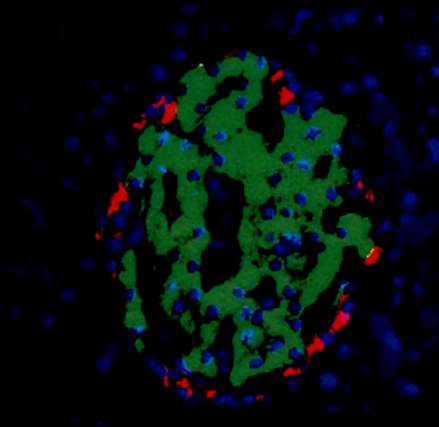


CONCISE SUMMARY

“In-vivo and in-vitro assessment of Oreocnide integrifolia (Gaud.)Miq. leaf extract on experimental diabetic models: Physiological, biochemical and molecular evaluations”.



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September, 2010



CONCISE SUMMARY

India is fast becoming diabetic capital with alarming increase in the number of diabetic subjects and fast progressing into a pandemic issue beyond epidemic proportions. Owing to the increasing trend of diabetes the world over, research is getting increasingly focused for the development of appropriate and effective therapeutics for this debilitating disease. Different avenues of treatment schedules ranging from diet control and exercise regimens to use of hypoglycemics and antioxidant agents and, herbal formulations are being intensely pursued. Though, diabetes is characterized by multiple manifestations, the three basic symptoms are excessive thirst, polyurea and polydypsea, essentially due to glucosuria. Based on the major causes, 2 classes of diabetes have been recognized: type 1 diabetes (Insulin dependent diabetes mellitus) and type 2 diabetes (Non-insulin dependent diabetes mellitus) though, other types of diabetes also exist. The type 1 diabetes need lifelong insulin therapy and balanced control between glucose intake and insulin dose (Lehninger et al., 1993). Insulin deficiency that characterises T1D patients is essentially due to destruction of insulin producing beta cells. Based on the cause, an autoimmune class and an idiopathic class of T1D have been identified. Whereas cell-mediated autoimmune destruction of the beta cells characterize the former, unknown multiple etiologies leading to beta cell dysfunction and destruction characterize the latter (Harris, 2000). Type 2 diabetes is a heterogeneous disorder characterized by two interrelated metabolic defects; insulin resistance, coupled with impaired insulin secretion by β -cells in the pancreas. Therefore, strategies that target these two mechanisms by improving insulin sensitivity and protecting β -cell function have become the focus of prevention efforts.

Since most of the modern medications used to treat diabetes are relatively effective are beset with long term complications, besides being more of single locus of action, alternative cost effective multipronged treatment schedules with no attendant side effects are of relevance in combating the globally rampant draconian disease. Traditional ethnic or folklore medicines practiced by humans from ancient times provide the much-needed solace. The traditional medicine practiced as Ayurveda in India from times immemorial needs a scientific relook and legitimate practical application. Herbs serve as excellent source of drugs and, many a drug currently in vogue for various disorders are derived directly or indirectly from plants. Alarcon-Aguilara et al. (1998) have provided ethno-medicinal information about nearly 800 plants that possess anti-diabetic properties. Several such herbs have been assessed by means of currently available experimental techniques (Saifi et al., 1971; Mukherjee et al., 1972; Coimbra et al., 1992; Kar et al., 1999; Jafri et al., 2000).

Active phytochemical ingredient such as alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycans, hypoglycans, guanidines, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions, have all been considered of significance in the treatment of diabetes. Even the commonly used hypoglycemic drug, metformin, has been derived from a herb, *Galela officinalis* and yet, practitioners are not inclined in exploring the therapeutic potentials of herbal formulations. Though oral hypoglycemic are the main stay of the current therapeutic schedule and are effective in controlling hyperglycemia, they have nevertheless adverse effects, and moreover, fail to alter the course of diabetic complications (Rang and Dale, 1991). Apart from insulin, modern modes of diabetic control include therapeutants like biguanides, sulphonylureas, thiazolidinediones and yet, no drug in vogue has been found

competent enough to ameliorate all diabetic complications, providing added impetus to look for new effective and easily accessible drugs.

