Antidiabetic properties of *Hibiscus rosa sinensis* L. leaf extract fractions on non-obese diabetic (NOD) mouse

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On fractionation the ethanolic extract of *H. rosa sinensis* leaves, 5 fractions were obtained. Of these, fraction-3 (F_3) and fraction-5 (F_5) were chosen for detailed investigation on non obese diabetic (NOD) mouse to study anti-diabetic properties because they were more active than others. Serum glucose, glycosylated hemoglobin, triglyceride, cholesterol, blood urea, insulin, LDL, VLDL, and HDL were estimated. Both fractions F_3 and F_5 on oral feeding (100 and 200 mg/kg body weight) demonstrated insulinotropic nature and protective effect in NOD mice. These fractions may contain potential oral hypoglycemic agent.

**Keywords**: Cholesterol, Glycosylated haemoglobin, *H. rosa sinensis*, Hyperglycemic, Insulin, VLDL

Estimates for global prevalence of diabetes for the year 2000 and projections for the year 2030 have been made. Top three countries to have highest number of people with diabetes are India (31.7 and 79.4), China (20.8 and 42.3) and U.S. (17.7 and 30.3) in millions respectively. Diabetes is classified mainly as Type 1 diabetes or insulin dependent diabetes mellitus (IDDM), Type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) and gestational diabetes mellitus (GDM). Type-1 diabetes that affects more than 5.3 million people worldwide. Approaches to the control of diabetes mellitus are aimed at prevention of hyperglycemia involving dietary manipulation and use of plant therapies.

More than 1200 plants are used worldwide in traditional medicine for their alleged hypoglycemic activity. Few reviews are published on medicinal plants useful for treatment of diabetes. Isolation of plant extracts for antiviral, antidiabetic and antioxidant properties have been reported.

Sachdeva and Khemani reported on hypoglycemic activity of *Hibiscus rosa sinensis*. Aqueous and alcoholic extracts of *H. rosa sinensis* leaf and flower were shown to possess hypoglycemic activity in diabetes induced rats. Vimala et al. reported the insulin secreting activity of *H. rosa sinensis* leaf extract in diabetes induced wistar rat.

The non-obese—diabetic (NOD) mouse has served as one of the primary models for type-1 diabetes in which new approaches for immunotherapy have been investigated. The NOD mouse spontaneously develops type-1 diabetes that has many immunological and pathophysiological similarities to human insulin dependent diabetes mellitus (IDDM). The autoimmune nature of diseases is suggested by lymphocytic infiltration of islets of Langerhans which precedes the destruction of insulin producing β-cell. The objectives of the present investigation is to fraction the leaf extract of *H. rosa sinensis* in ethanol and to screen the different fractions obtained on NOD mouse for antidiabetic activity.

**Materials and Methods**

*Extraction and fractionation* — Healthy disease free leaves of *H. rosa sinensis* were collected from the garden of the University campus. The authenticity of the plant was confirmed by a taxonomist of the Department of Botany, University of Mysore, Mysore, and a voucher specimen was preserved in the form of herbarium. Collected leaves were shade dried and ground in Waring blender. The ground powder was extracted in soxhlet apparatus using ethanol until the extract became colourless. The extract was dried using evaporator. The dried extract was further fractionated by dissolving in methanol:water (4:1) evaporated to one tenth the volume and filtered. The residue obtained was further dissolved in ethyl acetate and filtered to obtain two fractions i.e., ethyl acetate.
soluble fraction (F_1) and ethyl acetate insoluble fraction (F_2). The filtrate obtained was further extracted using acid base extraction. The filtrate was first acidified using 5% H_2SO_4 (pH 2) and extracted twice with chloroform. The remaining aqueous layer was made alkaline using 5% NaOH (pH 10) and later extracted twice using chloroform:methanol (3:1). The remaining aqueous layer was neutralized using 5% H_2SO_4. The five fractions obtained were, ethyl acetate soluble fraction (F_1), ethyl acetate insoluble fraction (F_2), chloroform fraction (F_3), the basic fraction (F_4) and neutral fraction (F_5).

The pilot experiment was conducted to test the hypoglycemic properties of the five fractions obtained. Since F_2 did not dissolve in tween-80 (1%), remaining fractions, F_1, F_3, F_4 and F_5 were dissolved in tween-80 (1%) with two concentrations of 100 and 200 mg/kg body weight and fed orally to NOD mice. F_1 did not show hypoglycemia whereas fractions F_3, F_4 and F_5 showed hypoglycemia at the termination of experiment (unpublished data). The F_4 was not obtained in sufficient quantity. Hence this fraction was not used for further study. Fractions F_3 and F_5 were chosen for detailed investigation to study anti-diabetic properties.

