Phytochemical Investigation and Anti-Hyperglycemic Effect of Traditionally Used Medicinal Plants in Azad Jammu and Kashmir

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Final Approval

It is certified that we have read the thesis entitled **"Phytochemical Investigation and Anti-hyperglycemic Effect of Traditionally Used Medicinal Plants in Azad Jammu and Kashmir"** submitted by Mr. Muhammad Shafiq Khan and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Biotechnology.

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In the name of Allah,

Most gracious, most merciful.

All praise be to Allah

Lord of all the worlds,

Most of beneficent, ever merciful,

Lord of the day of judgements

You alone we worship, and to you

Alone we turn for help.

Guide us (O Allah) to the path that is straight,

The path of those you have blessed,

Not of those who have earned your anger,

Nor those who have gone astray,

Al Quran: The opening Chapter

DEDICATION

This work is dedicated

to

The loving memory of my parents

who

Cherished this dream for me

DECLARATION

It is certified that work done on this Ph. D Biotechnology research thesis is purely conducted by me. I carried out the part of experimental work described in this thesis in Biotechnology Lab, Department of Biological Sciences, International Islamic University Islamabad (IIUI) Pakistan. Major part of research on animal model was performed in animal's house lab, National Institute of Health (NIH) Islamabad. The part of research study was also performed at Seoul National University (SNU) South Korea. Partial work have been conducted at Department of Biochemistry PMAS-UAAR, Pakistan. All the material is prepared by myself and has not been copied from anywhere; however, some test and figures used have been properly referenced. The conclusions are my own research after numerous discussions with my supervisor and co-supervisor. All the assistance and help received during the course of research have been duly acknowledged.

Date _____

Muhammad Shafiq Khan

I certify that the above statement is correct.

Prof. Dr. Zafar Mahmood Khalid

Supervisor

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ABBREVIATIONS

Abbreviation	Full form
DM	Diabetes mellitus
BGL	Blood glucose level
IDDM	Insulin dependent diabetes mellitus
NIDDM	Non-insulin dependent diabetes mellitus
GD	Gestational diabetes
FPG	Fasting plasma glucose
TZDs	Thiazolidinediones
WHO	World Health Organization
AJ&K	Azad Jammu and Kashmir
HLA	Human leukocytes antigen
OGTT	Oral glucose tolerance test
DKA	diabetic ketoacidosis
RAI	Rapid acting insulin
SAI	Short acting insulin
PPAR-γ	Peroxisomes proliferator activated receptor gamma

HONK	Hyperosmolar nonketotic diabetic
SZT	Streptozotocin
RC	*Rubia cordifolia*
LC	*Lespedeza cuneata*
ME	Methanolic extract
MERH	Methanolic extract of *Rumex hastatus*
MELC	Methanolic extract of *Lespedeza cuneatea*
MERC	Methanolic extract of *Rubia cordifolia*
MENG	Methanolic extract of *Nepeta grandiflora*
MESD	Methanolic extract of *Strobilanthes dalhousieanus*)
OECD	Organization for Economic Cooperation and Development
IBBC	Institutional Bioethics and Biosafety Committee
HFRC	Hexane fraction of *R. cordifolia*
EFRC	Ethylacetate fraction of *R. cordifolia*
BFRC	n-btanol fraction of *R. cordifolia*
AFRC	Aqueous fraction of *R. cordifolia*
HFLC	Hexane fraction of *L. cuneata*
EFLC	Ethyl acetate fraction of *L. cuneata*
BFLC	n-butanol fraction of *L. cuneata*
AFLC	Aqueous fraction of *L. cuneata*
NC	Normal control

TC	Toxicant control
SD	Standard drug
$FeCl_3$	Ferric Chloride
H_2SO_4	Sulphuric acid
HCl	Hydro chloric acid
AA	Ascorbic acid
GA	Gallic Acid
BW	Body weight
NIH	National Institute of Health
HPLC	High Performance Liquid Chromatography
S.D	Standard deviation
IBBC	Institutional Bioethics and Biosafety Committee
HF	*n*-Hexane Fraction
CF	Chloroform Fraction
EF	Ethyl Acetate Fraction
BF	*n*-Butanol Fraction
AF	Aqueous Fraction
IC_{50}	Half Maximal Inhibitory Concentration
RC	*Rubia cordifolia*
LC	*Lespedeza cuneata*

TPC	Total Phenolic Contents
µg/mg GAE	Microgram Per-Milligram Gallic Acid Equivalent
AA	Ascorbic Acid
QE	Quercetin Equivalent
ANOVA	Aanalysis of Variance
TFC	Total flavonoid content
mg/dL/wk	Milligram per deciliter per week
%	Percent
<	Less than
>	Greater than
°C	Degree Centigrade
g	Gram
mg/kg	Milligram Per Kilogram
mg	Milligram
µg/mL	Microgram Per Milliliter
µg	Microgram
g	Gram
mg/kg	Milligram Per Kilogram
mg	Milligram
g/wk	Gram per week
µL	Microliter

mg/mL	Milligram Per Mililiter
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
HPLC	High-performance liquid chromatography;
QTOF	Quadrupole-Time of Flight

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Muhammad Shafiq Khan

ABSTRACT

Plants are important source of medicines. *Rumax haustatus, Lespideza cuneata, Rubia cordifolia, Nepeta grandifolia and Strobilanthes dalhousianus* are traditionally used folk medicines to treat different diseases like diabetes, stomach disorder, liver complications, depression and skin diseases. There is no record found which shows the pharmacological investigation of these plants from Poonch AJ&K Pakistan. Current research was intended for screening of these selected medicinal plants *R. haustatus, L. cuneata, R. cordifolia, N. grandifolia* and *S. dalhousianus* to evaluate safety and antihyperglycemic activity in the mice. The most active antidiabetic plants (*L.cuneata* and *R.cordifolia*) were further investigated by fractionation using the solvents (n-hexane, ethyl acetate, n-butanol and aqueous) on increasing polarity basis. Crude methanolic extracts of these plants were prepared and investigated for acute toxicity test. Extract doses (250, 500 and 1000 mg/kg) treatment in mice for 24 hours did not show any behaviour change and lethality in mice showing that LD_{50} for extract of selected plants was beyond 1000mg/kg. Methanolic extract of selected plants were tested for antihyperglycemic effect in mice. Group one (G-I) treated as normal control while G. II to G.VIII intoxicated with alloxan monohydrate (100mg/kg) for 6 days after 2 days' interval to induce diabetes. G-III was given treatment of antihyperglycemic drug (Glibenclamide 0.5 mg/kg) while animals in group four to group eight (G. IV-G. VIII) were treated with methanolic extract of *R. haustatus* (MERH), *L. cuneata* (MELC), *R. cordifolia* (MERC), *N. grandifolia* (MENG), *S. dalhousianus* (MESD) with aerial parts (leaves and stem), respectively daily for four weeks. Blood glucose level (BGL) was significantly decreased ($P < 0.05$) due to alloxan induced hyperglycemia and normal increase in body weight (BW) on treatment of SD (0.5mg/kg) and *L.cuneata* and *R. cordifolia* (100 mg/kg) respectively in group G.III (SD), G.V (MELC) and G.VI (MERC). Methanolic extract of most active antihyperglycemic plants (*L. cuneata* and *R.cordifolia*) was subjected for further investigation. Fractions of solvents (*n*-hexane, ethyl acetate, *n*-butanol and an aqueous fraction) were analysed for antihyperglycemic effect in mice against alloxan-induced diabetes and DPPH antioxidant activity. High level of BGL due to Alloxan induced diabetes significantly ($P < 0.05$) decreased as compare to TC with the treatment of *R.cordifolia* ethyl acetate fraction (EFRC), n-butanol fraction of *R. cordifolia* (BFRC), ethyl

acetate fraction of *L.cuneata* (EFLC) and n- butanol fraction of *L.cuneata* (BFLC) in time dependent manner. Similarly, BW was normally increased with treatment of EF and n-BF of both plants comparable to SD. Antioxidant activity by DPPH assay of *R.cordifolia* and *L.cuneata*. The highest antioxidant activity was revealed by EFRC followed by BFRC and MERC (IC_{50} values 34.9, 36.86 and 38.19 µg/mL) of *R. cordifolia* and by BFLC followed by EFLC and MELC (IC_{50} values 30.89, 31.42 and 36.4µg/mL) of *L. cuneata*, that is comparable to standard ascorbic acid (IC_{50} 34.41) antioxidant activity. Antihyperglycemic activity and antioxidant potential is associated with phytochemicals (phenolics, flavonoids, saponins, terpenoids, Tanninsand alkaloids) identified during qualitative analysis. The compounds identified by HPLC anlysis (Mangiferine, purpurine, 2- methyl anthraquinone Kaempferol. and charatine) attributed to the antihperglycemic effect confirming the most active antidiabetic fractions against alloxan induced diabetes in mice. In conclusion; Over all our investigated plants exhibited antihyperglycemic effect against alloxan-induced pancreatic damaged mice. *R. cordifolia* and *L. cuneate* have most active antihyperglycemic effect against alloxan induced diabetes in mice due to ethylacetate and n-butanol fractions associated to antioxidant activity of phenolic and flavonoid contents.

INTRODUCTION

CHAPTER 1

INTRODUCTION

Insulin deficiency causes the Diabetes mellitus (DM) disorder during its production or its action or both. Long term hyperglycemia due to change in metabolic processes in the body is led by DM (Mukhtar *et al.*, 2019; Bastaki, 2005). If it is not treated properly then serious complications like muscular tissue damage, retinopathy (Arden and Ramsey, 2015; Bearse *et al.*, 2004), complications as neuropathy (Seki *et al.*, 2004), nephropathy (Looker *et al.*, 2003), heart disorders (Svensson *et al.*, 2004) and problems of ulceration (Wallace *et al.*, 2002) occur. Normal range of blood glucose level (BGL) according to body needs is controlled by insulin and glucagon that are pancreatic hormones. High level of BGL stimulates β-cells of Islets of Langerhans to secrete insulin. Insulin helps in absorption of sugar in muscles and red blood cells, out of the blood for the consumption during metabolic process. Glucagon, on the other hand in response of low BGL is secreted by α- cells of the pancreas, for the release of glucose from liver and muscles (Gupta and De, 2012).

Diabetes type 1 and type 2 are two main types based on etiology of disease (Alberti and Zimmet, 1998). In insulin dependent diabetes mellitus (IDDM type 1); no insulin secretion takes place and patients often need insulin replacement therapy for life. Type 1a (90% of type 1) and type 1b (10% of type 1) are two types of type-I insulin dependent diabetes mellitus (IDDM). Addison's disease, Grave's disease and Hashimoto's thyroiditis cause the damages of pancreatic β-cells and lead to type 1a (Atkinson and Maclaren, 1994, Betterle *et al.*, 1984). However, type 1b is idiopathic whose etiology is unidentified. Insulin deficiency in such type of patients leads to ketoacidosis (Mclarty *et al.*, 1990). In non-insulin dependent diabetes mellitus (NIDDM; type 2), there is abnormality in secretion insulin and its resistance (Defronzo *et al.*, 1992). It is predominant in elderly people (over 40 years). People with obesity, decreased body activity and interestingly suffer from NIDDM (Zimmet *et al.*, 1990). The disease is decreased by factors like dietry supplements, oral hypoglycemic agents and physical activities (Zimmet *et al.*, 2001). During the pregnancy, another type of diabetes called gestational diabetes (GD) onsets characterized by glucose intolerance (Bastaki, 2005).

Hyperglycemia in both type 1 and type 2 diabetes is due to loss of β-cells or their functions on involvement of genetics and environmental factors (Skyler *et al.*, 2017). Several diseases like pancreatitis, cystic fibrosis, Cushing's syndrome acromegaly lead to DM ultimately (Bastaki, 2005). DM is also induced by some drugs such as glucocorticoids, niacin and interferon (Pandit *et al.*, 1993).

In both types of diabetes; symptoms are same but differ intensively. Elevated blood-glucose levels are initial symptoms of untreated diabetic patients. Consequently, increased loss of glucose in urine, thirst dehydration and raised water consumption occur due to DM. There is loss in weight in spite of increase appetite and food intake during insulin deficiency characterized by fatigue, nausea and vomiting symptoms. Such patients develop bladder, skin, and vaginal infections. Blurred vision is also caused by DM. Moreover, very high sugar-levels lead to coma and even death (Gupta and De, 2012).

Measurement of fasting plasma glucose (FPG), at early morning is most common method for diagnosis of diabetes. FPG level less than 100 mg/dL in patients is considered normal; while those with 100-125 mg/dL; pre-diabetic and those having higher than 125 mg/dL are diabetic (Peters *et al.*, 1996). Recently, it is concluded that prediabetes or type 2 diabetes in children and adults can be diagnosed by A1C, FPG, or 2-h PG (Arsalanian *et al.*, 2018).

The main focus to treat diabetes is carried by regulating and decreasing blood glucose to normal level. Traditional and synthetic medicines stimulate β-cells of pancreas decrease the BGL by inhibiting other hormones that oftenly increase BGL. DM is treated by mechanisms like increase in glucose utilization in the body, reduction in glycogen release, improvement of blood circulation and resisting lipids peroxidation (Mankil *et al.*, 2006). Biguanides; are known drug that reduces hepatic gluconeogenesis and elevate the insulin-stimulated uptake and utilization of sugar. Biguanides are ineffective in the absence of insulin. Metformin is most important example. Certain enzymes are inhibited by alpha-glucosidase inhibitors, which are cause the metabolism of carbohydrates in the small intestine by reducing the absorption in the blood. Thiazolidinediones (TZDs) is another important class of oral antidiabetic agents which are potent to reduce the increased level of glucose in liver, skeletal muscles and adipose

tissue. Free fatty acids levels cause the β-cell death, TZDs can enhance β- cell function by lowering the free fatty acids (Koski, 2004). Non-sulfonylurease secretagogues are oral hypoglycemic that increases the secretion of insulin from active β-cells by similar mechanism but differently binding with β-cells receptors (Mayerson and Inzucchi, 2002).

Performance of nutritional assessment to monitor the diabetic patients with medical nutrition therapy by metabolic status, food intake, readiness to make changes and life style is made according to instructions with set goals. The set goals in nutritional therapy include balanced blood glucose level, concentration of lipids, cholesterol and triglyceride appropriately. Maintenance of proper balance of nutrients like carbohydrates, fats, proteins and calories is performed based on the individual's treatment plan food taken in proper planed program (Chiasson *et al.*, 2003).

For diabetic patients self-management, blood glucose monitoring and proper exercise are also critical components. Control of glucose, increase sensitivity to insulin uptake, minimizing the cardiovascular risk factors, weight loss and diabetes-related complications are further reduced by exercise. Diabetic patients can make adjustment to diet, medications and exercise by performing the self-monitoring of daily blood glucose levels (Kitabchi *et al.*, 2001).

Medicinal plants had been primary source for treatment of diseases. About 7000 plants species including lichens and trees have been used from time to time for medicinal purposes (Arrowsmith *et al.*, 2009). Herbs have been utilized to isolate or synthesize conventional drugs (Rates, 2001). Medicinal properties of 2000 plants heve been reported in Ayurveda (Kapoor, 2000). According to Chinese Pharmacopoeia, there are over 5700 traditional medicines, which had been derived from medicinal plants (Lu *et al.*, 2008). Estimate showed that 500 herbs are still utilized for therapeutic benefits in conventional medicines (Evans *et al.*, 2009).

There are 36 million people across the worlds that die due to non-communicable diseases such as cancer, diabetes, chronic lungs cancer and cardiovascular diseases. Different types of remedies are practiced all over the world to get rid of diseases. One of these is the traditional medicines. The sum total of skills, knowledge, practices based on

theories, believes and different cultures of the human experiences, for the maintenance of health and treatment of diseases are defined as traditional medicines (WHO, 2008). Modern medicines are discovered and developed by chemical methods and their approach for common person are costly and have side effects in the body when taken overdose. Pharmacologically tested plant's derived compounds are used in modern medicines. Effective standards are tested from natural sources in modern ways. There are about 120 active compounds, which are isolated from plants having therapeutic and traditional uses with 80% positive correlation (Fabricant and Norman, 2011). Drugs development and discovery in modern medicines and health depends upon medicinal plants. In process of drugs development pharmaceutical drugs are identified and brought to market (Lee *at al.*, 2012). In pharmaceutical industries, plants are mainly used to obtain compounds as natural sources. Medicinal plants have potential to produce drugs and their pharmacological use is reported to be of 20% of plants in the world (Farnaz *et al.*, 2010).

Pakistan is developing country and it depends on plants for shelter, food, agriculture, fodder and medicines (Shinwari, 2010). Doctors in Pakistan; due to lack of reliability and less knowledge about the medicinal plants; do not prescribe herbal drugs to patients. They consider that plant based compounds may be toxic (Badsha *et al.*, 2011). Medicinal plants in Pakistan have been studies from different regions. These medicinal plants are reported to have active compounds to treat various diseases. (Silva and Fernandes, 2010). Phenolic constituents have also antioxidant property (Rana *et al.*, 2016). Medicinal plants have antioxidant potential and *Cichorium intybus* is an important source of natural antioxidants (Rafique *et al.*, 2014). Different types of drugs are obtained from medicinal plants. In industrial countries, 80% people use traditional medicines and these are derived from plants which have medicinal value (Ahmad *et al.*, 2011).

Natural products are important source for drugs discovery and drugs development. Herbal preparations are important because of their cost effectiveness and least side effect and used for various treatments especially in rural areas (Arya *et al.*, 2011). Although the synthetic drugs are commonly used to treat diabetes however, they exhibit prominent side effects and those effects cannot be reversed. Plant based

medicines have less side effect that is why they are liked by most of the populations (Rao *et al.*, 2010).

Studies have shown that various anti-inflammatory agents and anti-oxidants are derived from plants and plants based drugs are non toxic and safe to use (Pavithra *et al.*, 2013). Numbers of human ailments are treated by herbal medicines since long and Indian ayurveda have already mentioned it in the literature. Several anti-diabetic plants are being used since prehistoric time in Indian traditional medicines, which have proven the anti-hyperglycemic effect (Gupta *et al.*, 2008).

Plants against hyperglycemia from Azad Jammu and Kashmir (AJ&K) are less studied from pharmacological point of view. Ethno-botanical survey shows a diverse flora, which can be studied or investigated experimentally for pharmacological investigations. Due to increasing rate of diabetes and urgent need of antihyperglycemic drugs having minimal side effects, present study is carried out to conduct phytochemical investigation and antihyperglycemic effects of traditionally used medicinal plants in Poonch Azad Jammu and Kashmir (AJ&K) Pakistan with following objectives.

1.1. Objectives of Current Study

The objectives of present study were:

1. To determine anti-hyperglycemic effect of methanolic extracts of selected plants (Poonch AJK) against alloxan induced hyperglycemic mice.

2. To study anti-hyperglycemic effect of different fractions of most active plants to determine most active fractions.

3. To carry out phyto-chemical analysis of methanolic extract and most active fractions of anti-hyperglycemic plants.

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

Metabolic dysfunctioning of fats, carbohydrates and proteins causes the chronic diabetes. There are two main types of diabetes mellitus (DM) i.e. idiopathic and secondary. Insulin dependent diabetes (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) are two types of idiopathic diabetes. IDDM is commonly (type 1) and NIDDM (type 2). Pancreatic β cells secrete insulin and regulates carbohydrate metabolism. After the absorption of food, particles into blood stream presence of carbohydrates stimulate pancreatic β cells to secrete insulin. Insulin facilitates the glucose particles to enter into body cell where it is metabolized and produce energy, which is used in various life process (Anon, 2010).

2.1. Classification of diabetes mellitus (DM)

2.1.1. Type 1 Diabetes

Importantly the major defect of type 1 diabetes is the deficiency of insulin where as failure of insulin secretion and insulin resistance are two main defects observed in type 2 diabetes (Anon, 2010). When β cells fail to secrete insulin due to destruction caused by macrophages and dendritic cells present in the surroundings of β cells antigens, then type 1 DM onsets. CD8+ T-cells causing beta-cell death in series of interleukin signaling. The autoimmune destruction to β cells lead to deficiency of insulin, causing the impaired glucose, protein, and lipid metabolism (Yoon *et al.*, 2005; Ozougwu, 2013; Ahmad, 2014).

Interaction of genetic and environmental factors also causes Type 1 DM. Multiple genes are involved that cause type 1 diabetes and one of the important gene is located on chromosome 6 and 8 polymorphisim on human leukocytes antigen (HLA) region that increase the genetic risk in type 1 diabetes development (Ulbelen *et al.*, 1977; Powers, 2015a).

2.1.2. Type 2 Diabetes

The resistance to insulin secretion is main abnormality in type 2 diabetes (Weyer *et al.*, 1999). When there is inadequate insulin secretion in the β-cells then chances of development of diabetes are enhanced and glucose metabolism is diminished than normal range in the blood (Triplitt *et al.*, 2014; Khan *et al.*, 2014). Several factors like insulin resistance deficiency and lifestyle factors i.e obesity, over eating and stress are responsible for causing Type 2 diabetes mellitus (Khan *et al.*, 1993; Kaku, 2010; Khan *et al.*, 2014).

2.1.3. Gestational Diabetes

Harrison's principle of internal medicines explains that 4% of pregnant women develop gestational diabetes (Powers, 2015a) In Gestational Diabetes mellitus (GDM) body increases the insulin secretion to normal range maintenance of glucose concentration. However, during the pregnancy GDM risks factor can decreased by observing the blood glucose (Triplitt *et al.*, 2014).

2.1.4. Diagnosis of Diabetes

Symptoms of DM and glucose concentration randomly; higher than range of 200 mg/dL are the criteria for diagnosis of DM and oral glucose tolerance test (OGTT) is another factor considered from plasma glucose concentration for 2 hours above 11.1 mmol/L (200 mg/dL) (Anon, 2010; Standard treatment Guidelines, 2014; Power 2015 a). Glycated hemoglobin A1C test (HbA1C) above 6.5 % is also considered in laboratory test for estimation of haemoglobin percentage attached to glucose (Anon, 2010; Powers, 2015a).

2.1.5. Signs and Symptoms

Polyuria, polyphagia and polydipsia which are more commonly found symptoms in type 1 diabetes mellitus. Due to excess concentration of glucose a hyperosmolar environment causing frequent urination. High plasma glucose concentration enhances the chances of dehydration, as a result patient water intake increases. The excess of plasma glucose cause serious effects and energy requirements are declined in the body so that food-eating requirements are also enhanced and in case of type 2 diabetes fatigue is major symptom in the patients (Standard treatment guidelines, 2014).

2.1.6. Screening for Diabetes Mellitus

DM of Type 1 recommendation is performed in high risk people and Type 2 in people having body mass index at 25 kg/m^2 or above. Following are the risk factors i.e. history of family, physical inactivity, cardiovascular diseases and high triglyceride level in the blood. The age of 40 years is also considered as risk factor even there are no symptoms of diabetes appear in the people and life style is mainly important during this age (Standard treatment Guidelines 2012, 2014).

2.2. Management of Diabetes Mellitus

Non-drug therapy

Patient education: Education to therapy is main factor considered during the management of diabetes. In case of emergency assistance patient should wear disease identification bracelet card or necklace.

Lifestyle changes: Loss of overweight and exercise are recommended to manage it in addition to walking and climbing the stairs (Standard treatment Guidelines, 2014; Power, 2015b).

Nutrition control: Low fats diet like fruit and vegetables are recommended to manage the diabetes which include a fresh fruit at one time, protein, vitamin C, one part of dark green vegetables and one yellow orange fruits (Standard Treatment Guidelines 2014; Powers, 2015b).

Drug therapy

It is difficult to treat the diabetes mellitus but proper management by using glucose lowering medications, healthy nutrition and exercise keeps the BGL in normal range. The biomarker to monitor the hyperglycemic patient is HbA1c is the marker whose target differs from young adult to elderly people with significant difference. Complications related to diabetes and education to understand the diseases related to cardiovascular disease play a key role to manage it. In the patients of type 1 diabetes the insulin is administered while

Type 2 diabetes is characterized mainly by insulin resistance and its treatment is performed by oral anti-hperglycemic medicines (Standard treatment guidelines, 2014; Powers, 2015b; Triplitt *et al.*, 2014).

Insulin Therapy

The injections of insulin is administered in typel diabetes which is mainly used in diabetic ketoacidosis (DKA) and pregnancy. The adverse reactions to brain damage occurs in insulin therapy in most cases. Rapid acting (RAI), short acting (SAI), intermediate acting insulin and long lasting insulin are important injections of insulin for diabetes (Powers, 2015b; Triplitt *et al.*, 2014). RAI allows physiological restore of functions and their action time is short having least risk of hyperglycemia after mealing (Power 2015 b; Triplitt ., 2014). SAI is administered before meal and is regular insulin molecule. SAI may cause early postprandial hyperglycemia and late postprandial hypoglycemia if given at meal time. Such type of insulin is used to manage diabetic ketoacidosis (Powers, 2015b; Triplitt *et al.*, 2014). Regular and rapid insulin in combination to intermediate acting insulin are used having predictable actions otherwise intermediate acting insulin is unpredictable used alone (Powers, 2015b; Triplitt *et al.*, 2014).

Oral Anti-diabetic Drugs

In order to maintain normal glucose level in the body and to prevent the complications due to increase concentration of glucose the oral anti-diabetic drugs are mainly applied to the diabetic patients. Oral therapy is prescribed and with continued diet restriction in case of type 2, diabetes (Triplitt *et al.*, 2014; Sumrall *et al.*, 2009). Hepatic output of glucose consumption by muscular tissues of periphery is reduced by biguanides drugs. The metformin drug is commonly used which has no direct effect on pancreatic beta cells. This drug declines the endogeneous insulin, plasma triglycerides and LDL-C by 8-15% requirements (Triplitt *et al.*, 2014; Sumrall *et al.*, 2009). Sulphonylureas are orally used hypoglycemic drugs that bind to receptors of sulfonylurea located on beta cells of pancreas (Triplitt *et al.*, 2014; Sumrall *et al.*, 2009). Alpha glucosidase inhibitors drugs are also suggested to treat the diabetes but they show competitive inhibition to α-glucosidase

enzymes that reduces the glucose excursions in post meal by declining the process of digestion and reduce the reabsorption of starch. Acorbose drug that inhibits the digestion of carbohydrates and reduces the glucose concentration about 30-50% in the patients (Sumrall *et al.*, 2009; Kennedy *et al.*, 2015).

