EVALUATION OF *IN-VITRO* AND *IN-VIVO* ANTIDIABETIC ACTIVITY OF *PULSATILLA NUTTALLIANA (Mill.)* LEAVES EXTRACT

A Dissertation submitted to

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY,

CHENNAI - 600 032

In partial fulfilment of the award of the degree of

MASTER OF PHARMACY

IN

BRANCH IV: PHARMACOLOGY

Submitted by

Name: SATHISHKUMAR. S

REG.No.261625215

Under the Guidance of

Dr. R. SHANMUGA SUNDARAM M.Pharm., Ph.D.,

HEAD OF THE DEPARTMENT

DEPARTMENT OF PHARMACOLOGY

J.K.K. NATTRAJA COLLEGE OF PHARMACY

KUMARAPALAYAM – 638183

TAMILNADU.

OCTOBER – 2018


CERTIFICATE

EVALAUTION CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"Evaluation of *In-vitro* and *In-vivo* Antidiabetic Activity of *Pulsatilla nuttalliana (Mill.)* Leaves Extract",** submitted to "**The Tamil Nadu Dr.M.G.R.Medical University – Chennai**", in partial fulfilment for the award of Degree of **Master of Pharmacy in Pharmacology,** is a bonafide work carried out by **Mr. SATHISHKUMAR. S, Reg.No. 261625215,** during the academic year 2017-2018 under my guidance and direct supervision in the Department of Pharmacology, J.K.K.Nataraja College of Pharmacy, Kumarapalayam.

Internal Examiner **External Examiner**

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Place: Komarapalayam
Date:

Dr.R.Shanmuga Sundaram, M.Pharm, Ph.D.
Vice Principal and Professor,
Head of the Department,
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy,
Kumarapalayam- 638 183.

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Place: Komarapalayam
Date:

Dr. R. Sambath Kumar, M.Pharm, Ph.D.
Principal and Professor,
Department of Pharmaceutics,
J.K.K. Nattraja College of Pharmacy.
Kumarapalayam- 638 183.

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Date:

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Vice Principal and Professor,
Head of the Department,
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy,
Kumarapalayam- 638 183.

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Department of Pharmaceutics,
J.K.K. Nattraja College of Pharmacy,
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Vice Principal and Professor,
Head of the Department,
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy,
Kumarapalayam- 638 183.

DECLARATION

I do hereby declared that the dissertation **"Evaluation of *In-vitro* and *In-vivo* Antidiabetic Activity of *Pulsatilla nuttalliana (Mill.)* Leaves Extract"** submitted to "**The Tamil Nadu Dr.M.G.R Medical University - Chennai",** for the partial fulfilment of the degree of **Master of Pharmacy** in **Pharmacology,** is a bonafide research work has been carried out by me during the academic year 2017-2018, under the supervision of **Dr. R. Shanmuga Sundaram, M.Pharm, Ph.D.**, Vice Principal and Head of the Department, Department of Pharmacology, J.K.K.Nattraja College of Pharmacy, Kumarapalayam.

I further declare that this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

Place: Kumarapalayam **Mr.SATHISHKUMAR.S**

Date: **Reg.no.261625215**

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Mr.SATHISHKUMAR.S

Reg.no.261625215

Dedicated to Parents,

Teachers &

My Family

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1.0 INTRODUCTION

1.1 Diabetes mellitus (DM)

Diabetes is a serious complex condition which can affect the entire body. Diabetes requires daily self care and if complications develop, diabetes can have a significant impact on quality of life and can reduce life expectancy. While there is currently no cure for diabetes, you can live an enjoyable life by learning about the condition and effectively managing it. It is a fast-growing global problem with huge social, health and economic consequences. It is estimated that in 2010 there were globally 285 million people (approximately 6.4% of the adult population) suffering from this disease. This number is estimated to increase to 430 million in the absence of better control or cure. An ageing population and obesity are two main reasons for the increase. Furthermore it has been shown that almost 50% of the putative diabetics are not diagnosed until 10 years after onset of the disease, hence the real prevalence of global diabetes must be astronomically high[1].

This high blood sugar produces the symptoms of frequent urination, increased thirst, and increased hunger. Untreated, diabetes can cause many complications. Acute complications include diabetic ketoacidosis and non ketotic hyperosmolarcoma. Serious long-term complications include heart disease, stroke, kidney failure, foot ulcers and damage to the eyes[2-7].

