STUDY ON ANTI-DIABETIC EFFECTS OF AQUEOUS EXTRACT OF SOME INDIGENOUS PLANTS AVAILABLE IN NORTH KARNATAKA AND THEIR INTERACTION WITH SOME HEAVY METALS

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By

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CERTIFICATE

This is to certify that the thesis entitled *"Study on Anti-diabetic effects of aqueous extract of some indigenous plants available in north Karnataka and their interaction with some heavy metals"* submitted for the award of Doctor of Philosophy in Chemistry is the original research work completed by Mr. Kailash S. Chadchan under my supervision and it has not been submitted to any other University wholly or in part for any other degree.

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DECLARATION

I hereby declare that the entire work embodied in this doctoral thesis has been carried out by me at Department of Chemistry, BLDEA's V. P. Dr. P. G. Halakatti College of Engineering and Technology, Vijayapur, Karnataka, India under the supervision of Dr. Swastika N. Das, Professor and Head, Department of Chemistry, BLDEA's V. P. Dr. P. G. Halakatti College of Engineering and Technology, Vijayapur, Karnataka, India. This thesis has not been submitted in part or full for the award of any degree / diploma of this or any other University.

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Date: ***Kailash S. Chadchan***

Place: *Vijayapur*

I dedicate this thesis to

My father

Shri. Sidramappa B. Chadchan

LIST OF ABBREVIATIONS

SYMBOLS	
ANS	Autonomic Nervous System
ATN	Acute Tubular Necrosis
ATP	Adenosine triphosphate
bpm	beats per minute
BRS	Baro Reflex Sensitivity
C	Centigrade
cGMP	cyclic Guanosine monophosphate
cm	centi meter
cNOS	constitutive Nitric Oxide Synthase
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DBP	Diastolic Blood Pressure
DDMP	2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one
dL	desi litre
DM	Diabetes Mellitus
DPX	Dibutyl phthalate and Xylene
e NOS	endothelial Nitric Oxide Synthase
ECG	Electrocardiogram
FBS	Fasting Blood Sugar
FFT	Fast Fourier Transform
g	Grams
g.b.wt	grams.body weight
GLUT2	Glucose Transporter 2
HR	Heart Rate
H	Hour
HF	High Frequency
HIF-1α	Hypoxia Inducible Factor-1α
HRV	Heart Rate Variability
i NOS	inducible Nitric Oxide Synthase
i.p.	Intraperitoneal
IDDM	Insulin Dependent Diabetes Mellitus
kg	kilo grams
LF	Low Frequency

MAP	Mean Arterial Pressure
mg	milli grams
mins	Minutes
mL	milli litre
NIDDM	Non Insulin Dependent Diabetes Mellitus
nm	Nanometer
NO	Nitric Oxide
NOS3	Nitric Oxide Synthase 3
OECD	Organization for Economic Cooperation and Development
OGTT	Oral Glucose Tolerance Test
pg	pico gram
PPAR	Peroxysome Proliferator –Activated Receptor
PPRE	Peroxysome Proliferator Responsive Elements
PSD	Power Spectrum Density
RMSSD	Square root of mean of square difference of successive RR intervals
SBP	Systolic Blood Pressure
SD	Standard Deviation
SDNN	Standard Deviation of Normal – Normal RR intervals
TBARS	Thiobarbituric Acid Reactive Substances
TC	Total Cholesterol
TG	Triglyceride
TZD	Thiazolidinedione
VEGF	Vascular Endothelial Growth Factor
VLF	Very Low Frequency
µg	micro gram

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ABSTRACT

Abstract

Background: Fenugreek (*Trigonella foenum graecum,* Methi), Kenaf (*Hibiscus cannabinus Linn,* Pundi), Chick pea (*Cicer arietinum Linn,* Chana) and Prickly lettuce (*Lactuca scariola Linn,* Hattaraki) leaves are few of indigenous plants which are routinely consumed by the people of north Karnataka region of India in the diet. Studies revealed on these plants noticed some potential anti-diabetic efficacies.

Objectives: To evaluate the *in vitro* glucose reduction capabilities and anti diabetic effects of *Trigonella foenum graecum, Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* extracts in alloxan induced albino rats with or without Ni^{2+} supplementation. Also to study the anti diabetic role of these plant extracts in relationship to oxygen dependent micro environment *in vivo* in diabetic experimental model through diabetic regulatory molecular mechanisms analyzed by NOS3, HIF-1α and VEGF protein expressions. Further, to evaluate electrophysiological interpretation to understand functional pathophysiology of cardiovascular system and histopathology of liver, kidney, heart and aortic tissues of experimental diabetic rats were studied with the supplementation of the most efficient anti diabetic plan extract.

Method: *In vitro* and *in vivo* tests on glucose regulatory systems, analysis of molecular markers as NOS3, HIF-1α and VEGF were carried out on plant extracts supplemented to the diets of alloxan-induced diabetic rats. Electrophysiological analysis like HRV, LF: HF ratio, baroreflex sensitivity (BRS) and histopathology of myocardial tissues and elastic artery were also examined on diabetic rats treated with *Lactuca scariola Linn*.

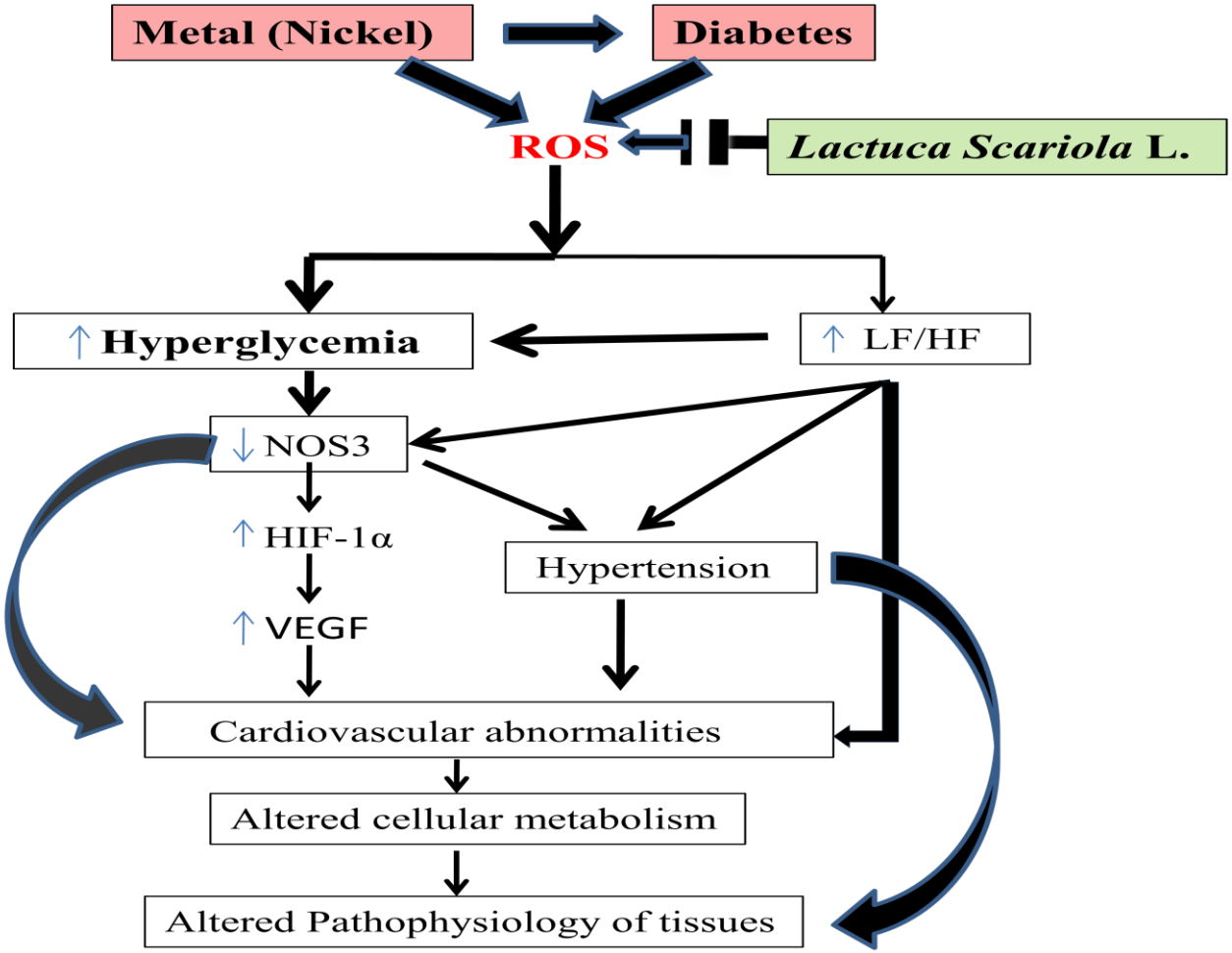
Results: Result indicates greater glucose reduction percentage in *Lactuca scariola Linn* as compared to *Trigonella foenum graecum.* Other plant extracts did not show any remarkable glucose reduction percentage *in vitro.* Result further showed the most beneficial role of *Lactuca scariola Linn* on glucose homeostasis in experimental diabetic rats. Studies on oxygen sensing molecular markers like NOS3, HIF-1α and VEGF reveals *Lactuca scariola Linn, Hibiscus cannabinus Linn* and *Cicer arietinum Linn* have beneficial role on expression of those proteins in diabetic rats as compared to diabetic rats without supplementation of any plant extract. Studies on cardiovascular impact on diabetes and subsequent supplementation of *Lactuca scariola Linn* showed better HRV, sympathovegal balance and baroreflex sensitivity in diabetic experimental rats as compared to without *Lactuca scariola Linn* supplementation. Study on histopathology

further confirms an improved architecture of myocardial tissues and elastic artery supplemented by *Lactuca scariola Linn*

Conclusion: These findings indicate *Trigonella foenum graecum* and *Lactuca scariola Linn* have anti diabetic properties, but *Lactuca scariola Linn* has greater beneficial effect on glucose homeostasis. Further it can be concluded that *Lactuca scariola Linn, Hibiscus cannabinus Linn* and *Cicer arietinum Linn* plant extracts have influence on oxygen sensing cell signaling mechanisms to protect against alloxan induced diabetic pathophysiology in albino rats. *Lactuca scariola Linn* is found to be the most effective anti diabetic and also a cardio protective.

Graphical Abstract

Graphical Abstract



Influence of *Lactuca scariola Linn* leaves extract on experimental diabetic model

Chapter 1

Introduction

1.1 Diabetes Mellitus

"Diabetes mellitus (DM), usually known as diabetes, is a group of metabolic diseases which result in a high blood sugar level over a prolonged period [1]." Diabetes causes many complications like heart disease, stroke, chronic kidney failure etc. if left untreated [2,3,4].

1.2 Types of Diabetes

Diabetes is either due to insufficient production of insulin by pancreas or the body cells fail to respond to the insulin produced [5]. Type 1 DM is because of the condition in which the pancreas produces less or no insulin and is referred to as "insulin dependent diabetes mellitus (IDDM)." Insulin is a hormone required for the body to transfer glucose from the bloodstream to the cells of the body [6]. Since, body produces insufficient insulin, the glucose converted from sugars and starch will not be carried to body cells and as a result the blood glucose level will be increased leading to type 1 diabetes. Type 2 diabetes in contrast is known as "non insulin dependent diabetes mellitus (NIDDM)." It is the most common form of diabetes. It is due to the condition in which body cells fail to respond to the produced insulin leading to an increase in the blood glucose level (Fig. 1.1). It is the most serious health problems around the world today accounting nearly up to 85-90% of all the diabetes cases [7].

1.3 Symptoms of diabetes

The most common symptoms of diabetes are increase in thirst, frequent urination, increased hunger, fatigue, blur vision, numbness of the feet or hands, unexplained weight loss etc [8].

Indications of type 1 diabetes may appear very soon i.e., in few weeks whereas, that of type 2 diabetes appear slowly i.e., over a period of few years and are very difficult to notice. Several people having type 2 diabetes don't show any symptoms at all.

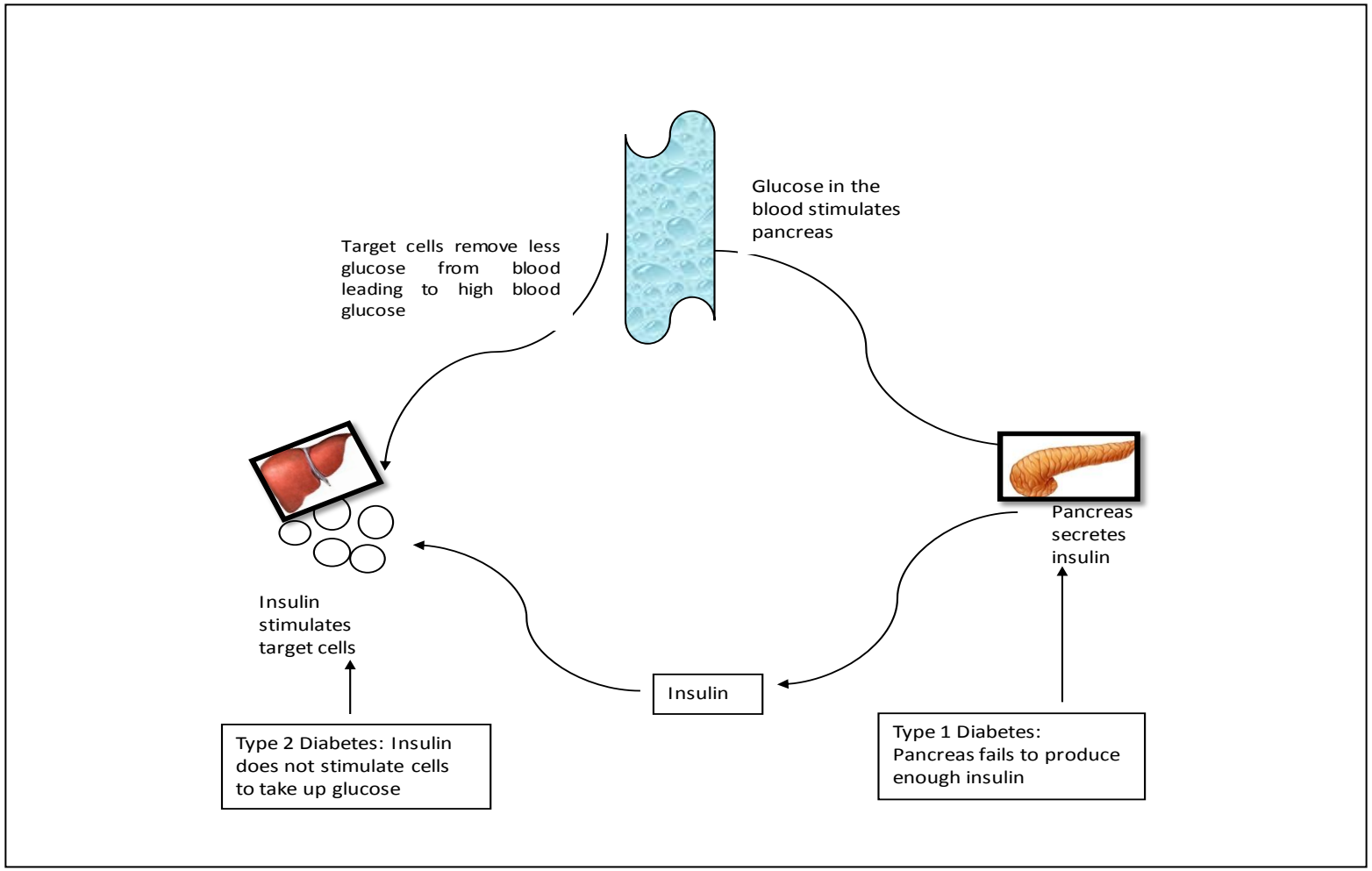


Fig. 1.1 Illustration of type 1 and type 2 diabetes

1.4 Causes of diabetes

Type 1 diabetes occurs due to the body's immune system attacks and destroys the β cells of pancreas which produce insulin. Scientists think type 1 diabetes is caused by genes and environmental factors that might trigger the disease [9].

Type 2 diabetes is caused by number of factors such as lifestyle, overweight, obesity, and physical inactivity. The people who are over weight due to less physical activity are more likely to develop type 2 diabetes. Extra weight also causes insulin resistance in type 2 diabetes. Extra belly fat is also linked to insulin resistance, type 2 diabetes and cardiovascular diseases [10].

1.4.1 Insulin resistance

Type 2 diabetes begins with insulin resistance i.e., muscles, liver and fat cells do not utilize the available insulin. Therefore, the body requires more insulin to help glucose enter cells. The insulin produced by the pancreas may not be sufficient enough and hence blood glucose levels rise.

1.4.2 Genes and family history

There are certain genes which are likely to cause type 2 diabetes. There are about 92 genes of type 2 diabetes mellitus. VEGF, HIF-1 α and NOS3 genes are considered as the most important diabetic regulatory genes [11]. Genes may increase the risk of type 2 diabetes by increasing a person's tendency to become overweight or obese.

This disease tends to run in families and occurs usually in these racial/ethnic groups like, Africans, Americans, Alaska Natives, American Indians, Asian Americans, Hispanics / Latinos, Native Hawaiians, and Pacific Islanders [12].

1.4.3 Molecular markers

Hyperglycemia decreases the availability of nitric oxide (NO) because of increase in the oxidative stress, reducing the efficacy of an endothelium derived vasodilator system which participates in vasculature homeostasis [13]. Nitric oxide syntase (NOS3) catalyze the production of nitric oxide from L-arginine in endothelial cells [14]. NOS3/eNOS gene plays a very major role in the susceptibility of type diabetes mellitus [15]. Researchers have shown that the impairment in neovascularisation due to hyperglycemia induced defect in activation of hypoxia inducible factor - 1α (HIF-1α), which is a transcription factor for regulating vascular endothelial growth factor (VEGF) expression [16]. In

patients with diabetes, VEGF may induce development of cardiac and limb vascular collateralization. VEGF plays a major role in mediating diabetic vasculopathy in many organs [17].

1.5 Heavy metals and diabetes

Diabetes mellitus, a serious metabolic disorder, is said to have affected more than 150 million people across the globe. The researchers of Asia have published their findings in 2009, stating that exposure to toxic heavy metals is one of the major contributors to this growing epidemic disease [18]. To fulfil the needs of the rising population rapid industrialization is taking place. The untreated effluent from the industries containing heavy metals as primary toxic pollutants is left to the nearby water bodies [19]. Along the sides of these water bodies agriculture lands also exist which get contaminated with these heavy metals. It is also interesting to know that heavy metals which are toxic to human health can accumulate in different parts of the plant as they are readily absorbed by the root system, resulting in retardation of plant growth [20]. On consumption of these plants directly or indirectly, leads to bioaccumulation of heavy metals in the human body damaging human organ functions and disrupting physiological homeostasis. The studies carried out on diabetes due to the effect of heavy metals *in-vitro, in-vivo* and on human subjects showed that the four major contributing heavy metals are Arsenic (As), Cadmium (Cd), Mercury (Hg) and Nickel (Ni). Arsenic causes insulin resistance, oxidative stress; Cadmium inhibits insulin release, damages insulin receptors, Mercury too inhibit insulin release resulting in a high blood glucose level. These heavy metals get accumulated in different biological samples of the people who are exposed to it causing diabetes [21]. Like other heavy metals, Nickel is an important industrial hazard and very few works have been done on its action on glucose regulatory mechanisms [22]. Some studies showed on Chinese adults, nickel has been associated with type 2 diabetes including insulin resistance [23]. In this study, an attempt is made to study the alterations of blood glucose level caused by the Ni (II) treatment on alloxan induced male diabetic rats.

1.6 Prevention / control of diabetes

Prevention of type 1 diabetes is not possible and prevention of type 2 diabetes is a very difficult task. However, changes in lifestyle can avoid serious problems of diabetes [24], which may lead to nerve, kidney and heart diseases. Some of the preventive tips from the "American diabetes Association" include,

i. Physical activity: Regular exercise helps to reduce weight, lower blood sugar level, and increase sensitivity to insulin which helps to keep blood sugar level in normal range.
ii. Dietary Fiber: Eating foods which are high in fiber help to reduce the risk of diabetes and heart disease.
iii. Lose extra weight: Overweight has many chances of leading to diabetes. Hence, losing the weight may reduce the risk of diabetes.

Oral medications: Sometimes the first treatments like physical activity, meal planning and weight loss may not be sufficient to decrease the blood glucose level to normal range. In that case, taking medicine, "oral anti diabetic medication" might help in lowering the blood glucose level. Either of the diabetes, type 1 or type 2 needs medication to keep the blood glucose level normal. The type of drug required depends on the type of diabetes one has like Sulfonylurea, biguanides etc.

In the case of type 1 diabetes, the most common medication given is insulin. The type of insulin needed depends on the severity of insulin depletion i.e.,

- Short acting insulin – Regular insulin (Humulin and Novolin)
- Rapid acting insulin – Insulin aspart (Novolog), Insulin glulisine (apidra), Insulin lispro (Humalog)
- Intermediate acting insulin – Insulin isophane (Humalin N and Novolin N)
- Long acting insulin- Insulin glargine (Lantus), Insulin detemir (Levemir)

In type 2 diabetes, usually oral medications are preferred. However, in some exception cases insulin is also supplemented in combination. Three classes of oral medications are recommended for type 2 diabetes that include,

Biguanides: These act to suppress hepatic glucose production. Metformin is the most commonly used biguanide for type 2 diabetes [25].

Thiazolidinediones (TZDs): These act to increase insulin sensitivity [26]. The most commonly used TZDs are rosiglitazone (Avandia), pioglitazone (Actos), troglitazone (rezulin).

Sulfonylureas: These act upon by increasing the plasma insulin concentrations [27]. The most commonly used sulfonylureas are glipizide, glibenclamide, glimepiride, gliclazide, glyclopyramide and gliguidone.

1.7 Contraindication of oral medication

Biguanides such as Metformin, Phenformin and Buformin which are used in the treatment of diabetes sometimes may have severe adverse effects on the health of human beings. These drugs have the risk of lactic acidosis, which is a condition where there is an increase of L-lactate in the body, which results in an excessively low pH **[**28, 29]. Several studies have reported that thiazolidinediones also produce numerous side effects like cardiovascular, hepatitis and liver damage risks [30]. On the other hand sulfonylureas have a very less side effects. However, all of these may contribute a little in causing weight gain. It can be seen that many of these oral medications which are in use to prevent or control diabetes may have one or the other kind of side effects, which may prove fatal in some cases. Hence, nowadays herbal medicines are being preferred to synthetic drugs.

1.8 Herbal medicine

The synthetic drugs despite offering an effective treatment options to cure this disease can have many adverse effects on the health such as, risk of liver disease, low blood sugar, weight gain, renal complications etc. Hence, in addition to the above mentioned treatments people are trying with numerous herbs and supplements to control diabetes. The increased scientific interest of the people and consumer demand has lead to the development of herbal products as dietary supplement. In developing countries, herbs are commonly used in the treatment of diseases [31]. These plant based alternatives may help in controlling blood sugar level, reduce insulin resistance and various other diabetes related complications. Some of these alternatives have shown promising results in animal studies. Many clinical studies conducted in recent times have shown potential links between herbal remedies and regulation of blood glucose level.

There are as many as 45000 plant species available in India, of which many are said to possess medicinal properties. Some of these plants which have medicinal properties are traditionally being used to treat diabetes [32]. Some of these plants are indigenous to

northern Karnataka region of India and are usually consumed by the people as dietary supplement. North Karnataka, locally known as Uttara Karnataka, is a geographical region which mostly consists of semi-arid plateau from 300 to 730 meters (980 to 2,400 ft) elevation that constitutes the northern part of the South Indian state of Karnataka.

1.8.1 Green leafy plants of North Karnataka

In this part of India, nearly about fifty one species of wild plants belonging to forty six genera are edible [33]. Some of these green leafy plants are grown indigenously in north Karnataka region. Recently researchers have shown a special interest on medicinal values of these leafy vegetables that grow exclusively in North Karnataka. The most commonly used and extensively grown green leafy plants of this region include fenugreek leaves (*Trigonella foenum graecum*), coriander leaves (*Coriandrum sativum Linn*), curry leaves (*Murraya koenigii*), Dill leaves (*Anethum graveolens*), Kenaf leaves (*Hibiscus cannabinus Linn.*), Prickly lettuce leaves (*Lactuca scariola Linn*), Spinach (*Spinacia oleracea Linn*), Chick pea leaves (*Cicer arietinum Linn*), and pig weed leaves (*Portulaca oleracea*). Some of these plants are believed to possess medicinal properties and some of these plants are even consumed to prevent or control diabetes. But the systematic scientific study has not been conducted. Hence, in this work an attempt is made to carry out the study on anti diabetic properties of some of these plants.

Chapter 2

Review of literature

2.1 Antihyperglycemic activity of oral medications

The commonly used biguanide i.e., Metformin reduces glucose absorption in the intestine. It increases uptake of circulating blood glucose by peripheral tissue and reduces hepatic glucose production. Insulin requirement during glucose metabolism will be reduced by metformin [34, 35].

Sulfonylureas: Earlier the most widely preferred oral anti-hyperglycemic medications were Sulfonylureas. These help in releasing insulin from the β cells of pancreas. Sulfonylureas bind to an ATP dependent K^+ channel on the cell membrane of β cells inhibiting tonic, hyperpolarizing out flux of K^+. This causes the electric potential over the membrane to be positive. This depolarization opens Ca^{2+} channels. Hence, increased intracellular Ca^{2+} leads to increase in the fusion of insulin granulae with the cell membrane and hence increases insulin secretion [36, 37].

Thiazolidinediones, also known as glitazones, bind to PPARγ, a type of nuclear regulatory protein involved in transcription of genes regulating glucose and fat metabolism. These PPARs take action on peroxysome proliferator responsive elements (PPRE) [38] influencing insulin sensitive genes resulting in a better use of glucose by the cells.

2.2 Classification and medicinal values of the plants

Many green leafy plants consumed by the people of northern Karnataka region as a dietary supplement are said to possess anti diabetic potential. The detailed classification of some of the plants used in this study as they are indigenous and readily available is as under:

Trigonella foenum graecum

Kingdom: Plantae

Order: Fabales

Family: Fabaceae

Genus: Trigonella

Species: *Trigonella foenum graecum*

Fig. 2.1 *Trigonella foenum graecum*

It is commonly known as fenugreek leaves and locally it is called as methi in Hindi, menthipalle in Kannada. It is an annual plant with leaves consisting of three small obovate to oblong leaflets (Fig. 2.1). India is the largest producer of fenugreek leaves. It is cultivated across the globe as a semiarid crop whose seeds are the common ingredient in dishes of South Asia. Its seeds are used as spice and fresh leaves as vegetable. Fresh fenugreek leaves forms the key ingredient in some of the Indian curries. In conventional medicine, fenugreek is used for proper digestion, induce labor and reduce blood sugar levels, although the evidence for these effects is lacking [39]. The seeds are commonly used to treat diabetes [40]. The work carried on Anti hyperglycemic effect of *Trigonella foenum graecum* seeds extract on alloxan induced diabetic rats showed that the seeds extract of fenugreek possess potential anti diabetic properties and can be used as alternative to treat diabetes [41]. Ethanolic extract of fenugreek leaves is said to possess significant anti diabetic property based on the study carried out on streptozotozin induced diabetic rats [42]. Another study is carried out in a rat model using *Trigonella foenum graecum* seed powder, wherein the results indicate the blood glucose level changes to near normal levels thereby managing the diabetic complications [43]. The works carried out on *Trigonella foenum graecum* show that it possesses anti diabetic properties. However, majority of the work carried out so far is by using seeds of the plant. The effect of its leaves or its extracts on type 1 / 2 diabetes has very limited information.

Hibiscus cannabinus Linn

Kingdom: Plantae

Order: Malvales

Family: Malvaceae

Genus: Hibiscus

Species: *Hibiscus cannabinus Linn*

Fig. 2.2 *Hibiscus cannabinus Linn*

It is commonly known as Indian hemp or Kenaf leaves in India and locally known as Pundipalle in Kannada, Gongura in Tamil/Telegu, Patsan and Pitwa in Hindi and Bengali respectively. It is a shrub with green to reddish stem height ranging from 1.5 to 4 m [44]. According to Joyner and Pate (1956) [45], there are two degrees of lobe formation in Kenaf. In one form it is found to be deep and compound in structure (Fig. 2.2), while in others it is very shallow and an entire leaf form is maintained. Leaves are two inches across, glabrous, cordate and the upper most part is 3-5 lobed. The tender leaves are consumed as vegetable in many parts of India especially in northern Karnataka region. The anti hyperlipidemic effect was studied using hydro alcoholic extract of Kenaf leaves in high fat diet fed rats, the results showed a strong anti hyperlipidemic activity and effective in reducing the levels of serum TC, TG, LDl-C and TBARS [46]. *Hibiscus cannabinus Linn* exhibits hepato protective activity against CCl_4 and paracetamol induced liver damages in rats [47]. The antioxidative activity of aqueous *Hibiscus cannabinus Linn leaves* extract was studied in rats with CCl_4 and paracetamol induced erythrocyte damage. The extract inhibits the growth of lipid peroxidation products in the plasma. The extracts also maintained superoxide dismutase and catalase antioxidant enzymes activity. *Hibiscus cannabinus Linn leaves* consumed in different parts of the world possess an erythrocyte protective activity against drug induced oxidative stress. The effect of methanolic leaves extract of *Hibiscus cannabinus Linn* on streptozotocin induced diabetic rats was found to have significant anti diabetic activity, which lowered the fasting blood glucose level significantly [48].

Cicer arietinum Linn

Kingdom: Plantae

Order: Fabales

Family: Fabaceae

Genus: Cicer

Species: *Cicer arietinum Linn.*

Fig.2.3 *Cicer arietinum Linn*

It is usually known as chick pea leaves, Bengal gram, Chana in Hindi, Kadli in Kannada, chhola in Bengali and its seeds are generally consumed in the diet. However, its young leaves are also eaten as cooked in some parts of the world, especially in India and Nepal. In malnourished populations, chick pea leaves can be supplemented as important dietary nutrients [49]. The plant grows 20–50 cm in height and has small feather like leaves on both side of the stem (Fig. 2.3). A number of chickpea varieties are cultivated across the globe. Bengal gram or 'Desichana' has small and a darker seed with a rough coat and is widely cultivated in northern Karnataka region of India. Other varieties known are Garbanzo bean (Kabuli chana), is light in colour with a smoother coat which is believed to be the variety from Kabul, Afghanistan [50]. Dark chickpeas contain plenty of fiber, which has numerous health benefits compared to that of 'Kabuli' legumes. The chick peas have beneficial effect on digestive tract because it has plenty of fibres. These also possess phytoestrogens which control the production of hormone that help in reducing chances of osteoporosis, breast cancer and hot flushes in women. Desi chana is good for patients with diabetes because of its rich protein and fibre content. It also has the ability to reduce triglycerides. A new flavanoid has been isolated from the seeds of *Cicer arietinum Linn* [51]. Though Chickpea is a good source of carbohydrates, protein, vitamins and minerals and forms a good source of dietary fibre [52, 53, 54] very few studies are available for its leaves. A preliminary research conducted shows that, consumption of Chickpea leaves lowers blood cholesterol [55].

Lactuca scariola Linn

Kingdom: Plantae

Order: Asterales

Family: Asteraceae

Genus: Lactuca

Species: *Lactuca scariola Linn.*

Fig. 2.4 *Lactuca scariola Linn*

It is also called as *Lactuca serriola L.* and normally known as prickly lettuce [56, 57]. It is known as Hattarki palle in Kannada, a local language. It is considered as weed of roadsides and field crops. It is the closest wild relative of cultivated lettuce (*Lactuca sativa L.*). *Lactuca scariola Linn* has a spineless reddish stem, containing milky latex, growing from 30 to 200 cm (12 to 79 in). *Lactuca scariola Linn* leaves are oblong often pinnate and gradually become smaller as they reach its top. Along the veins and leaf edges fine spines are present. The undersides have whitish veins (Fig. 2.4). Leaves when plucked, latex oozes out. Young *Lactuca scariola Linn* leaves are consumed in the form of salad, even though it has somewhat bitter taste [57]. The tender leaves are mild and make an excellent salad, but the plant is toxic and bitter to taste when grown fully. Consumption of these leaves in large quantities can cause gastric upsets. The juice of the leaves is used in the treatment of jaundice and it is useful for the pregnant women as it is rich in calcium and iron [33]. A new compound, triterpenoid saponin is isolated from the seeds of *Lactuca scariola Linn.* The compound "3-o-[β-D-galactopyranosyl-(1-3)-o-β-D-xylopyranosyl-(1-4)-o-α-L-rhamnopyranosyl]-oleanolic acid," showed antimicrobial activity against various bacteria and fungi [58].

2.3 Phytochemicals in the prevention of diabetes

Phytochemicals (in Greek, phyto means plant) are the plant chemicals which possess disease preventing properties. These are not the nutrients that are required by the human body for sustaining life. Earlier it was known that these chemicals are produced by plants to protect themselves. According to many researches conducted, phytochemicals influence the chemical processes of human body in many ways. Some of the phytochemicals are beneficial for health, while some phytochemicals like cocaine, nicotine, codeine etc are not.

2.3.1 Types of phytochemicals

Natural compounds include complex carbohydrates, alkaloids, glycopeptides, terpenoids, peptides and amines, steroids, flavonoids, lipids, coumarins, sulfur compounds, inorganic ions and others. The anti diabetic mechanisms are many, including direct competitive antagonism with insulin, stimulation of insulin secretion, stimulation of glycogenesis and hepatic glycolysis, adrenomimeticism, pancreatic β cell potassium channel blockers, cAMP (2nd messenger) stimulation, modulation of glucose absorption from the gut, among others. There are thousands of different phytochemicals, some of which are of particular interest in our study are listed below.

2.3.1.1 Polyphenols

These are the largest group of phytochemicals [59], which are considered as the powerful antioxidants. They are further split into several subgroups, mainly flavonoids.

Flavonoids

"Flavonoids are the naturally occurring polyphenolic compounds which are widely distributed in plants." There are as many as 4000 individual flavonoids. Subclasses of flavonoids include flavonols, flavanols, flavones and isoflavones. Flavonoids are considered to be strong antioxidants because of the presence of aromatic hydroxyl groups. They are the scavenger of reactive oxygen and nitrogen species, inhibiting peroxidation reactions [60, 61]. A flavonoid, epicatechin is reported to protect normal rat islets from alloxan, normalizing blood glucose levels and promotes regeneration of β cells in islets of alloxan treated rats [62]. Another flavonoid, quercetin was able to normalise the blood glucose level in alloxan induced diabetic rats [63]. Another experiment showed increase in the insulin release by 44–70% due to exposure of isolated rat islets to flavonoids like epicatechin or quercetin [64]. Experiment conducted on the treatment of quercetin on streptozotocin induced diabetic rats resulted in the increase of number of islets of pancreas, probably increased insulin release and also induced hepatic glucokinase enzyme [65].

Saponins

"Saponins are naturally occurring plant glycosides having soap like characteristics and produce lather when mixes with water [66, 67]." More than hundred families of plants both cultivated and wild plants contain saponins. The triterpenoid saponins are dominant in cultivated crops and steroid saponins are common in herbs [68]. Triterpenoids are present in beans, peas, tea, spinach etc. Steroid saponins are present in oats, capsicum,

tomatoes, fenugreek etc. The unique chemical structure of saponin produces foam when mixed with water. It binds with water as well as oils and fats i.e., saponins produce an emulsification of fat soluble molecules.

The chemical structure of saponins gives many health benefits such as, favourable effect on cholesterol, boosts the immune system and also has an antioxidant effect. A group of saponins found in legumes called group B soyasaponins as antioxidant moiety attached at C_{23} [69, 70]. This sugar residue DDMP, allows saponins to scavange superoxides by forming hydroperoxide intermediates, which prevent biomolecular damage by free radicals [71]. In an experiment conducted to study the anti diabetic activities of *Momordica charantia*, the potentially active compounds present were saponins, the fractions were characterised using LC-ToF-MS [72].

Triterpenes

"Triterpenes are the chemical compounds composing of three terpene units having the molecular formula $C_{30}H_{48}$." Triterpenes exist in many structures with more than 200 skeletons obtained from natural sources or through enzymatic reactions [73]. Triterpenes, also present in the form of triterpenoid saponins belong to saponin group of compounds are meant for defence mechanism [74]. Many experiments on triterpenes showed different explanation of its anti diabetic mechanism. Triterpenes inhibit enzymes involved in glucose metabolism, prevent insulin resistance, and normalize plasma glucose and insulin levels. They have strong antioxidant activity and inhibit the formation of advanced glycation end products [75].

2.4 How pH matters for GI absorption of dietary nutrients?

Different parts of gastro intestinal tract have different pH conditions because each stage of the digestive process requires different pH conditions. "Digestion is the process in which the food is broken down into components that can be readily absorbed by the body." The process starts with saliva, then passes down to the stomach, where enzymes break down the food further. Digestion and absorption of nutrients occurs in the intestine with the help of enzymes.

Enzymes secreted from intestine and pancreas breakdown carbohydrates to glucose, proteins to amino acids and fats to fatty acids and glycerol. Different enzymes are effective at different pH conditions. Components in saliva maintain the oral pH between 6.5 and 7.0, which is required for salivary amylase enzyme to break down carbohydrates.

The digestion of food in stomach, by the enzymes like pepsin require pH of about 2, while the enzymes, peptidase and maltase in the intestine require pH of about 7.5 [76].

Chapter 3

Aims and Objectives

3.1 Aims

To study the anti diabetic efficacies of the aqueous extract of some green leafy plants like *Trigonella foenum graecum, Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* in presence or absence of heavy metal (nickel). Also to find out the most potent anti diabetic efficacies among these four plants extracts with reference to diabetes regulatory molecular markers like sNOS3, sHIF-1α and sVEGF proteins expressions.

3.2 Objectives

- To evaluate the glucose reduction capabilities of aqueous extracts of fresh green leafy vegetables (*Trigonella foenum graecum, Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn*) at different pH (2.0, 7.0 and 9.0) conditions *in vitro* and also to identify the plant with greatest glucose reducing capabilities.
- To evaluate the effect of aqueous plant extracts with heavy metal Ni^{2+} supplemented glucose solution at different pH (2.0, 7.0 and 9.0) conditions *in vitro.*
- To evaluate the anti diabetic activity through glucose homeostasis by aqueous plant extracts of *Trigonella foenum graecum, Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* with and without Ni^{2+} supplementation in alloxan induced diabetic male albino rats *in vivo*. Further to identify the plant which is having most efficient anti diabetic activity *in vivo*.
- To evaluate possible anti diabetic role of these aqueous leaves extract of North Karnataka plants and their relationship with oxygen dependent micro environment *in vivo* in diabetic experimental model through diabetic regulatory molecular mechanisms analyzed by NOS3, HIF-1α and VEGF protein expressions.
- To identify the effect of most efficient anti diabetic plant extract of this study through glucose homeostasis and diabetes regulatory molecular markers and its further impact on electrophysiological interpretation to understand functional pathophysiology of cardiovascular system.

- Also to evaluate the effect of the most potent anti diabetic plant extract on histopathology of liver, kidney, heart and aortic tissues of experimental diabetic rats.

3.3 Hypothesis

We hypothesize that some of the green leafy vegetables consumed as a dietary supplement by the people of Northern Karnataka region of India have potential anti diabetic activity and are also beneficial for cardiovascular complications.

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Selection of plant materials

There are several green leafy vegetables which have potential medicinal values are readily available in most part of the north Karnataka region of India namely *Anethum graveolens Linn, Hibiscus cannabinus Linn, Amarantus paniculatus Linn, Amaranthus tenuifolius, Murraya koenigii, Coriandrum sativum, Lactuca scariola Linn., Cicer arietinum Linn., Trigonella foenum graecum, Spinacea oleracea* etc [77]. For the present study, we have selected four green leafy plants as they are widely consumed by the people of this region as a dietary supplement.

4.1.2 Collection and identification of plants

Fresh green leaves were collected either from the local market or directly from the agriculture field and were identified and authenticated as *Trigonella foenum graecum* (Fenugreek), *Hibiscus cannabinus Linn* (Kenaf), *Cicer arietinum Linn* (Chick pea) and *Lactuca scariola Linn* (Prickly lettuce) from the department of Botany, BLDEAssociation's S. B. Arts and K. C. P. Science college, Vijayapur [78].

4.1.3 Preparation of Plant extract

5 g of fresh leaves were washed thoroughly with distilled water and were soaked for few minutes. Soaked leaves were then added to 100 mL distilled water and heated in a water bath at 50^0C to 60^0C for about 1h and filtered using Whatman filter paper no. 1 in 100 mL measuring flask and made up to the mark using distilled water. Filtrate is used as a plant extract for carrying out the further work [79].

4.1.4 Chemicals

Preparation of Glucose solution

Stock solution: Dissolve 100mg of Glucose in 100mL of distilled water. Each mL of stock solution contains 1mg of Glucose.

Working standard (10mg/dL): Dilute 10mL of Stock solution to 100mL using distilled water in a measuring flask.

Working standard (20mg/dL): Dilute 20mL of Stock solution to 100mL using distilled water in a measuring flask.

Preparation of 0.1M acetate buffer solution (pH 2.0)

0.1M $CH_3COONa.\ 3H_2O$: Dissolve 1.36 g of $CH_3COONa.\ 3H_2O$ in 100mL distilled water.

0.1M CH_3COOH: Dilute 0.7mL of CH_3COOH to 100mL using distilled water in a measuring flask.

Add 1mL of 0.1M $CH_3COONa.\ 3H_2O$ and 99mL of 0.1M CH_3COOH and then adjusted to pH 2.0 by adding 0.1M HCl.

Preparation of 0.1M Phosphate Buffer solution (pH 7.0)

1. 1 M Phosphate buffer: Dissolve 0.0096g of $NaH_2PO_4.2H_2O$ and 0.008g of Na_2HPO_4 in 100mL distilled water.
2. 0.1 M Phosphate buffer: Dilute 1mL of 1M buffer solution to 10 mL using distilled water.

Preparation of 0.1M Phosphate buffer solution (pH 9.0)

0.1M Phosphate Buffer solution of pH 7.0 is adjusted to pH 9.0 by adding 0.1M NaOH solution.

Preparation of Glucose Oxidase-Peroxidase reagent

5mg of O-Dianisidine is dissolved in 0.2mL of methanol. Followed by the addition of 9.8mL of 0.1M buffer solution, 1 mg of Glucose Oxidase and 1mg of Peroxidase enzymes (Glucose oxidase from Aspergillus niger, Sigma, Horseradish Peroxidase, Merk).

Preparation of 6N HCl

Dilute 6.8mL of Conc. HCl to 10mL using distilled water.

Preparation of $NiSO_4$ Solution

Stock solution: Dissolve 100 mg of $NiSO_4$ in 1000mL of distilled water

Working Standard:

20mg/L: Dilute 20mL of the stock solution to 100mL using distilled water in a measuring flask

40mg/L: Dilute 40mL of the stock solution to 100mL using distilled water in a measuring flask

60mg/L: Dilute 60mL of the stock solution to 100mL using distilled water in a measuring flask

80mg/L: Dilute 80mL of the stock solution to 100mL using distilled water in a measuring flask

Preparation of 15% alloxan monohydrate

15g of Alloxan monohydrate dissolved in 100mL of distilled water for the treatment dosage of 15 mg / 100 g.b.wt. of rats.

4.1.5 Animals

Adult male albino rats of Wistar strain (*Rattus norvegicus*) which weighed 180-200 g were kept in animal house of "BLDE University's Shri. B. M. Patil Medical College, Hospital and Research Centre, Vijayapur, Karnataka, India," with 12h light and 12h dark cycles. "Shri. B. M. Patil Medical College, Hospital and Research Centre" is the sister institution under BLDE Association, Vijayapur, Karnataka, India. Three rats were housed in each metabolic wire cage (60cm x 30cm x 20cm). Hindustan Lever rat feed (Mumbai, India) was given as basal diet for rats during the experiments. The experiments were performed as per guidelines of "Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA)."

4.1.6 Ethics

The experiments using animals were performed as per the ethical norms approved by CPCSEA, "Ministry of Social Justice and Empowerment, Government of India," and ethical clearance granted by "Institutional Animal Ethical Committee, BLDE University, Vijayapur, Karnataka."

4.2 Methods

4.2.1 Phytochemical analysis

Preliminary phytochemical analysis of the aqueous leaves extracts is evaluated to find out the presence of various secondary metabolites such as alkaloids, saponins, triterpenes, flavonoids, tannins, steroids etc using standard procedures [80].

i. Test for alkaloids

Wagner's test: 2mL of extract is added to 2mL of freshly prepared Wagner's reagent. Reddish brown precipitate indicates presence of alkaloids.

Mayer's test: 2mL of aqueous extract is added to 2mL of freshly prepared Mayer's reagent. Cream coloured precipitate indicates presence of alkaloids.

Dragendorff's test: 2mL of extract is added to 2mL of freshly prepared Dragendorff's reagent. Orange coloured precipitate indicates presence of alkaloids.

ii. Test for Carbohydrates

Fehling's test: A mixture of 1mL of Fehling's A solution and 1mL of Fehling's B solutions was boiled. 2mL of aqueous extract was added to the above prepared solution and heated on a water bath for 10mins. Brick red precipitate confirms the presence of carbohydrates.

Molish's test: Few drops of α–naphthol solution was added to 2mL of aqueous extract and mixed well. Add Conc. H_2SO_4 along the sides of the test tube. Appearances of purple coloured ring at the junction of two layers indicate presence of carbohydrates.

Benedict's test: 5mL of Benedict's reagent added to 2mL of aqueous extract. Mixed well, boiled and cooled. Appearance of yellowish green precipitate indicates the presence of carbohydrates.

iii. Test for Flavonoids

Lead acetate test: 2mL of extract is added to 2mL of 10% Lead acetate solution. Yellow coloured precipitate indicates presence of flavonoids.

Mineral acid test: 2mL of extract is added to 2mL of Conc. H_2SO_4. Yellow colouration indicates presence of flavonoids.

iv. Test for Saponins

Libermann Burchard test: Dissolve the aqueous extract in acetic anhydride and add about 1mL of acetic acid and 1mL of chloroform. To this solution add Conc. H_2SO_4 though the sides of the test tube. Appearance of violet or purple coloured ring at the junction of two rings indicates the presence of saponins.

v. Test for Tannins

Ferric chloride test: Very dilute $FeCl_3$ is added gradually to aqueous extract. Appearance of olive green colour indicates the presence of Tannins.

Gelatin test: 2mL of aqueous extract is added to 1% gelatin solution in 10% NaCl. Immediate precipitation indicates the presence of Tannins.

vi. Test for Triterpenes

Libermann storch Morawski test: 2 mL of aqueous extract with 1 mL of warm acetic anhydride and a drop of Conc. H_2SO_4. Appearance of green colouration indicates the presence of Triterpenes.

Salkowski's test: 2mL of extract with 1mL of Chloroform is added to few drops of Conc. H_2SO_4. Appearance of yellow colour in chloroform layer indicates the presence of Triterpenes.

vii. Test for Steroids

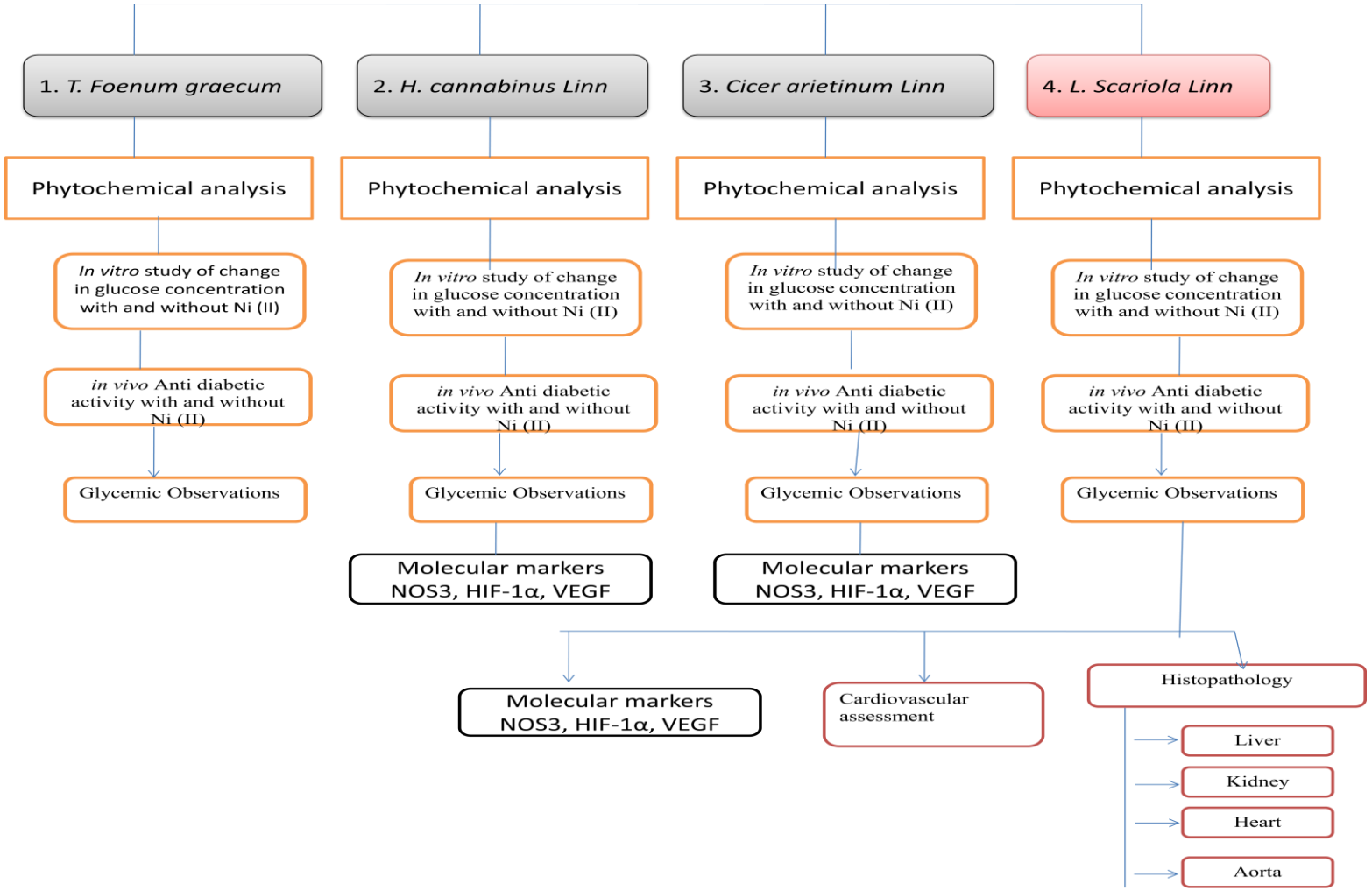
Libermann Burchard Sterol reaction: 1 drop of Conc. H_2SO_4 is added to 2 mL of aqueous extract in glacial acetic acid. Appearance of red colour indicates the presence of Steroids.

viii. Test for Carotenoids

Antimony trichloride: 2 mL of the extract is added to the mixture of Antimony trichloride in Chloroform. Appearance of blue colour indicates the presence of carotenoids.