Thiazolidinediones (TZDs) work by linkage with peroxisomes proliferator activated receptor gamma (PPAR-γ), which is found in fat, muscle and liver. TZDs are used to improve the insulin sensitivity. TZDs are used in combination with other anti-diabetic medicines or as monotherapy agents (Sumrall *et al.*, 2009; Kennedy *et al.*, 2015).

Meglitinides: They are often called short acting secretagogues, which absorbed rapidly. The vateglinide and repaglinide is commonly used drug of this class. Mechanism of action of these drugs is through insulin stimulation of pancreatic β- cells by interaction to potassium channels (Triplitt *et al.*, 2014; Sumrall *et al.*, 2009; Kennedy *et al.*, 2015). Dipeptidyl peptidase-4 (DPP-4) inhibitors are drugs, which prolong the half-life of GLP-1 in the blood (Triplitt *et al.*, 2014; Sumrall *et al.*, 2009).

2.3. Monitoring of Diabetes

By consistent monitoring mortality, rate and morbidity can be reduced significantly and other risk factors can be reduced which are associated to diabetes mellitus. Comprehensive education about nutrition, regular exercise and therapeutic regime to the patients reduces the diabetes in the patients (Standard treatment guidelines 2014; Powers, 2015 b).

2.4. Complications of Diabetes

Acute as well as chronic complications of DM can lead in body, which affect different organ systems. Hyperosmolar and hyperglycemic non- ketotic syndromes are main complications associated with diabetes whereas vascular (micro vascular and macro vascular) and nonvascular (loss of hair, gastro paresis, infections and skin complications)

complications are chronic types of diabetes (Triplitt *et al.*, 2014; Powers, 2015b; Standard treatment Guidelines 2012).

i- Diabetes ketoacidosis (DKA): DKA is a life threating diabetic disorder which demands more insulin. Younger people are mostly affected by DKA and time taken to develop such disorder is hours to days. The factors that lead to DKA are insulin omission, less administration of insulin, diseases like pancreatitis and myocardial infarction which are most commonly occur in Type 1 diabetes (Triplitt *et al.*, 2014; Powers, 2015b).

Symptoms: Loss of appetite, vomiting, dry skin, drowsiness, confusion, blurred vision and strong fruity breath odour are key symptoms of DKA (Triplitt *et al.*, 2014; Powers, 2015b).

Treatment: Insulin therapy, electrolyte replacement and oral or intravenous fluid replacement are applied to reverse DKA and it needs observations about blood pressure, pulse rate, respiration, mental state and fluids for 1-4 hours daily (Triplitt *et al.*, 2014; Powers, 2015b).

ii-Hyper osmolar non ketotic diabetes (HONK) is a syndrome which occurs in old age people having diabetes mellitus Type 2 and where it occurs due to insulin deficiency (Powers, 2015c; Standard treatment Guidelines, 2012).

Symptoms: Extreme dehydration, in consciousness and severe hyperglycemia are important symptoms which develop in days and weeks and there is rare acidosis and ketonemia with some gaps of anion metabolism (Powers, 2015c; Standard treatment guidelines, 2012).

Treatment: Fluid replacement may take to stabilize the hyperosmolar conditions and close monitoring of fluids in the patients and replacement of electrolytes is necessary which is taken with the intravenous insulin therapy (Powers, 2015c; Standard treatment guidelines, 2012).

2.5. Medicinal Plants

Various diseases are treated with application of medicinal plants since long in the human history and the key role is performed by natural compounds of these plants. Different parts of medicinal plants and natural products are used in many parts of the world for variety of human ailments (Dyubeni and Buwa, 2012; Maroyi, 2013). Medicinal plants play important role for antihyperglycemic effect by resorting the function of pancreatic tissues and increasing the absorption of insulin or inhibiting the glucose absorption through small intestine in addition to accelerating the carbohydrates metabolism. Although the diabetes is not completely treated but can be cured to certain extent that patient can live like normal lifestyles (Malviya *et al.*, 2010). The DM is serious health problem all over the world and is serious threat for human beings. The plants derived drugs have more efficacies to treat the diabetes and they have proven the anti-diabetic effects. Different studies have shown the hypoglycemic effects against diabetes. Certain plants contain antioxidant like glycosides, alkaloids, terpenoids, flavonoids, cartenoids, etc., having antidiabetic effect (Malviya *et al.*, 2010).

2.5.1. Bioactive Compounds in Medicinal Plants

Secondry metabolites derived from plants are known as bioactive compounds. These metabolites are not involved in metabolic activity of cell synthesis and they regarded as products of biochemical synthesis (Wadood *et al.*, 2013). These bioactive metabolites have important function in the living plants so most species of medicinal plants seem to be capable of producing various secondary metabolites (Amin *et al.*, 2013). Because of qualitative phytochemical analysis of different plant, fractions give rise to wide variety of bioactive metabolites. *Digitalis purpurea* and *Berberis aristata* plants contain metabolites such as saponins, alkaloids, glycosides, cumarins, phenols, tannins, triterpenoids and steroids (Shrestha *et al.*, 2015). The qualitative tests of *Samadera indica*, saponins, flavonoids, alkaloids, saponins, tannins and resins (Deepa *et al.*, 2015). The presences of bioactive metabolites in human and animal body elicit pharmacological or toxicological effects. Due to this reason, the identification and isolation of bioactive metabolites of plants being essential

in order to understand the nature of the secondary metabolites responsible for its medicinal property that will be therapeutically active and efficacious (Annapurna, 2015; Bernhoft, 2010). Flavonoids, proanthocyanidins, alkaloid, glycosides, tannins anthraquinones, terpenoids, phenylpropanoids, saponins, steroids and phenolic compounds are most important bioactive metabolites of plants (Bernhoft, 2010; Wadood *et al.*, 2013) as well as resins and lignins (Deepa *et al.*, 2015). Medicinal plants have known to treat variety of infectious conditions in human, due to the presence of the above mentioned secondary metabolites shown to have antimicrobial, antifungal and antioxidant properties (Belakhdar *et al.*, 2015; Paul *et al.*, 2012).

2. 5. 2. Plants Species with Anti-Diabetic Properties

People are familiar with plants based medicinal products since old ages (Subbulkshmi and Naik, 2001). Important drugs are also derived from plants and that is why medicinal are important source of drugs. Metformin is an important antihyperglycemic drug and is derived from *Galega officinalis* (Grover *et al.*, 2002). More than 800 plants are reported for anti-hyperglycemic effects (Alarcon-Aguilara *et al.*, 1988). Important compounds which are plants derivatives are alkaloids, glycosides, galactomannan, hypoglycans, steroids, terpenoids and flavonoids that have promising effect against hyperglycemia (Ivorra *et al.*, 1988; Maries and Farnsworth, 1995). Management of DM is perfomed by using mididicinal plants in the Native Americans Chinese (Vuksan *et al.*, 2000), South Americans (Garcia *et al.*, 2001) as well as in Asians (Subbulkshmi and Naik, 2001; Grover *et al.*, 2002).

2.5.3 Medicinal Plants with Anti-Hyperglycemic Properties

Various plants are used for treatmet of DM and important one that are reported in literature are discussed here. *Aloe barbadensis* is widely used plant to cure the Type 2 diabetes (Vogler and Ernst, 1999). Study shows that leaf extracts of *G. sylvestre* administered as conventional drug supplement in diabetic patients of Type 2 diabetes at 400 mg/day for 18 to 20 months have significantly reduced the level of BGL (Bhaskaran *et al.*, 2001). Gymnemic acids

which prevents the glucose uptake in the intestine is reported to present in leaf extracts of *G. sylvestre* (Shimizu *et al.*, 1999).

Momardica charantia is well known as bitter gourd. Bitter gourd is frequently used as an antihyperglycemic agent in Asian and Latin American countries (Ahmed *et al.*, 2001; Mirura *et al.*, 2001). It is reported from different studies that bitter guard possess anti-diabetic properties. The fruit has the ability to increase the cell's uptake of glucose and potentiate the effect of insulin (Grover *et al.*, 2002). The hypoglycemic effect is attributed to compounds present plants (Mcwhorter, 2001). *Ocimum tenuiflorum* containing a number of aromatic compounds and used as medicinal purposes worldwide. Basils, particularly *O. tenuit*/arum syn. *O. sanctum*, have been more extensively studied for their hypoglycemic properties (Grover *et al.*, 2002).

Fenugreek (*Trigonella foenum-graecum*) is a popular herb. In experimental animals' hypoglycemic activity of *T. foenum-graecum* has been reported (Grover *et al.*, 2002; Vats *et al.*, 2002). A dose response relationship between treatment effect of *T. foenum graecum* seed and blood glucose levels in rats has been reported earlier (Vats *et al.*, 2002). In an investigation, it is reported that SZT induced rat showed a significant decrease in blood glucose level by administration of *T. foenum graecum* on 15th and 30th day of experiments by 14.4 and 46.6% respectivel (Vats *et al.*, 2003). *Anacardium occidentale* has been used for treatment of diabetes and it has proven anti-hyperglycemic effect (Kamtchouing *et al.*, 1998; Sokeng *et al.*, 2001). Mice treated with leaves extract of *A. occidentale* have antihyperglycemic and renal protective effect against streptozotocin induced diabetes (Tedong *et al.*, 2006). It is reported from various studies that *A. squamosa* has antidiabetic activity. It inhibits the glucose output from liver and enhances uptake of insulin from pancreas and also increases the consumption of glucose in the muscles having the high safety (Gupta *et al.*, 2005).

Canavalia ensiformis DC. It is native plant of Central America and West Indies and is also reported to be cultivated in humid regions of Africa and Asia. The seeds are reported for antihyperglycemic effect and anti-hypercholestrolemic activities (Marfo *et al.*, 1990).

The aqueous extract of seeds have reduced the BGL in urine in diabetes mellitus patients (Rachel, 2003). *Canavalia ensiformis* DC which is shrubby plant and has high medicinal values is distributed in South India. It was considered as remedy for hyperglycemia (Asolkar *et al.*, 1992; Samani *et al.*, 2018). In experimental animals, this plant has anti-hyperglycemic effects in significant manners (Devaliya and Shirsat, 2017). *Cassia kleinii* having high medicinal value is used to cure hyperglycemia by folk people in South India, which has antihyperglycemic properties during experiments against alloxan-induced diabetes in rats. Drugs effect is associated to enhance the insulin uptake and acceleration of glucose metabolism (Babu *et al.*, 2002).

Catharanthus roseus mostly used as an anticancer agent, but hot water extract is also used to treat diabetes (Rasineni K *et al.*, 2010). It was reported that alcoholic extract of *C. roseus* show antihyperglycemic effect (Zhang *et al.*, 2015; Chattopadhyay *et al.*, 1991). *C. indica and C. rouses* are used in folk medicine for treatment of hyperglycemia in South East Asia and Africa commonly. Pectin a chemical compound found in fruit of *C. indica* plant is responsible for antihyperglycemic activity (Kumar *et al.*, 1993). Leaf juice of *C. rouses* showed significant antidiabetic activity and supports the traditional usage of the fresh leaves by folk people for the control of diabetes. (Nammi *et al.*, 2003). Hypoglycemic activity is exhibited by alcoholic extract of this plant. The mechanism of action is due to their β-cell restorative properties against alloxan-induced damage (Sivakumar, 2010). Ethanolic extract of *C. fenestratum* stem and *C. roseus* leaves have shown potent antihyperglycemic effect in alloxan-induced diabetic rats. *C. fenestratum* and *C. roseus* could therefore be used as an alternative remedy for diabetes mellitus and its complications. (Manoharan *et al.*, 2011).

Cocculus hirsutus Linn are bitter, acrid, laxative, demulcent, tonic and diuretic. Aquous extract of *C. hirsutus* leaves show antihyperglycemic in alloxan-induced diabetic mice. The hypoglycemic activity of aqueous extract of *C. hirsutus* due to lowering of serum glucose level in diabetic mice. Moreover, the extract prevents loss of body weight (Badole *et al.*, 2006). *Ficus hispida* Linn is commonly known as Daduri and is used for treatment of hyperglycemia. *F. hispida* is mostly found throughout India. Various studies on this plant have reported antidiabetic effects of different compounds obtained from *F. bengalensis*

(Geetha *et al.*, 1994; Cherian and Augusti, 1993). The hypoglycemic effect of water-soluble fraction of the alcoholic extract of *F. hispida* (bark) in normal and diabetic albino rats concluded that *F. hispida* is directly act on β-cells and decline the fasting BGL and alloxan induced hyperglycemic rats (Ghosh *et al.*, 2004).

Murraya koenigii Linn is commonly known as Curry patta is used as a spice in India. Its aqueous extract is reported to antihyperglycemic effect in mice against alloxan induced diabetes (Narayan *et al.*, 2005). About 60 days' diet treatment showed significant decrease in BGL (Khan *et al.*, 1995). It is observed that oral administration of different doses of *M. koenigii* leaves to diabetic rats show hypoglycemic activity (Yadav *et al.*, 2002). *M. koenigii* significantly lower the BGL and play an important role in glucose metabolism. (Kesari *et al.*, 2005).

Panax ginseng Linn. In Asian countries, *Panax ginseng* root has been used clinically in the treatment of type II diabetes. The plant possesses antihyperglycemic activity in vitro as well as in vivo animal studies. The ginsenoside play important role in hyporglycemic action and pharmacological effect on energy metabolism (Luo and Luo, 2008). *Syzyguim cumini* Linnhave been reported to show hypoglycemic activity and different parts of medicinal plants have been used against hyperglycemia in different Asian countries (Ayanagounder *et al.*, 2008). Hypoglycemic effect has been found in leaves, seeds, fruit and bark of *S. cumini* (Achrekar *et al.*, 1991).

2.5.4. Plants under Study

The traditionally used medicinal plants for treating various diseases are selected for this study are:

2.5.4.1 *Rumax haustatus*

Botanical name: *Rumex hastatus*

Vernacular name: khatimber, Churki, khati buti

Family: Polygonaceae

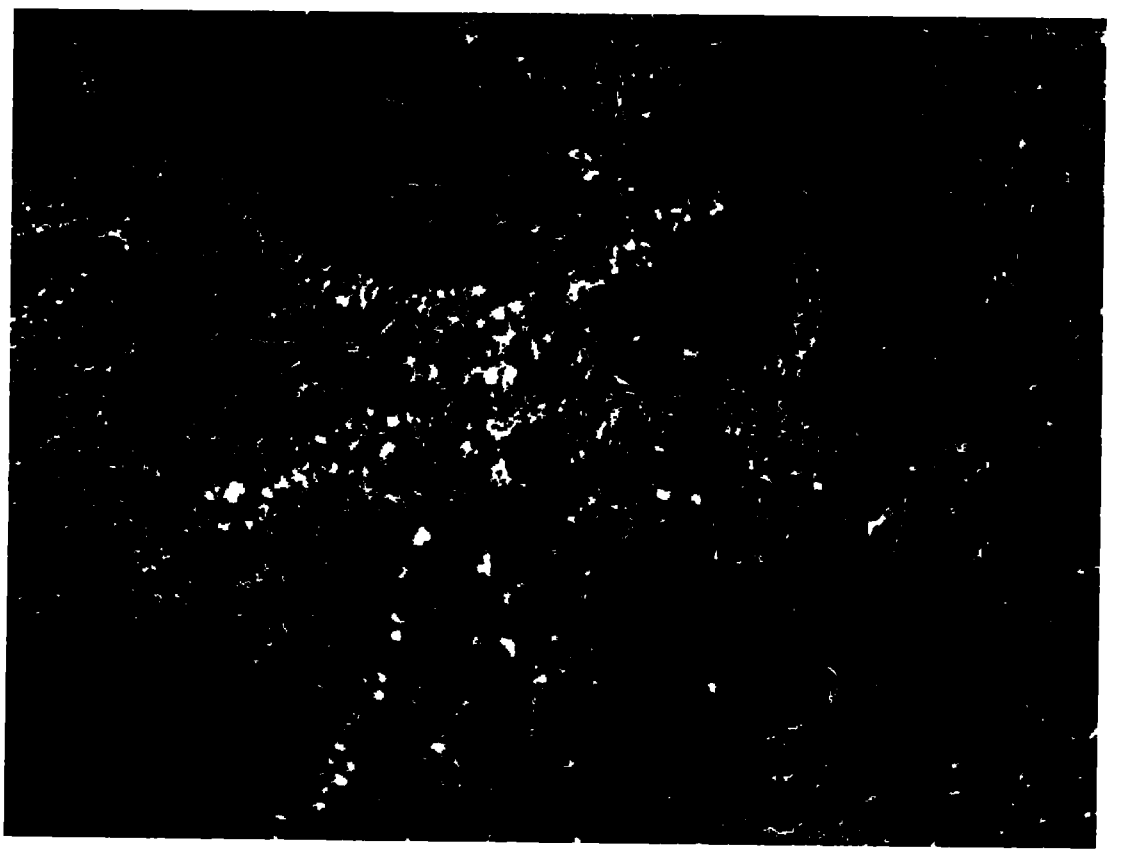

Figure 2.1: *Rumax haustatus* D. Don

Rumex haustatus D. Don (Polygonaceae) is a bushy shrub up to 90 cm high. It occurs chiefly on dry rocks and hillsides of western Himalayas from Kumaon to Kashmir, at altitudes between 300 and 2400 meters.

Medicinal use: All parts of this *R. hastatus* has been used as medicine, as tonic, as a laxative and used in rheumatism (Shinwari *et al.*, 2003). Different parts of *R. hastatus* has been reported to be used cure different diseases lik bleeding of lungs, piles bilious complaints and skin diseases. (Gorsi and Miraj, 2002). Various parts of this plant has also been used for diuretic, carminative, purgative and flavouring agent (Ullah and Rashid, 2007). *R. hastaus* has been used as a folk medicine against sexually transmitted diseases including AIDS (Vermani and Garg, 2001). Throat pain, constipation and skin disorders are treated with fresh rhizome of *R. hastatus* (Zhang *et al.*, 2009). Its seeds are reported to have cooling properties while shoots and leaves have diuretic properties (Haq *et al.*, 2011). Due to its pleasant taste, shoots and other parts like leaves are in food like pickles and chutnys (Irfan *et al.*, 2018). Roots are reported to used in rheumatism, backache, asthma cough and fever (Abbasi *et al.*, 2012; Abbasi *et al.*, 2010). The aerial parts (leaves and young shoots) are used as carminative, purgative, diuretic and in stomach problems (Murad *et al.*, 2011).

Previous pharmacological study: Previously, leaves of *R. hastatus* were evaluated for phenolic compounds (Shahreen *et al.*, 2011). It is thought that roots of *R. hastatus* might have antioxidant activity against free radicals. This plant is also used in traditional medicines (Singh *et al*, 2013; Alamgeer *et al.*, 2018).

2.5.4.2. *Lespedeza cuneata*

Botanical name: *Lespedeza cuneata*

Vernacular name: Koochi, Chinese bush clover, silky bushclover.

Family: Fabeacae

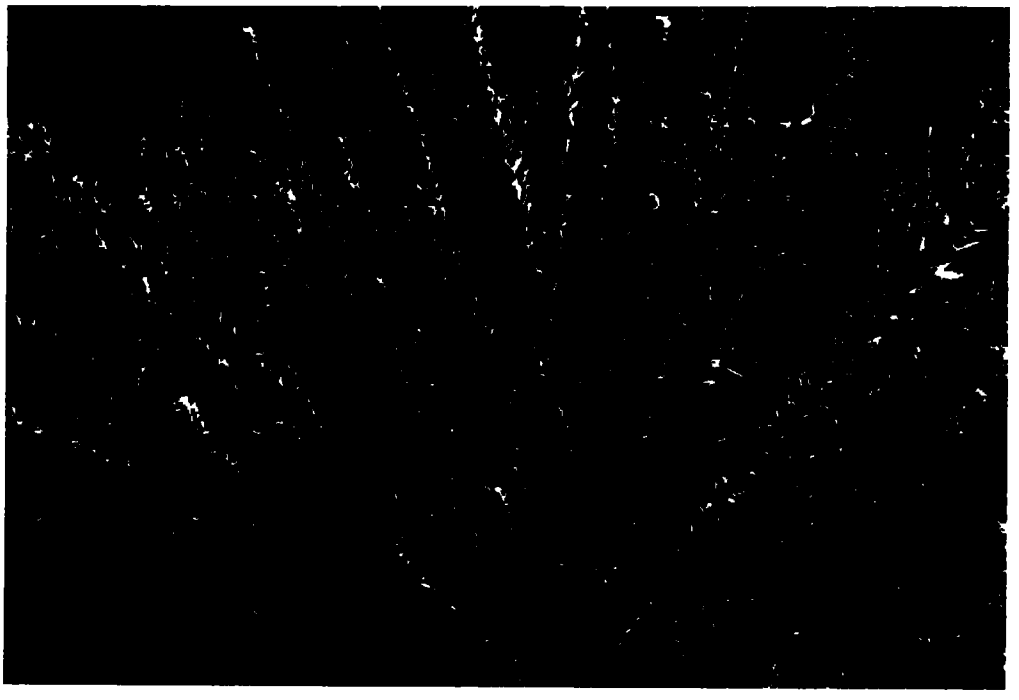

Figure 2.2: ***Lespedeza cuneata*** (Dum.Cours) G. Don

Lespedeza cuneata (G. Don) is common herb widely distributed in different countries of south East Asia and various states of America (Deng *et al.*, 2007).

Medicinal use: In Eastern Asia this plant is reported as herbal medicine for anthelmintic, depurative and tonic activities. It is also reported as natural deworming agent because it contains high-condensed tannin content (Shaik *et al.*, 2006; Lange *et al.*, 2006). In China diabetes type-II is reported to be treated by root and aerial parts of *L. cuneata* (Su, 1999). Hematuria, insomnia and malnutrition are also treated with *L. cuneata* (Xu PH., 2010). *L. cuneata* is commonly used for the treatment of different diseases like asthma, involuntary emission of semen, hyperglycemia and impotennce since long (Deng *et al.*, 2007).

Previous pharmacological study: Previous phytochemical such as pinitol, tannins, b-sitosterol, and flavonoids are reported to investigate from *L. cuneata* (Yong-Han and Su-Noh, 2008). Isoorientin Isovitexin, vicenin II Lucenin II, desmodin, trifolin hyperin and hirsutrin has also been reported from *L. cuneata.* Quercetin and kaempferol are important flavonoids that have been isolated from *L. cuneata* (Yoo *et al.*, 2015). Previous phytochemical studies have revealed flavonoids, sterols, triterpenoids (Kwon *et al.*, 2009;

Deng *et al.*, 2007) and phenylpropanoidglycosides (Zhou *et al.*, 2016) constituents showed antioxidant effects (Kim and Kim., 2007; Kim and Ryu, 2008; Cho *et al.*, 2009; Kim and Kim, 2010; Cho *et al*,. 2011) anti- inflammatory effects (Lee *et al.*, 2013) and antibacterial activities (Sang K *et al.*, 2011). Aerial part of this plant is reported to contain flavonoids, which showed protective effect against different disorders of kidney, liver and lungs (Kwon *et al.*, 2007; Kim and Sharma, 2016). It has been reported from various phytochemical studies that various flavonoids like (isovitexin, desmodin, isoorientin, lucenin II, vicenin II and homoadonivernith), O-glycosyl flavonols like (hyperin, trifolin, juglanin, hirsutrin and avicularin) and aglycones like (kaempferol aand qurercetin) have been isolated from aerial parts of *L. cuneata* extract (Kwon *et al.*, 2009; Matsuzaki *et al.*, 1990; Zhang *et al.*, 2016).

2.5.4.3 *Rubia cordifolia*

Botanical name: *Rubia cordifolia*

Vernacular name: Indian madder, manjistha Khornati

Family: Rubiaceae

Figure 2.3: ***Rubia cordifolia*** (Gand) Verdc

Distribution: It is distributed in South Asian, South East Asian countries and China. This plant is reported to be frequently present adjacent to rivers, streams, and upto 3750 m altitude from sea level in forests (Varier, 1995; Devi and Siril, 2014).

Medicinal uses: Indian folk medicines use the active parts (stem and roots) of *R. cordifolia*. Various parts of *R. cordifolia* are used as antiseptic, blood purifying agent, antidysentric agent and astringent. Hepatoprotective, antiviral and anti-rheumatic activities are also exhibited by *R. cordifolia* (Kamboj, 2000; Prajapati and Parmar, 2011). It is reported that root extract has been used against many diseases, i.e. pectoral disease, slow healing of broken bones, cough, hyperglycemia, Neuralgia, otopathy, hemorrhoids, splenopathy, arthritis, tubercular conditions of skin, general debility tuberculosis and urethrorrhoea (Devi and Siril., 2014). Leaves have antioxidant and antiviral activities (Bhat *et al.*, 2018: Bhatt and Kushwah, 2013). It is also reported that *R. cordifolia* is used as immunomodulator, hepetoprotective, nephroprotective, analgesic and diuretic. *R. cordifolia* exhibited potent

antioxidant activity against lead nitrate and radiation induced toxicity (Tripathi and Singh, 2007; Lodia and Kansala, 2012).

Previous Pharmacological study: Previously it is reported to study about wound healing and antioxidanssst activities (Bhatt and Kushwah 2013; Prajapati and Parmar, 2011). The phytochemical studies are reported about its stem and roots previously. On leaves of *R. cordifolia*; there is little research studies has been performed about their phytochemicals.

