Molecular and Biochemical Effect of Neem Extract On Experimental Diabetes

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Abstract: Return to nature is now a days target of view so the aim of this study to evaluate aqueous extract effects of Neem leaf on streptozotocin (STZ) experimental diabetic rats. Sixty male rats were divided equally into 4 groups, first group used as healthy normal control, second group injected with STZ (50 mg/kg.B.W) and act as control diabetic, third group injected I/P with STZ for induction of diabetes and treated with Neem leaf extract (200 mg/kg.B.W orally / day) for 30 days and the fourth group administrated with Neem leaf extract for 15 days before induction of diabetes, then during and after injection of STZ for 30 days Blood samples were collected at 15 & 30 days from onset of treatment. Serum was directed for estimation of glucose, lipid profile, insulin hormone, insulin receptor, HMG-coA reductase, cortisol and total IgE. Hepatic tissue subjected to PCR for stimulation insulin receptors. The recorded results revealed significant increase in glucose, cholesterol, TG, insulin and HMGcoA reductase in diabetic rats when compared to control. The treatment with Neem extract resulted in significant decrease in all these parameters whereas serum cortisol has no significant change in all groups, while HDL showed no change in diabetic. In conclusion Neem act as potent antidiabetic, antihyperlipidemic and has a good molecular activity on insulin receptor gene expression by PCR

Key words: diabetes, Neem, hormone, lipid profile and PCR for insulin receptors

I. Introduction

Medicinal plant plays an important role in management of diabetes especially in developing countries. The WHO has defined herbal medicine as finished labeled medicinal products that contain active ingredients as aerial or underground parts of plants or other plant material (Romila et al., 2010). Many minor components of foods such as secondary plant metabolites have been shown to alter biological processes which may reduce the risk of chronic disease in human (Chattopadhyay and Bondyopadhyay, 2005).

Neem, belongs to family Meliaceae. Which is one of the most useful medicinal plants (Kausik and Ranajet 2002). The blood sugar level lowering activity of Neem oil and leaf extracts has been reported in various models of diabetic animal (Mostofa et al., 2007).

Different parts of Neem have been reported to have antiseptic, wound healing and skin disease curing activity (Biswa et al., 2002). Studies reveal that, leaves water extract of Neem possess significant antinflammatory, antiserotonin, antifertility and hepatoprotective activities (Chattopadhyay et al., 1993).

Diabetes mellitus is a metabolic disorder in the endocrine system. This dreadful disease is found in all parts of the world and becoming a serious threat to man kind health. There are lots of chemical agents available to control and treat diabetic patient, but nowadays several medicinal plants have been investigated for their beneficial use in diabetes. The effect of these plants may delay the development of diabetic complications and correct the metabolic abnormalities. Many phyto constituents responsible for antidiabetic effects have been isolated from hypoglycemic plants (Ultar et al., 2011). Therefore, the aim of this research is to measure the beneficial effects of using Neem leaves decoction as a natural herb on some blood parameters and insulin receptors on experimentally-diabetic rats.

II. Materials and methods

Sixty male albino rats 12-16 weeks old weighted 150 – 200 gm were used in these experiments. Rats were kept at constant environmental and nutritional conditions throughout the experimental period. Water and feed were supplied ad-libitum.

Induction of diabetes:

Diabetes was induced by I/P injection of STZ at a dose of 50 mg/kg/b.w which dissolved in citrate buffer at pH 4.2 according to Ramanthan et al., (1999). A rise in blood glucose level was determined after 48 hr from injection of STZ.

Preparation of Neem leaves extract:

Freshly prepared Neem leaves at a dose (200 mg/kg/b.w) extract were obtained by decoction.

Animal grouping:

Animals were divided randomly into 4 groups (15 each). Animals placed in a separate cage.
Control group: injected only with citrate buffer.
Neem treated group: diabetic rats treated with Neem leaves extract at a dose of 200 mg/kg/b.w/day orally for 4 weeks (Halim, 2003).
Protective group: rats administrated Neem leaves extract before induction of diabetes by 2 weeks as well as during and post induction of diabetes /4 weeks.