Conc. HCl with Phenol: 2 mL of the aqueous extract is added to Conc. HCl containing Phenol. Appearance of dark blue colour indicates the presence of Carotenoids.

EXPERIMENTAL DESIGN



Note: plant extracts of 1, 2, 3, and 4 were gone under *in vitro* and *in vivo* study for glucose homeostasis with and without nickel. Further plant 2, 3, 4 were evaluated for diabetic molecular markers analysis (Plant 1 is already known anti diabetic with molecular understanding. Hence it was not taken into further analysis of diabetic regulatory gene expression in this study) [81]. Based on the observations, plant 4 was further analysed for its effect on cardiovascular system and peripheral tissue histopathology.

4.2.2 *in-vitro* study

4.2.2.1 Estimation of glucose at different pH conditions

The estimation of glucose concentration (*in vitro*) was determined by glucose oxidase - peroxidase enzymatic method at pH 2.0, 7.0 and 9.0 [82]. Briefly, 0.0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL and 1.0 mL of Standard Glucose solution (Working standard of 10mg/dL) was added to 1.5 mL, 1.0 mL, 0.8 mL, 0.6 mL, 0.4 mL, 0.2 mL and 0.0 mL of distilled water respectively in different test tubes. Followed by 0.5 mL of freshly prepared aqueous plant extracts and 1 mL of Glucose Oxidase-Peroxidase reagent were added to each test tube (Table 4.1) and incubated at 35^0C for about 40 to 50 mins. The reaction is completed by adding 2 mL of 6N HCl. Results were recorded at 540 nm using Elico SL210 UV-Vis Spectrophotometer. Change of glucose concentration was measured by the equation:

$$Concentration of Glucose = \frac{(Absorbance\ of glucose + Extract) X\ Initial\ Concentration\ of glucose}{Absorbance\ of glucose}$$

Table 4.1 Estimation of glucose at different pH *in vitro*

Sl. No	**Reagents**	**Blank**		**Std. Glucose solution + aqueous extract**				
		B_1	**B_2**	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**
1	Initial glucose concentration (mg / dL)	0.0	0.0	20	40	60	80	100
2	Working std. (mL)	--	--	0.2	0.4	0.6	0.8	1.0
3	Distilled water (mL)	1.5	1.0	0.8	0.6	0.4	0.2	0.0
4	Aqueous extract (mL)	--	0.5	0.5	0.5	0.5	0.5	0.5
5	Glucose oxidase-peroxidase reagent (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0

4.2.2.2 Estimation of glucose at different pH conditions with Nickel II

The estimation of glucose (*in vitro*) in the presence of nickel II of different concentrations was also determined at different pH conditions. 0.1 mL of $NiSO_4$ solution of different concentrations such as 20 mg/L, 40 mg/L, 60 mg/L and 80 mg/L were added in different testtubes, followed by 0.1 mL of standard glucose solution (working standard of 20mg/dL), 0.8 mL of distilled water, 0.5 mL of freshly prepared aqueous plant extracts and 1 mL of Glucose Oxidase-Peroxidase reagent were added to each test tube (Table 4.2). Then all the solutions were incubated at 35^0C for about 40 to 50 mins. The solutions are taken out from the incubator and then 2 mL of 6N HCl is added to complete the reaction. Results were recorded at 540 nm using Elico SL210 UV-Vis Spectrophotometer [79]. Reduction in the glucose concentration was measured using the equation:

$$Conc.\ of\ Glucose\ = \frac{(Absorbance of glucose + extract) X\ Initial\ Concentration\ of glucose}{Absorbance\ of glucose}$$

Table 4.2 Estimation of glucose at different pH with Ni^{2+} *in vitro*

Sl. No	**Reagents**	**Blank**		**Std. Glucose solution + aqueous extract**			
		B_1	**B_2**	**T_1**	**T_2**	**T_3**	**T_4**
1	Initial glucose concentration (mg/dL)	0.0	0.0	20	20	20	20
2	Working std. (mL)	--	--	0.1	0.1	0.1	0.1
3	$NiSO_4$ Solution (mL)	--	--	0.1 (20mg/L)	0.1 (40mg/L)	0.1 (60mg/L)	0.1 (80mg/L)
4	Distilled water (mL)	1.5	1.0	0.8	0.8	0.8	0.8
5	Aqueous extract (mL)	--	0.5	0.5	0.5	0.5	0.5
6	Glucose oxidase-peroxidase reagent (mL)	1.0	1.0	1.0	1.0	1.0	1.0

4.2.2.3 Spectral Analysis

The chemical behaviour of Ni^{2+} alone and in combination with different aqueous plant extracts at pH 7.0 and pH 9.0 (*in vitro*) were recorded using double beam UV-visible spectrophotometer (Elico SL 210; Elico Pvt, Ltd., Bangalore, Karnataka, India) [83].

4.2.3 *In vivo* study

4.2.3.1 Induction of Diabetes

Rats were made to fast overnight and diabetes was induced with a single intra peritoneal injection (i.p.) of freshly prepared 15% alloxan monohydrate solution (15 mg / 100 g bodyweight) in normal saline just before use. As alloxan is capable to produce hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution (i.p.) after 6 h. Then the rats were kept for the next 24 h with 5% glucose solution bottles in their cage to avoid hypoglycemia [84]. After 3 days blood samples were collected from the rat's tail vein. Fasting blood glucose levels were estimated by glucose oxidase - peroxidase enzymatic method after serum was separated and diabetes was confirmed [85]. Only the rats which showed hyperglycemia i.e., glucose level above 250 mg / dL were used for study.

4.2.3.2 Acute oral toxicity studies of extract

The acute oral toxicity study of extracts was performed as per the "organization for economic cooperation and development (OECD)" guidelines, draft guidelines 423 adopted on 17 December 2001 received from "committee for the purpose of control and supervision of experiments on animals (CPCSEA)", Ministry of Social Justice and Empowerment, Government of India [86]. Different doses of the aqueous leaves extracts from 5mg/100g.b.wt to 125mg/100g.b.wt were administered to the rats. The stepwise doses did not show any significant signs of toxicity. Hence, one tenth of this upper limit dose was selected for carrying out anti diabetic activity.

4.2.4 Glucose Homeostasis

4.2.4.1 Experimental groups

The experimental rats were divided into nine groups of six rats each. These nine groups were classified as, Group I to Group IV (non diabetic rats) and Group V to Group IX (diabetic rats). Supplementation and dosage of different group of rats is as shown in table 4.3.

Table 4.3 Experimental groups

Groups	**Supplementation**	**Dosage**
Group I	Normal Control	Distilled water
Group II	Non diabetic rats + aqueous extract	12.5 mg/ 100g.b.wt, orally
Group III	Non diabetic rats + Ni^{2+}	2.0mg / 100g.b.wt, i.p. [87]
Group IV	Non diabetic rats + Ni^{2+}+ aqueous extract	2.0mg / 100g.b.wt i.p. and 12.5 mg/ 100g.b.wt, orally
Group V	Diabetic control	Distilled water
Group VI	Diabetic rats + aqueous extract	12.5 mg/ 100g.b.wt, orally
Group VII	Diabetic rats + Ni^{2+}	2.0mg / 100g.b.wt i.p. [87]
Group VIII	Diabetic rats + Ni^{2+}+ aqueous extract	2.0mg / 100g.b.wt i.p. and 12.5 mg/ 100g.b.wt, orally
Group IX	Diabetic rats + Glipizide (ref. drug)	2.5 mg/kg [88]

4.2.4.2 Oral glucose tolerance test (OGTT)

Fasting blood sugar (FBS) level was measured in rats of all the groups. After half an hour of administration of aqueous leaves extract or Ni^{2+}or both or glipizide, rats of all nine groups were orally treated with glucose at 0.35 mg/100g.b.wt. Blood samples were collected from the rat tail vein just before the administration of glucose **(**0.00h) and at intervals of 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose loading. Blood glucose level was instantly measured using Accu check glucometer [89].

4.2.4.3 Sub chronic (13 days treatment) studies on leaves extract in diabetic rats

From the day of oral glucose tolerance test (OGTT), supplementation of aqueous leaves extracts orally (12.5mg/100g.b.wt) was continued every day in designed experimental groups (group II, group IV, group VI and group VIII) for 13 days. Similarly, Ni^{2+} (2.0 mg/100g.b.wt; i.p. on day 1, day 3, day 5, day 7, day 9, day 11 and day 13) was administered in group III, group IV, group VII and group VIII rats. Blood sample was collected from rat tail vein after half an hour of injection of aqueous leaves extract or Ni^{2+} treatment or both and glipizide treated rats on 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 9^{th}, 11^{th} and 13^{th} days in rats of all groups. Blood glucose level was immediately measured using Accu check glucometer [90].

4.2.4.4 Plasma Insulin and Insulinogenic Index:

The collected blood samples were transferred to microtubes containing heparin and then placed in an ice bath for about 20 mins and centrifuged at 4000 rpm for 7 mins. After centrifugation the plasma insulin concentrations were measured at 0 and 30 minutes of glucose administration by ELISA kits (ERINS, Thermo Fisher Scientific, Life technologies). Insulinogenic index was estimated after oral glucose (1g / kg b.wt.) administration. Then blood samples were collected with heparinised capillary tubes from the retro-orbital plexus at 0 and 30 minutes time interval for the measurement of blood glucose and plasma insulin concentrations[91]. Insulinogenic index was further calculated using the equation

$$\text{Insulinogenic index} = \frac{30\ \text{min plasma insulin} - \text{fasting plasma insulin}}{30\ \text{min blood glucose} - \text{fasting blood glucose}}$$

4.2.5 Analysis of molecular markers

Molecular markers were analysed on rats of different groups namely, group 1, control; group 2, Diabetic; group 3, diabetic + extract (*Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn*); group 4, diabetic + glipizide; using micro plate reader Merilyzer EIAQuant and micro plate washer Merilyzer EIAWash. For the determination of nitrosative stress, serum endothelial - NOS (e-NOS / NOS3) was estimated by ELISA kit (CSB-E08323r), Cusabio Biotech Co. Ltd. [92] and sNO was estimated by Greiss reagent, spectrophotometrically [93]. sVEGF was measured using ELISA kit (KT-EK0540, Boster Biological Company, USA) and sHIF-1α was estimated by ELISA kit (KT-17920, Kamiya, Biomedical Company, USA) [94].

Fig. 4.1 loading of sample during analysis of Molecular markers

Fig. 4. 2 Merilyzer EIAQuant

4.2.6 Electrophysiology Analysis of *Lactuca scariola Linn* supplemented rats.

Electrophysiology experiments were carried out on the rats of following the three groups,

I. Pre diabetic
II. Diabetic
III. Diabetic + *Lactuca scariola Linn*

The Wistar rats used for the study were anaesthetized with ketamine / xylazine injected intraperitonealy (i.p.) at a dose of ketamine 100mg/kg + xylazine 15mg/kg. Adequate anaesthesia was ensured by the total disappearance of pedal reflexes [95]. Systolic blood pressure (SBP) and diastolic blood pressure (DBP), pulse pressure (PP), mean arterial pressure (MAP) and heart rate were recorded.

4.2.6.1 Analysis of Heart Rate Variability (HRV)

ECG of the rats was recorded with PC based MP45 Biopac instrument connected through BSL 4.1 (Biopac Student Lab 4.1) software. Bipolar electrodes were attached to the upper and lower limbs of rats to record an ECG; HRV was analyzed by using Kubios HRV Software, version 2.0 developed by Department of Physics, University of Kuopio, Finland.

The analysis of HRV was done by three different methods namely, time-domain, frequency-domain and non linear methods.

4.2.6.2 Pre-processing methods

Any artifacts in the HRV interfere with the analysis of the signal. The artifacts within HRV signals are divided into technical and physiological artifacts. The most common issues in HRV analysis are motion artifacts and ectopic beats which can create considerable problems during HRV analysis. Hence, it has become an integral part of any analysis to include pre processing before RR intervals are acquired. However, RR intervals obtained from ECG recordings are imperfect in most cases, as they can contain a different number of abnormal beats, artifacts.

4.2.6.3 Removal of artifacts

Improper correction of artifacts in interbeat interval (IBI) data may lead to unreliable HRV results [96]. In kubios software there are two methods to correct artifacts from corrupted RR series namely, Automatic correction (Available in only Premium) and Threshold correction. In this study we have used threshold correction which compares

every beat interval against a local average RR, and finds the beat as artifact if it exceeds the specified threshold. The threshold value can be selected from Very Low (0.45 threshold in sec), Low (0.35 sec), medium (0.25 sec), Strong (0.15 sec), Very Strong (0.05 sec) and custom for setting a custom threshold in seconds.

4.2.6.4 Time domain methods

The time domain methods are applied directly to the series of successive RR interval values. A number of variables which determine the variability within the RR series also exist. The other parameters that are determined using RR interval values are Standard deviation of normal – normal RR intervals (SDNN) and Square root of mean of square difference of successive RR intervals (RMSSD). SDNN provides a major of total variability and RMSSD provides parasympathetic drive of ANS.

4.2.6.5 Frequency domain methods

In the frequency-domain methods, a spectrum estimate is calculated for the RR interval series. A power spectrum density (PSD) estimate is calculated for the RR interval series. FFT based methods are generally used to estimate PSD. The HRV spectrum is calculated with FFT based Welch's periodogram method. The frequency bands in case of short-term HRV recordings are of very low frequency (VLF, 0–0.04 Hz), low frequency (LF, 0.04–0.15 Hz), and high frequency (HF, 0.15–0.4 Hz). The frequency-domain measures extracted from the PSD estimate for each frequency band include absolute and relative powers of VLF, LF, and HF bands, LF and HF band powers in normalized units, the LF/HF power ratio, and peak frequencies for each band. In the case of FFT spectrum, absolute power values for each frequency band are obtained by simply integrating the spectrum over the band limits.

4.2.6.6 Non linear methods

Due to heart's complex control system, linear methods alone can not describe HRV. Hence, different nonlinear methods were also applied to HRV, to completely capture the characteristics of beat-to-beat variability.

4.2.6.7 Poincare plot

The Poincare plot is a graphical representation of the correlation between consecutive RR intervals, i.e. a plot of RR as a function of RR. The shape of the plot is quantified by fitting an ellipse into the data points oriented along the line of identity. The width and length of the ellipse are determined by the standard deviations of the point's

perpendicular to and along the line of intersection which are denoted by SD1 and SD2, respectively. SD1 measures short-term variability and SD2measures long-term variability.

4.2.6.8 Measurement of baroreflex sensitivity (BRS) test

Blood pressure (SBP, DBP) of rats were measured by using MP 45 Biopac; NIBP 200 A model. The baroreflex was tested with a pressor dose of phenylephine (PHE, bolus 8 μg/kg IV; Siga Chemical). The baroreflex was evaluated as the derivation of HR in function of the MAP variation (ΔHR/ΔMAP). There was an interval of at least 15 min between the infusions to allow basal values to recover.

Fig. 4.3 Electrophysiology experimentation using Biopac MP45

4.2.7 Histopathological studies of *Lactuca scariola Linn* supplemented rats

Histopathology of Liver, Kidney, Heart and Aorta

4.2.7.1 Tissue collection

After euthanasia, rats were kept in supine position and dissected. Kidney, liver, heart and aorta were collected and washed thoroughly in normal saline [97]. Histopathology of liver, kidney, heart and aorta was done. On the basis of routine histological techniques, the samples were put into paraffin and sections of 5 μm were taken from tissue blocks.

4.2.7.2 Tissue processing

Fixation: It is the first step during the preparation of histological section from a dead biological specimen. Tissues were kept in fixatives for nearly about 24 h. Most commonly used fixatives are formalin, Zenker's fluids and Bouin's fluid.

Washing: After fixation tissues were washed thoroughly under running tap water till fixative is completely removed from the tissue.

Dehydration, Clearing and Impregnation

As paraffin is not miscible with water of the tissues, it is not possible to imbibe the tissues with paraffin directly. Hence, dehydration is carried out in stages. In the first stage, tissues were immersed in alcohol which replaces water of tissues. Paraffin is not miscible with alcohol too; Xylene, Benzene, Cloroform and Toluene which are called clearing agents get mixed with both alcohol and paraffin. Hence, after alcohol, tissues were treated with one of these liquids and thereafter tissues were impregnated in melted paraffin (Table 4.4).

Embedding

Embedding enables small and delicate specimens to be surrounded by suitable material like paraffin that will support the tissues from all the sides without damaging the tissues. Then the tissues were made into sections of sufficiently thin slices without distortion.

Preparation of Blocks

It is done by using L-blocks which are metallic pieces. Two L-blocks are placed on a glass plate so that they make a rectangular space. The tissues were kept in the rectangular space and molten paraffin is poured on it which gets solidified slowly. Later L-blocks are removed.

Table 4.4 Manual tissue processing

Step	Treatment	Time interval in Hours
Fixation	Formalin	24
Dehydration	30 % Alcohol	1
	50 % Alcohol	1
	80 % Alcohol	1
	80 % Alcohol	1
	90 % Alcohol	1
	100 % Alcohol	1
	100 % Alcohol	1
	100 % Alcohol	1
Clearing	Xylene	1
Infiltration	Paraffin at 50^0 to 56^0C	1
	Paraffin at 50^0 to 56^0C	1
	Paraffin at 50^0 to 56^0C	1

Section cutting and staining

For histological preparation tissues were cut in to thin sections and then adjusted to desired thickness of 8-12mm. Sections were taken out with a soft brush. For Haematoxylin and Eosin staining, the tissues were subjected for treatment with various solvents as mentioned below,

1. Xylene 4 minutes
2. Absolute alcohol 2 minutes
3. 90% Alcohol 2 minutes
4. 70% Alcohol 2 minutes
5. 50% Alcohol 2 minutes
6. Distilled water 5 minutes
7. Haematoxylin 2 minutes
8. Then tissues were washed thoroughly under running tap water for 2-3 minutes. To confirm sufficient degree of staining, sections were observed under the microscope.
9. Excess amount of stain was removed by differentiation in acid alcohol (1% HCl in 70% alcohol). Blue staining of haematoxylin stain turns red by the acid.
10. Then the sections were washed with alkaline water for about 5 minutes to regain blue coloration. After blue coloration, the slides were again washed thoroughly under tap water.
11. The sections were then treated with 1% aqueous eosin for about 1-3 minutes
12. Then excess of eosin was washed in water.
13. Slides were then examined under the microscope. Cytoplasm, muscle cells appear deep pink, collagen fibers light pink, erythocytes and eosinophil appear orange-red.
14. Treatment of the sections was continued with 90% alcohol for 10-15 seconds then with absolute alcohol for 40-45 seconds followed by treating with Xylene until it's completely clear.
15. Lastly sections were made to mount in D. P. X.

4.2.8 Statistical analysis

- Mean and Standard deviation (SD) of different variables under the study were determined.
- To determine the significant difference between the groups One-way ANOVA was applied.
- Post-hoc test was used to determine the significant changes between two groups.
- Statistical significance was established at $p<0.05$

Chapter 5

Results

5.1 Phytochemical analysis

The preliminary phytochemical analysis of freshly prepared aqueous leaves extracts was carried out to identify various phytochemical constituents in them. Table 5.1 shows the phytochemical components present in experimental aqueous leaves extracts.

Table 5.1 Phytochemistry of *Trigonella foenum graecum, Hibiscus cannabinus Linn, Cicer aritinum Linn* and *Lactuca scariola Linn*

Sl No.	**Phyto constituent**	*Trigonella foenum graecum*	*Hibiscus cannabinus Linn*	*Cicer arietinum Linn*	*Lactuca scariola Linn*
1	**Test for alkaloids**	Negative	Negative	Negative	Negative
2	**Test for carbohydrates**	Negative	Negative	Negative	Negative
3	**Test for Flavanoids**	Positive	Positive	Positive	Positive
4	**Test for Saponins**	Positive	Positive	Positive	Positive
5	**Test for Tannins**	Negative	Positive	Negative	Negative
6	**Test for Triterpenes**	Positive	Positive	Positive	Negative
7	**Test for Steroids**	Negative	Negative	Negative	Positive
8	**Test for Carotenoids**	Negative	Negative	Negative	Negative

5.2 *Trigonella foenum graecum*

5.2.1 *In vitro* study

5.2.1.1 Effect of aqueous *Trigonella foenum graecum* leaves extract on glucose concentration at different pH conditions

The effect of aqueous *Trigonella foenum graecum* leaves extract on the concentration of glucose (*in vitro*) at pH 2.0, 7.0 and 9.0 has shown a significant decrease in the glucose concentration both in neutral and alkaline medium. However, the decrease in the concentration of glucose was greater in the alkaline medium i.e., at pH 9.0 compared to that at pH 7.0. But, it did not show any positive results in the acidic conditions i.e., at pH 2.0 (Fig. 5.1).

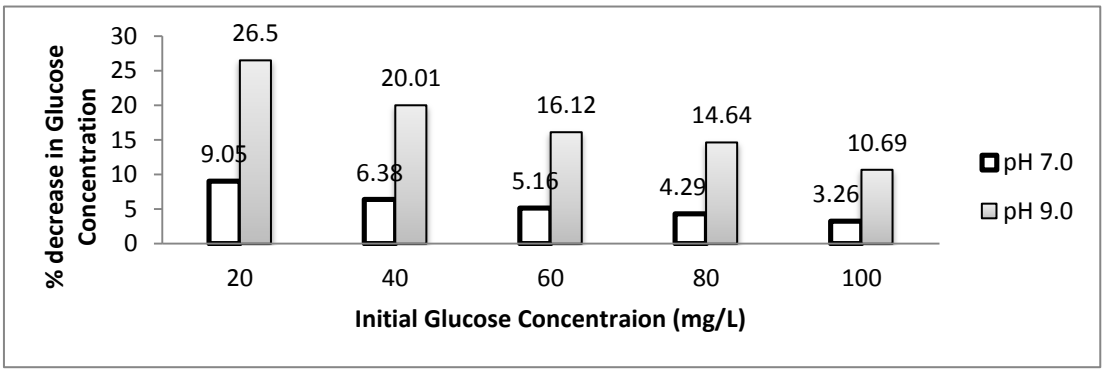


Fig. 5.1 % decrease in the glucose concentration at different pH conditions with *Trigonella foenum graecum*

5.2.1.2 Effect of aqueous *Trigonella foenum graecum* leaves extract on glucose concentration in the presence of Ni^{2+} at different pH conditions *in vitro.*

The effect of aqueous *Trigonella foenum graecum* leaves extract on the glucose concentration (*in vitro*) in the presence of heavy metal Ni^{2+} also showed the similar pattern i.e., greater decrease in the glucose concentration in the alkaline conditions. However, in the presence of Ni^{2+} the percentage decrease in the glucose concentration is comparatively less than that in its absence. It can also be seen that the concentration of Ni^{2+} has no significant effect on the decrease of glucose concentration (Fig. 5.2)

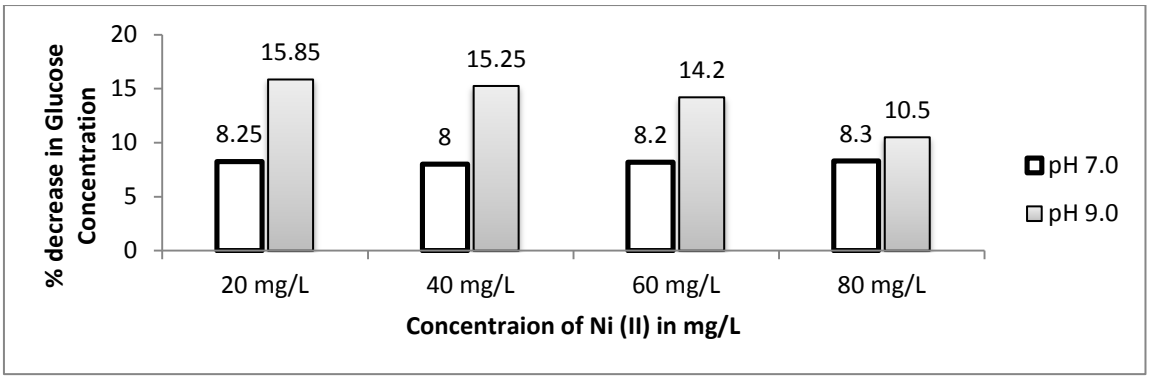


Fig. 5.2 % decrease in glucose concentration at different pH conditions with *Trigonella foenum graecum* in the presence of Ni^{2+}

5.2.1.3 Alteration of chemical behaviour of $Ni^{2+}$with *Trigonella foenum graecum* extract

The λ_{max} values of nickel at pH 7.0 and pH 9.0 were 393.5nm and 396.5nm respectively. In the presence of aqueous *Trigonella foenum graecum* leaves extract, the λ_{max} values of nickel made hypsochromic shift to 323.5nm and 323.0nm at pH 7.0 and pH 9.0 respectively (Table 5.2).

Table 5.2 λ_{max} and absorbance values of nickel sulphate and with *Trigonella foenum graecum* extract at different pH.

Sl. No	Chemical	Concentration	pH 7.0		pH 9.0	
			λ_{max} (nm)	Absorbance	λ_{max} (nm)	Absorbance
1	$NiSO_4$	6 mg/mL	393.5[a]	0.1294	396.5	0.1331
2	$NiSO_4$+ Fenugreek extract	6 mg/mL + 0.5 mL of extract	323.5	2.8252	323.0	2.9991

[a] Data shown as λ_{max} and absorbance values by Ni^{2+} alone and in combination with extract by using UV-VIS Spectrophotometer at pH 7.0 and 9.0.

5.2.2 *In vivo* study

Glucose homeostasis

5.2.2.1 Effect of aqueous *Trigonella foenum graecum* leaves extract on acute exposure to non diabetic and diabetic male rats (OGTT).

Fasting blood sugar was measured in rats of all the groups. Blood glucose levels were measured with the supplementation of aqueous *Trigonella foenum graecum* leaves extract or Ni^{2+} or both or Glipizide drug at intervals of 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose loading.

Non-Diabetic rats

The normal group I rats and aqueous extract supplemented group II rats showed similar OGTT results. Group III Ni^{2+} treated rats, showed increase in the blood glucose level. However, in group IV Ni^{2+} treated rats, a significant decrease in the blood glucose level was observed upon supplementation with aqueous leaves extract (Table 5.3).

Diabetic rats

The OGTT results of group V diabetic rats showed an increase in the blood glucose level. Upon supplementation with the aqueous leaves extract, significant decrease in the blood glucose level was observed in group VI diabetic rats. Similarly, the blood glucose level increased to a much higher level in the group VII diabetic rats due to Ni^{2+} treatment. In group VIII Ni^{2+} treated diabetic rats, the blood glucose level was significantly decreased on supplementation with aqueous leaves extract. However, the influence of aqueous extract in decreasing the blood glucose level is less compared to that of group IX rats which are treated with Glipizide drug (Table 5.4).

The blood glucose levels of both non diabetic and diabetic groups of *Trigonella foenum graecum* supplemented rats treated with $NiSO_4$ are depicted in fig 5.3. Figure clearly shows a steady increase in blood glucose level till the end of 1.0h in all the groups. In case of diabetic or Ni^{2+} treated or diabetic + Ni^{2+} treated groups, the oral glucose tolerance curve did not return back to near baseline values within 2h. Interestingly, rats of diabetic, Ni^{2+} treated or diabetic + Ni^{2+} treated groups when supplemented with *Trigonella foenum graecum* leaves extract have shown better glucose tolerance as compared to diabetic or nickel treated rats.

The significant decrease in blood glucose level with plant extract was also observed when the % change in glucose levels was calculated for different groups (Fig. 5.4).

Table 5.3 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Trigonella foenum graecum* extract on blood glucose level in normal (**non diabetic)** rats.

Period after glucose ingestion (hours)						
Treatment / Groups	FBS	0	0.5	1	1.5	2
I	$90.00 \pm 3.22^{a,1}$	$89.50 \pm 2.42^{a,1}$	$117.33 \pm 5.00^{b,1}$	$128.66 \pm 6.28^{c,1}$	$113.33 \pm 2.87^{b,1}$	$94.50 \pm 6.47^{a,1}$
II	$87.66 \pm 3.14^{a,1}$	$80.66 \pm 2.73^{b,2}$	$97.33 \pm 2.73^{c,2}$	$104.33 \pm 3.61^{c,2}$	$96.33 \pm 1.36^{c,2}$	$90.33 \pm 1.36^{a,1}$
III	$88.66 \pm 3.66^{a,1}$	$101.16 \pm 5.30^{b,3}$	$177.50 \pm 23.18^{c,3}$	$185.00 \pm 23.66^{c,3}$	$152.50 \pm 21.15^{d,3}$	$121.66 \pm 15.70^{e,2}$
IV	$85.00 \pm 3.22^{a,1}$	$96.33 \pm 3.14^{b,4}$	$113.66 \pm 6.77^{c,1}$	$124.33 \pm 7.28^{d,4}$	$115.00 \pm 7.09^{c,1}$	$106.66 \pm 5.95^{e,3}$

Treatment groups: I, normal control; II, *Trigonella foenum graecum* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Trigonella foenum graecum* leaves extract. Horizontal values are the average ± standard deviation (n=6) in each group till 2 h. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical values are variation in blood glucose level of four different groups at different time interval till 2 h. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

Table 5.4 Effect of acute exposure to $Ni^{2+}$and simultaneous supplementation with *Trigonella foenum graecum* leaves extract on blood glucose level in **diabetic** rats.

Period after glucose ingestion (hours)						
Treatment / Groups	FBS	0	0.5	1	1.5	2
V	257.66 ± 10.59 ^{a,1}	257.5 ± 10.36 ^{a,1}	322.83 ± 14.64 ^{b,1}	395.33 ± 7.39 ^{c,1}	375.66 ± 3.82 ^{c,1}	350.83 ± 7.35 ^{d,1}
VI	309.66±16.62 ^{a,2}	294.33±16.50 ^{b,1}	360.66±15.65 ^{c,1}	308.00±16.17 ^{a,1}	263.66±16.13 ^{d,1}	227.33 ± 11.5 ^{e,1}
VII	252.66 ± 10.19 ^{a,1}	278.50 ± 12.62 ^{b,1}	381.00 ± 12.48 ^{c,1}	402.00 ± 12.40 ^{d,1}	415.66 ± 14.05 ^{e,1}	405.00 ± 14.17 ^{d,1}
VIII	297.66 ± 16.93 ^{a,3}	241.33 ± 8.82 ^{b,1}	375.33 ± 9.47 ^{c,1}	322.66 ± 7.44 ^{d,1}	270.66 ± 7.71 ^{e,1}	238.33 ± 18.61 ^{b,1}
IX	251.83 ± 5.74 ^{a,1}	236.33 ± 11.91 ^{b,1}	275.00 ± 5.79 ^{c,1}	296.50 ± 4.37 ^{d,1}	216.66 ± 10.63 ^{e,1}	154.16 ± 20.35 ^{f,1}

Treatment groups: V, diabetic control; VI, diabetic+ *Trigonella foenum graecum* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic+Ni^{2+}+ *Trigonella foenum graecum* leaves extract; IX, diabetic + glipizide (0.25 mg/100 g). Horizontal values are the average ± standard deviation (n=6) in each group till 2 h. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical values are variation in blood glucose levels of five different groups at different time interval till 2 h. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

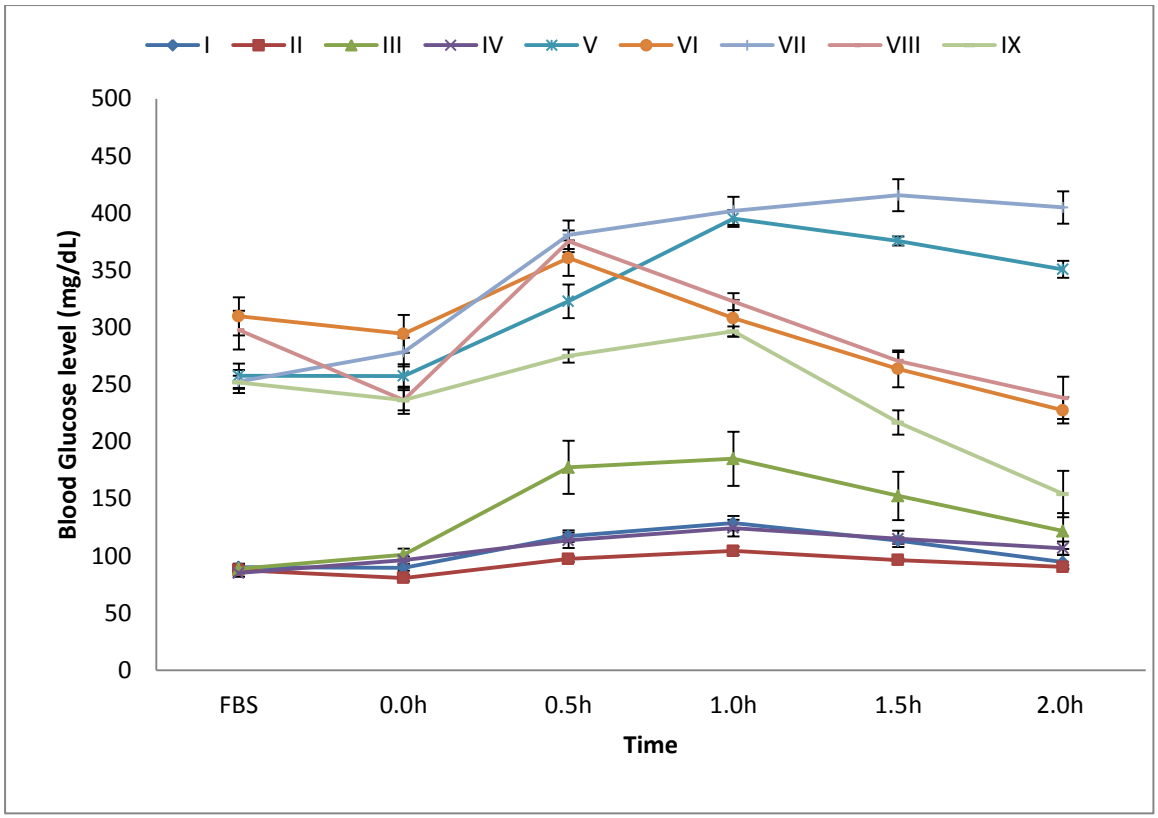


Fig. 5.3 OGTT curves of non diabetic and diabetic rats supplemented with aqueous *Trigonella foenum graecum* leaves extract

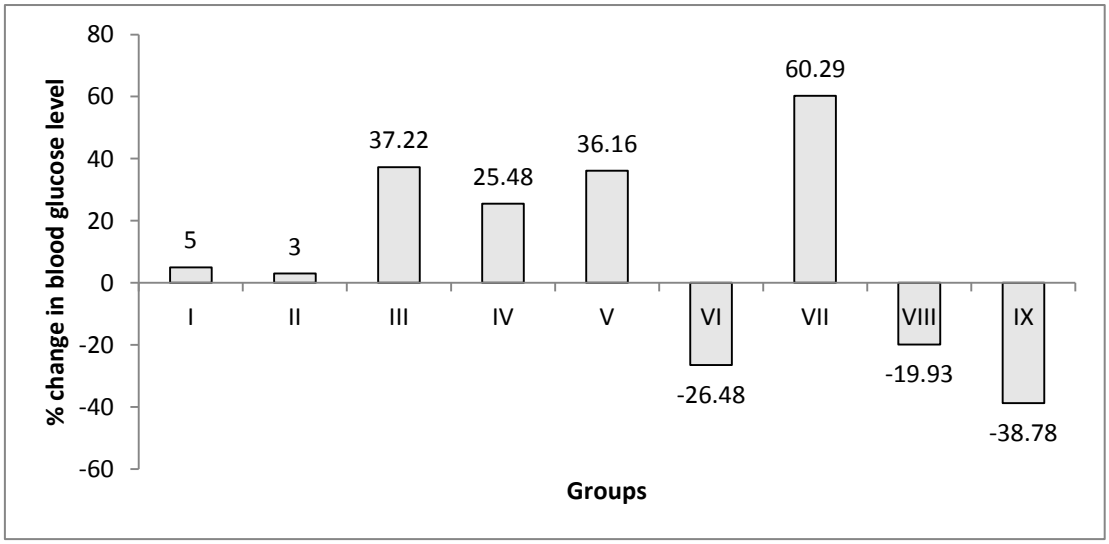


Fig. 5.4 % Change in blood glucose level at the end of 2.0 h of OGTT from the FBS with *Trigonella foenum graecum* in control and diabetic rats

Treatment groups: I, normal control; II, *Trigonella foenum graecum* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Trigonella foenum graecum* leaves extract; V, diabetic control; VI, diabetic + *Trigonella foenum graecum* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Trigonella foenum graecum* leaves extract; IX, diabetic + glipizide (0.25 mg / 100 g).

5.2.2.2 Effect of aqueous *Trigonella foenum graecum* leaves extract on the Sub chronic (13 days) exposure on non diabetic and diabetic male rats.

After the day of OGTT, the rats were continued with the treatment of the aqueous leaves extract and $NiSO_4$ in the designed experimental groups on every alternate day for a period of 13 days and simultaneously blood glucose level is monitored on every alternate day.

Non diabetic rats

Table 5.5 shows different blood glucose level for all groups of rats (group I to group IV). The group II rats which are supplemented with the extract showed similar results as that of group I control rats. The group III rats which are treated with Ni^{2+} for a period of 13 days showed an increase in the blood glucose level. However, group IV Ni^{2+} treated rats, upon supplementation with the aqueous leaves extract for a period of 13 days showed a significant decrease in the blood glucose level.

Diabetic rats

Table 5.6 shows different blood glucose level for all groups of rats (group V to group IX). The blood glucose level of group V diabetic rats was constantly increased over a period of 13 days. The Ni^{2+} treated diabetic rats of Group VII also showed an elevated blood glucose level similar to that of group V diabetic rats. However, significant decrease in the blood glucose level was observed due to supplementation of aqueous *Trigonella foenum graecum* leaves extract in diabetic rats of group VI and Ni^{2+} treated diabetic rats of group VIII. But, the diabetic rats of group IX which were treated with a known anti diabetic drug glipizide, showed a greater decrease in the blood glucose level compared to that of aqueous *Trigonella foenum graecum* leaves extract.

Fig. 5.5 depicts the percentage change in blood glucose level over a priod of 13 days in different group of rats. The percentage change in blood glucose level calculated in all the groups clearly shows a significant decrease of blood glucose level in groups VI and VIII rats due to supplementation of *Trigonella foenum graecum* leaves extract.

Table 5.5 Effect of sub chronic treatment with $Ni^{2+}$and simultaneous supplementation with *Trigonella foenum graecum* leaves extract on blood glucose level in normal (**non diabetic**) rats.

	Blood glucose levels (mg/dL)						
Treatment Groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day
I	89.33 ± 2.58 a,1	90.50 ± 2.50 a,1	91.83 ± 1.94 a,1	90.66 ± 1.75 a,1	89.83 ± 1.32 a,1	89.66 ± 1.03 a,1	91.00 ± 0.89 a,1
II	87.66± 3.14 a,1	86±4.09 a,1	87.66±4.92 a,1	87±3.22 a,1	86.33±4.92 a,1	87.33±4.92 a,1	88.66±3.72 a,1
III	88.66 ± 3.66 a,1	112.5 ± 8.80 b,2	134.16 ± 7.22 c,2	137.50 ± 7.03 c,2	138.50 ± 6.09 c,2	140.66 ± 5.78 c,2	142.16 ± 5.34 c,2
IV	85±3.22 a,1	93.66±4.03 b,1	106±7.79 c,3	111.66±6.83 d,3	111.6±6.94 d,3	118±6.44 e,3	121±8.53 e,3

Treatment groups: I, normal control; II, *Trigonella foenum graecum* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Trigonella foenum graecum* leaves extract. Horizontal values are the average ± standard deviation (n=6) in each group on every alternate day till 13th day. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group at different days. Vertical values are variation in blood glucose level of four different groups on each alternate day till 13th day. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

Table 5.6 Effect of sub chronic treatment with $Ni^{2+}$and simultaneous supplementation with *Trigonella foenum graecum* leaves extract on blood glucose level in **diabetic** rats.

	Blood glucose levels (mg/dL)						
Treatment Groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day
V	253.16 ± 7.22 ^{a,1}	262.66 ± 5.57 ^{b,1}	274.00 ± 9.87 ^{c,1}	295.66 ± 8.04 ^{d,1}	298.00 ±10.65 ^{d,1}	312.33 ±6.37 ^{e,1}	326.66 ±7.25 ^{f,1}
VI	309.66± 16.62 ^{a,2}	295.33±18.38 ^{b,2}	282.66± 12.20 ^{c,2}	265.66± 10.59 ^{d,2}	253± 10.07 ^{e,2}	242.33± 8.82 ^{f,2}	228.33± 8.06 ^{g,2}
VII	251.33 ± 7.55 ^{a,1}	266.33 ± 6.53 ^{b,1}	272.66 ± 6.12 ^{b,1}	293.00 ± 4.14 ^{c,1}	296.33 ± 3.88 ^{c,1}	311.83 ± 7.49 ^{d,1}	326.83 ± 2.48 ^{e,1}
VIII	297.66±16.93 ^{a,3}	285.66±13.66 ^{b,3}	273.66± 11.53 ^{c,1}	265± 9.67 ^{d,2}	253.33± 9.17 ^{e,2}	241± 9.07 ^{f,2}	234.33± 7.44 ^{g,2}
IX	254.56 ± 9.45 ^{a,1}	250.60 ±10.37 ^{a,4}	175.28 ± 9.87 ^{b,3}	142.49 ±10.05 ^{c,3}	125.28 ± 9.11 ^{d,3}	109.43± 10.28 ^{e,3}	94.24 ± 9.26 ^{f,3}

Treatment groups: V, diabetic control; VI, diabetic+ *Trigonella foenum graecum* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic+Ni^{2+}+ *Trigonella foenum graecum* leaves extract; IX, diabetic + glipizide (0.25 mg/100 g). Horizontal values are the average ± standard deviation (n=6) in each group on every alternate day till 13th day. Values with different superscript alphabets (a, b, c, d, e, f or g) are significantly different from each other ($p<0.05$) in the same group at different days. Vertical values are variation in blood glucose level of four different groups on each alternate day till 13th day. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

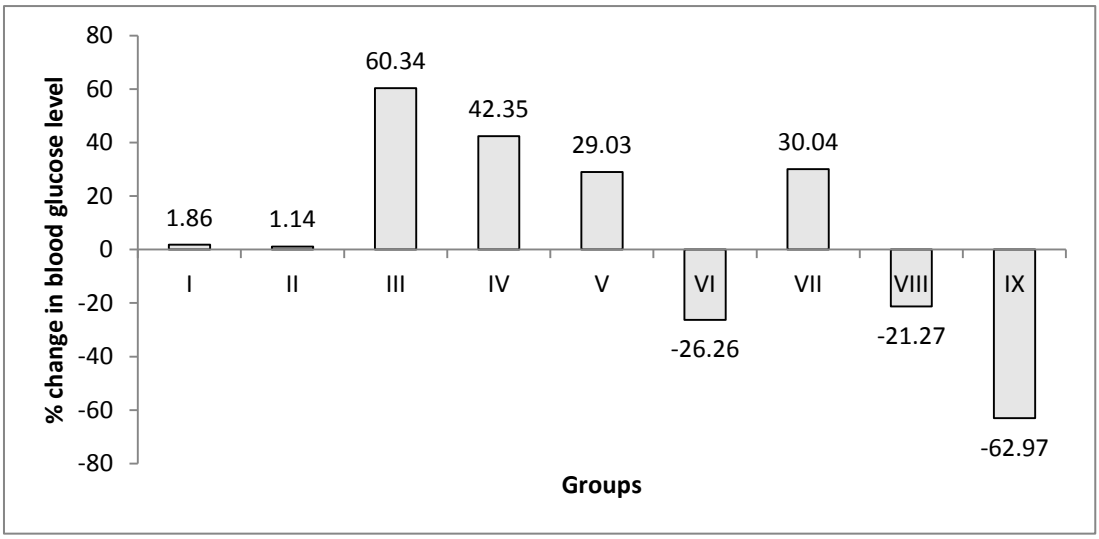


Fig. 5.5 % Change in blood glucose level at the end of 13 days of treatment from day 1 in control and diabetic rats

Treatment groups: I, normal control; II, *Trigonella foenum graecum* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Trigonella foenum graecum* leaves extract; V, diabetic control; VI, diabetic + *Trigonella foenum graecum* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Trigonella foenum graecum* leaves extract; IX, diabetic+glipizide (0.25 mg/100g).

5.3 Hibiscus cannabinus Linn.

5.3.1 *In vitro* study

5.3.1.1 Effect of aqueous *Hibiscus cannabinus Linn* leaves extract on glucose concentration at different pH conditions

The effect of aqueous *Hibiscus cannabinus Linn* leaves extract on the concentration of glucose at pH 2.0, 7.0 and 9.0 showed that the aqueous extract has very less influence on the decrease of glucose concentration both in neutral and alkaline conditions i.e., at pH 7.0 and pH 9.0 respectively. However, at pH 2.0 it did not show any results (Fig. 5.6).

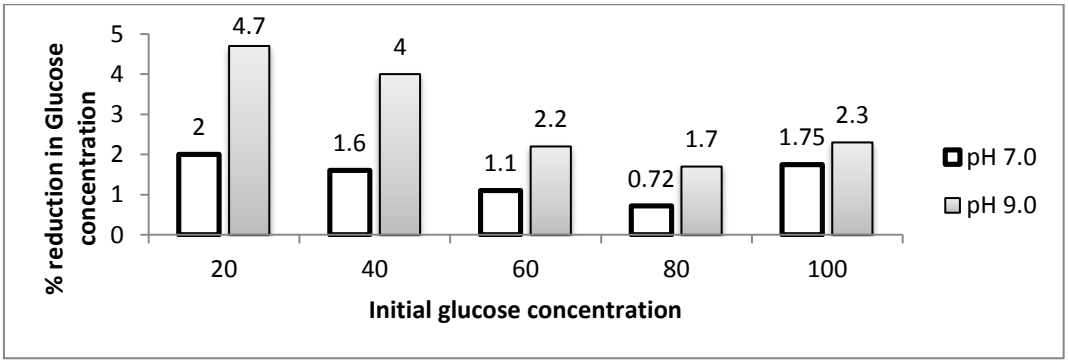


Fig. 5.6 % change in the glucose concentration at different pH conditions with *Hibiscus cannabinus Linn.*

5.3.1.2 Effect of aqueous *Hibiscus cannabinus Linn* leaves extract on glucose concentration in the presence of Ni^{2+} at different pH conditions *in vitro*

In the presence of heavy metal Ni^{2+}, the aqueous *Hibiscus cannabinus Linn* leaves extract didn't show significant changes in the glucose concentration (Fig. 5.7).

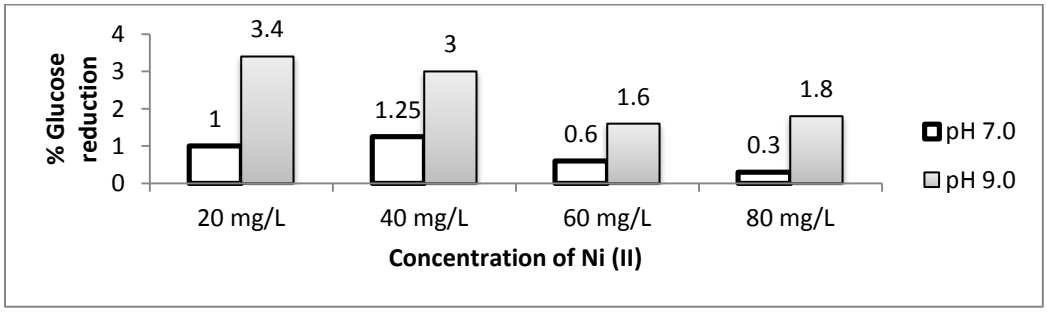


Fig. 5.7 % decrease in glucose concentration at different pH conditions in the presence of heavy metal Ni^{2+} and *Hibiscus cannabinus Linn.*

5.3.1.3 Alteration of chemical behaviour of $Ni^{2+}$with *Hibiscus cannabinus Linn* extract

There were no desired spectral changes of Ni^{2+} with *Hibiscus cannabinus Linn* leaves extract

5.3.2 *In vivo* study

Glucose homeostasis

5.3.2.1 Effect of aqueous *Hibiscus cannabinus Linn* leaves extract on acute exposure (OGTT) on non diabetic and diabetic male rats.

Fasting blood sugar was measured in rats of all the groups. Blood glucose levels were measured with the supplementaion of aqueous *Hibiscus cannabinus Linn* leaves extract or Ni^{2+} or both or Glipizide drug at intervals of 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose loading.

Non-Diabetic rats

Group I control rats and group II aqueous leaves extract supplemented rats showed similar OGTT results. Group III rats which are treated with Ni^{2+} showed an increased blood glucose level. However, group IV Ni^{2+} treated rats, on supplementation with an aqueous leaves extract caused no significant changes in the blood glucose level (Table 5.7).

Diabetic rats

The OGTT results of group V diabetic rats showed significant increase of blood glucose. On supplementation with the aqueous leaves extract in group VI rats, no significant decrease in the blood glucose level was observed. Similarly, the blood glucose level elevated to a much higher level in the group VII diabetic rats due to Ni^{2+} treatment. Upon supplementation with aqueous leaves extract, no considerable decrease in the blood glucose level was observed in group VIII Ni^{2+} treated diabetic rats. Whereas, a significant decrease in blood glucose level was seen group IX rats which are treated with Glipizide drug (Table 5.8).

The blood glucose level of both non diabetic and diabetic group of rats supplemented with *Hibiscus cannabinus Linn* and treated with $NiSO_4$ are depicted in fig 5.8. It can be seen from the figure that a steady increase in blood glucose level till the end of 1.0h in all the groups. In case of diabetic or Ni^{2+} treated or diabetic + Ni^{2+} treated groups, the oral glucose tolerance curve did not return back to near baseline values within 2h. However, the rats of diabetic, Ni^{2+} treated or diabetic + Ni^{2+} treated groups when supplemented with *Hibiscus cannabinus Linn* leaves extract also didn't show better glucose tolerance.

