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Evaluation of Immunomodulatory Potential of Two Naturally Occurring Bioactive Flavonoids: Luteolin & Apigenin

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ABSTRACT

AIM- The aim of the present investigation is to study the Immunomodulatory potential of two naturally occurring bioactive flavonoids: Luteolin & Apigenin. **MATERIAL & METHODS-** Fresh blood was collected from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in large volumes of Pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×109 cells/ml for immunization and challenge. The control group received normal saline solution. Group II was administered with only cyclophosphamide at the dose of 30 mg/kg, i.p. while different group rats received cyclophosphamide with both bioactive flavonoids (50 & 100 mg/kg) and *Ocimum sanctum* extracts (100 mg/kg) for 10 days. Red blood cell diluted fluid (Hayem's fluid) – 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200ml of distilled water. WBC diluting fluid or Turk's fluid was used as the diluents which can destroy RBC'S. WBC diluting fluid was prepared by mixing Glacial acetic acid, Gentian violet 1% and Water 95 ml. Haemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. **RESULTS-** The luteolin and apigenin produced a significant, dose-related increase in DTH reactivity in rats. Increase in DTH reaction in rats in response to cell dependent antigen revealed the stimulatory effect of luteolin and apigenin on T cells. Oral administration of luteolin and apigenin in 50 & 100 mg/kg for 7 days, and 10 min prior to carbon injection exhibited a dose-related increase in the clearance rate of carbon by the cells of the RES. Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the haemoglobin, RBCs, WBCs and differential leukocyte count. **CONCLUSION-** Many of the plant products exert some effect on immune system. They either enhance immune response to help body clear undesirable agents from body or suppress immune response to control deterioration in the body. Compounds that alter immune response are considered Immunomodulatory agent.

KEYWORDS-

Immunomodulatory Potential, Naturally Occurring Bioactive Flavonoids, Luteolin & Apigenin, Cyclophosphamide, Immune response

INTRODUCTION

Immunity is a homeostatic process, a sequence of delicately balanced complex, multicellular and physiologic mechanisms that allow an individual to make a distinction foreign material from "self" and neutralize and/or eliminate the foreign matter (Ali *et al.*, 2013). Immune cells in adult mammals are shaped primarily by the bone marrow, which serves both hematopoetic and immunopoetic functions. This organ is of paramount significance, as it is the only

lymphoid tissue capable of providing the complete restoration of primary lymphoid tissue (spleen, thymus, lymph nodes and liver), and therefore can alone prevent adult mammal death subsequent lethal irradiation. In most mammalian species, the bone marrow itself acts also as a primary and secondary lymphoid organ, regulating the production, differentiation, and maturation of lymphocytes (Babyminakshi *et al*., 2006).

Plant extracts used in traditional rehabilitation are being reviewed for their chemo protective and Immunomodulatory activities. Immunomodulators are biological response modifiers; exert their antitumor belongings by improving host defense mechanisms against the tumor. Immunomodulatory therapy could make available an alternative to conventional chemotherapy for a variety of diseased conditions, especially when host's defense mechanisms have to be activated beneath the conditions of impaired immune responsiveness or when a selective immunosuppression has to be induced in a situation, like inflammatory diseases, auto-immune disorders ad organ/bone marrow transplantation (Bellinghausen *et al*., 2001).

Although many drugs are used in ethno-medical practices in India, most of them are not pharmacologically evaluated. There is an imperative need to settle on the true therapeutic value of medicinal drug. The herb appears to be a safe drug. There is an urgent need to carry on studies to ascertain the therapeutic effectiveness of bioactive flavonoids is invaluable medicine to treat safe and non toxic herbal mediated health problems (Bhattacharya *et al*., 2000). The objective of the present effort is to study the effectiveness of some natural bioactive flavonoids for Immunomodulatory activity by different models.

MATERIALS & METHODS

Glass wares: Borosil and ASGI make glass wares were used.

