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Tectorigenin modulates immune responses via augmenting TH_1 and TH_2 immune responses

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Abstract
The immune system modulation by using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases. The present study investigates the role of different bioactive compounds in the modulation of immune responses. The study was carried out by using spleenocyte proliferation assays. Macrophage preparations were used for the determination of immunomodulatory potential of pure compounds (Iridin, Irigenin, Tectorigenin). The effect was determined by estimating the concentration of cytokines (IL8, IL10, TNF-α). The nitrite content in the peritoneal macrophages was done by using Griess reagent. Moreover, the enhancement in CD4 and CD8 cell populations was revealed by flow cytometry. The results showed that out of the tested compounds, only Tectorigenin potentially enhanced immune response via augmenting Th1/Th2 response in a dose dependent manner. This study may contribute to the traditional claims of *Iris* plant species based natural products used for the treatment of various types of infectious diseases.

Keywords: *Iris* plant, cytokines, nitric oxide, flow cytometry

Introduction

The immune system modulation, by using natural products has become a hot topic for scientists to investigate. Indian plants are used for the treatment of various diseases and are considered by many to be better over the conventionally used drugs which are expensive and have many side effects [1]. The immune system of our body protects us. Its integrity and efficiency is important during the treatment of diseases [2]. Inflammation is one of the first responses of the immune system to infection [3] Secondary metabolites from plants have profound immunomodulatory effect, including the activation of cell mediated immunity. The immunomodulatory potential of *Iris* plant has been earlier demonstrated [4]. The phytochemistry of the genus has been extensively investigated and found to be a rich source of flavones, isoflavones and quinones [5, 6]. Tectorigenin, one of the derivatives of [5, 7] dihydroxy-6-methoxyisoflavone, was isolated from the rhizomes of *Iris* plant. It is well known that 4 shows a wide variety of biological activities such as antioxidative [7], anti-inflammatory [8, 9], antimicrobiotic [10], and anticancer activities [11]. Tectorigenin has attracted interest as alternative estrogen and a modulator of estrogenic actions. Thus, it has extensively been studied for a potential role in many estrogen-dependent diseases, including osteoporosis [12].

The immense manifestation of natural products as immunomodulators either as immunostimulation or immunosupression have their own role and the need for better agents that exert these effects is need of the hour and becoming the field of major interest all over the world [13]. The first response of immune system to infetion is inflammation [14]. Inflammation is produced by eicosanoids and cytokines, which are released by injured or infected cells. Eicosanoids include prostaglandins that produce fever and the dilation of blood vessels associated with inflammation, and leukotrienes that attract certain white blood cells (leukocytes) [15, 16]. Common cytokines include interleukins that are responsible for communication between white blood cells; chemokines that promote chemotaxis; and interferons that have anti-viral effects, such as shutting down protein synthesis in the host cell [17]. Growth factors and cytotoxic factors may also be released. These cytokines and other chemicals recruit immune cells to the site of infection and promote healing of any damaged tissue following the removal of pathogens [18].

The present study evaluated the immunomodulatory potential of three isoflavones (iridin, irigenin, tectorigenin) on T-Lymphocytes (CD^4, CD^8), cytokines (IL10, IL8, TNF-alpha) and on nitrite content. Thus this study represents perspective of isoflavones as immunomodulatory agents from natural resources.

Material and methods

Animals

Healthy female Balb/c mice (18-22g) were procured from the indian institute of integrative medicine Jammu. The animals were kept under standard laboratory conditions: viz. humidity (25+2 c) and photoperiod of 12h. Commercial pellet diet and water were given ad libitum. Animal experiments were approved by institutional animal ethics committee.

Equipment and chemicals.

levamisole, cyclosporin-A (Sigma-Aldrich, India), wash buffer, mouse cytokine standards (IL-2, IFN-γ, IL-4, IL-5)

(BD Biosciences, USA) MTT, RPMI-1640, bovine serum albumin, were purchased from sigma chemicall Co, mo.,.USA. cytokine assay kit were purchased from R&D USA. Unless and otherwise specified, all the solvents were of analytical grade (Qualigens). Iridin, irigenin, tectorigenin standards were purchased from sigma.