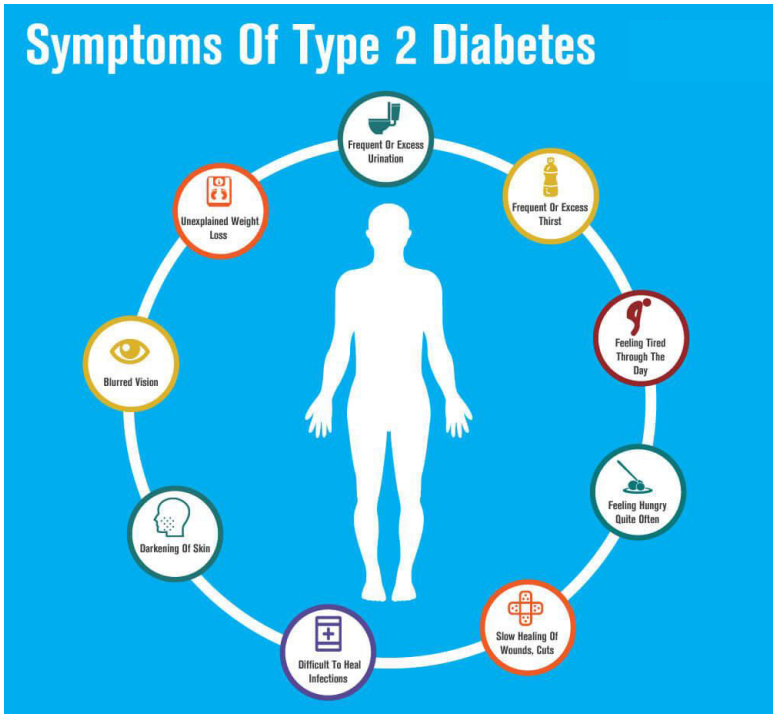
Indian physicians around the same time identified the disease and classified it as Madhumeha or "honey urine", noting but the urine would attract ants. This is possibly due to the diet and life-style of the ancient people, or because the clinical symptoms were observed during the advanced stage of the disease. Galen named the disease "Diarrhea of the urine" (diarrhea urinosa). The earliest surviving work with a detailed reference to diabetes is that of Aretaeus of Cappadocia (2nd or early 3rd century CE). Type 1 and type 2 diabetes were identified as separate conditions for

the first time by the Indian physicians Sushruta and Charaka in 400-500 CE with type 1 associated with youth and type 2 with being overweight. Effective treatment was not developed until the early part of the 20th century, when Canadians Frederick Banting and Charles Herbert Best isolated and purified insulin in 1921 and 1922. This was followed by the development of the long-acting insulin NPH in the 1940s.

1.2 Main Symptoms of Type II Diabetes

Indian physicians around the same time identified the disease and classified it as Madhumeha or "honey urine", noting but the urine would attract ants.

Figure No:1- Main Symptoms of Diabetes



1.3 Overview of the Most Significant Symptoms of Diabetes

The classic symptoms of untreated diabetes are weight loss, polyuria (frequent urination), polydipsia (increased thirst), and polyphagia (increased hunger). Symptoms may develop rapidly (weeks or months) in type 1 diabetes,

while they usually develop much more slowly and may be subtle or absent in type 2 diabetes.

Several other signs and symptoms can mark the onset of diabetes, although they are not specific to the disease. In addition to the known ones above, they include blurry vision, headache, fatigue, slow healing of cuts, and itchy skin. Prolonged high blood glucose can cause glucose absorption in the lens of the eye, which leads to changes in its shape, resulting in vision changes. A number of skin rashes that can occur in diabetes are collectively known as diabetic dermadromes.

People (usually with type 1 diabetes) may also experience episodes of diabetic Ketoacidosis, a type of metabolic problems characterized by Nausea, Vomiting and abdominal pain, the smell of acetone on the breath, deep breathing known as Kussmaul breathing, and in severe cases a decreased level of consciousness[8-17].

A rare but equally severe possibility is hyperosmolar nonketotic state, which is more common in type 2 diabetes and is mainly the result of dehydration.

1.4 Complications

All forms of diabetes increase the risk of long-term complications. These typically develop after many years (10–20), but may be the first symptom in those who have otherwise not received a diagnosis before that time. The major long-term complications relate to damage to blood vessels. Diabetes doubles the risk of cardiovascular disease and about 75% of deaths in diabetics are due to coronary artery disease. Other "macrovascular" diseases are stroke, and peripheral vascular disease.

The primary microvascular complications of diabetes include damage to the eyes, kidneys, and nerves. Damage to the eyes, known as diabetic retinopathy, is caused by damage to the blood vessels in the retina of the eye, and can result in

gradual vision loss and potentially blindness. Damage to the kidneys, known as diabetic nephropathy, can lead to tissue scarring, urine protein loss, and eventually chronic kidney disease, sometimes requiring dialysis or kidney transplant. Damage to the nerves of the body, known as diabetic neuropathy, is the most common complication of diabetes. The symptoms can include numbness, tingling, pain, and altered pain sensation, which can lead to damage to the skin. Diabetes-related foot problems (such as diabetic foot ulcers) may occur, and can be difficult to treat, occasionally requiring amputation. Additionally, proximal diabetic neuropathy causes painful muscle wasting and weakness[18-25].

There is a link between cognitive deficit and diabetes. Compared to those without diabetes, those with the disease have a 1.2 to 1.5-fold greater rate of decline in cognitive function.

1.5 Diagnosis

Diabetes mellitus is characterized by recurrent or persistent hyperglycemia, and is diagnosed by demonstrating any one of the following:

- Fasting plasma glucose level ≥ 7.0 mmol/l (126 mg/dl)
- Plasma glucose ≥ 11.1 mmol/l (200 mg/dl) two hours after a 75 g oral glucose load as in a glucose tolerance test
- Symptoms of hyperglycemia and casual plasma glucose ≥ 11.1 mmol/l (200 mg/dl)
- Glycated hemoglobin (Hb A1C) ≥ 6.5%.

A positive result, in the absence of unequivocal hyperglycemia, should be confirmed by a repeat of any of the above methods on a different day. It is preferable to measure a fasting glucose level because of the ease of measurement and the considerable time commitment of formal glucose tolerance testing, which

takes two hours to complete and offers no prognostic advantage over the fasting test. According to the current definition, two fasting glucose measurements above 126 mg/dl (7.0 mmol/l) is considered diagnostic for diabetes mellitus.