For countries like India with a high proportion of rural population, provision of modern medical healthcare is much in wanting due to economic constraints. Many of the common modern drugs used like, aspirin, anti-malarials, antioncogenics, digitalis etc are all plant derived and, in this context, effective anti-diabetic agents can be fashioned out of plants. Of about nearly 2,50,000 higher plants, hardly 1% has been screened for their pharmacological possibilities and, even very few have been tested for their anti-diabetic properties. Bioactive phytochemicals like, flavonoids and tannins have antioxidant effects besides hypoglycemic potentials (Larsen, 1988). It is highly prudent therefore to look for alternative options in the form of herbals for development of effective anti-diabetic formulations.

It is in this context, the present study on the extract of leaves of *Oreocnide integrifolia*, a plant used as folklore medicine for diabetes and hypertension in northeast India, has been undertaken. Overall, the study strives to address the following objectives:-

- 1) To evaluate the dose and duration dependent hypoglycemic and hypolipidemic potential of *Oreocnide integrifolia* (Gaud.) Miq aqueous leaf extract (OI) on streptozotocin induced type-1 diabetes in *Charles foster* rats.
- 2) To assess the potential of OI extract on experimentally induced hypertension and insulin resistance using high fructose fed *Charles foster* rats.

- 3) To evaluate the mechanism of of OI extract in terms of glucoregulation and its possible mediation through insulin signaling pathway and its role of leptin regulation. in high fat diet induced type-2 diabetic *C57BL/6J* mice.
- 4) To assess the phytochemicals present using chromatography techniques and to evaluate the insulin secretagogue potential of OI extract & its fractions through bioactivity guided approach using *in-vitro* models, and the role of bioactive fraction (s) *in-vivo*
- 5) To assess the role of active fraction(s) in terms of their beta-cell regeneration/neogenesis using experimental mice model of regeneration.

Chapter 1

The present study was undertaken to evaluate the dose and duration dependent hypoglycemic and hypolipidemic potential of *Oreocnide integrifolia* leaf extract on streptozotocin induced experimental diabetes. To, this end normoglycemic and diabetic animals were treated different doses (100,250,500 and 750) of aqueous extract of OI and compared with a standard antidiabetic drug, metformin over a period of 28 days. Blood glucose was monitored at weekly intervals while plasma insulin, Hb, HbA1c, Creatinine, Urea, lipid profile along with glycogen, glycogen phosphorylase, glucose-6-phosphatase and glycogen synthase were evaluated, while for histological observations HE staining and insulin immunohistochemistry was performed.

At the end of treatment period (day 28), OI extract (higher doses) lowered blood glucose levels in diabetic animals but, no significant changes were seen in normoglycaemic animals, suggestive of its antihyperglycaemic mode of action. Higher doses of OI extract treated diabetic animals registered significant increase in HbA1c levels. Total hemoglobin decreased in the diabetic group, possibly due to the increased formation of HbA1c whereas, the increase in hemoglobin levels in diabetic animals treated with OI extract may be due to decrease in blood glucose levels. OI extract treatment (500 mg/kg bw) in diabetic animals lowered cholesterol, triglyceride and LDL-C levels, and increased HDL-C levels. The underlying mechanism of lipid lowering role of OI extract may be by way of inhibited lipid absorption by saponins and phenolic substances present in the aqueous extract. The serum insulin levels were increased in extract treated rats which clearly attest to increased hormonal output overcoming the suppressive effect of STZ. The histological and immunohistochemical revelations suggest a potent protective effect of OI extract on β -cells of STZ diabetic rats. The increased number of islet cells and even

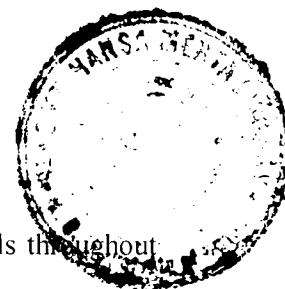
improved integrity of partially affected cells as seen in the routinely stained histological sections of pancreas as well as the increased insulin immunoreactivity in immunohistochemically stained sections are unambiguous documentary evidence of not only the protective effect of OI extract but, also its potential competence in promoting β -cell regeneration and/or proliferation. The present study revealed substantial recovery in hepatic and muscle glycogen stores on OI extract administration to diabetic rats. The recovery in tissue glycogen load is marked by decreased glycogenolysis which was marked by the significant decrement in the phosphorylase activity and further, decrease in hepatic G-6-Pase activity which attests to a moderated gluconeogenesis. Furthermore, OI extract treated diabetic rats showed pronounced decrease in the plasma levels of creatinine and urea suggestive of its renoprotective effect.

Overall, it can be concluded that, OI extract has potential anti- hyperglycemic and anti-hyperlipidemic effects along with competence to revert the metabolic derangements affecting carbohydrates and lipids and also bring about insulinogenic islet regeneration in STZ induced diabetic rats.

