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IN VITRO AND *IN VIVO* ANTIOXIDANT AND IMMUNOMODULATORY POTENTIAL EVALUATION OF ETHANOLIC EXTRACT OF *TINOSPORA CORDIFOLIA* IN EXPERIMENTAL DIABETES RAT MODEL

DEEPA P.M.[1]*, DIMRI UMESH[2], YATOO IQBAL[2] AND GOPINATH DEVI[2]
[1]Dept. of Veterinary Epidemiology and Preventive Medicine, CVAS, Pookode, Wayanad, Kerala
[2]Division of Medicine, Indian Veterinary Research Institute, Izatnagar, UP
*Corresponding Author: Email- deepapm@kvasu.ac.in

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Abstract- The objective of present study was to evaluate efficacy of 70% ethanolic extract of stem of *Tinospora cordifolia* in streptozotocin induced diabetic rats. 70% ethanolic extract of *Tinospora cordifolia* exhibited good *in vitro* antioxidant and immunomodulatory activity. Reduction of blood glucose was significant and non-significant increase in serum insulin levels in treated groups. *In-vivo* study of antioxidants exhibited significant decrease in malondialdehyde($P<0.05$), significant increase in Superoxide dismutase and reduced glutathione. Catalase activity was fond to be decreased on 14 and 21 days of treatment. *In vivo* immunomodulatory potential evaluation showed significantly increased cellular and humoral immune response in treated rats .On concluding, *In vitro* tests exhibited *Tinospora cordifolia*, a good antioxidant and immunomodulator and in *vivo* tests showed its efficacy in amelioration of *diabetes mellitus*.

Keywords- Diabetes, rats, *Tinospora cordifolia,* antioxidant, immunomodulatory.

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Introduction

Diabetes is a common disease affecting human health and life quality. Diabetes mellitus is a chronic disorder with hyperglycemia and can leads to complications like retinopathy, nephropathy, neuropathy and angiopathy. Chronic hyperglycemia may itself cause irreversible damage to beta cells [1]. Oxidative stress is suggested as mechanism underlying diabetes and diabetic complications [2]. The increase in the level of reactive oxygen species in diabetes could be due to the increased production or decreased destruction by nonenzymatic and enzymatic catalase (CAT), reduced glutathione and superoxide dismutase [3]. Antioxidants might therefore be helpful for treating diabetic patients and their complications. The experimental and clinical results suggest the importance of studies to further evaluate the potential values of antioxidants in prevention and treatment of diabetes and its complications [4].

Tinospora cordifolia (Willd.) (TC) belongs to the family Menispermaceae known for long in the Ayurvedic literature as therapeutic remedy for diabetes and other metabolic disorders [5]. Immunomodulatory and hepatoprotective properties of aqueous extract of *T. cordifolia* stem, in CCl_4 intoxicated Swiss albino mice, have been reported [6]. It was observed to modulate immune system by activating macrophages as obvious by increased secretion of lysozyme in treated macrophage cell lines [7].

The long asymptomatic period before the onset of chronic disease in diabetes presents opportunities for disease prevention. The use of antioxidant/immuno-modulators for management of early stages of diabetes may prevent the occurrence of the clinical stage. Hence, the present study was undertaken to evaluate the efficacy of 70 % ethanolic extract of *Tinospora cordifolia* in alleviation of *diabetes mellitus* in immune model of diabetes in rats induced by multiple streptzotocin protocol.

Materials and Methods

In *vitro* evaluation of plant extract

Stem of *Tinospora cordifolia* were dried and grinded. They were exhaustively extracted with 70 % ethanol under reflux condenser. The ethanolic extracts were filtered with Whatman filter paper (No.1) to free bigger particles. To make ethanol free it was kept in hot air oven at 50^0C for 48 hours. The dried form of extracts were kept in air tight polypacks and preserved at 4^0C for further use.

In vitro antioxidant potential evaluation of the extracts

The concentration of phenolics in plant extracts was determined using Folin Ciocalteu reagent method [8]. The FRAP assay was carried out according to procedure [9]. In this assay, the antioxidant activity was determined on the basis of the ability to reduce ferric (III) ion to ferrous (II) ion. Assay of Ascorbate –Iron (III) - catalysed phospholipids peroxidation was performed by modified method of [10]. In this test the ability of the extracts to scavenge hydroxyl radicals was determined.

