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Research Article

Cytokine Mediated Immunomodulatory Properties of Kaempferol-5-O-β-D-glucopyranoside from methanol extract of aerial parts of *Indigofera aspalathoides* Vahl ex DC

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ABSTRACT

The objective of the study was to evaluate the immunomodulatory effect of isolate compound Kaempferol-5-O-β-D-glucopyranoside from methanol extract of aerial parts of *Indigofera aspalathoides* Vahl ex DC. on Swiss albino rats by Cyclophosphamide induced myelosuppression, *In vivo* phagocytic activity by carbon clearance assay, Plaque forming cell (PFC) assay, flow cytometric studies for the estimation of T lymphocytes (CD3 and CD19) and Th1 cytokines (IL-2, IFN-γ and IL-4). Levamisole (50 mg/kg b.w.p.o) and Cyclophosphamide (30mg/kg b.w.i.p.) were used as immunostimulatory and immunosuppressive standard drugs. Administration of Kaempferol-5-O-β-D-glucopyranoside at the doses of 10, 20, 40, 50 mg/kg b. w. p. o. showed significant increase in the phagocytic index and in Plaque forming cell assay, and an significant protection against cyclophosphamide induced neutropenia and showed dose dependent increase in the production of T lymphocytes (CD3 and CD19) and Th 1 cytokines (IL-2, IFN-γ and IL-4). The maximum increase in the production of CD3, CD19, IL-2, IFN-γ and IL-4 at 50 mg kg b.w of the test compound was observed. The above results suggest that Kaempferol-5-O-β-D-glucopyranoside from methanol extract of aerial parts of *Indigofera aspalathoides* Vahl ex DC. can be further explored for the development of potent immunomodulatory drug.

Keywords: *Indigofera aspalathoides* Vahl ex DC; Kaempferol-5-O-β-D-glucopyranoside; immunomodulatory; T lymphocytes; cytokines

INTRODUCTION

Medicinal plants play a key role in the human health care. About eighty percent of the world populations rely on the use of traditional medicine, which is predominantly based on plant materials (Surendra et al., 2008) A large number of medicinal plants and their purified isolated metabolites have been shown to have beneficial therapeutic potential (Agbar et al., 2008). Immunomodulators are substances that alter the immune response by augmenting or reducing the ability of the immune system to produce antibodies or sensitized cells that recognize and react with the antigen that induced their production (Mans et al., 2000). The efficiency of immune system may be influenced by many exogenous and endogenous compounds. Apart from the natural mechanism, there are compounds that are capable of interacting with immune system, to up regulate or down regulate specific aspects of the

host response can be classified as immunomodulators and the mechanism involve with these is known as immunomodulation (Agarwal et al., 1999). Natural plant products comprise one of the most popular complementary and alternative medicine for inflammatory and immune disorders (Nicola et al., 2004). It is imperative to examine and unravel the immunological basis of the therapeutic activity of herbal products against autoimmune disorders as well as other conditions involving inflammation (Yamamoto et al 2009). Thus the medicinal plant products used as immunomodulators as possible therapeutic measure is becoming a new subject of scientific investigations and provide an alternative to conventional chemotherapy and prophylaxis of infection, for many autoimmune diseases, especially when the host immune is impaired (Mehta et al., 2009). Furthermore the presence of phytoconstituents such as lectins, peptides, flavonoids, proteins in plants have been reported to modulate the immune system in different experimental models (Srikumar et al., 2006). In recent times focus on plant research has increased around the world and a large body of evidence has been collected to show the immense activity of medicinal plants used in various traditional systems of medicine (Mali et al., 2010). One such plant selected for our study is *Indigofera aspalathoides* Vahl ex DC., a

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plant belonging to the family of papilionaceae, is a low under shrub with copiously spreading and terete branches. Traditionally the plant is very specific for viral hepatitis, secondary syphilis and for psoriasis (Balasubramanian et al., 2005). The plant shows the protective effect against CCl₄ induced hepatic damage in rats (Raj Kapoor et al 2006).

The aqueous extract of aerial parts of *Indigofera aspalathoides* Vahl ex DC., have the ability to counteract the adverse biological effects of carcinogens and its treatment enhanced the recovery from 20 – MCA – induced fibrosarcoma in rats due to its antioxidants and anti-neoplastic properties (Selvakumar et al., 2011). A flavones glycoside 5, 4'- Dihydroxy 6, 8 – dimethoxy 7 – O – rhamnol isolated from the stem of *Indigofera aspalathoides* Vahl ex DC. exhibited cytotoxicity activity against human cancer cell lines (Raj Kapoor et al., 2007). Although there are many research studies authenticating the bioactive effects of *I. aspalathoides*-Vahl ex DC., little is known about the immunomodulatory action. Thus the present study was mainly focused to study the immunomodulatory activity of Kaempferol-5-O-β-D-glucopyranoside in an animal model.

