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# Immunosuppressive and anti-cancer potential of aqueous extract of *Solanum Xanthocarpum*

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In this study whole plant aqueous extract of *Solanum Xanthocarpum* (HAESX) was investigated to assess its effect on humoral immune response along with interleukin-2 (IL-2) production and its expression in Wistar albino rats splenocytes culture. Anticancer potential of HAESX was investigated using rat lever hepatoma (N1S1 cancerous cell line). The effect of HAESX over humoral immune response was studied using four groups of five animals each (Group-I as control, Group -II orally fed with 125 mg/kg body weight, Group -III orally fed with 250 mg/kg body weight and Group -IV orally fed with 500 mg/kg body weight of HAESX). Quantification of IL-2 was done by sandwich ELISA and its expression was detected by the real time PCR. SRB assay (Sulforhodamine B) was done for detecting the effect of HAESX on N1S1 cell line. Dose dependent decrease in antibody titer was observed and production of IL-2 was also decreased significantly. Suppression of IL-2 production at 250 µg/mL and 500 µg/mL dose was also confirmed by the Real time PCR. Relative fold change in the expression of IL-2 gene was 592.22 and 10.77 at 250, 500 µg/mL HAESX concentrations respectively with respect to control. Dose dependent suppression of percent growth of N1S1 cells with increasing concentrations (10, 20, 40 and 80 µg/mL) of HAESX was found. It was concluded that *S. xanthocarpum* have the immunosuppressive, and anti cancer activity that can be further explore in treatment of various inflammatory and autoimmune disease.

**Keywords:** HAESX, Immunosuppressive, Interleukin-2, N1S1, *S. xanthocarpum*

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India is enriched with mega diversity, over 7500 medicinal plants including herbs and weeds have been enlisted and of which 2000 plants have been prized for their medicinal value in treating infectious diseases, metabolic disorders and immune diseases in humans and animals[1]. *Charaka Saṃhita* and *Sushruta Samhita* are two fundamental literatures of ancient Indian traditional systems of medicines that described the medicinal plants. During the last few decades the use of traditional systems of medicines has increased significantly in both developed and developing countries and presently it accounts for major part of the health care systems world wide[2,3]. People turn to traditional systems for complementary care because of its diversity, flexibility, availability, affordability and its widespread acceptance[4,5].

In traditional Indian and Chinese system of medicines Solanum plant species have been used for centuries as antiasthmatic, hepatoprotective, anti-inflammatory, anticancer, hypocholesterolaemic, antianaphylactic, antiandrogenic, antispermatogenic, insecticidal, antiaccelerator, antioxidant and many other diseases[6]. In Indian subcontinent many workers from time to time studied different species of genus Solanum plant and revealed that *S. xanthocarpum* and *Solanum nigrum* both have answers to treat the Asthma and atopic diseases such as hay fever and rhinitis[7,8]. Chinese worker found poysaccharide extracted from *S. nigrum* as antineoplastic agent[9,10].

Immunomodulatory properties of several medicinal plants used in Indian traditional medicines and other traditional systems have attracted many scientists to explore their effect on immune system of humans and animals[11,12]. Some of the common plants exhibiting immunomodulatory properties include *S. xanthocarpum, Tinospora cortifolia, Cyperus rotuntus, Operculina turpethium, Picrorrhiza curroa, Melia azadirachta*, *Ocimum tenuiflorum*, *Zingiber officinale*, *Adhatoda vasica*[13]. The Indian traditional system of medicine is lacking in answerable scientific basis such as mechanism of their actions, effects on host defense mechanism and how these herbal drugs

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cure metabolic disorders. It is now known that the cytokines and other informational bio molecules released by different cells essentially $T_H1$, $T_H2$, macrophages and NK-cells serve as the regulatory valve for immune responses and so can provide a mode of immunotherapy for readdressing variety of impaired responsiveness, autoimmune diseases and immune suppression[14,15].

The role of *S. xanthocarpum* known for its medicinal potential from ancient time in regulating or modulating the cytokines or other biomolecules for the betterment of immune system and for other defense mechanisms of the body is yet to establish emphatically so that this plant could be used scientifically for treating immunity related disorders.

## Materials and Methods

### Chemicals

Fetal Bovine Serum (FBS), Concanavalin A (Con A), TRI reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and RPMI-1640 medium were purchased from Sigma chemicals Co, USA. Rat IL-2 ELISA kit was obtained from R&D Systems, USA. Anti rat IgG-HRP conjugate antibody, revertaid reverse transcriptase and 2X SYBR green master mix were procured from Thermo Scientific, USA. Primers were designed from Xcelris, Ahmedabad, India.