In vitro immunomodulatory potential evaluation

In vitro immunomodulatory potential of extract was carried out by lymphocyte proliferation assay [11].

A total of 18 male wistar rats weighing about 150-200g were procured from the Laboratory Animal Resource section of IVRI, Izatnagar and used in the present study. They were maintained in standard environmental conditions of temperature (25±20C), relative humidity (55±10%) and 12 h dark/light cycle. They were fed with standard diet and water ad libitum. The present animal experimentation was in compliance with the guidelines laid out by the Institutional Animal Committee.

Diabetes was induced in 12 rats by intraperitoneal injection of a freshly buffered (0.1 M citrate, pH 4.5) solution of STZ at a dosage of 40 mg/kg body weight consecutively for 5 days. After 72 h of STZ administration, the tail vein blood was collected to find out fasting blood glucose level. Only rats with fasting blood glucose over 250 mg/dL were considered diabetic and included in the experiments. They were divided into three groups. Group I was kept as healthy controls which was injected with citrate buffer only. Group II was taken as positive control, which was given STZ injection only. Group III was treated with 70% ethanolic extract of stem of *Tinospora cordifolia,* 100 mg/kg/day PO once daily, starting on the day of injection of streptozotocin and continued for 3 weeks.

Fasting blood glucose and serum insulin (RIA) were estimated on days 0, 7, 14, 21 and 28 after induction of diabetes.

Estimation of Oxidative stress parameters in rats

About 2 mL of blood samples were collected from all rats from inner canthus of eye by using capillary tubes in a heparinised eppendorf tubes. After centrifugation at 3000 rpm for 10 min, the plasma and buffy coat was removed. Then, red blood cells were washed thrice in an ice- cold isotonic sodium chloride solution (NSS). The RBC pellet was diluted with ice-cold distilled water in 1:10 ratio for the preparation of 10 % hemolysate and rest of the RBC pellet was diluted with ice-cold NSS in 1:1 ratio to get RBC suspension, which was used, for GSH estimation. 10% hemolysate was used for the estimation of superoxide dismutase (SOD), lipid peroxidation (LPO) and catalase (CAT). Haemoglobin concentration of hemolysate was estimated by cyanohemoglobin method [12]. The lipid peroxides level in the RBC hemolysate was determined by the method of [13]. Reduced glutathione was estimated by DTNB method of [14] in RBC suspension. Catalase activity in hemolysate was estimated by using hydrogen peroxide as a substrate [15]. Superoxide dismutase was estimated [16].

Immune response evaluation in rats

Cellular and humoral immune responses were assessed in all rats. Humoral immune responses were evaluated [17]. Antibody titer was measured using micro-hemagglutination test [18]. Cellular immune response was assayed by footpad reaction method in rats using sheep red blood cells as antigen in Alserver's solution. On day 26 of the experiment, sheep RBC ($2.5x10^7$ cells) was injected in the sub-planter region of right hind paw of all rats of above-mentioned groups. Equal volume of phosphate buffered saline was injected in sub planter region of the left hind paw of all the rats, to compare as control. The reaction was assessed by measuring the increase in paw volume with help of a vernier's calliper after 48 h (on day 28 of the experiment) post challenge. The pre and post challenge difference in the thickness of footpad was expressed in mm and taken as a measure of delayed hypersensitivity reaction. The TLC count of the blood was determined by routine haematological method using Neubauer Chamber with haemocytometer. Differential leukocyte count was done as per procedure [19]. CD4+and CD8+ T cells in peripheral bold was determined by flow cytometry. Briefly, 100 µl of whole blood were added to 900 µl of working RBC lysis buffer. Mixed well and incubated at room temperature for 10 minutes. Centrifuged at 5000 rpm for 5 minutes. Discarded the supernatant. Again added 500 µl of working RBC lysis buffer mixed well and incubated for 10 minutes at room temperature. Centrifuged at 5000 rpm for 5 minutes and discarded the supernatant. To the pellet added 300µl of PBS and 20 µl of the combined monoclonal antibodies CD3: APC/ CD4: PE and CD 8: FITC. After incubation for 10 min at 25^0C in a dark room, measurement of CD3+CD4+ and CD3+CD8+ T cells was performed by gating lymphocyte areas on dot plots. Ten thousand cells were analysed and positive fluorescence was defined to exclude 98 % of the cells in the negative control.