Chemicals: All chemicals used were of analytical grade. Acetic acid S.D. Fines Chemicals; Bombay; Coomassie blue-Sigma Chemical Company, USA, DAB- Sigma Chemical Company, USA, DMSO- Sigma Chemical Company, USA, Dichlomethane Qualigens, Ethanol-Merck/Bengal Chemical; Ethyl acetate-Qualigens; EDTA-Qualigens; Methanol-Merck/Qualigens, Pet. ether, chloroform, methanol, water, molisch reagents, fehling solutions, Dilute hydrochloride, ferric chloride, glacial acetic acid, mercuric chloride, dragendroff reagents, millons reagents, hagers reagents, Ninhydrine reagents, SRBC, Precoated plates, silica gel, Indian ink etc.

Animals: Wistar Albino of either sex (150 to 200 g) was purchased from the CPCSEA approved vendors for *in-vivo* immunomodulatory activity. They were maintained under standard laboratory conditions at 25 ± 2°C and normal 12-hour light-dark cycle were used for the experiment. Commercial pellet diet and water were provided *ad libitum* throughout the course of study.

Procurement of Flavonoids

Luteolin and Apigenin were procured from the Yucca Chemicals, Mumbai as a gift sample and were analyzed for preliminary parameters for the identification.

EVALUATION OF IMMUNOMODULATORY ACTIVITY

Delayed-type hypersensitivity (DTH) response by SRBC

Animals were divided into six groups each having 6 animals. Group 1-received Vehicle, Group-2 received luteolin (50mg/kg), Group-3 received luteolin (100mg/kg), Group-4 received Apigenin (50mg/kg), Group-5 received Apigenin (100mg/kg), Group-6 received Standard drug, extract of *Ocimum sanctum.* (100mg/kg).

The both bioactive flavonoids were administered orally on day 0 and continued till day 7 of challenge (Cortes *et al*., 2013). One group of each extract contains 6 animals. *Ocimum sanctum* extract was used as a standard drug (Cohen *et al*., 2000).

Preparation of antigen

Fresh blood was collected from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in large volumes of Pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×109 cells/ml for immunization and challenge.

Delayed-type hypersensitivity (DTH) response

The rats were challenged by injection of 0.5×109 cells SRBCs in right hind foot pad. Foot thickness was measured after +24 and +48 h of this challenge. The differences obtained for pre- and post challenge foot thicknesses were taken for the measurement of DTH and were expressed in mm. The both bioactive flavonoids were administered orally on day 0 and continued till day 7 of challenge (Shivaprasad *et al*., 2006). One group of each extract contains 6 animals. *Ocimum sanctum* extract was used as a standard drug (Dar *et al*., 2015).

Phagocytic response of bioactive flavonoids

Animals were divided into six groups each having 6 animals.

Group 1-received Vehicle, Group-2 received luteolin (50mg/kg), Group-3 received luteolin (100mg/kg), Group-4 received Apigenin (50mg/kg), Group-5 received Apigenin (100mg/kg), Group-6 received Standard drug, extract of *Ocimum sanctum* (100mg/kg). The animals were treated from day 0 today 7 with the vehicle and luteolin and apigenin (50 & 100 mg/kg). *Ocimum sanctum* (100mg/kg) extract was used as a standard drug. On day 7, all the animals of the entire groups received the treatment of an intravenous injection of (0.3 ml per 30 g) Indian ink dispersion (pre-warmed at 37°C). The method was described by Cheng *et al*., (2005). The animals were treated from day 0 today 7 with the vehicle and different flavonoids. *Ocimum sanctum* extract was used as a standard drug. On day 7, all the animals of the entire groups received the treatment of an intravenous injection of (0.3 ml per 30 g) Indian ink dispersion (pre-warmed at 37°C). 50 µl of blood samples was collected from each animal by retro-orbital bleeding at an interval of 2 and 10 min after the injection of ink dispersion. Blood samples were added to 4 ml of 0.1% sodium carbonate solution to lyse the erythrocytes. Absorbance of the samples was measured at 675 nm using spectrophotometer (Davis and Kuttan, 2000; Devaki *et al*., 2013). Rate of carbon clearance (*K*) and phagocytic index (α) were calculated by using following formula:

$$\textbf{Rate of carbon clearance (K)} = \frac{\log OD2 - \log OD10}{T2 - T1}$$

Where OD2 is the log absorbance of blood at 2min; OD10 is log absorbance of blood at 10 min; T2 is the last time point of blood collection; T1 is the first time point of blood collection. Rate of carbon clearance and phagocytic index of treated group animals were compared with the control group animals.

