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Original Article

Effect of pterostilbene compared to tetrahydrocurcumin on insulin receptor status in type 2 diabetic rats: Studies on the binding of insulin to erythrocytes

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ABSTRACT

Pterocarpus marsupium has been used in the treatment of toothache, diarrhoea, heartburn, urinary tract infections, boils, sores and skin diseases. *P. marsupium* has been used for many years in the treatment of diabetes mellitus. Pterostilbene was found to be one of the active constituents in the extracts of the heartwood of *Pterocarpus marsupium.* Pterostilbene, a phenolic compound derived from resveratrol, possesses greater bioavailability than its parent compound due to the presence of two methoxyl groups. In this study, the beneficial effects of pterostilbene on diabetes, liver steatosis and dyslipidemia are summarized. Pterostilbene is a useful bioactive compound in preventing type 1 diabetes, insulin resistance and type 2 diabetes in animal models. Plants have received much attention as sources of biologically active substances. Tetrahydrocurcumin (THC) is a hydrogenation product of curcumin produced by reducing curcumin in an organic solvent using a metal catalyst. Erythrocytes have specific surface receptors, which have binding characteristics similar to those of the insulin receptor found in classical target tissues for insulin action. Streptozotocin (STZ) causes significant reduction in the number of receptors in erythrocytes and insulin target tissues. Erythrocytes from diabetic control rats show decreased ability to bind to insulin when compared with THC and pterostilbene-treated diabetic rats. High affinity (Kd1), low affinity (Kd2) and kinetic analyses revealed an increase in the average receptor affinity of erythrocytes from pterostilbene and THC-treated diabetic rats compared with diabetic control rats. These results suggest that acute alteration of the insulin receptor on the membranes of erythrocytes occurred in STZ diabetic rats. The effect of THC was more prominent than pterostilbene.

KEYWORDS: glucose, insulin binding sites, plasma insulin, tetrahydrocurcumin, pterostilbene.

INTRODUCTION

Diabetes mellitus is characterized by hyperglycemia, altered metabolism of carbohydrates, lipids and proteins along with an increased risk of complications. The mortality rate in patients with diabetes may be up to 11 times higher than those without the disease. Herbal treatment for diabetes focuses on goals and strategies for the treatment and prevention of diabetes and on achieving metabolic outcomes related to glycemia without any side effects. Alternative therapeutic approaches using phytochemicals and medicinal plants with antidiabetic activity have been researched extensively all over the world. The limitations of currently available oral antidiabetic agents in terms of efficacy/safety coupled with emergence of the disease into a global epidemic have encouraged a

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concerted effort all over the world to discover drugs that can manage diabetes more effectively [1].

The presence of insulin receptors in human erythrocytes and changes in the binding of insulin to human erythrocytes under a variety of physiological and pathological conditions have been well documented [2]. Human erythrocytes contain specific insulin receptors, which have binding characteristics similar to classical insulin target cells. These receptors have been extensively used to evaluate *in vitro* sensitivity of human subjects [3, 4]. Insulin is a key anabolic hormone that controls energy metabolism in mammals. Through binding to the insulin receptor, insulin regulates blood glucose levels by promoting glucose uptake to skeletal muscle and fat and inhibiting hepatic glucose production [5]. The insulin receptor in the human lymphocyte and placenta is a disulfide-linked oligomer of molecular weight (MW) 350,000, which on disulfide reduction yields subunits of MW 130,000 and 95,000 plus a variable subunit of 45,000 [6, 7].

A number of studies have shown that human erythrocytes can be used as a cellular model for assessing the status of insulin receptors in diabetes [8, 9]. Human erythrocytes are readily accessible and are therefore often used for studying insulin receptor regulation in states of altered insulin action [10]. The nature of binding of insulin to mature erythrocytes is similar to that of the binding of insulin to other cells in terms of affinity, temperature dependence, pH optimum, specificity and negative co-operativity [8]. Under steady state conditions, there appears to be a good correlation of insulin receptors in erythrocytes with those in other tissues [10]. The affinity of erythrocyte insulin receptors does show variation that parallels that in monocytes and exhibits acute modulation *in vivo* in response to intravenous infusions of insulin or glucose [11].

Erythrocytes should therefore be useful for indicating changes in other tissues, in terms of changes in insulin receptor affinity and long-term changes in receptor concentration [12]. Ligand-receptor binding studies are widely used for receptor characterization and in high throughput drug screening. Receptor binding is used to characterize receptors and to evaluate potential pharmaceutical agents by assessing their ability to interfere with specific binding of a radiolabelled ligand to its receptors.