Total IgG in serum samples of all rats was estimated using rat specific IgG ELISA kit (USCN life Science Inc). Intra assay coefficient of variation <10% and inter-assay coefficient of variation <12%.

Statistical analysis

The statistical significance was assessed using SPSS.17 and Graph Pad Prism version 4 Software (San Diego, California, USA). Results were expressed as Mean±SE. Data were analysed by two –way ANOVA for multiple comparisons followed by Bonferroni *post-hoc* test. A value of P<0.05 was considered to be statistically significant [20]

Results and Discussion

In *vitro* evaluation of plant extracts

Total phenol content of 70% ethanolic extract of *Tinospora cordifolia* was 56.28±1.4mg GAE/g. The effect of plant extract on iron (III) to iron (II) reducing activity was 1.8±0.3152 mmol Fe II/g. The ability of plant extract to inhibit the formation of 2- thiobarbituric acid reactive species (TBARS) by scavenging hydroxyl radicals generated by ascorbate-iron III at low concentration was 53.13±1.89%. The antioxidant capability of *T. cordifolia* was attributed to the presence of tannins and phenolics along with other compounds [21]. Lymphocyte stimulation index was compared with known mitogen concanavalin A (20µg/mL). *Tinospora cordifolia* exhibited a stimulation index comparable with con A. Previous studies reported immunostimulatory properties of *T. cordifolia* [22]. [Table-1].

Table-1 *In vitro evaluation of 70% ethanolic extract of Tinospora cordifolia*

FRAP(mmol Fe II/g)	1.8±0.31
TBRS(% inhibition)	53.13±1.89
Total Phenol(mg GAE/g)	56.28±1.4
Lymphocyte stimulation index	1.6±0.52

***In vivo* evaluation of extract**

Group III showed a significant decrease in fasting blood glucose (P<0.05) compared with positive controls on days 7, 14, 21 and 28 [Table-2]. Insulin concentration was found to be significantly (P<0.05) decreasing in positive control (group II) on different days. Group III revealed a non significant increase in serum insulin level compared with positive controls [Table-3]. This is in agreement with findings [23] who demonstrated that *T.cordifolia* administration in diabetic animals didn't cause any increase in serum insulin level but caused increase of hepatic glycogen synthase and decreased glycogen phosphorylase activity suggesting that the anti-diabetic activity of *T. cordifolia* is not through the insulin secretion by pancreatic beta cells but may be due to the increased entry of glucose into the peripheral tissues and organs like the liver.

Positive control animals (group II) revealed significantly higher (P<0.05) content of MDA in erythrocytes in comparison with healthy controls. Group III revealed significantly lowered (P<0.05) MDA on day 28. [Table-4]. Group II (positive control) animals exhibited significant decrease in GSH activity on different days. Group III revealed significant (P<0.05) increase of GSH level compared to positive control on day 28 [Table -5]. This finding is in agreement [24] who showed distinct increase in the level of glutathione, SOD and catalase in *T.cordifolia* treated rats as compared to sheep RBC and cyclophosphamide treated groups. There was significant (P<0.05) decrease in activity of SOD in group II (positive control) animals in different days. Group III showed significantly higher (P<0.05) activity of SOD on day 7 compared to positive controls and had comparable to that of healthy controls [Table-6]. In group II (positive control), activity of catalase enzyme was found to be significantly (P<0.01) increasing up to day 21 and found to be significantly lowered on day 28 [Table-7]. This is in agreement with findings of [25] who observed that the level of erythrocyte catalase activity in the diabetic rats was significantly greater than control rats. The increased catalase activity in diabetic rats could be due to higher production of H_2O_2. Hypoinsulinemia increases the activity of an enzyme, fatty acyl-CoA oxidase that initiates β oxidation of fatty acids resulting in the production of hydrogen peroxide [26]. In our study decrease of catalase enzyme on 28th day may be due to significantly decreased SOD activity on 28th day compared to healthy controls Group III showed a significant decrease of catalase level compared to positive control on day 14 and 21 and a non-significant increase of catalase enzyme on day 28 compared to positive control. *T.cordifolia* root extract administration resulted in decrease in level of plasma thiobarbituric acid reactive substance, ceruloplasmin and increased the level of glutathione and vitamin C in alloxan diabetic rats [27]. Group II (positive control) exhibited non-significant decrease in CMI response compared to healthy control. Group III exhibited a significantly increased cellular response in terms of increase in paw thickness [Table-8]. Group II animals showed significantly (P<0.05) lower haemagglutinating antibody titer as compared to healthy controls. Group III showed significant increase in humoral immune response compared with positive controls [Table-9]. Previous studies also reported *T.cordifolia* efficient in stimulating humoral immune response in a dose dependent manner [29,30].