Cyclophosphamide-induced myelosupression

Dose and Treatments

The control group received normal saline solution. Group II was administered with only cyclophosphamide at the dose of 30 mg/kg, i.p. while different group rats received cyclophosphamide with both bioactive flavonoids (50 & 100 mg/kg) and *Ocimum sanctum* extracts (100 mg/kg) for 10 days (Ezeani *et al*., 2017; Fernando *et al*., 2013).

Group 1: Served as control, received 10 ml/kg normal saline
Group 2: Received cyclophosphamide at the dose of 30 mg/kg, i.p
Group 3: Cyclophosphamide at the dose of 30 mg/kg, i.p + luteolin treated (50 mg/kg)
Group 4: Cyclophosphamide at the dose of 30 mg/kg, i.p+ luteolin treated (100 mg/kg)
Group 5: Cyclophosphamide at the dose of 30 mg/kg, i.p+ apigenin treated (50 mg/kg)
Group 6: Cyclophosphamide at the dose of 30 mg/kg, i.p+ apigenin treated (100 mg/kg)
Group 7: Cyclophosphamide at the dose of 30 mg/kg, i.p + *Ocimum sanctum* extracts (100 mg/kg)

On day 11, blood samples were collected from the retro-orbital plexus of individual animals and analyzed for hematological parameters (Giri KR., 2016).

Estimation of Hematological Parameters (Ige *et al*., 2011)

Red Blood Corpuscles

Red blood cell diluted fluid (Hayem's fluid) – 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200ml of distilled water.

Blood was sucked exactly up to the 20µl mark in the RBC pipette and the diluting fiuid was drawn immediately up to

the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 min for proper mixing. The Neubauer counting chamber was placed along with the cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops. One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2 – 3 minutes the counting chamber was put under the microscope and the ruled area was located. Erythrocytes were counted in the 5 squares of the counting area of 1 mm square. The number of cells in the 4-corner square was counted (Ige *et al*., 2011).

White Blood Corpuscles

WBC diluting fluid or Turk's fluid was used as the diluents which can destroy RBC'S. WBC diluting fluid was prepared by mixing Glacial acetic acid, Gentian violet 1% and Water 95 ml.

The method of counting is similar to RBC counting except that the count is made in 4 large (1 mm) cover squares of the Neubauer counting chamber.

The total number of cells in 4 squares is multiplied by a factor of 2500 to give the count/mm of blood (Ige *et al*., 2011).

Hemoglobin

Haemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube. 0.1 N HCl was added in the Haemoglobinometer upto the lowest marking. 20µl of blood was drawn up to 20µl in the Sahli's pipette. Adjusted the blood column carefully without bubbles.Wiped the excess of blood on the sides of the pipette by using a dry piece of cotton.Blown the blood into the acid solution in the graduated tube, rinsed the pipette well. Mixed the reaction and allow the mixture to stand at room temperature for 10 minutes. Diluted the solution with distilled water by adding few drops of water carefully and by mixing the reaction mixture until the colour matches the colour in the comparator. The lower meniscus of the fluid was noted and reading was noted in g/100ml (Ige *et al*., 2011).

STATISTICAL ANALYSIS

Data were expressed as the mean standard error of mean (S.E.M.) of the means and statistical analysis was carried out employing one-way ANOVA. Differences between the data were considered significant at $P < 0.05$.

RESULT

Delayed-type hypersensitivity (DTH) response by SRBC

The cell-mediated immune response of luteolin and apigenin were assessed by DTH reaction, i.e. foot pad reaction. As shown in Table, the luteolin and apigenin produced a significant, dose-related increase in DTH reactivity in rats. Increase in DTH reaction in rats in response to cell dependent antigen revealed the stimulatory effect of luteolin and apigenin on T cells.

