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Evaluation of immunomodulatory cells CD4+and CD8+and their ratio using alcoholic seed extract of *Trigonella foenum graecum* and alcoholic leaves extract of *Coccinia indica* by flow cytometry in streptozotocin-induced diabetic rats

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Abstract
The present study was taken up to evaluate the immunomodulatory efficacy of *Trigonella foenum graecum* and *Coccinia indica* individualy and in combination along with glibenclamide in streptozotocin induced diabetic rats for a period of 90 days. The various groups in this study included normal control (Group-I), diabetic control (Group-II), diabetic rats treated with glibenclamide (Group-III), diabetic rats treated with *Trigonella foenum graecum* (Group-IV), diabetic rats treated with *Coccinia indica* (Group-V), diabetic rats treated with *Trigonella foenum graecum and Coccinia indica* (Group-VI), diabetic rats treated with *Trigonella foenum graecum* and glibenclamide (Group-VII), diabetic rats treated with *Coccinia indica* and glibenclamide (Group-VIII). Diabetic rats treated with *Trigonella foenum graecum, Coccinia indica* and glibenclamide (Group-IX) respectively. There was significant variation in CD4+ and CD8+ values and in their ratio in diabetic rats when compared to normal control rats. The higher alleviation of the CD4+ and CD8+ values and in their ratio was observed in group which received combine *Trigonella foenum graecum* and *Coccinia indica* extracts. The groups which received individual extracts were showed moderate alleviation in CD4+ and CD8+ values and in their ratio whereas Group treated with glibenclamide did not show any significant improvement in the CD4+ and CD8+ values and in their ratio.

Keywords: Diabetes, *Trigonella foenum gaecum, Coccinia indica*, glibenclamide, streptozotocin

Introduction
Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia and disturbances in carbohydrate, fat and protein metabolism. These metabolic abnormalities result either from a deficiency of the blood sugar-lowering hormone insulin or from insulin resistance, a defect in the body's capacity to respond to insulin (Chandra *et al*., 2004) [4]. Thus diabetes mellitus is a complex, multi factorial disease associated with progressive deterioration of beta cell function and insulin resistance.

Although hundreds of traditional plants with anti diabetic effects have been identified, only a small number of them have been evaluated scientifically for their efficacy. The hypoglycaemic effect of some of the herbal extracts has been confirmed in human and animal models. However, the major drawback in usage of herbal medicine in modern medical practices is the lack enough of scientific and clinical data proving their efficacy and safety. There is a need for conducting clinical research, experimental evaluation in various animal models for their efficacy and safety, pharmacological and toxicological evaluation and long term studies. Also, there is a need for studies on replacement of oral antidiabetic treatment with herbal medicines having immunomodulatory action by experimental research in animal models. Hence, the present study was conducted with *Trigonella foenum graecum* and *Coccinia indica* commonly known as methi and Little gourd (kovai) respectively which are reported to possess hypoglycaemic and immunomodulatory effect (Raju *et al*., 2001; Srinivasan, 2006; Yu *et al*., 2006., Khalki *et al*., 2010) [15, 18, 26, 9] to evaluate their immunomodulatory effect individually and in combination in comparison with an oral antidiabetic drug glibenclamide.

Materials and Methods

Flow Cytometry

Materials

The monoclonal anti-mouse CD4 antibodies conjugated with Fluorescence isothyocynite (FITC) and anti-mouse CD8 antibodies conjugated with Phylloerythrin cyan-7 (PE-cy7) were procured from eBioscience, USA.

Flow cytometry tubes Sheath fluid (BD Biosciences, USA) Lysis buffer –Prepared 10 x lysis buffers by adding 9ml of distilled water to one ml of lysis buffer.