Animals—NOD mice were originally obtained from Center for Cellular and Molecular Biology (CCMB, CSIR), Hyderabad and maintained under standard environmental conditions of 12:12 h L:D cycle. Mice were fed with standard diet supplied by Ambruth Feeds Pvt Ltd Bangalore and water ad libitum. Healthy adult (30 weeks old) NOD mice of either sex weighing 25-30 g were selected for the study from the Central Animal Facility of the Department. All the selected animals showed the fasting blood glucose values ranging from 242 to 326 mg/100ml indicating hyperglycemia, the diabetic status. Experimental protocols were approved from Institutional Animal Ethics Committee (IAEC).

Experimental protocols—NOD mice were randomly divided into following 6 groups of 8 animals each:

Group 1 — control mice treated with vehicle alone.

Group 2 — mice treated with insulin (1 ml of Biphasic isophane insulin purchased from pharmaceutical company dissolved in 100 ml saline and 0.1 ml/mouse/day was injected, intraperitoneal).

Group 3 — mice treated with F_3 (100 mg/kg body weight).

Group 4 — mice treated with F_3 (200 mg/kg body weight).

Group 5 — mice treated with F_5 (100 mg/kg body weight).

Group 6 — mice treated with F_5 (200 mg/kg of body weight).

F_3 and F_5 were dissolved in tween-80 (1%) and fed orally to the animals of group 3–6 as indicated. The experiment was conducted for four weeks.

Biochemical analysis—Serum glucose levels were estimated using a glucometer (EZ Omnitest) every week to ascertain the status of diabetes in different groups of mice. Similarly body weight was recorded once in a week in every group. After 30 days animals were allowed to fast overnight with free access to water and autopsied under light ether anesthesia. The blood was collected from the carotid artery at the time of autopsy and centrifuged at 4°C, at 10000 rpm for 10 min; the separated serum was used for various biochemical analyses.

Serum glucose levels were estimated by Trinder’s method using GOD POD enzymatic kit. Glycosylated hemoglobin, triglycerides, HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol were estimated. Blood urea was estimated by urea-glutamate dehydrogenase (GLDH) method. Plasma insulin levels were determined in duplicate using insulin RIA Kit (Linco, St. Charles MO) with rat insulin as a standard.

Statistical analysis—Data were statically evaluated by using one-way ANOVA. Wherever the ANOVA values were found to be significant Duncan’s new multiple range test (DMRT) was applied (SPSS computer software). The values were considered significant when P < 0.05.

Results

Bodyweight—Body weight of group 1 animals remained constant without any gain whereas there was a significant increase in body weight of the animals of group 2-6 at the termination of the experiment (Table 1).

Serum glucose level in group 1-(NOD mice) exhibited hyperglycemia on the day of commencement of experiments (287 mg/dl) and remained in the diabetic state throughout the experiment (Fig. 1). Group 2 animals exhibited hyperglycemia (242.4 mg/100 ml) on the day of commencement of experiment but after
intraperitoneal daily injection of insulin resulted in bringing the blood sugar level to non-diabetic status (103.4 mg/100 ml).

Animals in groups—3 and 4 exhibited hyperglycemia with the fasting blood glucose values of 290 and 278.6 mg/100 ml respectively on the day of commencement of experiment. After oral feeding of F\(_3\) (100 and 200 mg/kg body weight) serum glucose level was brought to non-diabetic state of 94.4 and 90.2 mg/dl \((P < 0.05)\) respectively at the end of experiment. Similarly group 5 and 6 animals also exhibited hyperglycemia with the fasting blood glucose values of 280.8 and 326.6 mg/100 ml respectively on the day of commencement of experiment. After oral feeding of F\(_3\) and 200 mg (100 /kg body weight) serum glucose reached to non-diabetic state of 92.2 and 83.8 mg/dl \((P < 0.05)\) respectively at the termination of the experiment (Fig. 1). Since the fractions F\(_3\) and F\(_5\) showed promising hypoglycemic activity on NOD mice, the preserved blood was further used for biochemical analysis.

**Blood glycosylated haemoglobin (HbAlc) level**—Table 2 shows the levels of fasting glycosylated haemoglobin at the termination of experiment in all the groups. Control group showed maximum levels of glycosylated haemoglobin where as there was significant decrease in fasting glycosylated haemoglobin in insulin injected group. Oral administration of F\(_3\) and F\(_5\) for 30 days at two different doses (100 and 200 mg/kg body weight) resulted in significant decrease on fasting glycosylated haemoglobin in all the groups 3–6, especially 5 and 6.

**Plasma insulin**—Serum insulin level remained minimum in control group. Insulin level was enhanced significantly in group 2 animals (Table 2). Though F\(_3\) enhanced insulin level in group-3 and 4 animals, F\(_5\) resulted in insulin secretion on par with that of group-2 animals.