No significant % change in the blood glucose level can be seen at the end of 2.0h from the FBS with supplementation of the *Hibiscus cannabinus Linn* leaves extract (Fig. 5.9).

Table 5.7 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Hibiscus cannabinus Linn* extract on blood glucose level in normal (**non diabetic**) rats.

Period after glucose ingestion (hours)						
Treatment Groups	FBS	0h	0.5h	1h	1.5h	2h
Group I	$90.00 \pm 3.22^{a,1}$	$89.50 \pm 2.42^{a,1}$	$117.33 \pm 5.00^{b,1}$	$128.66 \pm 6.28^{c,1}$	$113.33 \pm 2.87^{b,1}$	$94.5 \pm 6.47^{a,1}$
Group II	$75.66 \pm 5.95^{a,2}$	$76.66 \pm 3.72^{a,2}$	$115 \pm 4.09^{b,1}$	$120.33 \pm 2.25^{c,2}$	$108 \pm 2.36^{d,1}$	$88.66 \pm 4.22^{e,2}$
Group III	$88.66 \pm 3.66^{a,1}$	$101.16 \pm 5.30^{b,3}$	$177.50 \pm 23.18^{c,2}$	$185.00 \pm 23.66^{c,3}$	$152.50 \pm 21.15^{d,2}$	$121.66 \pm 15.70^{e,3}$
Group IV	$84 \pm 5.36^{a,1}$	$99.66 \pm 0.51^{b,3}$	$116.66 \pm 4.92^{c,1}$	$140.66 \pm 5.46^{d,4}$	$122 \pm 6.44^{c,3}$	$108.66 \pm 7.71^{e,4}$

Treatment groups: Treatment groups: I, normal control; II, *Hibiscus cannabinus Linn* leaves extract; III, Ni2+; IV, Ni^{2+}+ *Hibiscus cannabinus Linn* leaves extract. Horizontal values are the average ± standard deviation (n=6) in each group till 2 h. Values with different superscript alphabets (a, b, c, d or e) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical values are variation in blood glucose level of four different groups at different time interval till 2 h. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

Table 5.8 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Hibiscus cannabinus Linn* extract on blood glucose level in **diabetic** rats.

Period after glucose ingestion (hours)						
Treatment Groups	FBS	0h	0.5h	1h	1.5h	2h
Group V	$257.66 \pm 10.59^{a,1}$	$257.5 \pm 10.36^{a,1}$	$322.83 \pm 14.64^{b,1}$	$395.33 \pm 7.39^{c,1}$	$375.66 \pm 3.82^{d,1}$	$350.83 \pm 7.35^{e,1}$
Group VI	$257 \pm 4.09^{a,1}$	$258 \pm 2.36^{a,1}$	$320 \pm 1.54^{b,1}$	$358.33 \pm 12.01^{c,2}$	$333.66 \pm 10.67^{d,2}$	$316.33 \pm 12.69^{b,2}$
Group VII	$252.66 \pm 10.19^{a,1}$	$278.50 \pm 12.62^{b,2}$	$381.00 \pm 12.48^{c,2}$	$402.00 \pm 12.40^{d,3}$	$415.66 \pm 14.05^{e,3}$	$405.00 \pm 14.17^{d,3}$
Group VIII	$251.33 \pm 26.83^{a,1}$	$251 \pm 32.53^{a,1}$	$356.6 \pm 73.67^{b,3}$	$377.66 \pm 10.74^{c,4}$	$348 \pm 31.03^{b,4}$	$319.66 \pm 21.47^{d,2}$
Group IX	$251.83 \pm 5.74^{a,1}$	$236.33 \pm 11.91^{b,3}$	$275.00 \pm 5.79^{c,4}$	$296.50 \pm 4.37^{d,5}$	$216.66 \pm 10.63^{e,5}$	$154.16 \pm 20.35^{f,4}$

Treatment groups: Treatment groups: V, diabetic control; VI, diabetic+ *Hibiscus cannabinus Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic+Ni^{2+}+*Hibiscus cannabinus Linn* leaves extract; IX, diabetic + glipizide (0.25 mg/100 g). Horizontal values are the average ± standard deviation (n=6) in each group till at 2 h. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical values are variation in blood glucose leve of five different groups at different time interval till 2 h. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

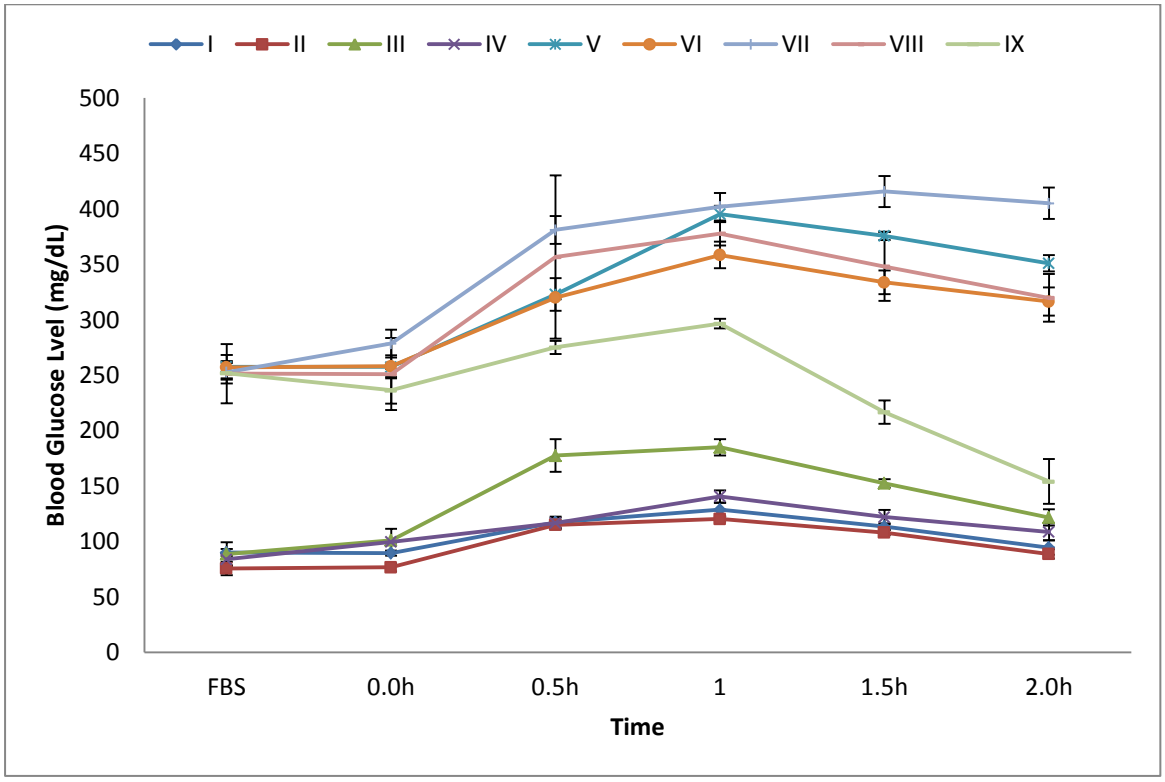


Fig. 5.8 OGTT curves of non diabetic and diabetic rats supplemented with aqueous *Hibiscus cannabinus Linn* leaves extract

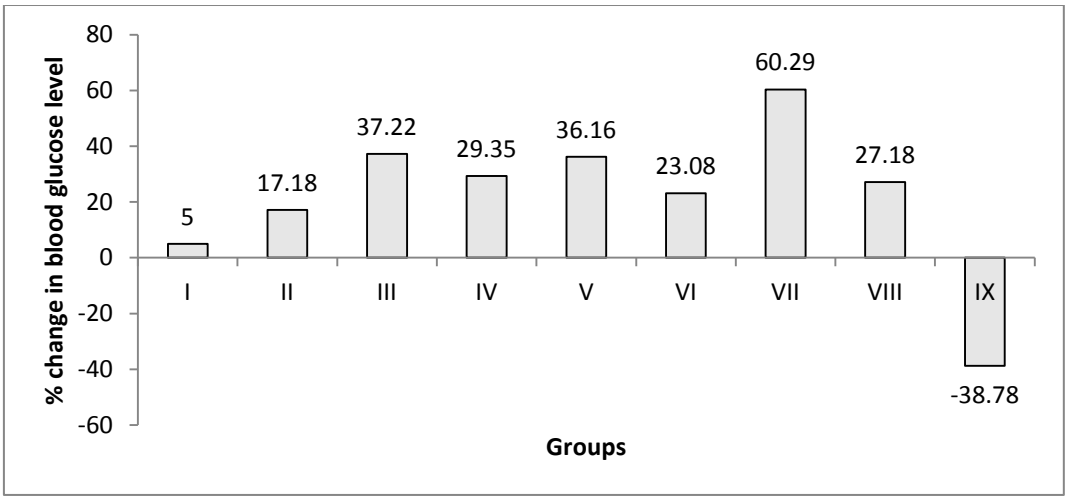


Fig. 5.9 % Change in blood glucose level at the end of 2.0 h of OGTT from the FBS with *Hibiscus cannabinus Linn*in control and diabetic rats

Treatment groups: I, normal control; II, *Hibiscus cannabinus Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Hibiscus cannabinus Linn* leaves extract; V, diabetic control; VI, diabetic + *Hibiscus cannabinus Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic+Ni^{2+}+ *Hibiscus cannabinus Linn* leaves extract; IX, diabetic + glipizide (0.25 mg / 100 g).

5.3.2.2 Effect of aqueous *Hibiscus cannabinus Linn* leaves extract on the Sub chronic (13 days) exposure on non diabetic and diabetic male rats *in vivo*.

After the day of OGTT, the rats were continued with the treatment of the aqueous leaves extract and $NiSO_4$ in the designed experimental groups on every alternate day for a period of 13 days and simultaneously blood glucose level is monitored on every alternate day.

Non diabetic rats

The results of sub chronic exposure showed that group II rats on supplementation with the aqueous leaves extract showed similar results as that of group I control rats. The group III rats which are treated with Ni^{2+} for a period of 13 days showed an increase in the blood glucose level. However, group IV Ni^{2+} treated rats did not show any significant decrease in the blood glucose level on supplementation of aqueous *Hibiscus cannabinus Linn* leaves extract for a period of 13 days (Table 5.9).

Diabetic rats

Group V diabetic rats showed an increased blood glucose level over a period of 13 days. The diabetic rats of Group VII which are treated with Ni^{2+} also showed an increased blood glucose level similar to that of group V rats. However, it is interesting to note that the diabetic rats of group VI and Ni^{2+} treated diabetic rats of group VIII showed no considerable decrease in the blood glucose level on supplementation of aqueous *Hibiscus cannabinus Linn* leaves extract (Table 5.10).

Fig. 5.10 depicts the percentage change in blood glucose level for a period of 13 days in different group of rats. When the percentage change in blood glucose level was calculated, no significant change in blood glucose was observed in group VI and group VIII rats which are supplemented with the plant extract.

Table 5.9 Effect of sub chronic treatment with $Ni^{2+}$and simultaneous supplementation with *Hibiscus cannabinus Linn* leaves extract on blood glucose level in normal (**non diabetic**) rats.

Treatment Groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day
Group I	$89.33 \pm 2.58^{a,1}$	$90.50 \pm 2.50^{a,1}$	$91.83 \pm 1.94^{a,1}$	$90.66 \pm 1.75^{a,1}$	$89.83 \pm 1.32^{a,1}$	$89.66 \pm 1.03^{a,1}$	$91.00 \pm 0.89^{a,1}$
Group II	$83 \pm 2.68^{a,1}$	$81.66 \pm 2.25^{a,1}$	$81.33 \pm 4.03^{a,2}$	$83 \pm 3.22^{a,2}$	$85 \pm 3.22^{a,1}$	$83.33 \pm 0.51^{a,1}$	$84 \pm 4.73^{a,1}$
Group III	$88.66 \pm 3.66^{a,1}$	$112.5 \pm 8.80^{b,2}$	$134.16 \pm 7.22^{c,3}$	$137.50 \pm 7.03^{c,3}$	$138.50 \pm 6.09^{c,2}$	$140.66 \pm 5.78^{c,2}$	$142.16 \pm 5.34^{c,2}$
Group IV	$84 \pm 5.36^{a,1}$	$102.66 \pm 5.46^{b,3}$	$109.66 \pm 9.00^{c,4}$	$110.66 \pm 6.59^{c,4}$	$112.33 \pm 10.36^{c,3}$	$111 \pm 15.51^{c,3}$	$108.33 \pm 12.90^{c,3}$

Treatment groups: I, normal control; II, *Hibiscus cannabinus Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Hibiscus cannabinus Linn* leaves extract. Horizontal values are the average ± standard deviation (n=6) in each group on every alternate day till 13th day. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group on different days. Vertical values are variation in blood glucose level of four different groups on each alternate day till 13th day. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

Table 5.10 Effect of sub chronic treatment with $Ni^{2+}$and simultaneous supplementation with *Hibiscus cannabinus Linn* leaves extract on blood glucose level in **diabetic** rats.

Treatment Groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day
Group V	$253.16 \pm 7.22^{a,1}$	$262.66 \pm 5.57^{a,1}$	$274.00 \pm 9.87^{b,1}$	$295.66 \pm 8.04^{c,1}$	$298.00 \pm 10.65^{c,1}$	$312.33 \pm 6.37^{d,1}$	$326.66 \pm 7.25^{e,1}$
Group VI	$257 \pm 4.09^{a,1}$	$250 \pm 0.89^{a,2}$	$252 \pm 6.75^{a,2}$	$241.33 \pm 3.61^{b,2}$	$231 \pm 3.22^{c,2}$	$222 \pm 6.26^{d,2}$	$218.66 \pm 10.74^{d,2}$
Group VII	$251.33 \pm 7.55^{a,1}$	$266.33 \pm 6.53^{b,1}$	$272.66 \pm 6.12^{b,1}$	$293.00 \pm 4.14^{c,1}$	$296.33 \pm 3.88^{c,1}$	$311.83 \pm 7.49^{d,1}$	$326.83 \pm 2.48^{e,1}$
Group VIII	$251.33 \pm 26.83^{a,1}$	$245 \pm 26.42^{a,2}$	$245.33 \pm 29.26^{a,2}$	$239.33 \pm 21.62^{b,2}$	$230.66 \pm 21.21^{b,2}$	$227.66 \pm 20.12^{b,2}$	$224.33 \pm 25.08^{b,2}$
Group IX	$254.56 \pm 9.45^{a,1}$	$250.60 \pm 10.37^{a,2}$	$175.28 \pm 9.87^{a,3}$	$142.49 \pm 10.05^{a,3}$	$125.28 \pm 9.11^{a,3}$	$109.43 \pm 10.28^{a,3}$	$94.24 \pm 9.26^{a,3}$

Treatment groups: V, diabetic control; VI, diabetic + *Hibiscus cannabinus Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+*Hibiscus cannabinus Linn* leaves extract; IX, diabetic + glipizide (0.25 mg/100 g). Horizontal values are the average ± standard deviation (n=6) in each group on every alternate day till 13th day. Values with different superscript alphabets (a, b, c, d, e, f or g) are significantly different from each other ($p<0.05$) in the same group on different days. Vertical valus are variation in blood glucose level of five different groups in each alternate day till 13th day. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

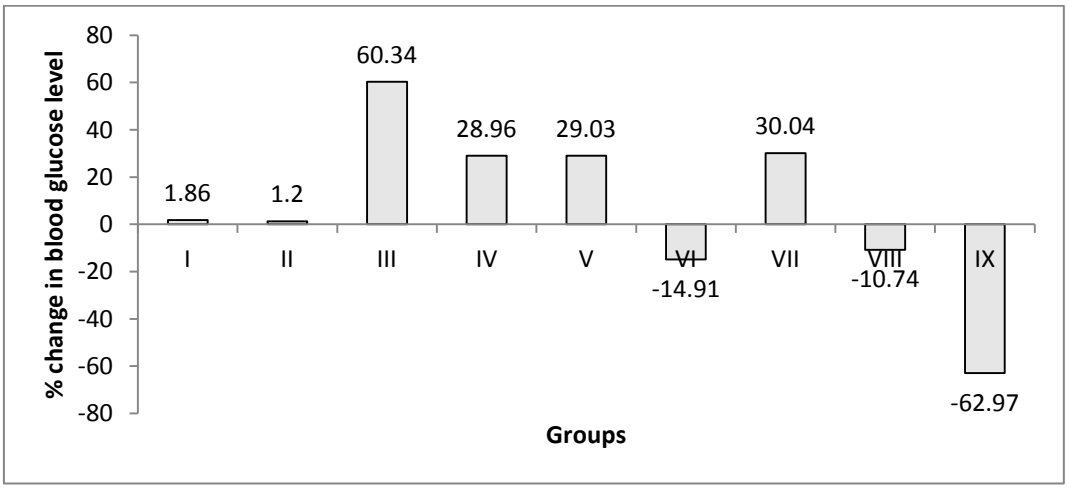


Fig. 5.10 % Change in blood glucose level at the end of 13 days of treatment from day 1 in control and diabetic rats

Treatment groups: I, normal control; II, *Hibiscus cannabinus Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Hibiscus cannabinus Linn* leaves extract; V, diabetic control; VI, diabetic + *Hibiscus cannabinus Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic+Ni^{2+}+ *Hibiscus cannabinus Linn* leaves extract; IX, diabetic+glipizide (0.25 mg / 100 g).

5.3.2.3 Effect of *Hibiscus cannabinus Linn leaves* extract on Fasting blood glucose, Fasting Plasma Insulin and Insulinogenic index.

As shown in table 5.11, after 13 days of sub chronic exposure, the diabetic rats showed an increased fasting blood glucose level (Group 2 vs Group 1). However, on supplementation of *Hibiscus cannabinus Linn extract* for a period of 13 days there was no significant change in the fasting blood glucose level (Group 3 vs Group 2). Whereas, on treating with glipizide drug there was a significant decrease in the fasting blood glucose level (Group 4 vs Group 2). Similarly, the fasting plasma insulin also decreased in diabetic rats (Group 2 vs Group 1). On supplementing the diabetic rats with the *Hibiscus cannabinus Linn extract* there was no significant change in the fasting plasma insulin (Group 3 vs Group 2). Whereas, with glipizide drug treatment the fasting plasma insulin was similar to the control rats (Group 4 vs Group 1). The insulinogenic index also increased in diabetic rats (Group 2 vs Group 1). On supplementation of the extract there was no significant change insulinogenic index (Group 3 vs Group 2). Whereas, on treating with glipizide drug the insulinogenic index was significantly decreased to control level (Group 4 vs Group 1).

Table 5.11 Effect of sub chronic (13 days) supplementation of *Hibiscus cannabinus Linn leaves* extract (12.5 mg/100 g. b.wt) on glucose homeostasis in diabetic rats.

Parameters	Control (Group 1)	Diabetic (Group 2)	Diabetic + *Hibiscus cannabinus Linn extract* (Group 3)	Diabetic + glipizide (Group 4)
Fasting blood glucose (mg/dL)	85.00 ± 5.56^{a}	180.45 ± 13.58^{b}	170.00 ± 23.30^{b}	105.23 ± 14.65^{d}
Fasting plasma Insulin (μg/L)	1.00 ± 0.17^{a}	0.66 ± 0.19^{b}	0.64 ± 0.10^{b}	0.97 ± 0.16^{a}
Insulinogenic – index	0.0043 ± 0.0005^{a}	0.0065 ± 0.0004^{b}	0.0062 ± 0.0004^{b}	0.0044 ± 0.0002^{a}

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Hibiscus cannabinus Linn extract*; 4, diabetic + glipizide. Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$).

5.3.3 Effect of *Hibiscus cannabinus Linn leaves* extract on sNOS3, sNO, sHIF-1α and sVEGF concentrations in diabetic rats.

After 13 days from the day 1, serum NOS3 decreased significantly in diabetic rats (Group 2 vs Group 1). However, diabetic rats supplemented with *Hibiscus cannabinus Linn extract* there was an increase in sNOS3 (Group 3 vs Group 2). Whereas, glipizide treatment showed remarkable improvement in the sNOS3 (Group 4 vs Group 2). Serum NO too showed the similar pattern as that of sNOS3 (Table 5.12). It can be seen that, the serum HIF-1α was significantly increased in diabetic rats (Group 2 vs Group 1). Even after the supplementation of *Hibiscus cannabinus Linn extract* still a significant increase in the sHIF-1α could be seen (Group 3 vs Group 2). Treatment of glipizide drug also didn't show any significant changes (Group 4 vs Group 3). It can also be seen that, there was a significant increase of serum VEGF in diabetic rats (Group 2 vs Group 1). However, the supplementation of *Hibiscus cannabinus Linn leaves* extract did not improve sVEGF level (Group 3 vs Group 2).

On further analysis of the data as % change difference reflects a greater improvement in sNOS3 after the treatment with *Hibiscus cannabinus Linn* (E1 vs E2). In the case of sNO % change improvement is very marginal (E1 vs E2). Whereas, figs. 5.13 and 5.14, reflect % change of serum HIF-1α and serum VEGF respectively, it has been clearly found that there is % change increase in sHIF-1α in diabetic rats treated with the *Hibiscus cannabinus Linn extract* (E2 vs E1). But sVEGF didn't show such alterations.

Table 5.12 Effect of sub chronic (13 days) supplementation of *Hibiscus cannabinus Linn leaves* extract (12.5 mg/100 g.b.wt) on serum NOS3, NO and HIF-1α, VEGF concentrations in diabetic rats.

Parameters	Group 1	Group 2	Group 3	Group 4
Serum NOS3 (nmol/mL)	7.13 ± 1.11^{a}	4.68 ± 1.53^{b}	5.64 ± 1.58^{c}	6.67 ± 2.34^{d}
Serum NO (µmol/L)	22.32 ± 3.04^{a}	10.45 ± 3.20^{b}	11.13 ± 2.11^{b}	17.36 ± 2.22^{d}
Serum HIF-1α (pg/mL)	150.21 ± 5.28^{a}	168.23 ± 12.32^{b}	178.10 ± 3.52^{c}	175.08 ± 8.15^{c}
Serum VEGF (pg/mL)	300.31 ± 5.65^{a}	423 ± 25.20^{b}	414.47 ± 6.51^{b}	378.17 ± 12.34^{d}

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Hibiscus cannabinus Linn extract*; 4, diabetic + glipizide. Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$)

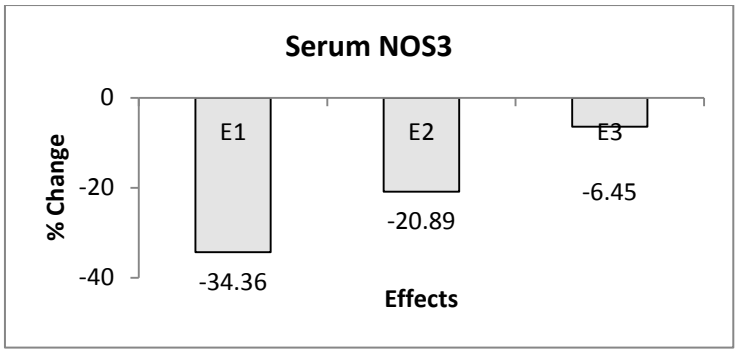


Fig. 5.11% change of Serum NOS3 in diabetic rats
E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

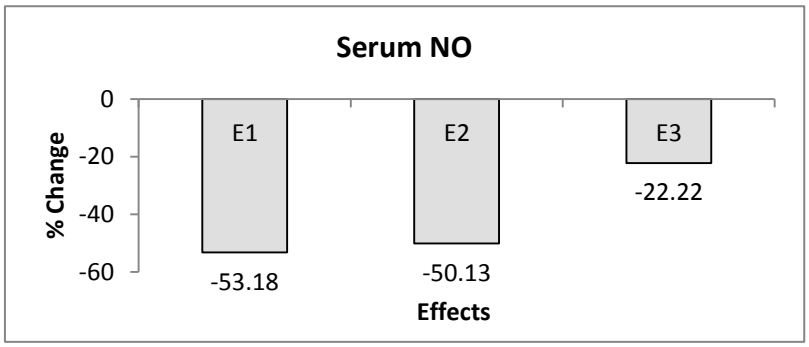


Fig.5.12% change of Serum NO in diabetic rats
E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

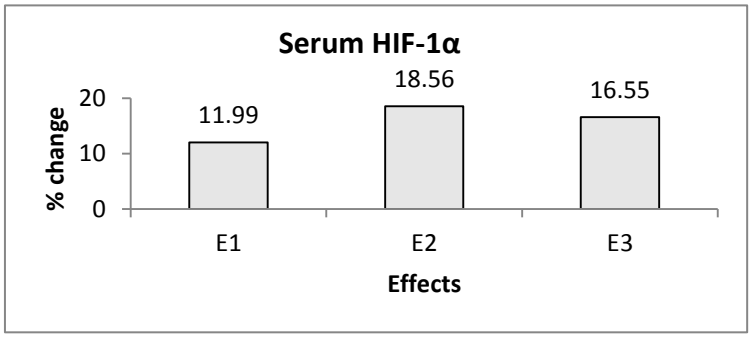


Fig.5.13% change of Serum HIF-1α in diabetic rats
E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

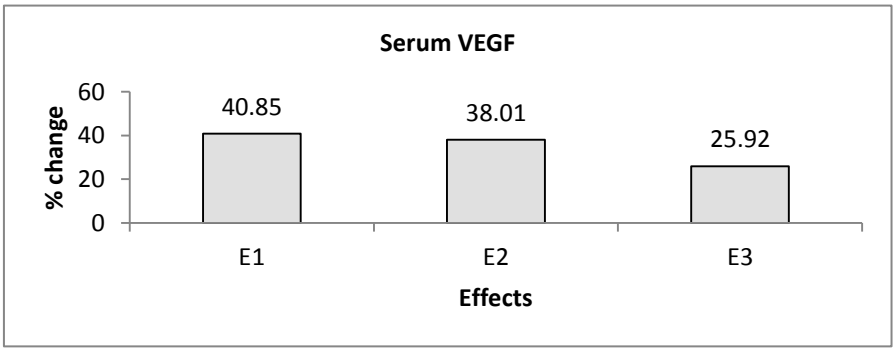


Fig. 5.14 % change of Serum VEGF in diabetic rats
E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

5.4 *Cicer arietinum Linn*

5.4.1 *In vitro* study

5.4.1.1 Effect of aqueous *Cicer arietinum Linn* leaves extract on glucose concentration at different pH conditions

Effect of aqueous *Cicer arietinum Linn* leaves extract on glucose concentration (*in vitro*) was studied by glucose Oxidase - Peroxidase enzymatic method at pH 2.0, 7.0 and 9.0. The results clearly show that the aqueous *Cicer arietinum Linn* leaves extract didn't show significant decrease of glucose concentration *in vitro* in any of the medium (Fig. 5.15).

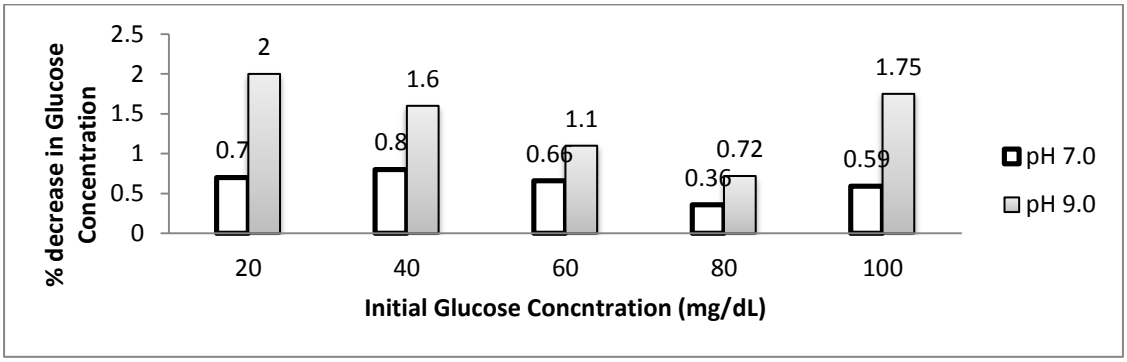


Fig. 5.15% change in the glucose concentration at different pH conditions with *Cicer arietinum Linn*

5.4.1.2 Effect of aqueous *Cicer arietinum Linn* leaves extract on glucose concentration in the presence of Ni^{2+} at different pH conditions.

The effect of aqueous *Cicer arietinum Linn* leaves extract on the glucose concentration (*in vitro*) in the presence of heavy metal Ni^{2+} was also studied similarly. The aqueous *Cicer arietinum Linn* leaves extract didn't show significant decrease in glucose concentration in the presence of heavy metal Ni^{2+} (Fig. 5.16).

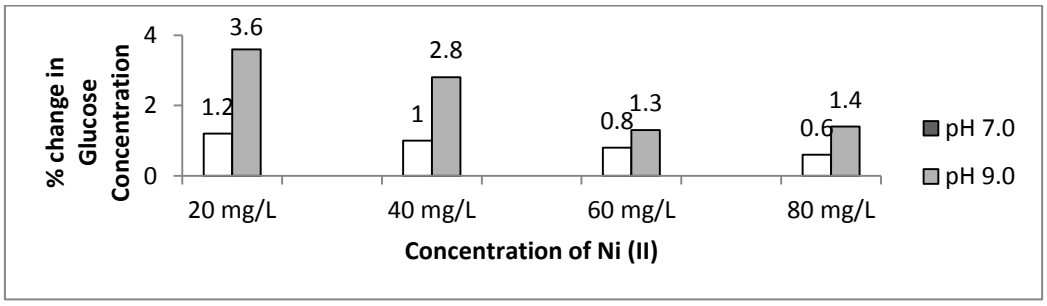


Fig. 5.16 % change of glucose concentration at different pH conditions in the presence of Ni^{2+} and *Cicer arietinum Linn*

5.4.1.3 Alteration of chemical behaviour of $Ni^{2+}$with *Cicer arietinum Linn* extract

There were no desired spectral changes of Ni^{2+} with *Cicer arietinum Linn* leaves extract

5.4.2 *In vivo* study

Glucose homeostasis

5.4.2.1 Influence of aqueous *Cicer arietinum Linn* leaves extract on acute exposure (OGTT) on non diabetic and diabetic male rats.

Fasting blood sugar (FBS) level was measured in rats of all groups. Blood glucose levels were measured with the treatment of aqueous *Cicer arietinum Linn* leaves extract or Ni^{2+} or both or Glipizide drug at intervals of 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose loading.

Non-Diabetic rats

Group II rats which are supplemented with aqueous leaves extract showed almost similar OGTT result as that of normal Group I rats. Group III rats which are treated with Ni^{2+} showed an increased blood glucose level. Similarly group IV Ni^{2+} treated rats too showed an increase in the blood glucose level even after supplementation of aqueous *Cicer aritinum L.* leaves extract (Table 5.13).

Diabetic rats

The OGTT results of group V diabetic rats showed a significant increase in the blood glucose level. The supplementation of aqueous leaves extract in group VI rats caused further increase in the blood glucose level. Similarly, the blood glucose level increased to a much higher level in the group VII rats due to Ni^{2+}treatment. Group VIII Ni^{2+}treated diabetic rats; on further supplementation of aqueous leaves extract didn't show any significant change of blood glucose level (Table 5.14).

The blood glucose level of both non diabetic and diabetic group of rats supplemented with *Cicer arietinum Linn* and treated with $NiSO_4$ are depicted in fig 5.17. It can be clearly seen from the figure that a steady increase in blood glucose level till the end of 1.0h in all the groups. In case of diabetic or Ni^{2+} treated or diabetic + Ni^{2+} treated groups, the oral glucose tolerance curve did not return back to near baseline values within 2h. However, the rats of diabetic, Ni^{2+} treated or diabetic + Ni^{2+} treated groups when supplemented with *Cicer arietinum Linn* leaves extract also did not show any better glucose tolerance.

No significant decrease in blood glucose level was observed with the supplementation of plant extract when the % change in blood glucose levels was calculated for different groups (Fig. 5.18).

Table 5.13 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Cicer arietinum Linn* extract on blood glucose level in normal (**non diabetic**) rats.

Period after glucose ingestion (hours)						
Treatment / Groups	FBS	0	0.5	1	1.5	2
I	$90.00 \pm 3.22^{a,1}$	$89.50 \pm 2.42^{a,1}$	$117.33 \pm 5.00^{b,1}$	$128.66 \pm 6.28^{c,1}$	$113.33 \pm 2.87^{b,1}$	$94.5 \pm 6.47^{a,1}$
II	$84 \pm 5.36^{a,1}$	$83 \pm 2.68^{a,1}$	$119.66 \pm 2.25^{b,1}$	$125 \pm 1.78^{b,1}$	$112.33 \pm 3.72^{b,1}$	$92.66 \pm 6.59^{a,1}$
III	$88.66 \pm 3.66^{a,1}$	$101.16 \pm 5.30^{b,2}$	$177.50 \pm 23.18^{c,2}$	$185.00 \pm 23.66^{c,2}$	$152.50 \pm 21.15^{d,2}$	$121.66 \pm 15.70^{e,2}$
IV	$85 \pm 3.22^{a,1}$	$104.66 \pm 4.92^{b,2}$	$121.33 \pm 4.03^{c,1}$	$147 \pm 4.09^{d,3}$	$136.33 \pm 3.14^{e,3}$	$125.33 \pm 3.38^{f,2}$

Treatment groups: Treatment groups: I, normal control; II, *Cicer arietinum Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Cicer arietinum Linn* leaves extract. Horizontal values are the average ± standard deviation (n=6) in each group till at 2 h. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical values are variation of blood glucose level of four different groups at different time interval till 2 h. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

Table 5.14 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Cicer arietinum Linn* extract on blood glucose level in **diabetic** rats.

Period after glucose ingestion (hours)						
Treatment Groups	**FBS**	**0**	**0.5**	**1**	**1.5**	**2**
V	$257.66 \pm 10.59^{a,1}$	$257.5 \pm 10.36^{a,1}$	$322.83 \pm 14.64^{b,1}$	$395.33 \pm 7.39^{c,1}$	$375.66 \pm 3.82^{d,1}$	$350.83 \pm 7.35^{e,1}$
VI	$253 \pm 33.66^{a,1}$	$251 \pm 32.53^{a,1}$	$331 \pm 33.11^{b,2}$	$372 \pm 30.29^{c,2}$	$351.33 \pm 30.65^{d,2}$	$339.66 \pm 30.01^{b,2}$
VII	$252.66 \pm 10.19^{a,1}$	$278.50 \pm 12.62^{b,2}$	$381.00 \pm 12.48^{c,3}$	$402.00 \pm 12.40^{d,1}$	$415.66 \pm 14.05^{e,3}$	$405.00 \pm 14.17^{d,3}$
VIII	$243.66 \pm 7.60^{a,2}$	$241.33 \pm 8.82^{a,3}$	$323.33 \pm 8.06^{b,1}$	$358.33 \pm 12.01^{c,3}$	$333.66 \pm 10.67^{d,4}$	$323.33 \pm 8.77^{b,4}$
IX	$251.83 \pm 5.74^{a,1}$	$236.33 \pm 11.91^{b,4}$	$275.00 \pm 5.79^{c,4}$	$296.50 \pm 4.37^{d,4}$	$216.66 \pm 10.63^{e,5}$	$154.16 \pm 20.35^{f,5}$

Treatment groups: V, diabetic control; VI, diabetic+ *Cicer arietinum Linn* leaves extract; VII, diabetic+Ni^{2+}; VIII, diabetic+Ni^{2+}+ *Cicer arietinum Linn* leaves extract; IX, diabetic+glipizide (0.25 mg/100 g). Horizontal values are the average ± standard deviation (n=6) in each group till at 2 h. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical values are variation in blood glucose level of five different groups at different time interval till 2 h. Values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times

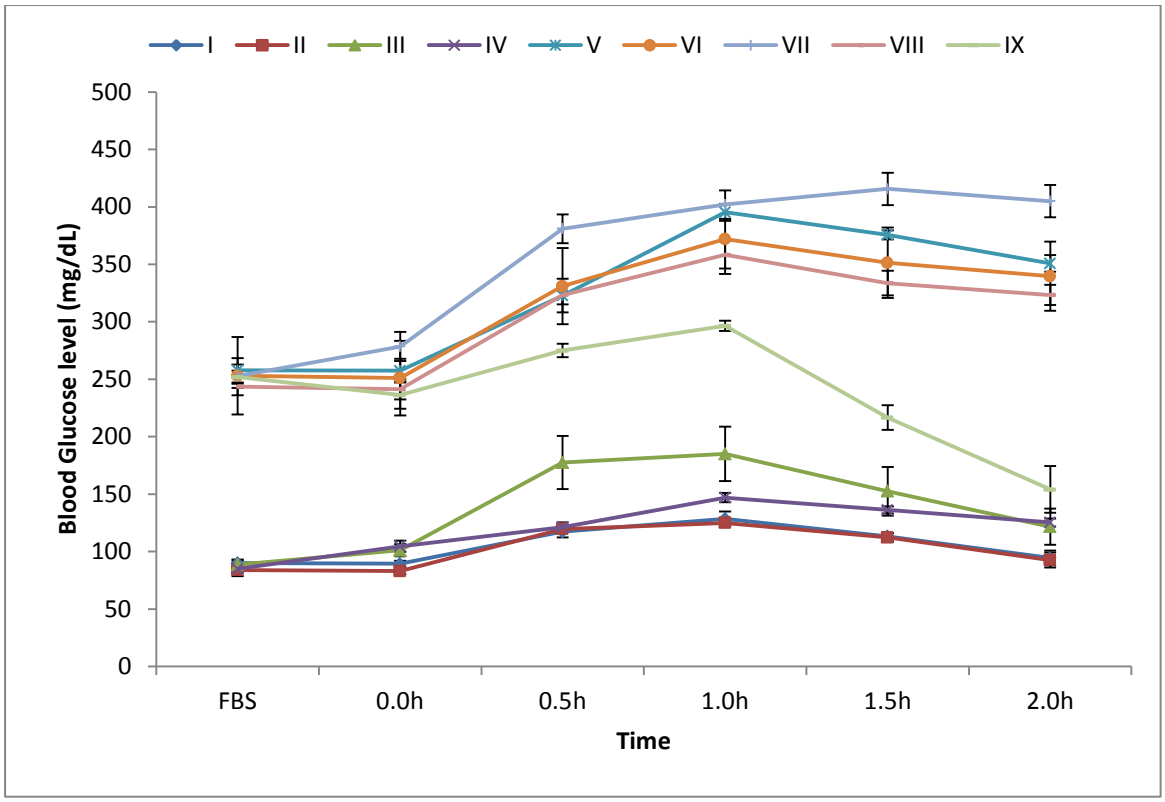


Fig. 5.17 OGTT results of non diabetic and diabetic rats supplemented with aqueous *Cicer arietinum Linn* leaves extract

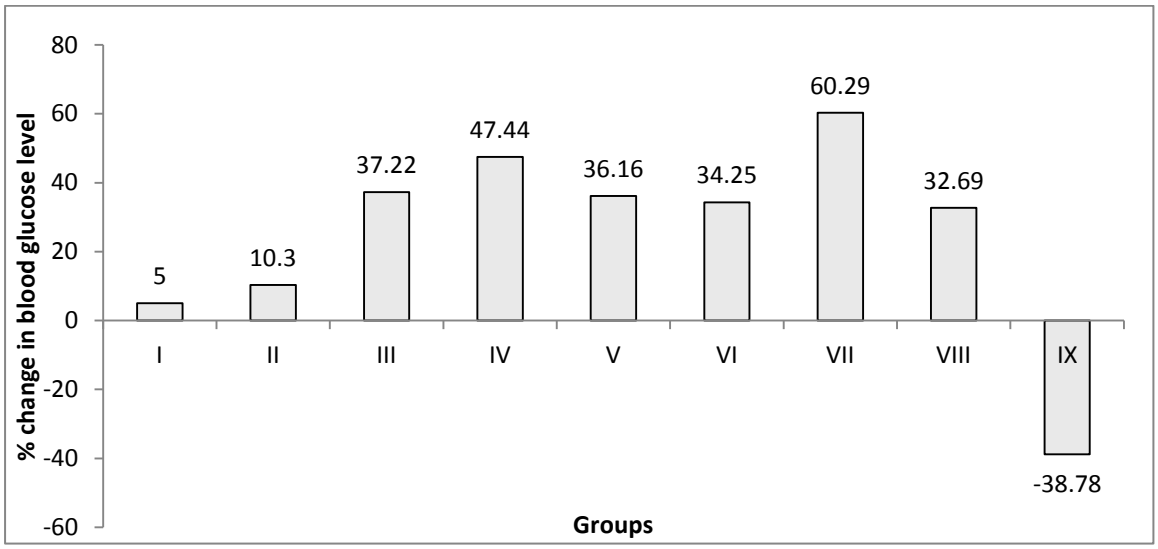


Fig. 5.18 %Change in blood glucose level at the end of 2.0 h of OGTT from the FBS with *Cicer arietinum Linn* in control and diabetic rats

Treatment groups: I, normal control; II, *Cicer arietinum Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Cicer arietinum Linn leaves* extract; V, diabetic control; VI, diabetic+ *Cicer arietinum Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Cicer arietinum Linn* leaves extract; IX, diabetic+glipizide (0.25 mg / 100 g).

5.4.2.2 Effect of aqueous *Cicer arietinum Linn* leaves extract on the Sub chronic (13 days) exposure on diabetic and non diabetic male rats.

After the day of OGTT, the rats were continued with the treatment of the aqueous leaves extract and $NiSO_4$ in the designed experimental groups on every alternate day for a period of 13 days and simultaneously blood glucose level is monitored on every alternate day.

Non Diabetic rats

The sub chronic exposure of group I and group II rats showed similar results. The group III rats on treating with Ni^{2+} for a period of 13 days showed an increase in the blood glucose level and group IV Ni^{2+} treated rats didn't show significant decrease in the blood glucose on prolonged supplementation of aqueous *Cicer arietinum Linn* leaves extract for a period of 13 days (Table 5.15).

Diabetic rats

Group V diabetic rats showed an increased blood glucose level over a period of 13 days. The diabetic rats of Group VII on treating with Ni^{2+} also showed an elevated blood glucose level similar to that of group V rats. However, the diabetic rats of group VI and Ni^{2+} treated diabetic rats of group VIII also showed slight increase in the blood glucose level on supplementation of aqueous *Cicer arietinum Linn.* leaves extract. But, the diabetic rats of group IX which on treating with a known anti diabetic drug, Glipizide showed a greater decrease in the blood glucose level (Table 5.16).

Fig. 5.19 depicts the percentage change in blood glucose level for a period of 13 days in different group of rats. The percentage change in blood glucose level calculated shows no significant change of blood glucose level in groups VI and group VIII rats which are supplemented with the plant extract.

Table 5.15 Effect of sub chronic treatment with $Ni^{2+}$and simultaneous supplementation with *Cicer arietinum Linn* leaves extract on blood glucose level in normal (**non diabetic**) rats.

	Blood glucose levels (mg/dL)						
Treatment Groups	**1st day**	**3rd day**	**5th day**	**7th day**	**9th day**	**11th day**	**13th day**
I	$89.33 \pm 2.58^{a,1}$	$90.50 \pm 2.50^{a,1}$	$91.83 \pm 1.94^{a,1}$	$90.66 \pm 1.75^{a,1}$	$89.83 \pm 1.32^{a,1}$	$89.66 \pm 1.03^{a,1}$	$91.00 \pm 0.89^{a,1}$
II	$83 \pm 2.68^{a,1}$	$81.66 \pm 2.25^{a,1}$	$83 \pm 4.64^{a,1}$	$83 \pm 3.22^{a,1}$	$85 \pm 3.22^{a,1}$	$85.33 \pm 3.61^{a,1}$	$84.33 \pm 5.24^{a,1}$
III	$88.66 \pm 3.66^{a,1}$	$112.5 \pm 8.80^{b,2}$	$134.16 \pm 7.22^{c,2}$	$137.50 \pm 7.03^{d,2}$	$138.50 \pm 6.09^{d,2}$	$140.66 \pm 5.78^{e,2}$	$142.16 \pm 5.34^{e,2}$
IV	$104.66 \pm 4.92^{a,2}$	$102.66 \pm 5.46^{a,3}$	$113 \pm 10.88^{b,3}$	$120.33 \pm 11.91^{c,3}$	$125.33 \pm 8.95^{c,3}$	$129.66 \pm 8.68^{c,3}$	$134.33 \pm 9.39^{d,3}$

Treatment groups: I, normal control; II, *Cicer arietinum Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Cicer arietinum Linn leaves* extract. Horizontal values are the average ± standard deviation (n=6) in each group in every alternate day till 13th day. Values with different superscript alphabets (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group at different days. Vertical values are variation in blood glucose level of four different groups in each alternate day till 13th day. Values with different superscript letters (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

Table 5.16 Effect of subchronic treatment with $Ni^{2+}$and simultaneous supplementation with *Cicer arietinum Linn* leaves extract on blood glucose level in **diabetic** rats.

	Blood glucose levels (mg/dL)						
Treatment Groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day
V	$253.16 \pm 7.22^{a,1}$	$262.66 \pm 5.57^{a,1}$	$274.00 \pm 9.87^{b,1}$	$295.66 \pm 8.04^{c,1}$	$298.00 \pm 10.65^{c,1}$	$312.33 \pm 6.37^{d,1}$	$326.66 \pm 7.25^{e,1}$
VI	$251 \pm 32.53^{a,1}$	$253.66 \pm 29.58^{a,2}$	$257.33 \pm 27.33^{a,2}$	$261 \pm 28.1^{b,2}$	$261.66 \pm 30.42^{b,2}$	$263.33 \pm 29.99^{b,2}$	$265 \pm 29.55^{b,2}$
VII	$251.33 \pm 7.55^{a,1}$	$266.33 \pm 6.53^{b,1}$	$272.66 \pm 6.12^{c,1}$	$293.00 \pm 4.14^{d,1}$	$296.33 \pm 3.88^{d,1}$	$311.83 \pm 7.49^{e,1}$	$326.83 \pm 2.48^{f,1}$
VIII	$241.33 \pm 8.82^{a,2}$	$245.33 \pm 7.28^{a,3}$	$249 \pm 6.44^{b,3}$	$251.66 \pm 4.92^{b,2}$	$254 \pm 5.58^{b,3}$	$253.66 \pm 5.39^{b,3}$	$255 \pm 5.36^{b,3}$
IX	$254.56 \pm 9.45^{a,1}$	$250.60 \pm 10.37^{a,2}$	$175.28 \pm 9.87^{b,4}$	$142.49 \pm 10.05^{c,3}$	$125.28 \pm 9.11^{d,4}$	$109.43 \pm 10.28^{e,4}$	$94.24 \pm 9.26^{f,4}$

Treatment groups: V, diabetic control; VI, diabetic+ *Cicer arietinum Linn* leaves extract; VII, diabetic+Ni^{2+}; VIII, diabetic+Ni^{2+}+ *Cicer arietinum Linn* leaves extract; IX, diabetic+glipizide (0.25 mg/100 g). Horizontal values are the average ± standard Deviation (n=6) in each group in every alternate day till 13th day. Values with different superscript alphabets (a, b, c, d, e, f or g) are significantly different from each other ($p<0.05$) in the same group at different days. Vertical values are variation in blood glucose level of five different groups in each alternate day till 13th day. In each column, values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

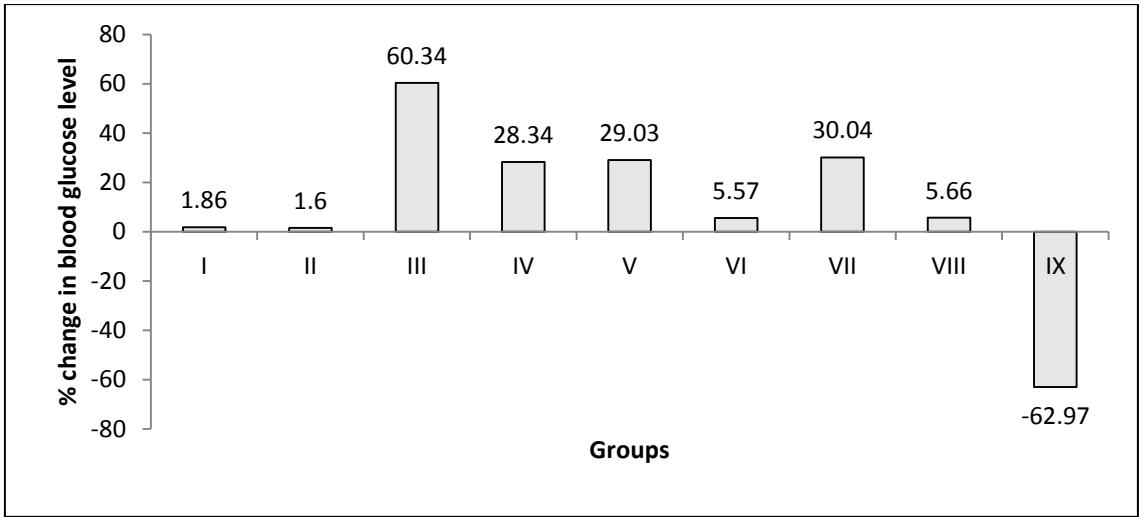


Fig. 5.19 % Change in blood glucose level at the end of 13 days of Sub chronic treatment

Treatment groups: I, normal control; II, *Cicer arietinum Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Cicer arietinum Linn leaves* extract; V, diabetic control; VI, diabetic+ *Cicer arietinum Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Cicer arietinum Linn* leaves extract; IX, diabetic + glipizide (0.25 mg / 100 g).

5.4.2.3 Effect of *Cicer arietinum Linn* laves extract on Fasting blood glucose, Fasting Plasma Insulin and Insulinogenic index

As shown in table 5.17, after 13 days of sub chronic exposure, the diabetic rats showed an increased fasting blood glucose level (Group 2 vs Group 1). However, upon treating with *Cicer arietinum Linn* extract for 13 days there was a slight decrease in the fasting blood glucose level (Group 3 vs Group 2). Whereas, glipizide drug treatment caused a significant decrease in the fasting blood glucose level (Group 4 vs Group 2). Similarly, the fasting plasma insulin also decreased in diabetic rats (Group 2 vs Group 1). On treating the diabetic rats with the aqueous *Cicer arietinum Linn* leaves extract there was no considerable change (Group 3 vs Group 2). The insulinogenic index was increased in diabetic rats (Group 2 vs Group 1). On treating the diabetic rats with the extract of *Cicer arietinum Linn* there wasn't any significant change (Group 3 vs Group 2). Whereas, on treating with glipizide drug the insulinogenic index was similar to that of control rats (Group 4 vs Group 1).