Table-2 *Effect of treatment on fasting blood glucose level (mg/dl) in different days (Mean±SE)*

Groups	0 day	3 day	7 day	14 day	21 day	28 day
Group I	$81.16±5.5^{aA}$	$82.16±3.75^{aA}$	$84.33±7.46^{aA}$	$88.33±5.9^{aA}$	$86±4.47^{aA}$	$83.66±4.2^{aA}$
Group II	$87.66±4.8^{aA}$	$179.83±8.53^{cB}$	$291±11.54^{dC}$	$353.5±20.07^{dD}$	$376.33±21.94^{dD}$	$384.66±18.88^{dD}$
Group III	$90.33±7.31^{aA}$	$163.33±14.92^{bcB}$	172.66±32.25	$189.5±22.02^{bB}$	$163.83±19.82^{bB}$	$161.66±14.55^{bcB}$

Table-3 *Effect on serum insulin levels (µU/ml) in different days (Mean±SE)*

Groups	0day	7 day	14 day	21 day	28 day
Group I	59.4±11.05^{aA}	53.68±11.96^{bA}	51.68±8.85^{cA}	55.88±10.79^{cA}	57.05±9.51^{cA}
Group II	52.11±755^{aB}	21.73±4.18^{aA}	17.31±3.34^{aA}	14.36±2.56^{aA}	11.55±1.8^{aA}
Group III	57.53±11.63^{aB}	27.63±12.56^{aA}	23.83±8.84^{aA}	24.8±8.19^{aA}	20.36±7.39^{aA}

Table-4 Efficacy of treatment on erythrocytic lipid peroxide level(nmol/mg Hb) (Mean±SE)

Groups	0 day	7 day	14 day	21 day	28 day
Group I	1.99±0.4^{aA}	1.96±0.33^{aA}	2.0±0.24^{aA}	2.12±0.35^{aA}	1.87±0.41^{aA}
Group II	1.96±0.3^{aA}	7.28±0.47^{cC}	6.18±0.44^{bB}	5.95±0.3^{cB}	5.43±0.39^{bB}
Group III	2.12±0.3^{aA}	3.94±0.56^{bB}	2.27±0.38^{aA}	3.47±0.58^{bB}	2.41±0.33^{aA}

Table-5 *Efficacy of treatment on reduced glutathione level (µmol /ml packed RBC) (Mean±SE)*

Groups	0 day	7 day	14 day	21 day	28 day
Group I	0.69±0.11^{aA}	0.72±0.07^{bA}	0.75±0.08^{bA}	0.73±0.12^{cA}	0.68±0.09^{cA}
Group II	0.74±0.08^{aB}	0.23±0.06^{aA}	0.16±0.03^{aA}	0.12±0.01^{aA}	0.1±0.2^{aA}
Group III	0.71±0.12^{aB}	0.6±0.12^{bB}	0.63±0.1^{bB}	0.51±0.13^{bcAB}	0.39±0.07^{bA}

Table-6 *Efficacy of treatment on SOD level (µmol MTT formazan/g Hb) (Mean±SE)*

Groups	0day	7 day	14 day	21 day	28 day
Group I	0.161±0.05^{aA}	0.168±0.007^{bA}	0.155±0.025^{aA}	0.161±0.02^{cA}	0.148±0.019^{bA}
Group II	0.159±0.021^{aB}	0.061±0.024^{aA}	0.05±0.008^{aA}	0.047±0.007^{aA}	0.025±0.005^{aA}
Group III	0.172±0.02^{aC}	0.167±0.024^{bC}	0.077±0.029^{aAB}	0.115±0.016^{bcBC}	0.046±0.01^{aA}

Table-7 *Efficacy of treatment on catalase level (µmol H_2O_2 decomposed/min/mg Hb)*