### Collection of plant

*S. xanthocarpum* plants collected in the month of February-March from Mathura (India) ( 27°33'05.0"N & 77°38'11.3"E) and its adjoining areas was identified and authenticated by Dr Anuradha Upadhye of Agharkar research institute, Pune (Boucher deposition no. 26). Whole plant was dried in shade. Coarsely powdered 200 g of SX was used for hot aqueous extraction by soxhlet apparatus at 100ºC for 8-10 h. The extracted solution was dried using rotary evaporator.

### Experimental animals

Wistar albino rats of both sexes weighing 60-100 g reared and maintained in central animal house, GLA University, Mathura were used for immunomodulatory studies.

### Determination of *In vitro* Humoral response against *Salmonella. typhimurium* 'O' antigen

Humoral immune response was studied using 4 groups of Wistar albino rats with 5 rats in each group by sensitizing them with *S. typhimurium* 'O' antigen which were subsequently fed with 125 mg, 250 mg & 500 mg/kg body weight of HAESX for 21 days.

### Preparation of *S. typhimurium* 'O' antigen for raising antisera[27]

Smooth colonies of *S. typhimurium* grown on Tryptose agar medium were selected and inoculated in nutrient broth. Inoculated broth was incubated for 8 h at 37°C and then centrifuged at 3000 rpm for 20 min and supernatant was discarded and pellet was washed with normal saline thrice and then boiled at 100°C for two h thirty min. This heated culture was then used as 'O' antigen for determination of humoral immune response in albino rats.

### Immunization of rats

Experimental Wistar albino rats were divided in 4 groups. In Group (I) as control, 'O' antigen was inoculated without feeding HAESX. Group II, III & IV were fed orally with HAESX respectively for 21 days and were immunized with 'O' antigen. One week after last dose of 'O' antigen, blood serum was collected for determining antibody titer in albino rats against 'O' antigen by indirect ELISA.

### Determination of antibody titer by indirect ELISA

Polystyrene micro titer plate (Nunc) are coated with Salmonella 'O' antigen and incubated overnight at 4°C. Following three washings with phosphate buffer saline (PBS) pH 7.2, 0.01M containing 0.05% tween-20 (PBS-T). Blocking was done using 1% bovine serum albumin dissolved in PBS, for 1½ h at 37°C. Diluted serum samples (1:10 to 1:10,240) were added from 1st to 11th well of micro titer plate, 12th well was used as blank. 100 µL horse radish peroxidase (HRP) conjugated rabbit anti rat immunoglobulin –G (IgG) and substrate solution TMB (1:20) were serially added to the wells of micro plate, after 15 min 50 µL of 1M sulphuric acid was added to each well to stop the colour development The optical density was measured at 450-570 nm. Each step is followed by 2 h incubation at 37°C and three washings with wash buffer was done after each step

### Splenocyte preparation & IL-2 Induction in presence of HAESX

Rat splenocytes were prepared as per the method suggested[27]. *In vitro* induction was done by Con A as mitogen (5 µg/mL) per well in 200 µL of $2x10^6$ spleen cells/mL in triplicate, cultured in RPMI-1640 medium supplied with 10% FBS. 20 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL and 500 µg/mL HAESX were added to respective wells of plate. The culture plate was incubated at 37°C for 48 h with

5% $CO_2$. Following incubation, culture supernatant was collected for quantitation of IL-2 using sandwich ELISA.

**Quantification of IL-2 using sandwich ELISA**

Cytokine were quantified by sandwich ELISA using the standard method. The collected supernatant from Con A activated splenocyte culture were thawed and used for ELISA. OD was measured at dual wave length 450-570 nm. Each experiment was performed in triplicate.

**Quantification of IL-2 cytokine expression by Real time PCR**

**Isolation of mRNA from spleen cells**

Splenocytes ($1x10^7$ cells/mL) were isolated from control rat and HAESX (250, 500 µg/mL) fed rat and cultured in RPMI-1640 medium on culture plates in presence of Con A (5 µg/mL). RNA was extracted from control and 250, 500 µg/mL HAESX treated spleen cells as per protocol of TRI Reagent.