MATERIALS AND METHODS

Plant material

Fresh aerial parts of *Indigofera aspalathoides* Vahl ex DC. was collected from the herbal garden of Tirunelveli district of Tamil Nadu during the month of January 2011. The plant material was identified and authenticated by Dr. J. Jayaraman, Director, Plant Anatomy Research Centre, West Tambarm, Chennai. Voucher specimen (No. PARC/2011/886) was preserved for future references.

Animals

Wistar albino rats (110-160 g) of either sex were used. Animals were acclimatized to the standard laboratory conditions of temperature, humidity and light. The animals had free access to water ad libitum and fed with pellet diet (Lipton India Ltd., Mumbai, India.) except 1 h before and during the experiments. All experimental procedures were carried out in strict accordance with the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA, 685/PO/02/a/CPCSEA/21-08-2002) and were approved by the Institutional Animal Ethics Committee (IAEC) from KMCH College of Pharmacy, Coimbatore (KMCH/PhD/17/2012-13, dated 21-12-2012).

Chemicals

Cyclophosphamide was procured from (Biochem – pharmaceutical industries Ltd. Mumbai), Carboxy methyl cellulose (Molychem, Mumbai, India), Rotring ink (Hamburg, Germany). FACS lysing solution, FACS permeabilizing solution, Golgi plug, FITC (Fluorescein isothiocyanate) labeled anti-CD3 monoclonal antibodies,

PE (Pycerytherin) labeled anti-CD19, IL-2, IFN-gamma and IL-4 monoclonal antibodies were purchased from B. D. Biosciences. All other reagents used were of analytical grade.

Extraction and isolation of compound

Collected fresh aerial parts of *I. aspalathoides* Vahl ex DC were cleaned, Powdered about (5kg) plant material was extracted with of 70% methanol (v/v) at 50 °C in Soxhlet apparatus (7 lt) for 72 h. The methanol extract on further liquid-liquid extraction with methyl acetate solvent yield an fraction which on further chromatographed using flash column on a silica gel (60-120 mesh, Merck, India) eluted with chloroform-methanol step gradient (starting with 100:0 to 4:1) yield 7 fraction. Fractions with sufficient yield were selected for further studies. Fraction 7 was chromatographed on a Sephadex LH-20 column eluted with methanol coupled with RP-ODS column gradient eluted with methanol-water (from 40% to 60%, v/v) which yielded a compound, with the help of spectral data and physico-chemical properties the compound was identified as Kaempferol-5-O-β-D-glucopyranoside used for treatment protocol for evaluation of immunomodulatory activity and their results are discussed below.

PHARMACOLOGICAL EVALUATION

Acute Toxicity Studies

Acute toxicity study for Kaempferol-5-O-β-D-glucopyranoside (3) was performed in Wistar albino mice as per OECD guideline 425 (OECD., 2001). Animals were divided into 3 groups of 3 animals in a group. Female, nulliparous and non-pregnant mice weighing between 18-22g was selected for this study. The animals were kept fasting overnight provided only with water. The dose progression study was carried out at three different dose levels of 100 mg/kg, 250 mg/kg and 500 mg/kg and observed for mortality during 48 h study period. The dose was administered only once for each group. The dose at which mortality was observed in two out of three mice was considered as toxic dose. All the animals were observed twice daily for health condition, morbidity and mortality for 14 days.

Selection of doses

The value of LD₅₀ for Kaempferol-5-O-β-D-glucopyranoside was calculated using acute oral toxicity study. As the lethal dose was found at 500 mg/kg b. w. p. o., 1/10th of the preceding dose of 50 mg/kg b.w. p. o. was taken as the test dose for further study.

Treatments Groups

Animals were divided into Seven groups of six animals each. Group – I (Control group) and Group – II (Cyclophosphamide group) received the vehicle 0.5 ml of 2% W/V CMC for 13 days. Group III – VI were administered Kaempferol-5-O-β-D-glucopyranoside (10, 20, 40, 50 mg/kg p.o.) and to the Group - VI were administered Levamisole (50 mg / kg p.o) daily for 13 days.

Cyclophosphamide induced myelosuppression

On 11th, 12th and 13th day, all the animals of each group except control were given Cyclophosphamide (30mg/kg i.p.), one hour after administration of respective dose. Blood samples were collected on the 14th day of experiment by retro orbital puncture and hematological parameters were studied for RBC, Hb, Platelets, total WBC counts and Differential leucocytes counts (Manjarekar et al., 2001).