Groups	0day	7 day	14 day	21 day	28 day
Group I	5.98±1.06^{aA}	6.49±1.24^{aA}	6.58±1.22^{aA}	6.18±1.44^{aA}	6.05±1.33^{bA}
Group II	6.16±0.53^{aB}	12.43±1.24^{cC}	10.95±0.71^{bC}	10.75±1.01^{bC}	2.12±0.52^{aA}
Group III	7.13±1.08^{aAB}	12.15±0.74^{cC}	6.84±1.04^{aB}	6.67±0.89^{aB}	4.04±0.83^{abA}

Table-8 *Effect of treatment on cell mediated immune response (Footpad thickness in mm)*

Groups	0 hr	48 hr	% increase in footpad thickness
Group I	0.056±0.006^{aA}	0.09±0.008^{aB}	66.06
Group II	0.053±0.008^{aA}	0.085±0.013^{aB}	40.1
Group III	0.06±0.005^{aA}	0.095±0.006^{aB}	64.06

Table-9 *Effect of treatment on humoral immune response (Mean±SE)*

Groups	21 days(primary)	28 days(secondary)
Group I	22±9.54^{aA}	36.33±20.72^{aB}
Group II	12±4.81^{aA}	20±4^{aA}
Group III	22±10.41^{aA}	38.66±20.46^{aB}

Serum IgG level of group II (positive control) animals was found to be non-significantly increased compared with healthy controls on different days. Type-1 diabetic children had higher serum IgG level compared to normal children and suggested the measurement of IgG levels can serve as surrogate marker to identify the risk for rapid development of diabetes [30]. *T. cordifolia* treated group revealed non-significant decrease in IgG levels compared to positive controls on day 28 [Table-10]. Group II (positive control) showed highly significant increase (P<0.01) in TLC on day 7 compared to healthy control (group I) and on subsequent days (day 14, 21, 28) revealed significant (P<0.01) reduction in TLC. In contrast to the present finding, [31] reported significantly higher TLC in streptozotocin induced diabetes in rats compared to control rats. Our finding is in agreement with [32] who observed that TLC in streptozotocin induced diabetic rats was significantly lower than healthy controls. Group III revealed highest leukocyte response on days 7, 14, 21 and 28 compared with group II and group I [Table-11]. Neutrophil count was found to be significantly higher (P<0.05) in group II (positive control) compared to group I (healthy control) on day 7, 14, 21 and 28 [Table-12]. This is in agreement with [33] who observed that STZ induced diabetic rats showed significantly increased neutrophils, monocytes and basophils but significantly decreased lymphocyte percentage compared to healthy control group. Group III showed a non significant decrease of neutrophil count in different days of treatment. Lymphocyte count showed significant (P<0.05) reduction in group II (positive control) compared with healthy controls (group I) on days 7, 14, 21 and 28. Group III revealed a non-significant increase in lymphocyte count in different days of treatment [Table-13]. In agreement with our finding, [34] reported that administration of *T.cordifolia* stem extract significantly increased the level of all leukocyte count. Group II (positive control) revealed significantly higher (P<0.05) monocyte count compared to healthy controls (group I). Group III showed significantly low (P<0.05) monocyte count compared to group II (positive control) [Table-14]. Group II (positive control) had significantly (P<0.05) lower CD4+T cells compared to healthy controls (group I). The ratio of CD4+/CD8+T cells was found to be significantly lower in group II (positive control) compared to group I (healthy control). There was significant (P<0.05) improvement in CD4+ T cells and CD4+/CD8+T cells ratio in group III compared to positive control [Table-15]. Analysis of lymphocyte markers of T cells (CD3+, CD4+, CD8+) in murine visceral leishmaniasis treated with *T.cordifolia* in combination with cisplatin showed enhancement in proliferation and differentiation of T lymphocytes [35].

Table-10 *Efficacy of treatment on serum IgG level (mg/ml) (Mean±SE)*

Groups	0 day	7 day	14 day	21 day	28 day
Group I	6.6±0.6^{aA}	6.58±0.73^{aA}	6.54±0.79^{aA}	6.19±0.44^{aA}	6.72±0.4^{aA}
Group II	6.84±0.51^{aA}	7.86±0.56^{aA}	7.7±0.67^{aA}	7.73±0.58^{aA}	7.8±0.47^{bA}
Group III	6.57±0.43^{aA}	7.64±0.63^{aA}	7.4±0.68^{aA}	7.14±0.59^{bA}	7.24±0.59^{bA}

Table-11 *Efficacy of treatment on total leukocyte count (103 cells/cu mm) (Mean±SE)*