**Real Time Polymerase chain reaction (qPCR) of cDNA**

The expression of IL-2 cytokine gene and β-actin as endogenous control in spleen cell were carried out by quantitative SYBR green real time PCR (qRT-PCR) in CFX96 Touch Real-time PCR detection system ( Biorad, USA) by using specific primer of β-actin 5'-TGGAGAAGAGCTATGAGCTGC-3'/5'-TCCACACAGAGTACTTGCGC-3'(315bp) and IL-2 5'-AGCTGTTGCTGGACTTACAGG-3'/5'-AATTC CACCACAGTTGCTGG-3'(307bp). All the reactions were set in 20 µL of reaction volume in 8 strip tubes in triplicate. In short, 1 µL of cDNA template (50 ng total RNA equivalent) for each sample was assayed in triplicate along with no template control (NTC) and non-reverse transcription control (NRT). The reaction mixture contain 10 µL of 2X Hot start Veri Quest™ Fast SYBR Green PCR Master Mix with Flurescein (USB, Affymetrix, USA), 1 µL (10 pmol) of forward and reverse primer. PCR condition were : hold at 50°C for 2 min, initial activation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 30 s. After 39 cycles of amplification, final extension was done at 72°C for 7 min followed by melt curve analysis starting from 65°C to 90°C for 20 min with continuous imaging by CCD camera.

Standardization and optimization of these primer were initially checked using conventional PCR using the same reaction condition as describe above (excluding melt curve analysis) and the products were verified by agarose gel electrophoresis in 1.5% TAE – Ethidium Bromide stained gel in the presence of 50 bp DNA ladder (Hi-media, MBT084-200LN) size run at 60 V for 1-2 h. The amplicons were analyzed by Quantity One® (Biorad) software.

**Real time QRT-PCR quantification**

Relative fold expression of IL-2 gene was determined by $2^{-\Delta\Delta CT}$ method[27]. The β-actin gene was taken as the endogenous control, and $\Delta C_T$ value were calculated by subtracting β-actin $C_T$ value from the $C_T$ value of specific target IL-2 cytokine for each sample. The average $\Delta C_T$ for sample collected from normal spleen cell was taken as the calibrator group ($\Delta C_T$ calibrator group) for each sample. Then $\Delta\Delta C_T$ value were calculated by subtracting the average $\Delta C_T$ for normal spleen cells from $\Delta C_T$ of normalized target values ($\Delta C_T$ target group).

***In vitro* anticancer activity of HAESX**:

For determining the *In vitro* anticancer activity of HAESX against the N1S1 rat hepatoma cell line was performed according to standard protocol[16]. Adriamycin was used as the standard drug and sulforhodamine was used to stain the cells. OD was measured at 510 nm by ELISA reader. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells X 100.

$$\% \text{ of control cell growth} = \frac{\text{mean OD sample} - \text{mean OD day 0}}{\text{mean OD negative control} - \text{mean OD day 0}} \text{ X } 100$$

% growth inhibition = 100 - % of control cell growth

% cell killed =

100 - mean OD sample/mean OD day 0 X 100

**Statistical analysis:**

Statistical analysis of data was done with one way analysis of variance (ANOVA) using SPSS version 20.0 software and DMRT at $p<.05$ and .01 to determine significant differences among treatment means.

**Results:**

**Determination of humoral immune response against *S. Typhimurium* (O) antigen**

Serum antibody titer in Wistar albino rats fed orally with different concentrations of HAESX (125 mg/kg/ 250 mg/kg/500 mg/kg) was $960.00^{ab}$±184.75, $800.00^{a}$ ±160 & $400^{a}$ ±138.56 respectively and dose dependent

decline in serum antibody titer was found with respect to control ($1600^{b}$±320.00) (Fig. 1).

### *In vitro* effect of HAESX on induction of IL-2 cytokine

Induction of IL-2 cytokine was evaluated by using Con A stimulated splenocytes in the presence of different concentrations of HAESX. Quantitation of IL-2 was done by ELISA using supernatant of splenocytes culture 48 h post treatment. This experiment revealed that IL-2 production suppressed with increase in HAESX concentration. High concentrations (500, 250 & 100 µg/mL) of HAESX induced 29%, 24.71% & 15.3% decrease in IL-2 respectively as shown in Fig. 2.

