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Research Article

Evaluation of the proliferative activity of aqueous extracts from three medicinal plants in murine spleen cells

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ABSTRACT

There are number of medicinal plant products (leaves, root, stem bark, seeds etc.) including its metabolites i.e. primary (protein) and secondary (flavonoids, terpenoids, glycosides etc.) that have been used as immunomodulatory (stimulatory, suppressive or adjuvant) agents. For the last several years, researchers focused on various immuno-pharmacological aspects especially its therapeutic action of these medicinal plants against various diseases. The objective of our study was to identify and evaluate the action of aqueous leaves extract from these medicinal plants i.e. *Azadirachta indica*, *Syzygium cumini* and *Prosopis spicigera* on the development of spleen cells (*ex vivo* cellular proliferation assay) from mice using hepatitis B vaccine containing surface antigen (HBsAg; 20 µg/ml) and also estimated its pro-inflammatory (IFN-gamma and TNF alpha) cytokines from spleen cell culture supernatant. For these studies, mice were immunized subcutaneously with HBsAg on day 0. After three days, spleens were excised aseptically and prepared single cell suspension (10^6 cells/ml) using variable doses of aqueous leaves extract (0.625-5 mg; 50 µl) incubated with or without HBsAg. After 48 h, cell culture supernatant was collected for the estimation of Th1 (IFN-gamma and TNF alpha) cytokines. However, cell proliferation was revealed by blue tetrazolium reduction assay (MTT) and cytokines by ELISA. The results showed these aqueous leaves extracts showed dose-dependent reduction of cell proliferation including pro-inflammatory cytokines. Overall, these medicinal plants showed immunosuppressive activity in *ex vivo* murine model based studies.

Keywords: *Azadirachta indica; Syzygium cumini; Prosopis spicigera*; Pro-inflammatory; Cytokines.

1. INTRODUCTION

Medicinal plant products are generally used in treating and preventing specific ailments and diseases that affect animals including human beings. Several metabolites (primary and secondary metabolites) of these medicinal plants were identified and used in curing various diseases and involved in production of new drugs using these plant products as a raw material [1-3]. Primary and secondary metabolites are constituents of medicinal plant products with antioxidant, anti-cancer, anti-allergic, anti-inflammatory properties etc. [4-7]. As per Ayurveda, these medicinal plants are responsible for maintaining good health and showed various medicinal uses [4, 5]. Today, there are at least 120 distinct chemical substances derived from various medicinal plant products that are considered

as important drugs currently in use in one or more countries in the world for various infectious diseases [1- 3]. Therefore, pharmaceutical companies have been motivated in order to develop new drugs against various diseases using medicinal plants [5, 6]. Thus, in the present work, medicinal plants with emphasis on its proliferative activity of spleen cells and estimation of cytokines from spleen cell culture supernatant using HBsAg.

Azadirachta indica (neem; family *Meliaceae*), medicinal plant widely used in the traditional Indian system of medicine and showed its various immunopharmacological activities. According to the literature, this medicinal plant products (leaves, root, stem, flowers seeds etc.) reported various immunobiologically active constituents e.g. azadirachtin, nimbin etc. [8-11]. In contrast, *Syzygium cumini* (Jambhul; glycoside *jamboline*; family *Myrtaceae*), medicinal plant are used to treat various ailments especially diabetes mellitus and showed various immunopharmacological activities i.e. anti-inflammatory, anti- cancer, anti-fungal etc. [12-15]. In addition, *Prosopis spicigera* (Shami; Family *Fabaceae*; alkaloid as spicigerin), medicinal plant showed several immunopharmacological activities, i.e., anti-inflammatory, antimicrobial, etc. [16, 17]. Overall, these medicinal plants that inspire to work on these immunological parameters (proliferation including cytokines estimation on murine spleen cells) for antigen specific immune response. The immunobiological properties of these medicinal plants have been linked to their capacity to inhibit or stimulate the activation of T cell, which is critically linked with proinflammatory cytokines. In order to evaluate the effect of these medicinal plants on inhibitory or stimulatory effect of T cell population using HBsAg and also determined the pro-inflammatory cytokines from spleen cell supernatant.