THC is one of the major colorless metabolite of curcumin. THC has been reported to exhibit the same physiological and pharmacological properties of curcumin [13]. Curcumin is rapidly metabolized during absorption from the intestine, yielding THC, which has shown the strongest antioxidant activity among all curcuminoids. Several studies in experimental animals indicated that THC also prevents cancer, protects against inflammation, atherosclerotic lesions, hepatotoxicity, nephrotoxicity and diabetes [1]. THC has been reported to produce significant antihyperglycemic effect [14]. Pterostilbene is a phenolic compound, which is found to be one of the major active constituents of *P. marsupium*. A short term (3 days) study suggests that pterostilbene in aqueous decoction of the heartwood of *P. marsupium* has the capability to reduce the plasma glucose in STZ-induced hyperglycemic rats [15]. An aqueous extract of heartwood of *P. marsupium* has been tested clinically and found to be effective in non-insulin dependent diabetes (NIDDM) patients. When administered to STZ-induced hyperglycemic rats, pterostilbene and marsupin, two of the major phenolic constituents in aqueous decoction of the heartwood of *P. marsupium*, significantly decreased plasma glucose [15]. In a previous study, the antidiabetic effect of pterostilbene and THC were compared in STZ-induced diabetic rats [16].

MATERIALS AND METHODS

Experimental design

In this experiment, rats were divided into 4 groups of 6 rats each. The effect of THC was compared with pterostilbene drug.

Group 1: Normal rats.

Group 2: Diabetic control rats.

Group 3: Diabetic rats given aqueous extract of THC (80 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 45 days [1].

Group 4: Diabetic rats given Pterostilbene (40 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 45 days [15].

No detectable irritation or restlessness was observed after each drug or vehicle administration. No noticeable adverse effect (i.e., respiratory distress, abnormal locomotion and catalepsy) was observed in any animals after the drug administration. At the end of 45 days, all the rats were killed by decapitation after inducing anaesthesia (Pentobarbitone sodium, (60 mg/kg)). Blood was collected in tubes containing potassium oxalate and sodium fluoride solution for the estimation of blood glucose, and plasma was separated for assay of insulin.

Animals

Studies were performed on adult male albino rats of Wistar strain weighing 180-220g. According to the experimental protocol approved by the Committee for Research and Animal Ethics of Annamalai University, animals were housed in cages and maintained at 24 ± 2 °C normal temperature and a 12 hour light/dark cycle. The animals were fed pellet diet (Lipton India Ltd., Mumbai) and water *ad libitum*.

Chemicals

THC and pterostilbene were a gift provided by Sabinsa Corporation, USA. Curcumin, STZ, insulin powder and pepstatin A were purchased from Sigma Chemical Co., (St Louis, MO), USA.

Experimental induction of type 2 diabetes

Streptozotocin (STZ) was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in normal physiological saline. Type 2 diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection of 65 mg/kg STZ, 15 min after the intraperitoneal administration of 110 mg/kg nicotinamide [17]. Hyperglycemia was confirmed by the elevated glucose levels in plasma, determined at 72 h. Animals with a glucose concentration of more than 250 mg/dl were used for the study.

Estimation of blood glucose

Blood glucose was estimated colorimetrically using commercial diagnostic kits (Sigma Diagnostics (I) Pvt. Ltd., Baroda, India) [18]. Glucose concentration was expressed as mg/dl.

Preparation of purified erythrocytes

The erythrocytes receptor assay was performed according to the method of Gambhir *et al*. with a modification [19]. The erythrocytes were separated using a percoll density gradient. Mononuclear leucocytes were separated from the erythrocytes by the use of pasteur pipettes. The erythrocytes were washed three times by centrifugation (4°C, 4500 rpm) in 10 ml of buffer G for 10 min. On each occasion, the supernatant was removed and the cells were re-suspended in buffer G and respun. After the final wash of the cells, the supernatant was removed and the cells were left in 4 ml of buffer G containing 1% human serum albumin. This suspension contained 4-6 x 10^9 cells/ml.

Binding of ^{125}I to erythrocytes cells

Erythrocytes (4.5x10^9 cells/ml) were incubated at 15 °C with ^{125}I-insulin (40 pg in 25 µl) with or without varying amounts of unlabelled insulin (0 to 0.5x10^5 ng) in a total volume of 0.5 ml. After 2.5 h of incubation, duplicate samples were placed in pre-chilled microfuge tubes along with the buffer and dibutylphthalate. Cell-bound and free insulin was separated by centrifugation at 7000 g at 4 °C for 10 min. The radioactivity in the cell pellet and supernatant was determined in a gamma counter (Electronics Corporation of India Limited, Hyderabad). The data was analyzed by Scatchard analysis [20]. The receptor affinity and receptor numbers were derived for the physiological range of insulin i.e. between 0.1 and 100 ng/ml. Non-specific binding is defined as the amount of radioactive insulin that remains bound in the presence of 10^5 ng/ml of unlabelled porcine insulin. All binding data were corrected for the non-specific binding to represent specific cell binding for purposes of comparison. Competitive binding curves were obtained for each erythrocyte suspension. From these curves, the insulin receptor affinity and number of the receptor sites were determined by the Scatchard analysis.

Cell binding analysis

The results of the binding studies are presented in

three ways: (1) the percentage binding of ^{125}I-insulin as a function of the total insulin concentration (competitive curve), (2) the bound-free insulin ratio plotted as a function of the bound insulin (Scatchard plot) and (3) the average affinity profile. The total binding capacity or concentration of the binding sites was derived from the point where the linear extrapolation of the curve intercepts the horizontal axis and this was used to calculate the number of receptor sites per cell [21].

Experimental data suggest that the insulin receptor consists of two binding sites that undergo negatively co-operative site-site interactions such that the affinity of the receptors for insulin is inversely related to the receptor occupancy.