### Real time PCR analysis for expression of IL-2 cytokine gene

Down regulated expression of IL-2 obtained in supernatant of splenocytes culture treated with 250 µg and 500 µg/mL was further confirmed by m-RNA expression using spleen cells harvested after 24 h employing real time PCR and results were compared with control using β-actin as housekeeping gene. In present study, results of Real time –PCR were analyzed by comparative $C_T$ values. Target gene (IL-2) was taken along with β-actin as housekeeping gene and fold expression of IL-2 gene in presence of 250, 500 µg/mL HAESX of was calculated with respect to control by $2^{-\Delta\Delta CT}$ method[27]. PCR efficiencies of β-actin and target gene IL-2 were assessed by estimating the standard curve for β-actin and IL-2. Cq value was found to be 20.18, 20.18 and 21.29 for β-actin gene from normal spleen cells and spleen cells treated with 250 µg/mL HAESX and 500 µg/mL HAESX respectively. Cq value for IL-2 gene from normal spleen cells and spleen cells treated with 250 µg/mL HAESX and 500 µg/mL HAESX were found to be 25.7, 28.2 and 31.11 respectively. Fold change in the expression of target gene IL-2 was 592.22 and 10.77 at 250, 500 µg/mL HAESX concentrations respectively with respect to control (untreated) IL-2 gene (Table 1).

**In *vitro* anticancer activity of HAESX:** Four concentrations (10, 20, 40 and 80 µg/mL) of HAESX were screened using N1S1 rat hepatoma cell line. Adriamycin was used as standard. Among the doses, 40 and 80 µg/mL HAESX were found to be effective against N1S1 rat hepatoma cell line (Fig. 3).

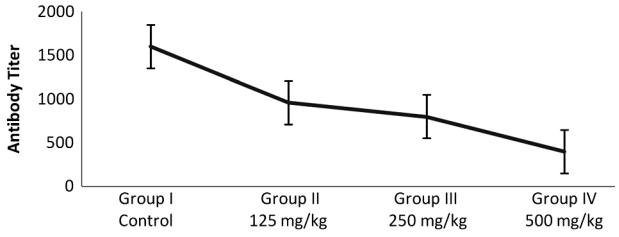


Fig. 1 — Effect of HAESX on Antibody titer

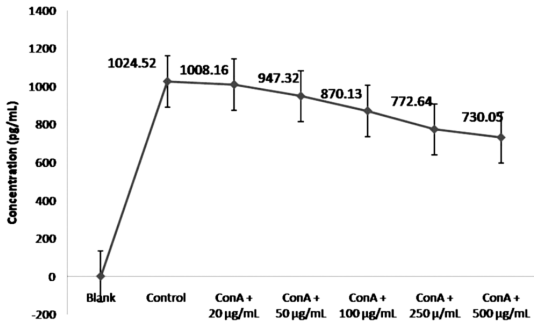


Fig. 2 — *In vitro* effect of HAESX on IL-2 induction

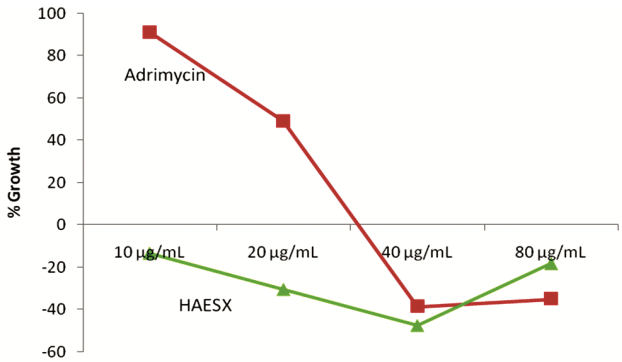


Fig. 3 — Effect of HAESX on rat hepatoma cell line N1S1

Table 1 — Expression of target gene (IL-2) in presence of 250 & 500 µg/mL HAESX with respect to IL-2 of untreated rat (control)

| S.N | Gene | Cq value/ $C_T$ value | Fold Change of target gene (IL-2)=$_2^{-\Delta\Delta CT}$ $\Delta\Delta CT$=(Target gene –Target internal control)-(Untreated control gene-Untreated internal control) |
|---|---|---|---|
| 1. | IL-2 control rat | 25.70 | - |
| 2. | IL-2, HAE treated rat (250 µg/mL) | 28.20 | 10.77 |
| 3. | IL-2, HAE treated rat (500 µg/mL) | 31.11 | 592.22 |
| 4. | β-actin control rat (internal control) | 20.18 | - |
| 5. | β-actin, HAE treated rat (250 µg/mL) | 20.75 | - |
| 6. | β-actin, HAE treated rat (500 µg/mL) | 21.29 | - |