2.5.4.4 *Nepeta grandiflora*

Botanical name: *Nepeta grandiflora*

Vernacular name: Catmint

Family: Lamiaceae

Figure 2.4: ***Nepeta grandiflora*** M. Bieb

Medicinal use: It is a good honey plant; decorative features. *N. grandiflora* is traditionally used as antitussive, anti-spasmodic, anti-asthmic, anti-diuretic and sedative agents and also used as a remedy for snake and scorpion bite. (Butt *et al.*, 2015).

Previous pharmacological study: Aerial parts of *N. grandiflora* contain different essential oils, steroids, tannins and flavonoids. Traditionally this plant is also used as folk medicine during anemia as substitute for tea. (Kovtun, 2004).

2.5.4.5 *Strobilanthes dalhousieanus*

Botanical name: *Strobilanthes dalhousieanus*

Vernacular name: Malol

Family: Acanthaceae

Figure 2.5: ***Strobilanthes dalhousianus*** **M. Bieb**

Distibution: It is present with wide distribution in Poonch, Bagh and forward Kohota Azad Kashmir, Pakistan (Khan *et al.*, 2012).

Medicinal use: Folk people of Kashmir mostly use its leaves and roots for treatment of stomach problems, liver complication and diabetes. Previous pharmacological investigation is not available about this plant in literature survey.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals and Solvents

Solvents (n-hexane, Ethylacetate, n-Butanol and Methanol) used in experiments were of analytical grade, purchased from registered chemical companies (Sigma Aldrich). While the solvents (orthophosphoric acid 85%, acetonitrile and methanol) of HPLC grade were also purchased from registered chemical companies (Sigma Aldrich). Standard compounds (Mangiferine, Purpurine, Kaempferol, Charatine and 2-methyl anthraquinone) and standard drug (alloxan) were also purchased from Sigma Aldrich.

3.2. Instruments (Equipments and glass wares)

Equipment used were rotary evaporator, spectrophotometer (UV-visible), glucometer, syringe nylon filter (0.45μm), mechanical grinder, laboratory centrifuge, filter papers (Whattman No.1), beakers, volumetric flasks, funnels, Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA), falcon tubes, glass vials, sonicator, digital balance, oven, Eppendorf, test tubes and cotton etc.

3.3. Study Design

Initially five selected plants were investigated for antihyperglycemic effect in mice model. Then most antihyperglycemic active one was further studied through fractionation process using different solvents on polarity basis. The most active plants were further investigated for antioxidant activities and phytochemical analysis.

3.4. Plants Material

3.4.1. Selection and Collection of Plants Material

Ethno medicinally important plants, used by folk people to treat diabetes and other diseases of various localities of Poonch Division of AJ&K were collected in the growth season (April to September). Standard procedure was used for preparation of herbarium sheets of plants (Khan *et al.*, 2012).

3.4.2. Identification of Plant Samples

Plants were identified by plant taxonomist (Dr. Azam, Associate Professor, Government Boys Degree College Hajira AJK). Correctly, identified specimens (*Rumex hastatus, Lespedeza cuneatea, Rubia cordifolia, Nepeta grandiflora* and *Strobilanthes dalhousieanus*) were deposited as voucher specimens (0441, 0442, 0443, 0444 and 0445 respectively) in the herbarium (Medicinal and aromatic plants of AJ&K) at Government Boys Degree College Abasspur AJ&K for future reference.

Table3.1. Plants used under study

S.N.	Botanical name	Vernicular name	Family	Parts used
1	*Rumex hastatus*	Khatimber	Polygonaceae	Aerial parts
2	*Lespedeza cuneatea*	Chines bush clover	Fabeacae	Aerial parts
3	*Rubia cordifolia*	Indian madder	Rubiaceae	Aerial parts
4	*Nepeta grandiflora*	Catmint	Lamiaceae	Aerial parts
5	*Strobilanthes dalhousieanus*	Malool	Acanthaceae	Aerial parts

3.5. Preparation of Methanolic Extract (ME) of Plants

Plants were allowed to dry at room temperature for ten days and then samples were grinded by mechanical grinder into powder form. Plants sample were separately packed into polythene bags to avoid moisture and contamination after weighing properly.

3.5.1 Extraction Procedure

The protocol (Elaloui *et al.*, 2016; Yeo *et al.*, 2014) was used to prepare methanolic extract (ME) of samples. Dried powdered samples (100g each) of plants were dipped in methanol (1:3) in five separate flasks (1000 mL each) and were kept for soaking for one week.Then each sample was separately filtered using filter paper. The process of extraction was repeated three times for separation of extracts. Under reduced pressure at 40 °C, whole filtrate of each plant was concentrated using rotary evaporater. All extracts were named as methanolic extract of *Rumex hastatus* (MERH), methanolic extract of *Lespedeza cuneatea* (MELC), methanolic extract of *Rubia cordifolia* (MERC), methanolic extract of *Nepeta grandiflora* (MENG) and methanolic extract of *Strobilanthes dalhousieanus* (MESD) for plant one to plant five (P1-P5) respectively.

3.6 Antihyperglycemic Activity of ME of Selected Plants

Antihyperglycemic activity of ME of each selected plant was tested according to standard procedure discussed below:

3.6.1 Experimental Animals

In this experiment 40 male albino mice (BALB/c) weighing about 28 ±5g, were purchased from National Institute of Health (NIH) Islamabad. Animals were housed in the stainless steel cages maintaining the conditions at 25±5 ^{0}C having 12 h light/dark cycles, in the experimental room of animal house of NIH Islamabad. Before start of experiment, all mice were allowed to acclimatize upto 7 days providing proper diet and water (Figure 3.1).

3.6.2. Acute Toxicity Test

Acute oral toxicity test of each selected plants (MERH, MELC, MERC, MENG and MESD) was carried out by the standard procedure and guideline adopted according to Organization for Economic Cooperation and Development (OECD Test 425, 2008) with standard procedure with slight modification (Ghazanfar *et al.*, 2014). Mice were kept fasting overnight before oral dosing. Selected doses (250, 500, 1000 mg/kg) of each plant extract were administered orally to tested mice and all tested mice were observed for 24 hours for behavioral, adverse changes and lethality.

3.6.3. Antihyperglycemic Effect of Selected Medicinal Elant extract (ME) Against Alloxan Induced Diabetic Mice

Dose selection: After testing the oral toxicity, one-tenth (1/10th) of maximum tolerated dose (1000 mg/kg b.w) of each plant extract was selected for further experimental process of antihyperglycemic activity.

Dose calculations: Normal saline (0.9%) was used to dissolve methanolic extract of each plant. Dose calculation was made according to guidelines laid by OECD-425. Calculations were following:

Dose calculation for normal saline: Daily maximum dose consumption of each mouse for experiment was calculated as:

The dose for one mouse (28g) per day required = 0.56 mL

5 mice in each group per day required as 5x 0.56 = 2.8 mL

Dose for 5 mice of 28 days required as 2.8 x28 = 78.4 mL

Plant extract dose calculation: Required dose of extract for 28 g mouse was 2.8 mg (100 mg/kg) per day. For 5 mice in each group the required dose was 14 mg; that was 392mg for 28 days. Total of 392 mg (0.392gm.) of each plant extract was weighed seperately and put into100 mL each, 5-falcon tubes separately. Then with the help of syringe; 84 mL of normal

saline solution was added in each falcon tube. Extract of each plant was properly dissolved using sonicator. Similar way was followed for alloxan dose calculation.

Standard antihyperglycemic drug dose calculation: Standard procedures (Vanitha *et al.*, 2013; Bukhari *et al.*, 2015) were used to calculate the dose of antihyperglycemic standard drug (Glibenclamide) for 5 animals of standard drug treatment group (GIII) with 0.5 mg/kg. Dose required for one mouse was 0.14mg/day. Required dose for 5 mice was 0.7 mg / day that was 19.6mg for 28 days. Total 19.6 mg standard drug was weighed and transferred into falcon tube (100 mL) with addition of 90 mL of normal saline solution in falcon tube using syringe. Sonicatorwas used for efficient dissolving of drug.

3.6.4. Experimental Design for Antihyperglycemic Activities

Institutional Bioethics and Biosafety Committee (IBBC) of the University (IIUI) through assigned number No. **IIU (BI&BT)/FBAS-IBBC-2016-06 dated July 13, 2016;** approved the experiment on mice and guideline laid by OECD-423 (adopted on 17th December 2001) were followed. Random division of animals into groups was performed (n=5). Group-I: Normal control group (NC); provided with standard diet for 28 days. Group-II: Toxicant control group (TC). Group-III: Standard drug (SD) treatment group. Groups IV- VIII: were plant methanolic extract treatments. Glibenclamide dose (0.5 mg/kg) in G-III was given according to protocols adopted by Issa *et al.*, (2015) following little modifications.

3.6.5. Treatment Procedure

Group-I: Normal control (NC) was provided with normal feed and water for 28 days.

Group-II: Toxicant control (TC): Dosed with Alloxan (100 mg/kg) by intraperitoneal injections for 6 days with 2 days interval. Group-III: Standard drug (SD) treatment group: Alloxan as in G-II+standard antihyperglycemic drug (Glibenclamide, 0.5 mg/kg/d) orally administered upto 28 days. Group-IV: Alloxan as in G-II + methonolic extract of plant one (MERH) orally administered daily (100 mg/kg) for 28 days. Group-V: Alloxan as in G-II + methonolic extract of plant two (MELC) orally administered daily (100 mg/kg) up to 28

days. Group-VI: Alloxan as in G-II+ methonolic extract of plant three (MERC) orally administered daily (100 mg/kg) up to 28 days. Group-VII: Alloxan as in G-II+ methonolic extract of plant four (MENG) orally administered daily (100 mg/kg) up to 28 days. Group-VIII: Alloxan as in G-II+ methonolic extract of plant five (MESD) orally administered daily (100 mg/kg) up to 28 days.

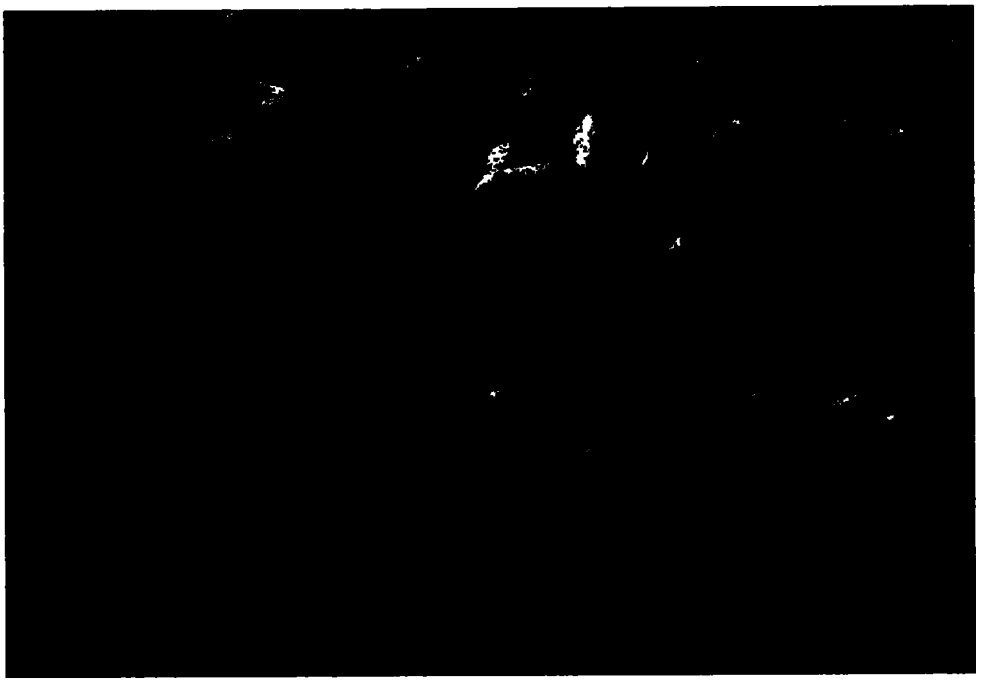

Figure 3.1: Mice acclamatized before experiment

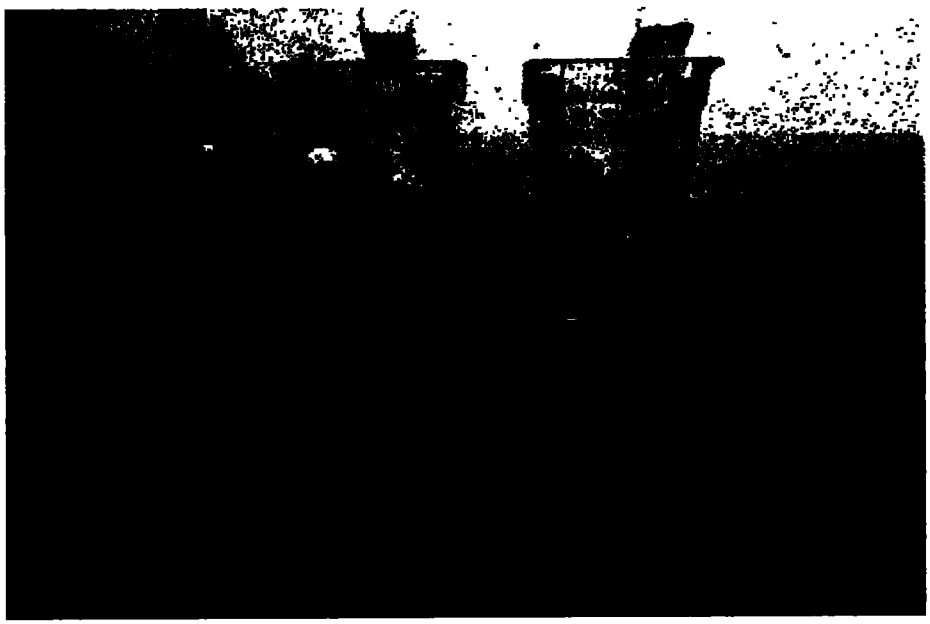

Figure 3.2: Mice sorted for assessment of antihyperglycemic effect of selected plants extract

Figure 3.3: Blood collection

3.6.6. Analysis for BGL and BW of Mice During Treatment with Methanolic Extract of Selected Plants

Blood glucose level (BGL) was assessed by glucometer method on 0,7th, 14th, 21st and 28th day during the experimental process (Gunjal *et al.*, 2016).

3.7. Shortlisting of Most Active Antihyperglycemic Plants

Most active antihyperglycemic plants were selected for further investigation based on experiments against alloxan-induced diabetes in mice.

3.8. Analysis of Shortlisted Most Active Antihyperglycemic Plants

3.8.1. Extraction of Shortlisted Most Active Plants

Preparation of more quantity of methanolic extract of most active plants *(R. cordifolia* and *L. cuneata*) was performed according to our previous method during the screening of plants in this study. For this purpose, 1kg (1000g) of each powdered plant material (*R. cordifolia* and *L. cuneata*) was treated for cold maceration process. 1kg (1000 gm) of each plant material was soaked in methanol with 1:3 (3000 mL) in a 5 liters round bottom flasks separately and kept for 4 days with daily shaking. Then the samples of each plant were filtered through filter paper. The process was repeated three times for maximum extraction. Whole extract was dried and made concentrated using rotary evaporator at 40 ^{0}C under reduced pressure. After that extract were weighted separately and were assigned number as methanolic extract (MERC*)* as plant A (P-A) and methanolic extract (MELC) as plant B (P-B).

3.8.2. Fractionation of Shortlisted Most Active Plants Extracts (MERC and MELC) and Their Antihyperglycemic Activities

3.8.2.1. Fractionation Process of MERC and MELC

Fractionation of methanolic extract of two shortlisted plants (*R. cordifolia* and *L. cuneata*) was performed according to standard procedure (Ul-Haq *et al.*, 2012). All plant extracts were fractionated by organic solvents (n-hexane, ethylacetate, n-butanol) and water separately on increasing polarity basis. ME of *R. cordifolia* and *L. cuneata* having mass of 100g each were suspended seperately in the distilled water (200 mL) to make aqueous suspension of ME of each plant. Four fractions i.e. n-hexane fraction (HF), ethyl acetate fraction (EF), n-butanol fraction (BF) and an aqueous fraction (AF) of aqueous suspension of each plant were formed with partition by separating funnel. During this process, n-hexane (3×200 ml) was added in aqueous suspension of ME of each plant in separating funnel and shaked vigorously. The n-hexane layer was separated and dried at 40 °C by rotary evaporator under reduced pressure. This was n-hexane fraction (HF). Now in aqueous portion ethyl acetate (3×200mL) was added in separating funnel and shaked vigrously. After separation of layers, i.e. upper layer of ethyl acetate; was dried by rotary evaporator at 40^{0}C under reduced pressure for each plant separately. This was ethyl acetate fraction (EF). Later on in remaining aqueous part of each sample, n-butanol (3×200mL) was added. Again two layers in separating funnel were developed for each sample as; an upper part of n-butanol and lower layer of aqueous portion. After separating both layers of each plant by separating funnel; samples were dried using rotary evaporator under reduced pressure at 40 ^{0}C. There were n-butanol fraction (BF) and an aqueous fraction (AF). All the fractions were weighed separately and labeled as methanolic extract of *R. cordifolia* (MERC), n-Hexane fraction of *R. cordifolia* (HFRC), ethylacetate fraction of *R. cordifolia* (EFRC), n-btanol fraction of *R. cordifolia* (BFRC), and aqueous fraction of *R. cordifolia* (AFRC). Similarly, methanolic extract of *L. cuneata* (MELC), n-Hexane fraction of *L. cuneata* (HFLC), ethyl acetate fraction of *L. cuneata* (EFLC), n-butanol fraction of *L. cuneata* (BFLC) and aqueous fraction

of *L. cuneata* (AFLC). To avoid contamination of samples the dried extracts were kept in refrigerator at 4^0C until further investigation.

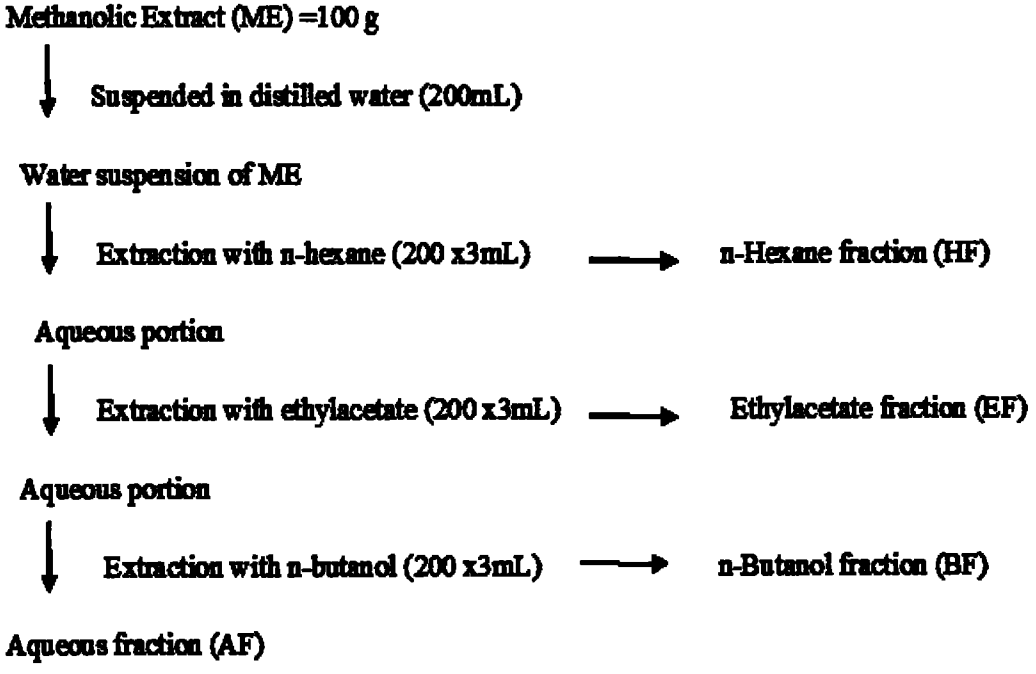

Fig 3.4: Fractionation scheme of methanolic extract (ME) (Ul-Haq *et al.*, 2012).

3.8.2.2. Anti-hyperglycemic Efects of Fractions of Shortlisted Active Plants (*R. cordifolia* and *L. cuneata*)

3.8.2.2.1. Experimental Animals

In current esperiment total 55 male albino mice of 28±5g purchased NIH Islamabad were kept in stainless steel cages providing 25±5 ^{0}C for 12 Hrs light/dark cycles conditions.

Dose selection and calculation: Dose of all selected fractions of both plants were taken as 1/10th of maximum tolerated dose (1000 mg/kg). The dose was calculated in the similar way as calculated previous experiment.

3.8.2.2.2. Experimental Design

Four fractions i.e HF, EF, BF and AF of ME of most active plants were tested for their antihyperglycemic effects. Experimental grouping was designed according to standard procedure (Surana *et al.*, 2008; Charan and Kantharia, 2013). Mice were divided into eleven group (n=5 in each) for treatment effect of both plant fractions. Normal control (NC), toxicant contro (TC) and standard drug (SD) control were common while the mice in plant treatment groups (IV-VII) were of two types e.g groups A (IV-VII) were treated with *R. cordifolia* fractions and Groups B (IV-VII) were treated *L. cuneata* fractions respectively. Animals in all groups were provided access with normal feed and water for 28 days.

3.8.2.2.3. Treatment Procedures with Different Fractions of Most Active Antihyperglycemic Plants

Group-I: (NC); Normal feed and water provided up to 28 days. Group-II: (TC): Alloxan (100 mg/kg) administered by intraperitoneal injections with 2 days' interval for 6 days. Group.III standard drug (SD) treated group: Alloxan as in group-II + standard drug (Glibenclamide 0.5 mg/kg) orally administered up to 28 days. Animals in Groups A (IV-VII) and B (IV- VII) were treated as (Fig 3.5):

A: Treatment with *R. cordifolia* fractions

Group A-IV: Alloxan as in group-II + treatment with n-hexane fraction of *R. cordifolia* (HFRC); oral administration of dose (100 mg/kg) once daily up to 28 days.

Group A -V: Alloxan as in group-II + treatment with ethyl acetate fraction of *R. cordifolia* (EFRC); oral administration of dose (100 mg/kg) once daily up to 28 days.

Group A -VI: Alloxan as in group-II + treatment with n-butanol fraction of *R. cordifolia* (BFRC); oral administration of dose (100 mg/kg) once daily up to 28 days.

Group A -VII: Alloxan as in group-II + treatment with aqueous fraction of *R. cordifolia* (AFRC) oral administration of dose (100 mg/kg) once daily up to 28 days.

B: Treatment with *L. cuneata* fractions

Group B-IV: Alloxan as in group-II + treatment with n-hexane fraction of *L. cuneata* (HFLC); oral administration of dose (100 mg/kg) once daily, up to 28 days.

Group B -V: Alloxan as in group-II + treatment with ethylacetate fraction of *L. cuneata* (EFLC); oral administration of dose (100 mg/kg) once daily, up to 28 days.

Group B -VI: Alloxan as in group-II + treatment with n-butanol fraction of *L. cuneata* (BFLC); oral administration of dose (100 mg/kg) once daily, up to 28 days.

Group B -VII: Alloxan as in group-II + treatment with aqueous fraction of *L. cuneata* (AFLC); oral administration of dose (100 mg/kg) once daily, up to 28 days. All animals were properly discarded after experiment (Fig 3.6).

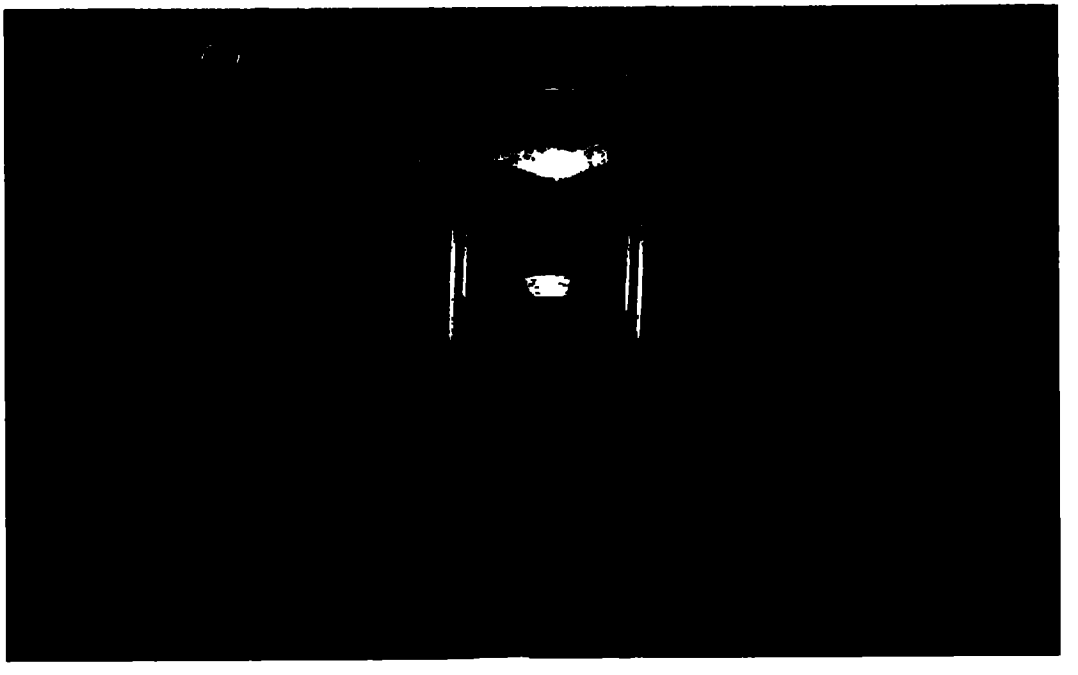

Figure 3.5: Mice sorted for treatment with different fractions of *R.cordifolia* (A) and *L.cuneata* (B)

Figure 3.6: Mice for discard after experiment

3.9 Analysis for Blood Glucose Level and Body Weight of Mice During Treatment with Fractions of *R. cordifolia* and *L. cuneata*

Glucometer was used to measure the blood glucose level (BGL) in all the groups with 7 days' interval (0, 7, 14, 21 and 28 days) during the experiment process with same procedure discussed earlier (Gunjal *et al.*, 2016).