Per the World Health Organization people with fasting glucose levels from 6.1 to 6.9 mmol/l (110 to 125 mg/dl) are considered to have impaired fasting glucose. People with plasma glucose at or above 7.8 mmol/l (140 mg/dl), but not over 11.1 mmol/l (200 mg/dl), two hours after a 75 g oral glucose load are considered to have impaired glucose tolerance. Of these two prediabetic states, the latter in particular is a major risk factor for progression to full-blown diabetes mellitus, as well as cardiovascular disease. The American Diabetes Association since 2003 uses a slightly different range for impaired fasting glucose of 5.6 to 6.9 mmol/l (100 to 125 mg/dl).

Glycated hemoglobin is better than fasting glucose for determining risks of cardiovascular disease and death from any cause.

The rare disease diabetes insipidus has similar symptoms to diabetes mellitus, but without disturbances in the sugar metabolism (*insipidus* means "without taste" in Latin) and does not involve the same disease mechanisms[25-27].

Table No: 1 Diagnostic Criteria for Diabetes Mellitus

Diagnostic Criteria for Diabetes Mellitus		
Test	**Cutoff**	**Comments**
A1C *	≥6.5 %	--
Fasting plasma glucose *	≥126 mg/dL (7.0 mmol/L)	No caloric intake for > 8 hours
2-hour plasma glucose *	≥200 mg/dL (11.1 mmol/L)	After 75 g glucose in water
Random plasma glucose	≥200 mg/dL (11.1 mmol/L)	In a patient with symptoms of hyperglycemia
*A positive test requires confirmation		

1.6 Avoidance and management

There is no known preventive measure for type 1 diabetes. Type 2 diabetes can often be prevented by a person being a normal body weight, physical exercise, and following a healthy diet. Dietary changes known to be effective in helping to prevent diabetes include a diet rich in whole grains and fiber, and choosing good fats, such as polyunsaturated fats found in nuts, vegetable oils, and fish. Limiting sugary beverages and eating less red meat and other sources of saturated fatcan also help in the prevention of diabetes. Active smoking is also associated with an increased risk of diabetes, so smoking cessation can be an important preventive measure as well.

Diabetes mellitus is a chronic disease, for which there is no known cure except in very specific situations. Management concentrates on keeping blood sugar levels as close to normal ("euglycemia") as possible, without causing hypoglycemia. This can usually be accomplished with diet, exercise, and use of appropriate medications (insulin in the case of type 1 diabetes; oral medications, as well as possibly insulin, in type 2 diabetes).

Learning about the disease and actively participating in the treatment is vital for people with diabetes, since the complications of diabetes are far less common and less severe in people who have well-managed blood sugar levels. The goal of treatment is an HbA1C level of 6.5%, but should not be lower than that, and may be set higher. Attention is also paid to other health problems that may accelerate the deleterious effects of diabetes. These include smoking, elevated cholesterollevels, obesity, high blood pressure, and lack of regular exercise. Specialised footwear is widely used to reduce the risk of ulceration, or re-ulceration, in at-risk diabetic feet.

1.6.1 Lifestyle:

People with diabetes can benefit from education about the disease and treatment, good nutrition to achieve a normal body weight, and sensible exercise, with the goal of keeping both short-term and long-term blood glucose levels within acceptable bounds. In addition, given the associated higher risks of cardiovascular disease, lifestyle modifications are recommended to control blood pressure.

1.6.2 Treatment options:

Metformin is generally recommended as a first line treatment for type 2 diabetes, as there is good evidence that it decreases mortality. Routine use of aspirin, however, has not been found to improve outcomes in uncomplicated diabetes. Angiotensin converting enzyme inhibitors (ACEIs) improve outcomes in those with DM while the similar medications angiotensin receptor blockers (ARBs) do not.

Type 1 diabetes is typically treated with combinations of regular and NPH insulin, or synthetic insulin analogs. When insulin is used in type 2 diabetes, a long-acting formulation is usually added initially, while continuing oral medications. Doses of insulin are then increased to effect.

In those with diabetes some recommend blood pressure levels below 120/80 mmHg; however, evidence only supports less than or equal to somewhere between 140/90 mmHg to 160/100 mmHg.

1.6.3 Surgical transplantation:

A pancreas transplant is occasionally considered for people with type 1 diabetes who have severe complications of their disease, including disease requiring kidney transplantation.

1.6.4 Therapy:

In countries using a general practitioner system, such as the United Kingdom, care may take place mainly outside hospitals, with hospital-based specialist care used only in case of complications, difficult blood sugar control, or research projects. In other circumstances, general practitioners and specialists share care in a team approach. Home telehealth support can be an effective management technique.

1.6.5 Exercise Regimen

Making healthy food choices is very important to help keep your blood glucose level under control. People with diabetes don't need to buy or prepare special foods. The foods that are best for someone with diabetes are excellent choices for everyone: foods that are low in fat, salt, and sugar, and high in fiber, such as beans, fruits, vegetables, and whole grains. These foods help you reach and stay at a weight that's good for your body. Regular physical activity is important for people with diabetes. Being physically active has been shown to improve blood glucose levels in older people whose levels are high. Exercise is especially good for people with diabetes because it helps control weight, helps insulin work better to lower blood glucose, is good for your heart and lungs, gives you more energy, Regular physical activity improves insulin resistance and lipid profile (reduction in triglyceride and increase in high-density lipoprotein (HDL)) and lowers blood pressure (although blood pressure will rise during exercise),the metabolic benefits in type 2 diabetes are lost within 3-10 days of stopping regular exercise, physical activity also protects against the development of type 2 diabetes.