Sterile distilled water Sterile phosphate buffer saline (PBS) – pH 7.4 Micro pipette and micro tips (10 µl, 50 µl and 1000 µl)

Procedure

Taken 50 µl of dipotassium EDTA added blood in four flow cytometry tubes Added 10 µl anti-mouse CD4 antibody in first tube, 10 µl anti-mouse CD8 antibody in second and 10 µl of both anti-mouse CD4 and CD8 in third tube and the fourth tube served as unstained control. The above tubes were used for setting up the flow cytometry for taking cell counts of test samples. In the test sample tubes added 10 µl of anti-mouse CD4 and 10 µl of anti-mouse CD8 in each tube. Incubated the tubes in dark at room temperature for 30 minutes. Added 2 ml of 10X lysis buffer to each tube and mixed properly. Incubated the tubes for 10 minutes at room temperature. After incubation, centrifuged the tubes at 1200 rpm for 10 minutes. Discarded the supernatant solution.

Added 2 ml of sterile PBS to each tube. Centrifuged the tubes at 1200 rpm for 10 minutes. Suspended the pellet in 400 µl of sheath fluid and recorded the cell counts in flow cytometry.

Data acquisition and post acquisition analysis was conducted using FACS Diva software version 6.1.3 (BD Biosciences, USA). The percentages of CD4+ and CD8+ T-lymphocytes were calculated after counting 10,000 events or cells per sample by flow cytometry. The CD4/CD8 ratio was calculated by dividing the percentage of CD4+ cells by percentage of CD8+ cells.

Results

Flow cytometry

Normal control Group-I

In the present study to evaluate the immunomodulatory effect of *Trigonella foenum graecum* and *Coccinia indica*, the blood samples collected at 90th day of the study were subjected for enumeration of CD4+ and CD8+ cells and CD4+ to CD8+ ratio by flow cytometry. The Flow cytometry analysis of control rats was 65.36 + 1.17 and 27.71+ 3.12 per cent for CD4+ and CD8+ cells respectively and the CD4+: CD8+ ratio was 2.02+ 0.08.

Normally the percentage of CD4+ T cell population of the immune system is higher compared to CD8+ cells. It is estimated to be approximately 65 per cent and 30 per cent for CD4+ and CD8+ cells respectively and 5 per cent of cells express neither CD4+ nor CD8+ molecules and said to be double negative. Normally, an elevated CD4+ count implies increased lymphocytic reactivity as helper cells predominate and a high CD8+ level implies depressed lymphocyte reactivity (Tizard, 2008) [20].

Diabetic control Group-II

In group-II (Diabetic control) rats on 90th day of study there was a significant decrease ($P \leq 0.001$) in CD4+ cell percentage and significantly increased ($P \leq 0.001$) CD8+ values compared to normal control. The ratio of CD4+:CD8+ was 0.65±0.04 which was significantly lesser ($P \leq 0.001$) compared to normal control.

Alterations in lymphocytes with decreased CD4+ count and increase in CD8+ cells are a common finding in both type I and type II diabetes (Hedman *et al.*, 2008). Since activation of T lymphocytes plays a pivotal role in initiating immune response and cell-mediated cytotoxic activity, inhibition of lymphocyte activation by diabetic state could evoke a clinically relevant immunosuppressive effect. In DM altered functions of different types of circulating immune cells, T lymphocytes and B lymphocytes have been reported (Yu *et al*., 2006) [26].

The decrease in CD4+ cells in diabetes could be attributed to several reasons such as oxidative stress, a common finding in diabetes; glucolipotoxicity due to chronic exposure to higher glucose; hypoinsulinism, depressed IL2 level and decreased response of lymphocytes to mitogen. White blood cells including lymphocytes and their functions are shown to be globally affected by ambient glucose concentrations (Alberti, 1977) [22]. It has been reported that the potential increase in basal levels of intracellular calcium in the lymphocytes of diabetic patients can lead to decreased release of IL2 and decreased response to mitogenic stimuli (Tripathi, 2010) [19]. In addition stress during diabetes has been shown to induce apoptosis of lymphocytes and Insulin to reduce apoptosis of the lymphocytes. Kitabchi *et al*. (1995) [10] reported that prevention of lymphocytic apoptotic cell death from various stimuli could be due to activation of phosphatidylinositol 3-kinase-Akt pathway (Hotchkiss and Karl, 2003) [8]. Experimental evidence suggests that antioxidant supplementation reduces oxidative stress in diabetics and improve insulin concentration and thus modulate immune cells. Venkatesha *et al*. (2011) [22] also reported that CD4+ CD25+ cell therapy delayed occurrence of several immunological diseases including diabetes in rat models.