Statistical analysis

All data were expressed as mean ± SD of number of experiments. The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS (SPSS, Cary, NC, USA) and the individual comparisons were obtained by Duncan's Multiple Range Test (DMRT) [22]. A value of $p<0.05$ was considered to indicate a significant difference between groups. Values sharing a common superscript do not differ significantly with each other at $p<0.05$. The data on insulin binding studies were analyzed by competition curve, Scatchard plot [23] and average affinity profiles. All values are expressed as mean ± SD.

RESULTS

Blood glucose and plasma insulin

Figures 1 and 2 show the level of blood glucose and plasma insulin in normal and experimental rats. The level of blood glucose significantly increased whereas the activity of plasma insulin significantly decreased in diabetic control rats. Oral administration of THC and pterostilbene to diabetic rats significantly decreased the blood glucose with significant increase in plasma insulin activity.

Competitive binding curves on the binding of insulin to its receptor on erythrocytes

Figure 3 summarizes the ability of non-radioactive insulin to competitively inhibit the binding of ^{125}I-insulin to the insulin receptor on the cell membranes of erythrocytes in rats treated with STZ, THC and pterostilbene. Comparison of the plots showed that the insulin receptor on the cell membranes of erythrocytes from rats treated with diabetic rats significantly binds to more 125-I-insulin than cells from the THC and pterostilbene

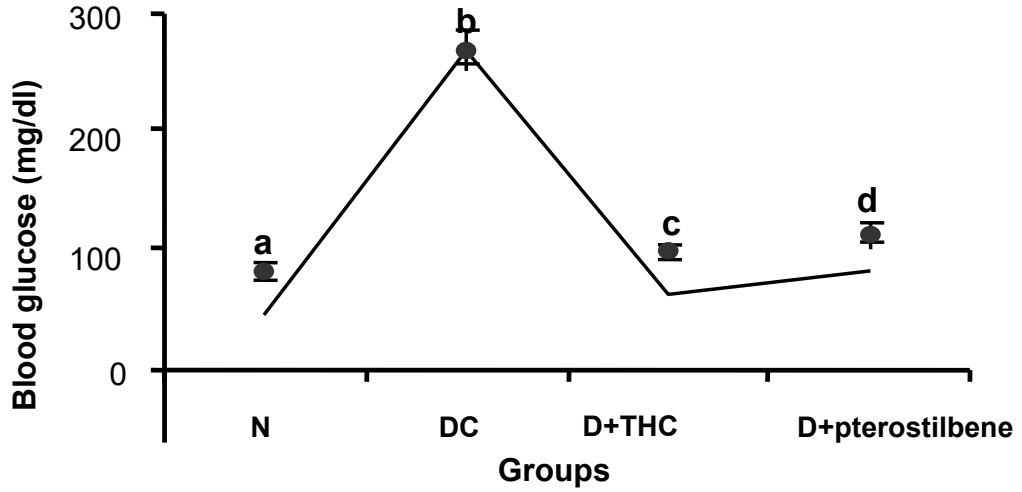

Figure 1. Effect of THC and pterostilbene on blood glucose of normal and experimental rats. Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). Duncan's procedure; Range for the level 2.95, 3.09, 3.20.

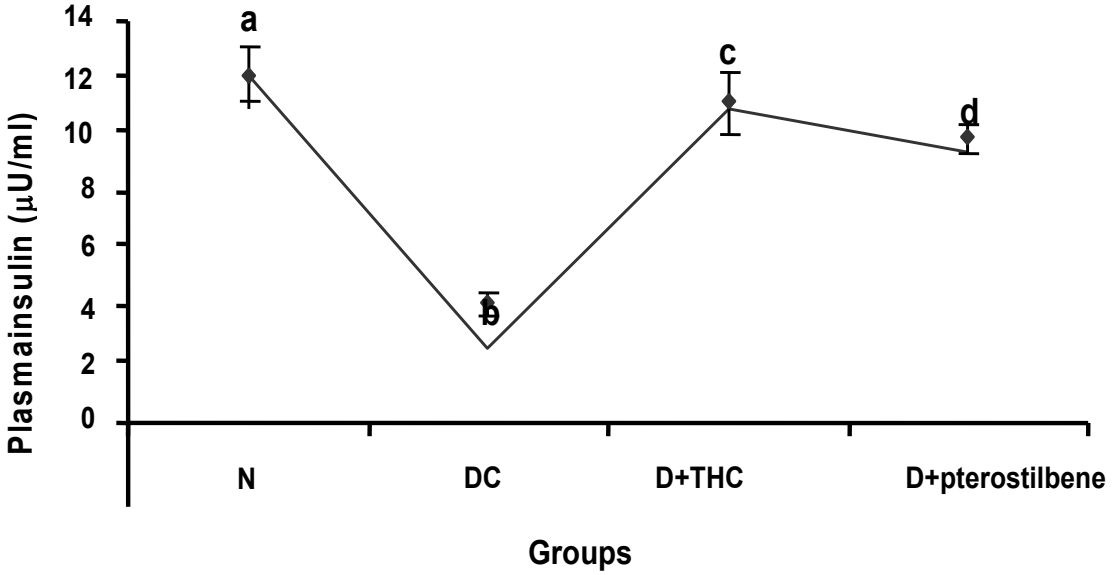

Figure 2. Effect of THC and pterostilbene on plasma insulin in normal and experimental rats. Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). Duncan's procedure; Range for the level 2.95, 3.09, 3.20.