Table 5.17 Effect of sub chronic (14 days) supplementation of *Cicer arietinum Linn* leaves extract (12.5 mg/ 100 g.b.wt) on glucose homeostasis in diabetic rats.

Parameters	Control (Group 1)	Diabetic (Group 2)	Diabetic + *Cicer arietinum Linn.* Extract (Group 3)	Diabetic + glipizide (Group 4)
Fasting blood glucose (mg/dL)	85.00 ± 5.56^{a}	180.45 ± 13.58^{b}	140.00 ± 25.20^{c}	105.23 ± 14.65^{d}
Fasting plasma Insulin (μg/L)	1.00 ± 0.17^{a}	0.66 ± 0.19^{b}	0.69 ± 0.12^{b}	0.97 ± 0.16^{a}
Insulinogenic – index	0.0043 ± 0.0005^{a}	0.0065 ± 0.0004^{b}	0.0061 ± 0.0002^{b}	0.0044 ± 0.0002^{a}

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Cicer arietinum Linn extract*; 4, diabetic + glipizide.

Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$).

5.4.3 Effect of *Cicer arietinum Linn* leaves extract on sNOS3, sNO, sHIF-1α and sVEGF concentrations in diabetic rats.

After 13 days from the day 1, the serum NOS3 decreased significantly in diabetic rats (Group 2 vs Group 1). However, diabetic rats supplemented with the extract *Cicer arietinum Linn*, no significant increase in serum NOS3 (Group 3 vs Group 2) was observed. Whereas, glipizide treatment showed remarkable improvement in the Serum NOS3 (Group 4 vs Group 2). Serum NO decreased significantly in diabetic group, which didn't change significantly on treating with *Cicer arietinum Linn* (Group 3 vs Group 2). However, on glipizide treatment, there was significant rise in the serum NO (Group 4 vs Group 2). It can be seen that, the serum HIF-1α was significantly increased in diabetic rats (Group 2 vs Group 1). On treatment with *Cicer arietinum Linn* extract there was still significant increase in the serum HIF-1α (Group 3 vs Group 2). Treatment of glipizide drug also didn't show any significant changes (Group IV vs Group 2). It can also be seen that, there was a significant increase of serum VEGF in diabetic rats (Group 2 vs Group 1). However, the supplementation of *Cicer arietinum Linn* leaves extract did not improve serum VEGF level (Group 3 vs Group 2) (Table 5.18). On further analysis of the data as % change difference (figs. 5.20 and 5.21) reflects a greater improvement in serum NOS3 after the treatment with *Cicer arietinum Linn* (E1 vs E2). In the case of serum NO % change improvement is very marginal (E1 vs E2). Whereas, fig. 5.22 and 5.23, reflects % change of serum HIF-1α and serum VEGF respectively, it has been clearly found that there is increase in % change of HIF-1α in diabetic rats treated with the *Cicer arietinum Linn* extract (E2 vs E1). But serum VEGF didn't show such alterations.

Table 5.18 Effect of sub chronic (13 days) supplementation of *Cicer arietinum Linn* leaves extract (12.5 mg/100 g.b.wt) on serum NOS3, NO and HIF-1α concentrations in diabetic rats.

Parameters	Group 1	Group 2	Group 3	Group 4
Serum NOS3 (nmol/mL)	7.13 ± 1.11^{a}	4.68 ± 1.53^{b}	5.96 ± 1.94^{c}	$6.67. \pm 2.34^{d}$
Serum NO (µmol/L)	22.32 ± 3.04^{a}	10.45 ± 3.20^{b}	12.02 ± 2.27^{b}	17.36 ± 2.22^{d}
Serum HIF-1α (pg/mL)	150.21 ± 5.28^{a}	168.23 ± 12.32^{b}	178.27 ± 14.41^{c}	175.08 ± 8.15^{c}
Serum VEGF (pg/mL)	300.31 ± 5.65^{a}	423 ± 25.20^{b}	417.47 ± 13.62^{b}	378.17 ± 12.34^{d}

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Cicer arietinum Linn* extract; 4, diabetic + glipizide.

Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$)

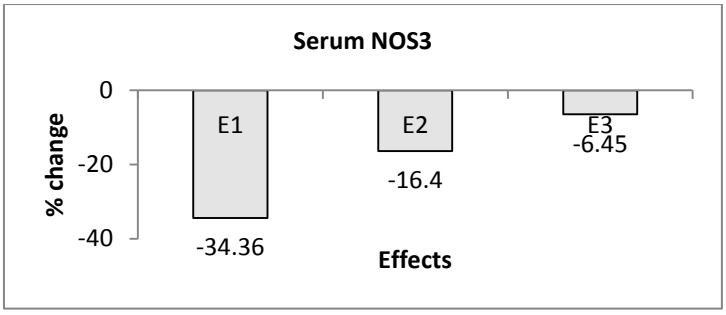


Fig.5.20 % change of Serum NOS in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

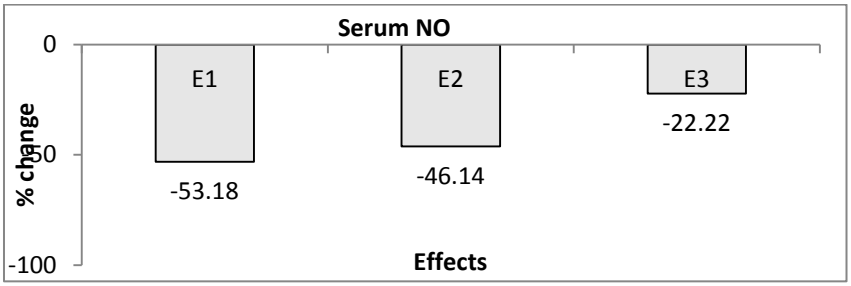


Fig.5.21 % change of Serum NO in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

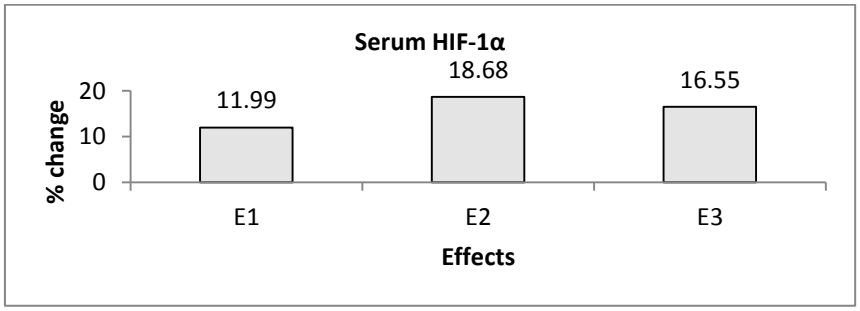


Fig.5.22 % change of Serum HIF-1α in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

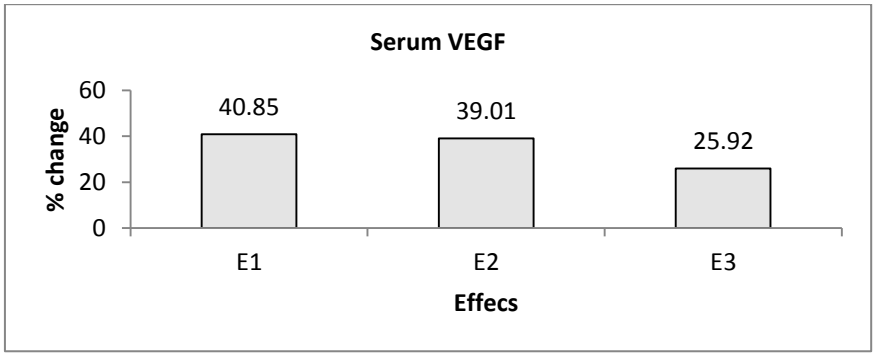


Fig.5.23 % change of Serum VEGF in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

5.5 *Lactuca scariola Linn*

5.5.1 *In vitro* study

5.5.1.1 Effect of aqueous *Lactuca scariola Linn* leaves extract on glucose concentration at different pH conditions.

The effect of aqueous *Lactuca scariola Linn* leaves extract on the reduction of glucose (*in vitro*) was studied by glucose oxidase - peroxidase enzymatic method at pH 2.0, 7.0 and 9.0. The results clearly show that the aqueous *Lactuca scariola Linn* leaves extract has greater glucose reducing capacity than other plants that were studied (Fig. 5.24).

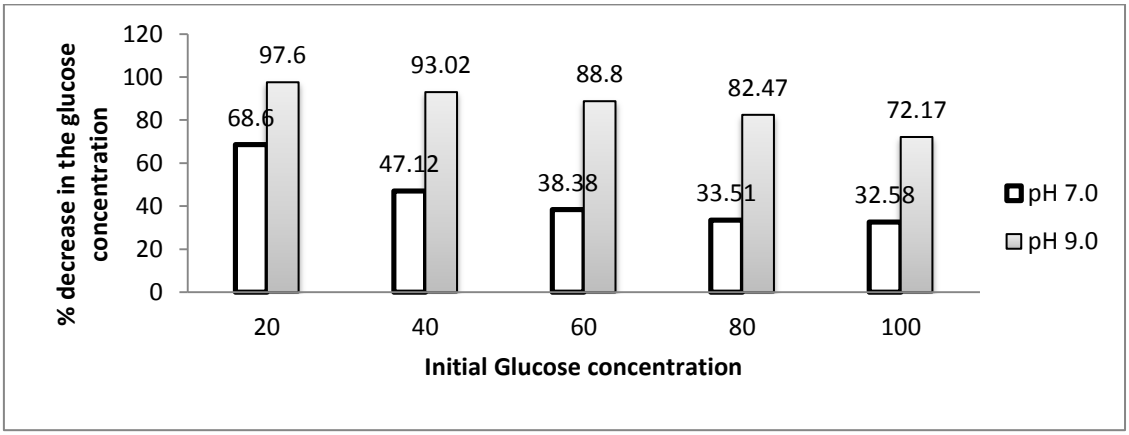


Fig. 5.24% change in the glucose concentration at different pH conditions with *Lactuca scariola Linn*

5.5.1.2 Influence of aqueous *Lactuca scariola Linn* leaves extract on glucose concentration in the presence of Ni^{2+}at different pH conditions *in vitro.*

On studying the effect of aqueous *Lactuca scariola Linn* leaves extract on the reduction of glucose (*in vitro*) in the presence of heavy metal Ni^{2+} the results showed a significant decrease in the glucose concentration at different pH conditions (Fig. 5.25).

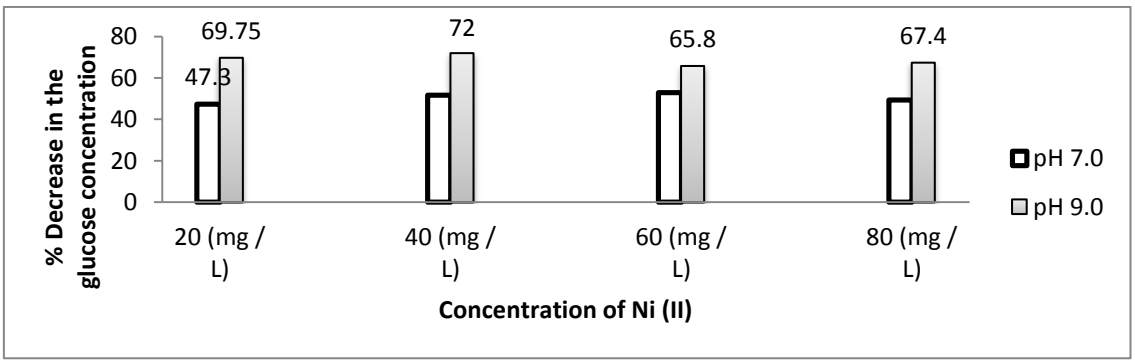


Fig. 5.25% change in the glucose concentration at different pH conditions in the presence of $Ni^{2+}$and *Lactuca scariola Linn*

5.5.1.3 Alteration of Chemical behaviour of Ni^{2+}at different pH *in vitro* with *Lactuca scariola Linn* leaves extract

The results revealed that the λ_{max} value of Ni^{2+}at pH 7.0 and 9.0 were 394.0 nm and 396.0 nm respectively. The combination of Ni^{2+} with aqueous extract *Lactuca scariola Linn* showed a hypsochromic shift to 330.0 nm and 306.0 nm at pH 7.0 and 9.0 respectively (Table 5.19).

Table 5.19 λ_{max} and absorbance values of Ni^{2+}alone and in combination with *Lactuca scariola Linn. Leaves* extract at different pH.

Solution Samples	**Concentration of samples**	**pH 7.0**		**pH 9.0**	
		λ_{max} (nm)	Absorbance	λ_{max} (nm)	Absorbance
Ni^{2+}	6 mg/mL	394.0[a]	0.1329	396.0	0.1274
Ni^{2+}+ extract	6 mg/mL + 0.5 mL of extract	330.0	4.3087	306.0	4.0569

Extract: *Lactuca scariola Linn.* leaves extract; [a] Data shown as λ_{max} and absorbance values by Ni^{2+}alone and in combination with extract by using UV-VIS Spectrophotometer at pH 7.0 and 9.0.

5.5.2 *In vivo* study

Glucose homeostasis

5.5.2.1 Influence of aqueous *Lactuca scariola Linn* leaves extract on acute exposure (OGTT) on non diabetic and diabetic male rats.

Fasting blood sugar (FBS) level was measured in rats of all the groups. Blood glucose levels were measured with the treatment of aqueous *Lactuca scariola Linn* leaves extract or Ni^{2+}or both or Glipizide drug at intervals of 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose loading.

Non-Diabetic rats

Group II rats which are treated with aqueous leaves extract showed almost similar OGTT result as that of normal Group I rats. Group III rats which are treated with Ni^{2+}showed an increased blood glucose level. Whereas, the blood glucose level of group IV Ni^{2+}treated rats was significantly decreased on supplementation with aqueous *Lactuca scariola Linn* leaves extract (Table 5.20).

Diabetic rats

The OGTT results of group V diabetic rats showed a significant increase in the blood glucose level. On supplementation of aqueous leaves extract to group VI rats there was a significant decrease in the blood glucose level. Similarly, the blood glucose level increased to a much higher level in the group VII rats due to Ni^{2+} treatment. In group VIII Ni^{2+} treated rats; on further supplementation with aqueous leaves extract there was a significant decrease in the blood glucose level (Table 5.21).

The blood glucose levels of both non diabetic and diabetic group of rats supplemented with *Lactuca scariola Linn* and treated with $NiSO_4$ are depicted in fig 5.26. It can be clearly seen from the figure that a steady increase in blood glucose level till the end of 1.0h in all the groups. In case of diabetic or Ni^{2+} treated or diabetic + Ni^{2+} treated groups, the oral glucose tolerance curve did not return back to near baseline values within 2h. Interestingly, rats of diabetic, Ni^{2+} treated or diabetic + Ni^{2+} treated groups when supplemented with *Lactuca scariola Linn* leaves extract have shown better glucose tolerance as compared to diabetic or nickel treated rats.

The significant decrease in blood glucose level with supplementation of plant extract was also observed when the % change in glucose levels was calculated for different groups (Fig. 5.27).

Table 5.20 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Lactuca scariola Linn* extract on blood glucose level in normal (**non diabetic**) rats.

Period after glucose ingestion (hours)						
Treatment Groups	**FBS**	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
I	$90.00 \pm 3.22^{a,1}$	$89.50 \pm 2.42^{a,1}$	$117.33 \pm 5.00^{b,1}$	$128.66 \pm 6.28^{c,1}$	$113.33 \pm 2.87^{b,1}$	$94.5 \pm 6.47^{a,1}$
II	$90.83 \pm 2.56^{a,1}$	$89.5 \pm 3.67^{a,1}$	$101.33 \pm 6.97^{b,2}$	$116.16 \pm 8.49^{c,2}$	$100.83 \pm 5.87^{b,2}$	$90.66 \pm 2.50^{a,1}$
III	$88.66 \pm 3.66^{a,1}$	$101.16 \pm 5.30^{b,1}$	$177.50 \pm 23.18^{c,3}$	$185.00 \pm 23.66^{c,3}$	$152.50 \pm 21.15^{d,3}$	$121.66 \pm 15.70^{e,2}$
IV	$89.66 \pm 4.76^{a,1}$	$95.33 \pm 4.08^{a,2}$	$113.83 \pm 3.97^{b,1}$	$138.83 \pm 4.95^{c,4}$	$125.16 \pm 6.79^{d,4}$	$116.66 \pm 7.44^{b,2}$

Treatment groups: I, normal control; II, *Lactuca scariola Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+} + *Lactuca scariola Linn* leaves extract. Horizontal values are the average ± standard Deviation (n=6) in each group till at 2 hours. Values with different superscript alphabets (a, b, c, d) are significantly different from each other ($p<0.05$) in same group till 2h. Vertical values are variation in blood glucose level of four different groups at different time interval till 2 hours. Values with different superscripts (1, 2, 3, 4) are significantly different from each other ($p<0.05$) at different times.

Table 5.21 Effect of acute exposure to Ni^{2+} and simultaneous supplementation with *Lactuca scariola Linn* extract on blood glucose level in **diabetic** rats.

Period after glucose ingestion (hours)						
Treatment Groups	**FBS**	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**
V	$257.66 \pm 10.59^{a,1}$	$257.5 \pm 10.36^{a,1}$	$322.83 \pm 14.64^{b,1}$	$395.33 \pm 7.39^{c,1}$	$375.66 \pm 3.82^{d,1}$	$350.83 \pm 7.35^{e,1}$
VI	$255.33 \pm 8.16^{a,1}$	$234.50 \pm 8.91^{b,2}$	$286.83 \pm 8.63^{c,2}$	$350.66 \pm 12.37^{d,2}$	$322.5 \pm 11.36^{e,2}$	$268.50 \pm 10.74^{f,2}$
VII	$252.66 \pm 10.19^{a,1}$	$278.50 \pm 12.62^{b,3}$	$381.00 \pm 12.48^{c,3}$	$402.00 \pm 12.40^{d,1}$	$415.66 \pm 14.05^{d,3}$	$405.00 \pm 14.17^{d,3}$
VIII	$256.00 \pm 8.48^{a,1}$	$263.00 \pm 9.6^{a,1}$	$324.00 \pm 7.12^{b,1}$	$382.66 \pm 10.98^{c,3}$	$381.33 \pm 9.72^{c,1}$	$368.00 \pm 6.81^{d,4}$
IX	$251.83 \pm 5.74^{a,1}$	$236.33 \pm 11.91^{b,2}$	$275.00 \pm 5.79^{c,2}$	$296.50 \pm 4.37^{d,4}$	$216.66 \pm 10.63^{e,4}$	$154.16 \pm 20.35^{f,5}$

Treatment groups: V, diabetic control; VI, diabetic + *Lactuca scariola Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Lactuca scariola Linn* leaves extract; IX, diabetic + glipizide (0.25mg/100g). Horizontal values are the average ± standard deviation (n=6) in each group till at 2h. Values with different superscript alphabets (a, b, c, d) are significantly different from each other ($p<0.05$) in same group till 2h. Vertical values are variation in blood glucose level of five different groups at different time interval till 2 hours. Values with different superscripts (1, 2, 3, 4) are significantly different from each other ($p<0.05$) at different times.

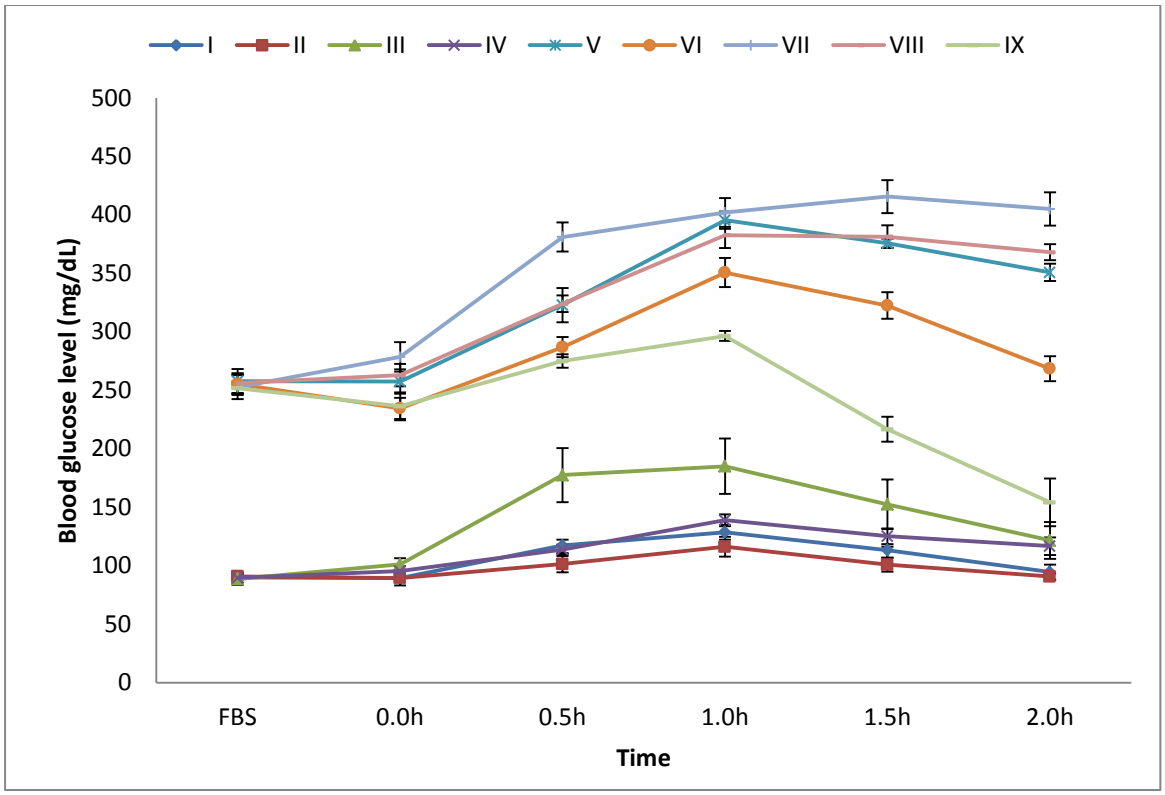


Fig. 5.26 OGTT results of non diabetic and diabetic rats supplemented with aqueous *Lactuca scariola Linn* leaves extract.

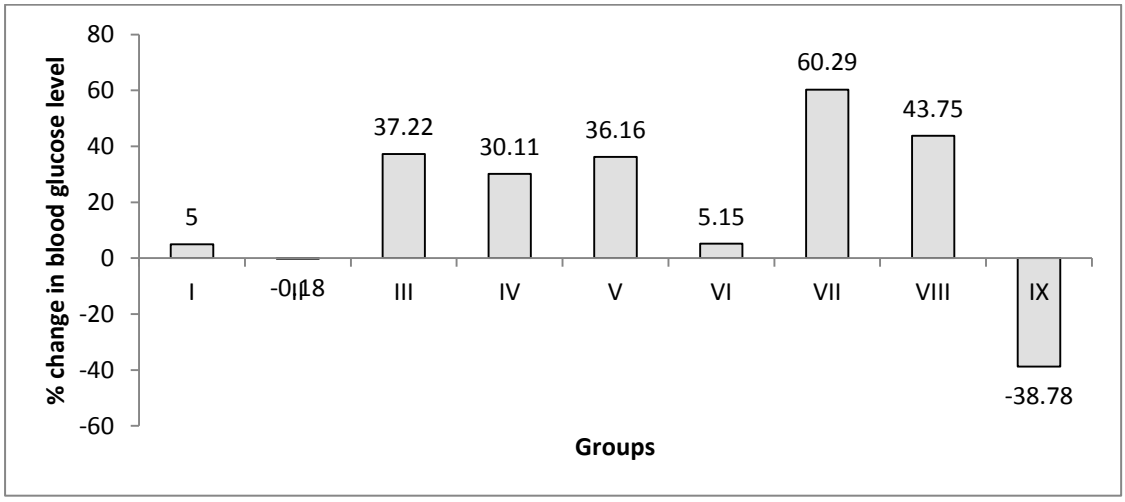


Fig. 5.27 % Change in blood glucose level at the end of 2.0 h of OGTT from the FBS with*Lactuca scariola Linn*in control and diabetic rats

Treatment groups: I, normal control; II, *Lactuca scariola Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+} + *Lactuca scariola Linn* leaves extract; V, diabetic control; VI, diabetic + *Lactuca scariola Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Lactuca scariola Linn* leaves extract ; IX, diabetic + glipizide (0.25mg/100g).

5.5.2.2 Influence of aqueous *Lactuca scariola Linn* leaves extract on the Sub chronic (13 days) exposure on diabetic and non diabetic male rats.

After the day of OGTT, the rats were continued with the treatment of the aqueous leaves extract and $NiSO_4$ in the designed experimental groups on every alternate day for a period of 13 days and simultaneously blood glucose level is monitored on every alternate day.

Non Diabetic rats

The results of sub chronic exposure of group I and group II rats showed similar results. The group III rats on treating with Ni^{2+} for a period of 13 days showed an increase in the blood glucose level and group IV Ni^{2+} treated rats on supplementation of *Lactuca scariola Linn* leaves extract for a period of 13 days, there was a significant decrease in the blood glucose level (Table 5.22).

Diabetic rats

Group V diabetic rats showed an increased blood glucose level over a period of 13 days. The diabetic rats of Group VII on treating with Ni^{2+} also showed an increased blood glucose level similar to that of group V rats. However, the diabetic rats of group VI and Ni^{2+} treated diabetic rats of group VIII showed a significant decrease in the blood glucose level on supplementation of aqueous *Lactuca scariola Linn* leaves extract. The blood glucose level was decreased to almost normal (Table 5.23).

Fig. 5.28 depicts the percentage change in blood glucose level for a period of 13 days in different group of rats. When the percentage change in blood glucose level was calculated, a significant decrease in blood glucose level was observed in group VI and group VIII rats which are supplemented with the plant extract.

Table 5.22 Effect of sub chronic 1reatment with $Ni^{2+}$and simultaneous supplementation with *Lactuca scariola Linn leaves* extract on blood glucose level in normal (**non diabetic**) rats.

	Blood glucose levels (mg/ dL)						
Treatment Groups	**1st day**	**3rd day**	**5th day**	**7th day**	**9th day**	**11th day**	**13th day**
I	$89.33 \pm 2.58^{a,1}$	$90.50 \pm 2.50^{a,1}$	$91.83 \pm 1.94^{a,1}$	$90.66 \pm 1.75^{a,1}$	$89.83 \pm 1.32^{a,1}$	$89.66 \pm 1.03^{a,1}$	$91.00 \pm 0.89^{a,1}$
II	$90.83 \pm 2.48^{a,1}$	$89.33 \pm 2.33^{a,1}$	$86.66 \pm 1.86^{a,1}$	$87.16 \pm 3.06^{a,1}$	$90.00 \pm 2.44^{a,1}$	$89.16 \pm 2.63^{a,1}$	$87.66 \pm 2.73^{a,1}$
III	$88.66 \pm 3.66^{a,1}$	$112.5 \pm 8.80^{b,2}$	$134.16 \pm 7.22^{c,2}$	$137.50 \pm 7.03^{c,2}$	$138.50 \pm 6.09^{c,2}$	$140.66 \pm 5.78^{d,2}$	$142.16 \pm 5.34^{d,2}$
IV	$89.83 \pm 4.16^{a,1}$	$106.50 \pm 6.50^{b,2}$	$116.83 \pm 2.99^{c,3}$	$120.66 \pm 3.76^{c,3}$	$122.16 \pm 3.76^{c,3}$	$120.00 \pm 4.42^{c,3}$	$116.83 \pm 2.71^{c,3}$

Treatment groups: I, normal control; II, *Lactuca scariola Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+}+ *Lactuca scariola Linn* leaves extract; Horizontal values are the average ± standard deviation (n=6) in each group in every alternate day till 13th day. Values with different superscript alphabets (a, b, c, d) are significantly different from each other ($p<0.05$) in same group at different days. Vertical values are variation in blood glucose level of four different groups in each alternate day till 13th day. Values with different superscripts (1, 2, 3, 4) are significantly different from each other ($p<0.05$) at different days.

Table 5.23 Effect of subchronic treatment with $Ni^{2+}$and simultaneous supplementation with *Lactuca scariola Linn* leaves extract on blood glucose level in **diabetic** rats.

	Blood glucose levels (mg/ dL)						
Treatment Groups	**1st day**	**3rd day**	**5th day**	**7th day**	**9th day**	**11th day**	**13th day**
V	$253.16 \pm 7.22^{a,1}$	$262.66 \pm 5.57^{a,1}$	$274.00 \pm 9.87^{b,1}$	$295.66 \pm 8.04^{c,1}$	$298.00 \pm 10.65^{c,1}$	$312.33 \pm 6.37^{d,1}$	$326.66 \pm 7.25^{d,1}$
VI	$252.50 \pm 10.32^{a,1}$	$235.16 \pm 6.33^{b,2}$	$219.83 \pm 4.53^{c,2}$	$196.00 \pm 4.89^{d,2}$	$178.33 \pm 8.16^{e,2}$	$157.50 \pm 8.75^{f,2}$	$141.33 \pm 9.45^{g,2}$
VII	$251.33 \pm 7.55^{a,1}$	$266.33 \pm 6.53^{b,1}$	$272.66 \pm 6.12^{b,1}$	$293.00 \pm 4.14^{c,1}$	$296.33 \pm 3.88^{c,1}$	$311.83 \pm 7.49^{c,1}$	$326.83 \pm 2.48^{d,1}$
VIII	$256.00 \pm 8.48^{a,1}$	$260.33 \pm 9.09^{a,1}$	$248.16 \pm 5.67^{b,3}$	$238.00 \pm 6.75^{b,3}$	$227.50 \pm 7.55^{c,3}$	$215.66 \pm 6.77^{d,3}$	$169.00 \pm 7.94^{e,3}$
IX	$254.56 \pm 9.45^{a,1}$	$250.60 \pm 10.37^{a,3}$	$175.28 \pm 9.87^{b,4}$	$142.49 \pm 10.05^{c,4}$	$125.28 \pm 9.11^{d,4}$	$109.43 \pm 10.28^{e,4}$	$94.24 \pm 9.26^{f,4}$

Treatment groups: V, diabetic control; VI, diabetic + *Lactuca scariola Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Lactuca scariola Linn* leaves extract; IX, diabetic + glipizide (0.25mg/100g). Horizontal values are the average ± standard deviation (n=6) in each group in every alternate day till 13th day. Values with different superscript alphabets (a, b, c, d etc.) are significantly different from each other ($p<0.05$) in same group at different days. Vertical values are variation of blood glucose level of five different groups in each alternate day till 13th day. Values with different superscripts (1, 2, 3, 4) are significantly different from each other ($p<0.05$) at different days.

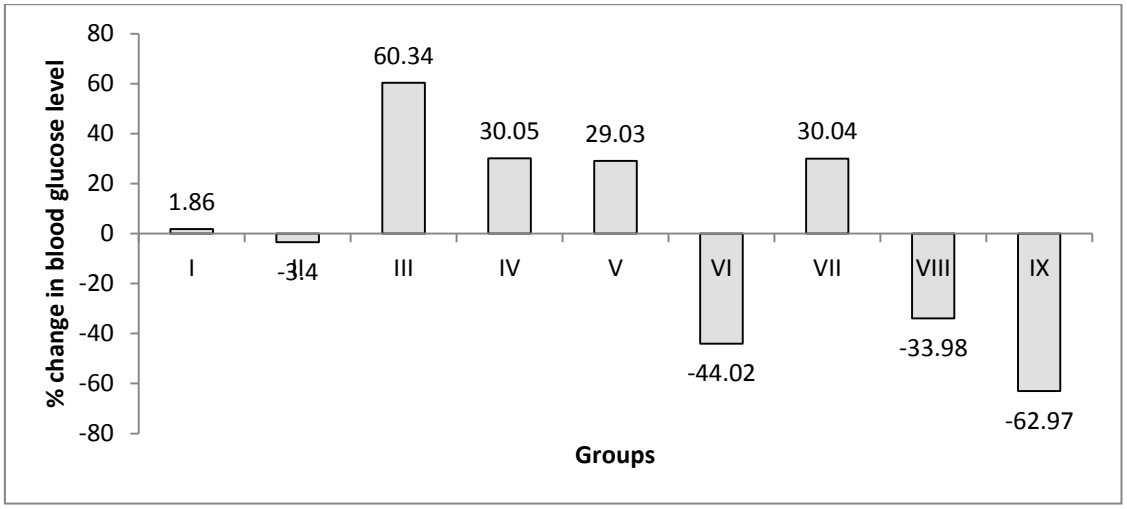


Fig.5.28% Change in blood glucose level at the end of 13 days of treatment from day 1 in control and diabetic rats

Treatment groups: I, normal control; II, *Lactuca scariola Linn* leaves extract; III, Ni^{2+}; IV, Ni^{2+} + *Lactuca scariola Linn* leaves extract; V, diabetic control; VI, diabetic + *Lactuca scariola Linn* leaves extract; VII, diabetic + Ni^{2+}; VIII, diabetic + Ni^{2+}+ *Lactuca scariola Linn* leaves extract ; IX, diabetic + glipizide (0.25mg/100g).

5.5.2.3 Effect of *Lactuca scariola Linn* on Fasting blood glucose, Fasting Plasma Insulin and Insulinogenic index.

As shown in table 5.24, after 13 days of sub chronic exposure, the diabetic rats showed an increased fasting blood glucose level (Group 2 vs Group 1). However, on supplementation of *Lactuca scariola Linn* extract for a period of 13 days there was a remarkable decrease in the fasting blood glucose level (Group 3 vs Group 2). Whereas, glipizide drug treatment also caused a similar kind of decrease in the fasting blood glucose level (Group 4 vs Group 2). Similarly, the fasting plasma insulin decreased significantly in diabetic rats (Group 2 vs Group 1). On supplementing the diabetic rats with the *Lactuca scariola Linn* extract there was no significant change (Group 3 vs Group 2). The insulinogenic index was increased in diabetic rats (Group 2 vs Group 1). On supplementation of *Lactuca scariola Linn* extract to diabetic rats there was no significant change (Group 3 vs Group 2). Whereas, on treating with glipizide drug the insulinogenic index was similar to the control rats (Group 4 vs Group 1).

Table 5.24 Effect of sub chronic (13 days) supplementation of *Lactuca scariola Linn* leaves extract (12.5 mg/100 g.b.wt) on glucose homeostasis in diabetic rats.

Parameters	Control (G-1)	Diabetic (G-2)	diabetic + *Lactuca scariola Linn* extract (G-5)	diabetic + glipizide (G-6)
Fasting blood glucose (mg/ dL)	85.00 ± 5.56^{a}	180.45 ± 13.58^{b}	110.00 ± 12.82^{d}	105.23 ± 14.65^{d}
Fasting plasma Insulin (µg/L)	1.00 ± 0.17^{a}	0.66 ± 0.19^{b}	0.65 ± 0.18^{b}	0.97 ± 0.16^{a}
Insulinogenic – index	0.0043 ± 0.0005^{a}	0.0065 ± 0.0004^{b}	0.0053 ± 0.0003^{c}	0.0044 ± 0.0002^{a}

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Lactuca scariola Linn* extract; 4, diabetic + glipizide. Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$).

5.5.3 Effect of *Lactuca scariola Linn* leaves extract on sNOS3, sNO, sHIF-1α and sVEGF concentrations in diabetic rats.

After 13 days from the day 1, the serum NOS3 decreased significantly in diabetic rats (Group 2 vs Group 1). However, diabetic rats supplemented with *Lactuca scariola Linn* extract there was a significant increase in serum NOS3 (Group 3 vs Group 2). Glipizide drug treatment also showed remarkable improvement in the Serum NOS3 (Group 4 vs Group 2). Serum NO which is decreased in diabetic group was significantly increased on the supplementation of *Lactuca scariola Linn* (Group 3 vs Group 2). However, on glipizide treatment, there was significant rise in the serum NO (Group 4 vs Group 2). It can be seen that, the serum HIF-1α was significantly increased in diabetic rats (Group 2 vs Group 1). On treatment with *Lactuca scariola Linn* extract there was a remarkable increase in the Serum HIF-1α (Group 3 vs Group 2). Treatment of glipizide drug also didn't show any significant changes (Group IV vs Group 2). It can also be seen that, there was a significant increase of serum VEGF in diabetic rats (Group 2 vs Group 1). However, the supplementation of *Lactuca scariola Linn* leaves extract did not improve serum VEGF level (Group 3 vs Group 2) (Table 5.25). On further analysis of the data as % change difference (fig. 5.29 and 5.30) reflects a greater improvement in serum NOS3 after the supplementation of *Lactuca scariola Linn* (E1 vs E2). In the case of serum NO, % change improvement is also significant (E1 vs E2). Whereas, fig. 5.31 and 5.32, reflects % change of serum HIF-1α and serum VEGF respectively, it has been clearly found that there is significant increase in % change of HIF-1α in diabetic treated with the *Lactuca scariola Linn* extract (E2 vs E1). But serum VEGF didn't show such alterations.

Table 5.25 Effect of sub chronic (13 days) supplementation of *Lactuca scariola Linn* leaves extract (12.5 mg/100 g.b.wt) on serum NOS3, NO and HIF-1α concentrations in diabetic rats.

Parameters	Group 1	Group 2	Group 3	Group 4
Serum NOS3 (nmol/mL)	7.13 ±1.11^{a}	4.68 ±1.53^{b}	6.45 ±2.16^{d}	6.67.±2.34^{d}
Serum NO (µmol/l)	22.32±3.04^{a}	10.45±3.20^{b}	15.11±1.75^{c}	17.36±2.22^{d}
Serum HIF-1α (pg/mL)	150.21±5.28^{a}	168.23±12.32^{b}	200.50±23.72^{d}	175.08±8.15^{c}
Serum VEGF (pg/mL)	300.31±5.65^{a}	423±25.20^{b}	423 ±25.50^{b}	378.17±12.34^{d}

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Lactuca scariola Linn* extract; 4, diabetic+ glipizide.

Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$)

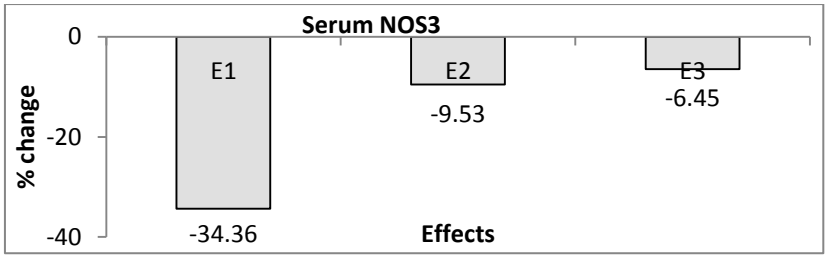


Fig.5.29 % change of Serum NOS3 in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

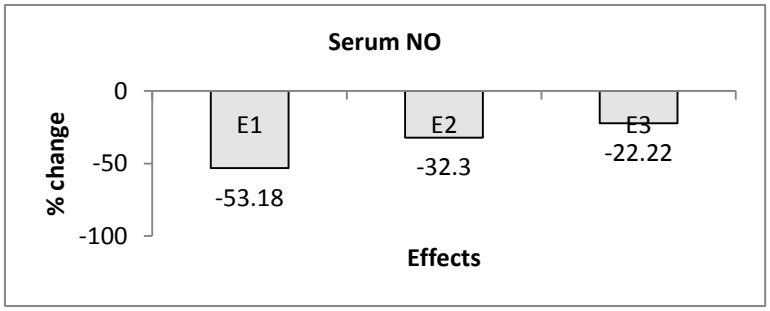


Fig.5.30 % change of Serum NO in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

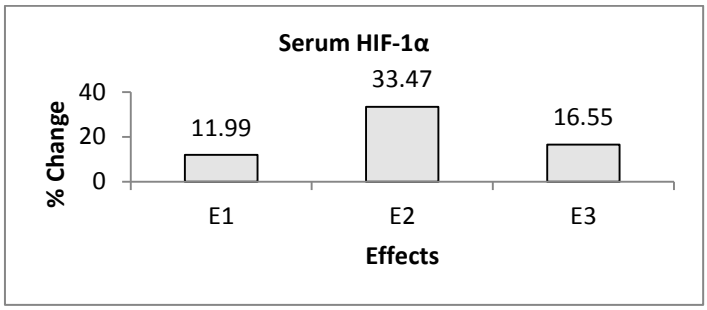


Fig.5.31 % change of Serum HIF-1αVEGF in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

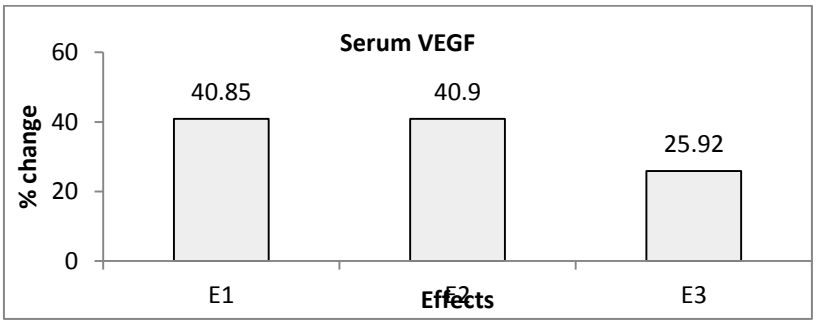


Fig.5.32 % change of Serum VEGF in diabetic rats

E1, Group1 vs Group II; E2, Group 1 vs Group III; E3, Group 1 vs Group IV

Comparative analysis of anti diabetic efficacy of four plants *in vivo*

As shown in the results, on supplementation of the diabetic male albino rats with various aqueous plant extracts for a period of 13 days i.e., sub chronic exposure, *Cicer arietinum Linn* leaves extract has not decreased the blood glucose level. Similarly, the aqueous extract of *Hibiscus cannabinus Linn leaves* also didn't significantly decrease the blood glucose level. However, the aqueous *Trigonella foenum graecum* and *Lactuca scariola Linn* leaves extract have shown remarkable decrease in the blood glucose level (Fig. 5.33).

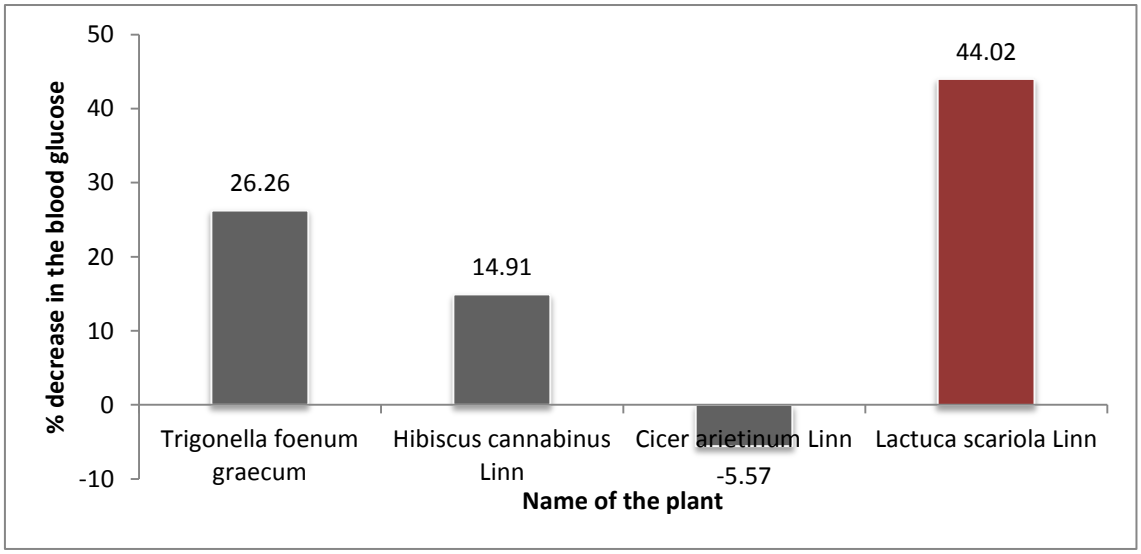


Fig. 5.33 Comparative % decrease in blood glucose level on sub chronic exposure of different extracts

As seen from the fig. 5.33, the aqueous leaves extract of *Lactuca scariola Linn* is found to be most efficient anti diabetic among the plants that were part of this work. Hence, to strengthen our work further, electrophysiology and histopathology studies were carried out with this plant.

Specific Electrophysiology and Histopathology analysis on diabetic rats supplemented with *Lactuca scariola Linn*

The aqueous leaves extract of *Lactuca scariola Linn* has shown greater anti diabetic potential than that of *Trigonella foenum graecum* leaves extract. As a result of this, we further extended our work to study the beneficial effects of *Lactuca scariola Linn* leaves extract on cardiovascular system *in vivo* and diabetic induced toxicities in liver, kidney, heart and aorta through histopathology measurements.

5.5.4 Electrophysiology Analysis of *Lactuca scariola Linn*

Electrophysiological analysis of *Lactuca scariola Linn* supplemented diabetic animals showed interesting observations in pre diabetic, diabetic and diabetic + *Lactuca scariola Linn* (14 days supplementation) rats. There was a significant reduction in the heart rate of diabetic rats when compared to that of pre diabetic rats. But remarkable improvement in the heart rate was observed in diabetic rats supplemented with *Lactuca scariola Linn* (Table 5.26).

Table 5.26 Mean arterial pressure (MAP), heart rate (H) and baroreflex sensitivity (sympathetic) in prediabetic (group 1), diabetic (group 2) and diabetic + *Lactuca scariola Linn* (14 days treatment)

Variables	G-1 (Pre diabetic)	G-2 (Diabetic)	G-3 (Diabetic + *L. scariolaL.*	P Value
SBP, mmHg	100.23 ± 10.23^{a}	146.23 ± 13.56^{b}	120.34 ± 12.23^{c}	0.0000
DBP, mmHg	74.45 ± 11.00^{a}	98.45 ± 11.54^{b}	80.45 ± 10.45^{a}	0.0000
PP, mmHg	25.78 ± 3.66	47.98 ± 3.84	39.89 ± 3.48	0.0000
MAP, mmHg	100.45 ± 15.34^{a}	124.50 ± 12.45^{b}	109.75 ± 12.55^{a}	0.0001
H, bpm	327.2 ± 72.9^{a}	274.92 ± 58.34^{b}	300.45 ± 23.56^{c}	0.0001

Values of different superscripts are significantly differ from each other ($p < 0.05$) (n=6)

Heart rate variability: Frequency domain results of HRV analysis (FFT spectrum) on rat 2 during pre diabetic, diabetic and diabetic with *Lactuca scariola Linn* supplementation for 14 days clearly shows the impact of *Lactuca scariola Linn* on HRV of rat 2 (Fig.5.34 and Kubios chart).

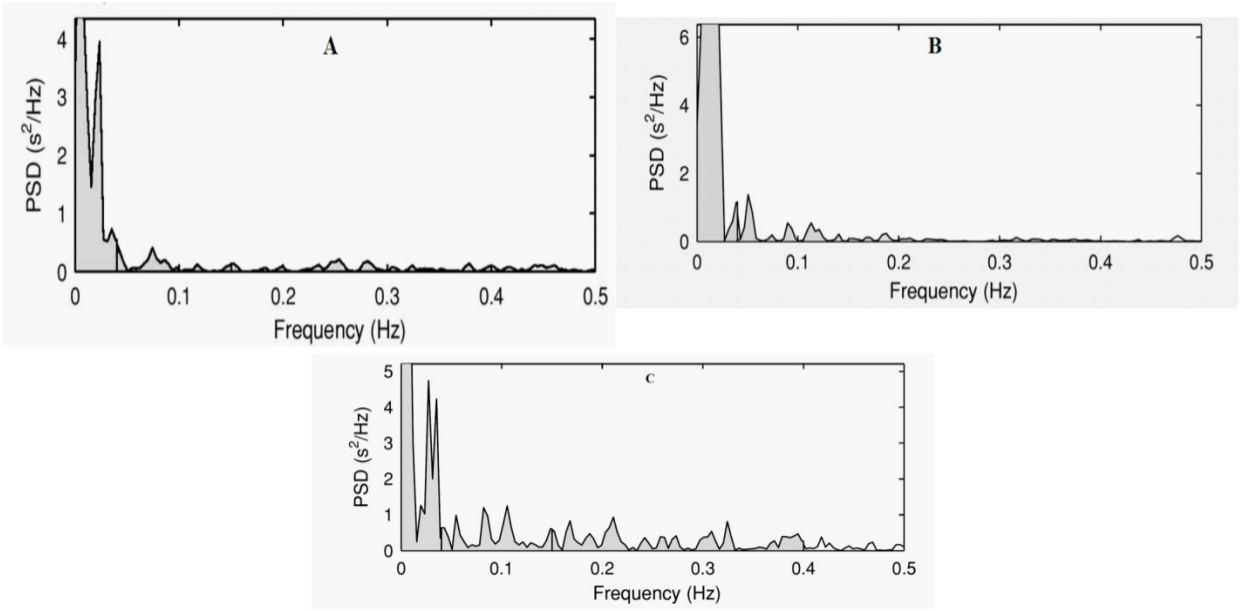


Fig. 5.34 Frequency domain results of HRV analysis (FFT Spectrum) on Rat 2 during Prediabetic (A), diabetic (B) and diabetic supplemented with Lactuca scariola Linn (C).