Elevated CD8+ T cell count in diabetes in conjunction with significantly low levels of CD4+ T cells would indicate acute critical illness with consequently an overload of the immune response. In such a situation, there is stimulation of suppressor CD8+ T cells and limitation of antigen induced lymphocyte proliferative responses by the production of suppressor factors. Additionally, the elevation of CD8+ T cells may be as a result of sequestration of CD4+ T cells to the pancreas in T2DM involving anti-islet T cell mediated pathogenic mechanisms (Yu *et al*., 2006) [26]. The chronic activation of lymphocytes due to glucose toxicity could increase CD8+ cells. Long standing hyperglycemia has been reported to result in chronic activation of lymphocytes and platelets due to activation of immune system and chronic systemic inflammation (Papatheodorou *et al.*, 2006) [13].

Glibenclamide group (III)

Flow cytometry analysis of rats treated with glibenclamide did not show any significant improvement in the CD4+ and CD8+ values and in their ratio compared to diabetic control. This finding indicated that treatment with oral antidiabetic drug though improves diabetic state with improvement in glucose, insulin, and other biochemical parameters, fails to improve the immune status and lacks immunomodulatory effect.

Treatment Groups IV, V, VI

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity. Many plants used in traditional

medicine have immunomodulating activities (Wagner and Proksh, 1985) [24]. Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles. Immunomodulation could be immunostimulation in a drug-induced immunosuppression model and immunosuppression in an experimental hyperreactivity model by the same preparation (Harsh *et al.*, 1969 and Butler *et al*., 2004) [6, 3]. Apart from being specifically stimulatory or suppressive, certain agents have been shown to possess activity to normalize or modulate pathophysiological processes and are hence called immunomodulatory agents. A number of medicinal plants as rasayanas, have been claimed to possess immunomodulatory activity (Veerapur *et al*., 2004) [21]. *Trigonella foenum graecum* and *Coccinia indica* are two such plants used in herbal medicine.

In the present study the combined treatment with *Trigonella foenum graecum* and *Coccinia indica* showed a significant improvement in the CD4+ and CD8+ cell counts compared to diabetic, glibenclamide and individual treatment with Trigonella and Coccinia extract groups. The results indicated that there is a synergistic effect in improving the T cell profile between Trigonella and Coccinia in the present study as individullay they failed to increase the number of CD4+ cells significantly. The fenugreek extract has been shown to increase in T-cell immune response significantly with activation of the CD4+ and CD8+ cells. In diabetes an elevated circulating inflamatory cytokines such as TNFα, IL-1β and IL-6 are observed in patients with hyperglycemia (Spranger *et al.*, 2003 and Manning *et al.*, 2008) [17, 12]. The cytokine TNFα has been reported to down regulate the peroxisome proliferator activator receptors PPAR-γ expression which modulates important metabolic events of the cell (Berger *et al*., 2005).

The aqeuous extract of *Trigonella foenum graecum* is known to decrease the levels of elevated TNFα in type-2 diabetic rats (Halagappa *et al.*, 2010) [5]. Trigonella seeds contain steroid, saponins compounds diasgenin, alkaloids and trigonelline compounds which upregulate PPAR-γ expression there by modulate inflammatory cytokine TNFα (Vishwakarma *et al.*, 2005 and Halagappa *et al.*, 2010) [23, 5]. The immunomodulatory effect of *Trigonella foenum graecum* could also be due to the antioxidant activity, hypoglycaemic effect and insulinotropic effect of its bioactive compounds (Smriti *et al.*, 2012) [16].

Coccinia indica has also been proved to be an immunomodulator due to its effect on haemopoietic tissue and its anti-inflamatory activity (Yadav *et al*., 2010) [25]. Anti-inflamatory activity of *Coccinia indica* has been specifically attributed to the cephalandrol, tritriacontane, lupeol, b-sitosterol, cephalandrine A, cephalandrine B, stigma-7-en-3-one, taraxerone and taraxerol, terpenoids, saponins, flavonoids, sterols present in the plant (Mandal *et al.*, 1992) [11]. In addition the insulinomimetic action of the triterpenes of Coccinia may also be contributory for the immunomodulation.