Table No 1: Effect of luteolin and apigenin on DTH response using SRBCs

S No.	Groups	Treatments	Dose	DTH Response (mm) 24 Hrs	DTH Response (mm) 48 Hrs
1	Group I	Control	10 ml/kg	0.22±0.04	0.14±0.05
2	Group II	Luteolin Treated	50 mg/kg	0.38±0.04*	0.32±0.02*
	Group III	Luteolin Treated	100 mg/kg	0.39±0.08*	0.34±0.07*
	Group IV	Apigenin Treated	50 mg/kg	0.78±0.06***	0.72±0.03***
	Group V	Apigenin Treated	100 mg/kg	0.80±0.02***	0.74±0.06***
4	Group VI	*Ocimum sanctum* Extract	100 mg/kg	0.85±0.04***	0.75±0.04***

Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to control ** p<0.01, compared to control. *** p <0.001, compared to control

Phagocytic response of Different Extracts

The method was described by Cheng *et al*., (2005). Oral administration of luteolin and apigenin in 50 & 100 mg/kg for 7 days, and 10 min prior to carbon injection exhibited a dose-related increase in the clearance rate of carbon by the cells of the RES. luteolin and apigenin (100 mg/kg) showed highest phagocytic index 6.46±0.11 & 6.61±0.16 respectively. The phagocytic index of control (group I) was 3.33±0.32.

Table No 2: Effect of luteolin and apigenin on Phagocytic Index

S No.	Groups	Treatments	Dose	Phagocytic Index
1	Group I	Control	10 ml/kg	3.33±0.32
2	Group II	Luteolin Treated	50 mg/kg	3.97±0.11*
	Group III	Luteolin Treated	100 mg/kg	4.15±0.13*
	Group IV	Apigenin Treated	50 mg/kg	6.46±0.11***
	Group V	Apigenin Treated	100 mg/kg	6.61±0.16***
3	Group VI	*Ocimum sanctum*	100 mg/kg	7.79±0.21***

Cyclophosphamide-induced myelosupression

Since apigenin showed best activity as compared to luteolin. So in this model, we have selected only apigenin for the determination of hematological parameters.

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the haemoglobin, RBCs, WBCs and differential leukocyte count. In case of cyclophosphamide induced myelosupression, there was decrease in the WBCs count in the control group. In treatment groups, the RBCs, HB and WBC count was found to be increased with $p<0.01$ and $p< 0.001$ at 50 & 100 mg/kg dose respectively on 11th day.

Table No 3: Effect of luteolin and apigenin on RBCs, WBCs and HB

S No.	Treatments	RBCs	WBCs	HB
		11th Day	**11th Day**	**11th Day**
1	Control	8.33±0.11	3.43±0.14	11.66±1.44
2	Cyclophosphamide	3.16±0.36**	0.96±0.23***	6.55±0.35**
3	Luteolin Treated (50 mg/kg)	6.15±0.54**	1.93±0.55**	8.93±0.67*
4	Luteolin Treated (100 mg/kg)	7.34±0.68**	2.94±0.76**	9.96±0.45*
5	Apigenin Treated (50 mg/kg)	6.93±0.74**	2.13±0.45***	9.35±0.22**
6	Apigenin Treated (100 mg/kg)	7.95±0.66**	2.92±0.74***	10.38±0.85**
7	*Ocimum sanctum* (100 mg/kg)	8.16±0.45**	3.35±0.88***	10.77±0.54***

Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to control ** p<0.01, compared to control. *** p <0.001, compared to control.

DISCUSSION

The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effectors molecules generated by activated cells. It is expected that theses nonspecific effects give protection against different pathogens

including bacteria, viruses, fungi etc. and constitute an alternative to conventional chemotherapy. The present aim of our study was to evaluate the pharmacological activity of different bioactive flavonoids (Islam *et al*., 2014).

Delayed type Hypersensitivity required the specific recognition of given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. DTH is a part of the process of graft rejection, tumor immunity and most important, immunity to many intracellular microorganisms. It can also be due to activation of complement, release of reactive oxygen or nitrogen species by activated phagocytes and pro-inflammatory cytokines (Jeong *et al*., 2013). Delayed type hypersensitivity (DTH) is antigen specific and cause erythema induction at the site of antigen infection in immunized animals. The histology of DTH can be different for different species but the general characteristics are influx of immune cells at the site of injection, macrophages and basophiles in rat's induction become apparent within 24-72 hrs (Liu *et al*., 2014).