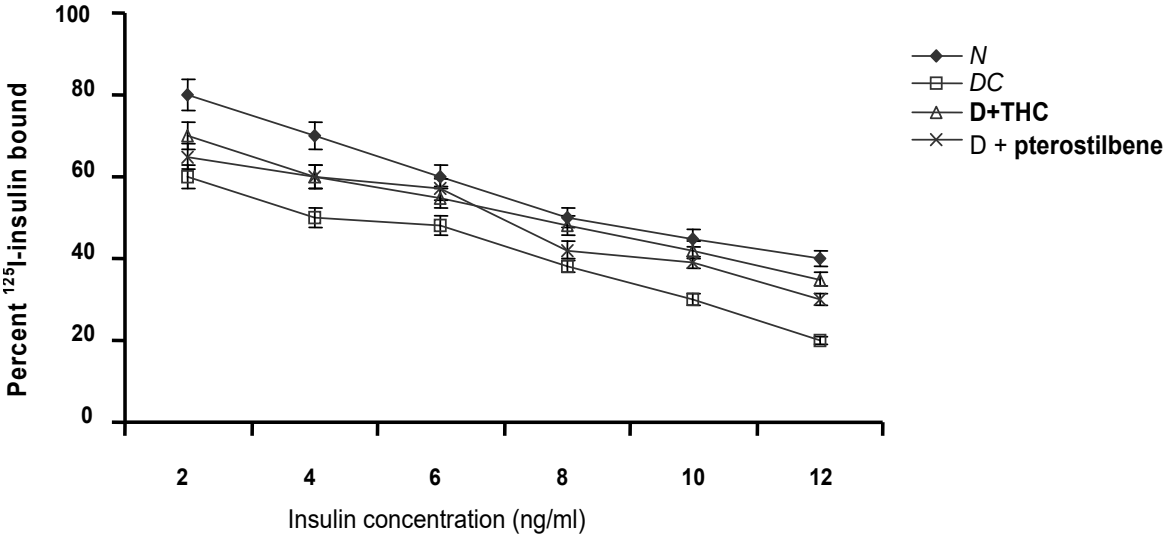

Figure 3. Competitive binding curves showing the effect of THC and pterostilbene on the binding of insulin to its receptor on erythrocytes of normal and experimental rats. Percentage of ^{125}I-insulin bound is plotted as a function of the unlabelled insulin concentration.

treated diabetic rats at unlabelled insulin concentrations. The percentage of ^{125}I-insulin bound to the insulin receptor on the cell membrane of erythrocytes of diabetic control rats (55.0 ± 2.8%) was significantly lower than the percentage of ^{125}I-insulin bound to those of THC (70.0 ± 3.5%) and pterostilbene (65.0 ± 3.3%)-treated diabetic rats at very low unlabelled insulin concentrations (0 and 1 ng/ml). Comparison of the competitive curves of percentage of ^{125}I-insulin bound to the insulin receptor on erythrocytes of the diabetic control and THC-treated diabetic rats showed

slopes that decreased steadily at values of 33.0 ± 1.1% and 42.0 ± 2.1%, respectively at an unlabelled insulin concentration of 10 ng/ml.

Bound/Free ratio

In this study, bound/free (B/F) ratio of the labelled hormone is expressed as a function of the bound hormone giving a scatchard plot for erythrocytes (Figure 4). Curvilinear plots were obtained for the diabetic control, THC and pterostilbene rats. A greater B/F ratio implies that there is more bound hormone than free. Comparison of the plots showed that insulin receptor on the cell membranes of erythrocytes for THC and pterostilbene-treated diabetic rats had maximum B/F values of 22.0 ± 1.0% and 19.0 ± 0.9%, respectively compared with 15.0 ± 0.85% of diabetic control rats.

Scatchard analysis of affinity profile and receptor binding sites in erythrocytes