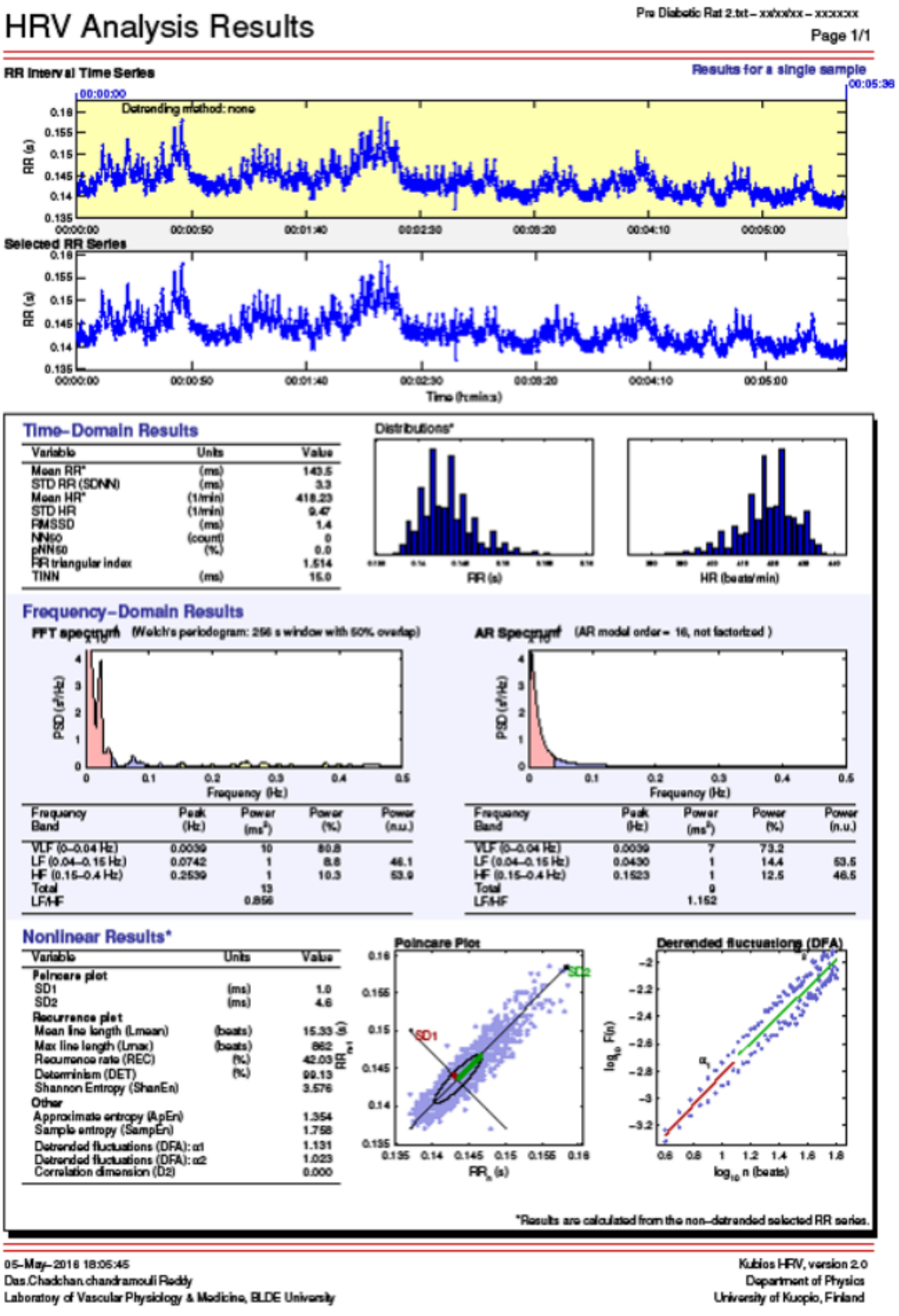


Fig. 5.35 Kubios chart of Pre diabetic rat 2

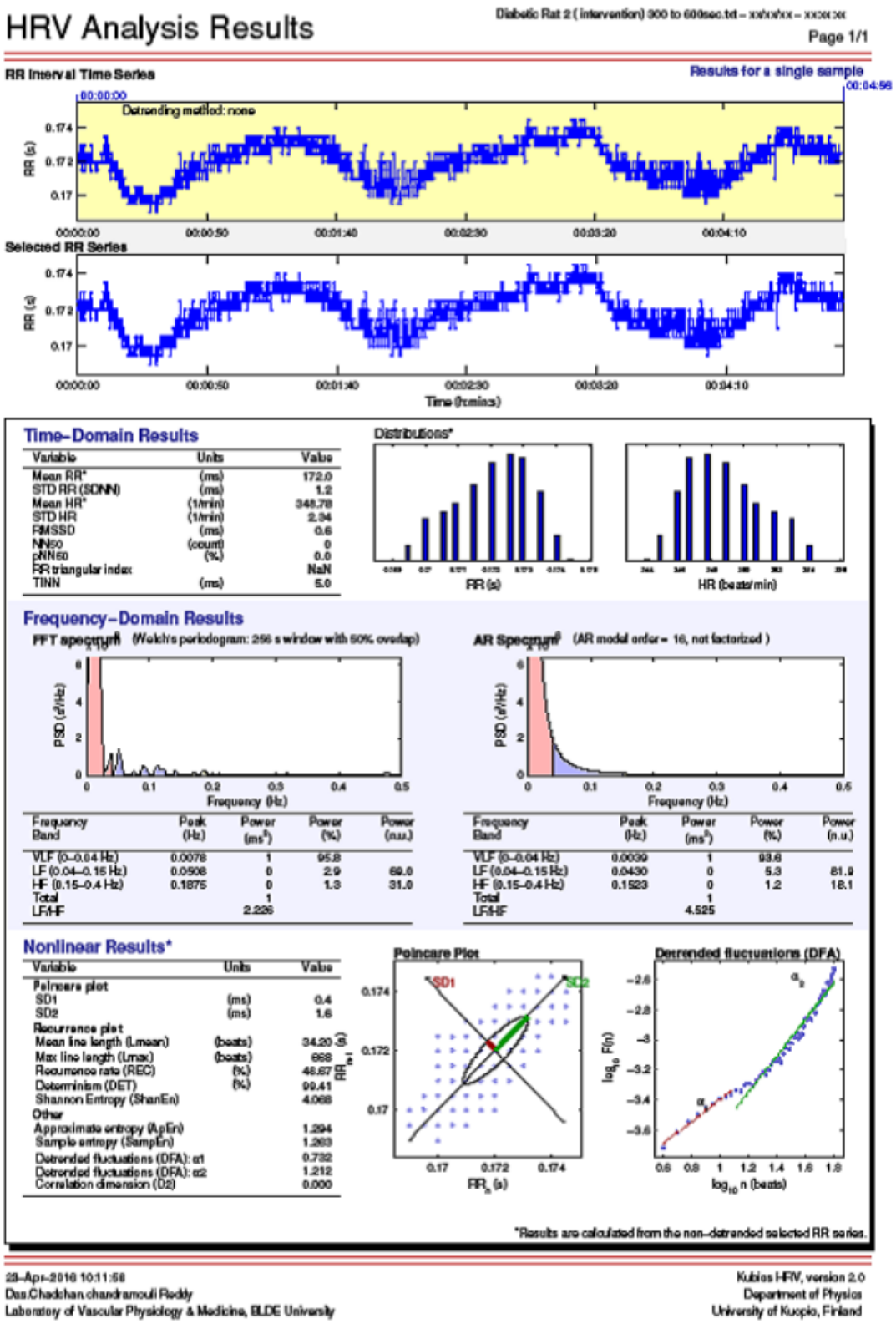


Fig. 5.36 Kubios chart of diabetic rat 2

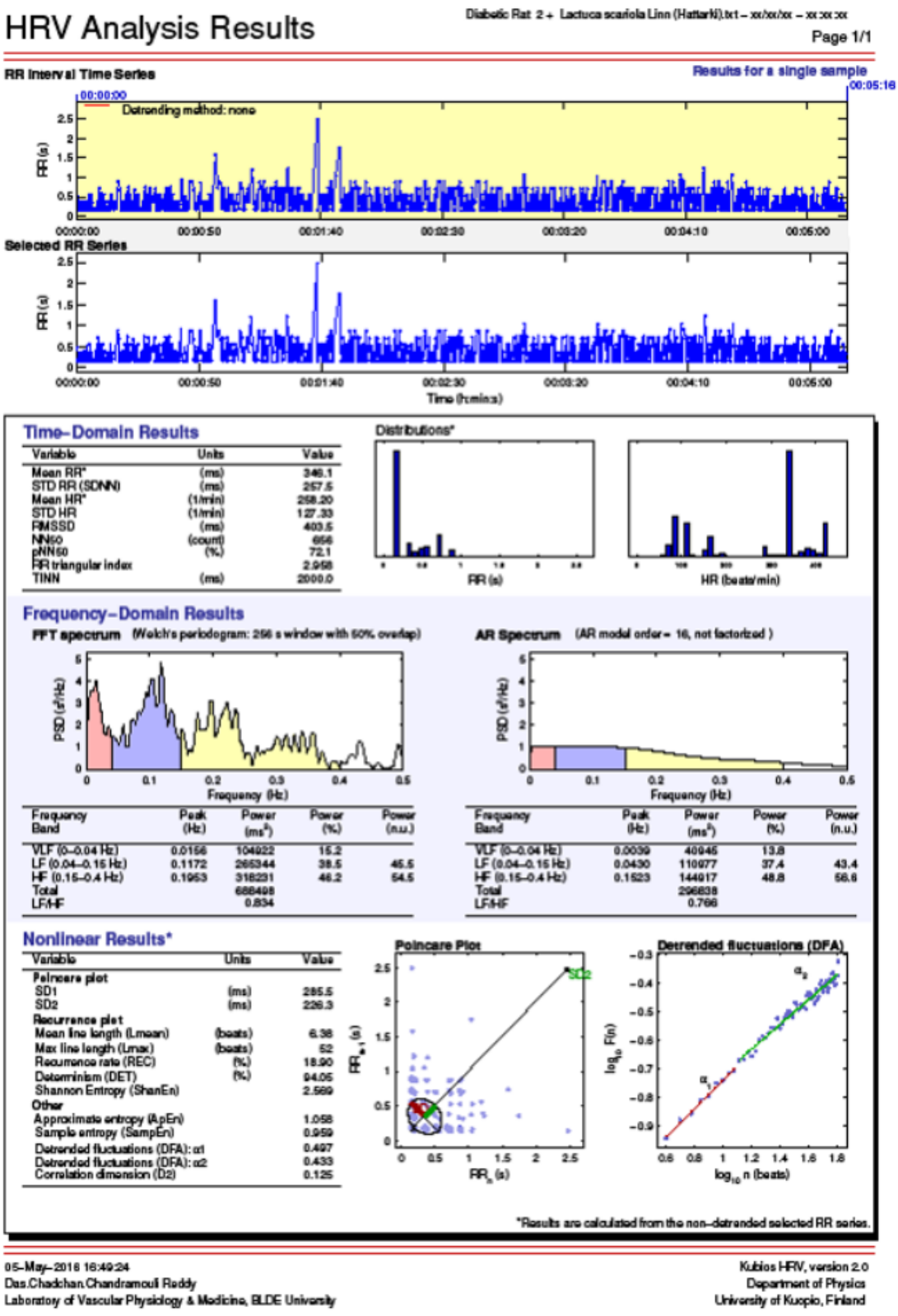


Fig. 5.37 Kubios chart of Diabetic rat 2 treated with *Lactuca scariola Linn*

Fig. 5.38 shows the HF and LF power (nu) of pre diabetic, diabetic and diabetic + *Lactuca scariola Linn* (14 days supplementation) rats. Result shows the decrease in HF power and increase in LF power of diabetic rats was remarkably improved due to supplementation of *Lactuca scariola Linn* for a period of 14 days.

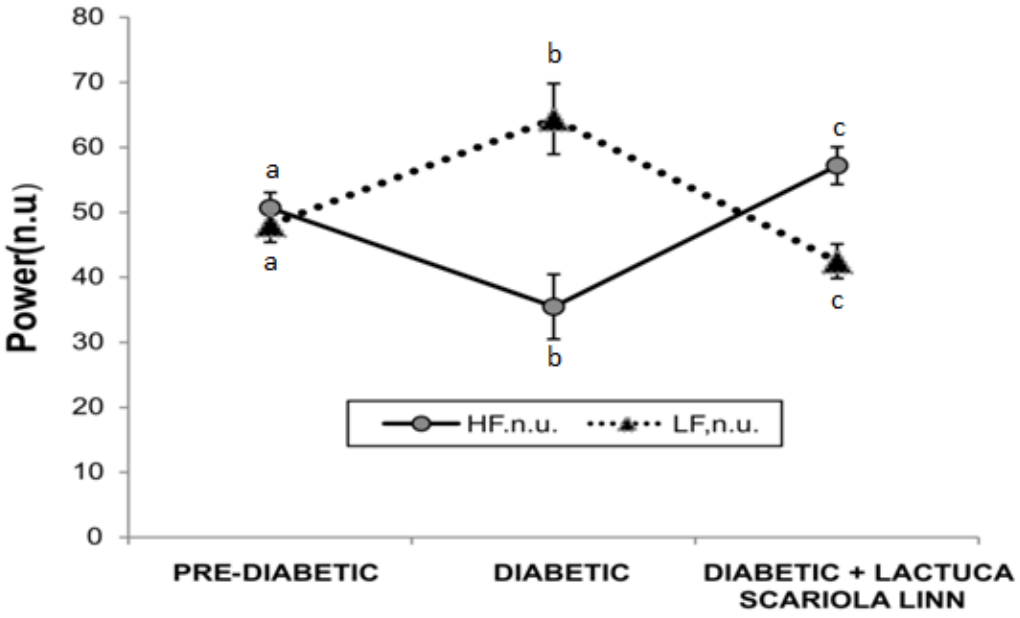


Fig. 5.38 Frequency domain results of HRV analysis (LF and HF power, n.u.) on same adult male rats (n = 6) during pre-diabetic, diabetic and after supplementation of *Lactuca scariola Linn* for 14 days. Values with different superscripts are statistically significant with each other at $p \leq 0.05$

It can be seen in fig. 5.39 that, there was an increase in LF and HF ratio in diabetic rats. However supplementation of *Lactuca scariola Linn* for 14 days showed a remarkable improvement LF and HF ratio.

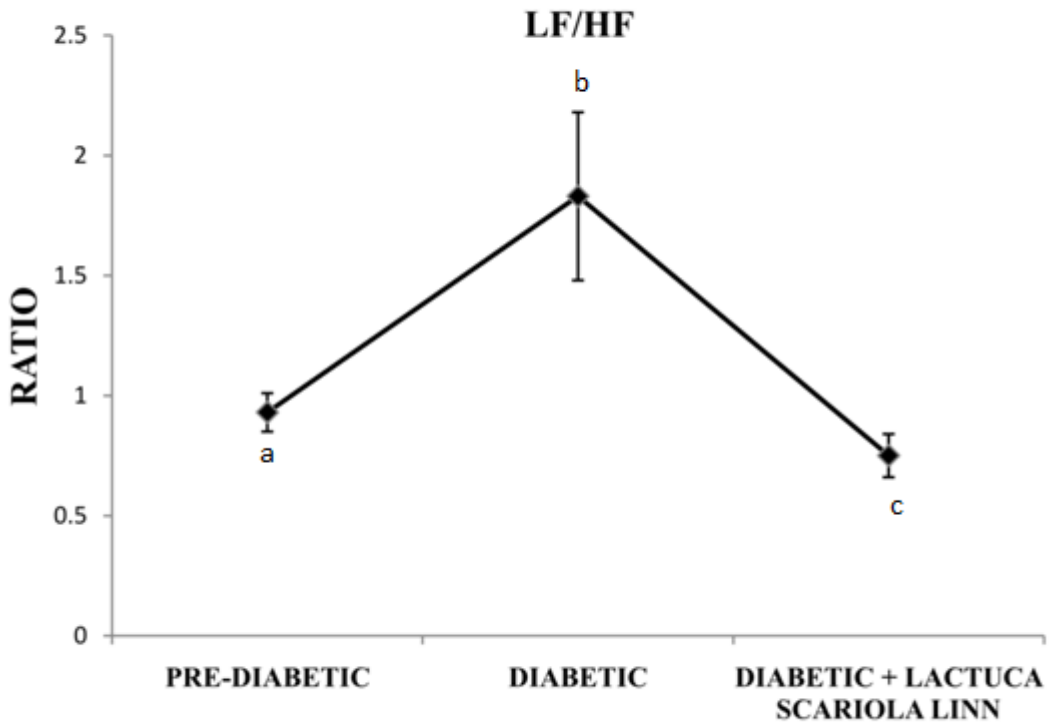


Fig. 5.39 Frequency domain results of HRV analysis (LF/HF ratio) on same adult male rats (n = 6) during pre-Diabetic diabetic and after supplementation of *Lactuca scariola Linn* for 14 days. Values with different superscripts are statistically significant with each other at $p \leq 0.05$

Blood Pressure and Baroreflex sensitivity (BRS): Increase in SBP, DBP and MAP was observed in diabetic rats. However, on supplementation with *Lactuca scariola Linn*, decrease in all three blood pressure parameters can be seen when compared to that of diabetic rats. BRS of diabetic rats was significantly reduced when compared to pre diabetic state. However, after supplementation of *Lactuca scariola Linn* to diabetic rats for a period of 14 days, showed a remarkable improvement in BRS, bringing it back to pre diabetic state (Fig.5.40).

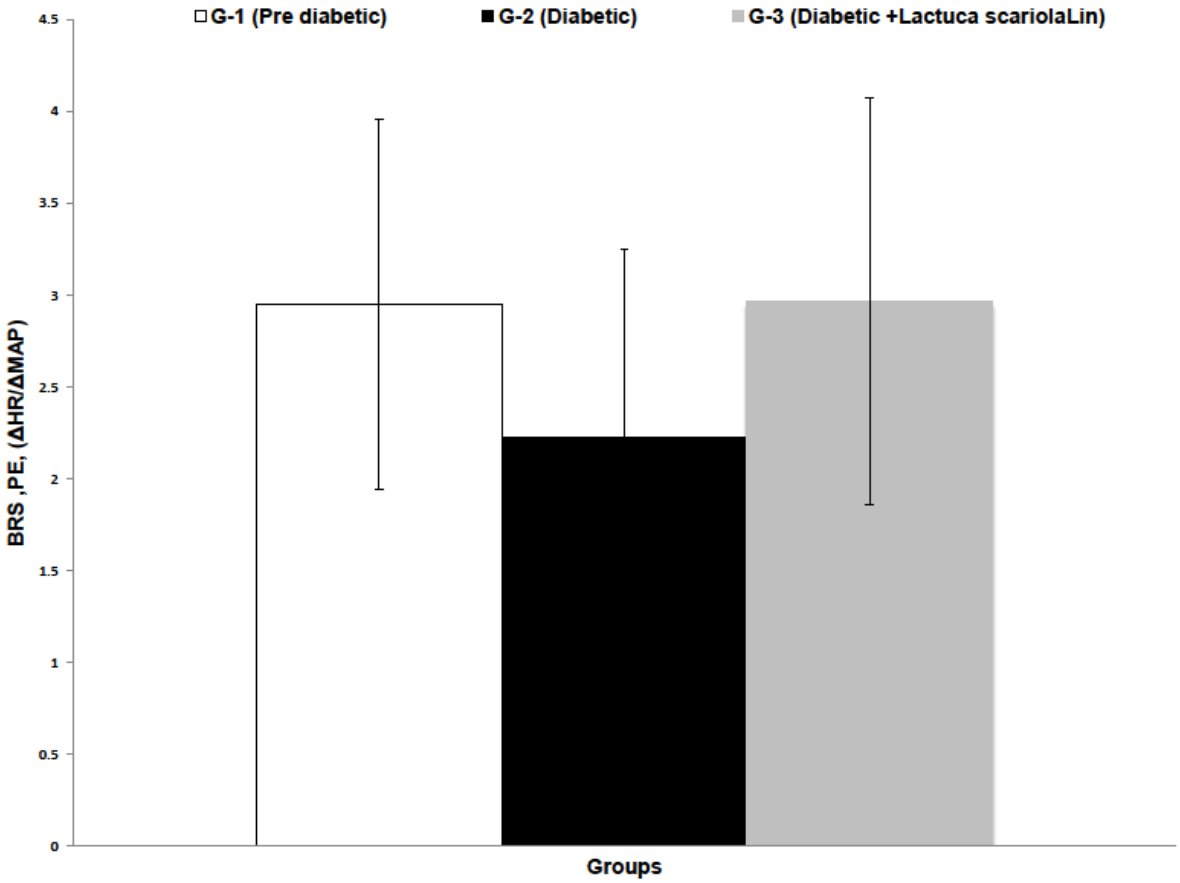


Fig. 5.40 Baroreflex sensitivity (BRS) in pre diabetic (group 1), diabetic (group 2) and diabetic + *Lactuca scariola Linn* supplemented rats (group 3).

5.5.5 Influence of *Lactuca scariola Linn* extract on Hepatorenal Architecture

5.5.5.1 Histopathology of Liver

Section studied under H and E stain showed normal hepatic parenchymal tissue which is having several hexagonal to pyramidal ***'Lobules'***. Each lobule consists of a central vein from which the hepatic plates radiate outwards towards the portal areas; 3 to 5 portal triads are located at the periphery of the lobule; containing branches of bile duct, portal vein and hepatic artery; and occasional mononuclear cells.

Group 1

In group I untreated rats, the histology structure of the liver of untreated rat (group I 10X & 45X) showed normal architecture, i.e., Cords of hepatocytes and blood containing sinusoids radiate from central vein to the peripheral portal triads. The central veins are lined by endothelial cells surrounded by a ring of collagen fibres. The sinusoids are lined by both endothelial cells and Kupffer cells both of which have inconspicuous flattened nuclei and ill-defined cytoplasmic margins. The hepatocytes are polygonal in shape with well defined borders. The nucleus is single, round and has a fine chromatin pattern with 1 to 2 clearly defined amphophilic prominent nucleoli. The cytoplasm is eosinophilic and finely granular (Fig. 5.41).

Group 2

In the case of group II (10X and 45X) diabetic rats, the majority of distorted 'lobular' architecture of liver parenchyma, hepatocytes were seemed to be little swollen, vacuolated microvesicular and eosinophilic cytoplasm beside increase in number of mitotic figures. Foci of fatty change and ballooning degeneration with necrosis of hepatocytes were found in zone 3 (centrilobular) areas. Portal area along with fibrous tissue found to be mildly proliferated and mixed acute and chronic inflammatory cells were found to be infiltrated (Fig. 5.41).

Group 3

However, in group III diabetic rats upon treating with L. scariola extract it can be shown that mild enlargement of hepatocytes. Periportal, mid zone and centrilobular appear to be normal. The central veins are lined by endothelial cells and appear to be slightly dilated (Fig. 5.41).

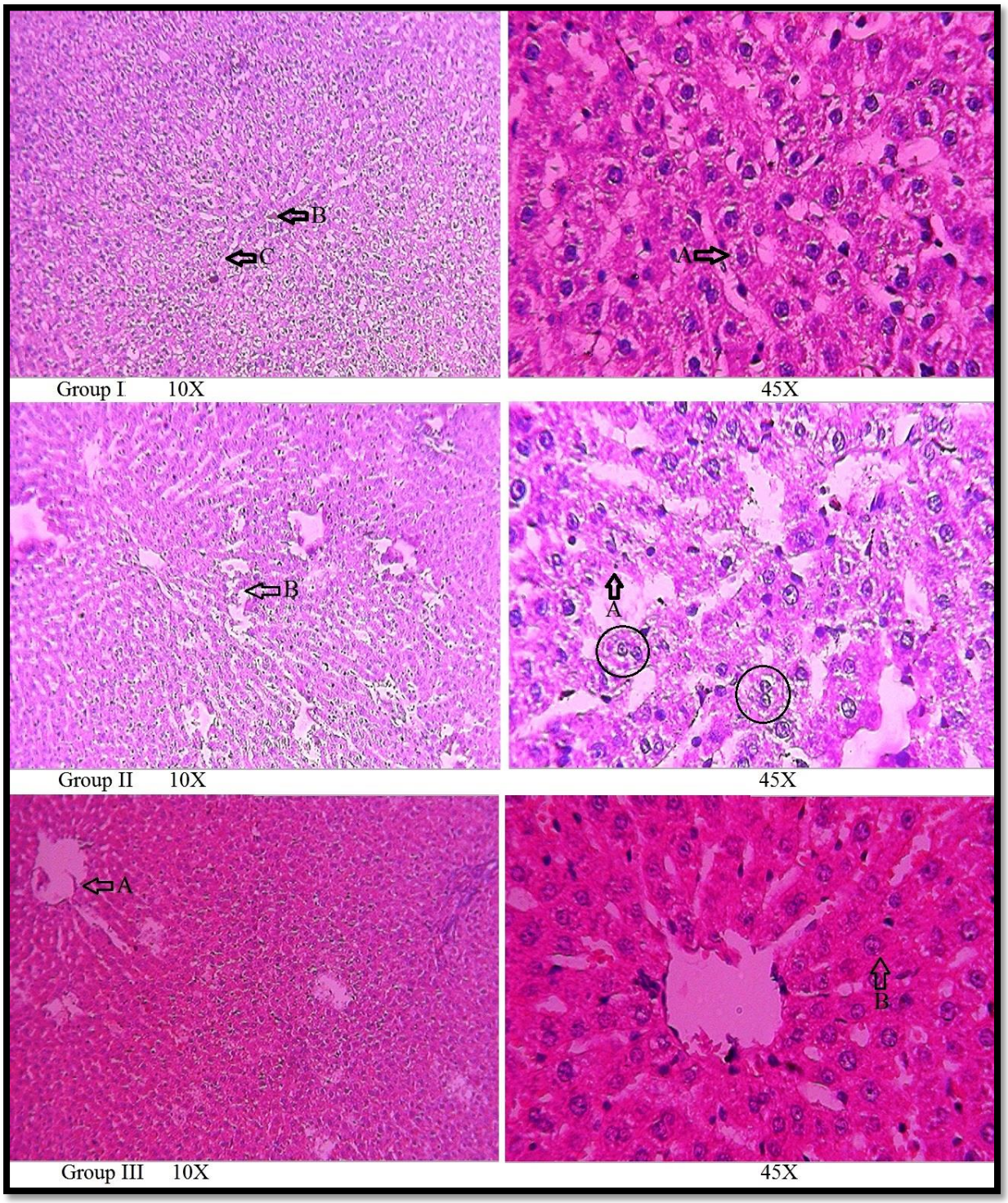


Fig. 5.41 H.E stain (10 x and 45x) of normal hepatic parenchymal tissue of pre diabetic (group 1): normal prediabetic rat liver showing normal architecture; diabetic (group 2): showing distorted 'lobular' architecture of liver parenchyma; diabetic + *Lactuca scariola Linn* (group 3): showing mild enlargement of hepatocytes, Periportal mid zone and Centrilobular appear to be normal.

5.5.5.2 Histopathology of Kidney

Group 1

Section studied under H and E stain shows normal renal parenchymal tissue which is composed of glomeruli and tubules separated by small amount of interstitial connective tissue containing peritubular capillaries. Each glomerulus is a spherical collection of interconnected capillaries within a Bowman's space lined by flattened parietal cells. The outer aspects of the glomerular capillaries are covered by a layer of visceral epithelial cells.It also shows that the capillary tufts and the mesangium appear to be normal.Tubules appear to be normal too. There is no evidence of tubular atrophy. Glomeruli seem to be normal in morphology and in cellularity (Fig. 5.42).

Group 2

In group II diabetic rats, it can be seen that Glomeruli are hypercellular with thickening of glomerular basement membrane and mesangial proliferation. Tubules show focal tubular basement membrane mild thickening with cloudy swelling (coagulation necrosis). The lumen shows eosinophilic protienacious (pink body), suggestive of Acute Tubular Necrosis (ATN). Interstitium is edematous with infiltration of inflammatory cells. Vessels are congested and sclerotic (Fig. 5.42).

Group 3

In group III diabetic rats which are treated with *L. scariola* extract, Glomeruli are hypocellular with thickening of glomerular basement membrane and show sclerotic changes. Tubules show focal tubular necrosis suggestive of Acute Tubular Necrosis (ATN) with regenerative epithelium. Interstitium is edematous with infiltration of inflammatory cells. Vessels are congested and sclerotic (Fig. 5.42).

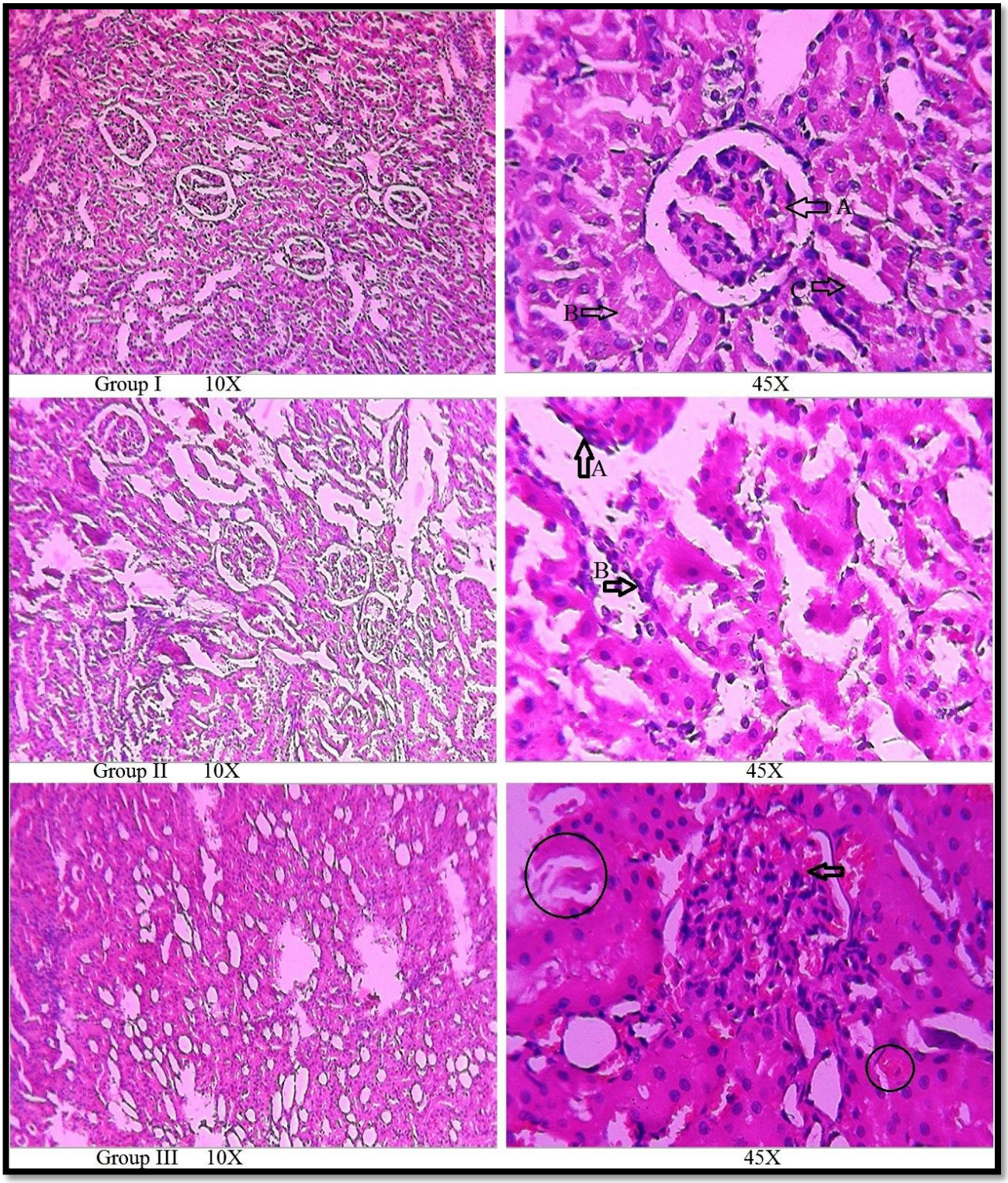


Fig. 5.42 H.E stain (10 x and 45x) of renal parenchymal tissue of pre diabetic (group 1): Glomeruli seem to be normal in morphology and in Cellularity.; diabetic (group 2): Glomeruli are hypercellular with thickening of glomerular basement membrane and mesangial proliferation; diabetic + *Lactuca scariola Linn* (group 3): Tubules show focal tubular necrosis suggestive of Acute Tubular Necrosis (ATN) with regenerative epithtelium.

5.5.5.3 Histopathology of Heart

Section studied under Hematoxylin and Eosin stain shows myocardium composed of branching and anastomosing striated muscle fibres arranged in parallel fashion, separated by capillaries. The myocardial fibres are connected to each other by *intercalated* discs.

Group 1

Microscopic architecture of myocardium shows prominent intercalated discs and centrally placed nucleus in normal group (Fig. 5.43).

Group 2

There is evidence of focal myocardial hypertrophy, degeneration and capillary congestion. However, there is no evidence of arteriosclerosis / atherosclerosis / calcification / aneurysm (Fig. 5.43).

Group 3

In *Lactuca scariola Linn* supplemented group, mild focal myocardial hypertrophy was observed. But, there is no evidence of arteriosclerosis / atherosclerosis / calcification / aneurysm (Fig. 5.43).

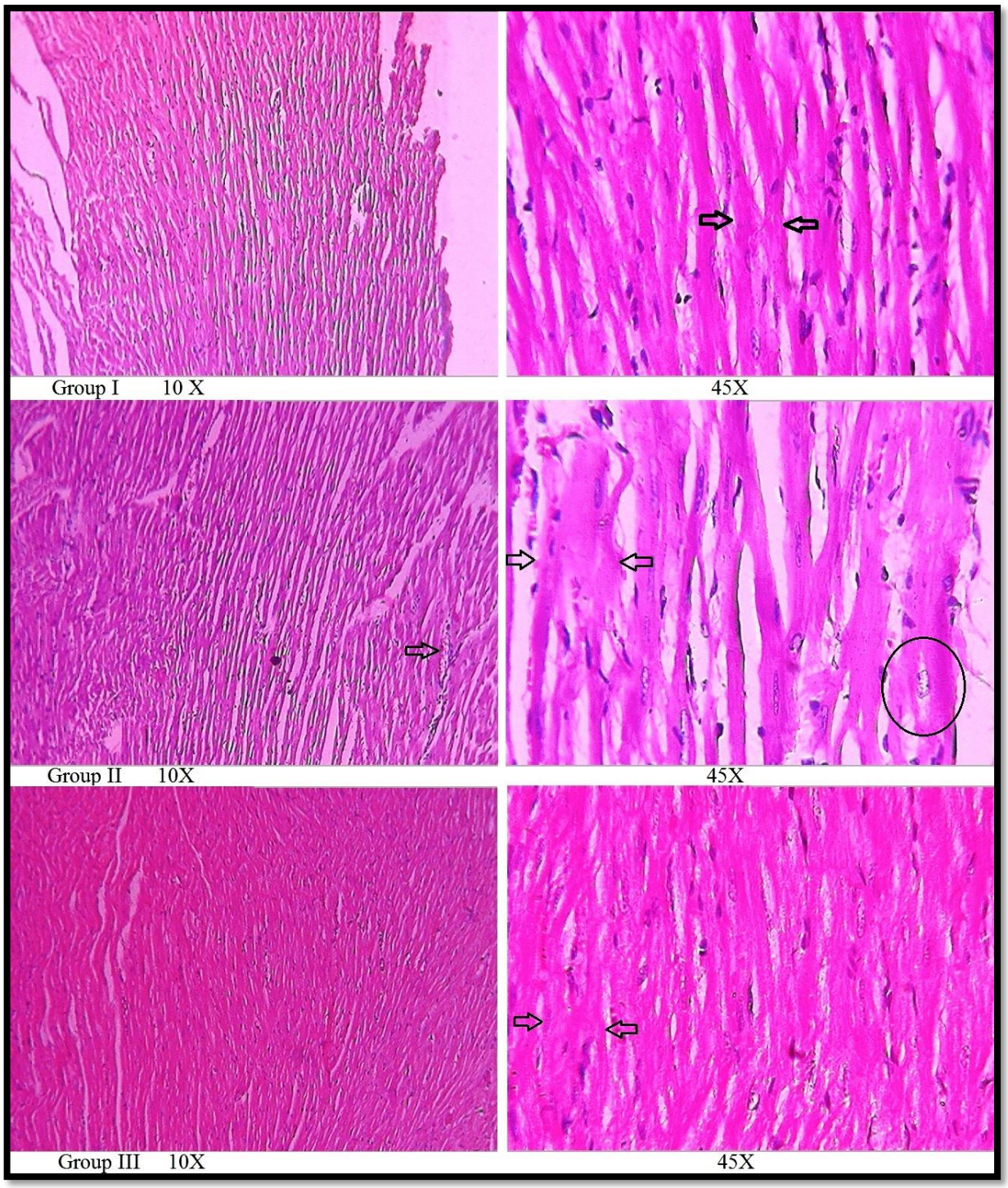


Fig. 5.43 H.E stain (10x and 45x) of ventricular muscle; pre diabetic (group 1): central placed nucleus, intercalated disk, branching and anatomising striated muscle, degeneration of capillary congestion; diabetic (group 2): myocardial hypertrophy; diabetic + *Lactuca scariola Linn* (group 3): focal myocardial hypertrophy.

5.5.5.4 Histopathology of Elastic Artery

Group 1

Section studied under H&E stain shows large sized artery consisting of inner tunica intima, middle tunica media and outer tunica adventitia. The tunica intima is lined by endothelial cells. The tunica media consists of smooth muscle cells with elastic fibres. The tunica adventitia is composed of loose connective tissue. The microscopic structure of elastic artery in group 1 shows the normal architecture of all the three layers, tunica intima, tunica media and tunica adventitia. There is no evidence of arteriosclerosis / atherosclerosis / arteritis / calcification / aneurysm / dysplasia (Fig. 5.44).

Group 2

The tunica intima is lined by endothelial cells with features of endothelial detachment, erosion and microulcerations. The tunica media which consists of smooth muscle cells with elastic fibres shows moderate thickening in diabetic group 2 (Fig. 5.44).

Group 3

The tunica intima lined by endothelial cells, shows mild thickening in *Lactuca scariola Linn* supplemented group 3. Tunica media is made up of smooth muscle cells with elastic fibres. The tunica adventitia is composed of loose connective tissue (Fig. 5.44).

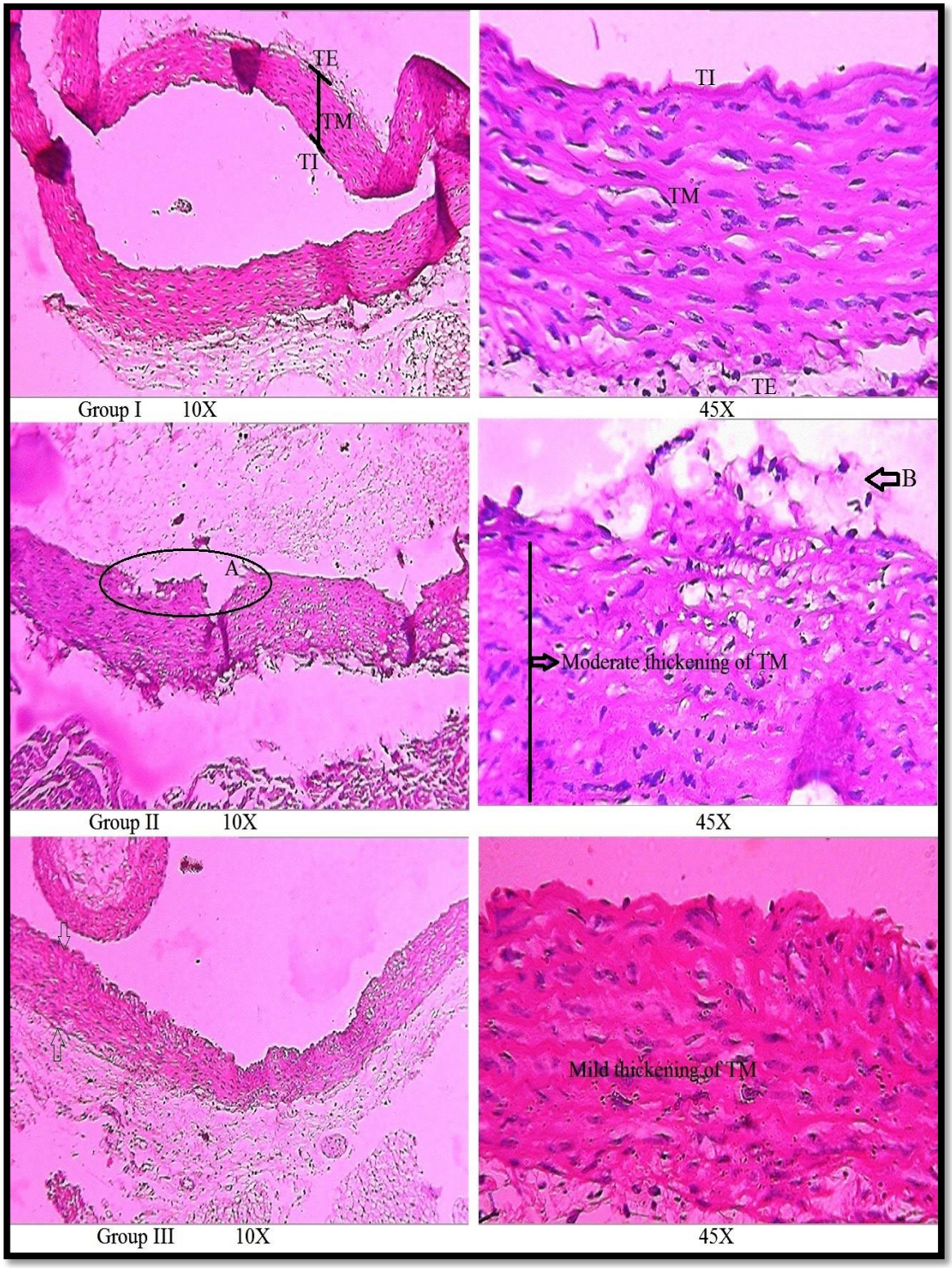


Fig. 5.44 H.E stain (10x and 45x) of elastic artery consisting of pre diabetic (group 1): normal Tunica intima, Tunica media and Tunica adventitia; diabetic (group 2): scattered epithelium with mild micro ulceration, moderate thickening of T.media; diabetic + *Lactuca scariola Linn* (group 3): mild thickening of T. intima

Chapter 6

Discussion

6.1 Phytochemical constituents

The preliminary phytochemical analysis of the aqueous plant extracts confirms the presence of some phytochemical constituents such as flavonoids, saponins, triterpenes etc which may have potential anti diabetic activity. *Trigonella foenum graecum* contains flavonoids, saponins and triterpenes. *Hibiscus cannabinus Linn* contains flavonoids, saponins, tannins and triterpenes. Similarly like *Trigonella foenum graecum*, *Cicer arietinum Linn* also contains flavonoids, saponins and triterpenes. Whereas, the aqueous leaves extract of *Lactuca scariola Linn* contains flavonoids, saponins and steroids.

6.2. *in vitro* and *in vivo* Glucose Homeostasis with and without nickel

6.2.1 Influence of plant extracts on glucose concentration *in vitro*.

The effect of aqueous plant extracts on changing the glucose concentration, *in vitro*, is determined by glucose oxidase - peroxidase enzymatic method. The results of the aqueous *Trigonella foenum graecum* leaves extract in changing the concentration of glucose *in vitro* clearly indicate that it has decreased the concentration of glucose significantly in both neutral and alkaline medium. It can also be seen from the results that the decrease in the glucose concentration is higher in the alkaline medium than in neutral medium. These results clearly show that pH of the medium does influence the plant extract in reducing the glucose concentration *in vitro*. Subsequently, in the presence of Ni^{2+} too the plant extract behaved similarly i.e., the glucose concentration was decreased significantly in both medium but the percentage of decrease in the glucose concentration is less in the presence of Ni^{2+}, which indicates that on treating with Ni^{2+}, the glucose reducing capability of the plant is effected. Because plants readily absorb nickel from the soil and unlike other non-essential trace elements, it moves through plant's circulatory system and gets accumulated in the leaves [98]. Nickel soluble compounds which are absorbed by plants through cation transport system, chelated nickel and inorganic nickel are transported through proteins such as permeases and through plant root cells via endocytosis respectively [99]. This nickel react with the components of plant, converting them to numerous nickel containing organic components which are highly toxic than the metal alone [100]. Even though the glucose reducing capability of the plant extract is

affected in the presence of Ni^{2+}, the decrease in the glucose concentration by the plant extract is significant, may be the results of spectral analysis reflect alteration of chemical behaviour of nickel in presence of *Trigonella foenum graecum* extract in both pH 7.0 and pH 9.0 *in-vitro,* which indicates that the *Trigonella foenum graecum* extract influences change of chemical behavior of nickel irrespective of pH of the environment possibly by molecular interaction between Ni^{2+} and antioxidant constituent of phytochemicals.

The aqueous extract of *Hibiscus cannabinus Linn* has very less influence on the reduction of glucose concentration *in vitro,* both in alkaline and neutral conditions. Like *Trigonella foenum graecum,* the *Hibiscus cannabinus Linn* also showed similar results with Ni^{2+} supplementation i.e., the % decrease in the glucose concentration is reduced in the presence of Ni^{2+}.

Our results also clearly reflect that the aqueous *Cicer arietinum Linn* leaves extract didn't show significant decrease in the glucose concentration *in vitro* with and without Ni^{2+} supplementation irrespective of pH of the medium.

Whereas, the *Lactuca scariola Linn* leaves extract decreased the glucose concentration to a much greater extent compared to that of other plants, interestingly, even in the presence of Ni^{2+} significant decrease in the glucose concentration was observed [101].

6.2.2. Anti diabetic effect of plant extracts *in vivo*.

After studying the effects of plant extracts with reference to glucose concentration *in vitro,* with or without Ni^{2+} supplementation, the work is extended to *in vivo* model through acute exposure (OGTT) followed by sub chronic (13 days) exposure of the plant extracts on diabetic rat models.

The results of OGTT and sub chronic exposure indicate that, the treatment of Ni^{2+} caused an increase in the blood glucose level both in non diabetic and diabetic group of rats which indicate a diabetic like response in glucose tolerance of nickel. These metals may get accumulated in different biological samples on getting exposed to it and may lead to increase in blood glucose level [102]. The studies carried out on diabetes due to the effect of heavy metals *in vitro, in vivo* and on human subjects show that nickel is one of the major contributors [103]. Our results are in accordance with nickel exposure can induce hyperglycemia [104]. Ni^{2+} induces hyperglycemia in both acute and sub chronic exposure conditions, which may be because of increased pancreatic glucagon release [105, 106]. Such alterations lead to a drastic drop in the insulin/glucagon plasma ratio [107].

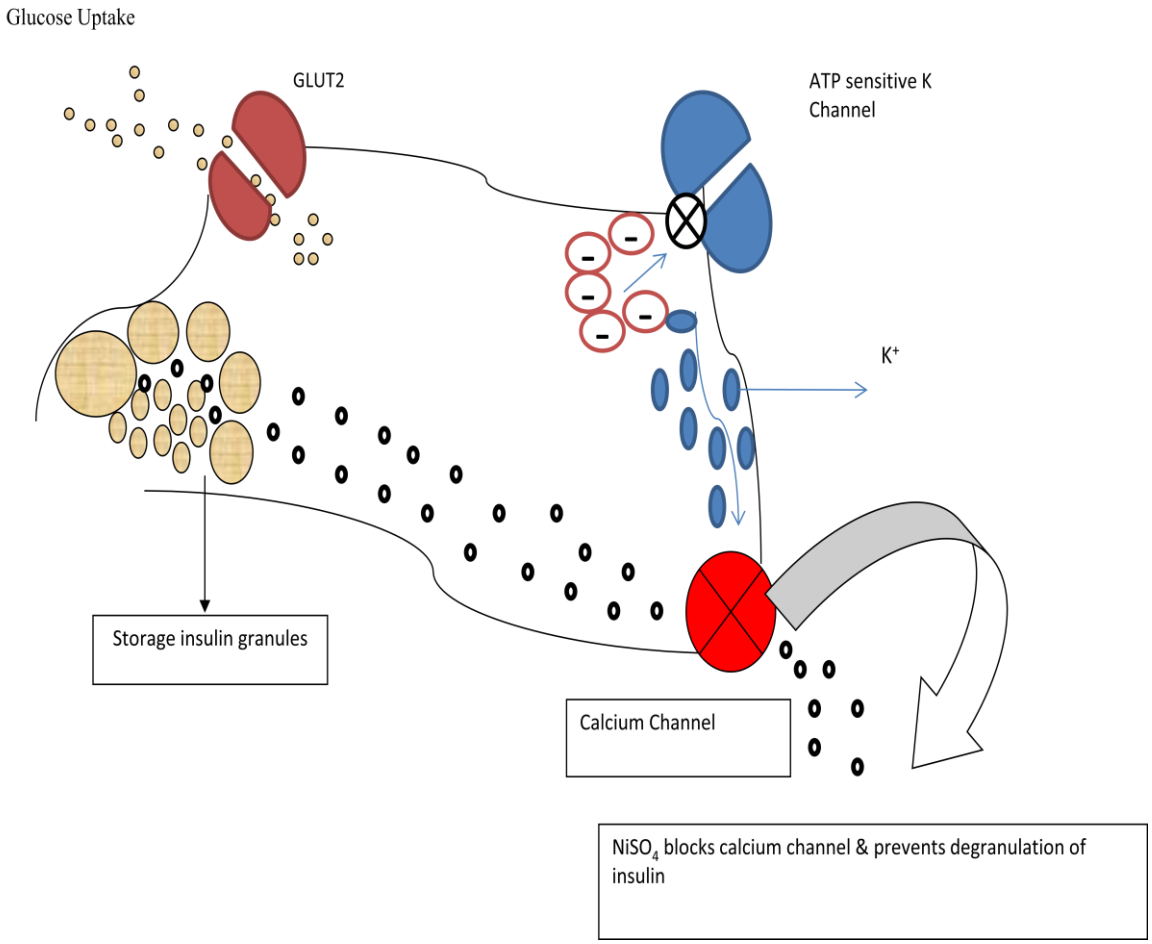


Fig. 6.1 Mechanism of insulin secretion and possible inhibition by nickel

The glucose that enters the bloodstream after consumption of food diffuses in the beta cell through GLUT-2 receptors. Glucose gets converted to Glucose-6-Posphate which subsequently oxidises to form ATP. This inhibits the ATP sensitive K^+ ion channels of the pancreatic beta cell to shut the channel gates. The closing of ATP - sensitive Potassium channel causes sustained polarization of the cell membrane which opens calcium channel on the membrane causing influx of the Ca^{2+} ions. The influx of the Ca^{2+} ions stimulates insulin secretion in the extracellular fluid outside the β cell from secretory vesicles and making its way into the blood stream. However, the $NiSO_4$ possibly blocks the calcium channel and prevents the release of insulin in the bloodstream (Fig. 6.1) [108, 109]. Thus, blood glucose level increases.