Antibodies, product of B-lymphocytes and plasma cells, are central to humoral immune responses. IgG and IgM are the major immunoglobulin's which are involved in the complement activation, opsonization and neutralization of toxins (Madakkannu *et al*., 2017). Immunoglobulin like IgM can overcome electric barrier and get cross-linking with red blood cells, which lead to subsequent agglutination. The augmentation of the humoral immune response to SRBCs by flavonoids as evidenced by an increased in the antibody titer in rats indicated that enhanced responsiveness of T and B lymphocyte subsets, which is involved in the antibody synthesis (Moazzam *et al*., 2013). The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation to antibody secreting plasma cells. In our study, luteolin and apigenin showed maximum activity in DTH model.

The carbon clearance assay was used to evaluate the effect on reticuloendothelial cell mediated phagocytosis (Prabu *et al*., 2013). When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. Rate of clearance of ink from blood is known as phagocytic index. The extract produced an increased in phagocytic index suggesting its effect on reticuloendothelial system. Stimulation of phagocytosis is influenced by the activation of macrophages; the activated macrophages secrete a number of cytokines, which in turn stimulate other immune cells (Sarjan *et al*., 2017).

The flavonoids activate the receptors to remove antigen (here the carbon particles) through pinocytosis as the antigen is very small. In case of mouse CRI, CRI2, CR3, CR3b and CR3bi are the main receptors. The phenols, flavonoids, terpennes and saponins as reported by Veroptta, 2001 are responsible to incite them, which in turn eliminate carbon particles or phagocyte phagocytosed made to assume the area of plasma membrane of neutrophil and monocytes increase but the microscopical examination of the blood of control and treated animals, show no change in size of monocytes either of cell. Neutrophils or monocytes, which are main phagocytic leucocytes, take up particles through minimum 40 receptors expressed on their surface. These receptors are for IgG complement, mannose and galactose terminated oligosaccharides. It is supposed that many of the receptors become active due to the exposure of the flavonoids. In our study, both flavonoids showed maximum activity against phagocytic index.

Triterpenoids and flavonoids have been studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, Immunomodulatory and tonic effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoa infections and above all, for their cytostatic effects (Uddin *et al*., 2012). The anti-inflammatory effects of triterpenoids are largely ascribed to their ability to inhibit arachidonate 5-lipoxygenase (5-LO) and human leukocyte elastase (HLE) as well as their potential for modulating the immune response by affecting complement and antibody production. Lipoxygenase is one of the most important enzymes that are involved in the synthesis of leukotrienes and this molecule is involved in various disorders i.e. hypersensitivity, such as asthma, arthritis, ulcerative colitis and disorders of the cardiovascular system *e.g.* shock and ischemia of the myocardium (Uddin *et al*., 2012). The complement is another system which may be influenced by triterpenoids.

Cyclophosphamide suppresses humoral, cellular, non-specific and specific cellular immune response. When animal was treated with cyclophosphamide then haemoglobin (Hb), RBC counts, WBC count, Lymphocyte % and Platelet count all

are reduced significantly (Uddin *et al*., 2012). Flavonoids in biological systems tend to adhere with the molecules of cyclophosphamide this causes to increase the size of the molecules and prevent its entry to the stem cells. As already stated that flavonoids are some more compounds are there which is not only negating the effect of cyclophosphamide, but also accelerating the total WBC and haemoglobin count. The luteolin and apigenin significantly produces the changes in WBCs, RBCs and hemoglobin levels. This suggests that the constituent of the plant preventing the access of cyclophosphamide to the stem cells so that synthesis of haemoglobin, WBC and RBC is not inhibited.

CONCLUSION

Many of the plant products exert some effect on immune system. They either enhance immune response to help body clear undesirable agents from body or suppress immune response to control deterioration in the body. Compounds that alter immune response are considered Immunomodulatory agent. Further detailed studies are required on the molecular basis which may be responsible for immunomodulatory activity. The present findings are significant for the development of alternative, inexpensive and perhaps safer strategies for the treatment of diseases.

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