Splenocyte proliferation assay

The spleens were collected under aseptic conditions, in HBSS, and minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension. Cells were centrifuged at 400 x g for 10 min at 40C. Erythrocytes were lysed with red cell lysis buffer for 5 min. After centrifugation (380×*g* at 4 0C for 10 min), the pelleted cells were washed 3 times in PBS, and resuspended in complete RPMI 1640 medium supplemented with 12mM HEPES (pH 7.1), 0.05mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. Cell numbers were counted with a haemocytometer by trypan blue dye exclusion technique. Cell viability exceeded 95% [16].

Splenocytes (2 x106 cells) were seeded into a 96-well flat-bottom microtiter plate in 100 μl complete medium. Thereafter, test materials along with Con A (5 μg/mL) to stimulate T-cell proliferation or LPS (10 μg/mL) to stimulate B cell proliferation were added giving a final volume of 200 μl. The plates were incubated at 37°C in 95% humidity at 5% CO2 in a CO2 incubator for 72 hrs. After 72 h, 50μl of MTT solution (5 mg/ml) was added to each well and the plates were incubated for 4 h. Thereafter, plates were centrifuged (1400×*g*, 5 min) and the untransformed MTT was removed. 200 μl of DMSO (192 μl DMSO with 8μl 1N HCl) was added to each well, and the absorbance was determined in an ELISA reader at 570 nm after 15 min.

IL-8, TNF-α and IL-10

IL-8, TNF-α and IL-10 were measured in spleen cell culture supernatants. Splenocytes were seeded into three to four wells of a 96-well flat-bottom microtiter plate (Nunc) at 2 x 106 cells/ml in 100 μl complete medium, thereafter variable doses of tectorigenin (1, 10 and 100 μg) along with Con-A (5 μg/mL) to stimulate T-cell proliferation or LPS (10 μg/mL) to stimulate B cell proliferation were added giving a final volume of 200 μl. The plates were incubated at 37°C in 95% humidity at 5% CO2 in a CO2 incubator for 48 hrs. After 48 h, culture supernatants were harvested and the measurement of cytokines in the culture supernatants was carried out using commercial kits (BD Opt EIA set) as per manufacturer's instructions by ELISA.

Nitrite assay

A volume of 10 ml of RPMI-1640 was injected into the peritoneal cavity of mice. After 5 min, the medium was taken out and centrifuged at 1800 × g for 10 min. at 40C. The cell pellet was re-suspended in RPMI 1640 medium. Macrophages (3 ×106) were seeded in 24-well culture plate in a CO2 incubator for 3 hrs. At the end of incubation period, non-adherent cells were removed and plates were further incubated for 48 hrs in presence of variable doses of tectorigenin (1, 10 and 100 μg) and LPS (1μg/ml). For the NO2- assay (nitrite content), 100 μl of culture media was incubated with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene-diaminein 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well micro plate [Green *et al.,* 1982]. Absorbance at 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard.

Lymphocyte immunophenotyping in spleen

The spleen (1/3 of the organ) collected from BALB/c mice was placed in PBS buffer (without Mg2+ and Ca2+) and stored on ice prior to preparation of single cell suspension. Splenic erythrocytes were lysed with red blood cell lysis buffer (BD Pharmingen). Cell suspensions were refrigerated (ca. 4 °C) before staining with antibodies. All reagents were purchased from BD Pharmingen. Thereafter, 2×106 cells were treated with variable doses of tectorigenin (1-100 μg) and incubated at 370C for 72 h. After 72 h incubation, cells were stained with conjugated anti-CD4 PE and anti-CD8 FITC antibodies. After staining with antibodies and 30 minute incubation at

room temperature, cells were washed and resuspended in PBS for flow cytometric analysis which was performed on a FACS Caliber flow cytometer equipped with Cell Quest software (Becton Dickinson) [17].

Statistical Analysis

Statistical analyses were performed by using statistical program Prism from GraphPad Software, Inc. (San Diego, CA USA). Each of the experiments was performed in triplicates. The results are expressed as Mean ± SD. *p* value of < 0.05 was considered significant. The inhibitory concentration (IC50) was calculated from dose–response curve obtained by plotting the percentage of inhibition versus the concentrations.