To analyze the affinity binding sites more precisely, the K_d, K_e and K_f were calculated. 'Dissociation constant of high affinity binding sites' (K_{d1}) significantly decreased in diabetic control rats ($1.0 \pm 0.08 \times 10^{-10}$ M^{-1}) and increased in diabetic rats treated with THC ($2.5 \pm 0.15 \times 10^{-10}$ M^{-1}) and pterostilbene ($2.0 \pm 0.10 \times 10^{-10}$ M^{-1}) (Figure 5A). Further 'dissociation constant of low binding sites' (K_{d2}) analysis (Figure 5B) showed significantly decreased affinity in diabetic control rats ($2.7 \pm 0.25 \times 10^{-8}$ M^{-1}) and significantly increased affinity in rats treated with THC ($17.0 \pm 1.0 \times 10^{-8}$ M^{-1}) and pterostilbene ($12.3 \pm 0.9 \times 10^{-8}$ M^{-1}). Similarly, analysis of the data shows that erythrocytes of rats treated with THC have an 'empty site' affinity (K_e) of $8.6 \pm 0.5 \times 10^{-8}$ M^{-1}, which begins to decrease with increasing occupancy of the receptor sites by ^{125}I-insulin, and the average receptor affinity progressively decreased to the 'filled site affinity' (K_f) of $2.7 \pm 0.15 \times 10^{-7}$ M^{-1} (Figure 5C). The comparable values of K_e and K_f for erythrocytes of rats administered with pterostilbene were $7.4 \pm 0.3 \times 10^{-8}$ M^{-1} and $2.0 \pm 0.15 \times 10^{-7}$ M^{-1}. The comparable values of K_e and K_f for erythrocytes of diabetic control rats were $6.0 \pm 0.4 \times 10^{-8}$ M^{-1} and $1.0 \pm 0.05 \times 10^{-7}$ M^{-1}, respectively. 'Receptor numbers' (B_{m1}) for K_{d1} was maximum in rats treated with THC (36.4 ± 3.0 f mol/mg) and pterostilbene (27.1 ± 1.8 f mol/mg) when compared with diabetic control rats (10.6 ± 0.5 f mol/mg). 'Receptor numbers' (B_{m2}) for K_{d2} also decreased in diabetic control rats (62.0 ± 4.0 f mol/mg) whereas THC (108.0 ± 8.0 f mol/mg) and pterostilbene (103.0 ± 8.0 f mol/mg) administration significantly increased the B_{m2} (Figure 6).

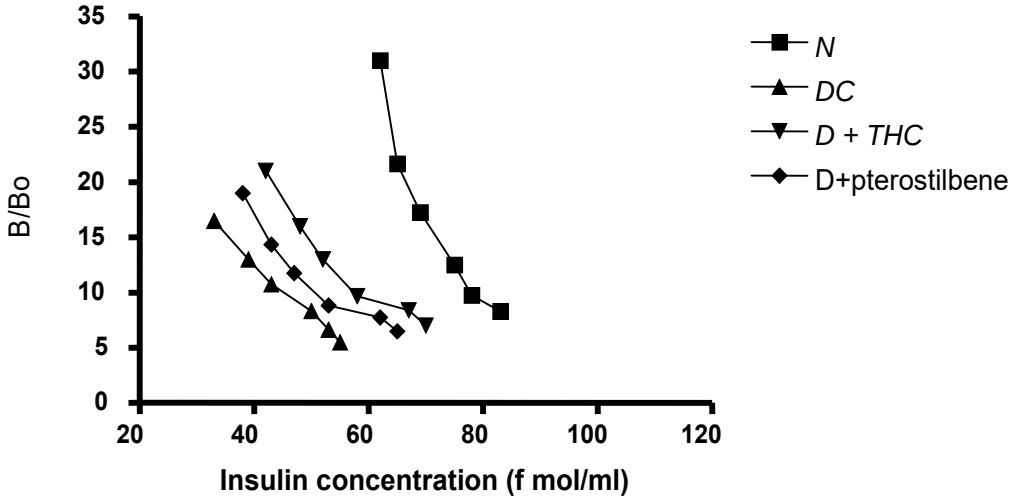

Figure 4. Scatchard plot showing the effect of THC and pterostilbene on the binding of insulin to its receptor on erythrocytes of normal and experimental rats. Bound/Free ratio is plotted as a function of the insulin bound (B).