1.6.6 HbA1C Test

The A1C test is used to detect type 2 diabetes and prediabetes but is not recommended for diagnosis of type 1 diabetes or gestational diabetes. The A1C test

is a blood test that reflects the average of a person's blood glucose levels over the past 3 months and does not show daily fluctuations. The A1C test is more convenient for patients than the traditional glucose tests because it does not require fasting and can be performed at any time of the day.The A1C test result are reported as a percentage. The higher the percentage, the higher a person's blood glucose levels have been. A normal A1C level is below 5.7 percent. An A1C of 5.7 to 6.4 percent indicates prediabetes. People diagnosed with prediabetes may be retested in 1 year. People with an A1C below 5.7 percent may still be at risk for diabetes, depending on the presence of other characteristics that put them at risk, also known as risk factors. People with an A1C above 6.0 percent should be considered at very high risk of developing diabetes. A level of 6.5 percent or above means a person has diabetes.

1.6.7 Plant Drugs

Allopathic medicines are very costly. In contrast, herbal medicines are very cheap. This cost effectiveness makes them all the more alluring. Herbal medicines can be brought without prescription and they are available in all most all health stores. Some herbs can even be grown at home. For certain ailments, herbal medicines are considered to be more effective than allopathic medicines.

Herbal medicines do not have any side effects, as they are free from chemicals. They are also milder than allopathic medicines. The natural detoxification process of the body is effectively enhanced by herbal medicines. They can be used to cleanse the colon, improve digestion and food absorption. Herbal medicines are also very good in boosting the immune system.

Herbal medicines are very effective in curing various digestive disorders like colitis, indigestion, peptic ulcers, and irregular bowel movements. These types of medicines are best for people who are allergic to various types of drugs. Herbal medicines are also effective in boosting the mental health. Most of the

ailments related to blood circulation like high blood pressure, varicose ulcers, and many others can be controlled through herbal medicine. Some herbal medicines are very good in reducing the cholesterol level in the blood stream. They are also used to treat coronary artery diseases. Herbal medicine can be used to reduce weight by regulating appetite.

1.6.8 Anti-diabetic drugs

Treating diabetes mellitus by lowering glucose levels in the blood. Traditional Medicines derived from medicinal plants are used by about 60% of the world's population. This review focuses on Indian Herbal drugs and plants used in the treatment of diabetes, especially in India. Diabetes is an important human ailment afflicting many from various walks of life in different countries. In India it is proving to be a major health problem, especially in the urban areas. Though there are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects, low cost and because of their natural origin.

Though different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes, there is increased demand by patients to use the natural products with anti diabetic activity. It is a considerable discharge of urine, for the most part excessive, of a violet smell and sweet taste and attended with great thirst and general debility.

In this disease, the animal salts are deficient in the urine, while sugar is secreted in considerable quantity, and these means are calculated to yield the former, and to counteract the later, at the same time that they are capable of correcting the morbid action of the digestive organs.

Diabetes mellitus is one of the common metabolic disorders characterized by hyperglycemia due to absolute or relative deficiency of insulin and results in

significant morbidity and mortality. Diabetes, by itself, increases the production of tissue damaging oxidative stress. Therefore, in diabetes the oxidative stress is referred as a case of double jeopardy for any beta cells that survive the disease.

1.6.9 Diabetes or Madhumeha

As per Ayurveda is a disease in which there is improper functioning of insulin and as a result sugar level in the blood increase. Diabetes may cause heart problem, kidney failure, blurred vision if not treated timely[28,29].

Diabetes mellitus is increasing alarmingly worldwide and is defined as the abnormal glucose tolerance which affects pancreatic beta cells functions and sensitivity leading to progression of diabetes and its related complications. It is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar level and an increased risk of vascular complications. It is the most common endocrine disorder in men and women, and the major public health problem of epidemic proportions, once believed to be a diseased of the west, is becoming an endemic to modernizing and urbanizing population in our country. Ayurvedic literature reveals that since the time of Charak and Sushrut many herbal medicines in different oral formulations have been recommended in Madhumeha (diabetes mellitus) and confident claims of cure are on record.

1.6.10 Types of Diabetes

Diabetes mellitus type 1 is a disease caused by the lack of insulin. Insulin must be used in Type I, which must be injected or inhaled.

Diabetes mellitus type 2 is a disease of insulin resistance by cells. Treatments include (1) agents which increase the amount of insulin secreted by the pancreas, (2) agents which increase the sensitivity of target organs to insulin and (3)

agents which decrease the rate at which glucose is absorbed from the gastrointestinal tract.

Patients suffering from this are therefore totally dependent on exogenous source of insulin while patients suffering from Type II diabetes (insulin independent) are unable to respond to insulin and can be treated with dietary changes, exercise and medication. Type II diabetes is the more common form of diabetes constituting 90% of the diabetic population[30].

2.0 LITERATURE REVIEW

Patil SB, et al (2013)[31]., studied the antidiabetic activity on ethanolic extracts of fruits of Terminalia chebula Retz. (EETC) Alloxan induced diabetic rats. EETC showed the nil toxicity upto 500mg/kg BW. The effect of EETC 200mg/kg BW was compared with Glibenclamide (600mg/kg BW). The histopathological changes caused after induction of alloxan showed the granular cytoplasma, dilation, shrunken nuclei and inflammation which were reduced after treatment of the EETC 200mg/kg BW. The present study on EETC has been confirmed that having the pharmacological action against the diabetic condition. It revealed the biological and pharmacological properties of Terminalia chebula Retz. (Haritaki). Terminalia chebula possesses antibacterial, antifungal, antiviral, antidiabetic, antimutagenic, anti-oxidant, antiulcer and wound healting properties. It also prevents cardiac damage and it is used for the treatment of kidney diseases. It is a mild safe and effective laxative in traditional medicine. Terminalia chebula and its phytoconstituents have therapeutic effects with no toxicity.