Groups	0 day	7 day	14 day	21 day	28 day
Group I	6.16±0.53^{aA}	6.72±1.32^{aA}	6.76±1.07^{aA}	7.3±1.01^{abA}	8.47±1.57^{bA}
Group II	7.47±1.79^{aA}	16.68±1.97^{bcB}	4.8±0.91^{aA}	4.45±0.75^{aA}	2.55±0.52^{aA}
Group III	7.71±1.36^{aA}	17.5±1.69^{bcB}	20.76±1.7^{bcB}	16.25±1.5^{efB}	13.73±1.63^{bcA}

Table-12 *Effect of treatment on neutrophil count (%) (Mean±SE)*

Groups	0 day	7 day	14 day	21 day	28 day
Group I	16.83±1.7^{aA}	17.33±2.47^{aA}	16.66±2.07^{aA}	19.33±2.74^{aA}	19.16±3.54^{aA}
Group II	19.5±1.23^{aA}	37.66±4.51^{bB}	41.33±3.72^{cBC}	42.16±5.27^{cBC}	44.66±6.36^{cC}
Group III	17.66±4.33^{aA}	32.33±4.31^{bB}	31.66±4.64^{bB}	29.83±4.03^{bAB}	26.83±3.87^{abAB}

Table-13 *Effect of treatment on lymphocyte count(%) (Mean±SE)*

Groups	0 day	7 day	14 day	21 day	28 day
Group I	79.5±1.36^{aA}	79.66±236^{bA}	79.5±2.91^{bA}	78.5±2.77^{bA}	78.33±3.72^{bA}
Group II	77.5±0.92^{aB}	58.83±4.5^{aAB}	54.66±3.9^{aAB}	53.5±5.33^{aAB}	51.66±6.1^{aA}
Group III	80.5±4.38^{aB}	67.16±5.14^{abAB}	68.33±5.81^{abAB}	68.83±4.06^{abAB}	70±3.94^{abAB}

Table-14 *Effect of treatment on monocyte count (%) (Mean±SE)*

Groups	0 day	7 day	14 day	21 day	28 day
Group I	1.83±0.47^{aA}	1.16±0.36^{aA}	1.33±0.22^{aA}	1.66±0.28^{aA}	1.5±0.22^{aA}
Group II	1.16±0.3^{aA}	2.5±0.42^{bAB}	3±0.36^{bB}	3.5±0.34^{bB}	3.16±0.54^{bB}
Group III	1.33±0.57^{aA}	1.16±0.39^{aA}	1.16±039^{aA}	0.83±0.2^{aA}	1±0.4^{aA}

Table-15 *Effect of treatment on CD4+/CD8+ Tcells ratio in peripheral blood (Mean±SE)*

Groups	CD4+Tcells (%)	CD8+ T cells (%)	CD4/ CD8 T cells ratio
GroupI	29.58±1.85^{b}	15.46±0.41^{a}	1.93±0.16^{b}
GroupII	20.61±1.32^{a}	24.06±1.15^{a}	0.85±0.03^{a}
GroupIII	32.87±3.45^{b}	22.23±1.7^{a}	1.47±0.09^{b}

Conclusion

In vitro antioxidant and immunomodulatory potential evaluation of 70 % ethanolic extract of *Tinospora cordifolia* revealed as good antioxidant and immuno-stimulator. Diabetic rats showed significant increase of fasting blood glucose and significant decrease of insulin compared to healthy control. *Tinspora cordifolia* treated group showed better response in reduction of fasting blood glucose, but showed no significant increase in insulin secretion. *In vivo* antioxidant potential revealed significant improvement in oxidant –antioxidant parameters in treated group after 4 weeks of therapy. *In vivo* immunomodulatory potential showed non-significant decrease in cellular immune response measured by DTH response to foot pad inoculation with sheep RBC, significant decrease in humoral immune response by HI with sheep RBC, non-significant increase in serum IgG levels by ELISA, significant decrease of total leukocyte count, significant increase of neutrophil count, significant decrease of lymphocyte count, significant increase of monocyte count, significant decrease of CD4+/CD8+ratio. Treated group showed high cellular and humoral immune response. Based on all the parameters studied 70% of ethanolic extract of *Tinospora cordifolia* is found to be effective in amelioration of *diabetes mellitus* especially in early stage of the disease.

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