Another explanation for Ni^{2+} induced rise of blood glucose level in male rats may be due to the involvement of nitric oxide mediated pathways [110]. Nickel causes an increase in cyclic guanosine monophosphate (cGMP) levels and constitutive nitric oxide synthase (cNOS) in the adrenals and brain or inducible nitric oxide synthase (iNOS) in the

pancreas by modulating the release of insulin from pancreas which leads to hyperglycemic condition. Probably because of these reasons there was an increase in the blood glucose level in some of the rats treated with nickel.

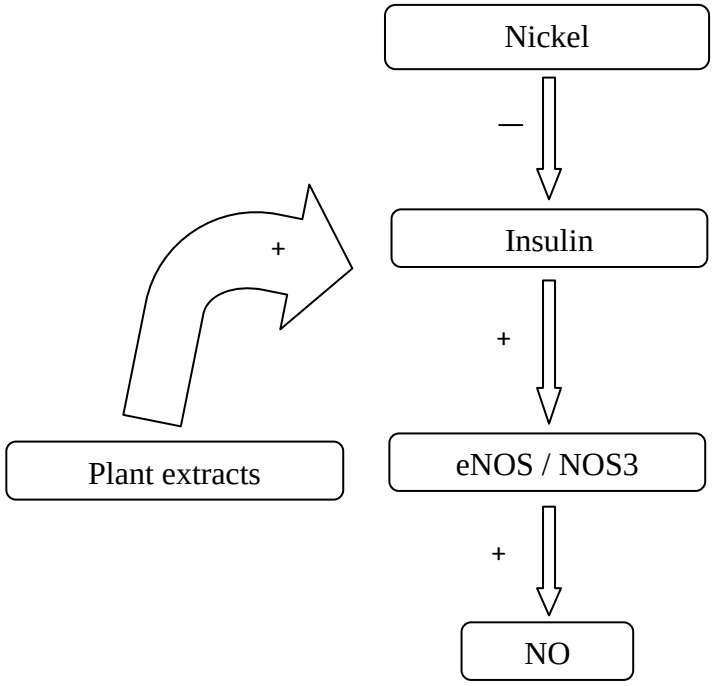


Fig. 6.2 Possible role of Plant extracts in nickel induced alteration of glucose homeostasis.

The OGTT and sub chronic exposure of *Trigonella foenum graecum* leaves extract showed that, the extract might possess anti diabetic potential. As shown in Fig. 5.4 the % change of blood glucose level is decreased to a significant extent on treating the non diabetic and diabetic rats with the extract. Because it is known that, the *Trigonella foenum graecum* have anti diabetic potential. *Trigonella foenum graecum* are traditionally being used not only in diabetes but also in high cholesterol, inflammation and gastrointestinal problems [111]. It's known that, alloxan completely destroys the pancreatic cells, our findings are contradictory to the suggestion that, sulphonylureas have anti diabetic mechanism of action in addition to effect of insulin secretion which helps in glucose uptake by the tissues [112]. The other mechanisms may be the plant extract increase glycogenesis, inhibiting gluconeogenesis in the liver [113].

The OGTT of *Hibiscus cannabinus Linn extract* didn't show significant decrease in the blood glucose level. However, the treatment of *Hibiscus cannabinus Linn* for 13 days period showed, decrease in % change of the blood glucose level of group IV non diabetic rats. Similarly, the % change in the blood glucose levels is decreased, but not to a

significant extent in diabetic rats of group VI and group VIII on treating with the *Hibiscus cannabinus Linn extract* over a period of 13 days. This clearly indicates that on prolonged treatment with the extract there was a slight decrease in the blood glucose level but not significantly i.e., the prolonged exposure to the extract might influence the pancreatic secretion of insulin from β cells of islets of Langerhans or the glucose uptake in the peripheral may be increased [114].

The OGTT and sub chronic treatment of *Cicer arietinum Linn* leaves extract on non diabetic and diabetic rats didn't show any significant decrease in the blood glucose level and ruled out any possibilities of having anti diabetic actions in *Cicer arietinum Linn* leaves extract. Perhaps this plant lacks the bioactive component responsible for hypoglycemic properties.

The *Lactuca scariola Linn* leaves extract showed a remarkable improvement in the blood glucose level. The elevated blood glucose level, may it be due to diabetes or due to nickel treatment, is significantly reduced almost to the normal level during OGTT and Sub chronic exposure of the *Lactuca scariola Linn* leaves extract. Compared to other plant extracts studied, *Lactuca scariola Linn* leaves decreased the blood glucose level to the greater extent. Among the four plants that were studied, *Trigonella foenum garecum* and *Lactuca scariola Linn* showed anti diabetic capabilities. The anti diabetic capabilities of these aqueous plant extracts may be due to the phytochemical constituents such as, flavonoids, saponins, triterpenes present in these extracts [115, 116]. Blood glucose level is commonly measured to check glycemic control mechanism. The decrease in blood glucose level may be due to increase in the pancreatic secretion of insulin by the β cells of islets of Langerhans. It is well known that flavonoids and saponins are bioactive anti diabetic components. Flavonoids are the polyphenolic compounds found abundantly in plants known to act on various molecular targets and regulate different signalling pathways in pancreatic β cells hepatocytes, adipocytes and skeletal myofibres [117, 118]. Flavonoids are able to terminate the free radicals and prevent oxidative stress [119]. Flavonoids present in some of the plants have shown to regenerate the damaged β-cells of pancreas in some of the studies. Saponins on the other hand influence structural alterations of glucose molecule though glycone and glucuronic acid [120]. Saponin present in plants has shown a protective effect on pancreatic islet cells and increase insulin secretion from the pancreatic β-cells. Saponin has the ability to reduce increased plasma blood glucose level making it beneficial to treat diabetes mellitus [121]. Similarly, triterpenes may also inhibit the enzymes involved in glucose metabolism to prevent the

development of insulin resistance thus normalizing insulin levels [122]. Hence, it can be predicted that the hypoglycemic activities of *Trigonella foenum graecum* and *Lactuca scariola Linn* extracts may be due to the effect of these bioactive components. There are a number of hypotheses to explain hypoglycemic effects of medicinal plants that include possible inhibitory impact on enzyme - insulinase which keeps insulin stable for longer durations or increase insulin sensitivity or increase peripheral glucose uptake mechanisms by inducing insulin mediated cell signalling pathways [123]. Beside these possible mechanisms, the antioxidant activities of the active compounds present in these medicinal plants also improve hepatic glucose regulatory system and decrease intestinal glucose uptake. These active compounds might also act at pancreatic level by protecting, regenerating and repairing pancreatic β cells by increasing the size and number of cells in the islets of Langerhans.

In our study, we have found significantly improved blood glucose levels in the diabetic rats which are supplemented with *Trigonella foenum graecum* and *Lactuca scariola Linn* both in acute (OGTT) or sub chronic conditions with and without nickel treatment. This finding may be because of an improvement in nickel induced oxidative stress and insulin resistance by *Lactuca scariola Linn* and *Trigonella foenum graecum* as well. Our study revealed a relationship between extracts and glucose metabolism, as there was increase in insulin sensitivity in diabetic rats treated with these extracts in both acute and subchronic conditions. Possibilities of decreased glucose uptake by gut due to plant extracts supplementation might not be ruled out. In our study, diabetic rats treated with Ni^{2+} did not show additional hyperglycemic response as compared to diabetic rats alone. On the other hand, supplementation with aqueous extracts to Ni^{2+} treated diabetic rats showed decreased blood glucose level in both OGTT and sub chronic exposures. It may be possible that the decrease of nickel induced oxidative stress and insulin resistance by extracts with concomitant increase of insulin sensitivity are the causes of this.

6.2.3. Effect of leaves extracts on Fasting blood glucose, Fasting Plasma Insulin and Insulinogenic index.

After the treatment of *Hibiscus cannabinus Linn* leaves on diabetic rats for a period of 13 days (Sub chronic exposure), there wasn't any significant change in the fasting blood glucose, plasma insulin and insulinogenic index. Similar is the case with *Cicer arietinum Linn* leaves extract. Our results showing increased fasting blood glucose and decreased fasting plasma insulin clearly indicate no hypoglycemic effects of these extracts. However, in the case of *Lactuca scariola Linn*, there was remarkable decrease in the

fasting blood glucose level showing its anti diabetic potential. But fasting plasma insulin didn't show any change. A possible influences of plant extracts (*Hibiscus cannabinus L., Cicer arietinum Linn* and *Lactuca scariola Linn*) on somatic tissues induced insulin sensitizing or insulin mimetic effect of polyphenolic compound in diabetes mellitus. This may suggest that these plant extracts have glucose lowering activity by acting and sensitizing insulin receptors. Probably polyphenolic compounds like tannins and flavonoids might play the protective role by different means including antioxidant activities, lipid regulations and glucose homeostasis in alloxan treated diabetic rats.

6.3 Molecular markers of diabetes mellitus (Protein expression) in *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* supplemented diabetic rats.

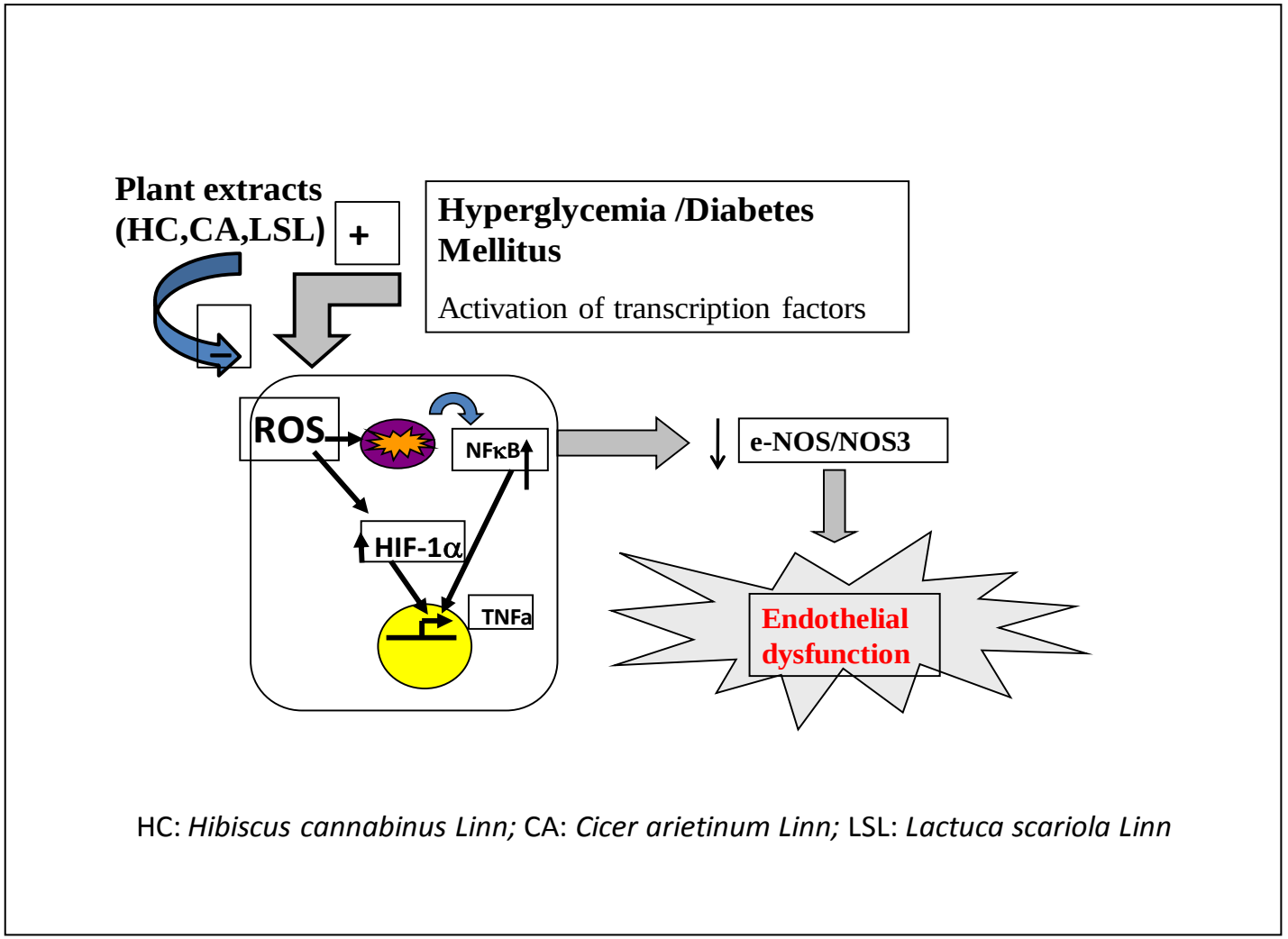
It is observed from our findings that the concentration of NOS3 in diabetic rats was significantly decreased, so is the NO level, as NOS3 is responsible for generation of NO in the vascular endothelium [124]. Several studies reported that, nitric oxide production decreases during diabetes. The decrease in nitric oxide may be due to pathogenesis of diabetic endothelial damage [125]. Endothelial cell NO inhibits proliferation of vascular smooth muscle cell [126]. Endotelium dependent vasodilatation damage in hyperglycemia in diabetic patients [127, 128] clearly suggests nitric oxide synthase (NOS) activity may be damaged in diabetic conditions. It was observed from the results that, the sub chronic (13 days) supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extract (12.5 mg/100 g body weight) on diabetic rats, there was an increase of sNOS3 and sNO significantly. Supplementation of *Lactuca scariola Linn* on diabetic rats showed a better improvement in sNOS3 and sNO level compared to other plants. sNO which is synthesized from L-arginine by nitric oxide synthase in different conditions provides vascular stabilities and reflects endothelial functions [129]. Decrease in sNOS3/e-NOS levels indicate vascular malfunctions and may cause coronary spasm. A clear association between sNOS3 and type 2 diabetes mellitus has already been established [130].

Studies on endothelial dysfunctions in diabetic animals showed a possible uncoupling of the VEGF from endothelial nitric oxide synthase (NOS3/eNOS) axis. This uncoupling phenomenon causes resultant increase in VEGF leading to excessive endothelial cellular growth. This alteration in VEGF-NO regulatory mechanism causes loss of vascular integrity in diabetic states [131]. Supplementation of *Hibiscus cannabinus Linn, Cicer*

arietinum Linn and especially *Lactuca scariola Linn* leaves extract, showed improvement in NOS3, NO and VEGF status in diabetic rats (Fig. 6.3). Further study on transcription factor showed that, hypoxia inducible factor-1α (HIF-1α), increased significantly in diabetic rats. However, supplementation of all the three plants extracts did not bring HIF-1α concentration back to normal. HIF-1α is usually expressed when the oxygen saturation in the cell is low and it is expressed to protect the cell from hypoxia induced injury by increasing the VEGF concentrations, suggesting the adaptive mechanism of protecting the cell at the time of complications related to diabetes especially in vascular systems [132]. Effects of sub chronic supplementation of all the three plants, particularly *Lactuca scariola Linn*, on HIF-1α provide a cellular adaptation against hyperglycemia induced alteration of cell signalling pathways. This protective mechanism is oxygen sensing intracellular mechanism and facilitates cardiovascular stabilities. The results from author's laboratory on molecular markers clearly indicate all the three plants have vascular protective benefits but *Lactuca scariola Linn* is observed to be better in its actions.

6.4 Electrophysiology interpretation on cardiovascular system

After carrying out the glucose homeostasis and molecular markers analysis in diabetic rats, the electrophysiological impact of *Lactuca scariola Linn* on vascular systems further evaluated. Decrease in HF power with concurrent increase of LF power in diabetic rats indicates dominance of sympathetic nervous system over parasympathetic functions. It is well known that the autonomic nervous system plays the most important role to control cardiovascular integrity. Alterations in heart rate, blood pressure, LF/HF ratio and baroreflex sensitivities (BRS) in diabetic rats clearly indicate autonomic malfunctions. Autonomic malfunctions due to increase in sympathetic adrenergic activity with decrease in vagal activities. Such abnormalities during diabetes may induce ventricular arrhythmia and even sudden death of patients [133].



HC: *Hibiscus cannabinus Linn;* CA: *Cicer arietinum Linn;* LSL: *Lactuca scariola Linn*

Fig.6.3 Plant extracts on diabetes Mellitus & cell signalling

Further studies from authors' laboratory on spectral analysis revealed augmented low frequency dominance in diabetic rat 2 as compared to pre diabetic status. Interestingly, sub chronic supplementation with *Lactuca scariola Linn* was found to improve the status of autonomic malfunctions. Hence it may be deduced from overall electrophysiological observations that the sub chronic supplementation of *Lactuca scariola Linn* extracts to diabetic rats indicates a possible beneficial revival of autonomic functions in diabetic rats. The improvement of HRV and BRS in diabetic rats supplemented with *Lactuca scariola Linn* extracts for 14 days, suggests its possible influence on parasympathetic vagal action on heart by AChE inhibition [134, 135].

It is well established that arterial baroreceptors which responds to arterial stretch, regulate both, sympathetic and parasympathetic actions on cardiovascular system and modulate ventricular stroke volume. Hence, the plant extract possibly act as cardio-protective agent influencing autonomic nervous system functions, regulating heart rate and blood pressure by altering afferent receptor mediated discharge which controls baroreflex sensitivities and subsequently ventricular functions [136] (Fig. 6.4).

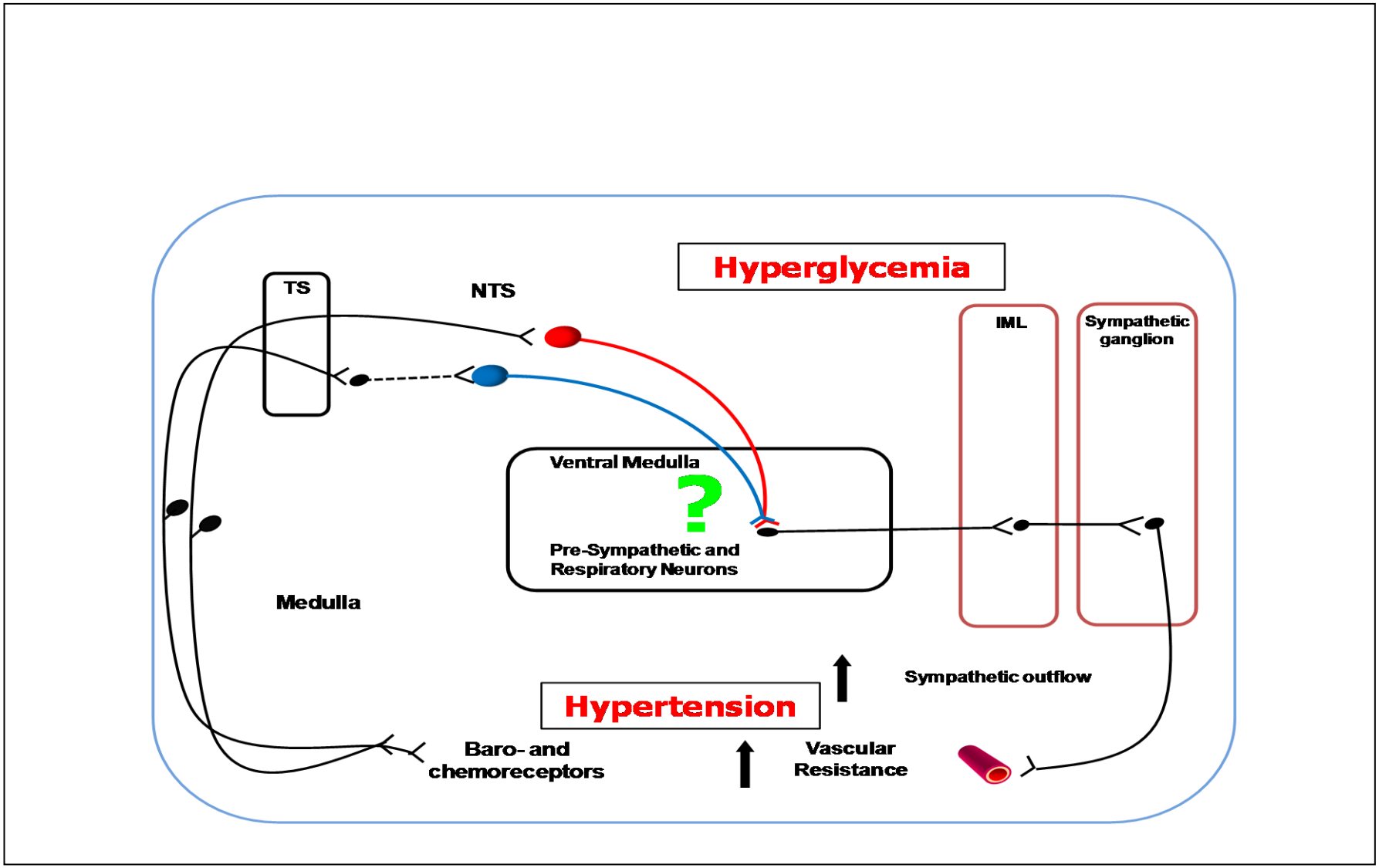


Fig. 6.4 Sympathetic over activity and hypertension in alloxan treated diabetic rats

6.5 Histopathology

6.5.1 Histopathology of Liver

The histological structure of normal untreated rat liver showed normal architecture (Fig. 5.37). In the case of diabetic rats' majority of the 'lobular' architecture of liver parenchyma distorted, little swollen hepatocytes, vacuolated micro vesicular and eosinophilic cytoplasm besides increase in number of mitotic figures. Mild proliferation of portal area along with fibrous tissue was noticed and mixed acute and chronic inflammatory cells were found to be infiltrated. These histopathology changes in diabetic rats may be due to hyperglycemia and accumulation of mucopolysaccharide deposits [137]. However, the diabetic rats on supplementation with *Lactuca scariola Linn* leaves extract, mild enlargement of hepatocytes has taken place. Periportal, mid zone and centrilobular appeared to be normal. The central veins lined by endothelial cells were appeared to be slightly dilated.

6.5.2 Histopathology of Kidney

Fig. 5.38, shows normal renal parenchymal tissue composed of glomeruli and tubules separated by small amount of interstitial connective tissue containing peritubular capillaries. Tubules appear to be normal with no evidence of tubular atrophy. Glomeruli seem to be normal in morphology and in cellularity. In diabetic rats glomeruli appears to be hypercellular with thickening of glomerular basement membrane and mesangial proliferation. Tubules show focal tubular basement membrane mild thickening with cloudy swelling (coagulation necrosis). The lumen shows eosinophilic protienacious suggestive of acute tubular necrosis (ATN), collectively may be due to deposition of mucopolysaccarides [138]. The study carried on lipid metabolism and glomerulo sclerosis in diabetes mellitus shows that the SREBP-1 expression is expression increases in diabetes which plays an important role in lipid synthesis leading to Triglyceride accumulation, mesangial expansion [139]. However, upon treating with *Lactuca scariola Linn* leaves extract, Glomeruli were hypocellular with thickening of glomerular basement membrane showing sclerotic changes. Tubules show focal tubular necrosis suggestive of acute tubular necrosis (ATN) with regenerative epithtelium.

6.5.3 Histopathology of Heart and artery

Histopathology findings of present study showed the structural organization of cardiac tissues was completely disturbed in alloxan induced diabetic rats. Myocardial tissues from ventricle showed ventricular hypertrophy and degeneration of capillaries in diabetic rats

and it also clearly shows no evidence of arteriosclerosis / atherosclerosis / calcification / aneurysm.

Similarly elastic artery in diabetic rats showed, the tunica intima lined by endothelial cells featuring endothelial detachment, erosion and micro ulcerations. Our present study finds significant increase of thickness in the tunica media which consists of smooth muscle cells with elastic fibres. Our results are in comparison with the previous works where it was mentioned that the smooth muscle cells proliferation in the tunica intima and tunica media takes place in aorta [140, 141]. But supplementation of *Lactuca scariola Linn* leaves extract for 14 days showed remarkable improvements of both vascular and myocardial architectures in diabetic rats. Hence it may be hypothesized that *Lactuca scariola Linn* possibly has a cardio-protective actions either by regulating glucose homeostasis in diabetes or controlling cardiac autonomic functions or it is possible with some antioxidant efficacies.

Chapter 7

Summary and Conclusion

7.1 Summary

The study was carried out to evaluate the anti diabetic efficacies of the aqueous plants extracts of north Karnataka region of India, in the presence or absence of heavy metal nickel. *In vitro* glucose reducing capabilities of the aqueous plant extracts (*Trigonella foenum graecum, Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn*) was evaluated by glucose oxidase-peroxidase method at different pH conditions (pH 2.0, 7.0 and 9.0) with or without heavy metal nickel. The aqueous extracts of *Trigonella foenum graecum* and *Lactuca scariola Linn* reduced the glucose concentration significantly at both pH 7.0 and 9.0 *in vitro* both in the presence and absence of nickel. However, the aqueous extract of *Lactuca scariola Linn* decreased the glucose concentration to the greater extent in both pH conditions in the presence and absence of nickel compared to that of *Trigonella foenum graecum.* It can also be seen from the results that the reduction in the glucose concentration by both *Trigonella foenum graecum* and *Lactuca scariola Linn* were found to be greater at pH 9.0, clearly indicating pH does influences the glucose reducing capabilities of the plant extracts. The extract of *Hibiscus cannabinus Linn* and *Cicer arietinum Linn* did not show any significant reduction in the glucose concentration in either of the pH conditions. Further, anti diabetic effects of these plant extracts was evaluated with and without nickel supplementation on alloxan induced diabetic male albino rats *in vivo,* through acute (OGTT) and sub chronic (13 days) exposure tests to plant extracts. The results of OGTT and sub chronic exposure tests of *Hibiscus cannabinus Linn* and *Cicer arietinum Linn* leaves extracts on alloxan induced diabetic male albino rats did not show any significant decrease in the blood glucose level. Interestingly, the extract of both *Trigonella foenum graecum* and *Lactuca scariola Linn* showed a significant decrease in the blood glucose level with and without nickel supplementation. *Lactuca scariola Linn* being remarkable in its effects decreased the blood glucose level to near normal. Our results clearly suggest that these plant extracts possess potential anti diabetic properties. The anti diabetic capabilities of these plant extracts are however lowered in the presence of nickel. The anti diabetic capabilities may be because of the presence of flavonoids, saponins, and triterpenes in these extracts. The phytochemical components probably influence to increase the pancreatic secretion of

insulin by the β cells of islets of Langerhans or decrease absorption rate of glucose from intestine.

It was also evaluated that the possible anti diabetic role of these aqueous plant extracts in relation with oxygen dependent micro environment in diabetic experimental model through molecular markers analysis of sNOS3, sHIF-1α, and sVEGF. It was observed from our findings that there was a significant decrease in the concentration of serum NOS3 and NO in diabetic rats which are the indication of endothelial dysfunctions in diabetes. Supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extracts showed a significant improvement of the concentration of NOS3 and NO level. Results further showed that the *Lactuca scariola Linn* supplementation had a greater improvement in the concentration of sNOS3 and sNO levels. The studies on endothelial dysfunctions in diabetic animals showed a possible uncoupling of the vascular endothelial growth factor (VEGF). This uncoupling phenomenon caused an increase in VEGF that leads to excessive endothelial cellular growth. Supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and especially *Lactuca scariola Linn* leaves extract, showed improvement in VEGF. The study on HIF-1α, showed a significant increase in diabetic rats. However, the supplementation of all the three plants extracts did not bring HIF-1α concentration back to normal. The *in vitro* and *in vivo* result showed the most efficient anti diabetic plant is found to be *Lactuca scariola Linn* which showed the greatest efficacies through glucose homeostasis and diabetes regulatory molecular mechanisms. To understand the molecular signalling efficacies and neurovascular performances of *Lactuca scariola Linn*, further studies on electrophysiology interpretation and functional pathophysiology of cardiovascular system with this plant extracts were done. Alterations in heart rate, blood pressure, LF/HF ratio and baroreflex sensitivities (BRS) in diabetic rats in the present study indicate autonomic malfunctions. The supplementation of aqueous *Lactuca scariola Linn* leaves extract for 14 days improved autonomic malfunctions. The overall electrophysiological observations showed the beneficial effect of autonomic malfunctions by the supplementation of *Lactuca scariola Linn* extract in diabetic rats. *Lactuca scariola Linn* extract act as cardio protective agent by influencing autonomic nervous system though regulating heart rate, blood pressure etc.. Further, to strengthen the work, the effect of *Lactuca scariola Linn* leaves extract was also studied on histopathology of liver, kidney, heart and aorta on alloxan induced diabetic rats. In diabetic rats, the lobular architecture of liver parenchyma was completely distorted. However, due to the supplementation of *Lactuca scariola Linn* extract the distorted parenchyma was appeared

to be near normal. Similarly, in diabetic rats, glomeruli were found to be hypercellular with thickening of the glomerulus. The supplementation of extract however, improved the renal histopathology. The histopathology findings of the structural organisation of cardiac tissues were completely disturbed in alloxan induced diabetic rats. Myocardial tissues from ventricle showed ventricular hypertrophy and degeneration of capillaries in diabetic rats but there were no evidences of arteriosclerosis. Similarly elastic artery in diabetic rats showed, the tunica intima which is lined by endothelial cells featuring endothelial detachment, erosion and micro ulcerations. Present study also finds significant increase of thickness in the tunica media. Supplementation with *Lactuca scariola Linn* for 14 days showed remarkable improvements of both vascular and myocardial architectures in diabetic rats. Hence it may be summarised that *Lactuca scariola Linn* possibly has a cardio-protective actions too besides having potential anti diabetic properties.

7.2 Conclusion

The *in vitro* study results at different pH conditions showed that the *Lactuca scariola Linn* leaves extract has the greatest glucose reduction capabilities in presence or absence of nickel supplementation as compared to other plant extracts i.e., *Trigonella foenum graecum, Hibiscus cannabinus Linn* and *Cicer arietinum Linn.* The treatment of Ni^{2+} alone *in vivo* caused a diabetic like response even in non diabetic rats in both OGTT and sub chronic exposures. Interestingly nickel treatment in diabetic rats also did not show any additional glucose impairments in experimental animals either in OGTT or in sub chronic exposures. The significant decrease in the blood glucose level caused by acute and sub chronic supplementation of *Lactuca scariola Linn and Trigonella foenum graecum* leaves extract clearly indicate that these plants possess potential anti diabetic properties. However, the anti diabetic effects in *Lactuca scariola Linn* were also found to be greater when it compared to *Trigonella foenum graecum*. Supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extract showed amelioratic effects on molecular markers like NOS3, NO and VEGF, which are potential regulatory gene for diabetes mellitus. Further it has been noted that *Lactuca scariola Linn* has the greatest influence on diabetic molecular markers like NOS3 and VEGF. HIF-1 α which is also considered as equally important transcriptional factor to regulate diabetes induced pathophysiology did not show any such beneficial effects after supplementation of all the three plant extracts including *Lactuca scariola Linn.* Further study on electrophysiology and histopathology clearly indicate a potential cardio protective action of *Lactuca scariola Linn* extract on alloxan treated diabetic experimental rats. Hence, it

can be stated that probably *Lactuca scariola Linn* and *Trigonella foenum graecum* plants of north Karnataka especially *Lactuca scariola Linn* extract has potential anti diabetic efficacies. In addition to that *Lactuca scariola Linn* leaves extract is also found to be cardio protective one.

Limitations of the study

- We could not isolate and identify the bioactive component responsible for the anti diabetic capabilities of the aqueous *Lactuca scariola Linn* leaves extract.

Scope for the future work

- Identification of the bioactive component responsible for the anti diabetic capabilities of the aqueous *Lactuca scariola Linn* leaves extract.
- To study the anti diabetic capabilities of the synthetic organic derivatives of the bioactive component isolated from the *Lactuca scariola Linn* leaves extract.

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List of awards

- Received third best Poster presentation award at KSTA, Bangalore and DST, Govt. of Karnataka sponsored Two Days National conference on "Science and Technology of Food and Nutrition" organized by Dept. of Botany, B.L.D.E.A's S. B. Arts and K.C.P Science college, Bijapur, Karnataka, India, in the month of February 2015
- Received best Oral presentation award at UGC sponsored two days National Seminar on "Building Awareness on Environment Protection" organized by Dept. of Chemistry, B.L.D.E.A's S. B. Arts and K.C.P. Science college, Bijapur, Karnataka, India, in the month of September 2014.

Presentations at National / International conference

- Presented paper entitled *"Nickel alters blood glucose level in male Wister rats: Possible effects of Trigonella foenum graecum, Hibiscus cannabinus Linn. and Cicer arietinum Linn"* in Fourth International Conference on Advanced Oxidation Processes, AOP-2016, held at BITS, Pilani, Goa Campus, Vasco Da Gama, Goa, India on 17th to 20th December 2016.
- Presented paper entitled *"Anti-diabetic effects of aqueous fenugreek (Trigonella foenum graecum) leaves extract in alloxan induced male diabetic rats treated with nickel (II)"* in ICC-2015, held at Uka Tarsadia Universiy, Bardoli, Surat, on 26th -28th December 2015.
- Presented paper entitled *"Anti-diabetic effects of aqueous prickly lettuce (Lactuca scariola Linn.) leaves extract in alloxan induced male diabetic rats treated with nickel (II)"* in KSTA Conference on Science and Technology for Food and Nutrition at Dept. Of Botany, B.L.D.E.A's S. B. Arts and K.C.P. Science college, Bijapur, Karnataka, India on 20th and 21st February 2015.
- Presented paper entitled *"Antidiabetic and in vitro glucose reduction properties of aqueous prickly lettuce (Lactuca scariola Linn) leaves extract in alloxan induced male diabetic rats"* in UGC sponsored Two-Day National Seminar on ''Building awareness on Environment Protection" held in the Dept. of Chemistry, B.L.D.E.A's S. B. Arts and K.C.P. Science college, Bijapur, Karnataka, India, on 12th and 13th September 2014.
- Presented paper entitled *"Alteration of chemical behaviour of nickel (II) and glucose reduction capabilities in presence of aqueous extract of fenugreek leaves (Trigonella graecum foenum) at different pH in vitro."* XXVth Annual Conference of The Physiological Society of India (PHYSICON-2013) held at Kamineni Institute of Medical Sciences, Narketpally, A.P., India, from 9th to 11th December 2013.

List of Publications

- Das, Kusal K; **Chadchan, Kailash S**; Reddy, R Chandramouli; Biradar, MS; Kanthe, Pallavi S; Patil, Bheemshetty S; Ambekar, Jeevan G; Bagoji, Ishwar B; Das, Swastika, Effects of Some Indigenous Plants of North Karnataka (India) on Cardiovascular and Glucose Regulatory Systems in Alloxan-Induced Diabetic Rats, *Cardiovascular & Hematological Agents in Medicinal Chemistry*, Vol 15, Issue 1, 2017, pp. 49-61.
- **KS Chadchan**, SN Das, JG Jargar, KK Das, A Comparative Study on Anti-diabetic Effects of Aqueous Trigonella foenum graecum, Hibiscus cannabinus Linn, and Cicer arietinum Linn extracts on Alloxan Induced Diabetic male albino rats, *Journal of Young Pharmacists*, Vol 9, Issue 2, 2017, pp. 230-233.
- **Chadchan KS**, Jargar JG and Das SN. Anti-diabetic effects of aqueous prickly lettuce (Lactuca scariola Linn) leaves extract in alloxan induced male diabetic rats treated with nickel (II). *Journal of basic and clinical physiology and pharmacology*, Vol 27, Issue 1, 2015, pp. 49-56.
- **Chadchan KS**, Das SN, Das KK, Aqueous extract of fenugreek (Trigonella foenum graecum) leaves and its interaction with heavy metal (Nickel II) at pH 7.0 and 9.0 with reference to glucose reduction capabilities in vitro, *Biomedicine*, Vol 34, Issue 1, 2014, pp. 104-108.

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RESEARCH ARTICLE

Effects of Some Indigenous Plants of North Karnataka (India) on Cardiovascular and Glucose Regulatory Systems in Alloxan-Induced Diabetic Rats

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Abstract: ***Background*:** Kenaf (*Hibiscus cannabinus Linn,* Pundi), Chick pea (*Cicer arietinum Linn,* Chana) and Prickly lettuce (*Lactuca scariola Linn,* Hattaraki) leaves are a few of indigenous plants which are routinely consumed by the people of north Karnataka in the diet. Studies on these plants showed some potential anti-diabetic efficacies.

***Objectives*:** To examine the effect of leaves extracts of *Hibiscus cannabinus Linn*, *Cicer arietinum Linn* and *Lactuca scariola Linn* on cardiovascular integrity, glucose homeostasis and oxygen sensing cell signaling mechanisms in alloxan induced diabetic rats.

***Method*:** *In vitro* and *in vivo* tests on glucose regulatory systems and molecular markers such as - NOS3, HIF-1α and VEGF were conducted in alloxan induced diabetic rats supplemented with all the three plant extracts. Electrophysiological analysis (HRV, LF: HF ratio, baroreflex sensitivity, BRS) and histopathogy of myocardial tissues and elastic artery were evaluated in diabetic rats treated with *L. scariola linn*.

Results: Out of these three plant extracts, *Lactuca scariola Linn* supplementation showed significant beneficial effects on glucose homeostasis and oxygen sensing cell signaling pathways in alloxan-induced diabetic rats. Furthermore, effects of sub chronic supplementation of *Lactuca scariola Linn* aqueous extracts showed significant improvement in sympatho-vagal balance in diabetic rats by increase of Heart Rate Variability (HRV) and regaining of Baroreflex Sensitivity (BRS). These results were also corroborated with myocardial and elastic artery histopathology of *Lactuca scariola Linn* supplemented diabetic rats.

Conclusion: These findings indicate an adaptive pathway for glucose homeostasis, oxygen sensing cell signaling mechanisms and cardio protective actions in alloxan – induced diabetic rats supplemented with *Lactuca scariola Linn* extracts.

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1. INTRODUCTION

Amidst the fast growing pharmaceutical industries, the need for traditional medicines for curing many diseases is still in demand. Nowadays, increased scientific interest and consumer demand lead to the development of herbal products as dietary supplements. The use of herbs to treat disease is common among developing countries [1]. A large number of plants used as food around the world possess many medicinal values. According to World Health Organization (WHO), approximately 25% of modern drugs used are derived from plants [2]. WHO also estimates that 80% of the population of Asian and African countries uses herbal medicines as primary healthcare [3].

Diabetes Mellitus commonly referred to as Diabetes is a global epidemic caused by a series of metabolic disorders resulting in a high blood sugar [4]. Studies conducted in India in the last decade have highlighted that not only the

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prevalence of diabetes is high but also it is increasing rapidly in the urban population [5]. Modern medicines or synthetic drugs, though effective to control this disease, adversely affect liver, kidney glucose homeostasis and body weight. Hence, nowadays plant-based alternatives are being preferred to these synthetic drugs for its prevention or control. There are many plants which are traditionally being used to treat diabetes. India has about 45000 plant species of which several thousand claims to possess medicinal properties and many are used to treat diabetes [6]. Some of these plants are indigenously grown in North Karnataka region of India, which are usually consumed in diet by the people of this region. North Karnataka, locally known as Uttara Karnataka, is a geographical region consisting of mostly semi-arid plateau from 300 to 730 meters (980 to 2,400 ft) elevation that constitutes the northern part of the South Indian state of Karnataka.

1.1. Green Leafy Plants of North Karnataka

In North Karnataka, India, fifty-one species of wild plants belonging to forty-six genera are edible [7]. Local people use leaves, stem, flowers, fruits, seeds and roots of these plants as a part of their diet. The most commonly used and widely cultivated green leafy plants of this region are fenugreek (*Trigonella foenum graecum*), coriander (*Coriandrum sativum L.*), curry (*Murraya koenigii*), Dill (*Anethum graveolens*), Kenaf (*Hibiscus cannabinus Linn*), Prickly lettuce (*Lactuca scariola Linn*), Spinach (*Spinacia oleracea L.*), Chick pea (*Cicer arietinum Linn*), and pig weed (*Portulaca oleracea*).

The green leafy plants used in this study are *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* as the tender leaves of these plants are consumed (raw or cooked) in this region. *Hibiscus cannabinus Linn* is commonly known as Kenaf leaves and is called as Pundi palle in the local language. It is a shrub with height ranging from 1.5-4m with a deeply lobed compound leaf structure, while in some cases shallow lobed entire leaf structure is maintained [8, 9] (Fig. **1A**). A study on antihyperlipidemic effect of hydro alcoholic extract of Kenaf leaves on high fat diet fed rats showed a strong dose dependent antihyperlipidemic activity and significantly reduced levels of serum TC, TG, LDl-C and TBARS [10]. It has also shown hepatoprotective activity against carbon tetrachloride and paracetamol induced liver damages in rats [11]. Another study on antidiabetic activity of methanolic extract of leaves of *Hibiscus cannabinus linn* in streptozotocin induced diabetic rats has shown significant antidiabetic activity [12]. *Cicer arietinum linn* is commonly known as chick pea, desi chana is locally called as Kadli palle. Its tender leaves are cooked and consumed in India and Nepal [13]. The plant grows to a height of 15-20 cm with feathery leaves on either side of the stem (Fig. **1B**). It is grown in several varieties cultivated across the world [14]. Chickpea seeds are a good source of carbohydrates, protein, vitamins, minerals and dietary fiber [15-17]. However, studies on its leaves are very few. *Lactuca scariola Linn* commonly known as prickly lettuce is locally known as Hattarki Palle [18, 19]. It has a spineless reddish stem, growing to 30 to 200 cm. The leaves are oblong often pinnate which get progressively smaller as they reach its top (Fig. **1C**). Fine spines are present along the veins and leaf edges. Its tender leaves are consumed as a salad, although it has slightly bitter taste [19] but the whole plant becomes bitter when fully grows and is toxic too.

The link between diabetes mellitus and cardiovascular functional abnormalities are well established hence any substance either drug or phytochemical, which effects blood glucose level possibly may have beneficial effects on cardiovascular system [20].

This article describes the effect of supplementation of leaves extract of *Hibiscus cannabinus Linn (*Kenaf). *Cicer arietinum Linn* (Chick pea) and *Lactuca scariola Linn* (Prickly lettuce) on glucose homeostasis, electrophysiological alterations related to cardiovascular systems and the functional activities of endothelial markers that includes oxygen sensing cell signaling mechanisms and histopathology of myocardial tissues and elastic arteries in alloxan-induced diabetic rats

2. MATERIALS AND METHODS

2.1. Collection and Identification of Plant

The fresh leaves of *Hibiscus cannabinus Linn (*Kenaf), *Cicer arietinum Linn* (Chick pea) and *Lactuca scariola Linn* (Prickly lettuce, Hattaraki pallye) collected from the local

Fig. (1). North Karnataka green leafy plants; **A**) *Hibiscus cannabinus Linn* (Pundi) **B**) *Cicer arietinum Linn* (Chana) **C**) *Lactuca scariola Linn* (Hattaraki).

market were authenticated by Dr. M. B. Mulimani, Associate Professor, Department of Botany, BLDEA's S. B. Arts and K. C. P. Science College, Vijayapur, Karnataka, India before further processing.

2.1.1. Preparation of Plant Leaves Extract

Fresh leaves of *Hibiscus cannabinus Linn* (Kenaf), *Cicer arietinum Linn* (Chick pea) and *Lactuca scariola Linn* (Prickly lettuce) weighing 5g each were washed thoroughly with distilled water and later crushed by electrical grinders. The crushed leaves were extracted with 100 mL distilled water at temperature 50-60 °C repeatedly, for 1 h. The final extracts were filtered using Whatmann filter paper no. 1. The filtrate was used as an aqueous plant leaves extract and orally administered to the animals by gastric intubation using a gavage (force feeding) during the experimental period.

2.1.2. Phytochemical Analysis

Preliminary phytochemical analyses of freshly prepared plant leaves extracts were carried out to identify the phyto constituents using standard procedures [21].

2.1.3. In Vitro Percentage Glucose Reduction at Different pH

The *in vitro* glucose reduction capabilities in percentage of all the three plant extracts were determined by oxidase-peroxidase enzymatic method at two different level of pH 7.0 and pH 9.0, respectively [22, 23]. In this process, 0.2 mL standard glucose solution (20 mg/dL) was added to 0.8 mL of triple-distilled water in a test tube followed by adding 0.5 mL of *Hibiscus cannabinus Linn (*Kenaf) leaves or *Cicer arietinum Linn* (Chick pea) leaves or *Lactuca scariola Linn* (Prickly lettuce) leaves. We have also added 1 mL of glucose oxidase reagent to all the test tubes and incubated at 35 °C for about 40 min. The chemical reaction was terminated by adding 2 mL of 6 N HCl. All the results were spectrophotometrically recorded at 540 nm. Percentage glucose reduction was measured using the following equation [24]:

$$\text{Concentration of glucose} = \frac{\text{Absorbance of glucose + Extract)}}{\text{(Concentration of glucose)}} \times \text{Absorbance of glucose}$$

2.2. Animals

Adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180-200g were housed in central animal house of BLDE University's Shri. B. M. Patil Medical College, Hospital and Research Centre, Vijayapur. All the animals were kept in the 12 h light: 12 h dark cycle. The rats were divided into six groups of six rats each. Three rats from each group were kept in each metabolic wire cage (60cm x 30cm x 20cm). Standard pellets obtained from Hindustan lever rat feed, Mumbai, India, were used as a basal diet during the entire experimental period. The control and all the experimental rats were provided food and drinking water *ad libitum*.

2.3. Chemicals

The chemicals used during the experiment are alloxan monohydrate (Spectrochem Pvt. Ltd., Mumbai), Tween 80 (S.D. Fine-chem limited, Mumbai). Accu-chek® Active Glucometer, Roche Diagnostic Corporation, Germany, Blood gluco-strips (Roche Diagnostic Pvt. Ltd., Mumbai, India). horseradish peroxidase (Merck Specialties Pvt. Ltd., Mumbai, Maharashtra, India), and glucose oxidase (Sigma Life Sciences 49180, St. Louis, USA). All other chemicals and reagents used were of analytical grade.

2.4. Acute Oral Toxicity Studies of Extracts

The acute oral toxicity studies of extracts were carried out as per the Organization for Economic Cooperation and Development (OECD) guidelines, draft guidelines 423 adopted on December 17, 2001 received from CPCSEA (Committee for the Purpose of control and Supervision of Experiments on Animals), Ministry of Social Justice and Empowerment, Government of India [25]. Administration of the stepwise doses of aqueous extracts of leaves of experimental plants, from 5mg/100g b.wt up to a dose of 125mg/100g b.wt., did not show any significant signs of toxicity in the tested rats. One-tenth of the upper limit dose was selected for the examination of anti-diabetic activity.

2.5. Experimental Groups

The experimental rats were divided into six groups of six rats each as shown in Table **1**.

Induction of diabetes: The overnight fasted rats were induced for diabetes with a single intra peritoneal injection (*i.p.*) of 15% freshly prepared alloxan monohydrate solution (15mg/100g b.wt) in sterile normal saline just before use. Since alloxan is capable of producing fatal hypoglycemia due to massive pancre-

Table 1. Experimental groups.

Group	Supplementation	Dosage
Group 1	Control	Distlled water
Group 2	Diabetic	Alloxan monohydrate, 15mg/100g bwt, *i.p* one dose and subsequently distilled water
Group 3	Diabetic + *Hibiscus cannabinus Linn*	12.5mg/100g bwt, orally
Group 4	Diabetic + *Cicer arietinum Linn*	12.5mg/100g bwt, orally
Group 5	Diabetic + *Lactuca scariola Linn*	12.5 mg/100 g, orally)
Group 6	Diabetic +antidiabetic drug, Glipizide (reference drug)	2.5 mg/kg

atic insulin release, rats were treated with 20% glucose solution (*i.p.)* after 6 h. The rats were then kept on 5% glucose solution for 24 h to prevent hypoglycemia. After three days, blood samples were collected from the tail vein. Serum was separated and fasting glucose levels were estimated by glucose oxidase-peroxidase enzymatic method to confirm diabetes [26]. Only the rats which have shown hyperglycemia (glucose level > 250 mg/dL) were selected for the study.

2.6. Sample Collections

Animals were sacrificed as per the guidelines of CPCSEA. Blood samples were collected from tail veins and retrorbital plexus of rats as per the needs of the study design. Ventricular tissues and elastic artery were dissected out from animals and preserved for histopathogical studies [25].

2.7. Glucose Homeostasis

2.7.1. Oral Glucose Tolerance Test (OGTT)

Fasting Blood Sugar Level (FBS) was measured in the rats of all groups. After 30 min of leaves extract administration, the rats of all six groups were orally treated with 0.35mg/100g b.wt. of glucose. Blood samples were collected from the rat tail vein just prior to glucose administration (0.0h) and at 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose infusion in each rat. Blood glucose levels were measured immediately by using glucometer [27].

2.7.2. Subchronic (14 days) Studies on Leaves Extract of Hibiscus cannabinus Linn (Kenaf), Cicer arietinum (Chick Pea) and Lactuca scariola Linn (Prickly Lettuce) with Glucose Regulation in Diabetic Rats

The day after OGTT, administration of extracts (12.5mg/100g b. wt, orally) was continued every day in designed experimental groups (group 3, group 4 and group 5) till 14 days. Blood samples were collected from the rat tail vein after 30 min of ingestion of plant extracts on 1st, 7th and 14th day in the rats of all groups. Group 6 diabetic rats were treated Glipizide (2.5 mg/kg) as reference drug for similar duration and blood samples were collected from tail vein on 1st, 7th and 14thday. Blood glucose levels were measured by using glucose oxidase peroxidase method accordingly [23].

2.7.3. Plasma Insulin and Insulinogenic Index

Blood samples of all the six groups were collected and transferred to heparin containing microtubes which were placed in an ice bath for 20 min and centrifuged for 7 min at 4000 rpm and then plasma insulin concentrations were also measured at 0 and 30 min after glucose administration by ELISA kits (ERINS, Thermo Fisher Scientific, Life technologies). Insulinogenic index were calculated after glucose (1g/kg b.wt.) was administrated orally followed by drawing of blood samples with heparinized capillary tubes from the retro-orbital plexus at 0 and 30 min interval for the measurement of blood glucose and plasma insulin concentrations. Insulinogenic-index was calculated by using the following equation [28].

Insulinogenic index = (30 min plasma insulin-fasting plasma insulin) / (30 min blood glucose - fasting blood glucose)

2.8. Molecular Markers Analysis

To determine nitrosative stress, we estimated serum endothelial-NOS (e-NOS/NOS3) by ELISA kit (CSB-E08323r), Cusabio Biotech Co. Ltd. [29] and serum NO by using Greiss reagent, spectrophotometrically [30]. Serum Vascular Endothelial Growth Factor (VEGF) was measured using commercially available ELISA kit (KT-EK0540, Boster Biological Company, USA). Serum HIF-1α estimated by using commercially available ELISA kit (KT-17920, Kamiya, Biomedical Company, USA) [31].