Gad MZ, et al (2006)[32]., evaluated phytoconstituents, bioefficacy and phytopharmacological activities of Terminalia chebula. This research shows various experimental studies conducted on bioactive compounds isolated from Terminalia chebula has prospective use in alleviating aging, cancer, various GIT disorders.

Swapnil B, et al (2013)[33]., reviewed *Pulsatilla nuttalliana* is a multipurpose medicinal plant. This review describes the prominence of *Pulsatilla nuttalliana* in therapeutics such as use of crude extract of plants for the amelioration of various diseases, morphology, and growth constrains, biochemical composition, biological activity in the field of plant tissue culture, natural products and nano-biotechnology.

Dzhafarova RE,, et al (2009)[34]., revealed the anti-diabetic properties of *Pulsatilla nuttalliana* stem extracts on Streptozotocin induced diabetic rats. The oral administration of various extracts (Hexane, Ethyl acetate and Methanol) of

Pulsatilla nuttalliana were found to have potent anti-diabetic activity that reduces blood sugar level in Streptozotocin induced diabetic rats.

Radhika S, et al (2011)[35]., studied the antidiabetic activity of *Pulsatilla nuttalliana* (Willd.) in Streptozotocin diabetic rats. The probable mechanism by which *Pulsatilla nuttalliana* may act as anti-hypoglycemic drugs is not through insulin secretion like sulfonyl ureas. It may be through some peripheral mechanisms, such as increasing the glycogen storage in the liver or decreasing the glucose release from the liver.

IshtiaqA, et al (2011)[36]., reviewed natural medicine from plant source used for therapy of diabetes mellitus. This review has been presented in a very interactive manner showing geographical region for availability, part of plant used, mechanism of action and phytoconstituents responsible for particular action, it will be of great importance who interested readers to easily identified and go for further research.Presented a review on medicinal plants with antidiabetic activity. The effects of this plant may delay the development diabetic complication and correct the metabolic abnormalities. This review work stimulates the researchers for further research on the potential use of medicinal plants having anti-diabetic potential.

Kuroda M, et al (2005)[37]., revealed the Pharmacognostic study of the leaves of Syzygium cumini Linn. The present investigation, the detailed pharmacognostic study of Syzygium cumini leaf is carried out to lay down the standards which could be useful in future experimental studies. evaluated the anti-diabetic, Phytochemical in Syzygium cumini (L). Skeels. (Myrtaceae). From the NMR data 4 different compounds Lupeol, 12 oleanen-3-ol-3β acetate, Stigmasterol, β-sitosterol were identified from n-hexane fraction of plant extract. These compounds have potential anti-diabetic activities which support the traditional use of the leaves as being remedy for treating diabetes. screened anti-diabetic activity of Syzygium cumini and its isolated compounds against streptozotocin induced diabetic rats. A compound

mycaminose was isolated from Syzygium cumini extract. The isolated compound 15mg/kg and ethyl acetate and methanol extracted compounds of Syzygium cumini leaves 200 and 400mg/kg was undertaken to evaluate the antidiabetic activity against streptozotocin induced diabetic rats.

Mondal SN, et al (2015)[38]., provided an overview on Cyperus rotundus. This study explore one of the cheapest and effective medicinal resources from this automatically growing plant all over india for the using thousands of arthritic conditions patients where more than 2% world population suffers from one kind or the other kinds of arthritis. tudied the morphological, microscopical and physiochemical investigation on the rhizomes of Cyperus rotundus Linn. The study includes Pharmacognostical evaluation by macroscopy, microscopy, powder analysis, fluorescence characteristic, WHO recommended physiochemical and Phytochemical procedures. evaluated the standardization parameters and invitro antidiabetic activity of Emblica officinalis fruits as per WHO guidelines. The TLC fingerprinting and fluorescence analysis of powdered fruits has been conducted and reported. The anti-diabetic activity is conducted by enzyme inhibition (α-glycosidase) in invitro methods on ethanolic extracts showed significant inhibition.

Grover JK***, et al (2002)[39].,*** studies in-vivo anti-diabetic activity of the methanolic and aqueous bark extract of the plant *Emblica officinalis* Gaertn. Results depicted that the maximum falloff blood glucose level of normal rats observed after 6 hrs during fasting blood glucose studies, with the dose of 250mg/kg identified as the most effective dose. The effect of bark extracts on serum lipid profile (cholesterol, triglycerides, HDL and LDL) were measured in diabetic rats. These evidence clearly indicates that the aqueous and methanolic extracs of *Emblica officinalis* stem barks have significant hypo-glycemic potential as well as anti-diabetic activity.

Rajnish Gupta, et al (2011)[40]., studied the glycemic effects of freeze dried Murraya koenigii. The finding of glycemic and Phytochemical studies suggest that the identified phytochemicals mightbe responsible for its glycemic effects and hence the freeze dried powder may be prescribed as adjunct to diatery therapy and drug treatment for managing diabetes mellitus. Studied the effect of extracts of Murraya koenigii leaves on the levels of blood glucose and plasma insulin in alloxan induced diabetic rats. Murraya koenigii Spreng showed significant reduction.