Effect of the characterized bioactive molecules on *in vitro* T & B cell proliferation:

The effects of isolated and characterised molecules were studied on *in vitro* T and B cell proliferation by splenocyte proliferation assay. From the results obtained, it was observed that out of the three molecules screened *in vitro*, *Tectorigenin* showed the stimulation index significantly higher than the reference immunostimulator (*Levamisole*). It enhanced the splenocyte proliferation at a selected dose of 10μg. The effect of *Tectorigenin* on Con-A and LPS-stimulated splenocyte proliferation is shown in Figure 1(Table 1). *Tectorigenin* caused profound lympho-activation which triggered significant (p<0.01) and concentration-dependent proliferation of naive murine splenocytes. Con-A (5 μg/mL) and LPS (10 μg/mL) stimulated splenocyte proliferation was significantly enhanced by tectorigenin in a dose related manner with the maximum effect at 100 μg/ml dose compared to the control group. On the basis of the above results of *in vitro* screening, tectorigenin was taken as the molecule of interest for the evaluation of its immunomodulatory activity.

Effect of *Tectorigenin* on IL-6, TNF-α and IL-10 in spleen cell culture supernatant.

In order to establish if Th1 and Th2 cytokines were involved in the immunostimulatory activity of tectorigenin, cytokine secretion patterns were analyzed in spleen cell culture supernatants. Tectorigenin caused a significant (P<0.01) dose dependent up-regulation of TNF-α, IL-6 and IL-10 cytokines:

maximum effect being at 100 µg/ml dose compared with the control group (Figure 2).

Effect of *Tectorigenin* on nitrite content in peritoneal macrophages

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues. Due to its involvement in these diverse systems, interest in measuring NO in biological tissues and fluids remains strong. One means to investigate nitric oxide formation is to measure nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of NO. This assay relies on a diazotization reaction that was originally described by Griess which uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO_2^- in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium.

Table 1: Effect of the characterized bioactive molecules on *in vitro* T & B cell proliferation.

Conc. (/ml)	Stimulation with Con A (2.5 µg/ml)	SI	Stimulation with LPS (2.5 µg/ml)	SI SI
Control	1.021 ± 0.04	-	0.972 ± 0.03	-
Levamisole (0.25 µg)	1.574 ± 0.03*	1.54	1.180 ± 0.02*	1.21
Iridin (10 µg)	1.039 ± 0.02	1.01	0.981 ± 0.05	1.00
Irigenin (10µg)	1.051 ± 0.03	1.02	0.988 ± 0.04	1.01
Tectorigenin (10µg)	**1.883 ± 0.04****	**1.84**	**1.495 ± 0.02****	**1.53**

Values are expressed as Mean ± S.E. (n=3).*p< 0.05 and**p< 0.01 vs. control. Results are expressed in stimulation Index (SI) which is calculated as: OD of Test molecule/ OD of Control.

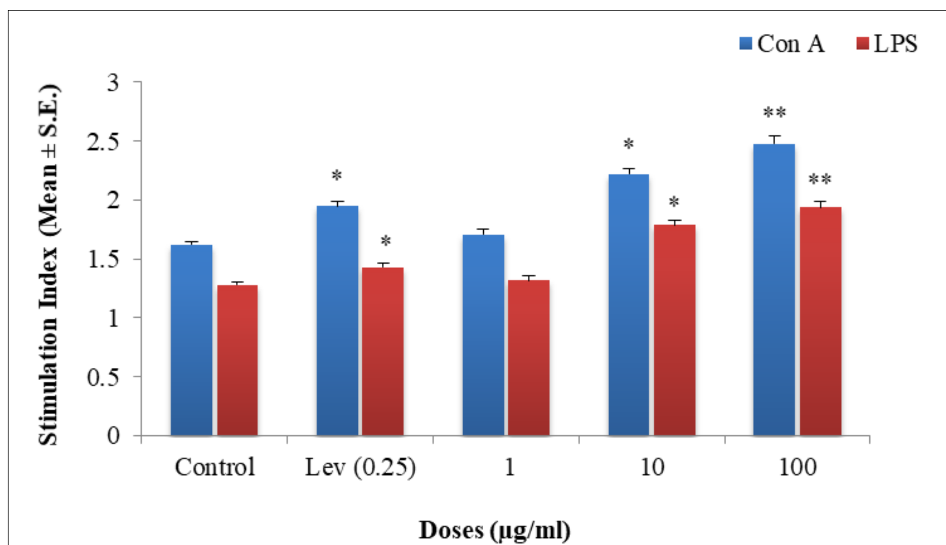

Fig 1

Fig 1: Effect of *Tectorigenin* (1–100 µg/ml) on proliferation of T and B lymphocytes. *Splenocyte proliferation is expressed as the stimulation index (SI). Data are mean ± S.E. of six animals. *p< 0.05 and ** p < 0.01 compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).*