3.9.1. Determination of Most Active Fractions

For *R. cordifolia* and *L. cuneata* fractions tested against alloxan-induced diabetes in mice, most active fractions of both plants were selected for further phytochemical analysis.

3.10. Bioactivity Determination (DPPH Assay for Antioxidant Activity)

Radical scavenging effect of methanolic extract and fractions of most active plants was determined with standard procedure (Labiad *et al.*, 2017; Gonzalez *et al.*, 2016) with some modifications using 2,2 diphenyl-1-picrylhydrazyl (DPPH) as free radical.

3.10.1. DPPH Assay and Calculation of IC_{50}

To determine the antioxidant activity, DPPH (6 mg) solution was prepared by dissolving it in100 mL of methanol. This DPPH solution (2800 μL) was mixed in each plant samples (200 μL) solution, adding in glass vials leading the final concentration of 150, 110, 70, 35, 25 and10 (μg /mL) respectively. All the samples were shaken well and kept at room temperature (25^0C-28^0C) for 1 hour. Measurement of absorbance was carried at 517nm using spectrophotomer. DPPH (2800 μL) solution and mixture of methanol (200 μL) taken as negative control while positive control was ascorbic acid standard while methanol as blank to check the activity of sample. Antioxidant activity (%) was measured according to formula given below and calculation of IC_{50} was mase by linear regression of standard ascorbic acid by graphic method.

$\%A\ A = [(Abc - Abs) \div Abc] \times 100$

"Abc" was absorbance of control and "Abs" means absorbance of test sample.

3.11. Phytochemical Analysis

3.11.1. Qualitative Phytochemical Analysis

Qualitative analysis was performed by standard procedures (Saleem *et al.*, 2018; Labiad *et al.*, 2017).

I. Tests for Flavonoids: Methanolic extract and most active fractions of both active plants i.e *R. cordifolia* (MERC, EFRC, and BFRC) and *L. cuneata* (MELC, EF LC and BFLC)

solutions were added with few pieces of magnesium ribbon and with the addition of few drops of conc. HCl. Pink colour appearance showed the presence of flavonoids.

II. Tests for Phenols a Tannins: 2ml (2%) $FeCl_3$ solutionwas added in 2ml of extract sample. Blue green colour showed the presence of phenol while black color showed the presence of tannins in the samples.

III. Tests for Saponins: 2 ml sample of plant was added with 5 ml distilled water in test tube and shaked it. Saponins shown were in form of foam formation.

IV. Tests for Terpenoids: 2 ml of chloroform was added in each extract sample then evaporated to dry, then 2ml of conc. H_2SO_4 was added and heating for two minutes, grayish colour appearance showed the presence of terpenoids while absence of grayish colour confirmed the absence of terpenoids.

V. Tests for Alkaloids: 2 ml of 1% HCl was added in extract samples of each plants followed by heating and addition of Mayer's and Wagner's reagents to this mixture. Turbity of precipitates showed the presence of alkaloids.

3.11.2. Quantitative Phytochemical Analysis

Phytochemical quantification of ME and active fractions of shortlisted antihyperglycemic active plants (*R. cordifolia* and *L. cuneata*) was performed for total phenolic contents (TPC) and total flavonoids contenta (TFC) with standard procedure (Sembiring *et al.*, 2018).

3.11.2.1. Assay for Total Phenolic Content (TPC)

TPC was estimated according to Folin-Ciocalteu method. Using Gallic acid (GA) as standard the absorbance was measured at 765nm. Firstly prepared GA solution then serial dilutions (500, 250, 125, 50, 25, 10, 5 and 2.5(μg/mL) respectively) were prepared from the stock solution. Similarly, the solution of all samples were prepared. Absorbance of all samples including gallic acid solutions was measured at 765 nm. Ploted the calibration curve of GA from values obtained by the difference of absorbance of tested GA solutions valuses and values of blank absorbance of GA. During this method, 4μL of extract solution +180μL

distilled water +4μL Folin-Ciocalteu reagent afterwards (all this was done in three replicates using 96-well plate) for each sample extract. Reagent was added and then shaked it vigorously. Aqueous sodium carbonate (2%) solution (12μL) of was added after 4 minutes and then the mixture was allowed to stand in dark with intermittent shaking. Blank was prepared with 4 μL extract solution +180 μL distilled water + 12μL of aqueous sodium carbonate (2%) solution, used against the test samples while reading absorbance at 765 nm. Calculation TPC concentration was performed by equation constructed from standard GA calibration curve. Results were expressed as μg per mg of GAE equivalent (μg/mg GAE) of extract.

$y = 0.005X - 0.011$, ($R^2 = 0.987$).

3.11.2.2. Assay for Total Flavonoid Contents (TFC)

Aluminum chloride method was used to evaluate total flavonoid content (TFC) with described method (Sembiring *et al.*, 2018) using quercetin as the standard. Different concentrations (500, 250, 125, 50, 25, 10, 5 and 2.5 (μg/mL)) of quercetin solution were prepared using methanol. Standard solutions were tested similar to samples using aluminium trichloride (2% $AlCl_3$). The measurement of absorbance was made at 415 nm against the blank quercetin solution. Quercetin calibration curve was plotted for the values obtained by subtracting the blank values from tested quercetin values. During this assay, 100 μL of extracts solution were added in the 100μL of 2% aluminium trichloride in methanol (all this was performed in three replicates using 96-well plate). Absorbance was read at 415 nm against blank samples (100-μL extract solution along with 100-μL methanol) after 40 minutes. Concentration of flavonoid compounds was calculated according to equation obtained from standard quercetin calibration curve. The results were expressed as μg /mg QE of extract. $y = 0.005X - 0.054$, ($R^2 = 0.962$).

3.12. Identification of Compounds

3.12.1. HPLC of Methanolic Extract of *R. cordifolia* (MERC) and *L. cuneate* (MELC) and their Most Active Fractions

HPLC was performed for ME and most active fractions of antihyperglycemic plants (*R. cordifolia* and *L. cuneata*) for the identification of bioactive chemical compounds. HPLC was carried by co-elusion with reference standard compounds (Ijaz *et al.*, 2019).

Reference Standards: In present investigation, 3 standards of phenolic compounds mangiferine, purpurine and Charatin were used for HPLC profiling of *R. cordifolia* and Kaempferol, mangiferine, purpurine and 2-methyl anthraquinone for *L. cuneata.*

Plant Samples: The samples for analysis were methanolic extracts of *R. cordifolia* and *L. cuneata* (MERC and MELC) and their most active antidiabetic fractions (EFRC, BFRC, EFLC and BFLC).

Preparation of Samples: All samples (plant extract and standards) were prepared in HPLC grade methanol (1mg/ml)) in amber Eppendorf tubes to avoid from effect of light. Solutions were sonicated (10 mint) for proper mixing. Nylon membrane filters (0.45μm) were used for filtration of samples in separate Eppendorf tubes.

Apparatus: The HPLC equipment used was a LC-2010 C HT system (Shi-madzu, Kyoto, Japan), having quaternary low pressure gradient pump with an auto-sampler, degasser, block-heating type column oven, UV detector set at 254nm and a Shi-maddu LC-solution workstation. An agilent 1200 HPLC (Agilent technologies Santa Clara CA) was equipped with an online vacuum degasser, a Quat pump, an automated injection valve, a thermostated column compartment; a DAD and an Agilent Chem Station were selected to analyze samples.

Conditions of HPLC: HPLC using C_{18} column (250 mm×4.6 mm i.d., 5 µm, Waters, MA, USA) in tandem with a Phenomenex C_{18} guard cartridge (4.0 mm×3.0 mm, Phenomenex, Torrance, CA). Column was eluted by using acetonitrile and water in 18 min. The flow rate was set at 1.0 mL/min while the column temperature was set at 30°C. Spectra were recorded from 190 to 400 nm while the chromatogram was acquired at 254 nm.

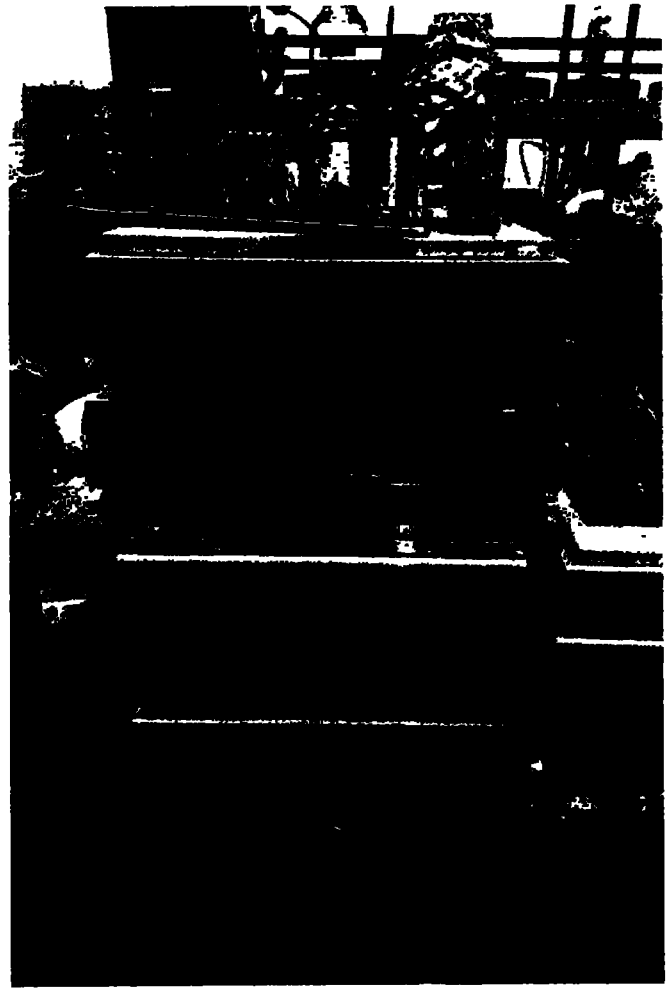

Figure 3.7: HPLC Instrument used for qualitative analysis of plants extracts and their active fractions

3.13. Statistical Analysis

Data was expressed as mean values ± standard error of means (S.E) with five animals in each group. Comparison between the groups was performed by one-way analysis of variance (ANOVA) using Statistics 8.1, followed by Tukey HSD test with $P < 0.05$ considering statistically significant (Khan *et al.*, 2020). Calculation of mean and standard deviation of DPPH assay and TPC and TFC was performed from calibration curve constructed by Excel 2010.

RESULTS

CHAPTER 4

RESULTS

4.1. Acute Oral Toxicity Test

Results of acute oral toxicity test showed that there was no behavioural change in the mice with test doses (250, 500, and 1000 mg/kg) of each plant extract. There was not any lethality in mice during 24 hours' observation. Mice remained normal up to 14 days observations.

4.2. Antihyperglycemic Effect of Methanolic Extract (ME) of Selected Plants

The effect of different treatments on blood glucose level (BGL) of mice is represented in table 4.1 while the body weight is represented in table 4.2 below. The BGL in the group 1(normal control) remained 96.00±0.65, 96.86±0.56, 98.44±0.64, 99.10±1.54, 98.60±0.93 (mg/dL/wk) from day zero up to 28th day respectively. The blood glucose level of group II (Diabetic control) was significantly increased 100.40±0.62, 256.15±0.78, 274.10±0.96, 294.40±0.74, 325.80±1.44 (mg/dL/wk) with the treatment of alloxan (100 mg/kg/wk) as compared to normal control group (G. I). BGL with the treatment of standard drug (Glibenclamide 0.5 mg/kg) and methanolic extract of selected plants (*R. haustatus, L. cuneata, R. cordifolia, N. grandifolia* and *S. delhousiana* with the dose of 100 mg/kg) in the treatment groups (G.III- G.VIII), after alloxan induced diabetes; was significantly reduced as compared to diabetic control group generally. This effect was highly significant ($P < 0.05$) on time dependent manner i.e after 14th day treatment as shown in table 4.1 and figure 4.1. The BGL was almost same before the treatment of standard drug and selected plant extract respectively on day seven. The comparison of blood glucose level among treatment groups (G.IV-G. VIII) of selected plants extract showed that G.V and G.VI highly reduced the blood glucose level which is comparable to standard drug (Glibenclamide) treatment (G.III) while the G.IV, VII and VIII have shown the similar effect with less reduction of BGL which was increased due to alloxan induction.

The table 4.2 showed the effect of different treatments on body weight (BW) in the group I (normal control) significantly increased, 26.14±0.67, 27.30±0.62, 30.80±0.75, 32.70±0.54, 36.32±0.58 (g/wk) from day zero up to 28th day respectively. The body weight in-group II (TC) was significantly decreased 27.40±0.92, 26.90±0.83, 25.54±0.66, 24.50±0.63, 22.80±0.68 (g/wk) with the treatment of alloxan (100 mg/kg) as compared to normal control group (G.1). BW with the treatment of standard drug (Glibenclamide 0.5mg/kg) and methanolic extract of selected plants (*R. haustatus, L. cuneata, R. cordifolia, N. grandifoliaand S. delhousiana* with the dose of 100 mg/kg) in the treatment groups (G.III-G.VIII) respectively, after alloxan induced diabetes; was significantly increased as compared to diabetic control group. This effect was highly significant ($P < 0.05$) on time dependent manner i.e after 14th day treatment as shown in table 4.2 and figure 4.2. The body weight was almost same before the treatment of standard drug and selected plant extract respectively on day seven. The comparison of blood glucose level among treatment groups (G. IV-G. VIII) of selected plants extract showed that in G. V and G.VI the body weight increases with passage of time, which is comparable to standard drug (Glibenclamide) treatment (G.III) while the G. IV, G.VII and G.VIII have shown the similar effect with reduction in BW which was decreased due to alloxan induction.

Table 4.1. Effect of methanolic extract of selected plants on blood glucose level of alloxan induced diabetic mice

S. No	Treatment	Blood Glucose concentration (mg/dL)				
		00 day	7th day	14th day	21st day	28th day
GI	Normal control	96.00±0.65^{b}	96.86±0.56^{d}	98.44±0.64^{f}	99.10±1.54^{g}	98.60±0.93^{f}
GII	DC(Alloxan 100mg/kg)	100.40±0.62^{ab}	256.15±0.78^{bc}	274.10±0.96^{c}	294.40±0.74^{c}	325.80±1.44^{b}
GIII	Diabetic+SD(0.5mg/kg)	99.10±1.10^{ab}	260.32±1.14^{ab}	220.54±0.78^{e}	192.62±1.42^{f}	151.51±1.01^{de}
GIV	Diabetic+MERH(100mg/kg)	96.70±0.75^{b}	252.20±1.35^{c}	274.73±1.14^{bc}	300.71±0.90^{ab}	312.90±1.44^{b}
GV	Diabetic+MELC(100mg/kg)	100.20±1.59^{ab}	256.40±1.24^{bc}	230.31±0.84^{d}	220.33±1.04^{d}	205.22±0.72^{cd}
GVI	Diabetic+MERC(100mg/kg)	98.30±1.44^{b}	259.80±1.19^{ab}	225.40±0.75^{e}	197.92±0.76^{e}	169.13±1.21^{de}
GVII	Diabetic+MENG(100mg/kg)	103.50±0.81^{ab}	258.30±2.02^{ab}	279.12±1.96^{bc}	297.91±1.16^{bc}	316.64±1.44^{b}
GVIII	Diabetic+MESD(100mg/kg)	96.66±0.88^{b}	263.10±1.74^{a}	286.62±0.92^{a}	303.62±1.25^{a}	316.53±1.07^{b}

Values are expressed as mean± SE (n=5) letters a, b, c... show the significant ($P < 0.05$) difference and same letters do not have the significant ($P > 0.05$) difference.

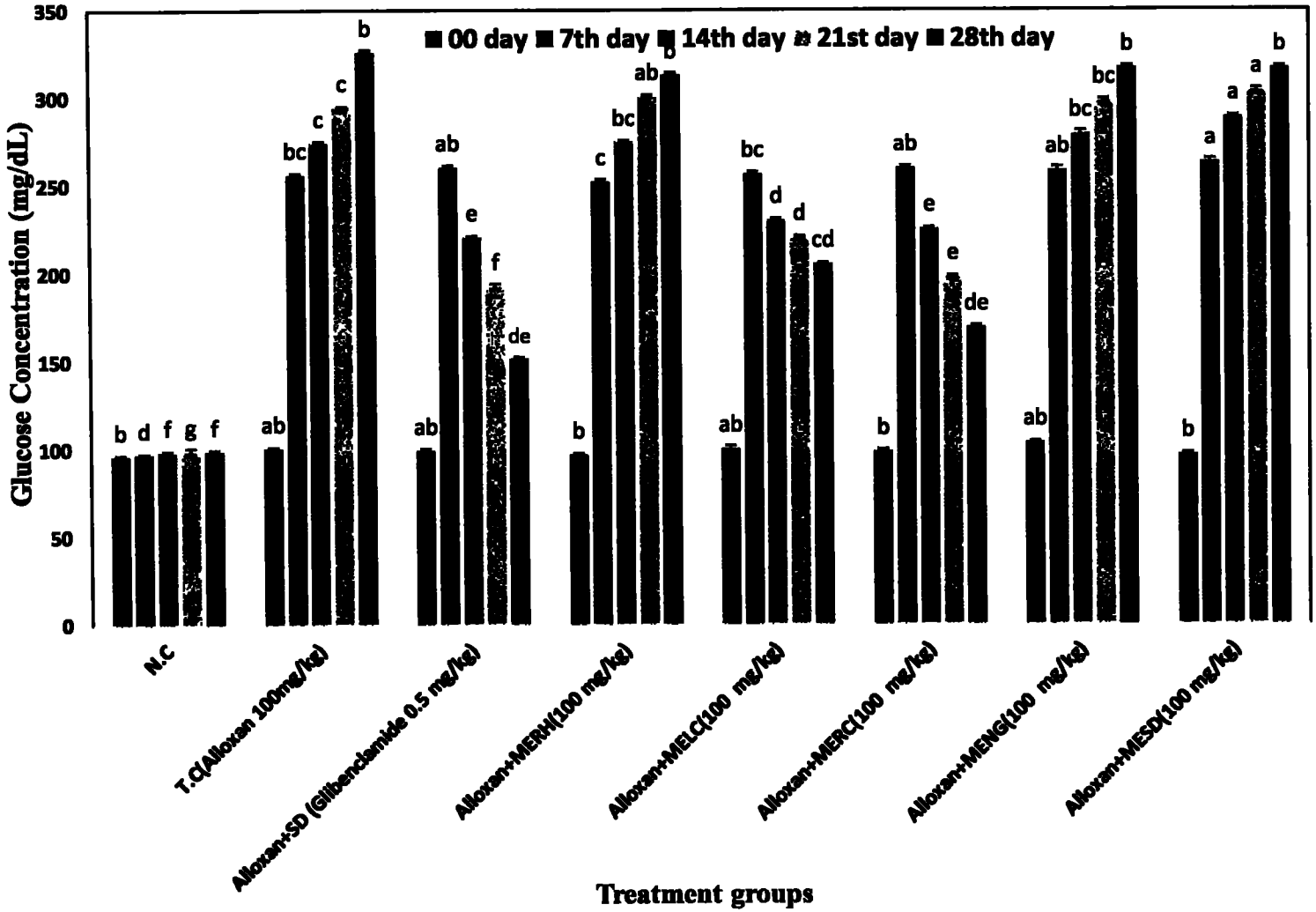

Figure 4.1: Effect of methnolic extract of selected plants on blood glucose level of alloxan induced diabetic mice

Table 4.2. Effect of methanolic extract of selected plants on body weight (BW) of alloxan induced diabetic mice

S. No	Treatment	Body weight (g)				
		00 day	7th day	14th day	21st day	28th day
GI	Normal control	26.14±0.67^{a}	27.30±0.62^{ab}	30.80±0.75^{ab}	32.70±0.54^{a}	36.32±0.58^{a}
GII	DC(Alloxan 100mg/kg)	27.40±0.93^{a}	26.90±0.83^{ab}	25.54±0.66^{c}	24.50±0.63^{b}	22.80±0.68^{d}
GIII	Diabetic+SD(0.5mg/kg)	27.70±1.30^{a}	28.40±1.51^{ab}	30.56±1.23^{ab}	31.40±1.13^{a}	33.90±0.73^{ab}
GIV	Diabetic+MERH(100mg/kg)	28.96±0.97^{a}	28.46±0.99^{ab}	27.40±0.87^{abc}	26.70±0.99^{b}	25.60±0.76^{cd}
GV	Diabetic+MELC(100mg/kg)	27.92±1.16^{a}	28.70±1.11^{ab}	30.40±0.94^{ab}	31.30±0.87^{a}	33.42±0.0.59^{b}
GVI	Diabetic+MERC(100mg/kg)	29.30±0.66^{a}	29.78±0.68^{ab}	31.14±0.726^{ab}	32.10±0.29^{a}	33.70±0.46^{ab}
GVII	Diabetic+MENG(100mg/kg)	25.26±0.81^{a}	25.26±0.81^{b}	24.30±0.768^{c}	23.80±0.77^{b}	22.80±0.64^{d}
GVIII	Diabetic+MESD(100mg/kg)	28.14±0.77^{a}	27.86±0.80^{ab}	26.92±0.72^{bc}	26.4±0.73^{b}	25.7±0.37^{c}

Values are expressed as mean± S.E (n=5) letters a, b, c… show the significant ($P < 0.05$) difference and same letters show non-significant ($P > 0.05$) difference.

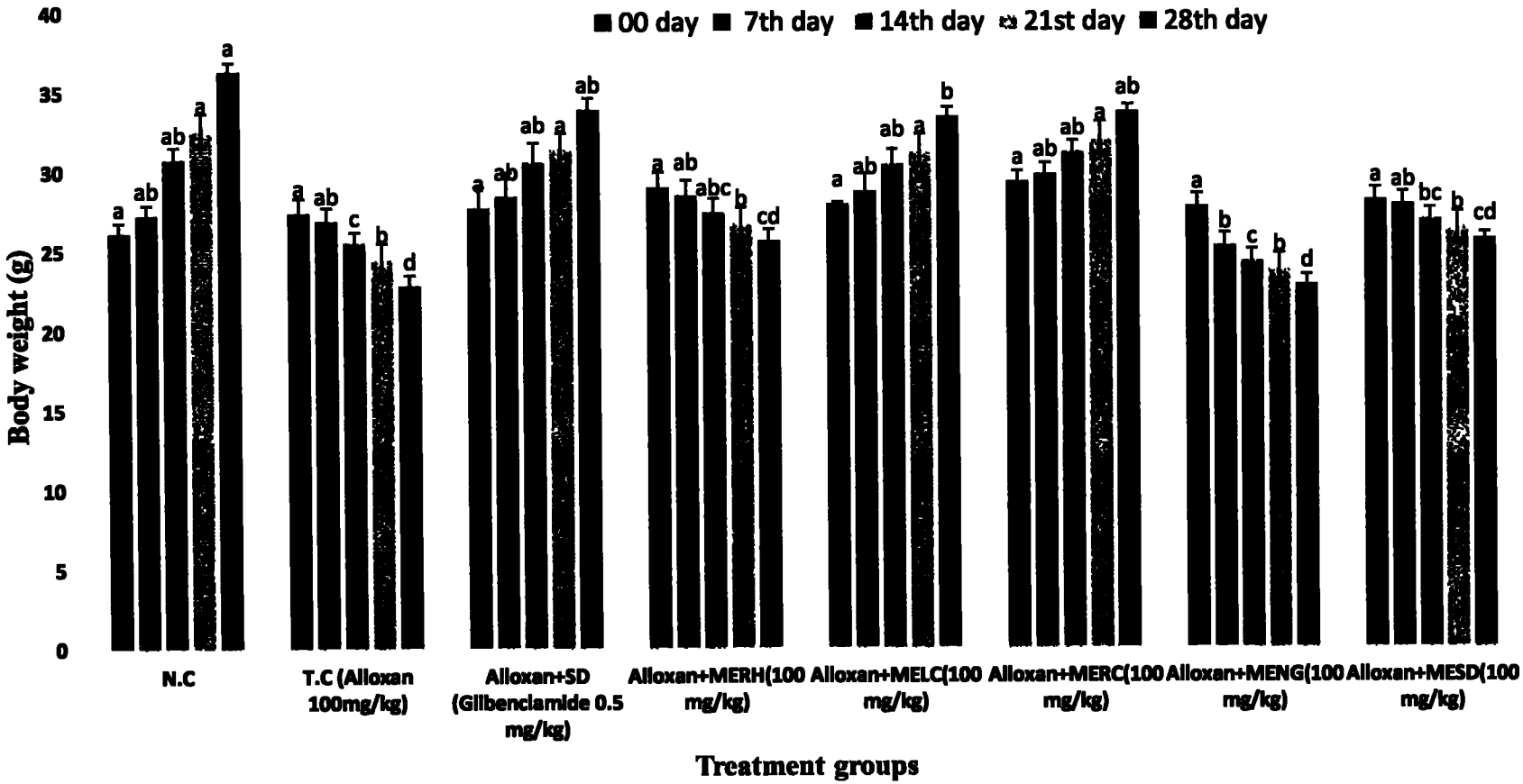

Figure 4.2: Effect of methnolic extract of selected plants on body weight of alloxan induced diabetic mice

Valuses are expressed as mean± S.E (n=5) letters a, b, c… show the significant (P<0.05) difference and same letters show non-significant (P>0.05) difference.

