human whole blood for determined its antigen specific immune response using HBsAg and OVA. The results showed that this aqueous showed enhancement of antibody production against specific protein antigens but inhibiting antigen specific immune response in infected lysed human whole blood, thereby supporting its antimicrobial properties. This immunopharmacological activity could be possible due to the presence of alkaloids, flavonoids, saponins and phenols that are present in the aqueous extract which is determined qualitatively.

In addition, apart from secondary metabolites, qualitative parameters were also evaluated for the presence of protein content in aqueous leaves extract using SDS PAGE. The data revealed that protein (around 30 - 40 kDa and 100 kDa) including secondary metabolites are responsible for inhibiting antigen specific immune response. As per the literature, 30- 40 kDa protein were purified and sequenced but showed identical N-terminal sequence which is not included in sequence databases. In addition, mixed lymphocyte assays also revealed that only 40 kDa protein is responsible and showed immunosuppressive capability[16] whereas 100 kDa represents aristolochic acids. According to the literature, it revealed that precipitation of 100 kDa protein for Aristolochic acid i.e. protein precipitation studies with anti-aristolochic acid antigens[17].

Hepatitis vaccine antigen (HBsAg) and OVA (weak antigen) were used as coating antigen and tested variable doses of aqueous extract of *Aristolochia bracteolate* for determining IgG anti-HBsAg and anti-OVA titre. When aqueous leaves extract were exposed to these two specific protein antigens, the results showed that there is a dose dependent change in antibody production which is determined through Elisa. Additionally, there was strong correlation between aqueous leaves extract of *Aristolochia bracteolate* and conventional specific protein antigen related to antibody (IgG) production which is determined through Elisa assay. In other words, *Aristolochia bracteolate* could be a potent enhancer of B cell production.

CONCLUSION

Aqueous leaves extracts of *Aristolochia bracteolate* exhibited significant antimicrobial activity in infected lysed human whole blood using HBsAg and OVA. This aqueous extract also showed improvement in antibody (B cell) production against these specific protein antigen and so might be of value in T cell mediated disorders especially autoimmune disease. Further investigation is in necessary to determine the exact phytoconstituents or secondary metabolite that are responsible for inhibiting T cell proliferation and enhanced B cell production.

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