2. MATERIALS AND METHODS

2.1. Collection and preparation of plant material

The studied medicinal plants i.e. leaves of *Azadirachta indica, Syzygium cumini* and *Prosopis spicigera* were collected from the garden of Vidya Pratishthan's School of Biotechnology, VSBT) Baramati, District Pune, Maharashtra, India. For evaluation of proliferative activity of various medicinal plants in murine spleen cells using HBsAg. These plants were dried at room temperature (1-2 h) in a shady region after removing the dust particles using tap water and distilled water. Afterwards, dried leaves were macerated and prepared fine powder. Finally, the powder was macerated and dissolved in phosphate buffered saline (PBS, pH 7.2- 7.4). Collect the samples and centrifuged at 10,000 rpm for 10 minutes at 4 °C and then collect the supernatant for the estimation of secondary metabolites through high performance thin layer chromatography (HPTLC) and measured splenocyte proliferation assay including Th1 cytokine estimation.

2.2. HPTLC studies

To determine the presence or absence of secondary metabolites (qualitatively and quantitatively) in the aqueous leaves extracts of three medicinal plants i.e. *Azadirachta indica, Syzygium cumini* and *Prosopis spicigera* were subjected to battery of chemical tests using standard protocols [18]. For HPTLC studies, aqueous extract redissolved in chromatographic grade methanol and water (1 ml) which was used for sample application on pre-coated silica gel 60 F 254 TLC plates. For aqueous leaves extract of *Azadirachta indica, Syzygium cumini* and *Prosopis spicigera,* solvent system was Ethyl Acetate: n Butanol in the ratio of 6:4. Application of bands of aqueous leaves extract was carried out (4mm in length and 1µl in concentration) using spray technique. Samples were applied on pre-coated silica gel 60 F254 TLC plates (20 x 10 cm) with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software. These HPTLC studies revealed the presence of *Azadirachta indica* (Azadirachtin, 2.2%; retardation factor (Rf) value of terpenoid, 0.96 and saponin, 0.38); *Syzygium cumini* (Rf value of terpenoids, 0.96 and flavonoids, 1.78) and *Prosopis spicigera* (Rf value of glycosides, 3.26; terpenoids, 0.96 and saponin, 0.34-0.46)

2.3. Spleen cell collection

This study was conducted in accordance with

the ethical standards for research involving animals, with the prior approval of Savitribai Phule Pune University, Research Ethics Committee (CPCSEA Reg. no. 1814/PO/ERE/S/15/CPCSEA). Male Swiss mice (18-22 g; n=10) were immunized on day 0 subcutaneously with HBsAg (20 µg/ml; 10 µl dissolved in 190 µl PBS). On day 4, spleen cells were obtained aseptically (killed by cervical dislocation) and prepared single cell suspension using froster slides. The cell concentration of spleen cells was determined in a Neubauer chamber and the verification of cell viability by the trypan blue exclusion test. Only cell suspensions with viability more than 90% were used.

2.4. Splenocyte proliferation assay and estimation of Th1 cytokines by Elisa

The cell culture was performed in microtiter plates (96 well, flat bottom tissue culture plates, Himedia, India) with incubation of spleen cells (10^6 cells/ml) and aqueous leaves extracts (0.625, 1.25, 2.5 and 5 mg/ml; 50 µl) of *Azadirachta indica, Syzygium cumini* and *Prosopis spicigera* in the presence of HBsAg (20 µg/ml, 10 µl) for 48 h at 37 °C and in 5% CO_2. After 48 h, supernatant was collected after centrifuging and estimate its Th1 (IFN-gamma and TNF alpha) profile by Elisa.

For splenocyte proliferation assay, after 48 h incubation, supernatant was collected for analysis of Th1 cytokines and then add equal quantity of fresh medium is added in 96 well plates. The assay revelation was performed by blue tetrazolium reduction assay (MTT), with the addition of 10 µL of MTT at 2.5 mg/ml. Incubate the plate for 3- 4 h at CO_2 incubator and then add 100 µL of DMSO (dimethyl sulfoxide) was added to each well. The plate was incubated for 1-2 h at CO_2 incubator and optical density (OD) determined at 540 nm (Perkin Elmer). Cell response to HBsAg was used as a positive control for cell proliferation. The colorimetric method of MTT reduction can be used to measure cytotoxicity, proliferation and even cell activation, since the determination of the activity of mitochondrial dehydrogenase of living cells directly and proportionally represents the number of cells. All the results were expressed as the mean and standard error of three experiments performed in triplicates [19].