Chapter 2

The present study evaluates the folklore use of *Oreocnide integrifolia* (Gaud.) Miq (OI) leaf extract used by the people of northeast India and scientifically validates the ethnomedicinal claims against diabetes associated with hypertension in an experimental rat model. In this context, an animal model for experimental metabolic syndrome was developed wherein, Male *Charles foster* rats were fed high fructose diet (60 g/100 g) for a period of 6 weeks for induction of insulin resistance coupled with hypertension. Animals were divided into five groups (a) control, (b) fructose control, (c) fructose + OI 250, (d) fructose + OI 500 and (e) fructose + Metformin. Biochemical parameters like plasma levels of glucose, insulin, cholesterol, triglycerides, lipoproteins, free fatty acids along with glucose tolerance and insulin response tests were evaluated. Mean arterial blood pressure was measured at the end of 6-week period and the animals were further challenged with acetylcholine, phenylephrine, isoprenaline or adrenaline to assess the vascular reactivity *in vivo*.

The observations made on fructose fed rats after 6 weeks of high fructose feeding essentially confirmed the significant expressions of various manifestations of metabolic syndrome like hyperglycemia, hyperinsulinemia and insulin resistance, glucose intolerance, dyslipidemia and hypertension. OI supplemented group exhibited significant decrease in plasma glucose and insulin titers, which were comparable to that metformin treated rats. Analysis of glucose tolerance pattern and AUC of glycemia during 120 min test period in control and experimental animals revealed that, fructose fed rats developed glucose intolerance. AUC of glucose during OGTT rose to 40% in fructose fed rats as compared to control rats. OI extract supplemented rats showed lower glucose elevation and faster glucose disposal rates thereby displaying significant improvement ($P < 0.001$) in glucose tolerance pattern. During insulin



response tests, fructose fed rats showed constant decrease in plasma glucose levels throughout the 120 min period while, OI extract treated groups displayed improved recovery rates attributable to improved insulin sensitivity. Moreover, OI extract supplementation decreased plasma levels of cholesterol (23.4%, 29.3%), triglycerides (45.8%, 55.5%), LDL-C (42.5%, 51.4%), VLDL-C (46.1%, 55.2%) and FFA (35%, 40.7%) and increased HDL-C levels (37.5%, 40.82%) in fructose fed animals. Fructose feeding caused a significant increase in mean arterial blood pressure in this model and consistent with the reports of other workers, whereas OI extract supplemented rats showed significant decrease in arterial blood pressure and was even better than metformin treated animals. Pressor responses to phenylephrine and adrenaline increased significantly ($p<0.001$) at end of 6 week period and decreased significantly ($p<0.001$) when challenged with isoprenaline or acetylcholine in fructose fed rats while OI supplemented groups displayed significant ($p<0.001$) increase in vascular responses to acetylcholine and isoprenaline significant decrease ($p<0.001$) in vasoconstrictor responses when challenged with phenylephrine and adrenaline. These studies find correlation with other workers and the possible role of OI extract to abrogate 5-HT mediated hypertensive changes. Overall, this is the most effective alternative mode of therapy and in this context, the present finding on the potent efficacy of OI extract in preventing all manifestations of metabolic syndrome is of great value.

Chapter 3

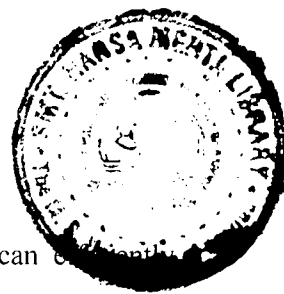
The present study was designed to evaluate the possible antihyperglycemic, antihyperlipidemic action and mechanism of glucoregulation of OI extract, when supplemented simultaneous to high fat diet. To this effect, C57BL/6J mice, a clinically relevant experimental model for type – 2 diabetes were fed with high fat diet for duration of 24 weeks. The experimental animals received 3% aqueous extract of OI simultaneously mixed with feed. Animals were sacrificed on completion of treatment period and parameters related to glucoregulation (Plasma glucose, serum insulin, [^{14}C] Glucose Oxidation in liver and muscle, glycogen, OGTT, IRT, hepatic m-RNA expression of glucokinase, G6Pase and PEPCKase), component proteins of insulin signaling pathway (IRS-1, AKT, cytosolic and membrane bound GLUT-4) and histopathological alterations in liver together with serum lipid profile, insulin and leptin levels and immunolocalization of leptin in pancreatic islets and PPAR- γ mRNA expression in adipocytes were undertaken. By 24 weeks, high fat diet alone fed group of mice showed type-2 diabetic manifestations marked by hyperglycemia to the tune of 200%, hypoinsulinemia to the extent of 20% and an altered higher level of glucose homeostasis and insulin sensitivity as recorded by the glycemic changes during glucose tolerance and insulin response tests (GTT and IRT). Attendant metabolic alterations were manifested in the form of reduced peripheral glucose oxidation and hepatic glucokinase mRNA expression, coupled with increased hepatic glucose-6-phosphatase and phosphoenol pyruvate carboxykinase mRNA expressions. Insulin resistance (IR) was clearly evidenced from the much higher glucose elevation rate seen in both GTT and IRT. IRS-1 expression in muscle (cytosolic fraction) was decreased significantly ($P < 0.001$) in diabetic mice compared to controls while OI extract supplemented animals depicted higher near normal level when compared to diabetic animals. However, AKT-1 expression (cytosolic