In vivo phagocytic activity by carbon clearance assay

Colloidal carbon solution, Rotring ink (Hamburg, Germany) was diluted with normal saline (1:8), and injected (0.01 ml/g body weight) was via tail vein to each albino rats 24 h after last dose. Blood samples were drawn from retroorbital plexus under ether anesthesia at 2 and 15 min after injection. Blood (25 μ l) was mixed with 0.1 % sodium carbonate (2 ml) for the lysis of erythrocytes OD was recorded at 660 nm. The phagocytic index (K) was calculated by using following equation: $K = (\ln OD1 - \ln OD2) / (T2 - T1)$, where OD1 and OD2 are the optical densities at times T1 and T2, respectively (Miller et al., 1991).

Antigen

Sheep red blood cells (SRBC) suspension was collected in Alsever's solution and were washed three times with pyrogen free sterile normal saline (0.9% NaCl, w/v). After adjusting the cells count to 5 \times 10⁹ cells/ml, SRBC was used for sensitization and challenge at required time schedule.

Plaque forming cell (PFC) assay

The assay was performed according to the method reported by Jerne and Nordin. After immunizing the animals with SRBC on day 0, they were treated as stated above for 5 consecutive days. Briefly, the spleen cells of SRBC immunized extract treated rats were separated in RPMI-1640 medium, washed twice and suspended in same medium. Glass petridishes were layered with 1.2% agarose in 0.15 M Sodium chloride to form bottom layer. A mixture of 2 mL agarose (0.6%) in RPMI-1640 medium (42°C), 0.1 mL suspension of 20% SRBC and 1 \times 10⁶ spleen cells in a volume of 0.2 mL was poured over the bottom layer of agarose followed by an incubation period of 90 min at 37 °C. A 2 mL

quantity of 1:9 diluted fresh rabbit serum was added to petridish and plates were reincubated for 40 min to allow the formation of plaques. The numbers of plaques were counted immediately and values were expressed as counts per 10⁶ spleen cells (Deborah et al., 2009).

Flowcytometric studies

For Flow cytometric studies, experimental animals were sensitized by injecting 5 \times 10⁹ SRBC/ml intraperitoneally (i.p) on day 0. Drug administration was carried out as stated above till next 6 consecutive days. On day 7 animals were challenged by injecting same concentration of SRBC and on day 8, blood was collected from retro-orbital plexus of animals under ether anesthesia in EDTA coated tubes for lymphocyte immunophenotyping and intracellular cytokines estimation.

Lymphocyte immunophenotyping

Immuno-phenotyping focuses on lymphocyte populations involved in acquired immunity and specific molecules present on the cell surface that defines characteristics of lymphocytes such as state of activation or functional capabilities. Lymphocyte subsets were measured by immunofluorescent antibody staining of whole blood and subsequently analyzed using two color flow cytometry (Becton & Dickinson, UK). Murine monoclonal antibodies conjugated to a fluorochrome and directed against receptors CD3 and CD19 were used for the study. FITC-labeled anti-mouse CD3 monoclonal antibody and PE-labeled anti-mouse CD19 monoclonal antibody were added directly to 100 μ l of whole blood, which was then lysed using whole blood lysing reagent (BD Biosciences). Following the final centrifugation, samples were resuspended in phosphate-buffered saline (pH, 7.4) and analyzed directly on the flow cytometer (LSR, BD Biosciences) using Cell Quest Pro Software (BD Biosciences) (Khan et al., 2006).

Intracellular cytokines estimation

100 μ l of whole blood was taken in falcon tubes and red blood cells were lysed by adding whole blood lysing reagent (BD Biosciences). After washing in Phosphate Buffer Saline (PBS), cells were permeabilized using permeabilizing solution and incubated with anti-mouse IL-2, anti-mouse IFN-gamma and anti-mouse IL-4 for

Table 1: Effect of Kaempferol-5-O- β -D-glucopyranoside on cyclophosphamide induced myelo suppression

Groups	RBC (10 ⁶ /mm ³)	Hb (g%)	Platelet(10 ⁵ /mm ³)	WBC (10 ³ /mm ³)
I	6.422 \pm 0.021	8.731 \pm 0.016	6.368 \pm 0.071	4.217 \pm 0.213
II	5.149 \pm 0.070*	8.320 \pm 0.118*	4.323 \pm 0.048*	2.321 \pm 0.298*
III	5.921 \pm 0.138*	9.103 \pm 0.115*	5.819 \pm 0.139*	3.710 \pm 0.176*
IV	5.237 \pm 0.148*	9.170 \pm 0.086*	5.317 \pm 0.170*	3.120 \pm 0.180*
V	5.277 \pm 0.128*	9.376 \pm 0.086*	5.347 \pm 0.160*	3.607 \pm 0.180*
VI	5.859 \pm 0.127*	9.419 \pm 0.030*	5.350 \pm 0.129*	3.701 \pm 0.047*
VII	7.821 \pm 0.031*	9.981 \pm 0.073*	8.368 \pm 0.027*	5.972 \pm 0.813*