Sampling:
Blood samples were collected from all animals twice after 2 and 4 weeks from induction of diabetes. While the protected group collected another sample just before induction of diabetes. Samples were collected after overnight fasting from medial canthus of the eyes. Blood samples were centrifuged at 3000 r.p.m for serum separation which collected in clean tubes and directed for estimation of glucose (Trinder, 1969), total cholesterol (Frings and Dunn., 1970) triacylglycerol (Bucolo and David., 1973), high density lipoprotein (HDL) (Finley et al., 1978), and hydroxymethyleneptutryle CoA reductase (HMG-CoA) (Cighetti et al., 1988). Liver tissue were collected after 4 weeks and kept directly at -80°C to to be used for insulin receptor expression evaluation (Schmittgen and Livak., 2008).

ELISA technique
ELISA was used for estimation of cortisol (Burtis 1994), IgE (Ishizaka, 1982), insulin hormone (Frosig 2007) and Insulin receptors (Cloud, 2013)

PCR
Total cellular RNA was extracted from tissue using a RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to manufacturer’s protocol. Real-time PCR was done using QuantiTect SYBR® Green RT-PCR kit (Qiagen, Valencia, CA, USA) according to the manufacture’s protocol.
The real-time PCR cycling program consisted of reverse transcription at 48°C for 30 min, initial PCR activation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, and the dissociation curve was added to the protocol wherever necessary. The real-time PCR assay was done using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA).
The amount of change in gene expression was calculated from the obtained cycle threshold (CT) values provided from real-time PCR instrumentation using the 2-∆∆CT calculation, where ∆CT indicates the CT changes in target gene in comparison with the reference (house-keeping) gene (Livak and Schmittgen., 2001 and Schmittgen and Livak., 2008).

III. Statistical analysis
The obtained data were statistically analyzed and the significant difference between groups was evaluated by analysis of variance (ANOVA) using SPSS (Statistical Package for Social Science, 1999; ver.10). The significance among the samples was compared at ≤ 0.05, and T- test was applied according to (Snedecor and Cochran., 1982)

IV. Results
Table (1): Effect of Neem leaves extract on serum level of glucose (mg/dl), total cholesterol (mg/dl), triacylglycerol (mg/dl), HDL-c (mg/dl) and HMGCoA reductase (pg/ml) on rats before induction of diabetes by 15 days.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Glucose</th>
<th>Cholesterol</th>
<th>TG</th>
<th>HDL</th>
<th>HMGCoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.7±2.7</td>
<td>80.6±3.4</td>
<td>41.8±6.3</td>
<td>37.1±6.5</td>
<td>223.8±25.3</td>
</tr>
<tr>
<td>Protective</td>
<td>73.0±2.6**</td>
<td>81.4±8.8</td>
<td>68.2±4.4**</td>
<td>36.1±2.0</td>
<td>175.8±16.6</td>
</tr>
</tbody>
</table>

Mean value± standard error.
Superscript with different letters in the same column showed significant difference while same letters showing non significant difference

Table (2): Effect of Neem leaves extract on serum level of cortisol (ng/ml), IgE (ng/ml), insulin (uIU/ml), and insulin receptors (ng/ml) on rats before induction of diabetes by 15 days.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Cortisol</th>
<th>IgE</th>
<th>Insulin</th>
<th>Insulin receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.5±0.33</td>
<td>17.3±1.1</td>
<td>12.4±0.8</td>
<td>1.95±0.45</td>
</tr>
<tr>
<td>Protective</td>
<td>1.7±0.14</td>
<td>6.7±0.18**</td>
<td>12.5±1.2</td>
<td>1.5±0.23</td>
</tr>
</tbody>
</table>

Mean value± standard error.
Biochemical Effect of Neem Extract On Experimental Diabetes Gene Expression

Superscript with different letters in the same column showed significant difference while same letters showing non significant difference

Tables (1 and 2) revealed that, rats administrated Neem leaves extract for 15 days before induction of diabetes (protective group) showed a significant decrease in serum glucose and IgE level when compared with control rats, while TG level was significantly increased in protected group. Other parameters showed non-significant changes when compared with control.