4.3. Antihyperglycemic Effect of Different Fractions of *R. cordifolia*

The effect of treatment of different fractions of *R. cordifolia* on BGL of alloxan induced diabetic mice is represented in table (4.3) while on body weight is represented in table (4.4) below. The BGL in the group I (NC) remained 95.80±1.19, 98.10±0.85, 99.30±1.32, 98.40±1.22, 99.36±0.88 (mg/dL/wk) from day zero up to 28th day respectively. The BGL of group II (TC) was significantly increased 97.86±1.32, 253.01±1.22, 263.52±1.13, 283.20±1.53, 313.44±1.27 (mg/dL/wk) with the treatment of alloxan (100 mg/kg) as compared normal control group I (NC). BGL with the treatment of standard drug (Glibenclamide 0.5 mg/kg) and different fractions of *R. cordifolia* (HFRC, EFRC, BFRC and AFRC) with the dose of 100mg/kg) in the treatment groups (G.III- G.AVII), after alloxan induced diabetes; was significantaly reduced as compared to diabetic control group generally. This effect was highly significant ($P < 0.05$) on time dependent manner i.e after 14th day treatment as shown in table 4.3 and figure 4.3. The blood glucose level was almost same before the treatment of standard drug and *R. cordifolia* fractions respectively on day seven. The comparison of blood glucose level among treatment groups (G.AIV-G. AVII) of *R. cordifolia* fractions showed that G. AV and G.AVI highly reduced the blood glucose level which is comparable to standard drug (Glibenclamide) treatment (G.III) while the G. AIVand G. AVII have shown the similar effect with less reduction in BGL which was increased due to alloxan induction.

The table (4.4) showed the effect of different treatments on body weight of mice. The body weight in the group (NC) significantly increased, 28.10±0.71, 28.90±0.75, 31.30±0.86, 32.62±0.93, 35.30±0.77 (g/wk) from day zero up to 28th day respectively. The body weight in group II (Diabetic control) was significantly decreased 29.00±1.23, 28.50±0.1.03, 27.10±0.83, 25.32±0.39, 23.62±0.54 with the treatment of alloxan (100 mg/kg/wk) as compared to normal control group I (NC). Body weight with the treatment of standard drug (Glibenclamide 0.5 mg/kg/wk) and different fractions of *R. cordifolia* (HFRC, EFRC, BFRC, AFRC with the dose of 100 mg/kg/wk) in the treatment groups (G.III- G. AVII), after alloxan induced diabetes; was significantaly increased as compared to diabetic control group generally. This effect was highly

significant ($P < 0.05$) on time dependant manner i.e after 14th day treatment as shown in table (4.4) and figure (4.4). The body weight was almost same before the treatment of standard drug and different fractions respectively on day seven. The comparison of blood glucose level among treatment groups (G.A IV-G. AVII) of selected plants extract showed that in G. AV and G.AVI the body weight increases with passage of time which is comparable to standard drug (Glibenclamide) treatment (G.III) while the G.AIV and G. AVII have shown the similar effect with reduction in body weight like group two (G-II).

Table 4.3 Effect of different fractions of *R. cordifolia* on blood glucose level of alloxan induced diabetic mice

S. No	Treatment	Blood glucose concentration (mg/dL)				
		00 day	7th day	14th day	21st day	28th day
G.I	Normal control	95.80±1.19^{b}	98.10±0.85^{c}	99.300±1.32^{d}	98.40±1.22^{e}	99.36±0.88^{e}
G.II	DC(Alloxan 100mg/kg)	97.86±1.32^{ab}	253.01±1.22^{b}	263.52±1.13^{b}	283.20±1.53^{b}	313.44±1.27^{a}
G.III	Diabetic+SD(0.5mg/kg)	96.98±1.19^{ab}	256.64±1.78^{ab}	228.10±1.18^{c}	187.40±0.95^{d}	154.64±1.22^{d}
G.AIV	Diabetic+ HFRC (100mg/kg)	99.70±1.04^{ab}	255.04±2.34^{ab}	272.70±1.22^{a}	291.24±1.68^{a}	309.6±1.08^{a}
G.AV	Diabetic+EFRC((100mg/kg)	102.18±1.51^{ab}	259.80±1.64^{ab}	231.82±1.74^{c}	210.50±0.93^{c}	178.02±1.04^{c}
G.AVI	Diabetic+BFRC(100mg/kg)	99.18±1.70^{ab}	262.50±1.27^{ab}	232.00±1.04^{c}	212.80±0.92^{c}	197.82±1.39^{b}
G.AVII	Diabetic+AFRC(100mg/kg)	97.54±1.26^{ab}	254.66±2.06^{b}	276.10±2.94^{a}	292.80±2.05^{a}	310.9±1.15^{a}

Valuses are expressed as mean± S.E (n=5) different letters (a, b, c…) show the significant (P<0.05) difference and same letters show non-significant (P>0.05) difference.

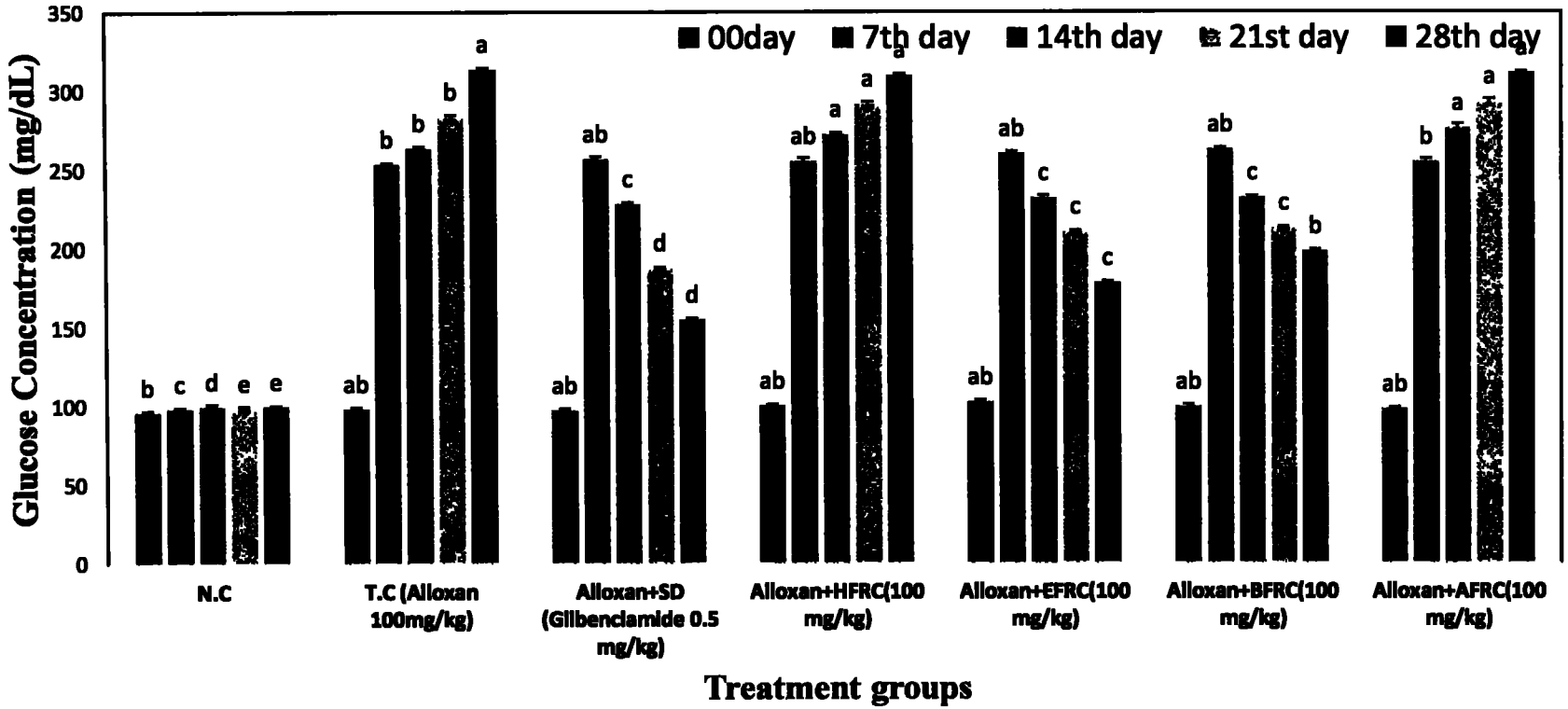

Figure 4.3: Effect of different fractions of *R. cordifolia* on blood glucose level (BGL) of alloxan induced diabetic mice
Values are expressed as mean± S.E (n=5) letters a, b, c... show the significant (P<0.05) difference and same letters show non-significant (P>0.05) differences.

Table 4.4. Effect of different fractions of *R. cordifolia* on body weight of alloxan induced diabetic mice

S. No	Treatment	Body weight (g)				
		00 day	7th day	14th day	21st day	28th day
G.I	Normal control	28.10±0.71^{a}	28.90±0.75^{a}	31.30±0.86^{a}	32.62±0.92^{a}	35.30±0.77^{a}
G.II	DC(Alloxan 100mg/kg)	29.00±1.22^{a}	28.50±0.1.02^{a}	27.10±0.83^{bc}	25.32±0.39^{b}	23.62±0.54^{b}
G.III	Diabetic+SD(0.5mg/kg)	27.60±0.81^{a}	27.86±0.87^{a}	30.04±1.31^{abc}	31.76±1.62^{a}	34.8±2.29^{a}
G.AIV	Diabetic+ HFRC (100mg/kg)	28.00±1.38^{a}	27.50±1.16^{a}	26.50±1.09^{c}	26.10±1.07^{b}	25.10±0.90^{b}
G.AV	Diabetic+ EFRC (100mg/kg)	27.80±1.02^{a}	28.18±1.02^{a}	29.66±0.94^{abc}	30.76±0.54^{a}	33.6±0.53^{a}
G.AVI	Diabetic+BFRC(100mg/kg)	27.2±0.78^{a}	28.34±0.64^{a}	30.52±0.41^{abc}	31.12±0.23^{a}	33.2±0.17^{a}
G.AVII	Diabetic+AFRC(100mg/kg)	28.08±0.45^{a}	27.6±0.37^{a}	26.60±0.24^{bc}	25.56±0.46^{b}	24.52±0.53^{b}

Valuses are expressed as mean± S.E (n=5) letters a, b, c... show the significant (P<0.05) difference and same letters show non-significant (P>0.05) differences.

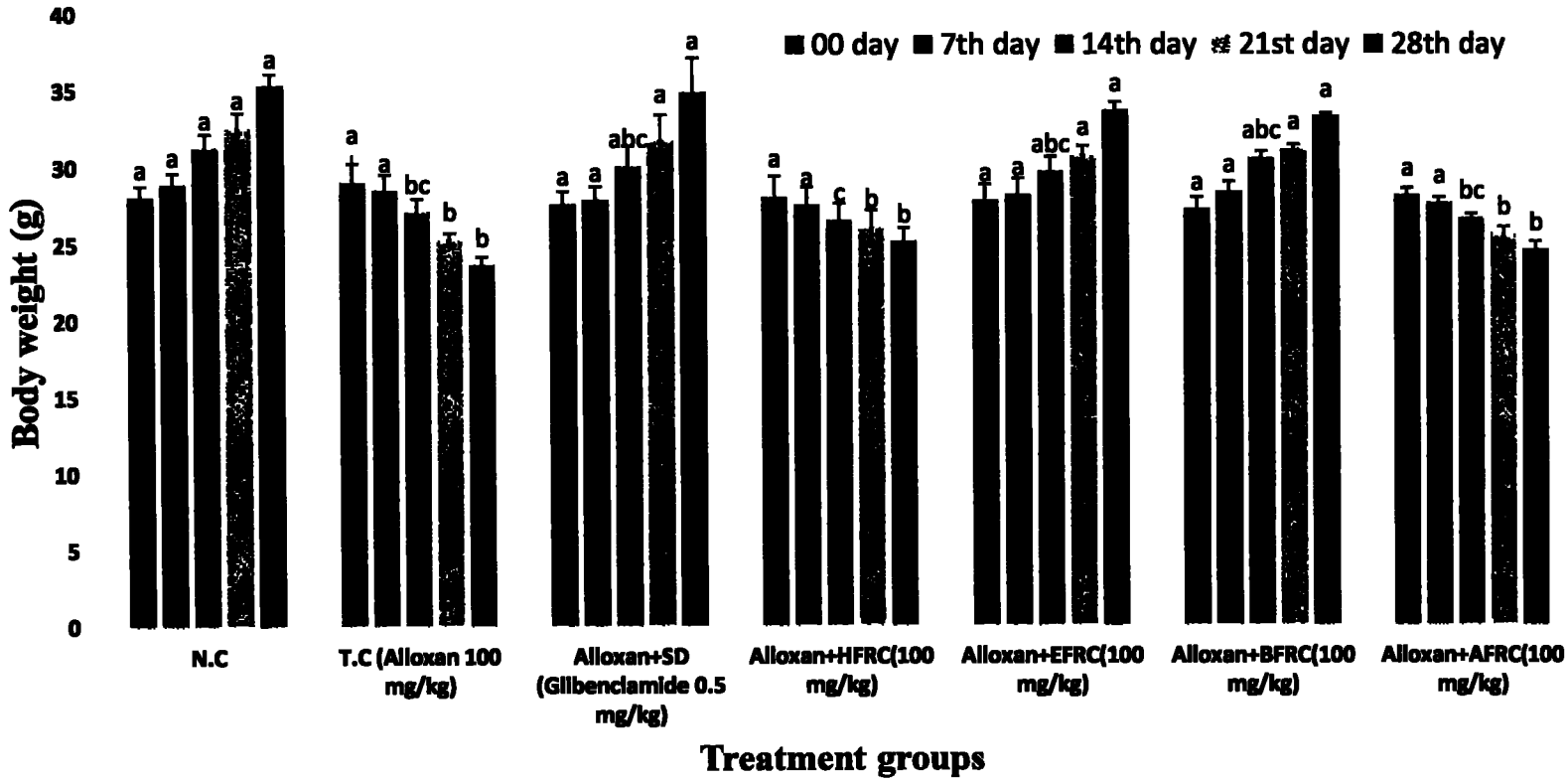

Figure 4.4: Effect of different fractions of *R. cordifolia* on body weight (BW) of alloxan induced diabetic mice

Values are expressed as mean± S.E (n=5) letters a, b, c... show the significant (P<0.05) difference and same letters show non-significant (P>0.05) differences.

4.4. Antihyperglycemic Effect of Different Fractions of *L. cuneata*

The effect of treatment of different fractions of *L. cuneata* on BGL of alloxan induced diabetic mice is represented in table 4.5 while on BW is represented on in table 4.6 below. The BGL in the group I (NC) remained 95.80±1.19, 98.00±0.85, 98.48±1.42, 98.40±1.23, 98.26±0.73 (mg/dL/wk) from day zero up to 28th day respectively. The BGL of group II (TC) was significantly increased 97.86±1.32, 253.00±1.22, 263.52±1.13, 283.20±1.53, 313.44±1.27 (mg/dL/wk) on treatment of alloxan (100 mg/kg) as compared normal control group (G. I). BGL with treatment standard drug (Glibenclamide 0.5 mg/kg) and different fractions of *L. cuneata* (HFLC, EFLC, BFLC and AFLC) with the dose of 100mg/kg) in the treatment groups (G.III- G. BVII), after alloxan induced diabetes; was significantly reduced as compared to diabetic control group generally. This effect was highly significant ($P < 0.05$) on time dependent manner i.e after 14th day treatment as shown in table 4.5 and figure 4.5. The blood glucose level was almost same before the treatment of standard drug and *L. cuneata* fractions respectively on day seven. The comparison of blood glucose level among treatment groups (G.BIV-G. BVII) of *L. cuneate* fractions showed that G.BV and G.BVI highly reduced the blood glucose level which is comparable to standard drug (Glibenclamide) treatment (G.III) while the G. BIVand G. BVII have shown the similar effect with less reduction in body weight which was decreased due to alloxan induction.

The table 4.6 showed the effect of different treatments on body weight of mice. The body weight in the group I (normal control) significantly increased, 27.94±0.80, 28.90±0.75, 31.30±0.86, 32.62±0.93, 35.30±0.77 (g/wk) from day zero up to 28th day respectively. The body weight in-group II (Diabetic control) was significantly decreased 28.06±0.61, 27.10±0.68, 26.14±0.69, 25.08±0.50, 23.62±0.54 (g/wk) with the treatment of alloxan (100 mg/kg) as compared normal control group I (NC). Body weight with treatment of standard drug (Glibenclamide 0.5 mg/kg) and different fractions of *R. cordifolia* (HFLC, EFLC, BFLC, AFLC with the dose of 100 mg/kg) in the treatment groups (G.III- G. BVII), after alloxan induced diabetes; was significantly increased as compare to diabetic control group II (TC) generally. This effect was highly significant ($P < 0.05$) on time dependent manner

i.e. after 14th day treatment as shown in table 4.6 and figure 4.6 below. The body weight was almost same before the treatment of standard drug and different fractions respectively on day seven. The comparison of blood glucose level among treatment groups (G.BIV-G. BVII) of selected plants extract showed that in G.BV and G.BVI the body weight increases with passage of time which is comparable to standard drug (Glibenclamide) treatment (G.III) while the G. BIVand BVII have shown the similar effect with reduction in body weight which was decreased due to alloxan induction.

Table 4.5. Effect of different fractions of *L. cuneata* on blood glucose level of alloxan induced diabetic mice

S. No	Treatment	Glucose concentration(mg/dL)				
		00 day	7th day	14th day	21st day	28th day
G.I	Normal control	95.80±1.19^{a}	98.00±0.85^{b}	98.48±1.42^{e}	98.40±1.22^{f}	98.26±0.75^{e}
G.II	DC(Alloxan 100mg/kg)	97.86±1.32^{a}	253.00±1.21^{a}	263.52±1.13^{b}	283.20±1.53^{b}	313.44 ±1.27^{a}
G.III	Diabetic+SD(0.5mg/kg)	96.98±1.19^{a}	256.64±1.78^{a}	228.1±1.18^{d}	187.40±0.95^{e}	154.64±1.22^{d}
G.BIV	Diabetic+ HFLC (100mg/kg)	98.52±0.35^{a}	254.48±0.86^{a}	278.44±0.86^{a}	298.00±1.27^{a}	308.62±1.90^{a}
G.BV	Diabetic+ EFLC((100mg/kg)	99.00±0.72^{a}	254.70±1.32^{a}	234.84±1.32^{c}	216.40±1.34^{c}	191.22±1.40^{b}
G.BVI	Diabetic+BFLC(100mg/kg)	97.82±0.55^{a}	255.60±1.04^{a}	225.90±1.04^{d}	205.70±1.24^{d}	183.10±1.91^{c}
G.BVII	Diabetic+AFLC(100mg/kg)	98.12±1.13^{a}	254.2±1.52^{a}	276.04±1.52^{a}	294.54±1.01^{a}	308.12±0.98^{a}

Values are expressed as mean± S.E (n=5) different letters (a, b, c…) show the significant (P<0.05) difference and same letters show non-significant (P>0.05) differences.

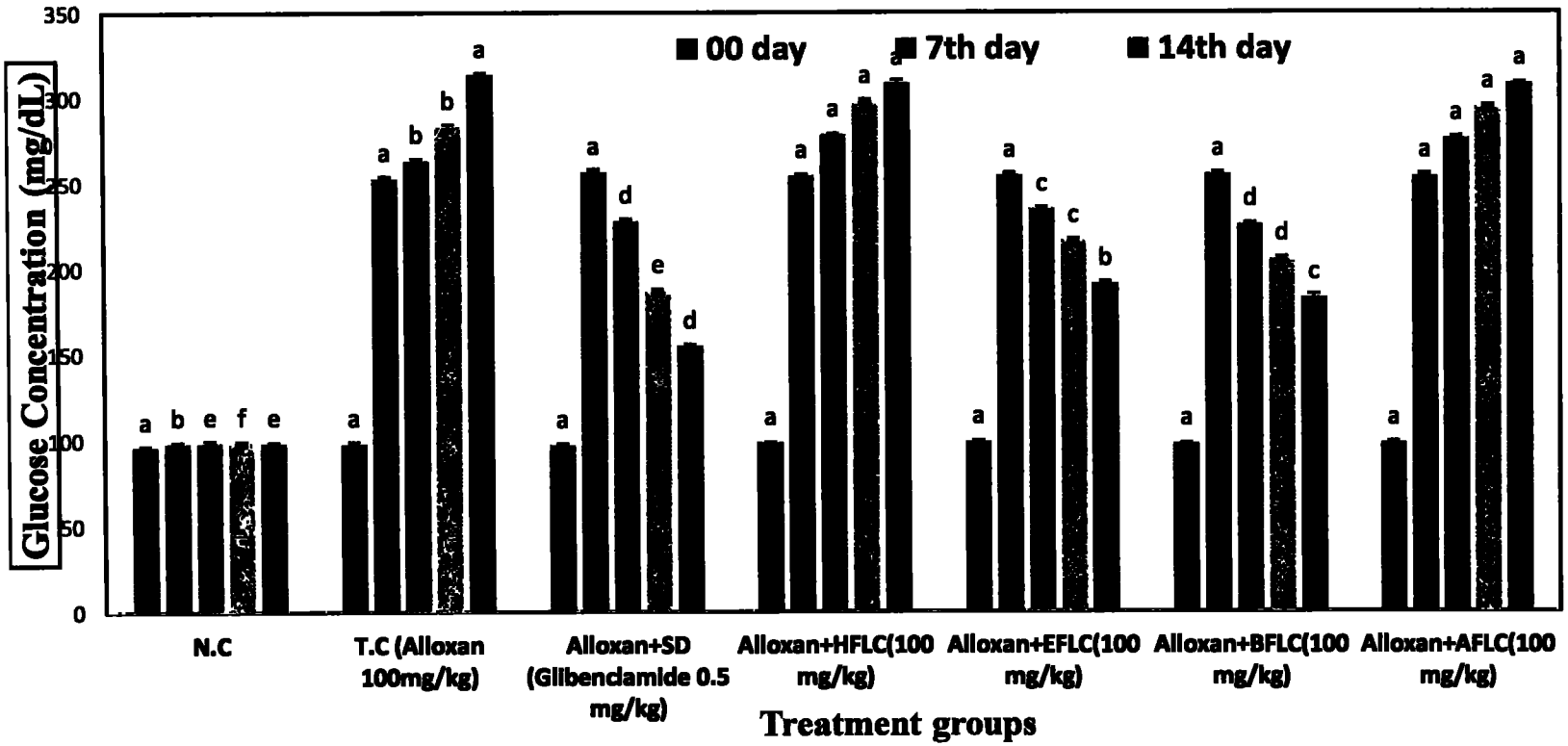

Figure 4.5: Effect of different fractions of *L. cuneata*on blood glucose level (BGL) of alloxan induced diabetic mice

Values are expressed as mean± S.E (n=5) letters a, b, c... show the significant (P<0.05) difference and same letters show non-significant (P>0.05) difference.

Table 4.6 Effect of different fractions of *L. cuneata* on body weight of alloxan induced diabetic mice

S. No	Treatment	Body weight (g)				
		00 day	7th day	14th day	21st day	28th day
G.I	Normal control	27.94±0.80[a]	28.90±0.75[a]	31.30±0.86[a]	32.62±0.93[a]	35.30±0.77[a]
G.II	DC(Alloxan 100mg/kg)	28.06±0.61[a]	27.10±0.68[a]	26.14±0.69[b]	25.08±0.50[b]	23.62±0.54[b]
G.III	Diabetic+SD(0.5mg/kg)	28.04±0.69[a]	28.80±0.80[a]	30.00±0.62[a]	32.48±0.35[a]	34.70±0.46[a]
G.BIV	Diabetic+ HFLC (100mg/kg)	26.86±0.53[a]	26.18±0.55[a]	25.30±0.58[b]	24.66±0.56[b]	23.92±0.41[b]
G.BV	Diabetic+ EFLC((100mg/kg)	27.02±0.76[a]	28.34±0.94[a]	29.96±1.08[a]	31.50±0.79[a]	34.42±0.53[a]
G.BVI	Diabetic+BFLC(100mg/kg)	27.20±0.78[a]	28.50±0.55[a]	30.52±0.41[a]	31.56±0.23[a]	33.76±0.46[a]
G.BVII	Diabetic+AFLC(100mg/kg)	28.50±0.42[a]	27.30±0.56[a]	26.40±0.40[b]	25.36±0.53[b]	24.32±0.61[b]

Values are expressed as mean± S.E (n=5), different letters (a, b, c...) show the significant ($P<0.05$) difference and same letters show non-significant ($P>0.05$) difference.