Combined treatment groups with glibenclamide (VII, VIII and IX)

Among the combined treatment groups (VII, VIII, IX) the improvement in CD4+ and CD8+ cells and their ratio was significantly higher in groups IX rats treated with Trigonella, Coccinia along with glibenclamide half dose compared to Group VII and VIII. The improvement in T cell profile of group IX was comparable to that of Group VI treated with Trigonella and Coccinia combindly. The improvement in T cell profile could be attributed to the combined synergistic effect of plant extracts and not due to glibenclamide as the glibenclamide alone failed to improve the CD4+ cell number.

Thus in the present study the antioxidants present in the herbal preparation may be responsible in bringing about immune modulation. Antioxidants could modulate the functions of the neutrophils, their opsonising capacity and T and B cell proliferation response. Herbal preparations are said to affect the immune reactions through their anti–inflammatory actions. In most cases the therapeutic efficiency of these plants may, in part, be mediated via their influence on the immune response. This could be attributed to decrease in the number of lymphocytes in circulation due to lymphocytolytic effect of STZ. Adeghate *et al.* (2010) [1] and Pragathi, (2011) [14] observed lymphocytolysis in the spleen of the diabetic control animals due to STZ.

Table 1: The mean (±SE) values of CD4+, CD8+ cells and their Ratio (percentage) of different groups at day 90 of the study.

Groups	Days Post Treatment		
	CD4+	CD8+	Ratio CD4+/CD8+
Group I	68.36±1.17^{a}	27.71±3.12^{a}	2.02±0.08^{a}
Group II	37.30±1.26^{b}	52.36±0.57^{b}	0.71±0.03^{b}
Group III	38.75±1.54^{b}	50.01±0.93^{b}	0.76±0.05^{b}
Group IV	43.98±3.78^{b}	48.97±4.12^{b}	0.93±0.15^{c}
Group V	41.34±2.29^{b}	48.22±1.09^{b}	0.86±0.07^{c}
Group VI	55.83±0.53^{c}	37.18±1.66^{c}	1.51±0.07^{d}
Group VII	43.48±4.17^{b}	50.05±3.90^{b}	0.91±0.17^{c}
Group VIII	32.31±4.87^{b}	61.72±4.57^{cd}	0.40±0.20^{e}
Group IX	54.31±4.66^{cd}	41.12±4.11^{ce}	1.38±0.22^{df}

The means with at least one common superscript are not significantly different ($P \leq 0.001$).