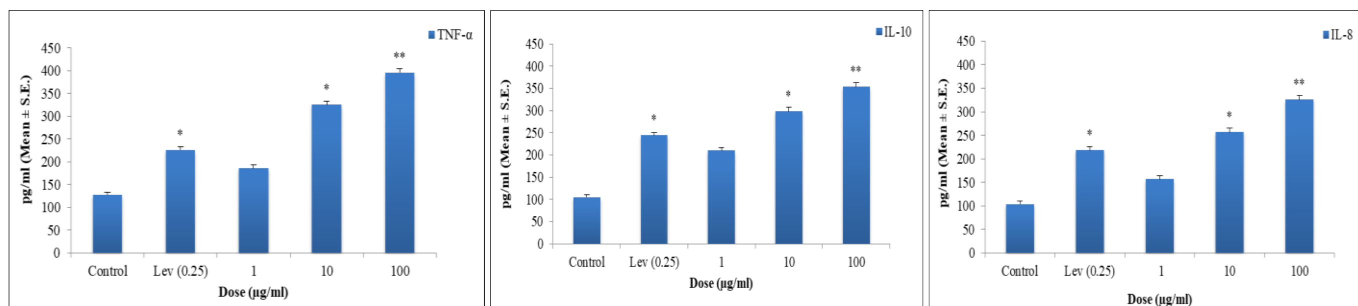

Fig 2

Figure 2 Effect of *Tectorigenin* on IL-8, TNF-α and IL-10 release in spleen cell culture supernatant. *IL-8, TNF-α and IL-10 concentrations were measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer. Data are mean ± S.E. of six animals. *p< 0.05 and **p < 0.01 compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).*

The effect of *Tectorigenin* on nitrite production is shown in Figure 3. This effect on macrophage function was assessed

by measuring the amount of nitrite produced from peritoneal macrophages. Griess reagent was used to measure the nitrite levels, the stable end-product of NO metabolism. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and the results are expressed in µM. Increasing doses of tectorigenin (1-100 µg/ml) significantly enhanced the nitrite content in peritoneal macrophages in a dose related manner. The maximum effect was observed at 100 µg/ml dose compared with the control group.

Lymphocyte immunophenotyping in spleen

The effect of *Tectoreginin* was observed on the population of T and B-cell surface markers like CD4 and CD8 in the splenocytes prepared from the spleen of mice treated with variable doses of tectoreginin for 72 h. Results are depicted in Figure 4. *Tectoreginin* showed a dose dependent increase in both CD4 and CD8 population at the doses of 1, 10 and 100 µg: maximum response being at 100 µg dose compared with the control group.

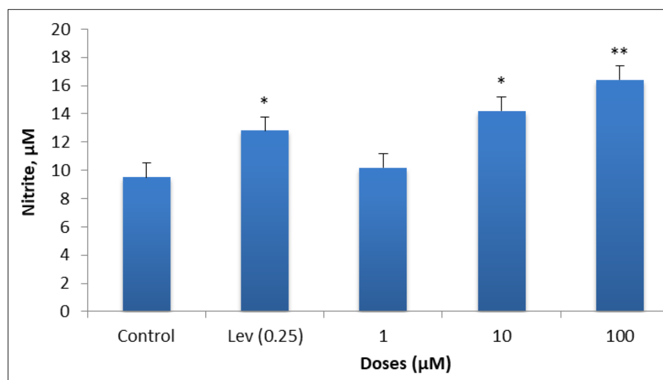

Fig 2

Figure 3. Effect of tectorigenin (1-100 µg/ml) on nitrite content in macrophages. *Macrophages (3×10⁶ cells/well) were cultured and stimulated with LPS (1 µg/ml) for 48 h. The supernatants were used for nitrite assay using Griess reagent. Results are expressed in µM. Data are mean ± S.E. of six animals. *p< 0.05 and ** p <0.01 compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).*