For cytokine estimation, mouse IFN-gamma and TNF alpha (ELISA kits) were purchased from BD Biosciences, India and performed on triplicate samples of supernatants derived from spleen cell cultures. These experiments were conducted as per the manufacturer's instructions [19, 20]. The sensitivity of the assay lends itself to measurement of even very low frequencies of analyte producing cells.

3. RESULTS

3.1. Splenocyte proliferation assay

This study examines the potential immunosuppressive effects of these medicinal plants on splenocyte proliferation assay as shown in Fig. 1. For these studies, HBsAg used as standard for T cell activation. Cells were co-cultured with HBsAg along with variable doses of aqueous leaves extract. We found that these medicinal plants showed remarkably decline in splenocyte proliferation at higher doses as compared to standard (HBsAg). When cells were cultured with HBsAg, it showed enhance cellular proliferation as compared to control.

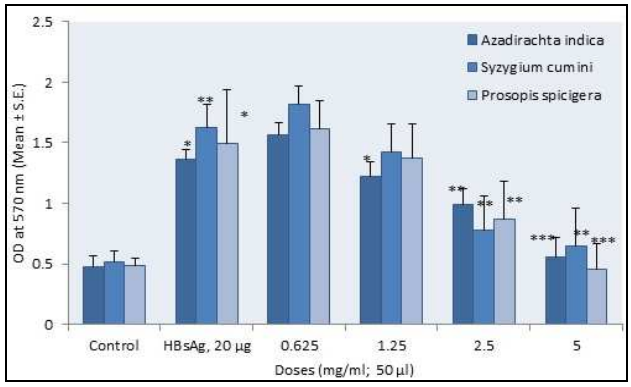

Figure 1. Splenocyte proliferation assay. Swiss mice were immunized subcutaneously with HBsAg on day 0. On day 4, splenocytes were collected and cultured with variable doses of these three medicinal plants in presence of HBsAg for 72 h as described in materials and methods section. Optical density (OD) was measured at 570 nm. Values are expressed as Mean ± S.E. The difference between control versus standard and standard versus variable doses of three medicinal plants is determined through one way ANOVA test. *P <0.05; **P<0.01 and ***P<0.001.

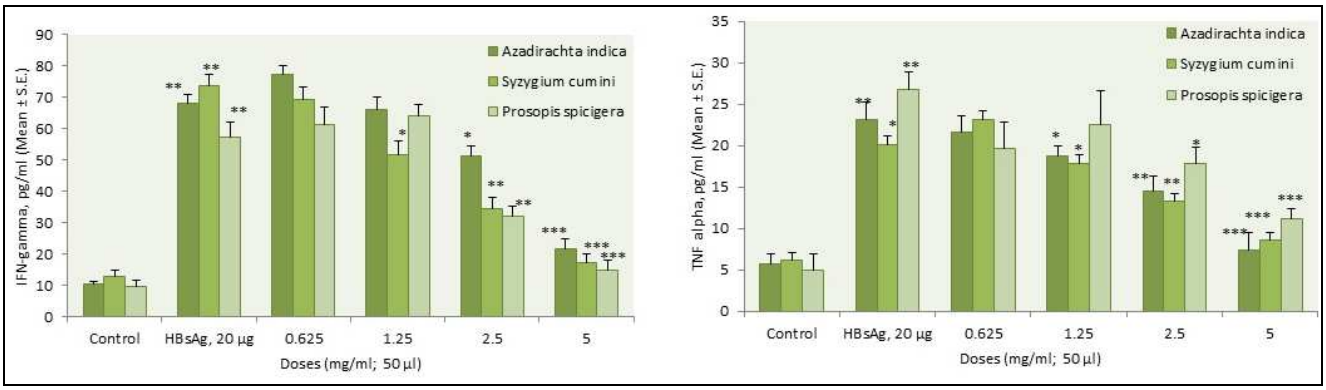

Figure 2. ELISA (Th1 cytokines i.e. IFN-gamma and TNF alpha). Swiss mice were immunized subcutaneously with HBsAg on day 0. On day 4, splenocytes were collected and cultured with variable doses of these three medicinal plants in presence of HBsAg for 48 h and then supernatant was collected for the estimation of Th1 cytokines. Values are expressed in pg/ml as Mean ± S.E. The difference between control versus standard and standard versus variable doses of three medicinal plants is determined through one way ANOVA test. *P <0.05; **P<0.01 and ***P<0.001.