N= 6, values were expressed as Mean \pm SEM, *p value <0.05 statistically significant; Group I; vehicle control group, Groups II; Cyclophosphamide group, III, IV, V and IV; treatment group, Group VII; standard group.

the duration of 30 min in dark. After incubation, cells were given three washes of PBS and after final washing; cells were acquired directly on Flowcytometry (Bani et al., 2005). Calculation of percentage activity: %Activity= Mean Values of Control – Mean Values of Treated x 100/Mean Values of Control

RESULT AND DISCUSSION

Immunomodulatory agents of plant origin enhance the immune responsiveness of an organism against a pathogen by specifically or nonspecifically activating the immune system. The evaluation of plants products that either promote or inhibit immunocyte proliferation is crucial to the study of immunomodulation and drug discovery (Nudo et al., 2011). Bone marrow, a site of continued proliferation of blood cells, concern mainly the cells for its immune activity. In fact bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs and makes more sensitive, organ damage which results in loss of stem cells and inability to regenerate new blood cells by bone marrow, leads to thrombocytopenia and leucopenia (Sanjeevet al., 2013). Cyclophosphamide (Group II) induced bone marrow suppression (myelosuppression), and it is counteracted by increase in RBC, total WBC platelet counts, Hb % and DLC in the Kaempferol-5-O-β-D-glucopyranoside and Levamisole (10, 20, 40, 50 and 50 mg/kg) treated group (Group III & VII).

The results indicate modulation of bone marrow activity, namely suppression when used cyclophosphamide alone and stimulation to counteract the cyclophosphamide induced myelosuppression in pretreated Kaempferol-5-O-β-D-glucopyranoside treated group (Table 1 & 2). Phagocytosis indicates an potent immune defence mechanism in which leukocytes play an important role in the removal of pathogenic microorganisms, malignant cells, foreign bodies, tissue debris or injured cells. The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The reticulo endothelial system (RES) is a diffuse system consisting of phagocytic cells (Gokhale et al., 2003). Cells of the RES play important role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is go-

verned by an exponential equation. Kaempferol-5-O-β-D-glucopyranoside appeared to enhance the phagocytic function by exhibiting a dose related increase in clearance rate of carbon by the cells of the reticulo-endothelium system. The results of this assay are presented in Table 3. The phagocytic index (K) was highly significant (P<0.05) for test treated group as compared to control and cyclophosphamide group.

Number of antibody secreting cells from spleen was determined using plaque forming cell assay. Since spleen contributes immensely to the humoral as well as cellular arms of immune system, its role in generation of antibody secreting cells was studied. However, Kaempferol-5-O-β-D-glucopyranoside (10, 20, 40, 50 mg/kg) and Levamisole (50 mg/kg) produced significant enhancement in number of cells secreting antibodies against SRBC, which served as specific antigen (Table 3). The pharmacologically active mediators released from the granules exert biological effects like vasodilatation and smooth muscle contraction that may be either systemic or localized depending on the extent of mediator released (Katzung et al., 2006).

Cytokines are a diverse group of products mainly of immune cells that play a vital role as signaling molecules in cellular communication. Components of both innate and adaptive immune systems may respond to certain antigens via the process of inflammation. It is a rapid and non-specific process that initiates prior of specific immune response. In the inflammatory response assay the capability of test compounds to modulate inflammatory cytokines was assessed under the conditions of induced inflammation. Inflammatory response is characterized by increased blood supply carrying more leukocytes and plasma molecules, increased permeability of capillaries allowing exudation of plasma proteins and increased migration of leukocytes to the affected tissue (Male et al., 2007). Kaempferol-5-O-β-D glucopyranoside showed dose related increase in the production of CD3, CD19, IL-2, IFN-γ and IL-4 but the maximum increase was seen at 50 mg/kg and statistically significant (P < 0.001) when compared against control group (Table 4). In case of standard drugs Cyclophosphamide showed decrease in the production of CD3, CD19, IL-2, IFN-γ and IL-4 when compare to control. In this consideration the observed

Table 2: Effect of Kaempferol-5-O-β-D-glucopyranoside on Differential leukocyte count