fraction) of control, diabetic or OI treated group did not show any significant difference. Cytosolic fraction showed no significant changes in Glut-4 protein expression in either control or experimental groups of animals. However, membrane glut-4 expression was remarkably reduced ($P < 0.0001$) in diabetic animals whereas there was a conspicuous maintenance of Glut-4 expression in OI supplemented group though yet lesser than in control animals. OI extract offset most of the diabetic alterations as evident from the significantly lower hyperglycemia and near normal GTT and IRT curves, and further, glucose elevation rates under both these tests were significantly lower in OI extract supplemented mice than in diabetic animals. Further, Diet induced T2D alterations was also evident in the form of increased body weight, greater feed efficiency, adiposity, dyslipidemia, hypoinsulinemia, hyperleptinemia, increased immunoreactivity for leptin in pancreatic acini and reduced PPAR γ expression in adipose tissue of BL 6 mice. Except for PPAR γ expression, all other alterations are reversed to a greater extent by OI extract supplementation. These observations such as noted minimal decrease in plasma insulin titre bespeak of both an insulinogenic action, as well as insulin sensitivity potentiating effect of OI extract. Since type 2 diabetes is a complex metabolic disorder affecting multiple loci like hyperglycemia, impaired insulin action/ secretion, dyslipidemia etc. and has complicated expression patterns by multiple genetic background and varied interactions with environmental conditions, its ameliorative or therapeutic intervention need to address to all the above multifaceted manifestations. The results of the present study are suggestive of OI extract as an alternative therapeutics to counteract the manifestations of type -2 diabetes.

Chapter 4

The present study was carried out to evaluate the phytochemicals present in OI extract and with the help of solven partition chromatography, isolate bioactive fraction in terms of their insulin secretion and glucose uptake potential. Qualitative phytochemical analysis showed the presence of phenols, flavonoids, saponins, tannins, terpenoids, sterols (Fig. 1b: F), carbohydrates, while alkaloids, anthraquinones and amino acids were found to be absent. Quantitative analysis revealed the presence of phenols (64.81 ± 1.51 mg/g), flavonoids, (72.8 ± 1.87 mg/g), tannins (22.76 ± 0.72 mg/g), and saponins (106.4 ± 2.81 mg/g). Moreover HPTLC fingerprinting and their derivitization for different class of phytochemicals revealed presence of alkaloids, steroids and terpenoids, TLC and HPLC fingerprint of flavonoids rich fraction revealed at least 4 flavonoids, when assessed with known standard markers. Analysis of non-polar compounds was carried out using chromatography and GC-MS indicating the presence of Hentriacontane derivatives.

Glucose stimulated insulin secretion (GSIS) of OI extract was carried out in isolated mouse pancreatic islets and demonstrated significant dose dependent effects in insulin release. For bioactivity guided assays, RINm5F (rat insulinoma) and C2C12 (mouse myoblast) cell line were used as experimental *in-vitro* models to screen different fractions for their insulin secretion ability in presence of glucose and their glucose uptake potential in presence of insulin. Flavonoid rich fraction (FRF) exhibited maximal potential in terms of these bioactivities. Further, FRF displayed significant potential in terms of GSIS and increasing intracellular calcium and cAMP levels even in presence of a phosphodiesterase inhibitor, IBMX. The findings finds correlation with the fact that the potentiating insulin secretagogue action of FRF could be mediated through GLP-1 action as, flavonoids are known to increase GLP-1 level as well as inhibit dipeptidyl