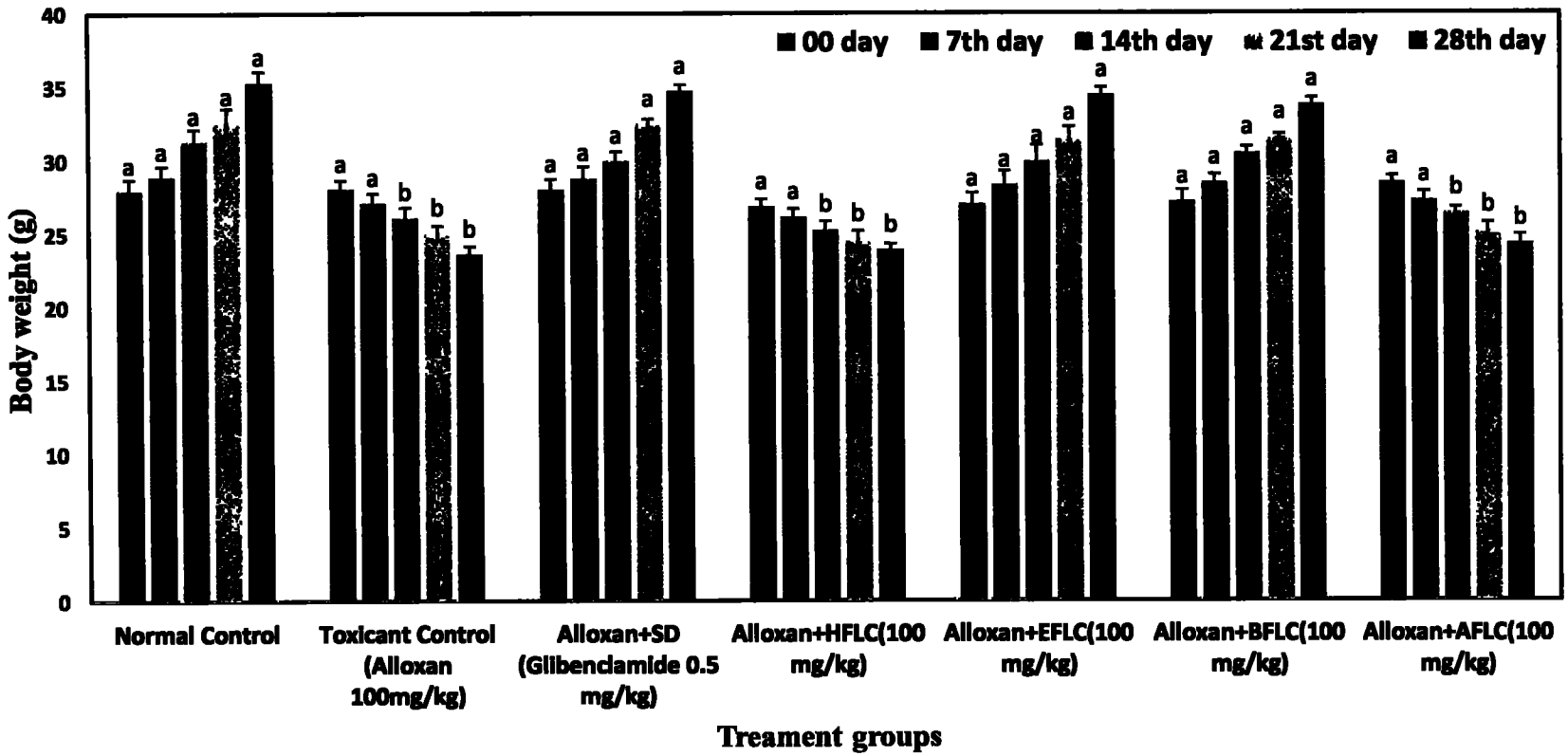

Figure 4.6: Effect of different fractions of *L. cuneata* on BW of alloxan induced diabetic mice

Values are expressed as mean± S.E (n=5) letters a, b, c… show the significant (P<0.05) difference and same letters show non-significant (P> 0.05) difference.

4.5. Antioxidant Activity of Methanolic Extracts and Fractions of Shortlisted Active Plants (DPPH radical scavenging assay)

DPPH assay for ME and fractions of *R. cordifolia,* and *L. cuneata* was determined using ascorbic acid (standard antioxidant compound). Absorbance of selected concentrations (10, 25, 35, 70, 110 and 150 (μg/mL) of ascorbic acid and samples were measured. Calibration curve for ascorbic acid and of test samples was plotted to determine the percent inhibition. IC_{50} value was calculated from regression equation.

$$Y = 0.711X+25.51, \qquad R^2 = 0.976$$

4.5.1. DPPH Radical Scavenging Activity of *R. cordifolia* and *L. cuneata*

When concentration of sample is increased then percent (%) radical scavenging effect of *R. cordifolia* is also increased (Table 4.7) that showed concentration dependent effect both by standard antioxidant ascorbic acid (AA) and samples. Methanolic extract (MERC) as well as its different fractions (HFRC, EFRC, BFRC and AFRC) exhibited the radical scavenging effect (% inhibition). AA showed the highest activity (126.3±0.70) followed by EFRC (123.11±0.85), BFRC (120.53±1.36), MERC (119.00±0.87), AFRC (109.10±0.85) and HFRC (106.77±1.66) shown below (Table 4.7). Standard ascorbic acid concentrations (10, 25, 35, 70, 110 and 150 (μg/mL) was used to make calibration curve for calculation of IC_{50}. The samples (MERC, HFRC, EFRC, BFRC, AFRC and AA) have the IC_{50} value of 38.19, 48.52, 34.9, 36.86, 44.88 and 34.41 (μg/mL) respectively. EFRC showed lowest IC_{50} value 29.92 μg/m L. The order of decreasing IC_{50} values of MERC and its different fractions is; HFRC> AFRC> MERC> BFRC> EFRC (having IC_{50} values of 48.52μg/mL 44.88μg/mL, 38.19μg/mL, 36.86μg/mL, and 34.9μg/mL) respectively. Standard ascorbic acid have IC_{50} value 34.41 μg/mL.The highest antioxidant activity is exhibited by sample; having lowesr IC_{50} value. Similarly, the scavenging effect (%) of *L. cuneata* is also increased with increasing concentration of sample (Table 4.7). MELC as well as its different fractions (HFLC, EFLC, BFLC and AFLC) have shown high

scavenging effect (% inhibition). AA showed highest scavenging effect (126.3±0.70) followed by BFLC (125.13±1.03), EFLC (124.37±1.21), MELC (120.13±0.81), AFLC (110.41±1.43) and HFLC (108.33±2.08) as shown by below (Table 4.7). Standard ascorbic acid concentrations (10, 25, 35, 70, 110 and 150 (μg/mL) were used for calculation of IC_{50} by construction of calibration curve. The tested samples (MELC, HFLC, EFLC, BFLC, AFLC and AA have the IC_{50} value of 36.4, 47.08, 31.42, 30.89, 47.49 and 34.41 (μg/mL) respectively. BFLC showed lowest IC_{50} value 30.89μg/m L. The order of decreasing IC_{50} values of MELC and its different fractions is; AFLC> HFLC> MELC> EFLC> BFLC (having IC_{50} values of 47.49 μg /mL 47.08μg/mL, 36.4μg/mL, 31.42μg/mL, and 30.89μg/mL) respectively showing that samples with lowest IC_{50} value showed highest antioxidant activity.

Table 4.7: Percent (%) DPPH radical- scavenging activity of *R. cordifolia* and *L. cuneata* extract/fractions and ascorbic acid (A.A) with IC_{50} values

S. No	Plant Extract /Fraction	Radical scavenging effect (%) at different concentrations (µg/mL) SD						IC_{50} (µg/mL)
		10	25	35	70	110	150	
1	MERC	24.40±0.55	35.73±1.50	48.27±0.06	88.00±0.92	103.67±1.47	119.00±0.87	38.19
2	HFRC	19.73±0.31	30.27±1.14	43.47±1.29	76.50±1.32	92.20±0.92	106.77±1.66	48.52
3	EFRC	26.11±1.11	36.20±0.80	52.20±1.01	91.57±1.25	107.00±1.80	123.11±0.85	34.9
4	BFRC	25.10±1.15	36.10±1.05	49.87±1.21	89.13±2.00	105.10±1.15	120.53±1.36	36.86
5	AFRC	23.13±1.03	32.30±1.21	44.27±1.32	78.47±1.55	96.50±1.32	109.10±0.85	44.88
6	MELC	26.23±0.80	36.30±0.96	49.43±1.40	89.30±0.62	105.07±0.90	120.13±0.81	36.4
7	HFLC	20.27±0.95	31.50±0.70	45.23±0.75	77.13±0.81	92.07±1.83	108.33±2.08	47.08
8	EFLC	28.40±0.53	38.07±1.10	53.97±1.27	97.17±1.11	107.20±0.72	124.37±1.21	31.42
9	BFLC	27.07±1.10	37.57±1.60	52.53±1.29	95.03±1.12	108.50±0.62	125.13±1.03	30.89
10	AFRC	21.10±1.01	31.17±1.26	40.60±1.68	77.07±2.10	96.30±0.76	110.41±1.43	47.49
11	AA	26.12±0.98	42.04±0.01	52.2±1.11	85±1.00	106.02±1.03	126.3±0.70	34.41

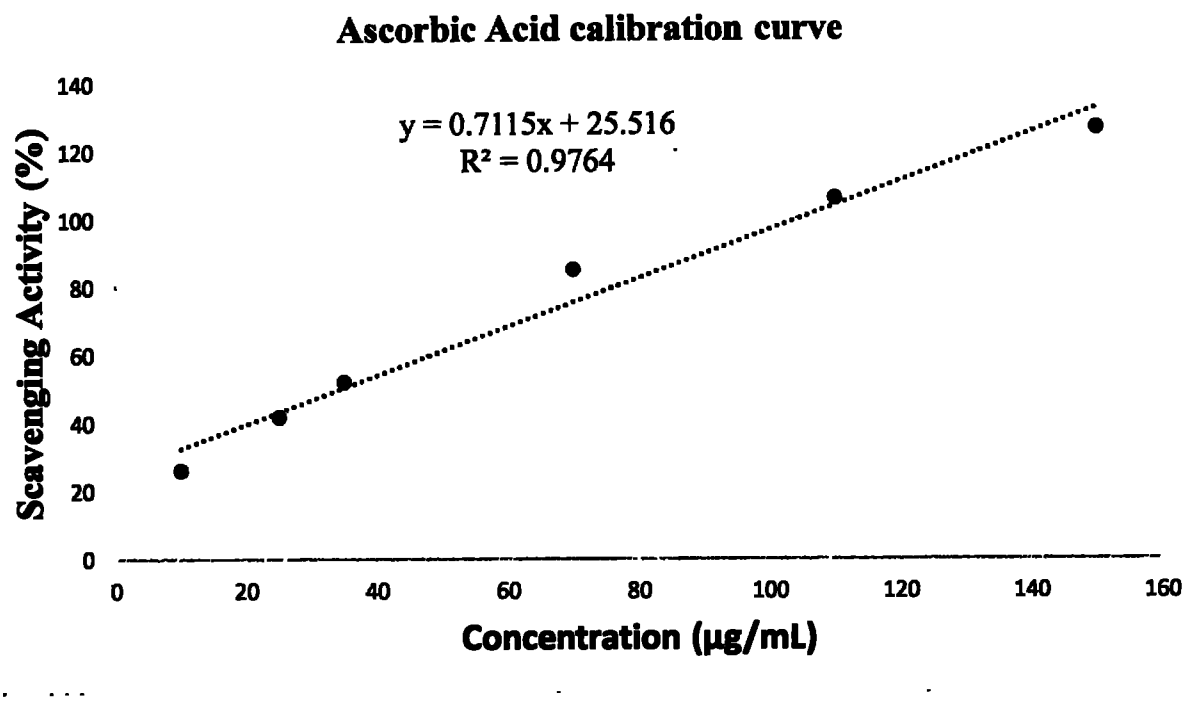

Fig 4.7: Ascorbic Acid standard curve for IC_{50}

4.6. Phytochemical Analysis (Qualitative)

Results of qualitative analysis of *R. Cordifolia* methanolic extracts (MERC), *L. cuneata* methanolic extracts (MELC) and their most active fractions (EFRC, BFRC and EFLC, BFLC) are represented in table (4.8)

The results show that in case of *R. cordifolia* alkaloids, phenols, flavonoids, tannins, saponins and terpenoids are present in methanolic extract (MERC). The ethyl acetate fraction (EFRC) showed the presence of phenols, flavonoids and saponnins where as alkaloids tennin and terpenoids were found absent. The n-butanol fraction of *R. cordifolia* (BFRC) showed the presence of phenols and flavonids, saponins and terpenoids while alkaloids and tannins were found absent. The results show that in case of *L. cuneata* alkaloids, phenols, flavonoids, tannins, saponins and terpenoids are present in methanolic extract (MELC). Its ethylacetate fraction (EFRC) showed the presence of alkaloids, flavoniods, saponins and terpenoids while phenols and tannins were found to be absent. The

n-butanol fraction of *R. cordifolia* (BFRC) showed the presence of phenols, flavonoids, tannins and terpenoids while alkaloids and saponins were found absent as shown in table (4.8) below.

Table 4.8. Qualitative analysis of phytochemicals

Phytochemicals	Plants /extract					
	R. cordifolia			*L. cuneata*		
	MERC	EF RC	BFRC	MELC	EF LC	BFLC
Alkaloids	+	−	−	+	+	−
Phenols	+	+	+	+	−	+
Flavonoids	+	+	+	+	+	+
Tannins	+	−	−	+	−	+
Saponins	+	+	+	+	+	−
Terpenoids	+	−	+	+	+	+

MERC= Methanolic extract of *R. cordifolia*, EFRC= Ethyl acetate fraction of *R. cordifolia*, BFRC= n-Butanol fraction of *R. cordifolia*, MELC= Methanolic extract of *L. cuneata*, EFLC= Ethyl acetate fraction of *L. cuneata* and BFLC= n-Butanol fraction of *L. cuneata*.

+ (Present) − (Absent

4.7. Quantative Analysis (Total phenolic contents and Total Flavonoid contents)

4.7.1. Total Phenolic Contents (TPC)

The total phenolic contents (TPCμg/mg GAE) were calculated according to standard calibration curve of Gallic acid (G.A μg/mL) as represented in fig. (4.8) below. The results show the presence of phenolic contents of *R. cordifolia* and *L. cuneata* in table (4.9) and (4.10) below. The results show that TPC in MERC, EFRC and BFRC have 99.732±0.938, 207.306±0.730 and 119.120±0.893 (µg/mg GAE) respectively. Similarly, the results of *L. cuneata* show the presence of phenolic contents in table (Table 4.10) below. The results show that TPC in MELC, EFLC and BFLC have 101.269±1.039, 80.699±0.542 and 204.184±0.986 (µg/mg GAE) respectively.

Table 4.9 TPC of *R. cordifolia*

Sr. No	Plant/Extract	TPC (µg/mg GAE), Mean ± S.D
1	MERC	99.732±0.938
2	EFRC	207.306±0.730
3	BFRC	119.120±0.893

MERC= Methanolic extract of *R. cordifolia*, EFRC= Ethyl acetate fraction of *R. cordifolia* and BFRC= n-Butanol fraction of *R. cordifolia*.

Table 4.10 TPC of *L. cuneata*

Sr. No	Plant/Extract	TPC (µg/mg GAE), Mean ± S.D
1	MELC	101.269±1.039
2	EFLC	80.699±0.542
3	BFLC	204.184±0.986

MELC= Methanolic extract of *L. cuneata*, EFLC= Ethyl acetate fraction of *L. cuneata* and BFLC= n-Butanol fraction of *L. cuneata*.

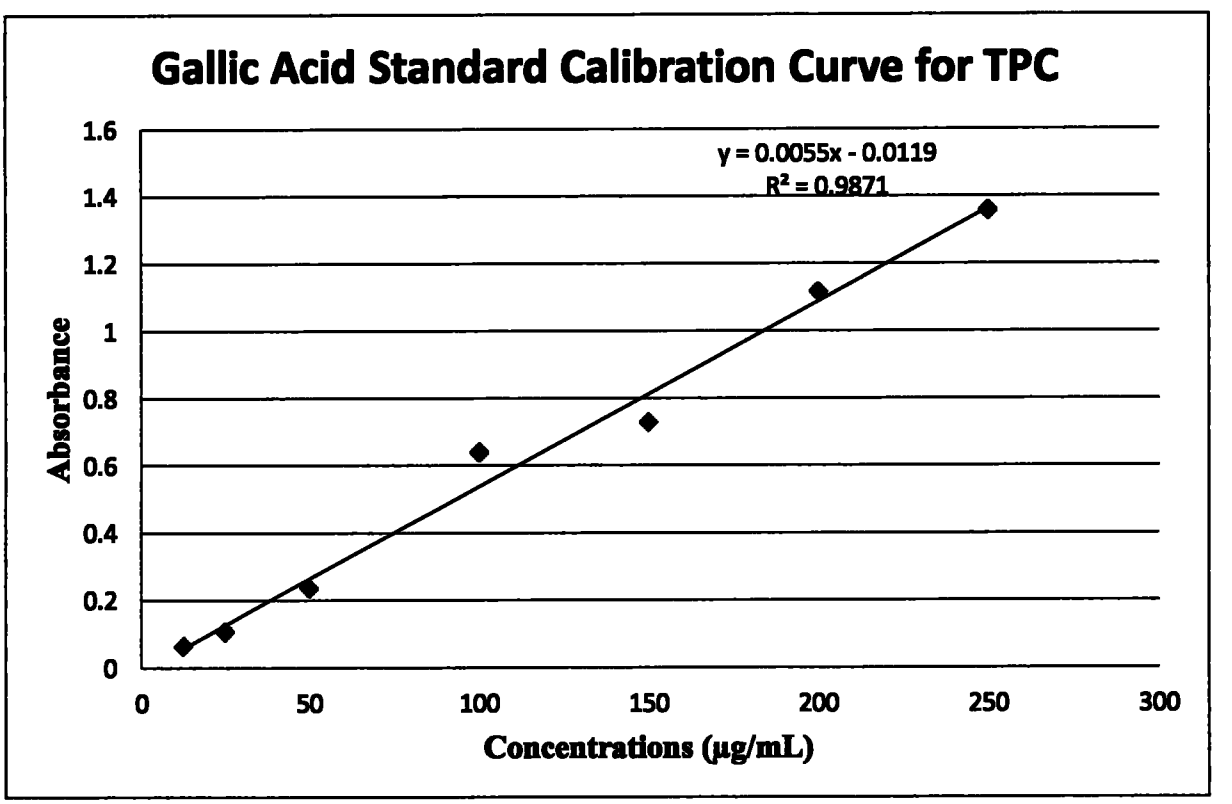

Figure 4.8: Gallic acid calibration curve

4.7.2. Total Flavonoids Contents (TFC)

The contents of total flavonoids (TFC μg/mg QE) were calculated according to standard calibration curve of Quercetin (µg/mL) as represented in figure 4.9 below. The results show the presence of total flavonoids contents (TFC) of *R. cordifolia* and *L. cuneata* in table 4.11 and 4.12 below. The results show that TFC in MERC, EFRC and BFRC have 96.564±0.996, 195.224±0.940 and 76.848±0.519 (µg/mg QE) respectively. Similarly, the results show the presence of total flavonoids contents (TFC) of *L. cuneata* in table 4.12 below.The results show that TFC in MELC, EFLC and BFLC have 86.376±1.012, 95.410±9.873and 203.970±0.359 (µg/mg QE) respectively.

Table 4.11. TFC (µm/mg QE) of *R. cordifolia*

Sr. No	Plant/Extract	TFC (µg/mg QE), Mean ± S.D
1	MERC	96.564±0.996
2	EFRC	195.224±0.940
3	BFRC	76.848±0.519

MERC= Methanolic extract of *R. cordifolia*, EFRC= Ethyl acetate fraction of *R. cordifolia* and BFRC= n-Butanol fraction of *R. cordifolia.*

Table 4.12. TFC of (µm/mg QE) *L. cuneata*

Sr. No	Plant/Extract	TFC (µg/mg QE), Mean ± S.D
1	MELC	86.376±1.012
2	EFLC	95.410±9.873
3	BFLC	203.970±0.359

MELC= Methanolic extract of *L. cuneata*, EFLC= Ethyl acetate fraction of *L. cuneata* and BFLC= n-Butanol fraction of *L. cuneata*.

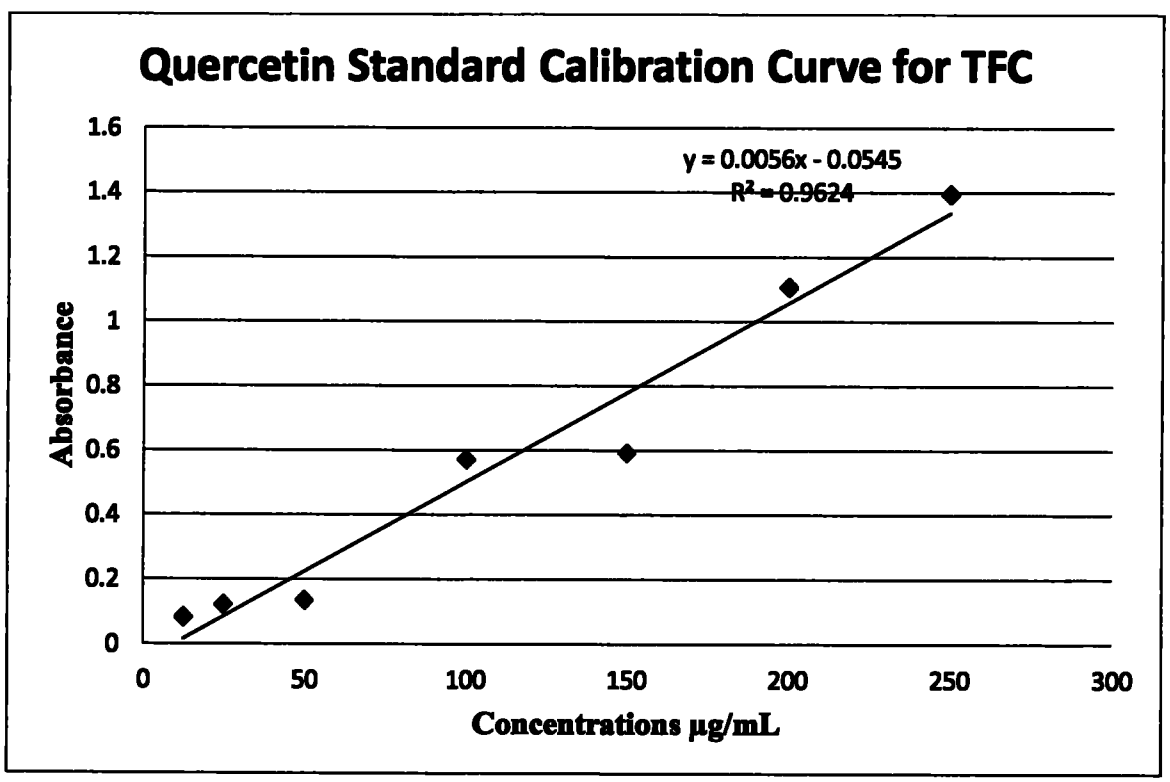

Figure 4.9: Quercetin Calibration Curve

4.8. HPLC of *R. cordifolia*

HPLC of most active plant (*R. cordifolia*) against antihyperglycemia is presented in figure (figure 4.13, figure 4.14, and figure 4.15) below. The chromatograms of standard compounds are shown in figures (figure 4.10, figure 4.11, and figure 4.12) below. Mangiferines, Purpurine and Charatine were three standard compounds used whose retention time is 9.102, 12.400, and 31.814 (min). There is shown the presence of mangiferine in all the samples while the purpurine is present in methanol extract and ethylacetae fractions whereas the charatine is absent in all fractions.

Table 4.13. HPLC of identified compounds in *R. cordifolia*

Sr. No	Extract	Compound identified	R.T (mint) of identified comp.	R.T (mint) standards
1	MERC	Mangiferine	9.158	9.102
		Purpurine	12.398	12.400
2	EFRC	Mangiferine	9.125	9.102
		Purpurine	12.494	12.400
3	BFRC	Mangiferine	9.102	9.102

MERC= Methanolic extract of *R. cordifolia*, EFRC= Ethyl acetate fraction of *R. cordifolia* and BFRC= n-Butanol fraction of *R. cordifolia*.