2.9. Electrophysiology Analysis

Electrophysiology experiments were performed on following three groups of rats:

I. Pre dibetic II. Diabetic and III. Diabetic + *Lactuca scariola Linn.*

2.9.1. Anaesthesia and Adaptation of Animals

The experiments were performed in ketamine / xylazine anaesthesized male Wistar rats (ketamine 100 mg/kg + xylazine 15 mg/kg, i.m.) [32]. Anesthesia was maintained through entire period of experiments to avoid painful stimuli evoking noticeable motor or cardiovascular responses during surgery.

2.9.2. Measurement of Heart Rate Variability

Rats ECG was recorded by using MP45 Biopac instrument with PC based BSL 4.1 (Biopac Student Lab 4.1) software. Bipolar electrodes were attached to the upper and lower limbs of the rats to record an ECG; HRV was analyzed by using Kubios HRV Software, version 2.0 developed by Department of Physics, University of Kuopio, Finland. Analysis of HRV was performed by calculating 600 RR intervals of HRV parameters. The following HRV parameters were measured: RR interval duration (ms), Low Frequency (LF) power (baroreceptor activity, or sympathetic and parasympathetic activity together), High-Frequency (HF) power (corresponding to parasympathetic activity) and Low Frequency (LF) and High Frequency power ration (LF/HF).

2.9.3. Measurement of Baroreflex Sensitivity (BRS) Test

Blood pressure (SBP, DBP) of rats was measured by using MP 45 Biopac; NIBP 200 A model. The baroreflex was tested with a pressor dose of phenylephrine (PHE, bolus 8 μg/kg IV; Sigma Chemical). The baroreflex was evaluated as the derivation of HR in function of the MAP variation (ΔHR/ΔMAP). There was an interval of at least 15 min between the infusions to allow basal values to recover [32].

2.10. Animal Sacrifice

At the end of the last treatment with all the aqueous plant extracts (*Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn*) the animals were sacrificed after overnight fasting by decapitation between 09.00 AM and 11.30 AM to avert the circadian rhythm [33]. Blood was collected in centrifuge tubes, kept at room temperature for about 2 h and centrifuged at 1500 × g for 15 min to collect serum. Serum was then used for the estimations of glucose, insulin, NOS3, HIF-1α and VEGF.

2.11. Histopathology of Myocardial Tissues and Elastic Arteries

After proper dissection of animal, ventricle and elastic artery were isolated immediately and fixed in 10% neutral buffered formalin solution for 24 h [34].

The fixed tissues were processed routinely and then embedded in paraffin, sectioned to 3–5 μm thickness, deparaffinized, and rehydrated using standard techniques [34]. The extent of diabetes induced necrosis was evaluated by assessing morphological changes in all above mentioned organ sections stained with hematoxylin and eosin (H and E), using standard techniques. Photomicrographs were captured with the help of personal computer connected Magnifying Image Processing System (MIPS).

2.12. Ethics

All the animal experiments were conducted in accordance with the ethical norms approved by CPCSEA, Ministry of Social Justice and Empowerment, Government of India, and ethical clearance was granted by Institutional Animal Ethical Committee, BLDE University, Vijayapur, Karnataka to Prof. Kusal K. Das [25].

2.13. Statistical Analysis

The controls and experimental data of all the studied animals were analyzed for evaluating the range of significance. Results were expressed as mean ± SD values for each group. The significance of inter-group differences was calculated by one way analysis (ANOVA) followed by 'post hoc t test'.

3. RESULTS

3.1. Phytochemical Analysis

The preliminary phytochemical analysis of aqueous leaves extracts confirmed the presence of flavonoids, saponins, tannins and triterpenes in *Hibiscus cannabinus Linn* leaves extract. *Cicer arietinum Linn* contains flavonoids, saponins and triterpenes, whereas *Lactuca scariola Linn.* Leaves extract confirms the presence of flavonoids, saponins and Steroids.

3.1.1. In Vitro Percentage Glucose Reduction at Different pH

It was, observed, as shown in Table **2**, that all the three plant extracts (*Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn)* reduced percent glucose concentration at both, pH 7.0 and pH 9.0, respectively. Among the three plants, *Lactuca scariola Linn* leaves extract showed maximum glucose reduction (98.0%) at pH 9.0. Results are tabulated in Table **2**.

Data are shown as percent reduction in glucose concentrations *in vitro* by leaves extracts of: *Hibiscus cannabinus Linn, Cicer arietinum Linn and Lactuca scariola Linn* ex-

Table 2. ***In vitro*** **hypoglycemic effect of** ***L. scariola*** **leaves extract alone at different pH.**

Sl.No	Solution Sample	pH 7.0	pH 9.0
1.	Glucose + *Hibiscus cannabinus Linn extracts*	20.5%	30.5%
2.	Glucose + *Cicer arietinum Linn*	35.5%	50.0%
3.	Glucose + *Lactuca scariola Linn*	68.0%	98.0%

Table 3. Effect of acute supplementation with ***Hibiscus cannabinus Linn, Cicer arietinum Linn*** **and** ***Lactuca scariola Linn*** **leaves extract (12.5 mg/100 g body weight) on blood glucose level (Oral Glucose Tolerance) in diabetic rats.**

Treatment Group	FBS	0.0h	0.5	1.0h	1.5h	2.0h
Control(G- 1)	$87.5 \pm 10.34^{a,1}$	$87.00 \pm 8.45^{a,1}$	$110.00 \pm 8.45^{b,1}$	$125.5 \pm 10.45^{c,1}$	$108.5 \pm 7.45^{b,1}$	$88.5 \pm 9.45^{a,1}$
Diabetic(G -2)	$235.50 \pm 20.5^{a,2}$	$244.50 \pm 15.5^{a,2}$	$340.50 \pm 35.5^{b,2}$	$400.50 \pm 40.5^{c,2}$	$450.50 \pm 45.5^{d,2}$	$430.50 \pm 38.5^{c,2}$
Diabetic + *Hibiscus cannabinus Linn* (G-3)	$245.50 \pm 30.5^{a,2}$	$250.50 \pm 24.5^{a,2}$	$325.50 \pm 30.5^{b,3}$	$385.50 \pm 50.5^{c,3}$	$400.50 \pm 40.5^{d,3}$	$380.50 \pm 30.5^{c,3}$
Diabetic + *Cicer arietinum Linn* (G-4)	$240.50 \pm 25.5^{a,2}$	$248.50 \pm 25.5^{a,2}$	$315.50 \pm 25.5^{b,3}$	$380.50 \pm 20.5^{c,3}$	$400.50 \pm 35.5^{d,3}$	$345.50 \pm 30.5^{e,4}$
Diabetic + *Lactuca scariola Linn* (G-5)	$245.50 \pm 30.5^{a,2}$	$247.50 \pm 35.5^{a,2}$	$290.50 \pm 30.5^{b,4}$	$300.50 \pm 20.5^{c,4}$	$285.50 \pm 20.5^{b,4}$	$283.50 \pm 24.5^{b,5}$
Diabetic + glipizide(G-6)	$234.50 \pm 20.5^{a,2}$	$240.50 \pm 15.8^{a,2}$	$275.50 \pm 30.5^{b,5}$	$280.50 \pm 20.00^{b,5}$	$220.50 \pm 30.00^{c,5}$	$185.67 \pm 18.00^{d,6}$

Treatment groups: diabetic control; diabetic + *Hibiscus cannabinus Linn;* diabetic + *Cicer arietinum Linn;* diabetic + *L. scariola Linn* leaves extract; diabetic + glipizide (0.25 mg/100 g). Horizontal values are the mean ± SD of six observations in each group till at 2 h. In each row, values with different superscript letters (a, b, c or d) are significantly different from each other ($p < 0.05$) in the same group till 2 h. Vertical columns indicate variation of blood glucose levels among six different groups at different time interval till 2 h. In each column, values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p < 0.05$) at different times.

tracts as measured by using glucose oxidase-peroxidase method at pH 7.0 and 9.0.

3.2. Glucose Homeostasis

3.2.1. Oral Glucose Tolerance Test

Group 1 (control) rats showed the typical normal OGTT response i.e, glucose level increased at 1.0 h and decreased to base line after 2.0h of glucose treatment. In group 2 diabetic rats, the glucose level steadily increased till 1.5 h (on glucose treatment) with slight decrease at 2.0h. The group 3 and 4 (diabetic + *Hibiscus cannabinus Linn and diabetic + Cicer arietinum Linn*) rats did not show any significant decrease in the glucose level after 1.5h till 2.0 h of the glucose treatment. The group 5 (*Lactuca scariola Linn*) rats showed significant decrease in blood glucose levels at all the time intervals and almost reached its baseline at 2h which indicates improved glucose tolerance. The glucose tolerance tests also showed that glipizide (as a reference drug) produced a decrease in blood glucose levels at all the time intervals after glucose administration (Table **3**).

Subchronic (14 days) studies on leaves extract of *Hibiscus Cannabinus Linn* (Kenaf), *Cicer Arietinum* (Chick pea) and *Lactuca scariola Linn* (Prickly lettuce) in diabetic rats: It was observed that the concentration of glucose in group 1 normal control rats remained unchanged till the 14th day. Group 2 diabetic rats showed progressive rise in blood glucose concentration till 14th day. Group 3 diabetic rats treated with *Hibiscus cannabinus Linn* extract did not show any significant decrease in blood glucose level whereas group 4 diabetic rats treated with *Cicer arietinum Linn* extract showed a significant decrease in blood glucose level. Group 5 rats which were treated with *L. scariola Linn* leaves extract showed a significant reduction in blood glucose concentrations (Fig. **2**).

3.2.2. Plasma Insulin and Insulinogenic Index

Table **4** shows fasting plasma glucose, insulin and insulinogenic index of experimental animals. Results indicate that subchronical (14 days treatment) exposure of supplementation of *Cicer arietinum Linn and Lactuca scariola Linn* has significant effect on fasting blood glucose levels in diabetic rats. No significant changes were observed in fasting plasma insulin levels in all the experimental groups when compared with diabetic group. However, insulinogenic index was significantly increased in all the groups of diabetic rats except *Lactuca scariola Linn* supplemented group which showed a significant decrease in insulinogenic index.

3.3. Analysis of Molecular Markers

Table **5** shows the effect of sub chronic (14 days) supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extracts (12.5 mg/100 g body weight) on serum NOS3, nitrite, HIF-1α and VEGF concentra-

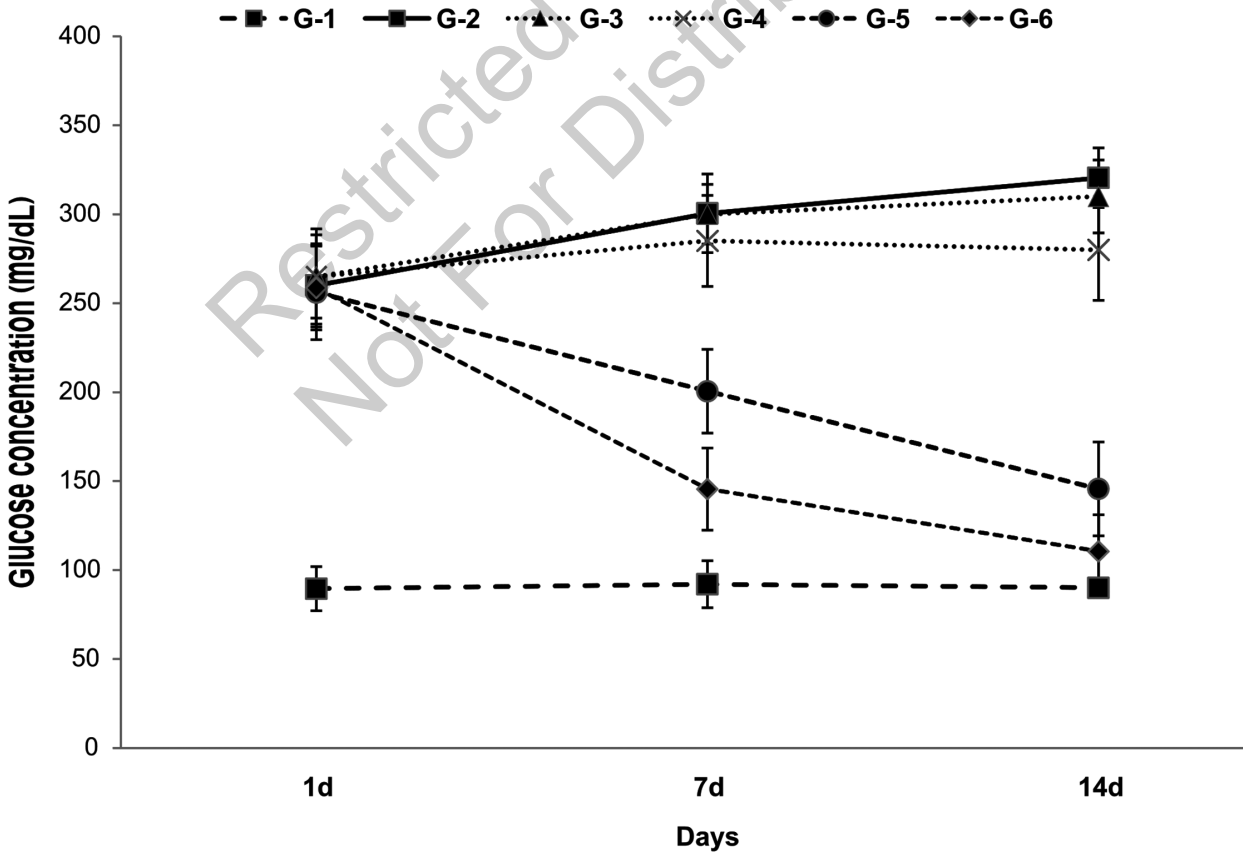

Fig. (2). Effect of sub chronic (14 days) supplementation of leaves extracts (12.5 mg/100 g body weight)) on blood glucose level in diabetic rats (day 1, 7 and 14). Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Hibiscus cannabinus Linn* extract; 4, diabetic +, *Cicer arietinum Linn.* Extract; 5, diabetic + *Lactuca scariola Linn* extract; 6, diabetic + glipizide (0.25 mg/100 g).

tions in diabetic rats. Group 2 diabetic rats showed a significant decrease of serum NOS3 and nitrite levels with increased levels of serum HIF-1α and VEGF concentrations. Supplementation with *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extracts showed significant increase in serum NOS3 and nitrite levels along with increased levels of serum HIF-1α as compared to diabetic rats. VEGF concentrations in all the three plant extracts supplemented rats remained unchanged as compared to group 2 diabetic rats.

3.4. Electrophysiology Analysis

Electrophysiological analysis of *Lactuca scariola Linn* supplemented diabetic animals were observed in pre diabetic, diabetic and diabetic + *Lactuca scariola Linn* (14 days supplementation) rats. Heart rate of diabetic rats significantly improved (decrease LF and HF ratio)as compared to pre-diabetic state but these diabetic rats supplemented with *Lactuca scariola Linn* showed remarkable improvement (Table **6**).

3.4.1. Heart Rate Variability

Fig. **3** depicts the HF and LF power (nu) of rats during pre diabetic, diabetic and diabetic + Lactuca scariola Linn (14 days supplementation) status. Results show the decrease of HF power and increase of LF power in diabetic rats which significantly improved after 14 days supplementation with *Lactuca scariola Linn*. Further HRV analysis showed an increased LF and HF ratio in diabetic group of rats but an improvement of LF/HF ratio was noticed in diabetic rats supplemented with *Lactuca scariola Linn* for 14 days (Fig. **4**).

3.4.1.1. Blood Pressure and Baroreflex Sensitivity (BRS)

Results show that SBP, DBP and MAP were increased in diabetic rats whereas rats supplemented with *Lactuca scariola*

Table 4. Effect of sub chronic (14 days) supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extract (12.5 mg/100 g body weight) on glucose homeostasis in diabetic rats.

Parameters	Control (G-1)	Diabetic (G-2)	Diabetic + *Hibiscus cannabinus Linn* Extract (G-3)	Diabetic +, *Cicer arietinum Linn.* Extract (G-4)	Diabetic + *Lactuca Scariola Linn* Extract (G-5)	Diabetic + Glipizide (G-6)	't' Value	df	'p' Value
Fasting blood glucose (mg/dl)	85.00 ± 5.56[a]	180.45 ± 13.58 [b]	170.00 ± 23.30 [b]	140.00 ± 25.20 [c]	110.00 ± 12.82 [d]	105.23 ± 14.65[d]	4.54	5	0.0000
Fasting plasma Insulin (µg/L)	1.00 ± 0.17 [a]	0.66± 0.19 [b]	0.64 ± 0.10[b]	0.69 ± 0.12 [b]	0.65 ± 0.18 [b]	0.97 ± 0.16 [a]	1.38	5	0.0478
Insulinogenic - index	0.0043 ± 0.0005 [a]	0.0065 ± 0.0004 [b]	0.0062 ± 0.0004[b]	0.0061 ± 0.0002 [b]	0.0053 ± 0.0003 [c]	0.0044 ± 0.0002 [a]	2.85	5	0.002

Horizontal values are the mean ± SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$).

Table 5. Effect of sub chronic (14 days) supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extract (12.5 mg/100 g body weight) on serum NOS3, nitrite and HIF-1α concentrations in diabetic rats.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	't' Value	df	'p' Value
Serum NOS3 (nmol/mL)	7.13 ± 1.11[a]	4.68 ± 1.53[b]	5.64± 1.58[c]	5.96± 1.94[c]	6.45 ± 2.16[d]	6.67.± 2.34[d]	2.55	5	0.0015
Serum Nitrite (µmol/L)	22.32± 3.04[a]	10.45± 3.20[b]	11.13± 2.11[b]	12.02± 2.27[b]	15.11± 1.75[c]	17.36± 2.22[d]	3.24	5	0.0001
Serum HIF-1α (pg/mL)	150.21± 5.28[a]	168.23± 12.32[b]	178.10± 3.52[c]	178.27± 14.41[c]	200.50± 23.72[d]	175.08± 8.15[c]	10.32	5	0.0000
Serum VEGF (pg/mL)	300.31± 5.65[a]	423± 25.20[b]	414.47± 6.51[b]	417.47± 13.62[b]	423 ± 25.50[b]	378.17± 12.34[d]	9.42	5	0.0000

Treatment groups: 1, control; 2, Diabetic; 3, diabetic + *Hibiscus cannabinus Linn* extract; 4, diabetic +, *Cicer arietinum Linn.* Extract; 5, diabetic + *Lactuca scariola Linn* extract; 6, diabetic + glipizide (0.25 mg/100 g). Group1, control; group 2, diabetic; group 3, diabetic + *Hibiscus cannabinus Linn*, group 4, *Cicer arietinum Linn*, group 5, *Lactuca scariola Linn*, group 6, diabetic + glipizide.

Horizontal values are the mean • } SD of six observations in each group. In each row, values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.0$.

Linn showed a decrease in all three blood pressure parameters as compared to diabetic rats. BRS was significantly reduced in diabetic rats as compared to pre diabetic state whereas after 14 days of supplementation with *Lactuca scariola Linn* to diabetic rats showed BRS back to pre diabetic state (Table **6**) (Fig. **5**).

3.5. Histopatholgy of Myocardial Tissues and Elastic Arteries

3.5.1. Myocardium

Microscopic architecture of myocardium showing prominent intercalated discs and centrally placed nucleus in normal group. In case of diabetic group (2), focal myocardial hypertrophy and degeneration and capillary congestion were observed, and in treated group a mild focal myocardial hypertrophy was observed (Fig. **6**).

3.5.2. Elastic Artery

Microscopic structure of Elastic artery. All the three layers tunica intima, media and adventitia showing normal architectures in group 1. The tunica intima is lined by endothelial cells with features of endothelial detachment, erosion and microulcerations.

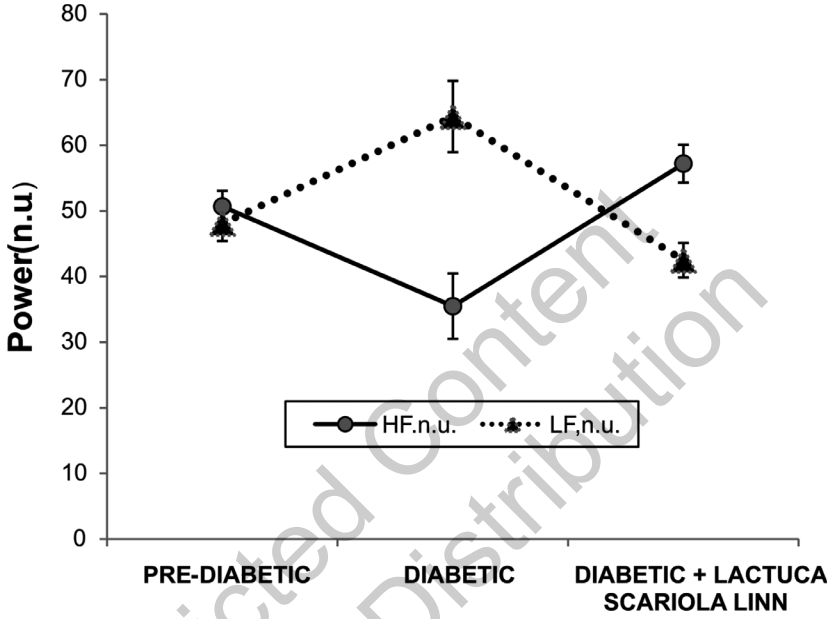

Fig. (3). Frequency domain results of HRV analysis (LF and HF power, n.u.) on same adult male rats (n = 6) during pre-diabetic, diabetic and after supplementation of Lactuca scariola Linn for 14 days. Values with different superscripts are statistically significant with each other at $p \leq 0.05$.

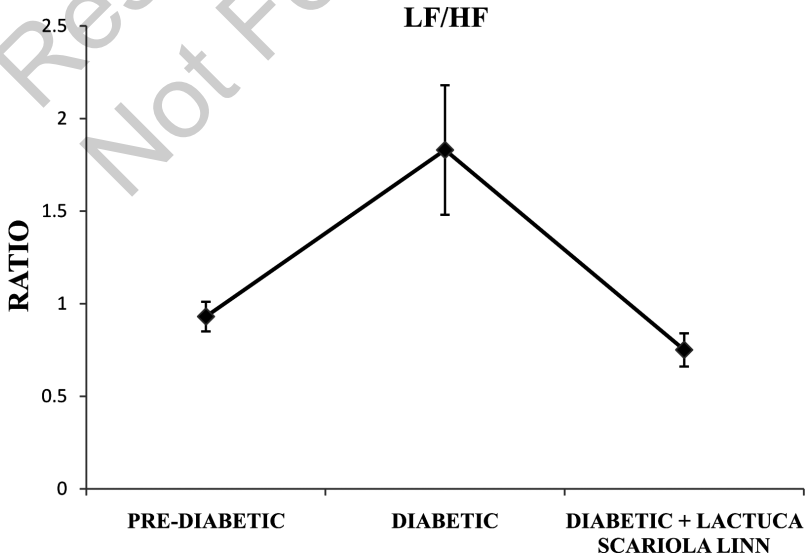

Fig. (4).Frequency domain results of HRV analysis (LF/HF ratio) on same adult male rats (n = 6) during pre- Diabetic and after supplementation of Lactuca scariola Linn for 14 days. Values with different superscripts are statistically significant with each other at $p \leq 0.05$.

Table 6. Mean Arterial Pressure (MAP), Heart Rate (HR) and baroreflex sensitivity (sympathetic) in prediabetic (group 1), diabetic (group 2) and diabetic + *Lactuca scariola Linn* (14 days treatment).

Variables	G-1 (Pre diabetic)	G-2 (Diabetic)	G-3 (Diabetic + *Lactuca scariola Linn*	P Value
SBP, mmHg	100.23 ± 10.23[a]	146.23 ± 13.56[b]	120.34 ± 12.23[c]	0.0000
DBP,mmHg	74.45 ± 11.00[a]	98.45 ± 11.54[b]	80.45 ± 10.45[a]	0.0000
MAP, mmHg	100.45 ± 15.34[a]	124.50 ± 12.45[b]	109.75 ± 12.55[a]	0.0001
HR, bpm	327.2 ± 72.9[a]	274.92 ± 58.34[b]	300.45 ± 23.56[c]	0.0001

Values of different superscripts significantly differ from each other ($p < 0.05$)

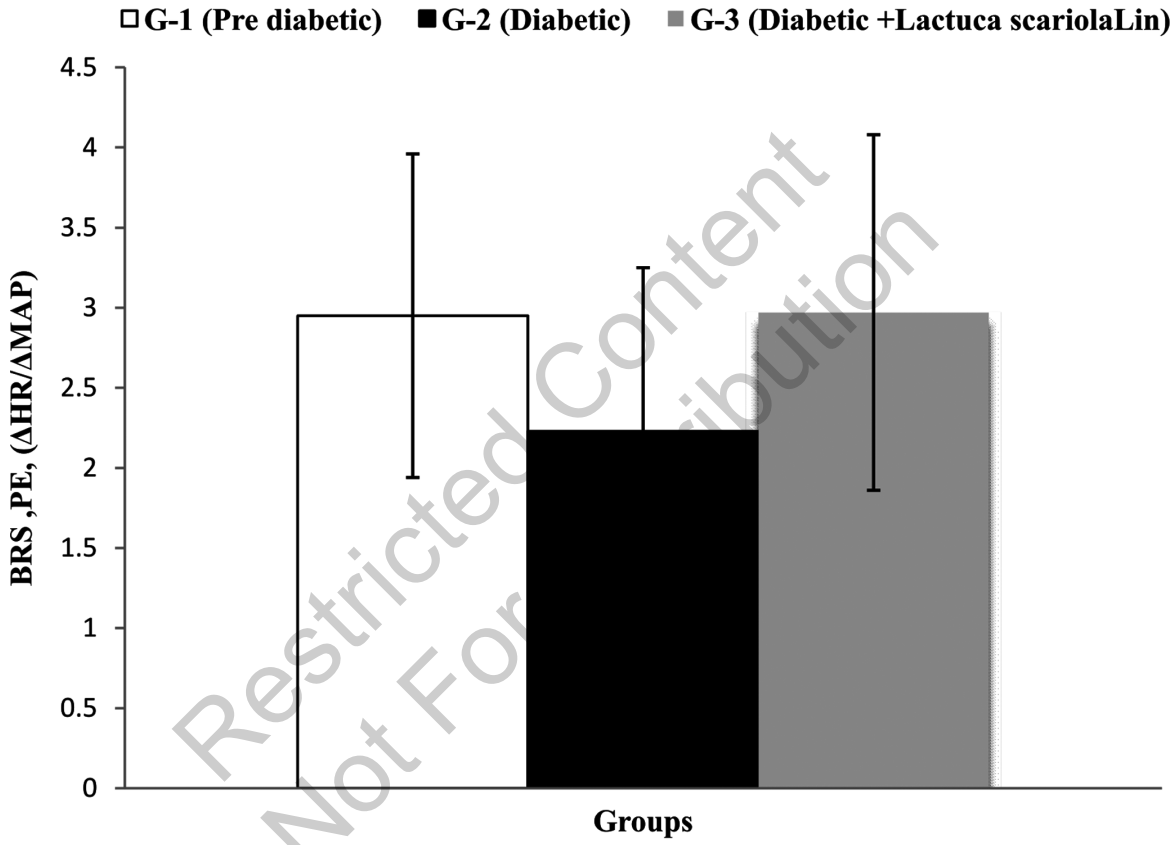

Fig. (5). Baroreflex Sensitivity (BRS) in pre diabetic (group 1), diabetic (group 2) and diabetic + *Lactuca scariola Linn* supplemented rats (group 3).

The tunica media consists of smooth muscle cells with elastic fibres and shows moderate thickening in diabetic group 2.The tunica intima is lined by endothelial cells and shows mild thickening in treated group 3 (Fig. **7**).

4. DISCUSSION

Results from *in vitro* studies show percent change reduction in glucose concentration by all the three plant extracts in pH 7.0 and pH 9.0 but percent change reduction was highest by *L. scariola* extracts. Results indicate the possible alteration of molecular behavior of glucose in the presence of all the three plants, especially in case of *Lactuca scariola Linn.* Results also showed higher percent reduction of glucose concentration at alkaline pH 9.0. (Table **2**).

It was found that the elevated glucose concentration of alloxan treated rats remained around 250 mg/dL at fasting conditions, which appears like type 2 diabetes (150 mg/dl – 250 mg/dl). It also suggests a partially functional impairment of pancreatic Langerhans islets cells [28]. Results from glucose tolerance tests indicated that all the three plant extracts showed improved glucose tolerance however, effect of *Lactuca scariola Linn* showed normalization of impaired glucose tolerance rats. The results of OGTT studies with acute supplementation corroborated with the sub chronic studies (14 days plants extracts supplementation on diabetic rats) of all the three plant extracts supplemented rats. *Lactuca scariola Linn* supplementation for 14 days in diabetic rats showed significant decrease in glucose levels, almost near to control values (Fig. **2**). The results suggest that the *Lactuca scariola Linn* leaves extract has anti diabetic properties. Possibly,

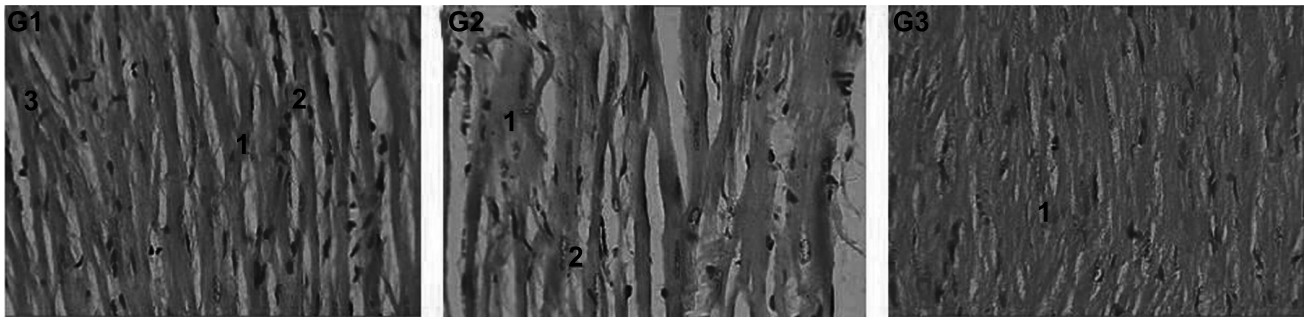

Fig. (6). H.E stain (45x) of ventricular muscle; pre diabetic (group 1): central placed nucleus, intercalated disk, branching and anastomosing striated muscle, degeneration of capillary congestion; diabetic (group 2): myocardial hypertrophy; diabetic + *Lactuca scariola Linn* (group 3): focal myocardial hypertrophy.

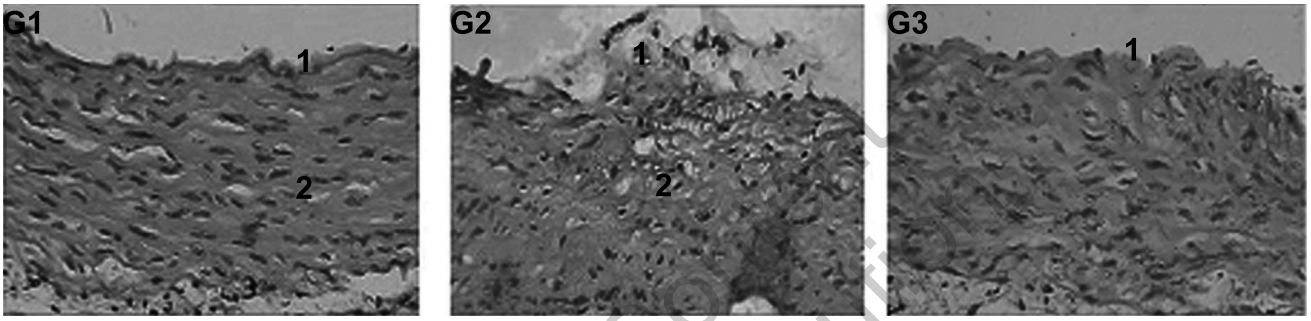

Fig. (7). H.E stain (45x) of elastic artery consisting of pre diabetic (group 1): normal Tunica intima, Tunica media and Tunica adventitia; diabetic (group 2): scattered epithelium with mild micro ulceration, moderate thickening of T.media; diabetic + *Lactuca scariola Linn* (group 3): mild thickening of T. intima.

many poly phenolic compounds including flavonoid, triterpenes and saponin like bioactive substances in these plants may be acting as hypoglycemic agents (Table **3**). These bio active compounds may be affecting insulin signaling mechanisms of pancreatic islet cells or may be altering molecular configuration of glucose by converting it to glycone and glucuronic acid [23, 35, 36]. It has been reported that the flavanoids play a major role in maintaining blood glucose levels, glucose uptake, insulin secretion and modulating immune function to prevent specific types of diabetes mellitus [37]. The consumption of flavanoids or flavanoid rich foods reduces the risk of diabetes mellitus. On the other hand, saponins have the ability to reduce increased plasma blood glucose, hence making it useful in treating diabetes mellitus [38]. The hypoglycaemic effect of saponin is through restoration of insulin response, improvement in insulin signalling, increase plasma insulin and induction of insulin release from the pancreas [38]. There are several hypotheses to explain hypoglycemic effects of many medicinal plants that include a possible inhibitory impact on enzyme- insulinase which keeps insulin stable for longer durations or increases insulin sensitivity or increases peripheral glucose uptake mechanisms by inducing insulin mediated cell signaling pathways [39]. Beside these possible mechanisms, the antioxidant actions of the active compounds of these medicinal plants also improve hepatic glucose regulatory system and decrease intestinal glucose uptake. Also, these active compounds may act at pancreatic level by protecting, regenerating and repairing pancreatic beta cells by increasing the size and number of cells in the islets of Langerhans [39].

Observations on molecular markers in sub chronic (14 days) supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and *Lactuca scariola Linn* leaves extract (12.5 mg/100 g body weight) on diabetic rats show significant increase of serum NOS3 and NO (Table **5**). The improvement in NOS3 and NO levels were found to be the maximum in *Lactuca scariola Linn* supplemented diabetic rats. Nitric oxide which synthesized from L-arginine by nitric oxide synthases in different conditions provides vascular stabilities and reflects endothelial functions [40]. Decrease in NOS3/e-NOS levels indicates vascular malfunctions and may cause coronary spasm. A clear association between NOS3 and type 2 diabetes mellitus has already been established [41].

Studies on endothelial dysfunctions in diabetic animals showed a possible uncoupling of the vascular endothelial growth factor (VEGF) from endothelial nitric oxide synthase (NOS3/eNOS) axis. This uncoupling phenomenon causes resultant increase in VEGF which leads to excessive endothelial cellular growth. These alterations in VEGF-NO regulatory mechanism supposedly cause loss of vascular integrity in diabetic states [42]. Supplementation of *Hibiscus cannabinus Linn, Cicer arietinum Linn* and especially *Lactuca scariola Linn* leaves extract showed improvement in NOS3, NO and VEGF status in diabetic rats (Table **5**). Further study on hypoxia inducible factor-1α (HIF-1α), a transcription factor showed a significant increase in diabetic rats. Supplementation of all the three plants extracts did not bring HIF-1α concentration back to normal. HIF-1α is usually expressed during low oxygen saturation in the cell and it is expressed to protect the cell from hypoxia induced injury by enhancing VEGF concentrations, suggesting adaptive mecha-

nism to protect the cell during diabetes related complications especially in vascular systems [43]. Subchronic treatment with all the three plants, particularly *Lactuca scariola Linn,* effects on HIF-1α provide a cellular adaptation against hyperglycemia induced alteration of cell signaling pathways. This protective mechanism is oxygen sensing intracellular mechanism and facilitates cardiovascular stabilities. The results from author's laboratory on molecular markers clearly indicate that all the three plants have vascular protective benefits but *Lactuca scariola Linn* is observed to be better in its actions (Table **5**).

After the glucose homeostasis and molecular marker analysis in diabetic rats are carried out, the electrophysiological impact of *Lactuca scariola Linn* on vascular systems and histopathology evaluations of myocardial tissues and elastic arteries are further evaluated.

Decrease in HF power with concurrent increase of LF power in diabetic rats indicates dominance of sympathetic nervous system over parasympathetic functions. It is well known that the autonomic nervous system plays the most important role to control cardiovascular integrity. Alterations in HR, blood pressure, LF/HF ratio and Baroreflex Sensitivities (BRS) in diabetic rats, clearly indicate autonomic malfunctions. Autonomic malfunctions are due to increase in sympathetic adrenergic activity and decrease in vagal activities. Such abnormalities in diabetics may induce ventricular arrhythmia and even sudden death of patients [44].

Further studies from authors' laboratory on spectral analysis revealed augmented low frequency dominance in diabetic rat 2 as compared to pre diabetic status. Interestingly, sub chronical supplementation with *Lactuca scariola Linn* was found to improve the status of autonomic malfunctions (Figs. **3, 4** and **5**). Hence, it may be deduced from overall electrophysiological observations that supplementation of *Lactuca scariola Linn* extracts sub chronically to diabetic rats indicate a possible beneficial revival of autonomic functions in diabetic rats. The improvement of HRV and BRS in diabetic rats supplemented with *Lactuca scariola Linn* extracts for 14 days suggests its possible influence on parasympathetic vagal action on heart by AChE inhibition [45, 46].

It is well established that arterial baroreceptors which respond to arterial stretch, regulate both, sympathetic and parasympathetic actions on cardiovascular system and modulate ventricular stroke volume. Hence, the plant extracts possibly act as cardio-protective agents influencing autonomic nervous system functions, regulating HR and blood pressure by altering afferent receptor mediated discharge which controls baroreflex sensitivities and subsequently ventricular functions [47] (Table **6**) (Fig. **5**).

Histopathogical findings of myocardial tissues from ventricle showed ventricular hypertrophy and degeneration of capillaries in diabetic rats. Similarly elastic artery showed moderate thickening of the tunica media in diabetic rats. But supplementation with *Lactuca scariola Linn* for 14 days

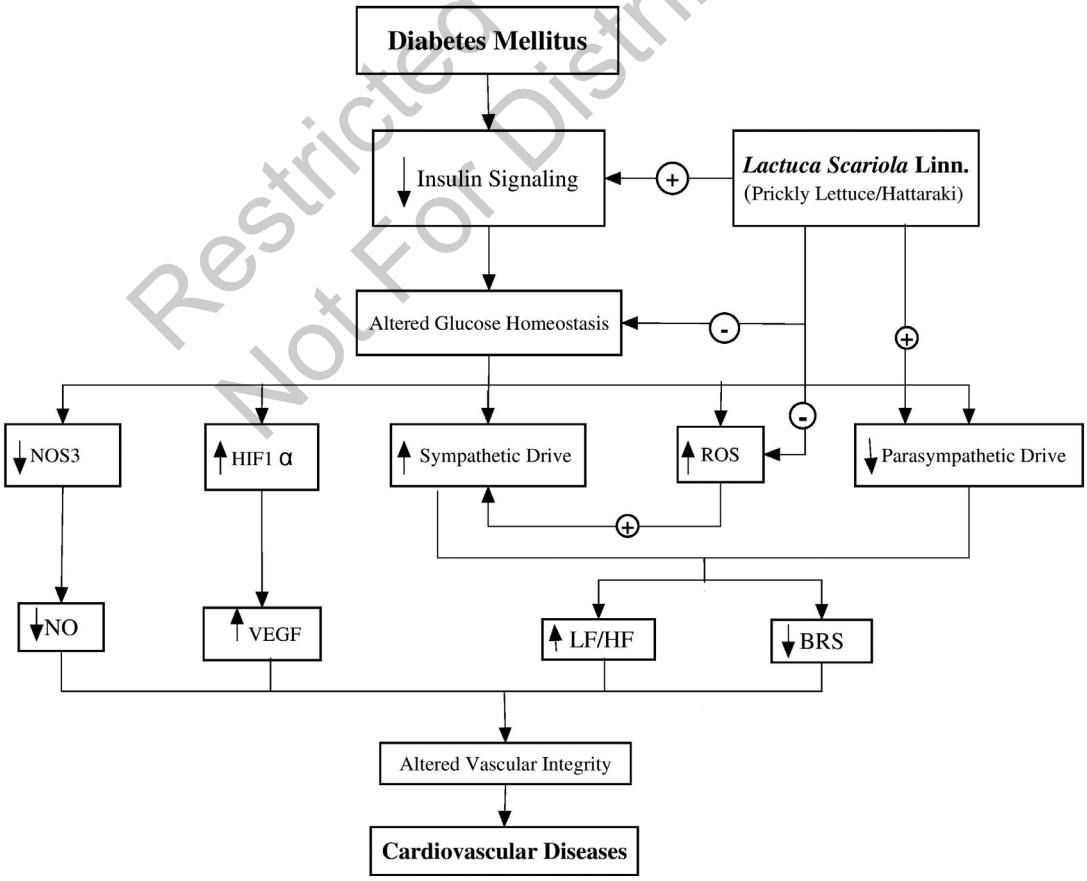

Fig. (8). Postulated mechanisms of actions of *Lactuca scariola Linn* (prickly lettuce or hattaraki) on diabetic rats in terms of cardiovascular actions.

showed remarkable improvements of both vascular and myocardial architectures in diabetic rats. Hence, it may be hypothesized that *Lactuca scariola Linn* possibly has a cardioprotective actions either by regulating glucose homeostasis in diabetes or controlling cardiac autonomic functions or it is possible with some antioxidant efficacies (Figs. **6** and **7**).

CONCLUSION

The results of acute and sub chronic exposure of leaves of *Lactuca scariola Linn* (prickly lettuce or hattaraki) showed significant anti-diabetic efficacies besides cardio protective effect; possibly through insulin regulatory mechanisms or controlling autonomic nervous system functions or altering oxygen sensing cell signalling pathways. Histopathology of myocardial tissues and elastic artery supported these observations. The possible mechanisms are depicted in Fig. (**8**). The mechanism shows a possible insulin signaling mechanism of *Lactuca scariola Linn.* along with stimulation on oxygen sensing pathways like HIF1α and NOS3. Beside these the figure also postulates a possible pathway to improve sympathetic- vegal balance by *Lactuca scariola Linn* treatment on experimental animals, which ultimately benefit cardiovascular functions in diabetic rats.

The beneficial effects of *Hibiscus cannabinus Linn (*Kenaf) and *Cicer arietinum* (Chick pea) on leaves glucose homeostasis and oxygen sensing cell signalling regulatory mechanisms were less significant compared to beneficial effects of *Lactuca scariola Linn* (prickly lettuce or hattaraki) leaves.

LIST OF ABBREVIATION

TBARS	=	Thiobarbituric Acid Reactive Substances
HRV	=	Heart Rate Variability
LF	=	Low Frequency
HR	=	High Frequency
BRS	=	Baroreflex Sensitivity
NIBP	=	Non Invasive Blood Pressure
AChE	=	Acetyl Choline Esterase

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

IAEC was taken (vide 2.12).

HUMAN AND ANIMAL RIGHTS

Vide 2.12.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Original Article

A Comparative Study on Anti-diabetic Effects of Aqueous *Trigonella foenum graecum, Hibiscus cannabinus Linn,* and *Cicer arietinum* extracts on Alloxan Induced Diabetic Male Albino Rats

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ABSTRACT

Objective: To compare anti-diabetic effect between the different aqueous extracts of green leafy plants locally available in North Karnataka region of India on alloxan induced diabetic male albino rats. **Materials and Methods:** The preparation of aqueous extracts, preliminary phytochemical analysis and toxicity screening test of 3 aqueous extracts was done by using standard protocol. To study anti-diabetic activity experimental rats were divided into five groups *viz.* Group I (Control), Group II (Diabetic, Alloxan monohydrate, 15mg/100g bwt, *i.p.*), Group III (Diabetic with *Trigonella foenum graecum*), Group IV (Diabetic with *Hibiscus cannabinus Linn*) and Group V (Diabetic with *Cicer arietinum*). All above extracts were supplemented with same dose *i.e.* 12.5mg/100g bwt, orally. The blood glucose levels were evaluated in all the above experimental groups after acute (OGTT) and sub chronic (2 weeks) supplementation. **Results:** Our results depicts statistically significant decreased blood glucose level in Group III rats after both acute and sub chronic supplementation whereas in Group IV rats only after sub chronic supplementation when compared with Group II rats. But, Group V rats not showed any significant change in both acute as well as sub chronic exposure when compared with Group II rats. **Conclusion:** *Trigonella foenum graecum* and Hibiscus cannabinus Linn. leaves may be used as a dietary supplement in diabetic patients.

Key words: *Trigonella foenum graecum, Hibiscus cannabinus Linn., Cicer arietinum*

Key message: Aqueous *Trigonella foenum graecum* leaves extract can be used as a dietary supplement for controlling blood glucose level.

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INTRODUCTION

Trigonella foenum graecum (Methi), *Hibiscus cannabinus Linn.* (Pundi) and *Cicer arietinum* (Channa) leaves are usually consumed by the people as a dietary supplement and available locally in North Karnataka of India. *Trigonella foenum graecum* leaves is available throughout the year, whereas, *Hibiscus cannabinus Linn.* and *Cicer arietinum* leaves are seasonal, which are available only during the months of November to March. There are many Medicinal plants of India which are known to have anti-diabetic potential.[1] Similarly plants like *Nerium indicum* possess antidiabetic activity[2]. *Hibiscus cannabinus Linn.* and *Trigonella foenum graecum* leaves which are commonly used as a dietary supplement also known to possess anti-diabetic activities[3] while that of *Cicer arietinum* leaves remains unknown. The synthetic drugs which are used to control diabetes mellitus have many side effects; hence, replacement of these drugs by plant based alternatives is essential. Many herbal medicines possess anti-diabetic properties.[3] Hence, the present work is carried out to compare anti-diabetic effect between the different aqueous extracts of *Trigonella foenum graecum, Hibiscus cannabinus Linn.* and *Cicer arietinum* leaves on alloxan induced diabetic male albino rats exposed to both acute and sub chronic supplementation.

MATERIALS AND METHODS

Collection and Identification of Plants

The fresh *Trigonella foenum graecum, Hibiscus cannabinus Linn.* and *Cicer arietinum* leaves were collected from the local market and were authenticated[4] by Dr. M. B. Mulimani, Associate Professor, Department of Botany, BLDEA's S. B. Arts and K. C. P. Science College, Vijayapur, Karnataka, India.

Preparation of Plant Extracts

The 5g of fresh leaves were washed thoroughly with distilled water, crushed and extracted with 100mL distilled water at 50-60^{0}C temperature for 1 hr. The resulting extract was filtered using Whatmann filter paper no. 1. Filtrate is used as an aqueous plant extracts and orally fed to the rats. The extracts of all the plants were prepared with similar protocol.

Phytochemical analysis

Preliminary phytochemical analysis of freshly prepared plant extracts was carried out to identify the phyto constituents using standard procedures.[5]

Animals

Adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180-200g were housed in central animal house of BLDE University's Shri. B. M. Patil Medical College, Hospital and Research Centre, Vijayapur with 12 hr light: 12 hr dark cycle. The rats were divided into five groups

of six rats each. Three rats were kept in each metabolic wire cage (60cm x 30cm x 20cm). Standard pellets obtained from Hindustan lever rat feed, Mumbai, India, were used as a basal diet during the experimental period. The control and experimental rats were provided food and drinking water *ad libitum*.

Chemicals

The chemicals used during the experiment are alloxan monohydrate (Spectrochem Pvt. Ltd., Mumbai), Tween 80 (S.D. Fine-chem limited, Mumbai). Accu-chek® Active Glucometer, Roche Diagnostic Corporation, Germany, Blood gluco-strips (Roche Diagnostic Pvt. Ltd., Mumbai, India). All other chemicals and reagents used were of analytical grade.

Acute oral toxicity studies of extract

The acute oral toxicity studies of extracts were carried out as per the OECD guidelines, draft guidelines 423.[6] Administration of the stepwise doses of aqueous extracts of leaves from 5mg/100g b.wt up to a dose of 125mg/100g b.wt caused no significant signs of toxicity in the tested rats. One-tenth of the upper limit dose was selected for the examination of anti-diabetic activity.

Experimental Groups

The experimental rats were divided into five groups of six rats each as shown in table 1.

Induction of diabetes

The overnight fasted rats were induced for diabetes with a single intra peritoneal injection (*i.p.*) of 15% freshly prepared alloxan monohydrate solution (15mg/100g b.wt) in sterile normal saline just before use. Since alloxan is capable of producing fatal hypoglycemia due to massive pancreatic insulin release, rats were treated with 20% glucose solution (*i.p.)* after 6hr. The rats were then kept on 5% glucose solution for 24 hrs to prevent hypoglycemia. After three days, blood samples were collected from the tail vein. Serum was separated and fasting glucose levels were estimated by glucose oxidase-peroxidase enzymatic method to confirm diabetes.[7] Only the rats which have shown hyperglycemia (glucose level > 250 mg/dL) were selected for the study.

Oral glucose tolerance test (OGTT)

Fasting blood sugar level (FBS) was measured in the rats of all groups. After 30min of leaves extract administration, the rats of all five groups were orally treated with 0.35mg/100g b.wt. of glucose. Blood samples were collected from the rat tail vein just prior to glucose administration (0.0hr) and at 0.5hr, 1.0hr, 1.5hr and 2.0hr after glucose loading. Blood glucose levels were measured immediately by using glucometer.

Determination of blood glucose level

After the day of OGTT, administration of extracts (12.5mg/100g b. wt, *orally*) was continued every day in designed experimental groups (group III, group IV and group V) till 13 days. Blood sample was collected from the rat tail vein after 30 minutes of ingestion of plant extracts on 1st, 3rd, 5th, 7th, 9th, 11th, 13th day in the rats of all groups. Blood glucose levels were measured by using glucometer.

Statistical analysis

The statistical data, obtained from all the control and experimental samples analyzed for evaluating the range of significance. Results expressed as mean ± SD values were calculated for each group. To determine the significance of inter-group differences, one way analysis (ANOVA) followed by 'post hoc t test' were done.

Ethics

All the animal experiments were conducted according to the ethical norms approved by CPCSEA, Ministry of Social Justice and Empowerment, Government of India, and ethical clearance was granted by Institutional Animal Ethical Committee, BLDE University, Vijayapur.