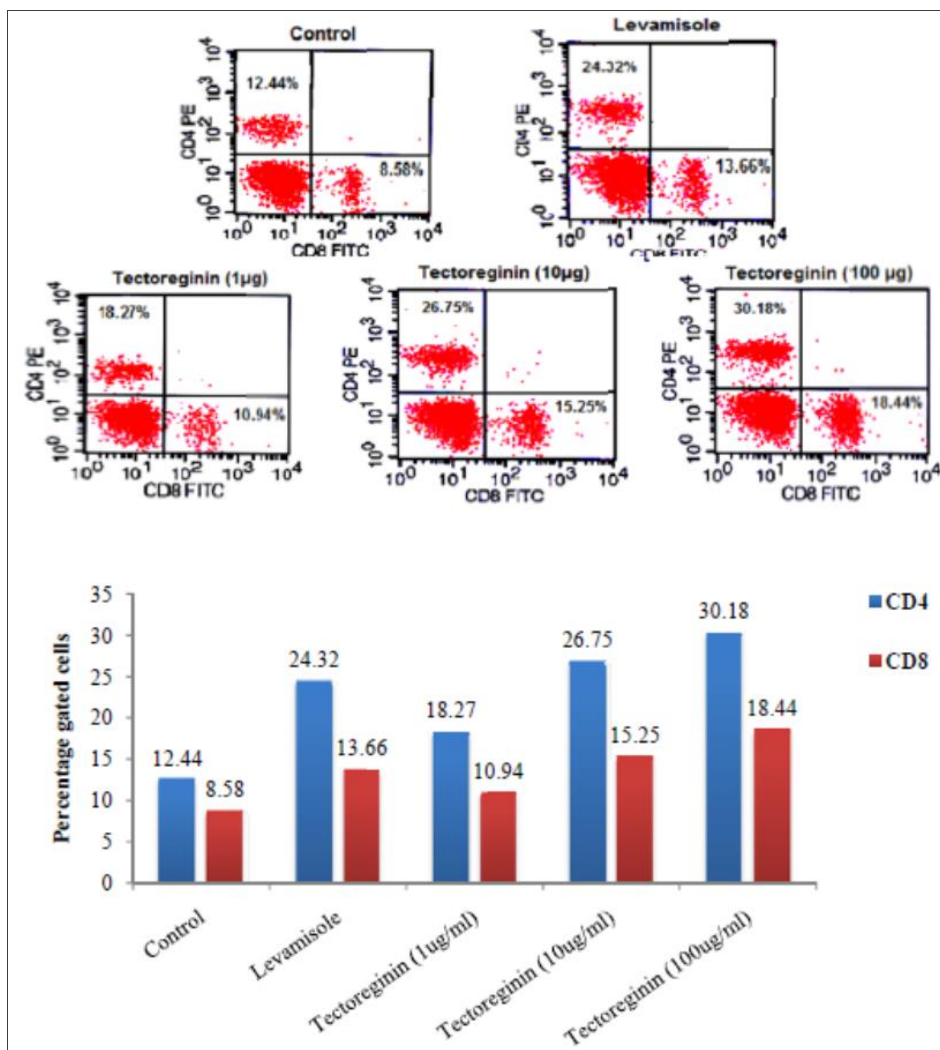

Fig 4: Flow cytometric analysis of splenocytes from BALB/c mice treated with variable doses of tectorigenin. *Cells were labeled with CD4 (PE-conjugated monoclonal antibody) and CD8 (FITC-conjugated monoclonal antibody) and analyzed in flow cytometer using cell quest software.*