## Discussion

Traditional use of medicinal herbs and weeds has been practice for the treatment of humans and animal diseases. However, the progress is not substantial due to doubts in the minds of scientific and medical fraternity regarding safety, efficiency, and quality of herbal drugs[17]. This may be due to lack of clinical experiments, molecular based scientific evidences and sub standardization of herbal drugs in terms of quality and efficacy. Therefore phytotherapy has not achieved the status parallel to modern medicines. In view of problem of drug resistance, there has been renewed interest in natural and herbal medicines worldwide[18]. This also be due to attention of scientist on the clinical experiments and exploration of importance of biomolecules and their signal networks on the lines of modern medicine systems by adopting latest methodologies and technologies. Modern medicine system does not cure all ailments specially chronic and degenerative diseases, immunological disorders and autoimmune diseases. There is alarming increase in incidence of respiratory diseases such as asthama, hay fever, rhinitis, chronic obstructive pulmonary disease (COPD) lung infection, lung cancer etc. resulting in respiratory distress ranging from acute to chronic conditions hampering working capability and disability of persons to perform effective working. In addition respiratory distressed persons become prone to several degenerative and metabolic disorders[19].

In the present study herbal medicines were used in order to contain allergens' and pollutants' associated respiratory disorders. Modern drugs used to treat asthma and other related diseases have adverse effects on body organ and systems as well as on metabolism of the host[20]. Allopathic drugs relieve symptoms slowly for shorter period and their continued use have ill effects on heart such as dilation of left ventricle and weakening of myocardium which fail to pump blood with its full strength[21]. In Ayurveda medicinal plants viz. Argemone, Cassia, Clerodendrum, Eclipta, Euphorbia, Ficus etc[22], reported to have medicinal value in respiratory infections, of these medicinal plants, *S. xanthocarpum* was selected for scientific based detailed study as it has several phytochemicals that show the pharmacological activity[23].

Present study includes assessment of humoral immune response (antibody mediated) against somatic 'O' antigen of *S. typhimurium* by indirect ELISA test. Dose dependent significant suppression ($p<0.05$) in serum antibody titer was recorded in rats fed orally with increasing concentrations of HAESX (Fig. 1). This shows that HAESX has inhibitory effect on the antibody production which is in general monitored by $T_H2$ lymphocytes. Several investigators[24,25,26] narrated the immunomodulatory role of *S. nigrum* by studying antibody production as well as induction of cell mediated immune response but not at the molecular level that influence these immune responses through inflammatory messanger molecules referred to as cytokines, interleukin and chemokines. In our opinion no study has been done showing effect of *S. xanthocarpum* or its components on cytokine induction and expression.

In our current study, levels of IL-2 in HAESX treated rat splenocyte cell culture supernatants were determined by an enzyme linked immune sorbent assay (ELISA) using commercially available kit. A Dose dependent significant suppression ($p<.01$) in level of IL-2 cytokine in comparison to control was observed (Fig. 2). The major source of IL-2 is the $CD4^+T$ cell ($T_H$) specifically $T_H1$ subset. Physiological function of IL-2 is to promote the activation and proliferation of T cell and natural killer cells in autocrine and paracrine manner. IL-2 also plays a role in suppressing T cell induced immune response[27]. The recent additional evidences suggest that IL-2 is not only as an activator of immune response but also participate as a key mediator of immune tollerence[28], in these study it was explained that effects of essential fatty acid on down regulation of IL-2 and concluded that fatty acids show the anti-inflammatory actions by inhibiting the IL-2 production. This study was in agreement with our study that showed the down regulation of IL-2. It might be possible the HAESX might have the essential fatty acid that do the same function. Although this *in vitro* system can not accurately depict the kinetics of inflammatory process in the body of the host, it provides a plate form at least to understand in terms of cytokine mRNA expression, protein synthesis and release by Con A stimulated splenocytes. This investigation may have good relevance with respect to the potential of HAESX in regulating proinflammatory cytokines production.

Anticancer activity of HAESX using N1S1 rat hepatoma cell line was also evaluated. Different concentrations (10, 20, 40 and 80 μg/mL) of HAESX were used to treat N1S1 rat hepatoma cell lines. 40 μg/mL and 80 μg/mL concentrations inhibited the

cell growth by 38.7% and 35.7% respectively as compared to control (Fig. 3). Antimetastatic activity of *S. xanthocarpum* was evaluated by using lung cell line[29]. Our work was in agreement with Several other workers who reported anti cancer activity of *S. xanthocarpum,* and *S. nigrum*[30,31,32].

## Conclusion

This study was conceptualized to understand immunomodulation with respect to cytokines expression. Present study provided molecular based scientific evidence to support Indian system of medicine for the use of *Solanum* plant species as potential medicinal value in treating chronic inflammatory diseases that include respiratory diseases (Asthma, Hay fever, Rhinitis, pulmonary edema), malignancy and many other degenerative and immunological ailments.

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