RESULTS

Phytochemical analysis

The preliminary phytochemical analysis of aqueous extract of *Trigonella foenum graecum* leaves confirms the presence of flavonoids, saponins, triterpenes and steroids. Similarly, the preliminary phytochemical analysis of aqueous extract of *Hibiscus cannabinus Linn*. leaves showed the presence of flavonoids, saponins, triterpenes and Tannins. Whereas, the preliminary phytochemical analysis of aqueous extract of *Cicer arietinum* leaves revealed the presence of flavonoids, saponins and triterpenes (Table 2).

Acute oral toxicity studies

In acute toxicity study, aqueous extracts of leaves showed non-significant toxicity sign when observed for the parameters during the first 4 hrs and followed by daily observations for 14 days and mortality was also not observed; the drugs were found to be safe at the tested dose level of 125 mg/100g b. wt. One-tenth of this dose level was taken as effective dose. All the extracts were experimented at the same dose of 12.5 mg/100g b. wt. In order to ascertain a scientific base for the usefulness of these plants in the treatment of diabetes, it was decided to evaluate experimental design of antidiabetic activity by following glucose tolerance test.

Oral glucose tolerance test

Group I rats showed the typical normal OGTT response i.e, glucose level increased at 1.0hr and decreased to base line after 2.0hr of glucose treatment. In group II diabetic rats, the glucose level kept on increasing till 1.0hr of the glucose treatment with slight decrease at 2.0hr. The Group III rats which are treated with *Trigonella foenum graecum* leaves extract have shown significant decrease in the glucose level, less than the fasting blood glucose level after 2.0hr of the glucose treatment. The group IV and group V diabetic rats which are treated with *Hibiscus cannabinus Linn.* and *Cicer arietinium* extracts respectively did not show any positive response even after 2.0hr of the glucose supplementation (Figure 1).

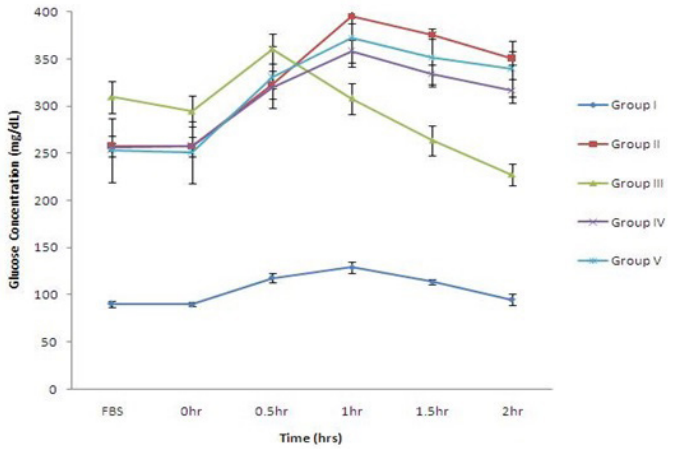

Figure 1: Effect of acute exposure of *T. graecum, H. cannabinus Linn* and *C. arietinum Linn*. leaves extract on blood glucose level in diabetic rats.

Sub chronic effects (13 days) of leaves extracts and glucose level

The concentration of glucose in Group I normal control rats remained constant till the 13th day. Group II diabetic rats showed progressive rise of blood glucose concentration till 13th day. Group III diabetic rats which are treated with *Trigonella foenum graecum* extract showed a significant decrease in blood glucose level. Similarly, Group IV diabetic rats which are treated with *Hibiscus cannabinus Linn.* extract also showed a significant decrease in blood glucose level. Whereas, Group V rats which are treated with *Cicer arietinum* leaves extract did not show any positive response (Figure 2).

DISCUSSION

It can be depicted from the observations of OGTT results (Figure. 1), that aqueous extract of *Trigonella foenum graecum* leaves reduced greater glucose concentration, where as *Hibiscus cannabinus Linn.* and *Cicer arietinum* did not show any positive response. The results of sub chronic exposure of plant extracts were quite interesting. *Trigonella foenum graecum* leaves extract showed a significant decrease in the blood glucose level after 13 days treatment. Interestingly, *Hibiscus cannabinus Linn.* which did not show any positive response during OGTT, showed a significant decrease in the blood glucose level on sub chronic exposure indicating anti diabetic potential on prolonged treatment. However, the percentage reduction in the blood glucose level is less than that treated with *Trigonella foenum graecum* extract (Figure 3). It was reported that the chemical components such as flavonoids, saponins and triterpenes are beneficial to control diabetes.[8] The preliminary Pytochemical analysis of *Trigonella foenum graecum* and *Hibiscus cannabinus Linn* leaves confirms the presence of these components. The flavanoids play a major role in maintaining blood glucose levels, glucose uptake, insulin secretion and modulating immune function to prevent specific diabetes mellitus.[9]

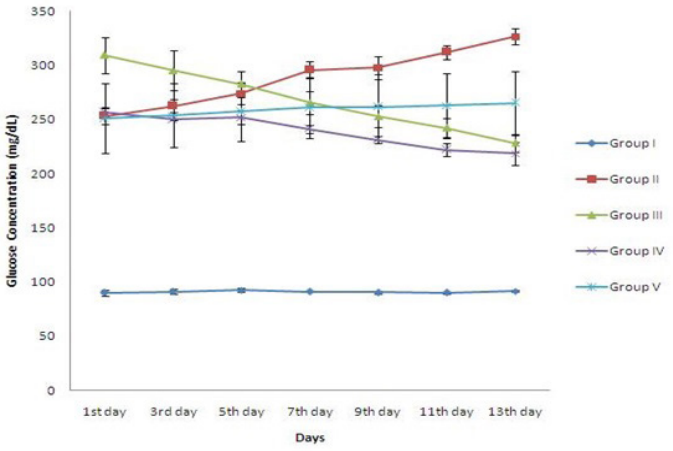

Figure 2: Effect of sub chronic exposure of *T. graecum*, *H. cannabinus Linn* and *C. arietinum Linn*. leaves extract on blood glucose level in diabetic rats.

Table 1: The experimental rats were divided into following groups

Group I	Normal control
Group II	Diabetic control
Group III	Diabetic rats are treated with Trigonella foenum graecum extract (12.5mg/100g, orally)
Group IV	Diabetic rats are treated with Hibiscus cannabinus Linn extract (12.5mg/100g, orally)
Group V	Diabetic rats are treated with Cicer arietinum extract (12.5mg/100g, orally)

Table 2: Phytochemical analysis of various plant extracts

Sl.No.	Phytochemical Constituents	Name of the test	Trigonella foenum graecum	Hibiscus. Cannabinus Linn.	Cicer arietinum
1.	Alkaloids	Mayer's test,	-	-	-
		Dragondorff's test,	-	-	-
		Wager's test	-	-	-
2.	Carbohydrates	Molish's test	-	-	-
		Fehling's test	-	-	-
		Benedict's test	-	-	-
3.	Flavonoids	Lead acetate test	+	+	+
		Mineral acid test	+	+	+
4.	Saponins	Libarmann Burchard test	+	+	+
5.	Tannins	Gelatin test	-	+	-
		Ferric chloride test	-	+	-
6.	Triterpenes	Libermann storch Morawski test	+	+	+
		Salkowski's test	+	+	+
7.	Steroids	Libermann Burchard sterol reaction	+	-	-
8.	Caretenoids	Antimony trichloride	-	-	-
		Conc. HCl with phenol	-	-	-

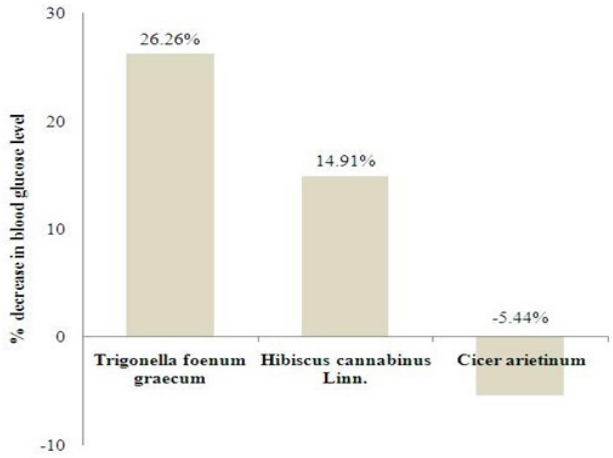

Figure 3: % decrease in blood glucose level on sub chronic exposure of plant extracts in male diabetic rats.

The consumption of flavanoids or flavanoid rich foods reduces the risk of diabetes mellitus. On the other hand, Saponins have the ability to reduce increased plasma blood glucose, hence, making it useful in treating diabetes mellitus. The hypoglycaemic effect of saponin is through restoration of insulin response, improvement in insulin signalling, increase plasma insulin and induction of insulin release from the pancreas.[10] Similarly, the presence of Triterpenes inhibit enzymes involved in glucose metabolism and prevent the development of insulin resistance thus normalising insulin levels.[11] However, the preliminary phytochemical analysis of *Cicer arietinum* leaves confirms the presence of flavonoids, saponins and triterpenes but it did not show any antidiabetic activities, probably because the bio active component responsible for hypoglycaemic activities might not be present in the leaves extract.

CONCLUSION

The acute and sub chronic exposure results obtained from this study reveal that *Trigonella foenum graecum* leaves has significant anti-diabetic potential. The *Trigonella foenum graecum* leaves may be used as a substitute for synthetic drugs to treat diabetic patients. On prolonged treatment with *Hibiscus cannabinus Linn*. leaves extract showed reduction in the blood glucose level, it can also be used as a anti-diabetic drug with lesser potential. Whereas, the *Cicer arietinum* leaves extract, which did not show any positive results, cannot be used as an anti-diabetic drug.

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CONFLICT OF INTEREST

There is no conflict of Interest.

ABBREVIATION USED

T. graecum: Trigonella foenum graecum; **H. cannabinus:** Hibiscus cannabinus Linn.; **C. arietinum:** Cicer arietinum Linn.

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Anti-diabetic effects of aqueous prickly lettuce (Lactuca scariola Linn.) leaves extract in alloxan- induced male diabetic rats treated with nickel (II)

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Anti-diabetic effects of aqueous prickly lettuce (*Lactuca scariola* Linn.) leaves extract in alloxan-induced male diabetic rats treated with nickel (II)

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Abstract

Background: Hattaraki pallye or prickly lettuce (*Lactuca scariola* Linn.) is one among several green leafy plants that grow in north Karnataka; it is usually consumed by the people of this region and is found to be antidiabetic in nature. The objective of this study is to evaluate hypoglycemic activities of supplementation with aqueous extract of prickly lettuce (*L. scariola*) leaves in vivo in acute and subchronic exposure with or without nickel (II) along with its glucose reduction capabilities with or without nickel (II) at pH 7.0 and 9.0 in vitro.
Methods: Percentage glucose reduction (in vitro) was determined by glucose oxidase-peroxidase enzymatic method at pH 7.0 and pH 9.0 using UV-Vis spectrophotometer. Hypoglycemic activities of *L. scariola* were carried out in alloxan-induced male diabetic rats at both acute and subchronic exposure.
Results: The results showed a significant alteration in the λ_{max} value of Ni (II) in combination with *L. scariola* leaves extracts at both pH 7.0 and 9.0. The aqueous extract also produced a significant reduction in the glucose concentration at pH 7.0 and pH 9.0 even in presence of Ni (II) in vitro. *Lactuca scariola* leaves in either acute or subchronic supplementation showed a greater glucose tolerance and hypoglycemic regulation of blood sugar in diabetic rats with or without nickel (II) treatments.
Conclusions: *Lactuca scariola* leaves can be a substitute for synthetic drugs to treat diabetic patients.

Keywords: hypoglycemia; *Lactuca scariola* Linn. extract; nickel (II); pH alteration.

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Introduction

Hattaraki pallye or prickly lettuce (*Lactuca scariola* Linn.) is among several green leafy plants that grow in north Karnataka and is consumed by the people of this region; it is found to be antidiabetic in nature. It is wildly grown in cultivated fields as a weed and is considered to have lots of medicinal values; notably, it exhibits analgesic, sedative, and diuretic properties and is also used to combat insomnia [1]. As a result, it is the most popular of all salad vegetables [2]. Heavy metals like nickel are considered one of the important industrial hazards which are found to induce diabetes mellitus among the people who are exposed to it [3]. It is found to alter insulin response. Most of the synthetic drugs which are routinely used to control diabetes seem to have many side effects; therefore, there is a need to replace these with various plant-based alternatives. Many herbal medicines that control diabetes are listed in the Ayurvedic literature [4]. As the digestive tract consists of different pH environments (acidic in stomach, alkaline in intestine and pH 7.4 in blood), the present work was undertaken to study the glucose-reduction capabilities of *L. scariola* alone and in combination with nickel (II) at two different pH conditions in vitro; also, hypoglycemic activities of *L. scariola* in alloxan-induced diabetic rats exposed to nickel (II) in vivo are carried out.

Materials and methods

Collection and identification of plant

The fresh prickly lettuce (*L. scariola*) leaves were collected locally from the market and were authenticated [5] by Dr. M.B. Mulimani, Associate Professor, and Dr. Paramanna D, Associate Professor, Department of Botany, B.L.D.E.A's S.B. Arts and K.C.P. Science College, Bijapur, Karnataka, India.

Preparation of plant extract

Fresh prickly lettuce (*L. scariola*) leaves (5 g) were washed thoroughly with distilled water, crushed and extracted with 100 mL distilled water at temperature 50–60 °C repeatedly, for 1 h. The resulting extract was filtered using Whatmann filter paper no. 1. The filtrate was used as an aqueous plant extract and orally administered to the animals by gastric intubation using a gavage during the experimental period.

Phytochemical analysis

Freshly prepared *L. scariola* extract was evaluated to find out the phytochemical constituents using standard procedures (Table 1) [6].

Spectral analysis

The chemical behavior of nickel (II) alone and in combination with *L. scariola* extracts at pH 7.0 and pH 9.0 (in vitro) were recorded using double-beam UV-visible spectrophotometer (Elico SL 210; Elico Pvt, Ltd., Bangalore, Karnataka, India).

In vitro percentage glucose reduction at different pH

The percentage glucose reduction (in vitro) was determined by glucose oxidase-peroxidase enzymatic method [7, 8] at pH 7.0 and pH 9.0. Briefly, 0.2 mL standard glucose solution (20 mg/dL) was added to 0.8 mL of triple-distilled water in a test tube followed by adding 0.5 mL of *L. scariola* extracts and 1 mL of glucose oxidase reagent to the test tube and incubated at 35 °C for about 40 min. The reaction was terminated by adding 2 mL of 6 N HCl. Results were spectrophotometrically recorded at 540 nm. Percentage glucose reduction was measured using the following equation:

$$\text{Concentration of glucose} = \frac{\text{Absorbance of glucose} + \text{Extract}}{\text{Absorbance of glucose}} \times \text{Concentration of glucose}$$

A similar procedure was followed to determine the percentage glucose reduction in combinations of nickel (II) and *L. scariola* extracts. Each of the experiments was repeated 10 times.

Animals

Adult male albino rats of Wistar strain (*Rattus norvegicus*) weighing 180–200 g were housed in central animal house of BLDE University's Shri. B.M. Patil Medical College, Hospital and Research Centre, Bijapur, with 12 h light and 12 h dark cycles. The rats were divided into nine groups with each group having six rats. Three rats were kept in each metabolic wire cage (60 cm×30 cm×20 cm). Standard pellets obtained from Hindustan Lever rat feed (Mumbai, India) were used as a basal diet during the experimental period. The control and experimental rats were provided food and drinking water ad libitum.

Chemicals

Following is the list of chemicals used: alloxan monohydrate (Spectrochem Pvt. Ltd., Mumbai, Maharashtra, India), glipizide (Sun Pharma Ltd., Mumbai, Maharashtra, India), Tween 80 (S.D. Fine-Chem Limited, Mumbai, Maharashtra, India), Accu-chek® Active Glucometer (Roche Diagnostics, Mannheim, Germany), blood gluco-strips (Roche Diagnostics, Mannheim, Germany), horseradish peroxidase (Merck Specialties Pvt. Ltd., Mumbai, Maharashtra, India), and glucose oxidase (Sigma Life Sciences 49180, St. Louis, USA). All other chemicals and reagents used were of analytical grade.

Acute oral toxicity studies of extract

The acute oral toxicity studies of extracts were carried out as per the Organization for Economic Cooperation and Development guidelines, draft guidelines 423 adopted on 17 December 2001 received from CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Social Justice and Empowerment, Government of India [9]. Administration of the stepwise doses of aqueous extracts of prickly lettuce (*L. scariola*) leaves from 5 mg/100 g body weight up to a dose of 12.5 mg/100 g body weight caused no considerable signs of toxicity in the tested animals. One tenth of the upper limit dose was selected as the level for examination of antidiabetic activity.

Experimental groups

The experimental rats were grouped as shown in Table 1 [10, 11].

Table 1: Experimental groups.

Groups	Supplementation	Dosage
Group I	Normal control	
Group II	Non diabetic rats+*L. scariola* extract	(12.5 mg/100 g, orally)
Group III	Non diabetic rats+nickel (II)	(2.0 mg/100 g body weight i.p.)
Group IV	Non diabetic rats+nickel (II)+*L. scariola* extract	(2.0 mg/100 g body weight; i.p.) and (12.5 mg/100 g, orally)
Group V	Diabetic control	
Group VI	Diabetic rats+*L. scariola* extract	(12.5 mg/100 g, orally)
Group VII	Diabetic rats+nickel (II)	(2.0 mg/100 g body weight; i.p.)
Group VIII	Diabetic rats+nickel (II)+*L. scariola* extract	(2.0 mg/100 g body weight; i.p.) and (12.5 mg/100 g, orally)
Group IX	Diabetic rats+antidiabetic drug, Glipizide (reference drug)	2.5 mg/kg

Induction of diabetes

The rats were made to fast overnight, and diabetes was induced with a single intraperitoneal injection (i.p.) of 15% freshly prepared solution of alloxan monohydrate (15 mg/100 g body weight) in sterile normal saline just before use. Since alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution (i.p.) after 6 h. The rats were then kept for the next 24 h on 5% glucose solution bottles in their cages to prevent hypoglycemia [12]. After 3 days blood samples were collected from the tail vein. Serum was separated, and fasting glucose levels were estimated by glucose oxidase-peroxidase enzymatic method [13] to confirm diabetes. Only those rats which showed hyperglycemia (glucose level >250 mg/dL) were selected for the study.

Oral glucose tolerance test

Fasting blood sugar level (FBS) was measured in the rats of all groups. Thirty minutes after administration of *L. scariola* leaves extract, or nickel (II), or both, or glipizide treatment, the rats of all nine groups (non-diabetic: groups I to IV and diabetic: groups V to IX) were orally treated with glucose at 0.35 mg/100 g body weight [14]. Blood samples were collected from the rat tail vein just prior to glucose administration (0.0 h) and at 0.5 h, 1.0 h, 1.5 h and 2.0 h after glucose loading. Blood glucose levels were measured immediately by using a glucometer.

The *L. scariola* leaves extract and glucose regulation on alloxan-induced diabetic rats for 2 weeks

After the day of oral glucose tolerance test (OGTT), administration of extracts (12.5 mg/100 g body weight, orally) was continued every day in designed experimental groups (group II, group IV, group VI and group VIII) till 13 days. Similarly, nickel (II) (2.0 mg/100 g body weight; i.p. on day 1, day 3, day 5, day 7, day 9, day 11 and day 13) was administered in group III, group IV, group VII and group VIII rats. Blood sample was collected from the rat tail vein after 30 min of either ingestion of plant extracts or nickel (II) treatment or both and glipizide treated rats on the 1st, 3rd, 5th, 7th, 9th, 11th and 13th days in the rats of all groups. Blood glucose levels were measured by using a glucometer.

Statistical analysis

The statistical data obtained from all the control and experimental samples were analyzed to evaluate the range of significance. Results expressed as mean±SD values were calculated for each group. To determine the significance of inter-group differences, one-way analysis of variance followed by post hoc t test was done.

Ethics

All the animal experiments were conducted according to the ethical norms approved by CPCSEA, Ministry of Social Justice and Empowerment, Government of India, and ethical clearance was granted by the Institutional Animal Ethical Committee, BLDE University, Bijapur.

Results

Phytochemical analysis

The preliminary phytochemical analysis of aqueous extract of prickly lettuce leaves (*L. scariola* extract) revealed the presence of flavonoids, saponins and steroids (Table 2).

Spectral analysis

The results revealed that the λ_{max} value of nickel (II) at pH 7.0 and pH 9.0 were 394.0 nm and 396.0 nm, respectively. The combination of nickel (II) with aqueous extract of *L. scariola* showed a hypsochromic shift to 330.0 nm and 306.0 nm at pH 7.0 and pH 9.0, respectively (Table 3).

In vitro percentage glucose reduction at different pH

It was noticed that the aqueous extract of *L. scariola* significantly decreased percentage glucose level at pH 7.0 and pH 9.0 with or without combination of nickel (II) in vitro as tabulated in Table 4.

Table 2: Phytochemical analysis of *L. scariola* extracts.

Sl. no.	Phytochemical constituents	Name of the test	Aqueous extract
1.	Alkaloids	Mayer's test	–
		Dragondorff's test	–
		Wager's test	–
2.	Carbohydrates	Molish's test	–
		Fehling's test	–
		Benedict's test	–
3.	Flavonoids	Lead acetate test	+
		Mineral acid test	+
4.	Saponins	Libarmann Burchard test	+
5.	Tannins	Gelatin test	–
		Ferric chloride	–
6.	Triterpenes	Libermann storch Morawski test	–
		Salkowski's test	–
7.	Steroids	Libermann Burchard sterol reaction	+
8.	Caretenoids	Antimony trichloride	–
		Conc. HCl with phenol	–

Table 3: λ_{max} and absorbance values of nickel (II) alone and in combination with *L. scariola* leaves extract at different pH.

Solution samples	Concentration of samples	pH 7.0		pH 9.0	
		λ_{max}, nm	Absorbance	λ_{max}, nm	Absorbance
Nickel (II)	6 mg/mL	394.0[a]	0.1329	396.0	0.1274
Nickel (II)+extract	6 mg/mL+0.5 mL of extract	330.0	4.3087	306.0	4.0569

Extract: *Lactuca scariola* leaves extract; [a]Data shown as λ_{max} and absorbance values by nickel (II) alone and in combination with extract by using UV-VIS Spectrophotometer at pH 7.0 and 9.0.

Table 4: In vitro hypoglycemic effect of *L. scariola* leaves extract alone and in combination with nickel (II) at different pH.

S. no.	Solution samples	pH 7.0	pH 9.0
1.	Glucose+extract	68.6%[a]	97.6%
2.	Glucose+extract+nickel (II)	47.3%	69.75%

Extract: *Lactuca scariola* leaves extract; [a]Data are shown as percent reduction of glucose concentrations in vitro by extract alone and in combination with nickel (II) using glucose oxidase-peroxidase method at pH 7.0 and 9.0.

Acute oral toxicity studies

In acute toxicity study, aqueous extracts of *L. scariola* leaves showed non-significant toxicity sign when observed for the parameters during the first 4 h and followed by daily observations for 14 days, and mortality was also not observed; the drug was found to be safe at the tested dose level of 12.5 mg/100 g body weight. One tenth of this dose level was taken as effective dose. All the extracts were tested at the same dose of 12.5 mg/100 g body weight. In order to ascertain a scientific base for the usefulness of this plant in the treatment of diabetes, it was decided to evaluate the experimental design for antidiabetic activity by following glucose tolerance test and in vitro glucose reduction.

Oral glucose tolerance test

The effect of *L. scariola* extracts on glucose tolerance test in non-diabetic and diabetic rats are shown in Tables 5 and 6, respectively. At 1.0 h after glucose administration in normal rats (group I), the peak of blood glucose level increased rapidly from the fasting value. Subsequently, the values began to decline during the next 60 min to reach its near-baseline value after 2 s of glucose administration. The *L. scariola* extract-treated non-diabetic rats (group II) showed similar results as the normal rats (group I). The blood glucose levels of extract-treated non-diabetic rats insignificantly decreased compared to normal rats at all time intervals after glucose administration. Interestingly, nickel (II)-treated group III rats showed a diabetic-like OGTT result with its peak glucose concentration at 1 h, and even at 2 h glucose concentration did not return to baseline. In the case of group IV rats (nickel (II)+extract), blood glucose concentration showed a remarkable improvement

Table 5: Effect of acute exposure to nickel (II) (2.0 mg/100 g body weight) and simultaneous supplementation with *L. scariola* leaves extract (12.5 mg/100 g body weight) on blood glucose level in normal rats.

		Non-diabetic model				
		Period after glucose ingestion, h				
Treatment groups	FBS	0.0	0.5	1.0	1.5	2.0
I	90.00±3.22^{a,1}	89.50±2.42^{a,1}	117.33±5.00^{b,1}	128.66±6.28^{c,1}	113.33±2.87^{b,1}	94.5±6.47^{a,1}
II	90.83±2.56^{a,1}	89.5±3.67^{a,1}	101.33±6.97^{b,2}	116.16±8.49^{c,2}	100.83±5.87^{b,2}	90.66±2.50^{a,1}
III	88.66±3.66^{a,1}	101.16±5.30^{b,1}	177.50±23.18^{c,3}	185.00±23.66^{c,3}	152.50±21.15^{d,3}	121.66±15.70^{e,2}
IV	89.66±4.76^{a,1}	95.33±4.08^{a,2}	113.83±3.97^{b,1}	138.83±4.95^{c,4}	125.16±6.79^{d,4}	116.66±7.44^{b,2}

Treatment groups: I, normal control; II, *Lactuca scariola* leaves extract; III, nickel (II); IV, nickel (II)+*L. scariola* leaves extract. Horizontal values are the mean±SD of six observations in each group till at 2 h. In each row, values with different superscript letters (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical columns indicate variation of blood glucose levels among four different groups at different time interval till 2 h. In each column, values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

Table 6: Effect of acute exposure to nickel (II) (2.0 mg/100 g body weight) and simultaneous supplementation with *L. scariola* leaves extract (12.5 mg/100 g body weight) on blood glucose level in diabetic rats.

				Diabetic model		
				Period after glucose ingestion, h		
Treatment groups	FBS	0.0	0.5	1.0	1.5	2.0
V	257.66±10.59^{a,1}	257.5±10.36^{a,1}	322.83±14.64^{b,1}	395.33±7.39^{c,1}	375.66±3.82^{d,1}	350.83±7.35^{e,1}
VI	255.33±8.16^{a,1}	234.50±88.91^{b,2}	286.83±8.63^{c,2}	350.66±12.37^{d,2}	322.5±11.36^{e,2}	268.50±10.74^{f,2}
VII	252.66±10.19^{a,1}	278.50±12.62^{b,3}	381.00±12.48^{c,3}	402.00±12.40^{d,1}	415.66±14.05^{d,3}	405.00±14.17^{d,3}
VIII	256.00±8.48^{a,1}	263.00±99.6^{a,1}	324.00±7.12^{b,1}	382.66±10.98^{c,3}	381.33±9.72^{c,1}	368.00±6.81^{d,4}
IX	251.83±5.74^{a,1}	236.33±11.91^{b,2}	275.00±5.79^{c,2}	296.50±4.37^{d,4}	216.66±10.63^{e,4}	154.16±20.35^{f,5}

Treatment groups: V, diabetic control; VI, diabetic+*L. scariola* leaves extract; VII, diabetic+nickel (II); VIII, diabetic+nickel (II)+*L. scariola* leaves extract; IX, diabetic+glipizide (0.25 mg/100 g). Horizontal values are the mean±SD of six observations in each group till at 2 h. In each row, values with different superscript letters (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group till 2 h. Vertical columns indicate variation of blood glucose levels among five different groups at different time interval till 2 h. In each column, values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different times.

in OGTT (Table 5). In the case of group V (diabetic control) rats, the peak glucose concentration in OGTT showed at 1 h, and even at 2 h it showed typical diabetic response, whereas in group VI (diabetic+*L. scariola* extract), rats showed declined blood glucose levels at all the time intervals and almost reached its baseline at 2 h. In the case of group VII (diabetic+nickel (II)), a greater diabetic OGTT response as compared to diabetic alone was noticed at all the given times, whereas in group VIII (diabetic+nickel (II)+*L. scariola* extract), rats showed improved diabetic response by reducing glucose concentration in OGTT at all the given times as compared to group VII. Further, we found that glipizide (as a reference drug) produced a fall of blood glucose levels at all the time intervals after glucose administration in group IX (diabetic+glipizide) rats (Table 6). The results of each group were significantly different at different time intervals from each other ($p<0.05$).

Subchronic effects (13 days) of *L. scariola* leaves extract and glucose regulation on alloxan-induced diabetic rats

Group III (non diabetic+nickel (II)) rats showed clear hyperglycemia as compared to the non-diabetic control rats (group I). The *L. scariola* extract significantly lowered elevated blood glucose levels in group IV (nickel (II)+*L. scariola* extract) as compared to only nickel (II)-treated non-diabetic (group III) rats (Table 7). It was also noticed that there was a progressive hypoglycemic effect of extracts in group IV rats till the 13th day. Diabetic nickel (II)-treated group VII rats showed progressive rise of blood glucose concentration till the 13th day, but in group VIII (diabetic+nickel (II)+*L. scariola* extract), remarkable progressive reduction in glucose concentrations was noticed. Group VIII rats also showed a reduction of blood glucose

Table 7: Effect of subchronic treatment with nickel (II) (2.0 mg/100 g body weight) and simultaneous supplementation with *L. scariola* leaves extract (12.5 mg/100 g body weight) on blood glucose level in normal rats.

				Non-diabetic model			
				Blood glucose levels, mg/dL			
Treatment groups	1st day	3rd day	5th day	7th day	9th day	11th day	13th day
I	89.33±2.58^{a,1}	90.50±2.50^{a,1}	91.83±1.94^{a,1}	90.66±1.75^{a,1}	89.83±1.32^{a,1}	89.66±1.03^{a,1}	91.00±0.89^{a,1}
II	90.83±2.48^{a,1}	89.33±2.33^{a,1}	86.66±1.86^{a,1}	87.16±3.06^{a,1}	90.00±2.44^{a,1}	89.16±2.63^{a,1}	87.66±2.73^{a,1}
III	88.66±3.66^{a,1}	112.5±8.80^{b,2}	134.16±7.22^{c,2}	137.50±7.03^{c,2}	138.50±6.09^{c,2}	140.66±5.78^{d,2}	142.16±5.34^{d,2}
IV	89.83±4.16^{a,1}	106.50±6.50^{b,2}	116.83±2.99^{c,3}	120.66±3.76^{c,3}	122.16±3.76^{c,3}	120.00±4.42^{c,3}	116.83±2.71^{c,3}

Treatment groups: I, normal control; II, *Lactuca scariola* leaves extract; III, nickel (II); IV, nickel (II)+*L. scariola* leaves extract. Horizontal values are the mean±SD of six observations in each group in every alternate day till 13th day. In each row, values with different superscript letters (a, b, c or d) are significantly different from each other ($p<0.05$) in the same group at different days. Vertical columns indicate variation of blood glucose levels among four different groups in each alternate day till 13th day. In each column, values with different superscript letters (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

concentration on the 13th day which is actually below the baseline value from the 1st day (Table 8). The reference drug glipizide lowered the blood glucose level in diabetic rats (group IX) significantly, bringing it nearly back to the normal. The values are significantly different from each other ($p<0.05$) in the same group at different days.

Discussion

Our results reflect alteration of chemical behavior of nickel (II) in combination with aqueous *L. scariola* extracts at both pH 7.0 and pH 9.0 in vitro, which indicates that the aqueous *L. scariola* extract influences the change of chemical behavior of nickel (II) irrespective of the pH of the environment, possibly by molecular interaction between nickel (II) and the antioxidant constituent of phytochemicals [15]. It can be seen from the observations of Table 3 that aqueous *L. scariola* extracts reduced greater glucose concentration in alkaline pH 9.0 as compared to neutral pH 7.0 in vitro. Similarly, it was noticed that in the presence of nickel (II) supplementation in vitro, percentage reduction of glucose concentration is also greater at pH 9.0 as compared to pH 7.0. Hence, it can be postulated from our study that pH of the environment influences glucose reduction capabilities of aqueous *L. scariola* extracts with or without the presence of heavy metals like nickel (II). The results of OGTT and chronic blood glucose levels clearly indicate that *L. scariola* extract showed hypoglycemic activity. Blood glucose level is commonly measured to check glycemic control mechanism. In the present study, *L. scariola* extract has shown significant reduction in the blood glucose level. The results were significant with ($p<0.05$) with different groups at different time intervals and different days. This could be due to increase in the pancreatic secretion of insulin by the β cells of islets of Langerhans. It is well known that flavonoids and saponins are bioactive antidiabetic components. The phytochemical screening of aqueous *L. scariola* extracts revealed the presence of these bioactive components. Flavonoids act on various molecular targets and regulate different signaling pathways in pancreatic β cells [16]. Perhaps saponin influences structural alterations of glucose molecule through glycone and glucuronic acid [17]. Hence, it can be predicted that the hypoglycemic activities of *L. scariola* extracts may be due to the effect of these bioactive components. Nickel (II) induces hyperglycemia in both acute and subchronic exposure conditions, which could be due to an increased pancreatic release of glucagons [18, 19]. Such alterations were found to lead to a drastic drop in the insulin/glucagon plasma ratio [20]. Another explanation for the nickel-induced rise of blood glucose level in male rats could be an involvement of nitric oxide-mediated pathways [21]. Nickel causes an increase in the level of cyclic guanosine monophosphate and constitutive nitric oxide synthase in the adrenals and brain or inducible nitric oxide synthase in the pancreas by modulating the release of insulin from pancreas, finally leading to hyperglycemic condition. In our study, we found a significant improvement in blood glucose levels in diabetic rats treated with both nickel (II) and *L. scariola* when compared to only nickel-treated diabetic rats in acute (OGTT) or subchronic conditions. This finding may be due to an improvement of nickel-induced

Table 8: Effect of subchronic treatment with nickel (II) (2.0 mg/100 g body weight) and simultaneous supplementation with *L. scariola* leaves extract (12.5 mg/100 g body weight) on blood glucose level in diabetic rats.

							Diabetic model
							Blood glucose levels, mg/dL
Treatment groups	**1st day**	**3rd day**	**5th day**	**7th day**	**9th day**	**11th day**	**13th day**
V	$253.16\pm7.22^{a,1}$	$262.66\pm5.57^{a,1}$	$274.00\pm9.87^{b,1}$	$295.66\pm8.04^{c,1}$	$298.00\pm10.65^{c,1}$	$312.33\pm6.37^{d,1}$	$326.66\pm7.25^{d,1}$
VI	$252.50\pm10.32^{a,1}$	$235.16\pm6.33^{b,2}$	$219.83\pm4.53^{c,2}$	$196.00\pm4.89^{d,2}$	$178.33\pm8.16^{e,2}$	$157.50\pm8.75^{f,2}$	$141.33\pm9.45^{g,2}$
VII	$251.33\pm7.55^{a,1}$	$266.33\pm6.53^{b,1}$	$272.66\pm6.12^{b,1}$	$293.00\pm4.14^{c,1}$	$296.33\pm3.88^{c,1}$	$311.83\pm7.49^{c,1}$	$326.83\pm2.48^{d,1}$
VIII	$256.00\pm8.48^{a,1}$	$260.33\pm9.09^{a,1}$	$248.16\pm5.67^{b,3}$	$238.00\pm6.75^{b,3}$	$227.50\pm7.55^{c,3}$	$215.66\pm6.77^{d,3}$	$169.00\pm7.94^{e,3}$
IX	$254.56\pm9.45^{a,1}$	$250.60\pm10.37^{a,3}$	$175.28\pm9.87^{b,4}$	$142.49\pm10.05^{c,4}$	$125.28\pm9.11^{d,4}$	$109.43\pm10.28^{e,4}$	$94.24\pm9.26^{f,4}$

Treatment groups: V, diabetic control; VI, diabetic+*L. scariola* leaves extract; VII, diabetic+nickel (II); VIII, diabetic+nickel (II)+*L. scariola* leaves extract; IX, diabetic+glipizide (0.25 mg/100 g). Horizontal values are the mean±SD of six observations in each group in every alternate day till 13th day. In each row, values with different superscript letters (a, b, c, d, e, f or g) are significantly different from each other ($p<0.05$) in the same group at different days. Vertical columns indicate variation of blood glucose levels among five different groups in each alternate day till 13th day. In each column, values with different superscript numbers (1, 2, 3 or 4) are significantly different from each other ($p<0.05$) at different days.

oxidative stress and insulin resistance by *L. scariola*. Our study revealed a relationship between *L. scariola* and glucose metabolism, as there was increase in insulin sensitivity in diabetic rats treated with *L. scariola* in both acute and subchronic conditions. Possibilities of decreased glucose uptake by gut due to *L. scariola* extract supplementation might not be ruled out. In our study, diabetic rats treated with nickel (II) did not show additional hyperglycemic response as compared to diabetic rats alone. On the other hand, supplementation with *L. scariola* to nickel (II)-treated diabetic rats showed decreased blood glucose level in both OGTT and subchronic exposures. It may be possible that the decrease of nickel-induced oxidative stress and insulin resistance by *L. scariola* with concomitant increase of insulin sensitivity are the causes of this.

Conclusions

In vitro and in vivo results obtained from this study reveal that *L. scariola* leaves have significant hypoglycemic and antidiabetic potential. The *L. scariola* leaves may be used as a substitute for synthetic drugs to treat diabetic patients. The present study has also opened avenues for further research, especially with reference to the different dose studies and development of potent formulation for diabetes mellitus from *L. scariola* leaves.

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Fenugreek (*Trigonella foenum graecum*) leaves extract and its interaction with heavy metal (Nickel II) with reference to glucose reduction capabilities *in-vitro.*

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Abstract

Introduction: Heavy metals like nickel may be considered as one of the important industrial hazard which is found to induce diabetes mellitus among people who are exposed to it. It is found to alter the insulin response.

Objectives: To evaluate the glucose reduction capabilities of aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves and its interaction with nickel at pH 7.0 and 9.0 *in-vitro.*

Materials and Methods: Spectral analysis of Ni (II) alone and in presence of fenugreek extract was recorded at pH 7.0 and pH 9.0 using UV-Vis spectrophotometer at room temperature of 25^0 C. Glucose reduction capabilities of aqueous extract of fenugreek leaves alone and in supplementation with nickel sulphate were also evaluated at pH 7.0 and 9.0 by glucose oxidase method.

Results: The λmax value of Ni showed hypsochromic shift from 393.5 and 396.5 to 323.5 and 323.0 at pH 7.0 and pH 9.0 respectively in the presence of aqueous fenugreek extract. The aqueous extract of fenugreek leaves has produced significant reduction in the glucose concentration at pH 7.0 and pH 9.0 even in presence of heavy metal like Ni (II) *in-vitro.*

Conclusion: The aqueous extract of fenugreek leaves is capable to change the chemical behaviour of heavy metals like Ni. The study also revealed a strong glucose reducing properties of fenugreek with or without nickel supplementation *in vitro*. Interestingly such glucose reducing characteristics were found to be higher in alkaline medium (pH 9.0).

Keywords: Nickel, fenugreek (*Trigonella foenum graecum*) extract, pH alterations, glucose reduction.

Introduction

Nickel is a trace metal that is necessary for the survival of bacteria, plants and animals. It is a hard, bright, silvery-white metal that is present in soil, water, cocoa and chocolate, nuts, dried beans, peas, soya beans, spinach, lettuce, oatmeal, grains, fruits and other vegetables, leguminous seeds, egg, and milk (1). Heavy metals like nickel may be considered as one of the important industrial hazard which is found to induce diabetes mellitus among people who are exposed to it (2). It is found to alter the insulin response. As most of the synthetic drugs which are routinely used to control diabetes seem to have many side effects; hence these synthetic drugs are being replaced by various plant based alternatives. Many herbal medicines that control diabetes are enlisted in the Ayurvedic literature (3). There are several leafy plants which grow in north Karnataka, usually consumed by the people of this region are found to be antidiabetic in nature (4). Menthe palle or fenugreek (*Trigonella foenum graecum*) is one among these. As fenugreek (*Trigonella foenum graecum*) usually consumed orally as diet passes through various pH environment in gut, specifically alkaline pH in small intestine where absorption of nutrients, drugs, and heavy metals take place hence, the present study was undertaken to evaluate the alteration of chemical behaviour of heavy metal (Nickel II) in presence of aqueous extract of fenugreek leaves and also to evaluate the glucose reduction capabilities of aqueous extract of fenugreek leaves at pH 7.0 and 9.0 *in-vitro in presence of nickel II.*

Materials and Methods

Collection and identification of plant: The fresh fenugreek (*Trigonella foenum graecum*) leaves were collected locally from the market and authenticated by the Department of Botany, S. B. Arts and K. C. P. Science College, Bijapur, Karnataka.

Preparation of extract: 5g of fresh fenugreek (*Trigonella foenum graecum*) leaves were washed with distilled water and were soaked for few minutes in water. Soaked leaves were then added to 100ml of distilled water and heated at 50° C in a water bath for about 1 hour and filtered. Filtrate is used as a plant extract to carry out the work.

***Phytochemical analysis*:** Freshly prepared aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves was evaluated to find out the presence or absence of vact of fenugreek (*Trigone*sing standard phytochemical procedures.

Analysis of chemical behaviour of nickel (II) in-vitro: Spectral analysis of analytical grade nickel at pH 7.0 and pH 9.0 were recorded and spectral analysis of nickel in combination with aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves were also recorded using double beam UV visible spectrophotometer (Elico SL 210).

Estimation of glucose concentration in-vitro: In another experiment, the glucose concentration was determined by glucose oxidase method at pH 7.0 and pH 9.0. For this experiment 0.2 ml standard glucose solution (10.0 mg/dL) was added to 0.8ml of distilled water in a test tube. 0.5ml of aqueous fenugreek extract and 1.0ml of glucose oxidase reagent were added to the test tube and incubated at 35° C for about 40 mins. The reaction is terminated by adding 2 ml of 6N HCl. Results were spectrophotometrically recorded at 546nm [5]. Concentration of glucose was measured using the relation,

$$\text{Concentration of glucose} = \frac{\text{Absorbance of glucose+Extract}}{\text{Absarbance of glucose}} X \text{ Concentration of Glucose}$$

Similar procedure was followed to determine the concentration of glucose in presence of nickel sulphate (20 mg/L) and aqueous fenugreek (*Trigonella foenum graecum*) extract combination. Each of the experiment were repeated for 10 times. The entire work was carried out in the Department of Chemistry of BLDEA's Dr.P.G.Halakatti College of Engineering and Technology, Bijapur, Karnataka, India.

Table 1
Phytochemical analysis of aqueous extract of fenugreek (*Trigonella foenum graecum)* leaves

S.No.	Phytochemical Constituents	Name of the test	Aqueous extract
1.	Alkaloids	Mayer's test,	-
		Dragondorff's test,	-
		Wager's test	-
2.	Carbohydrates	Molish's test	-
		Fehling's test	-
		Benedict's test	-
3.	Flavonoids	Lead acetate test	+
		Mineral acid test	+
4.	Saponins	Libarmann Burchard test	+
5.	Tannins	Gelatin test	-
		Ferric chloride	-
6.	Triterpenes	Libermann storch Morawski test	+
		Salkowski's test	+
7.	Steroids	Libermann Burchard sterol reaction	+
8.	Caretenoids	Antimony trichloride	-
		Conc. HCl with phenol	-

Table 2
λ max and absorbance values of nickel sulphate and with fenugreek (*Trigonella foenum graecum*) extract at different pH.

S.No.	Chemical	Concentration	pH 7.0		pH 9.0	
			λmax (nm)	Absorbance	λmax (nm)	Absorbance
1	Nickel sulphate	6 mg/mL	393.5	0.1294	396.5	0.1331
2	Nickel sulphate +Fenugreek extract	6 mg/mL + 0.5 mL of extract	323.5	2.8252	323.0	2.9991

Table 3 % reduction in the concentration of Glucose with or without nickel in presence of fenugreek (*Trigonella foenum graecum*) extract at different pH.

Sample (n=10)	pH 7.0	pH 9.0	t value	p value
Glucose + Fenugreek extract	9.68 ± 0.76 (%)	24.20 ± 2.24(%)	18.65	0.0000
Ni + Glucose + Fenugreek extract	8.15 ± 0.12 (%)	14.28 ± 2.06 (%)	7.86	0.0000

Students t test were done between the values of pH 7.0 and 9.0.

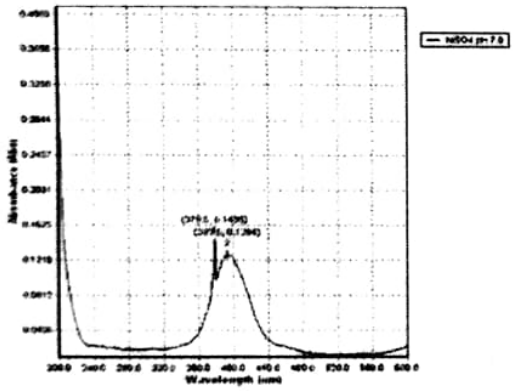

Fig. a. λ max value of $NiSO_4$ at pH 7.0

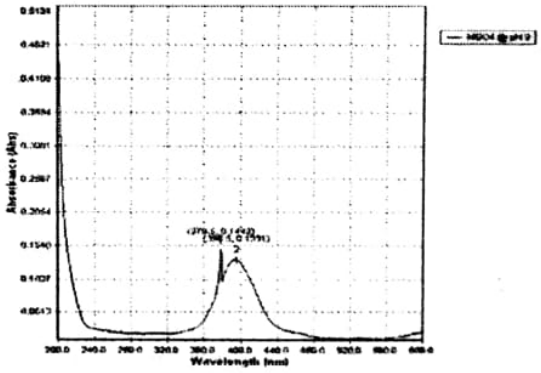

Fig. 1b. λ max value of $NiSO_4$ at pH 9.0

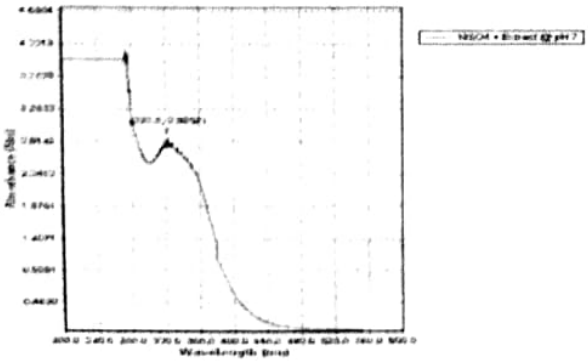

Fig. a. λ max value of $NiSO_4$ with extract at pH 7.0

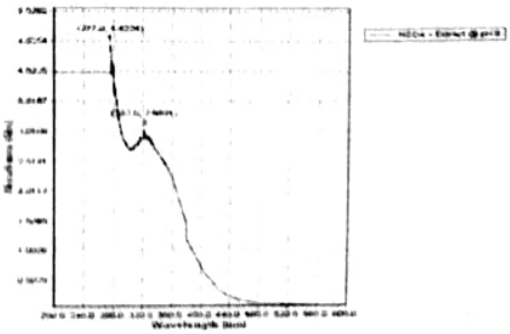

Fig. 2b. λ max value of $NiSO_4$ with extract at pH 9.0

Results

Phytochemical analysis: The preliminary phytochemical analysis of the aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves revealed the presence of saponins, flavanoids, triterpenoids and steroids (Table 1).

Alteration of chemical behaviour of Nickel (II) in presence of fenugreek extract: The results revealed that λ_{max} values of nickel at pH 7.0 and pH 9.0 are 393.5nm and 396.5nm respectively. Whereas, in the presence of aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves, the λ_{max} values of nickel made hypsochromic shift to 323.5nm and 323.0nm at pH 7.0 and pH 9.0 respectively (Fig. 1a to Fig. 2b & Table 2).

Reduction in the glucose concentration: It was also noticed that the aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves can significantly decrease glucose concentration at pH 7.0 and pH 9.0 with (p= 0.0000) or without Ni (II) (p=0.0000) *in-vitro* (Table 3).

Discussion

Plants are known to absorb nickel readily from the soil and unlike other non-essential trace elements, nickel moves through plant's circulatory system and accumulates in leaves, seeds and roots (6). However, accumulation of nickel in leaves is less than that in seeaves were then added to 100ml th various soluble components of plant and gets converted into number of Ni-containing organic complexes which are highly toxic than the inorganic metal Ni (6). Thisresults reflect alteration of chemical behaviour of nickel in presence of fenugreek (*Trigonella foenum graecum*) extract in both pH 7.0 and pH 9.0 *in-vitro,* which indicates that the fenugreek (*Trigonella foenum graecum*) extract influences change of chemical behaviour of nickel irrespective of pH of the environment possibly by molecular interaction between Ni(II) and antioxidant constituent of phytochemicals (6). It can be depicted from the observations of Table 3, that fenugreek (*Trigonella foenum graecum*) extract reduced greater glucose concentration in alkaline pH 9.0 as compared to neutral pH 7.0 (24.20% vs 9.68%) *in-vitro*. Similarly, it was noticed that in presence of nickel supplementation *in-vitro,* % reduction of glucose concentration is also greater at pH 9.0 as compared to pH 7.0 (14.28% vs 8.15%). Hence it can be postulated from our study that pH of the environment influences glucose reduction capabilities of fenugreek (*Trigonella foenum graecum*) extracts with or without presence of heavy metals like nickel. It was reported that the chemical components of fenugreek (*Trigonella foenum graecum*) leaves include flavonoids, saponins and triterpenes are beneficial to control diabetes (7). This report very much corroborates with our findings from phytochemical analysis (Table 1). Perhaps saponins in fenugreek influences structural alterations of glucose molecule through glycone and glucuronic acid (8). From the phytochemical analysis of fenugreek (*Trigonella foenum graecum*) compound we may further interpret that the capabilities to attach various sugar molecules by the triterpene unit of fenugreek (*Trigonella foenum graecum*) might be the possible cause of glucose reduction capabilities of fenugreek (*Trigonella foenum graecum*) with or without heavy metal nickel (II) (8).

Conclusion

The aqueous extract of fenugreek (*Trigonella foenum graecum*) leaves is capable to change the chemical behaviour of heavy metals like nickel. Perhaps, some antioxidant substances of fenugreek (*Trigonella foenum graecum*) interacting with nickel (II) change its chemical nature. It is also interesting that pH of the solution practically has no interference in change of chemical behaviour of nickel (II) in presence of aqueous extract of fenugreek leaves (*Trigonella foenum graecum*). The study also revealed a strong glucose reducing properties of fenugreek leaves (*Trigonella foenum graecum*) with or without nickel supplementation *in-vitro* which was interestingly found to be dependent on pH as % reduction of glucose concentration was greater at pH 9.0.

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