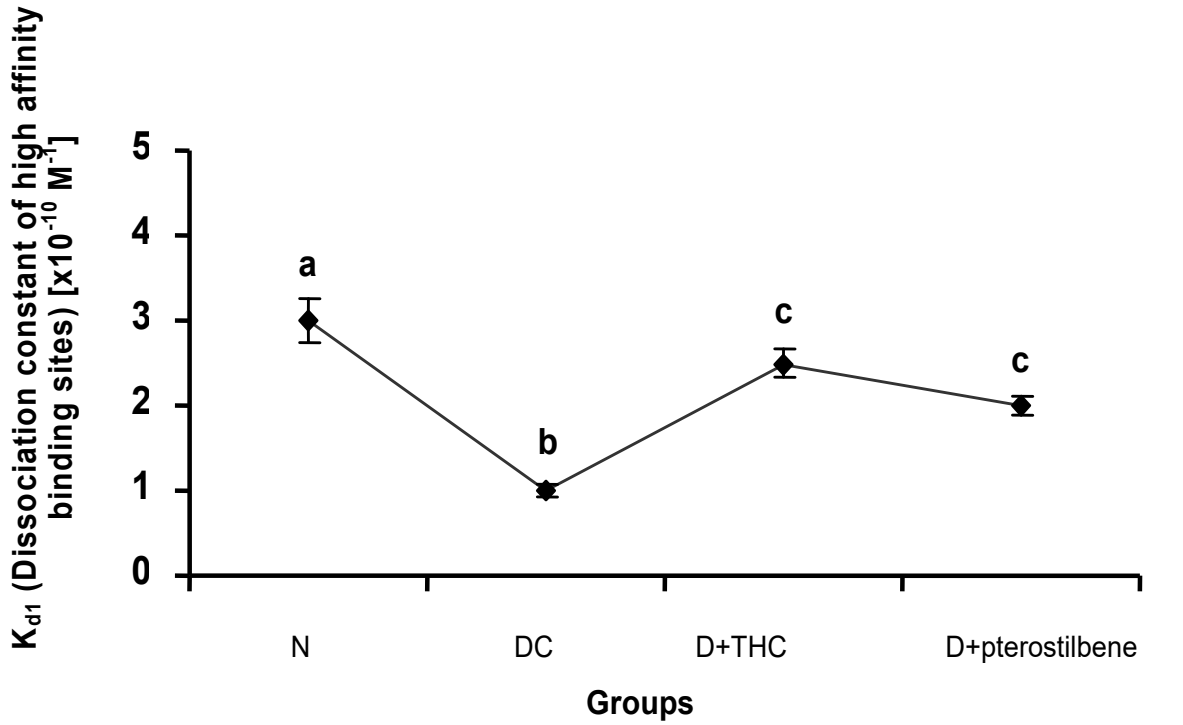

Figure 5A. Effect of THC and pterostilbene on K_{d1} of high affinity binding sites' profile in erythrocytes of normal and experimental rats. Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). Duncan's procedure; Range for the level 2.95, 3.09, 3.20.

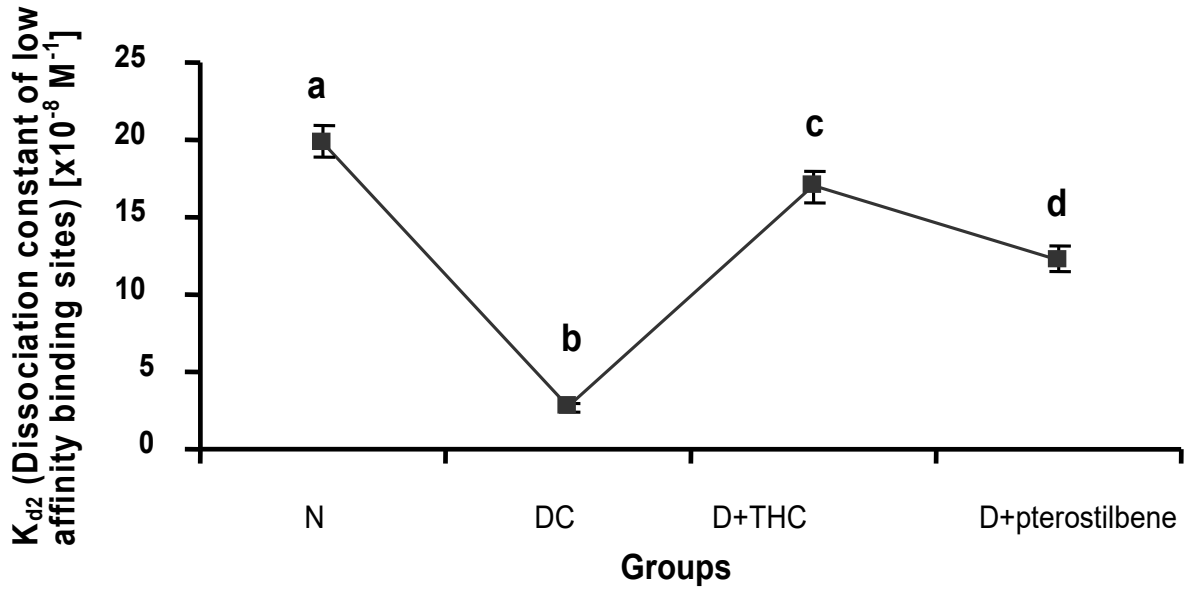

Figure 5B. Effect of THC and pterostilbene on Kd2 of low affinity binding sites' profile in erythrocytes of normal and experimental rats. Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). Duncan's procedure; Range for the level 2.95, 3.09, 3.20.