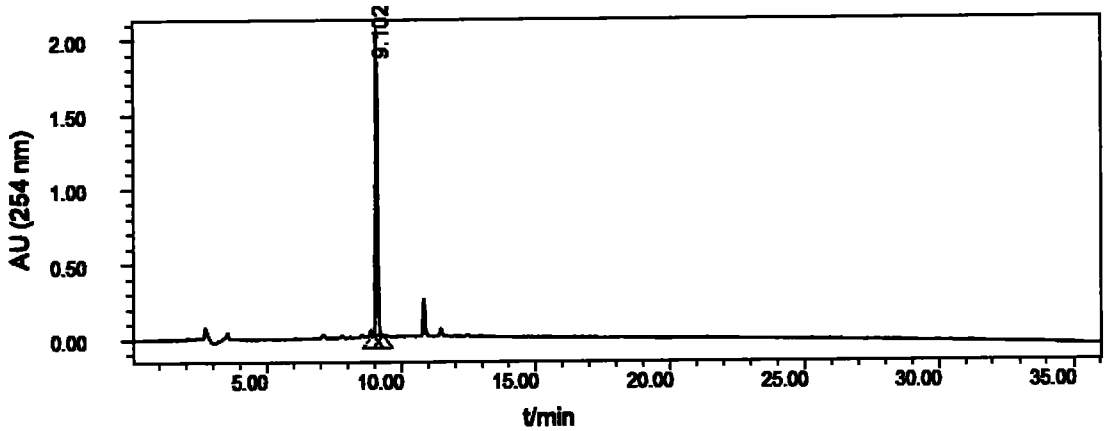

Figure 4.10: Chromatogram of standard-1(Mangiferine)

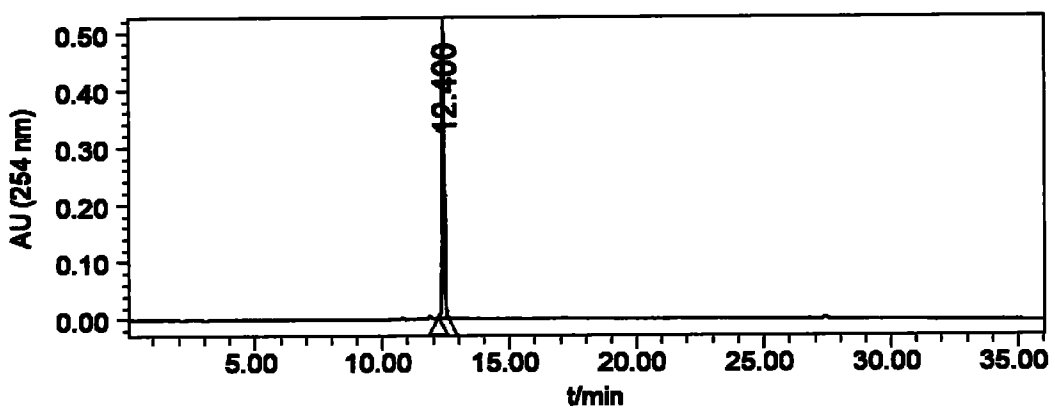

Figure 4.11: Chromatogram of standard-2 (Purpurine)

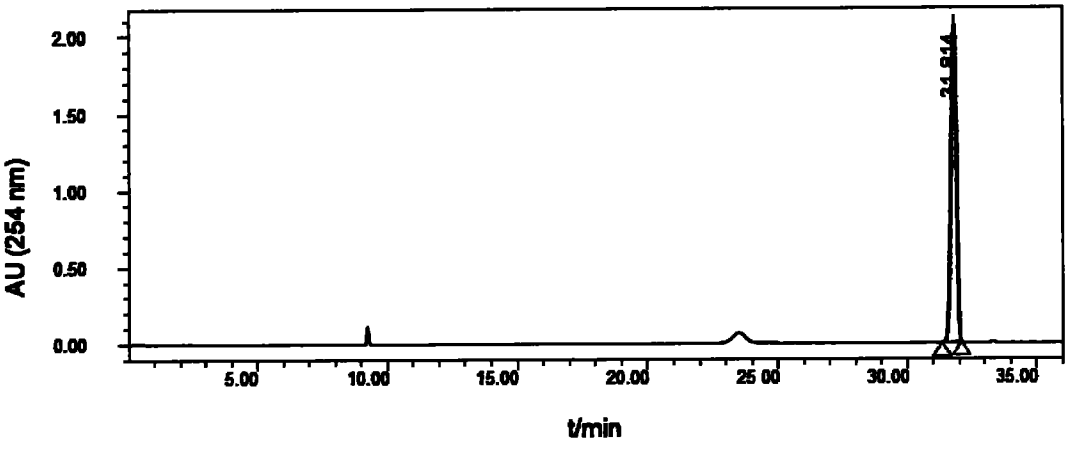

Figure 4.12: Chromatogram of standard-3 (Charatine)

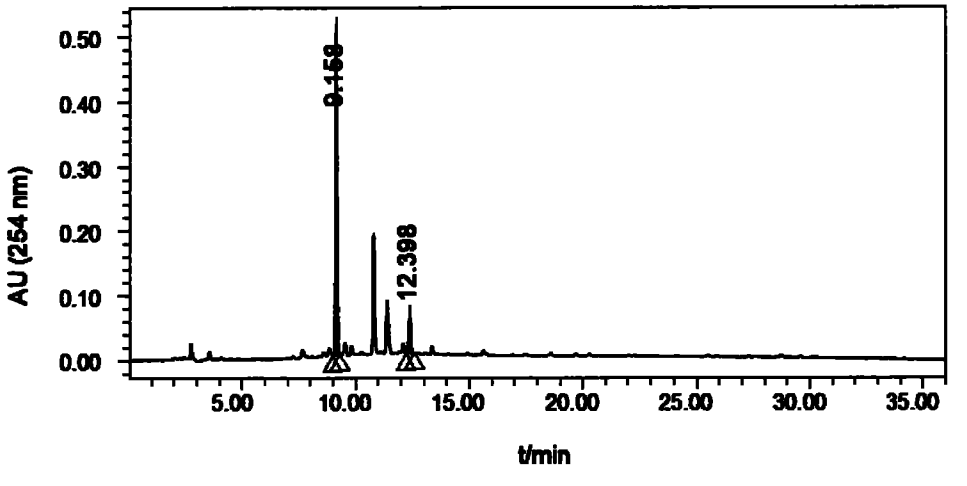

Figure 4.13: Chromatogram of MF *R. cordifolia*

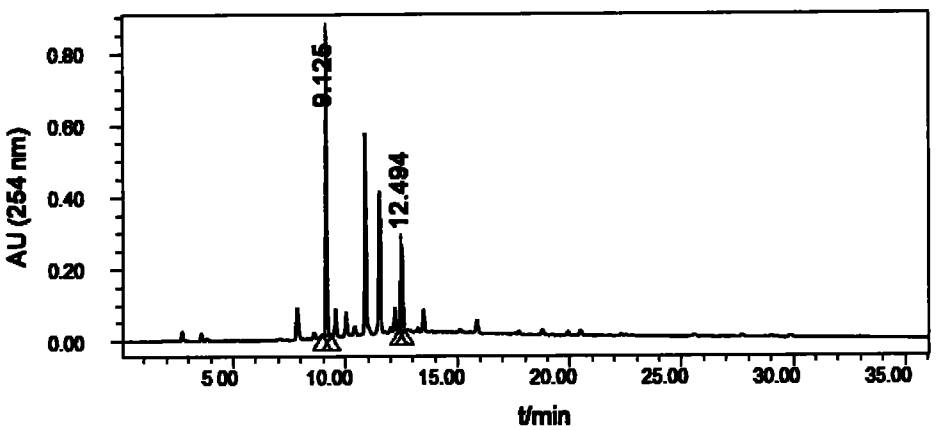

Figure 4.14: Chromatogram of EF *R. cordifolia*

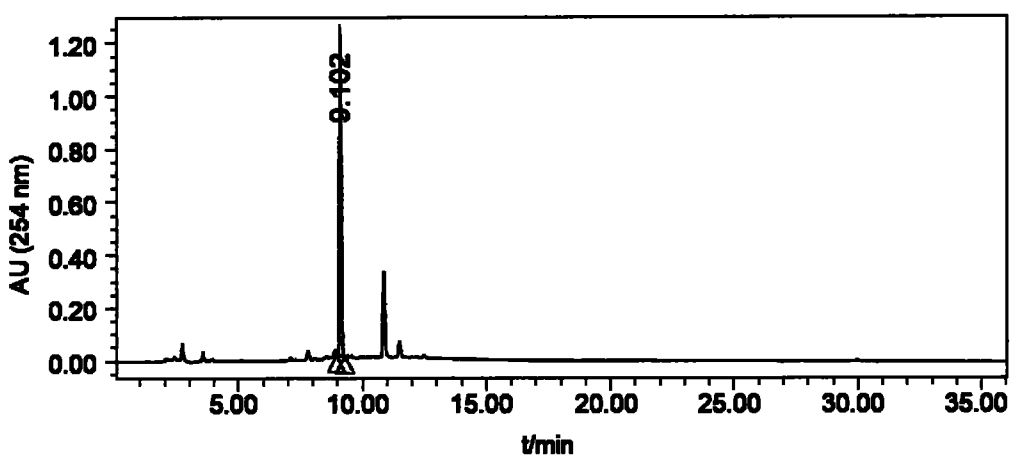

Figure 4.15: Chromatogram of BF *R. cordifolia*

4.9. HPLC of *L. cuneata*

HPLC tests of *L. cuneata* indicates the presence of kaemfperol, mangiferine and purpurine in methanolic fraction. The mangiferine, 2-methyle anthraquinone and purpurine retention times are matched with peaks in n-butanol fraction. Whereas, none of these compounds were identified in ethylacetae fraction or in very minute amount. The chromatograms of standard compounds (Kamferol, Mangiferines, 2-methyl anthraquinone and Purpurine) are shown in figures (figure 4.16, 4.17, 4.18, and 4.19 respectively) and that of identified compounds in figures 4.20-4.22 in table (Table 4.14) below.

Table 4.14. HPLC of identified compounds in *L. cuneata*

Sr. No	Extract	Compound identified	R.T(min) of identified comp.	R.T (min) of standards
1	MELC	Mangiferine	9.095	9.195
		Purpurine	12.058	12.280
		Kaempferol	10.855	10.999
2	EFLC	-	-	-
3	BFLC	Mangiferine	9.095	9.195
		Purpurine	12.058	12.280
		2-methyl anthraquinone	11.490	11.647

MELC= Methanolic extract of *L. cuneata*, EFLC= Ethyl acetate fraction of *L. cuneata* and BFLC= n-Butanol fraction of *L. cuneata*.

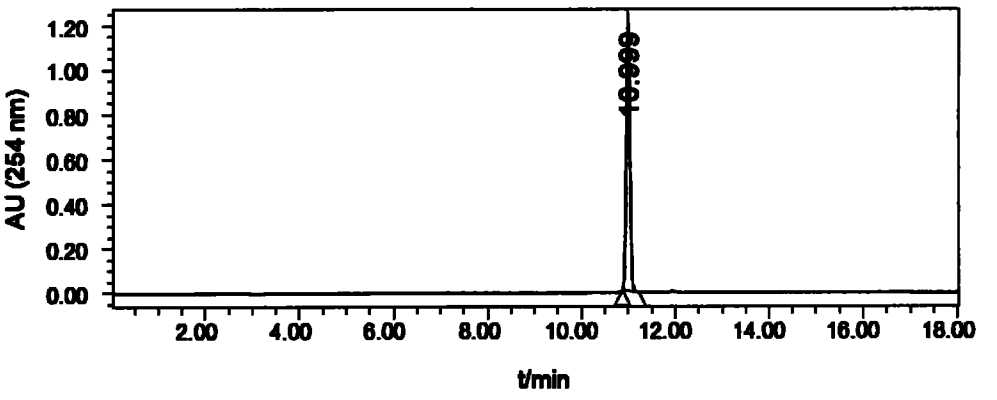

Figure 4.16: Chromatogram of standard-1 (Kaempferol)

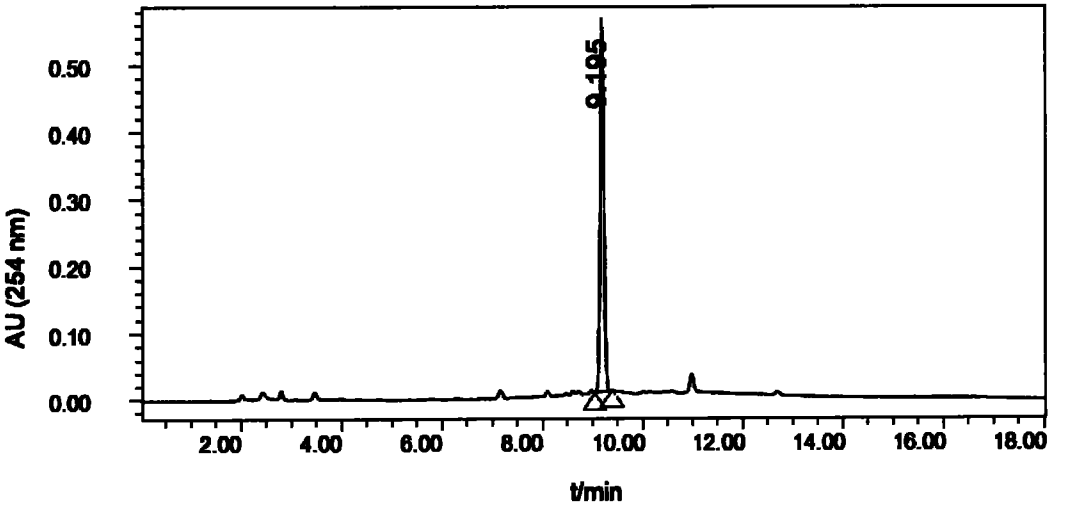

Figure 4.17: Chromatogram of standard-2 (Mangiferine)

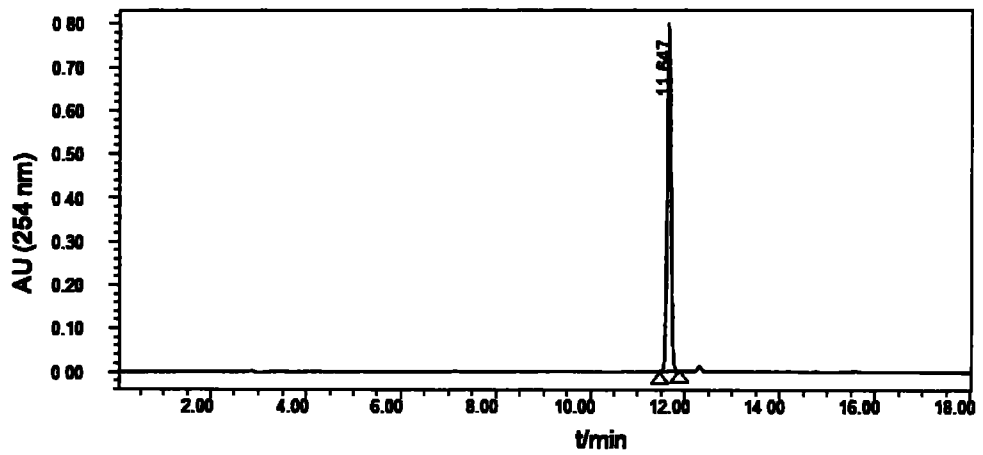

Figure 4.18: Chromatogram of standard-3 (2-methyl anthraquinone)

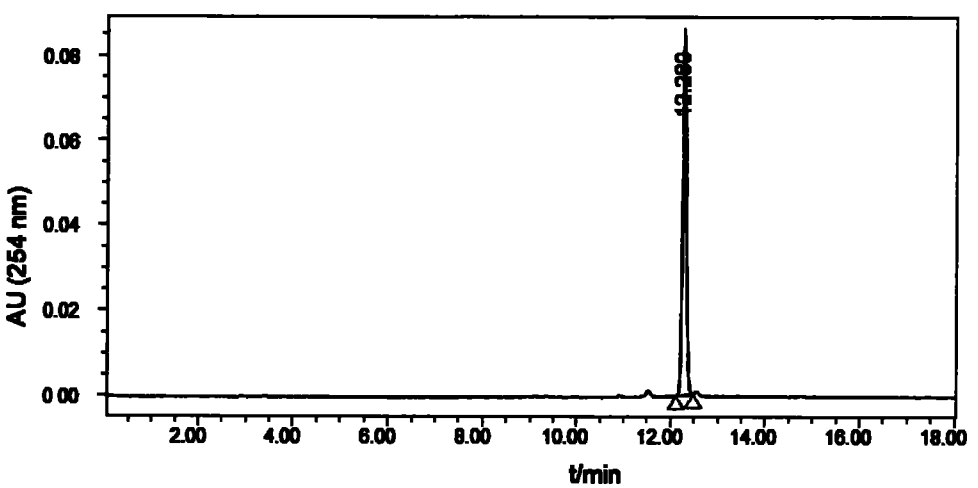

Figure 4.19: Chromatogram of standard-4 (Purpurine)

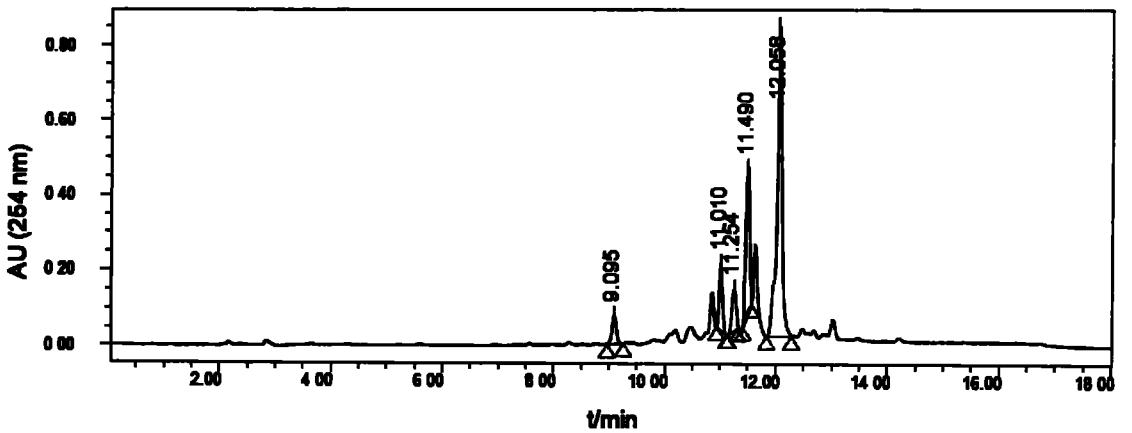

Figure 4.20: Chromatogram of ME *L. cuneata*

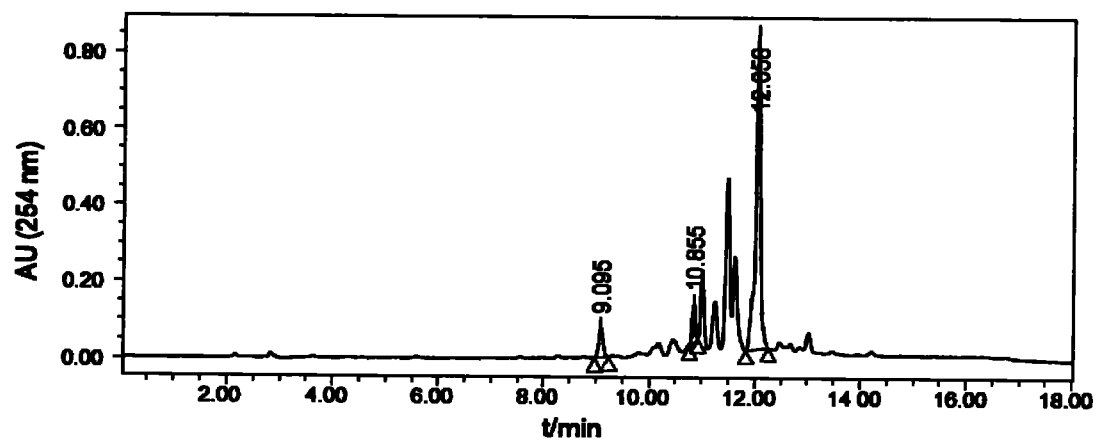

Figure 4.21: Chromatogram of BF *L. cuneata*

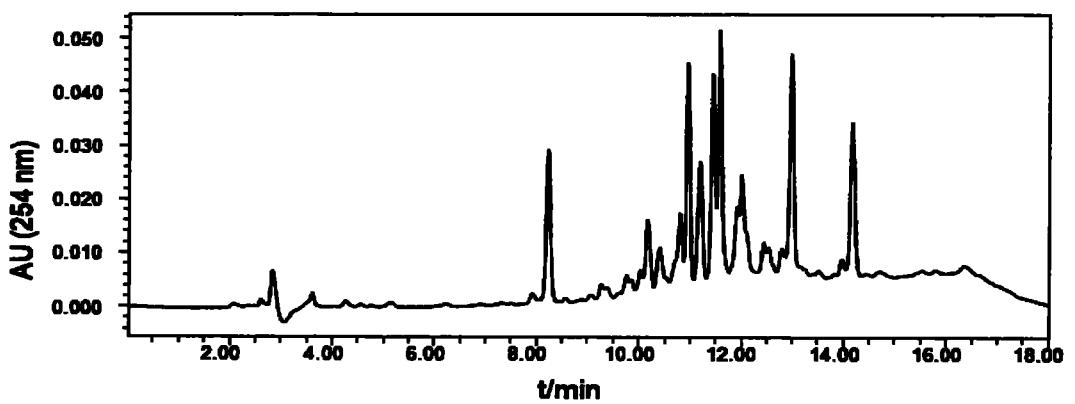

Figure 4.22: Chromatogram of EF *L. cuneata*

DISCUSSION

CHAPTER 5

DISCUSSION

5.1. Discussion

Diabetes is a common metabolic disorder which is worldwide present and mostly occurring in developing countries and leading cause of premature deaths and disability. Number of diabetic people increasing since 1980 due to diabetic type 2 and other factors like obesity and overweight; and the number of cardiovascular diseases have also increased the death rate up to 2.2 million associated to high BGL. The management of diabetes is much cost-effective and hectic in poor population of the under developed countries. The synthetic drugs are cost-effective and approach to common people is not easy that is why the mortality rate due to diabetes is high. These synthetic drugs also impair the body metabolites and cause serious side effects. Plants based medicines to treat diabetes are practiced now a days by pharmaceutics and health care professionals all over the world. Possible management of diabetes is urgent to reduce the mortality rate (Chang *et al.*, 2013). Due to easily availability of herbal medicines and low cost, developing countries population (80% people) rely mostly on plant based medicines. There is recommendation by WHO to include the plant based medicines for health care programs with proven safety and efficacy. (WHO, 2011). Keeping the importance of medicinal plants under consideration and urgent need of safe anti-diabetic medicines, current study was designed to explore anti-diabetic plants of Azad Jammu and Kashmir Pakistan.

In the present study of early phase of investigation, it was found that there was no mortality in mice treated for acute oral toxicity test of selected traditionally used medicinal plants (*R. hastatus, L. cuneatea, R. cordifolia, N. grandiflora* and *S. dalhousieanus)*. It was observed that mice treating for diabetes with doses (250, 500 and 1000 mg/kg) of each selected plant methanolic extract did not show any behavioral change or lethality and mice remained normal. The LD50 value of extract for plants under study was found to be beyond

the 1000 mg/kg revealed that this dose is safe. Plants infusions and extracts to treat various diseases are administered by traditional herbal practitioners (Ghazanfar *et al.*, 2014).To investigate the pharmacologically effectiveness of these traditionally used antihyperglycemic plants (*R. hastatus, L. cuneatea, R. cordifolia, N. grandiflora* and *S. dalhousieanus*) after safe oral toxicity test, present study was further elaborated for detail investigation on the basis of solvents extraction method using in vivo mouse model experimentation to screen the most active anti-hyperglycemic plants from these selected plants and further analysis for anti-diabetic effect by fractionation of most active plants on basis of increasing polarity of solvents. For testing of plants for anti-diabetic effect in mouse model is more accepted and has resemblance to human being (Hammeso *et al.*, 2019). On the basis of preliminary screening of selected plants for antihyperglycemic effect against alloxan induced diabetes in mice it was proved by results that *S. dalhousieanus* have not shown any antidiabetic effect but *R. hastatus* and *N. grandiflora* have shown less antidiabetic effect as compare to *L. cuneatea* and *R. cordifolia.* These plant have none significant ($P > 0.05$) difference in treatment groups (G.IV, VII and VIII) having less/no reduction in BGL, that was increased due to alloxan induced toxicity as compare to diabetic control mice(G-II), while the plants (*L. cuneatea* and *R. cordifolia*) in the treatment groups (G.V and G.VI) highly reduced the blood glucose level which is comparable to standard anti-diabetic drug (Glibenclamide) treatment (G.III) and normal control mice (G-I), have shown highly significant ($P < 0.05$) difference with most active anti-diabetic effect (Table 4.1 and Figure 4.1). So the most active plants that have shown potential high antidiabetic effect against alloxan induced diabetic mice might be due to nature of phytochemical contents and their separation in the methanolic extract or the part of respective plant used (Belayneh and Birru, 2018). The alloxan induction in mice have caused the effect on pancreatic β- cells damage to mimic the insulin production that enhanced the increasing level of blood glucose on 7th day. The treatment with selected plants extract caused the recovery of pancreatic cells damage. The recovery effect of plants (*R. cordifolia* and *L. cuneata*) in treatment groups (G-V, G-VI) is significant on treatment duration (14, 21 and 28th days) respectively showing the time dependent effect against alloxan induced diabetes (Belayneh and Birru, 2018).

The anti-diabetic effect of our selected plants extract is further elaborated by the analysis of body weight of mice (Table 4.2, Figure 4.2). Normal mice (G-I) have increase in average body weight during the whole experimental duration while the alloxan induced toxicity caused the mildly reduction of weight (g) of mice as represented in toxicant control group (G-II). Similar behaviour of treatment effect is shown by plant extract in treatment groups (G-IV, G-VII and G-VIII). The treatment effect with standard antihyperglycemic drug (Glibenclamide 0.5mg/kg) kept the increase in body weight (g) of mice (G-III). The plants extract (*R. cordifolia* and *L. cuneata*) in treatment groups (G-V and G-VI) also increased the body weight of mice comparable to normal group (G-I) and standard antihyperglycemic drug (G-III) as compare to toxicant control group (T.C. G-II). The treatment effect of these two plants is time dependent showed the antihyperglycemic effect. Similar investigation was performed by Ezzat *et al.*, (2014) in which evaluation of methanolic extract of aerial parts of some plants (*Cleome ramosissima*, *Barleria bispinosa* and *Tribulus macropterus*) was performed for their antihyperglglycemic and antihyperlipidimic effect against streptozotacin (STZ) induced diabetic mice by dose of 500 mg/kg. These plants showed the highest reduction in BGL in significant manners by *C. ramosissim* followed by *B. bispinosa* and *T. macropterus* upto twenty eight days treatment where the insulin level was significantly increased in plasma by treatment of *C. ramosissima* and *T. macropterus* by 100.06% and 189.9% respectively and reduced the low density lipoprotein cholesterol(LDL-C). The effect of these plants extract increased both utilization and tolerance of glucose in mice (Ezzat *et al.*, 2014). A study performed earlier on anti-diabetic potential of an aqueous stem bark extract of *Ficus sycomorus* showed remarkable anti-diabetic effect on alloxan-induced diabetic mice that caused the reduction of BGL in dose-dependent manner (Njagi *et al.*, 2012). Earlier study about methanolic extract of *Olea europaea* leaves (MEO) on streptozotocin (STZ) induced diabetes in rats; results exhibited strong antihyperglycemic and antioxidant potential (Bencheikh *et al.*, 2016) These investigations show that the methanolic extract of plants possess the antihyperglycemic effect against toxicity induced diabetes in mice which strongly supports our studies about effect of selected plants methanolic extract against alloxan induced diabetes in mice.