Discussion

Plant secondary metabolites serve as a major reserve of drug precursors, drug prototypes and pharmacological probes. There are several plant extracts or "*phytomedicines*" in clinical trials for the treatment of various diseases including cancers. With the availability of new hyphenated analytical methods drug discovery from plants has been accelerated to a large extent paving way to establish new approaches for future medicines. *Iris* species are considered as one of the most important medicinal plants owing to their huge reserves of secondary metabolites viz. flavanoids, isoflavanoids, quinones, triterpenoids, flavones and xanthones. The most essential class among these secondary metabolites are those of flavanoids and isoflavanoids-the entities known to have varied medicinal properties ranging from anti-bacterial or anti-inflammatory to anti-cancer activities. Cancer cell survival not only depends on the tumor cell phenotype but is majorly defined by communication between the tumor cells and the surrounding cells—the microenvironment. The tumor microenvironment consists of tumor cells, fibroblasts, leukocytes, bone marrow-derived cells, blood and lymphatic vascular endothelial cells. The interplay between these cell types determines the fate of tumor cell survival and progression. Recent evidence indicates that the microenvironment provides essential cues to the maintenance of cancer initiating cells and to promote the seeding of cancer cells at metastatic sites. Furthermore, inflammatory cells and immunomodulatory mediators present in the tumor microenvironment polarize host immune response toward specific phenotypes impacting tumor progression. A growing number of studies demonstrate a positive correlation between angiogenesis, carcinoma-associated fibroblasts, and inflammatory infiltrating cells and poor outcome, thereby emphasizing the clinical relevance of the tumor microenvironment to aggressive tumor progression. Thus, the dynamic and reciprocal interactions between tumor cells and cells of the tumor microenvironment plan events critical to tumor evolution toward metastasis, and many cellular and molecular elements of the microenvironment are emerging as attractive targets for therapeutic strategies [156]. Aggressive inflammation is associated with the process of tumorigenesis whereas number of reports suggests that progression of tumor is mainly due to immune-suppressive microenvironment around the tumor cells, indicating that immune-editing might be one of the ways to restrict cancer growth. These tumor cells are capable of modulating the anti-cancer phenotype of immune cells and convert them into more of a tumor supporting cells. In such scenario, immune cells would need an external impetus so as to produce an immuno-aggressive phenotype to overcome the tumor load. We tested the efficacy of *Iridin*, *Irigenin* and *Tetorigenin* in modulating immune cells T-cells, B-cells and macrophages. Preliminary experiments showed that out of the three molecules screened *in vitro*, *Tectoreginen* at 100mM was able to differentiate splenocytes more efficiently compared to that of known immunostimulatory drug *Levamisole.* This was further supported by simulation of CD4+ and CD8+ populations of splenocytes by *Tectorigenin*. *Tectorigenin* also significantly activated peritoneal macrophages in a dose related manner compared with the control group as evident by enhanced nitrite release, which further supports its immunostimulatory activity. The presence of tumor specific T cells, particularly CD8+ cells in systemic circulation are necessary for tumor regression however their efficacy is largely governed by their replenishment in the tumor microenvironment. IL-10 is among those cytokines that regulates the intra-tumoral expansion of CD8+ cells, responsible for tumor regression. IL-10 is believed to modulate the tumor microenvironment to such a state where the tumor promoting immune cell phenotypes are disdained and tumor cells are vulnerably exposed to circulating CD8+ cells [**19**] Likewise TNF-alpha targets the tumor-associated vasculature (TAV) by inducing hyperpermeability and destruction of the vascular lining [**20**].

IL-8, also known as neutrophil chemotactic factor, is primarily secreted by macrophages and cells with toll-like receptors and induces chemotaxis in target cells, primarily neutrophils, basophils, and T-cells causing them to migrate toward the site of injury or infection. Thus, IL-10 may be regarded as an important constituent of the cellular milieu regulation growth of cancer cells. While cytokines viz. IL-8 and TNF-α augment inflammatory reaction; IL-10 polarize the immune reaction towards the T helper 2 (Th2) pathway [21].

After establishing the anti-proliferative and immuno-stimulatory effect of *Tectorigenin* on cancer cell lines and splenocytes, we evaluated the effect of *Tectorigenin* on cytokine release by splenocytes. To do so, we checked the levels of three important cytokines viz IL-8, IL-10 and TNF-alpha, manipulating tumor microenvironment. It was found that *Tectorigenin* increased secretions of IL-8, TNF-α and IL-10 in a dose dependent manner favoring tumor inhibiting immune cell phenotypes.

Thus, out the three molecules isolated from *Iris* rhizomes, *Tectorigenin* seems to be a candidate anti-cancer/ immuno-adjuvant drug owing to its immune-stimulatory and anti-proliferative properties. It could be argued that the mechanism for the anti-proliferative/ immunostimulatory action of *Tectorigenin* could be the consequence of induced NO-release in targets cells. The multifaceted roles of nitric oxide have well been established in anti-proliferative/ immunostimulatory scenarios. While NO induces both cytostasis and cytotoxicity to tumor cells, it also activates cells of the immune system [22, 23, 24]. Since, NO-activation is controlled by complex programming of signaling circuits in the less understood tumor microenvironment, further research is required for precise evaluation of specific targets for *tectorigenin*.

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