3.0. PLANT PROFILE

Figure No 2. Plant of *Pulsatilla nuttalliana -I*

Figure No 3. Plant of *Pulsatilla nuttalliana-II*

3.1 Plant Description and Characteristics

Kingdom : *Plantea*

Clade : *Angiosperms*

Family : *Ranunculaceae*

Genus : *Pulsatilla*

Species : *Pulsatilla nuttalliana*

The Pulsatilla approaches more nearly to its botanical characters. It flourishes especially in the dry and sandy bluffs which form the bed of the Mississippi. The flower is pale purplish. "The odour of the dried plant is rather faint, being slightly camphoraceous, the taste of the dried flowers simply sweetish and herbaceous, that of the leaves more astringent with very slight acrimony. The taste and to some extent the odour of the fresh plant are both acrid and irritating" (Hale). A. W. Miller, a pharmaceutist (referred to by Hale), analyzed P. nutt. The extracted substances had an acrid, almost caustic taste, and well-marked camphoraceous odour. When volatilized they produced an irritating, pungent vapour, affecting the eyes and causing sneezing.

3.1.1 Phytochemical Constituents

The analysis revealed the following constituents:

(1) Organic Grape-sugar, Gum, Resin, an Alkaloid and Anemonic acid, Quercetin, Kaempferol.

(2) Inorganic Sulphate of Potash, Carbonate of potash, Chlorate of Potassium, Carbonate of Lime, Magnesia, and "A Proto-Salt of Iron."

3.1.2 Scientifically Proved Activities

Dr.W. H. Miller (brother of A.W. M.), an allopathists, claimed to have used *P. nutt. With success in many chronic eye affections, particularly catarrh, amaurosis, and corneal opacities, cutaneous eruptions, and secondary syphilis. Hale instituted the first homoeopathic research, and Burt was the first prover. The symptoms have a strong resemblance to those of P. nig., and cases cured with P. nutt. are mostly such as would be amenable to P. nig. A patient of mine was incidentally cured of a tendency to catch colds by taking P. nutt. for some weeks as a prophylactic against measles. Burnett cured with it a case of deafness and oedema of left upper eyelid. The proving showed a powerful effect on the menstrual function, and Hale reports many cures of retentio mensium: A young lady, formerly subject to retardation of menses, had: Constant chilliness, cold hands and feet, loss of appetite, sour eructations, nausea after meals, hemicrania, toothache, melancholy, general malaria. Menses two weeks late, took a chill at the time they were due, when she had precursory symptoms, no symptoms of menses now. P. nutt. 1 in water every two hours. After the first dose the menses came on and the constitutional symptoms cleared off. Plethoric, usually healthy young woman, had menses delay two weeks. Continual severe headache, a heaviness and fullness, worse moving or stooping, sight dim, complete blindness on stooping or rising suddenly, weight in uterine region severe aching extending to back, worse evening. Hands and feet cold, weakness in lower limbs. Failed to relieve. P. nutt. Ix, 5 drops every three hours, brought speedy improvement. Next day menses came on profusely but without pain, two days before the expiration of the eighth week. The Pulsatilla flying pains were very noticeable in the proving. Hale reports this case: strong, healthy looking man had wandering rheumatic pains chiefly in dorsum of right foot, loins, thighs, chest, arms, head. The head pain was a dull, heavy pressure in vertex, nearly constant, with occasional sharp pains. Some fever but no local inflammation of joints or muscles. Urine scanty, depositing lithates. Acidity of stomach. Appetite good. Bowels

normal. P. nutt. Ix cured in three days. Some Peculiar Symptoms are: Home-sick feeling. Trembling weakness, weariness, heaviness, Snapping noise in ears. Fidgety feet. Colic before and after stool. Colic after eating a pear. Stiffness of fingers. Hands hot and dry (a constant symptom). The symptoms are worse coming in from open air, after eating, eating a pear, at night, by warmth, by reading, on urinating, on walking. better Walking in open air, rubbing with flesh-brush, scratching.

4.0 AIM AND OBJECTIVES

The aim of the study is evaluate the antidiabetic activity of *Pulsatilla nuttalliana* leaves extract

Diabetes, often referred to by doctors as diabetes mellitus, describes a group of metabolic diseases in which the person has high blood glucose (blood sugar), either because insulin production is inadequate, or because the body's cells do not respond properly to insulin, or both.

In recent years there has been a tremendous increase in demand for herbal drugs due to its safety, efficacy and better therapeutic results Due to its safety, efficacy and better therapeutic results. Due to its economic pricing as compared to synthetic or allopathic drugs which have several therapeutic complication.

As we know that everything in this world change time by time since thousands of year the ear was of ayurveda or herbal origin drug. But last few decades it was replaced by allopathic system of medicine, which was rapidly accepted work wide but latter due to its lots of adverse effect and safety profile and the people are more believing in natural origin drug.

5.0 PLAN OF THE WORK

- Gathering and authentification of the genuine plant material.
- Shade drying and granulation of the dried leaves.
- Sequential extraction of the plant material using petroleum ether, ethyl acetate and ethanol.
- Phytochemical analysis of active extract.
- Acute toxicity study.
- *In-vitro* antidiabetic study using α-amylase inhibition assay.
- *In-vivo* antidiabetic study of active extract.

6.0 MATERIALS AND METHODS

6.1 Plant collection and authentification

The leaves of *Pulsatilla nuttalliana* were collected from Anthiyur forest area, Tamilnadu and which was authenticated.