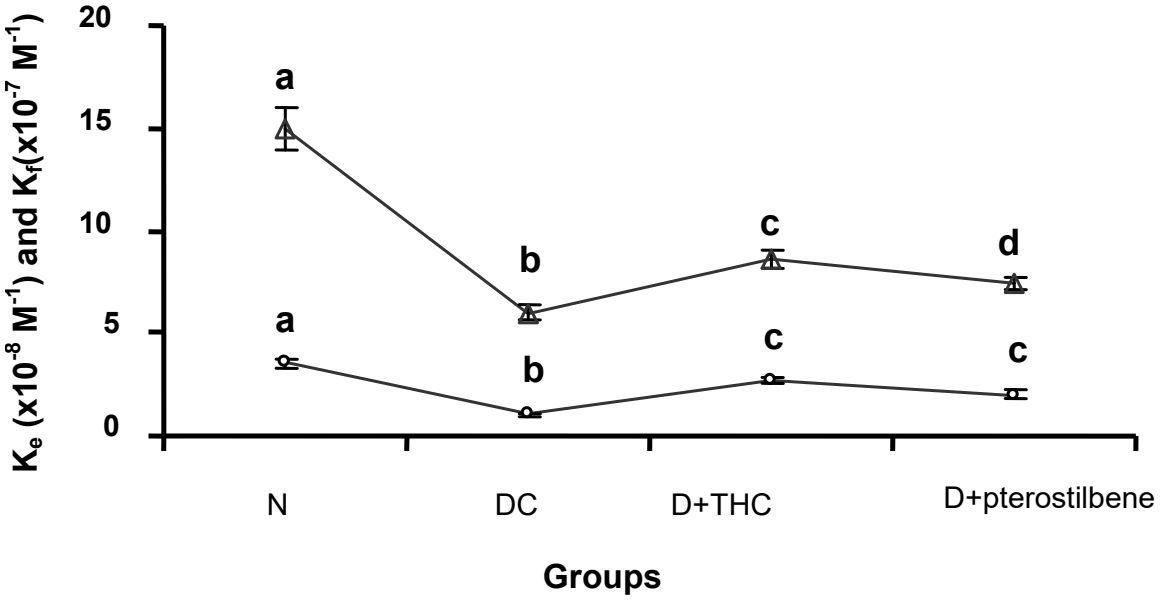

Figure 5C. Effect of THC and pterostilbene on K_e and K_f – Affinity constant for empty receptors (△) and Affinity constant for filled receptors (○) in erythrocytes of normal and experimental rats. Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). Duncan's procedure; Range for the level 2.95, 3.09, 3.20.

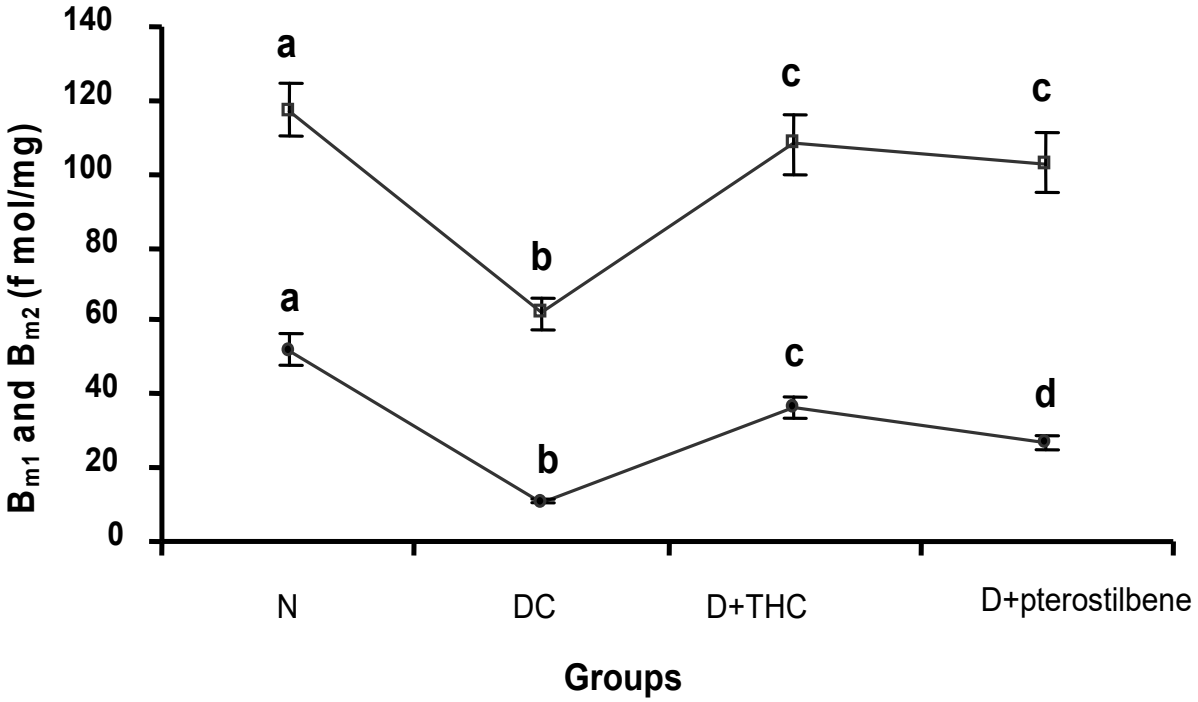

Figure 6. Effect of THC and pterostilbene on B_{m1} and B_{m2} - Number of high (●) and low (□) affinity binding sites in erythrocytes of normal and experimental rats. Values are given as mean ± SD from 6 rats in each group. Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT). Duncan's procedure; Range for the level 2.95, 3.09, 3.20.

DISCUSSION

Diabetes mellitus is a metabolic disorder of heterogeneous aetiology [24]. Insulin binding to receptors is the first event in insulin action, and this first step represents a major control point for insulin effects *in vivo*. Insulin binding to receptors is not a fixed biologic process but is subjected to modulation by alterations in either receptor or affinity. Insulin receptors have been demonstrated in cells of a large variety of tissues from different animal species. It has been shown that binding of insulin receptors in different tissues is heterogeneous, which may be due to a multiple class of receptors, to negative co-operation between receptors or combination of both [25]. Many studies have shown decreased insulin binding in diabetes mellitus [26].

STZ caused significant reduction in the receptors of erythrocytes and insulin target tissues. In the present investigation, treatment with THC and pterostilbene showed significant antihyperglycemic activity. This is probably indicative of the efficacy of the plant. In addition the present study showed that STZ causes decrease in the number of insulin binding sites and administration of THC and pterostilbene increases the number of binding sites.