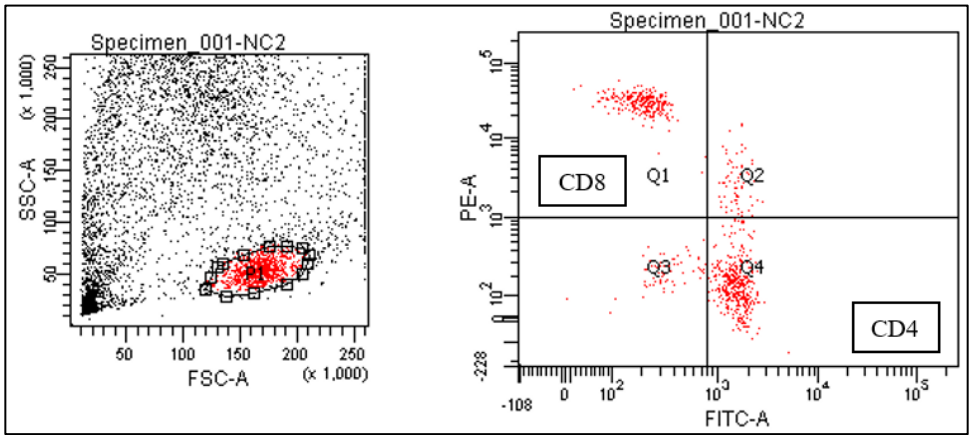

Fig 1: Flow cytometry results of normal control animal

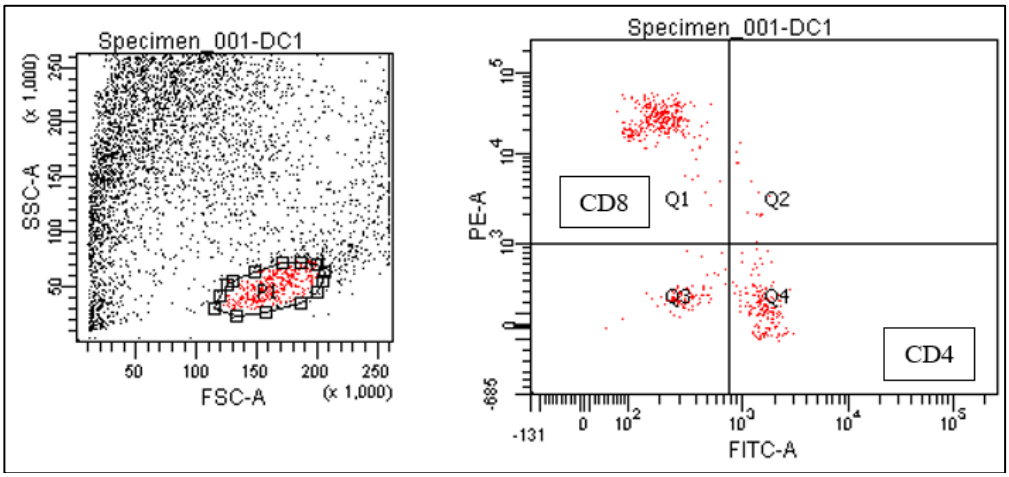

Fig 2: Flow cytometry results of diabetic control animal

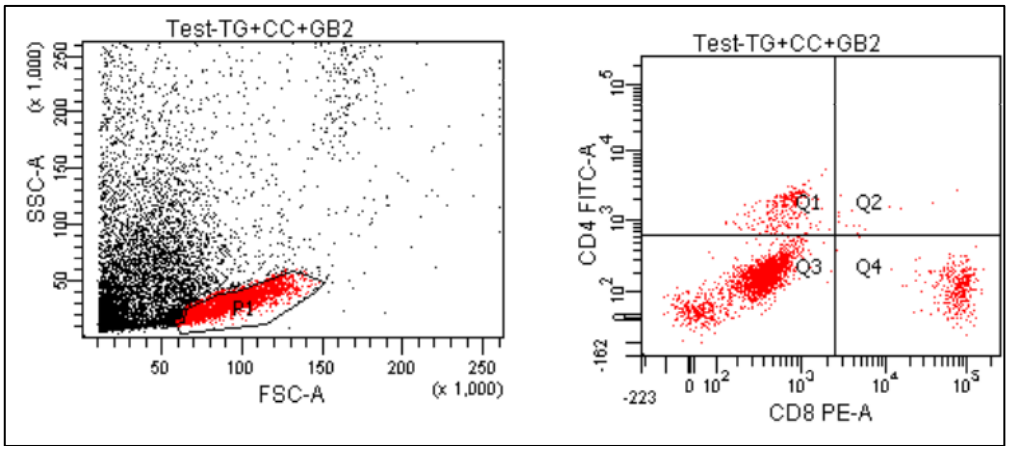

Fig 3: Flow cytometry results of diabetic animal treated with combination of *Trigonella foenum graecum*, *Coccinia indica* and glibenclamide (Group IX)

Summary and Conclusion

The Flow cytometry analysis of group-II (Diabetic control) rats showed significantly lower CD4+ and significantly higher CD8+ values compared to normal control. The rats of treatment groups (III to IX) showed improvement in values of CD4+ and CD8+ values and in their ratio when compared to diabetic control animals. Compared to other treatment groups, among these, groups IV, VI and IX showed better improvement.

The *Trigonella foenum graecum* and *Coccinia indica* extracts have a good antioxidant effect compared to glibenclamide and have a synergistic effect in alleviating the CD4+ and CD8+ values and in their ratio induced by STZ and there is a synergistic effect between the two herbal extracts in improving CD4+ and CD8+ values and in their ratio.

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