6.2 Preparation of coarse powder and Extraction technique

The leaves were shade dried at room temperature for 10 days. Then these were milled into powder by mechanical grinder. This powder was sequentially extracted to their increasing polarity with Petroleum ether, Ethyl acetate, Ethanol respectively. About 500gm of powdered leaves was uniformly packed into a thimble in a soxhlet apparatus and extracted with 1000ml Petroleum ether, Ethyl acetate and Ethanol, respectively. Constant heat was provided by Mantox heater for recycling of the solvent. The process of extraction continues for 1-2 hours for each solvent. The excess solvent was evaporated and the dried extracts were kept in refrigerator at 4ºC for their future use in phytochemical analysis and pharmacological screenings.

6.3 Preliminary phytochemical screening of ethanolic leaves extract of *Pulsatilla nuttalliana*

The ethanolic leaves extract of *Pulsatilla nuttalliana* was used for testing preliminary phytochemical screening in order to detect major chemical groups.

6.3.1 Test for carbohydrates

- Molisch's test: Dissolved small quantity of 300mg alcoholic and dried leaves extract powder of Pimenta dioica separately in 4ml distilled water and filtered. The filtrate was subjected to Molisch's test.

- Fehling's test: Dissolve a small portion of extract in water and treat with Fehling's solution.

- Phenols test: The extract was spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the spot and was exposed to ammonia vapours.

6.3.2 Test for flavanoids

- Shinoda test: To 2 to 3ml of extract, a piece of magnesium ribbon and 1ml of concentrated HCl was added.

- .Lead acetate test: To 5ml of extract 1ml of lead acetate solution was added.

6.3.3 Test for tannins

- Braemer's test: To a 2 to 3ml of extract, 10% alcoholic ferric chloride solution was added.

6.3.4 Test for steroid/terpenoid

- Liebermann-Burchardt test: To 1ml of extract, 1ml of chloroform, 2 to3ml of acetic anhydride and 1 to 2 drops of concentrated Sulphuric acid are added.

6.3.5 Test for alkaloids

- Draggendorf's test: A drop of extract was spotted on a small piece of precoated TLC plate and the plate was sprayed with modified Draggendorf's reagent.

- Hager's test: The extract was treated with few ml of Hager's reagen.

- Wagner's test: The extract was treated with few ml of Wagner's reagent.

6.3.6 Tests for Glycosides

- Legal's test: Dissolved the extract [0.1g] in pyridine [2ml], added sodium nitroprusside solution [2ml] and made alkaline with Sodium hydroxide solution.

6.3.7 Test for Saponins

- Foam test: 1ml of extract was dilute with 20ml of distilled water and shaken with a graduated cylinder for 15 minutes.

6.3.8 Test for Anthraquinones

- Borntrager's test: About 50 mg of powdered extract was heated with 10% ferric chloride solution and 1ml of concentrated HCl. The extract was cooled, filtered and the filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia.

6.3.9 Test for Amino acids

- Ninhydrin test: Dissolved a small quantity of the extract in few ml of water and added 1ml of ninhydrin reagent.

6.3.10 Test for fixed oils and fats

Press small quantity of the petroleum ether extract between two filter paper.

Note: the results for the above experiments can be noted as follows.

- If the response to the test is high it can be noted as +++which indicates that the particular group is present as the major class.
- If the response is average then note it as ++ indicates the presence in moderate quantity.
- If the response is very small then note it as + indicating the presence of only in traces.
- If no response is then negative.

6.4 Thin layer chromatographic profiling

6.4.1 Practical requirements

- Stationary phases
- Glass plates
- Mobile phase
- Preparation and activation of TLC plates
- Application of sample
- Development tank
- Development technique
- Detecting or visualizing agents

6.4.2 Glass plates

Specific dimensions like 20 cm*20cm (full plate), 20cm*10cm (half plate), 20cm*5cm (quarter plate) can be used. It should be good quality and can withstand temperature used for drying.

6.4.3 Preparation and activation of TLC plates

Mixture of stationary phase and water forms slurry. TLC plates prepared by pouring, dipping, spraying or spreading. In pouring technique, slurry prepared and poured on the glass plate. Slurry spread uniformly on glass plate. Plates are dried in oven. In dipping technique two plates dipped in slurry, separated and dried. Disadvantage is that large quantity slurry required.

6.4.4 Spreading technique

In this technique TLC spreader is used. The glass plates are stacked on base plate. Slurry poured in the reservoir of TLC spreader. Thickness adjusted using knob in the spreader. 0.25 mm is normally used thickness for analytical purposes. Spreader rolled on the plates and air drying the plates. Plates are activated by keeping at 100 degree Celsius to 120 degree Celsius for one hour. Activated plates can be stored in thermostatically controlled oven for further use.

6.4.5 Application of sample

The concentration of sample of standard should be minimum for good spots. 2- 5ug of 1% solution of standard or sample spotted using capillary tubes. Spots should be 2 cm above the base of plate and spotting area should not be immersed in mobile phase. At least 4 spots can be spotted on quarter plate.

6.4.6 Development tank

The developing tanks require more solvent for developing tank –having hump in the middle and require less solvent. It should be lined inside with filter paper moistened with mobile phase

6.4.7 Mobile phase

The solvent or mobile phase depends on various factors.