Table (3): Effect of Neem leaves extract on serum level of glucose(mg/dl), total cholesterol(mg/dl), triacylglycerol (mg/dl), HDL-c (mg/dl) and HMGCOA reductase (pg/ml).on STZ induced diabetes in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Glucose</th>
<th>cholesterol</th>
<th>TG</th>
<th>HDL-c</th>
<th>HMGCOA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.7± 2.66*</td>
<td>80.63 ± 1.9*</td>
<td>41.83± 6.30*</td>
<td>37.1± 6.54*</td>
<td>223.8 ± 15.44*</td>
</tr>
<tr>
<td>Control diabetic</td>
<td>245.5± 16.18*</td>
<td>121.4 ± 4.19*</td>
<td>85.17± 5.57*</td>
<td>69.4 ± 1.93*</td>
<td>386.5 ± 11.39*</td>
</tr>
<tr>
<td>Neem treated</td>
<td>129.9 ± 2.15*</td>
<td>112.2± 6.32**</td>
<td>50.3 ± 3.33**</td>
<td>41.07± 3.12**</td>
<td>150.4 ± 3.44*</td>
</tr>
<tr>
<td>protective</td>
<td>96.6 ± 7.07*</td>
<td>101.1 ± 5.55*</td>
<td>55.6 ± 2.23*</td>
<td>55.7 ± 2.9**</td>
<td>235.7 ± 3.15*</td>
</tr>
</tbody>
</table>

Table (4): Effect of Neem leaves extract on serum level of cortisol (ng/ml), IgE (ng/ml), insulin (uIU/ml), and insulin receptors (ng/ml) on STZ induced diabetes in rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Cortisol</th>
<th>IgE</th>
<th>Insulin</th>
<th>Insulin receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.8± 0.3*</td>
<td>7.33 ± 1.1*</td>
<td>12.37± 0.84*</td>
<td>1.9± 0.43**</td>
</tr>
<tr>
<td>Control diabetic</td>
<td>2.1± 0.06*</td>
<td>7.6± 1.08*</td>
<td>22.53± 2.54*</td>
<td>2.8 ± 0.41*</td>
</tr>
<tr>
<td>Neem treated</td>
<td>1.07 ± 0.7*</td>
<td>6.6± 1.30*</td>
<td>13.77± 0.89*</td>
<td>1.77± 0.05*</td>
</tr>
<tr>
<td>Protective</td>
<td>1.4 ± 0.07*</td>
<td>6.9± 0.43*</td>
<td>9.0 ± 1.2</td>
<td>1.6± 0.15**</td>
</tr>
</tbody>
</table>

Table (5): Effect of Neem leaves extract on liver insulin receptor by RT. PCR.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>Insulin receptors(RT. PCR)</th>
</tr>
</thead>
</table>

Mean value± standard error. Superscript with different letters in the same column showed significant difference while same letters showing non significant difference

Table (3) revealed significant increase in serum total IgE level after 2 weeks. While rats administrated Neem leaves extract for 15 days before induction of diabetes (protective group) showed a significant decrease in serum total IgE level when compared with control rats, while TG level was significantly increased in protected group. Other parameters showed non-significant changes when compared with control.

Table (4) diabetic rats showed non-significant increase of serum cortisol concentration when compared with control. Neem treatment to diabetic rats showed non-significant decrease all over the period of experiment. Moreover, diabetic and treated rats revealed non significant increase in serum total IgE level after 2 weeks. While after 4 weeks IgE level was significantly increased in diabetic rats. Administration of Neem leaves extract to diabetic rats resulted in a significant decrease in serum total IgE.

Diabetic rats revealed a significant increase in serum insulin level after 2 and 4 weeks. Administration of Neem leaves extract revealed a significant decrease when compared to diabetic rats and return to normal level when compared to control group.

Diabetic rats showed non significant increase in serum insulin receptors when compared with control. While rats administrated with Neem revealed a significant decrease after 2 weeks when compared with diabetic rats.
Biochemical Effect of Neem Extract On Experimental Diabetes Gene Expression

<table>
<thead>
<tr>
<th></th>
<th>Mean value± standard error.</th>
<th>Superscript with different letters in the same column showed significant difference while same letters showing non significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1±0.03</td>
<td></td>
</tr>
<tr>
<td>Control diabetic</td>
<td>0.04± 0.00³</td>
<td></td>
</tr>
<tr>
<td>Neem group</td>
<td>0.09±0.00</td>
<td></td>
</tr>
<tr>
<td>Protective group</td>
<td>0.08±0.00</td>
<td></td>
</tr>
</tbody>
</table>

Table (5): Similarly, expression of hepatic insulin receptor showed down-regulation in all groups when compared with control as detected by real-time PCR. Administration of Neem leaves as protected or treated groups reveal an initiation of gene expression when compared with diabetic control.