Using erythrocytes as the study tool, it was shown that in STZ-induced diabetic rats there is a decreased specific binding of insulin to erythrocyte insulin receptors. This appears to be mainly due to a significant decrease in the receptor concentration per cell and also due to a marginal decrease in the affinity of the receptor. As insulin levels decrease in diabetic control rats, insulin binding to receptors also decreases [27, 28]. It has been reported that phasic insulin-dependent diabetes (Malnutrition related diabetes mellitus) is characterized by decreased red and white cellular binding to insulin in addition to decreased production of insulin [29].

The data in Figure 3 clearly demonstrate that insulin binding is decreased at lower non-radioactive insulin concentration, but the curves converge at the higher non-radioactive insulin levels. This pattern suggests a change in receptor affinity. The decrease in insulin binding attributed by a decrease in receptor affinity was confirmed by the decrease in the average affinity (K) for erythrocytes.

To investigate whether the decrease in insulin binding was attributed to the decrease in the number of receptor sites per cell, the Scatchard plot for the data was analyzed. In this method of analysis, the X-intercept represents the number of insulin receptor sites per cell. Calculations revealed that there was a decrease in the number of receptor sites per erythrocyte in diabetic control rats compared with THC and pterostilbene-treated rats. Therefore, the decrease in insulin binding due to the acute effects of STZ is primarily as a result of the decrease in the number of receptor sites per cell.

The co-operative interactions among receptor sites can be explained in terms of “negative co-operativity” model. Negative co-operativity is a frequent occurrence in hormone-receptor systems in which there are site-site interactions, resulting in a decrease in the apparent affinity of receptor for insulin when fractional saturation of the receptor increases. According to this model and calculation of the number of receptor sites using Scatchard analysis, the decreased insulin binding observed is primarily due to the decrease in the number of receptor sites. It can be suggested that the lower number of receptor sites per cell in rats treated with STZ–nicotinamide, could be the result of primary alteration in the receptor or was secondary to some other alterations in the integrity of the membrane (generation of free radicals) [30]. STZ may damage erythrocytes in the short term with direct effects on membrane structure, membrane fluidity, cross-linking and function. The molecular nature and site-site interactions of the receptor sites may include mechanisms such as intra-molecular changes in tertiary or quaternary structure of the receptor, association and dissociation of the receptor molecules, clustering of receptors in the membrane or phase transitions in the membrane itself. These changes in membrane integrity could be responsible for the decreased number of receptor sites per cell. The resulting membrane dysfunction can impair transport of glucose across the cell membrane resulting in the observed hyperglycemia.

The contributing effect of any alteration in receptor affinity was evaluated by average affinity. There was a significant decrease in the empty site affinity for erythrocytes supporting the concept

that there can be alterations in both receptor affinity and receptor sites, both contributing to the decreased insulin binding. Some of the insulin receptor on the cell membranes of erythrocytes from rats treated with STZ may be affected by the cytotoxic effect and may become desensitized.

Desensitization is associated with a total lack of insulin effect despite remaining insulin receptors. Several possibilities exist to explain the mechanism of changing receptor affinity and desensitization. Firstly, fluidity may be an important factor in the modulation of insulin binding and action. Secondly, the insulin receptor may be covalently associated with another protein that modulates receptor affinity. It is therefore a possibility that free radicals released from STZ may alter the interaction of insulin with its receptors, thus affecting the ability of insulin to differentially regulate its receptor and this regulator protein [31]. Free radicals especially NO generated from STZ may also affect the formation of the insulin receptor complex. A third possibility is that the receptor undergoes some form of post-translational modification that alters binding and signal transmission properties [32]. The post-translational modification could involve a change in the redox state of the receptor. The insulin receptor is composed of major sub-units linked by disulfide bonds to various oligomeric forms. Reduction of the oxidized forms of the receptors could modify the affinity of insulin [33].

The advantage of using circulating blood cells for investigating the receptor status in humans and animals is that they are more easily accessible than cells of primary insulin target organs, such as adipocytes and muscles [34, 35]. STZ-induced change in this binding may be associated with specific change in the receptor. The present study suggests that THC and pterostilbene potentiate insulin secretion and increase the binding of insulin to the insulin receptors. Oral hypoglycemic agents especially sulphonylurea compounds have been reported to improve insulin receptor status [29, 36, 37]. In the present study, administration of THC and pterostilbene increased the plasma insulin levels with improvement in insulin binding to isolated erythrocyte insulin receptors. The factors responsible for such a rapid improvement in insulin action *in vivo* may be multiple. Therefore erythrocytes represent a more uniform population of cells capable of same receptor-mediated function as adipocytes, providing a clearer reflection of the insulin receptor status in target tissues.

CONCLUSION

The study results suggest that acute alteration of the insulin receptor on the membranes of erythrocytes occurred in STZ diabetic rats. Treatment with pterostilbene and THC significantly improved specific insulin binding to receptors, with receptor number and affinity binding reaching to near normal levels. The effect of THC is more prominent than that of pterostilbene.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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