Evaluation about antihyperglycemic effect of aqueous extract of *L. cuneata* against streptozotocin (STZ)-induced diabetes in rats is reported which this extract increased the secretion of insulin by regenerating the damaged β -cells of pancreas and decreased serum blood glucose significantly. Results of our investigation demonstrated the beneficial effect on streptozotocin (STZ)-induced damaged pancreatic β- cell in mice (Kim *et al.*, 2016). Anti-diabetic effect of *R. cordifolia* Linn (Rubiaceae) aqueous root extract on streptozotacin (STZ) induced diabetic rat model is earlier reported. *R. cordifolia* root extract exhibited significant antihyperglycemic activities on streptozotacin (STZ) induced hyperglycemic rats by 2 weeks of treatment. The beneficial effect of *R. cordifolia* root extract treatment might be due to different types of active principles with diverse range of biological activities (Baskar *et al.*, 2006). To investigate the pharmacological activities of plants extracts there is systematic approach which best evaluates any biological activity. To further investigate the most active antihyperglycemic plants from selected plant under study; solvent-solvent partition/fractionation was applied to separate the phytoconstituents into respective fraction based on increasing polarity of solvents. The most active antihyperglycemic plants (*R. cordifolia* and *L. cuneata*) fractions i.e n-hexane fraction of *R. cordifolia*, ethyl acetate fraction of *R.cordifolia*, n-butanol fraction of *R. cordifolia*, aqueous fraction of *R. cordifolia* (HFRC, EFRC, BFRC, AFRC) and n-hexane fraction of *L. cuneata*, ethyl acetate fraction of *L. cuneata*, n-butanol fraction of *L. cuneata*, aqueous fraction of *L. cuneata* (HFLC, EFLC, BFLC, AFLC) separated the constituents of methanolic extracts (MERC and MELC) of *R. cordifolia* and *L. cuneata*. Solvent-solvent fractionation technique on the basis of their polarity of solvents from the methanolic extract of *Carissa opaca* leaves; showed strong antioxidant activity by n-hexane, ethylacetate, n-butanol and aqueous fractions (Sahreen *et al.*, 2017). Our results about antihyperglycemic fractions of most active plants *R. cordifolia* (HFRC, EFRC, BFRC, AFRC) and *L. cuneata* (HFLC, EFLC, BFLC, AFLC) have shown significant ($P< 0.05$) reduction in blood glucose level (BGL).

The results of *R. cordifolia* fractions against alloxan induced diabetes show that all the fractions (HFRC, EFRC, BFRC, AFRC) treatment (100 mg/kg) have shown the significant reduction effect on BGL (Table 4.3 and Figure 4.3) that was elevated due to

alloxan induction in mice as compare to toxicant control group (T.C-G.II) on time dependent manner (14, 21 and 28 days).The highest effect in treatment groups (G-AV and G-AVI) was shown by polar fractions (EFRC and BFRC) on day 28 significantly reducing the elevated level of BGL nearly similar to standard antihyperglycemic drug (Glibenclamide 0.5 mg/kg) treatment (G.III). The more significant anti-diabetic effect of these fractions to reduce the elevated level of BGL is due to separation of phytochemicals in these solvent fractions as compare to other fractions (HFRC and AFRC) during solvent-solvent partition. The anti-diabetic effect of these fractions is also supported by measurement of body weight (BW) of mice during the whole treatment period (Table 4.4 and Figure 4.4). The normal BW (31.30±0.860, 32.62±0.92 and 35.30±0.76 g) on 14, 21 and 28th day was decreased (27.10±0.833, 25.32±0.393 and 23.62±0.543 g) on alloxan induction. On treatment with standard drug (glibenclamid) and *R. cordifolia* fractions (HFRC, EFRC, BFRC and AFRC), BW (g) was increased in treatment groups (G-III,G-AV and G-AVI) significantly ($P< 0.05$) on day 14, 21 and 28 respectively as compared to toxicant control group (G.II) and comparable to SD (G.III), but was no significant ($P> 0.05$) increase in body weight of groups under treatment (G-AIV and G-AVII). These results showed that the EFRC and BFRC have strong anti-hyperglycemic effect against alloxan induced toxicity in mice. The effect of these fractions (EFRC and BFRC have detoxified the effects of alloxan induced toxicity and kept the BW increase in normal way. The fractions (HFRC and AFRC) have less effect on alloxan induced toxicity and BW (g) remained decreasing during the treatment.

The results of *L. cuneata* fractions against alloxan induced diabetes show that all the fractions (HFLC, EFLC, BFLC, AFLC) treatment (100 mg/kg) have shown the significant reduction effect on BGL (Table 4.5 and Figure 4.5) that was elevated due to alloxan induction in mice as compare to toxicant control group (T.C-G.II) on time dependent manner (14, 21 and 28 days). The highest effect in treatment groups (G-BV and G-BVI) was shown by polar fractions (EFLC and BFLC) on day 28 showing the highly significant effect to reduce the elevated level of BGL nearly similar to standard antihyperglycemic drug (Glibenclamide 0.5 mg/kg) treatment (G.III). The highest anti-diabetic effect of these fractions to reduce the elevated level of BGL is due to separation of phytochemicals in these

solvent fractions as compare to other fractions (HFLC and AFLC) during solvent-solvent partition. The anti-diabetic effect of these fractions is also supported by measurement of body weight (BW) of mice during the whole treatment period (Table 4.6 and Figure 4.6). The normal BW (31.30±0.860, 32.62±0.92 and 35.30±0.76 g) on 14, 21 and 28th day was decreased (27.10±0.833, 25.32±0.393 and 23.62±0.543 g) on alloxan induction. On treatment with standard drug (glibenclamid) and *L. cuneata* fractions (HFLC, EFLC, BFlC and AFLC), BW (g) was increased in treatment groups (G-III, G-BV and G-BVI) significantly ($P< 0.05$) on day 14, 21 and 28 respectively as compared to toxicant control group (G.II) and comparable to SD (G.III), but there was none-significant increase ($P> 0.05$) in BW(g) in treatment groups (G-BIV and G-BVII). These results showed that the EFLC and BFLC have strong anti-hyperglycemic effect against alloxan induced toxicity in mice. The effect of these fractions (EFLC and BFLC have detoxified the effects of alloxan induced toxicity and kept the BW increase in normal way. The fractions (HFLC and AFLC) have less effect on alloxan induced toxicity and BW (g) remained decreasing during the treatment. The study effect about aqueous and ethyl acetate extract of leaves for *Senna singuena* by alloxan induced diabetes showed the reduction in BGL that was increased on toxicity induction in alloxan in swiss albino mice by recovering the necrosis of pancreatic cells (Njogu *et al.*, 2017). Another study showed the similar findings by Arika *et al.*, (2015) and Karau *et al.*, (2012) who reported that *Lippia javanica* and *Pappea capensis* exhibited anti-hyperglycemic effect. The possible mechanism of antidiabetic activity of *R. cordifolia* and *L. cuneata* is regenerating ability to damaged pancreatic β-cells and stimulated the secretion of insulin from regenerated or remnant β- cells (Mukundi, 2015). Our findings about antidiabitic activity of most active fractions of *R. cordifolia* (EFRC and BFRC) and *L. cuneata* (EFLC and BFLC) are strongly supported by these findings discussed in earlier studies.

Biological activities of medicinal plants are correlated to their antioxidant activity. Therapeutic potential of plants is contributed to their antioxidant activities. DPPH assay is an important tool for the analysis of plant extracts (Sahreen *et al.*, 2017). The results of methanolic extract (MERC) as well as its different fractions (HFRC, EFRC, BFRC and

AFRC) showed effective free radical scavenging effect (% inhibition). The antioxidant scavenging effect of extract samples of *R. cordifolia was* increased with increase in concentration (Table 4.7). There is highest scavenging activity (%) of EFRC (123.11±0.85) followed by BFRC (120.53±1.36), MERC (119.00±0.87), AFRC (109.10±0.85) and HFRC (106.77±1.66) as indicated in the table (4.7). Our results of antioxidant effects of samples (MERC, HFRC, EFRC, BFRC, AFRC and AA having order of decreasing IC_{50} values of MERC and its different fractions is; HFRC > AFRC > MERC > BFRC> EFRC (having IC_{50} values of 48.52μg/mL 44.88μg/mL, 38.19μg/mL, 36.86μg/mL, and 34.9μg/mL) respectively. The IC_{50} results of BFRC and EFRC are very close to IC_{50} value of standard antioxidant ascorbic acid is 34.41 μg/mL. Results prove that lower is IC_{50} value; has highest antioxidant activity. The fractions (HFRC, EFRC, BFRC and AFRC) tested for DPPH assay showed that EFRC and BFRC have the strong antioxidant activity.

In our results, radical scavenging effect (%) of *L. cuneata* is also increased with increasing concentration of sample (Table 4.7). Samples of methanolic extract (MELC) as well as its different fractions (HFLC, EFLC, BFLC and AFLC) exhibited free radical scavenging effect (% inhibition). Highest scavenging effect of test samples of *L. cuneata* (MELC, HFLC, EFLC, BFLC and AFLC) was shown by EFLC and BFLC in concentration dependent manner. In case of antioxidant effects of above samples (MELC, HFLC, EFLC, BFLC, AFLC and AA), the IC_{50} value is decreased (36.4, 47.08, 31.42, 30.89, 47.49 and 34.41 (μg/mL) respectively showing increasing antioxidant activity. The order of decreasing IC_{50} values of MELC and its different fractions is; AFLC> HFLC> MELC> EFLC> BFLC (having IC_{50} values of 47.49 μg /mL 47.08μg/mL, 36.4μg/mL, 31.42μg/mL, and 30.89μg/mL) respectively. BFLC and EFLC showed lowest IC_{50} value (30.89μg/m L and 31.42μg/mL) confirming the highest antioxidant activity against DPPH free radical in our findings, comparable to IC_{50} value of ascorbic acid (34.41 μg/mL). These findings showed that the phytoconsituents separated in ethylacetae fractions (EF) and n-butanol fraction (BF) of *R. cordifolia* and *L. cuneata* have strong antioxidant activity. These are intermediate polar fractions as compare to aqueous fraction (AF) and n-hexane fraction (HF) of these

investigated plants. Earlier studies on antioxidant activity of phenolic and flavonoid fractions of *Cleome gynandra* and *Maerua angolensis* showed radical (DPPH) scavenging activity by different fractions (n- hexane, dichloroform, acetonitril, ethyle acetate, methanolic and n-butanol fraction) of each plant. The highest activity was shown by n-butanol fraction (BF) and ethylacetate (EF) while lowest activity was exhibited by dichloromethane and n-hexane fraction (Meda *et al.*, 2013). Similar study about antioxidant activity by DPPH assay showed the strong antioxidant activity by n-butanol (BF) of both plants (Meda *et al.*, 2010). Our estimation of *R. cordifolia* and *L. cuneata* fractions by DPPH assay is similar in which results are closely correlated to findings performed earlier (Meda *et al.*, 2010). In the study about antioxidant activity of *Conocarpus erectus* L. leaves n-butanol extract (BF) showed the scavenging activity for DPPH which was comparable to antioxidant activity of ascorbic acid (Hussein, 2016).

During the investigation on leaves extracts of *Gaultheria procumbens* L. it was concluded that systemtic phytochemical evaluation and compounds profiling by different fractions (diethyl ether, ethyl acetate, *n*-butanol and water fractions) showed the dose dependant effect etate and n-butanol fractions showed highest activities at concentration of 100 µg/mL (Michel *et al.*, 2014). These findings of antioxidant activity strongly support our investigations about antioxidant activity of fractions of selected plants (*R. cordifolia* and *L. cuneata*). *Carissa opeca* leaves were evaluated for antioxidant profile in which chloroform, n-hexane, n-butanol, ethylacetate and aqueous extract were investigated by DPPH assay in which all the fractions have strong antioxidant scavenging effect with highest activity of methanolic fraction followed by chloroform fraction, n-hexane fraction,aqueous fraction, ethylacetate and n-butanol fraction (Sahreen *et al.*, 2017) which is little contrast to our results in which order of antioxidant activity is EFRC > BFRC > MERC > AFRC > HFRC for *R. cordifolia* and BFLC> EFLC, MELC> HFLC>AFLC for *L. cuneata* by DPPH assay. This might be the nature of phytoconstituents and part of plant used as well as the climatic effect and geographic locality of plants.

Standardized procedure is of significance for the crude extraction of plants to get desired portion for therapeutic purpose and remove the unwanted parts by treating with selective solvent (Pendli *et al.*, 2014). Biological activities are associated to quality of phytochemicals like phenolic and flavonoids, tannins, terpenoids, saponins and alkaloids. Our investigation about methanolic extract of *R. cordifolia* and *L. cuneata* (MERC and MELC)) and their most active fractions (EFRC, BFRC and EFLC, BFLC) screening, showed the variety of phytochemicals. Alkaloids, phanols, flavonoids, tannins, saponins and terpenoids are present in methanolic extract (MERC) while ethylacetate fraction (EFRC) showed the presence of phenols, flavonoids and saponins whereas tannins and terpenoids were absent. The n-butanol fraction of *R. cordifolia* (BFRC) showed the presence of phenols and flavonoids, saponins and terpenoids which are more variety as compare to EFRC and alkaloids and tannins are shown absent. Similarly, in *L. cuneata*; alkaloids, phenols, flavonoids, tannins, saponins and terpenoids were found present in methanolic extract (MELC). Its ethylacetate fraction (EFRC) contained alkaloids, flavonoids, saponins and terpenoids but phenols and tannins were not separated by this solvent. In n-butanol fraction of *R. cordifolia* (BFRC) there were present phytochemicals like phenols, flavonoids, tannins and terpenoids. In this n-butanol fraction (BFRC) alkaloids and saponins were not present (Table 4.8). Phytochemicals like alkaloids, phenols, flavonoids, tannins, saponins and terpenoids, carbohydrate and steroids are very valuable and they play important physiological actions in the body used for treatment of diseases (Pendli *et al.*, 2014). Previously screening of biologically active compounds like glycosides, anthraquinones, saponins, steroids, flavonoids and phenols from various solvent extracts of root, stem and leaf in *R. cordifolia* was perfomed by Pendli *et al.*, (2014). Screening of similar phytochemicals also in our investigation showed that methanolic extract, ethylacetate and n-butanol has highest potential to separate the required biologically important constituents from medicinal plants. These phytochemicals have strong anti bacterial, anti-inflammatory, anti-diarrheal and anticancerous activites (Liu *et al.*, 2002; Bachaya *et al.*, 2009). Variety of compounds i.e., phenolic compounds, saponins, alkaloids, flavonoids (such as apigenin-7-0-glucoside) and starch are contained in medicinal plants (Hule *et al.*, 2011). Flavonoids are

important for their anti-diabetic activity along with alkaloids (Firdous, 2014). Variety of anthraquinones, naphthoquinones, triterpenoids and cyclic hexapeptides had been identified from this plant that possess anti-oxidation, anti-inflammatory, immunomodulatory and anti-tumor activities (Shan *et al.*, 2016). These results of *R. cordifolia* revealed that these plants with methanolic extract (MERC) and their solvent fractions (EFRC and BFRC) are important for the preparation of drugs to treat the different diseases including the diabetes. Screening of phytochemicals from methanolic extract and its fractions by solvents (n-hexane, ethyl acetate and n-Butanol) of *L. cuneata* led to identification of steroids, flavonoids, phenolics, carotenoid, lignin and hexose on the basis of their spectroscopic data (Min *et al.*, 2016) Tannins, saponins, terpenoids and alkaloids are identified in our investigation from aerial parts of *L. cuneata* which were not screened earlier. The screening of these phytochemicals from *L. cuneata* in our investigation is important for drugs discovery.

The important phytochemicals of plants are phenolic and flavonoids, which play important role in pharmaceutical development. The quantitative analysis of polyphenolic compounds is essential part to know about their total content in the respective part of plants. Our results about TPC (µg/mg GAE) of *R. cordifolia* methanolic extract and its most active fractions (MERC, EFRC and BFRC) showed 99.732±0.938, 207.306±0.730 and 119.120±0.893 (µg/mg GAE) while *L. cuneata* and its active fractions (MELC, EFLC and BFLC) showed 101.269±1.039, 80.699±0.542 and 204.184±0.986 (µg/mg GAE) respectively. This showed that highest TPC (µg/mg GAE) were present inEFRC (207.306±0.730) followed by BFRC (119.120±0.893 µg/mg GAE) and MERC (99.732±0.938 µg/mg GAE) in *R. cordifolia* (Table 4.9). The TPC (µg/mg GAE) in *L. cuneata* were highest in BFLC (204.184±0.986) followed by MELC (101.269±1.039) and EFLC (80.699±0.542) respectively (Table 4.10). The order of presence of TPC in both plants are EFRC > BFLC > BFRC > MELC > MERC > EFLC. The highest content in EFRC, BFLC and BFRC showed that phenolic compounds are more separated in ethyl acetate

fraction of *R. cordifolia* and then n-butanol fraction (BF) of both plants (*R. cordifolia* and *L. cuneata*).

Results of total flavonoids (TFC µg/mg QE) of *R. cordifolia* (MERC, EFRC and BFRC) showed 96.564±0.996, 195.224±0.940 and 76.848±0.519 (µg/mg QE) respectively (Table 4.11) while the results of TFC of *L. cuneata* (MELC, EFLC and BFLC) were 86.376±1.012, 95.410±9.873and 203.970±0.359 (µg/mg QE) respectively (Table 4.12). There were highest TFC (µg/mg QE) in EFRC (195.224±0.940) followed by MERC (96.564±0.996) and BFRC (76.848±0.519) of *R. cordifolia* showing that flavonoids are more separated in ethyl acetate solvent during the fractionation of methanolic extract. Similarly the results of *L. cuneata* of TFC (µg/mg QE) showed that highest TFC is present in BFLC (203.970±0.359) followed by EFLC (95.410±9.873) and MELC (86.376±1.012) showing the more TFC separated in n-butanol solvent and then by ethyl acetate solvent during fractionation of methanolic extract. The variations in TPC of same plant in different solvents may be due to the nature of solvents polarities and also the nature of phenolic compounds (Sembiring *et al.*, 2018). Different plants have different phytochemicals present and in our studied plant the contents are different in both plants (*R. cordifolia* and *L. cuneata*) in solvent, and this difference is also due to plants belong to two different families also and different parts used (Sembiring *et al.*, 2018) and geographical regions and nutrition of plants (Zhang *et al.*, 2017). In another study, same method of calculation of TPC and TFC was performed by Aryal *et al.*, (2019) in vegetables from Nepal (*Basella alba, Cassia tora, Alternanthera sessilis, Digera muricata,, Leucas cephalotes, Portulaca oleracea, Ipomoea aquatica* and *Solanum nigrum*) in methanolic extract in which *A. sessilis* had highest phenolic contents with smallest in *B. alba* but flavonoids were high in *P. oleracea* while the least was recorded for *I. aquatica* which showed that there was higher contents of these phytochemicals as compare to methanol extract for *R. cordifolia* and *L. cuneata* in our investigation. This difference is quiet representation that geographical regions, type of plants and extraction methods have effect on plants secondary metabolites contents. The solvents and extraction procedure are important factor for dissolving the plant compounds

(Siddhuraju *et al.*, 2003). The plant has valuable compounds in polar solvent extract than none polar solvents due to hydroxyl group so the separation of our compound (TPC and TFC) from selected plants (*R. cordifolia* and *L. cuneata*) in methanol is due to its polarity. The partition of these compounds on their polarity basis having different extractive capacities (Russo *et al.*, 2015).

Identification of phytochemicals is an important parameter for knowing the exact role of plants metabolites with biological activities. Pure compounds have more prominent biological activities than crude extract or complex compounds when they are not separated. In our results about analysis of polyphenolic compounds from most active anti-hyperglycemic plant (*R. cordifolia* and *L. cuneata*), HPLC tests confirmed the presence of mangiferine in all samples of *R. cordifolia* (MERC, EFRC and BFRC). The purpurine is present in methanolic extract and ethylacetate fraction of *R. cordifolia* (Figure 4.13 and Figure 4.14) only whereas the charatine is absent in all samples of *R. cordifolia* (Table 4.13). Similarly, the HPLC of *L. cuneata* showed the presence magfirine and purpurine in MELC (Figure 4.20) and its BFLC (Figure 4.21) while Kaempferol is identified in MELC (Figure 4.20) and 2-methyl anthraquinone in BFLC (Figure 4.21). Methanolic extract should show good antidiabetic activity whereas BFLC highly significantl hypoglycemic activity. The antidiabetic effect of EFLC might be due to presence of other phenolic and flavonoid compounds, which may present in it because these standards are not eluded from EFLC (Figure 4.22). Rubiadin (1, 3-Dihydroxy-2-methylanthracene-9, 10-Dione), which is a dihydroxy anthraquinone, and possesses effective antioxidant property and prevents lipid peroxidation was identified from roots and aerial parts by HPLC methodology (Mughees *et al.*, 2017). In earlier study three steroids, nine flavonoids, two phenolics, one carotenoid, one lignin and one hexose on the basis of their spectroscopic data were isolated and identified in *L. cuneata* by Min *et al.*, (2016). Phytochemicals like carbohydrate, alkaloids, amino acids, saponins, glycosides, phenolic compound and tannins were found as a major constituent in *R. cordifolia* Linn. Root, which play important role for biological activities like antitoxin, antiseptic, antimutagenic, anticarcinogenic, antidiabetic and antioxidant

agents (Gupta and Gupta, 2017). Physiochemical characterization of any drug is important before the any pharmaceutical activity. Chemical characterization of *R. cordifolia* showed that it has proteins, glycosides, phenols, flavonoids and tannins in an earlier investigation (Siddiqui *et al.*, 2011). According to Kumar *et al.*, (2019) purpurin is also present in traditionally used plants in Himalayan region and these plants are used for treating varieties of diseases. The oral administration of aqueous root extract (1g/kg/day for 8 weeks) of *R. cordifolia* showed antihyperglycemic effect in mice due to presence of Cordifoliol, cordifodiol, rubiacordone, purpurin, alizarin compounds (Verma *et al.*, 2016; Baskar *et al.*, 2006). In our investigation, purpurin is identified from ethylacetate fraction and methanol extract of *R. cordifolia* showing the evidence that antihyperglycemic effect is due to chemical compound, purpurin in association with other phenolic and flavonoids. Naturally, mangiferin is predominant in *Mangifera indica*. Daily oral administration of 400 mg/kg *M. indica* extract in laboratory animals resulted in decrease of serum glucose by some 60 mg/dL compared to control in 15 days (Madhuri *et al.*, 2017). In our investigation about identification of compounds, it is evidence that mangiferin identification in both the fractions *R. cordifolia* (EFRC and BFRC) and its methanolic extract (MERC) exerted the anti-hyperglycemic effect by 100 mg/kg orally administered once daily in mice up to 28 days' treatment after alloxan induced diabetes in mice for 6 days. Investigation on *R. cordifolia* in another study was carried out to evaluate the diuretic activity of the hydroalcoholic extract of roots in rats to support its folklore claim about diuretic problems and its results showed the potential diuretic activity (Pawar *et al.*, 2009). *R. cordifolia* is traditionally used as an anti-inflammatory, antiseptic, and galactopurifier and during the investigation of its anticancerous activities it was proved that its methanolic extract has strong anticancer effect (Shilpa *et al.*, 2012). Kaempferol, mangiferine, purpurine and anthraquinones are commercially available compounds having antidiabetic potential. These are used as standards for detecting the compounds in methanolic extract, ethyl acetate and n-butanol fractions of *L. cuenetta* plant which is traditionally used for the treatment of type-2 diabetic. Antihyperglycemic effect of our investigated plants is associated to synergistic effect of phytochemicals such as phenolic and flavonoids present in *R. cordifolia* and *L.*

cuneata. Diabetes is increasing health problem globally and there is urgent need for drugs to treat it. Literature review shows that there is not antidiabetic effect of *R. cordifolia* reported from Poonch region of Azad Jammu and Kashmir Pakistan and in our investigation has been performed first time in, *in vivo* animal model.

5.2. Conclusion

Present investigation has been made to identify the potential plants in the alloxan induced diabetes in *in vivo* experiment. During present investigations on antihyperglycemic effect of selected plants against alloxan induced diabetes provide the evidence that *R. hustatus, N. grandiflora* and *S. dalhousieanus* have poor antidiabetic activity while *R. cordifolia* and *L. cuneata* have hypoglycaemic activity and strong anti-diabetic effect to reduce the increased concentration of BGL in mice. *R. cordifolia* and *L. cuneata* have stimulation effect on β cells of pancreas to secrete the insulin. The most active anti-diabetic plants in our investigation showed that the solvents fractions (ethylacetate fraction and n-butanol fraction) of *R. cordifolia* and *L. cuneata* have significant anti-diabetic effect on alloxan induced diabetic mice. This effect is due to the antioxidant activity of these solvents fractions that have been demonstrated by DPPH assay. The anti-diabetic activity of two most active plants (*R. cordifolia* and *L. cuneata*) is associated to antioxidant activity and phytochemical contents like phenolic and flavonoids, specifically the compounds, mangiferine, purpurine (in *R. cordifolia*), kaempferol and 2-methyl anthraquinone (in *L. cuneate*) identified by HPLC profiling (Table 4.13 and 4.14) respectively. The other phytochemicals such as tannins, terpenoids, alkaloids and saponins may have synergistic effect against alloxan induced diabetes in mice. Furthermore, the results obtained from this study confirmed that LD_{50} of all the selected plants are safe for treating the diabetes and they are non-toxic to mice up to 1000 mg/kg of body weight. This investigations on *R. cordifolia* and *L. cuneata* studies give assurance that the both plant species have anti-diabetic and antioxidant effects that supports or makes justification about the reported folkloric use of *R. cordifolia* and *L. cuneata* to treat the diseases of diabetes in northern region of AJ& K, Pakistan. In brief conclusion from our investigation we say that *R. cordifolia* has the most active anti-hyperglycemic effect in

alloxan induced (100 mg/Kg) mice due to synergistic effect of phytochemicals like purpurin and mangiferin present in its fractions and methanolic extract.

However, it is recommended that:

- Further studies about the toxicity of the plant species should performed to enhance the safe use of *R. cordifolia* and *L. cuneata.*
- Clinical and pharmacological studies may be investigated to know the exact mechanism of action of the antihyperglycemic effects of these selected plants under current studies.
- Isolation, identification and structural elucidation of phytochemicals from *R. cordifolia* and *L. cuneata* could be assessed.

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CHAPTER 6

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