V. Discussion
Diabetes is a metabolic disease in which a person has high blood glucose either because the body does not produce enough insulin or the body cells do not respond to insulin that is produced (Kumar et al., 2011). Experimentally induced diabetic rats showed significant increase in serum glucose level which may be attributed to the body tissue don’t respond very well to insulin signal and therefore don’t metabolize the glucose in diabetic rats (Richard., 2007). Furthermore, Rosa et al., (2011) attributed the increase of blood glucose in STZ diabetic rats to the nitrosourea derivatizes that has diabetogenic activity due to its ability to induce oxidative stress and damage to β-cells by producing free radicals of oxygen, nitrogen mono oxide and reducing intracellular NAD and NADP.

Pretreatment with Neem leaf prevented the rise in blood levels as compared to control. These indicated that Neem could be beneficial in diabetes mellitus as controlling blood sugar or may also be helpful in delaying the onset of the diabetes due to its pharmacological activity. (Khosla et al., 2000).

Administration of Neem leaves extract to diabetic rats exhibit a significant decrease in serum level after 2 and 4 weeks these results came in accordance with (Boby et al., 2003) that proved Neem extract increase the peripheral utilization of glucose and decrease in insulin resistance. A possible mechanism is to inhibit action of epinephrine on glucose metabolism resulting in increase utilization of peripheral glucose (Chattopadhyay, 1996). Aqueous extract of Neem leaves significantly decrease blood sugar level and prevent adrenalin as well as glucose – induced hyperglycemia. When aqueous leaf extract fed orally it produce hypoglycemia in normal rats and blood glucose level in experimentally STZ induced diabetes in rats. These results are similar to the results of this study. (Rao et al., 2012).

The recorded data revealed a significant increase in serum level of total cholesterol, triacylglycerol, and HDL-c in diabetic rats these could be attributed to the link between diabetic dyslipidemia and insulin resistance which is the precursor of type 2 diabetes and these factors raises risk of heart attack and stroke (Richard, 2007). However, insulin activates enzyme lipoprotein lipase which hydrolyses triacylglycerol under normal condition. Destruction of beta cells lead to depletion of plasma insulin, which resulted in hyperlipidemia and hypercholesteremia caused derangement of metabolic abnormalities (Rasineni et al., 2010). Moreover, the elevated levels might be due to the stimulation of hepatic triacylglyceride synthesis as a result of free fatty acid influx (Maghrani et al., 2004). A significant decrease in serum level of cholesterol, TG and HDL-c agreed with Kumar et al., (2010) who reported that, repeated administration of Neem has beneficial effect on hyperlipidemia associated with hypoglycemia. Furthermore, Chattopadhyay and Bandyopadhyay., (2005) demonstrated that, treatment with Neem leaves extract to diabetic rats revealed a significant decrease in total cholesterol and TG while return HDL to normal level these may be explained that, the chemical analysis of Neem leaf extract contain compounds including Quercetin -3-O-B-D-glucoside, Myricetin-3-rutinoside, Quercetin-3-o-rutinoside, Kaempferol-3-o-rutinoside, Kaempferol-3-O-B-D-glucoside, Quercetin-3-O-L-rhamnoside either wholly or partially may be responsible for antihyperlipidemic activity. In addition, it contains Nimbidin which significantly reduce serum cholesterol that helps to reduce blood clots. It also causes dilatation blood vessels and may be responsible for helping as hypotensive and improve blood circulation (Host. 2012).

Purohit, (1999) recorded that; Neem leaf extract feed to cholesterol fed rabbit resulted in reduction in serum total cholesterol, and HDL-c this indicated that Neem has antiatherogenic or hypolipidemic microconsituent. In this study the significant increase in HMGCoA reductase in diabetic rats agreed with (Magni et al.,1992) who exhibited that, the percentage of the active thiolic dephosphorylated form was higher in these diabetic animals than control suggesting a response of the liver to the decrease in both total and specific activity of HMGCoA reductase.