**Blood triglycerides**—The triglycerides levels significantly decreased in groups 3-6 compared to group-1 animals (Table 2). However the levels were enhanced significantly in insulin treated mice compared to that of control group.

### Table 1 — Effect of *H. rosa sinensis* (HLE) fractions on body weight of experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial body weight</th>
<th>Final body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- (NOD diabetic control)</td>
<td>27.8±0.25</td>
<td>27.1±0.19</td>
</tr>
<tr>
<td>2- Insulin injected NOD</td>
<td>30.1±0.40</td>
<td>31.7±0.40</td>
</tr>
<tr>
<td>Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3- HLE.F(_3) (100mg/kg/bw)</td>
<td>29.0±0.42</td>
<td>32.2±0.18</td>
</tr>
<tr>
<td>4- HLE.F(_3) (200mg/kg bw)</td>
<td>28.8±0.58</td>
<td>32.4±0.36</td>
</tr>
<tr>
<td>5- HLE.F(_5) (100mg/kg bw)</td>
<td>29.0±0.35</td>
<td>32.2±0.3</td>
</tr>
<tr>
<td>6- HLE.F(_5) (200mg/kg bw)</td>
<td>28.3±0.20</td>
<td>32.1±0.4</td>
</tr>
</tbody>
</table>

All values of final body weight are significant at \(P<0.05\) except in group 1 vs initial body weight

### Table 2 — Effect of alcohol leaves extract of *H. rosa sinensis* fractions on serum glucose, glycosylated haemoglobin, insulin, triglyceride, cholesterol and blood urea levels of experimental groups at the termination of experiment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood glucose (mg/dl)</th>
<th>Glycosylated haemoglobin (%)</th>
<th>Insulin (µl/l)</th>
<th>Triglycerides (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Blood urea (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- (NOD diabetic control)</td>
<td>281.6±3.7</td>
<td>9.54±0.18</td>
<td>0.144±0.01</td>
<td>80.8±0.37</td>
<td>78.2±0.58</td>
<td>61.4±0.51</td>
</tr>
<tr>
<td>2- (insulin injected NOD mice)</td>
<td>103.7±2.13</td>
<td>6.44±0.13</td>
<td>0.336±0.01</td>
<td>98.4±2.5</td>
<td>76.8±2.03</td>
<td>38±1.00</td>
</tr>
<tr>
<td>3- (HLE.F(_3) 100mg/kg/bw)</td>
<td>94.4±1.86</td>
<td>8.16±0.33</td>
<td>0.262±0.01</td>
<td>58.2±0.58</td>
<td>68±0.71</td>
<td>57.4±0.75</td>
</tr>
<tr>
<td>4- (HLE.F(_3) 200mg/kg bw)</td>
<td>90.2±2.46</td>
<td>7.32±0.25</td>
<td>0.286±0.02</td>
<td>38.6±0.81</td>
<td>30.4±0.5</td>
<td>54.4±0.51</td>
</tr>
<tr>
<td>5- (HLE.F(_5) 100mg/kg bw)</td>
<td>92.2±2.63</td>
<td>7.3±0.0</td>
<td>0.324±0.01</td>
<td>64.4±0.24</td>
<td>49.6±0.58</td>
<td>46.9±0.24</td>
</tr>
<tr>
<td>6- (HLE.F(_5) 200mg/kg bw)</td>
<td>83.8±3.15</td>
<td>6.5±0.14</td>
<td>0.382±0.01</td>
<td>59.2±0.37</td>
<td>47.2±0.37</td>
<td>37.2±0.37</td>
</tr>
</tbody>
</table>

All values are significant at \(P<0.05\) vs diabetic control (Gr.1)

HLE- *H. rosa sinensis* leaf extract
**Cholesterol**—There was no significant variation in cholesterol in control group and insulin injected group (Table 2). After oral treatment with F₃ and F₅ at two different doses (100 and 200 mg/kg body weight) the cholesterol level of group 3-6 showed significant reduction compared to that of control group.

**Blood urea**—The blood urea in control group showed higher values whereas it remained significantly lower in all the remaining groups of animals (Table 2).

LDL value remained higher in group-1 animals. There was a significant decrease in LDL value in group 2-6 animals (Fig. 2).

The levels of VLDL in different groups of experimental animals at the termination of experiment remained higher in group 2 animals (Fig. 2). Group 3-6 experimental animals exhibited significantly lower level of VLDL compared to that of group 1 animals. Fig. 2 also shows the levels of HDL in different groups of experimental animals at the termination of the experiment. HDL level remained higher significantly in all the groups compared to that of group-1 animals.

**Discussion**

Many studies on type-1 diabetes use animal models by inducing diabetes by injecting animals with streptozotocin or alloxan whereas the present investigation reports on NOD mice which spontaneously develops type-1 diabetes and has similar features to those of human type-1 diabetes.