Groups	Neutrophil (%)	Lymphocytes (%)	Eosinophil (%)	Basophils (%)	Monocytes (%)
I	24.58±0.06	66.33 ± 0.47	3.04 ± 0.003	1.780±0.410	1.05±0.003
II	12.65±0.04**	42.16 ± 0.47**	1.33 ±0.007**	0.319±0.320**	0.185±0.004**
III	25.45±0.06**	70.4 ± 0.05 **	2.13 ± 0.004*	0.510±0.223**	0.732±0.006**
IV	24.48±0.03**	71.61 ± 0.06 **	2.16±0.006**	0.521±0.210**	0.816±0.006**
V	24.07±0.07**	72.26 ± 0.16**	2.24±0.005**	0.420±0.223**	1.943±0.006**
VI	25.04±0.07**	71.36 ± 0.16**	2.94±0.005**	0.531±0.223**	1.423±0.006**
VII	26.26±0.03**	70.41 ± 0.07 **	2.98±0.004**	1.932±0.223**	1.94 ± 0.003**

N= 6, values were expressed as Mean ± SEM, **p value <0.05 statistically significant; Group I; vehicle control group, Groups II; Cyclophosphamide group, III, IV, V and IV; treatment group, Group VII; standard group.

Table 3: Effect of Kaempferol-5-O-β-D-glucopyranoside on Phagocytic index and Plaque forming cells/10⁶ cells

Groups	Phagocytic Index	Plaque forming cells/10 ⁶ cells
I	0.062 ± 0.040	284.00 ± 5.83
II	0.059 ± 0.035**	299.87 ± 7.93**
III	0.072 ± 0.034**	307.01 ± 4.83**
IV	0.074 ± 0.025**	312.52 ± 8.32**
V	0.081 ± 0.025**	335.65 ± 6.87**
VI	0.085 ± 0.004**	384.32 ± 5.65**
VII	0.089 ± 0.004**	402.54 ± 6.88**

N= 6, values were expressed as Mean ± SEM, **p value <0.05 statistically significant; Group I; vehicle control group, Groups II; Cyclophosphamide group, III, IV, V and IV; treatment group, Group VII; standard group.

Table 4: Lymphocyte immune phenotyping and intercellular cytokines estimation by flowcytometry

Groups	CD ₃	CD ₁₉	IL-2	IFN-γ	IL-4
	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E
I	28.24±0.79	35.41±1.00	2.66±0.08	2.10±0.15	1.07±0.11
II	22.19±1.34**	24.11±1.27**	2.06±0.09**	2.04±0.07**	1.09 ±0.05**
III	50.17±1.79**	62.90±1.82**	4.04±0.12**	3.18±0.16**	2.30±0.13**
IV	51.44±1.83**	64.00±1.80**	4.32±0.09**	3.28±0.17**	2.41±0.09**
V	52.24±1.90**	65.11±2.12**	4.21±0.10**	3.32±0.15**	2.90±0.14**
VI	55.27±1.90**	68.11±2.12**	4.80±0.10**	4.36±0.15**	3.40±0.14**
VII	59.09±2.56**	69.16±2.66**	5.69±0.43**	4.82±0.33**	3.94±0.27**

N= 6, values were expressed as Mean ± SEM, **p value <0.001 statistically significant; Group I; vehicle control group, Groups II; Cyclophosphamide group, III, IV, V and IV; treatment group, Group VII; standard group.

enhancement in inflammatory response, granulocyte adhesion by treatment may occur under the influence of increased expression of above cytokines.

The result is owing to a mechanism related to phagocytosis by macrophages. The process of phagocytosis by macrophages includes opsonisation of the foreign particulate matter with antibodies and complement C3b, leading to a more rapid clearance of foreign particulate matter from the blood. Kaempferol-5-O-β-D-glucopyranoside were found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of cytokines (Xing et al., 1999). The overall effects of these cytokines are to recruit macrophages into the area and activate them, promoting increased phagocytic activity with increased concentration of lytic enzymes for more effective killing.

CONCLUSION

In the above study, Kaempferol-5-O-β-D-glucopyranoside an isolated compound from aerial parts of methanolic extract of *Indigofera aspalathoides* Vahl ex DC. significantly increased phagocytic index, Plaque forming cell assay, and also significantly increase the production of lymphocytes (CD3) intracellular cytokines (IL-2 and IFN-γ). So, further studies can be performed to explore the Kaempferol-5-O-β-D-glucopyranoside to develop an immunomodulatory drug which will be effective and having no side effect.

Conflict of interest statement

We declare that we have no conflict of interest.

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