On other hand, rats administrated Neem leaf extract (protective group) showing significant decrease in serum HMGCoA reductase activity. These results are in harmony with Bopanna et al., (1997) who reported a significant decrease in the concentration of serum lipid, glucose and enzymes activity of ALP, LDH, G6P and HMGCoA reductase due to antidiabetic and antihyperlipidemic effect of Neem.
This study investigated that, serum cortisol level showed non significant change in diabetic rat and treated group. These results agreed with Gholap and Kar (2004) suggested that, Neem could exhibit hypoglycemic activity without altering the serum cortisol concentration. This may be due to plant extracts may potentially regulate corticosteroid induced diabetes mellitus. Some chronic diseases as diabetes and cancer are associated with production of free radicals and advanced glycation end product (Jane, 2006).

A significant increase of serum insulin and non significant increase in insulin receptors in diabetic rats may be due to that, pancreas can still produce the insulin hormone eventually insulin production dwindles in type 2 diabetes (Ian, 2008). Furthermore, Colen (2013) recorded a new hormone called Betatrophin that will cause mice to produce insulin – secreting by beta cells up to 30 times than normal rate. The new beta cells only secret insulin which offering the potential for the natural regulation of insulin and great reduction in the complication associated with diabetes.

Krishnapuran et al., (2013) recorded that, insulin-resistant states in obesity or diabetes are often associated with a decrease in the binding of insulin to insulin receptors, and/or a decrease in insulin-stimulated activation of IRS, which may diminish the effectiveness of anti-diabetic agents dependent on this signaling. Administration of Neem leaf extract demonstrated a significant decrease in serum insulin level and non significant decrease in serum insulin receptors. These results agreed with Joseph, (1999) who reported that, drinking a simple cup of tea out of leaves Neem extract significant and consistently reduced insulin requirements for nonkeytonic, insulin fast and insulin sensitive form of diabetes. Furthermore, Klaus, (2007) indicated that, number of insulin – dependant diabetic were able to reduce their insulin considerably when treated with Neem leaf extract and Neem oil in which the general impression is that Neem may enhance insulin receptor sensitivity and may work well on type 2-diabetes. Next, we studied the biosynthesis of the insulin receptor. Real time PCR referred that insulin receptors were down regulated in diabetic patient when compared to control diabetic and treated group these results agreed with (William 1978) who recorded that, down regulation reduction in cellular receptor expression for specific hormone done when the concentration of that hormone is increased. Obesity and diabetes are associated with decrease expression of insulin receptor. An inverse relation ship between insulin concentration and insulin receptor number (Olefsky 1978). Furthermore, Khan et al., (1978) demonstrated that only pathogenic condition in which a decrease in insulin receptor has been definitily shown to be result of insulin resistance and hyperinsulinism. Moreover, increased cortisol level might cause insulin receptor down-regulation (William 1978).

Administration of Neem to diabetic rats improved gene expression of insulin receptors when compared with control diabetic these results agreed with sharma (2013) who recorded that Neem enhance insulin receptor sensitivity, help in blood circulation by dialing blood vessels and decrease glucose level. Diabetic rats showed a significant increase in the serum IgE level after 4 weeks. These results came in accordance with Madacysy et al., (1983) who reported that total IgE level higher than normal in diabetic children this may be due to existence of symptoms of free atrophy in IDDM that proved autoimmune pathogenesis of insulin-dependant diabetes. Moreover, a high level of IgE was found in diabetes, autoimmune disease, and arthritis .it was further revealed that people who have increase level of IgE showed increase myoglobin and insulin (Binie, 2009) .The mast cell activator immunoglobulin IgE may serve as a novel inflammatory that associate with the risk of pre-diabetes and diabetes mellitus (Wang.,2011).

Neem extract administration revealed decrease in IgE level this may be attributed to presence of nimbidin which one of Neem leaves consistituent that has potent diuretic and anti-inflammatory properties (Mitra et al., 1978). In addition Neem leaf and its consistients have been demonstrated to exhibit immunomodulatory, anti-inflammatory and antioxidant properties (Subapriya and Nagini; 2005). From this study we recommended with treating diabetes mellitus with plant derived compounds as Neem which are accessible and do not require laborious pharmaceutical synthesis and has no side effects.

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Biochemical Effect of Neem Extract On Experimental Diabetes Gene Expression


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