Control mice did not gain any bodyweight and this was associated with hyperglycemia throughout the experiment (Table 1 and Fig. 1). Whereas all the remaining groups gained significant body weight either with treatment of insulin (group 2) or with fractions F₃ (group 3 and 4) and F₅ (group 5 and 6). Stanley et al. and Grover et al. demonstrated characteristic loss of body weight after induction of diabetes with streptozotocin in sharp contrast to oral administration of plant extract of *T. cordifolia* which caused significant increase in body weight.

Deficiency of insulin hormone resulted in the impairment of glucose homeostasis in group 1 mice resulting in hyperglycemia throughout the experiment (Fig. 1 and Table 2). Patients with type-1 diabetes have to take exogenous insulin for survival. In the present investigation, group 2 animals were injected with insulin bringing the blood glucose level to non-diabetic status. Groups 3-6 animals received fractions F₃ and F₅ with different concentrations which improved insulin secretion resulting in homeostasis of blood glucose, thus approaching non-diabetic status (Fig. 1). It is evident from Table 2, that fraction-5 enhanced insulin secretion comparatively better than fraction 3. Several investigators have recommended that glycosylated hemoglobin be used as an indicator of metabolic control of diabetes since glycohemoglobin levels approach normal values in diabetics in metabolic control. Glycosylated hemoglobin remained maximum in group 1 animals whereas there was a significant reduction in all the remaining groups after treatment with either insulin or the fractions, F₃ and F₅. F₅ with 200 mg/kg body weight oral treatment reduced the glycosylated hemoglobin level on par with insulin injected mice (Table 2).

The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia. Administration of F₃ and F₅ during experimental period reduced triglyceride and cholesterol levels significantly in groups 3-6. However F₃ with 200 mg/kg body weight oral administration reduced triglycerides and cholesterol levels considerably (Table 2). Interestingly in group 2 animals receiving insulin injection there was enhancement in triglycerides and cholesterol levels which remained on par with control group. Though exogenous insulin reduced the blood sugar level, it has a side effect on triglyceride and cholesterol level.

Elevated levels of urea are seen during increased protein breakdown in renal disorders like glomerular nephritis and chronic nephritis. In the present investigation, control group animals exhibited elevated levels of blood urea whereas after treatment
with F3 and F5 there was a significant reduction in blood urea levels. F5 (200 mg/kg body weight oral feeding) reduced the blood urea on par with insulin injected group (Table 2).

High levels of LDL and VLDL are atherogenic, as opposed to HDL which transports cholesterol from peripheral tissues to liver and then for excretion. Therefore, HDL has protective effect. LDL and VLDL levels were significantly higher in control mice compared to those of groups 3-6 animals. VLDL slightly enhanced in insulin injected group whereas LDL values reached minimum in F3 treated animals; VLDL values reached minimum in F3 treated groups. Similarly the levels of HDL were enhanced in F3 and F5 treated groups even better than the insulin injected group. Thus improvement in glycemic control followed by fall in LDL and VLDL levels after treatment with F3 and F5 could be attributed to therapeutic efficacy of these fractions in NOD mice. Laakso et al. and Laakso showed improved glycemic control followed by fall in VLDL production in diabetic patients after treatment with oral hypoglycemic agents. Earlier studies have showed higher concentration of LDL and lower concentration of HDL cholesterol in diabetic patients. Hypcholesteremetric drugs decrease LDL cholesterol and enhance HDL cholesterol. This seems to be true of F3 and F5 treatments also.

Subash-Babu et al. reported on methanolic extract and n-hexane fraction of Ichnocarpus fructescence (IFML extract) leaf on streptozotocin induced diabetic rats. Type-1 diabetic patient needs exogenous insulin injection for metabolic control; However the discovery of orally active molecules with insulin like effect could lead to alternative therapies. Hypoglycemic plants act through a variety of mechanisms such as improving insulin sensitivity, augmented glucose dependent insulin secretion and stimulating regeneration of islets of Langerhans in diabetic rats. Babu et al. reported the antidiabetic activity of ethanol extract of Crassia kleini reported in diabetes induced rats. C. kleini extract acts like metformin, an oral hypoglycemic agent that did not influence serum insulin level in diabetic and in normo-glycemic rats.

The crude extract of Hibiscus rosa sinensis was reported to have insulin secreting activity in diabetes induced Wistar rats. In the present investigation, fractions from crude extract were isolated and it is evident that both the fractions (F3 and F5) have insulinotropic effect as also protective effect in NOD mice. However, it is obvious from above studies that F3 with 200 mg/kg body weight oral administration has more insulinotropic effect thereby indicating that, it may contain a source of potential new oral hypoglycemic agent. However, isolation of the active component is in progress.

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