3.2. Th1 cytokines estimation

The effect of these medicinal plants using HBsAg on spleen cell culture supernatant for the estimation of Th1 (IFN-gamma and TNF alpha) cytokines as shown in Fig. 2. We found that these medicinal plants showed decline in Th1 cytokine profile at higher doses as compared to control.

4. DISCUSSION

Studies with medicinal plant based products involved various immunopharmacological assays for the screening of various metabolites with subsequent evaluation of the fractions and isolated compounds responsible for immunobiological activity. Recently, cell proliferation model based studies using MTT and thymidine based assay has been a target of study in the search for new therapeutic agents of natural origin. The activation of the humoral (B cell) and cellular (T cell) immune response in this model promotes cell proliferation with an increase or decreased in the number of cells present in the culture within a defined period and can be identified through MTT reduction by mitochondrial dehydrogenase of living cells [19, 20].

The aqueous leaves extract of these medicinal plants were used in this study and reduced HBsAg cell proliferation in a dose dependent manner. HBsAg used as standard for this study and activate T lymphocytes *in vitro*. Thus, the results obtained with the aqueous leaves extract of these medicinal plants suggest that the components present in this aqueous leaves extract (phytochemical investigations revealed the presence of terpenoids, flavonoids etc.) may suppress T lymphocyte proliferation, supposedly inhibiting the cellular immune response. It should be noted that the study of this model against mitogens (LPS, Con A etc) or specific protein antigens e.g. HBsAg should be able to evaluate the effect of these aqueous extract on other cell types involved in the defense process [19, 20]. However, the preliminary data obtained in our study provides guidance for biomonitored follow up these studies with aqueous leaves extract that have shown promising results.

The rationale for these immunopharmacological based studies related to Th1 and Th2 cytokines which suggests that cytokines produced by Th1 cells i.e. IFN-gamma and TNF alpha (pro-inflammatory cytokines), indicator of cell mediated (T cell) immunity and played a critical role in the pathogenesis of persistent inflammation. In contrast, cytokine products of Th2 cells i.e. IL-4, labeled as anti-inflammatory agent. As per the literature, secondary metabolites i.e. flavonoids, terpenoids, saponin, glycosides, phenolics etc. that are present in the aqueous leaves extract which is determined qualitatively and quantitatively through HPTLC and showed potent immunopharmacological activities such as anti-inflammatory, anti-viral, anti-diabetic properties [9-12]. Apart from this, immunomodulatory study of aqueous leaves extract of these three medicinal plants and determined its effects on

cell proliferation using specific methodologies i.e. splenocyte proliferation assay and molecular techniques (e.g. Elisa) may contribute to the elucidation of the mechanisms involved in the effects observed. The evaluation of immunomodulatory effect of these aqueous extract can be determined by proliferation and cytokines estimation from cell culture supernatant whether it showed stimulatory or suppressive effect. In this study, the results showed aqueous leaves extract of three medicinal plants showed inhibitory action on cell proliferation using HBsAg and Th1 cytokines from cell culture supernatant and possessed inhibitory effects on the cellular (T cell-mediated) immunity.

In short, the need for control (untreated) and maintained an equilibrium between various immunopharmacological activities that involved suppression or stimulation of immune response for proper functioning or maintaining the immune system, has promoted the identification and characterization of medicinal plant products with immunomodulatory activity. The characterization of medicinal plant products which enable identification of compounds that are responsible for the most promising activities observed i.e. stimulating or inhibitory action on the proliferation of murine spleen cell by aqueous leaves extract of these medicinal plants as well as the mechanisms underlying these immunobiological properties.

5. CONCLUSION

Th1 responses are a feature of a number of human and experimental disease states including cancer. The results of this study suggest that the possibility that a wide range of Th1 cytokines derived from spleen cell culture supernatant including proliferation that showed potentially a broad spectrum of immune activities may be targeted by aqueous leaves extract of these medicinal plants and emphasize the need to more precisely define the immunosuppressive properties of this potentially highly useful therapeutic modality.

AUTHORS' CONTRIBUTION

This work was carried out in collaboration between two authors. AG designed the study, wrote the protocol and interpreted the data, 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. The final manuscript has been read and approved by both authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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