peptidase IV (DPP-IV), the enzyme that metabolises GLP-1, both of which can be upregulated by FRF. FRF has been shown to upregulate insulin secretion from pancreatic islets in presence of glucose. Furthermore, antioxidant and cytoprotective role of FRF was evaluated by exposing islets to 2mM streptozotocin (STZ) stress for 8 hrs. Presence of FRF in the medium along with STZ depicted a dose dependent reversal of all the cytotoxic manifestations with the highest dose of 250µg/ml normalizing most of the measured parameters (reactive oxygen species, lipid peroxidation and mitochondrial membrane potential) except for peroxynitrite and NO formation, suggesting the need for a probably higher dose to completely nullify the NO and peroxynitrite generation potential of STZ. The herein observed anti-oxidant, anti-ROS and NOs generation potentials and maintenance of mitochondrial membrane potential found substantial support from the reported anti-oxidant potential of flavonoids.

Further, scrutinization of FRF was done using a multidose STZ mice model in a dose dependent manner. FRF decreased plasma glucose and increased insulin titres when administered for a period of 28 days. Cytoprotective effect of FRF was well evidenced by the histological appearance of islets from STZ+FRF mice compared to STZ mice. Further, proof for the cytoprotective effect of FRF is provided by its anti-apoptotic potential as seen from the TUNEL assay and the noticeable insulin immunoreactivity.

Overall, the flavonoid mixture has shown to have significant insulin secretagogue, insulinomimetic and cytoprotective effects, as confirmed through the *in vitro* and *in vivo* models of evaluation.

Chapter 5

Insulin deficiency is the prime basis of all diabetic manifestations and strategies that can trigger β -cell regeneration/ neogenesis would be of pivotal significance in therapy and cure of diabetes. Therefore, agents which can either trigger proliferation of β -cells or induction of neogenesis of β -cells from precursors would play an important role in reversing diabetic complication. To test this hypothesis, we evaluated the role of flavonoid rich fraction (FRF) using an mice model of experimental regeneration. To this end, BALB/c mice were subjected to subtotal pancreatectomy (70%) and simultaneously supplemented with FRF for a period of 7, 14 and 21 days post pancreatectomy. Sham operated and Px mice were sacrificed at predefined time points (day 7, 14 and 21 post px) and plasma glucose levels, insulin titres. With immunohistochemistry and confocal microscopy we scored BrdU incorporation in islets (day 7, 14, 21 px), ducts (day 7 px), acini (day 7 px) and non-islet cells (day 7 px) along with Pdx-1 expression. H&E staining was performed for histological assessment of pancreas. Further, we used Taqman based probe duplex quantitative real time pcr to assess transcript levels of Ins-1, Ins-2, Reg3-a, Reg 3g and NgN-3.

Pancreatectomised mice treated with FRF extract showed significantly lower hyperglycaemia, which showed a slow but gradual decline through one to three weeks. Histological data demonstrated more prominent islet like buds getting organised around pancreatic ducts by day 7 treated with FRF. Both insulin reactivity as well BrdU labelling seemed to be higher in general compared to α -cells and acinar cells but with a significantly higher reactivity and labelling in Px + FRF pancreas through week 1 to week 3 post Px. There was significant temporal increase in insulin immunostaining, which was more prominent in Px + FRF treated pancreas and also demonstrated increased Pdx-1⁺ nuclei during day 7 post

pancreatectomy. Both the proinsulin transcripts showed a significant decrement seven days post Px with a relatively lesser decrement in Px + FRF pancreas. Expressions are increased gradually over 14 and 21 days post Px with relatively greater expression in FRF treated pancreas while 3- α and Reg3- γ transcripts were over-expressed maximally at day 7 post Px with FRF treated mice pancreas showing significantly greater expression. Both the transcripts decreased gradually to reach lowest levels by day 21 post Px with the levels in Px + FRF being relatively higher at all times. Pdx-1 and NgN-3 expression of both transcripts were the feature at day 7 post Px with relatively greater expression in Px + FRF mice pancreas. Transcript levels of both genes decreased thereafter through day 14 to reach the lowest levels at day 21, with the transcript levels in Px+ FRF pancreas being relatively higher at all time periods.

Overall, the present findings indicate islet neogenesis as the mode of β -cell regeneration in pancreatectomized BALB/C mice and provide evidence for flavonoidal rich fraction of OI extract to have enhancing influence on islet neogenesis and greater β -cell regeneration.