- Nature of substance
- Nature of stationary phase
- Mode of chromatography
- Separation to be achieved. Analytical or preparative

6.4.8 Procedure

Commercial sheet precoated with silica gel are available. Select a solvent by testing out the samples in various solvents. Dissolve a small quantity of ethanolic extract of *Pulsatilla nuttalliana* leaves of the unknown in different flask containing solvents of different polarity. Place the TLC plates, the spotted side down in to the chamber so that the lower the pencil line about the solvents. Remove the plate from the development chamber and allowed to dry. Plate is placed under UV light, dark spots are observed.

6.4.9 Quantitative analysis

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

Whereas, R_f = Retardation factor

6.5 *Invitro* Antidiabetic Activity of *Pulsatilla nuttalliana* Leaves Extracts

6.5.0 Alpha-Amylase Inhibition Assay

6.5.1 Chemicals or Reagents

Potato starch, trichloroacetic acid, Folin-Ciocalteau reagents were purchased from SD Fine Pvt. Ltd., Mumbai, 3,5-dinitrosalicylic acid, Tris buffer, linoleic acid, ammonium molybdate, were purchased from Hi-Media Pvt. Ltd., Mumbai, α-amylase, α-glucosidase enzymes, xanthine oxidase, quercetin, hypoxanthine, pyrocatechol were purchased from SRL Pvt. Ltd., Mumbai. Glucose assay kit from Agappe diagnostic Pvt. Ltd., Kerala, Acarbose was obtained from Bicon Pvt. Ltd., Chennai, ferrozine, (2'2-azobis (2-amidino propane) dihydrochloride), butylated hydroxy toluene from Loba Cheme. All other chemicals used in the study were obtained commercially and were of analytical grade.

6.5.2 Instrument used

UV-visible Spectrometer (Systronic double beam- UV-2201).

6.5.3 Preparation of extract

Leaves extraction used in *invitro* and *invivo* studies were prepared by using suitable solvents (Carboxy methyl cellulose).

6.5.4 Experimental procedure for α-amylase inhibition assay

A total of 500 µl of test samples and standard drug (100-1000µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitro salicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle.

Calculation of 50% Inhibitory Concentration (IC_{50})

The concentration of the plant extracts required to scavenge 50% of the radicals (IC_{50}) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by

$$I\ \% = \frac{(Ac-As)}{Ac} \times 100$$

whereas,

Ac = Absorbance of the control

As = Absorbance of the sample.

6.6 Acute Oral Toxicity Study

In a research study when a drug is administered to a biological system there will be some interactions may happen .In most case these are desired and useful, but many effects are not advantageous. Acute, subacute and chronic toxicity studies are performed by the manufacturers in the investigation of a new drug. Acute toxicity is involved in estimation of LD_{50} (It is the lethal dose (cuasing death)to 50% of tested group animals)[54].

6.6.1 LD_{50} (Median Lethal Oral Dose)

LD $_{50}$ (median lethal oral dose) is a statistically derived oral dose of a substance that can be expected to cause death in percent of animals when administered by the oral route. The LD_{50} value is expressed in terms of weight of test substance per unit weight of animal (mg/kg)

In this study acute toxicity study was carried out in wistar albino rats. The procedure was followed by using OECD 423(Acute toxic class method).The rats are fasted overnight, prior to dosing. The three dose levels are administered by the help of oral feeding needle over the prior of 24 hours .After the drugs has been administered, food may be withheld for a further 3-4 hours in rats. The purpose of sighting study is to allow selection of the appropriate starting dose for main study. The test substance is administered to a single animal in a sequential manner following from the fixed dose levels of 5, 50, 300 and 2000mg/kg. The interval between dosing of each level is determined by the mortality/onset, duration and severity of toxic signs over the period of 24 hours, special attention given during the

first 4 hours. Four hours after the drug administration, provide the food and water for 14 days and daily observed some parameters such as food intake, water intake, mortality, onset, Duration and severity of toxic signs. The animal weight is recorded on weekly once. On the day fourteen all the animals are sacrificed, to isolate the organs and observe the histopathological changes. Based on the mortality result of sighting is decided and carried out with five animals per dose level (5 or 50 or 300 or 2000mg/kg).Based on the mortality result on 14^{th} day of observation, the doses for invivo study are selected.

6.7 *In-vivo* Antidiabetic Activity of *Pulsatilla Nuttalliana* Leaves Extract in Streptozotocin Induced Diabetic in Wistar Rats.

Wistar rats (150- 200 grams) of both sexes were used in the study and were obtained from the Animal house, Jkkncp, Kumarapalayam. The studies were performed with the approved by the institutional animal ethical committee, and these animals were used to evaluate Antidiabetic activity of *pulsatilla nuttalliana* leaves extract. Prior to the experiment the rats were housed in a clean polypropylene cages (6 rats/ cages) for a period of 7 days under standard temperature (25 - 30^{O} c), relative humidity (45 – 55%), dark / light cycle (12 /12 hrs).. The animals were put in overnight fasting were deprived of food for 16 hrs but allowed free access of water.

6.7.1 Chemicals:

Streptozotocin from Loba Chemie. Standard Glibenclamide (Daonil) from Aventis Pharma. Ethanol (Analytical grade) and 5% Dextrose solution Glucose Estimation Kit from Gluco Dr Super sensor

6.8 Hypoglycemic Test

Groupings were done as follows:

- Group I served as control – Carboxy Methyl Cellulose (CMC) 1% (0.3ml\100g rat),
- Group II served as negative control – Streptozotocin (100 mg/kg, I.P)
- Group III served as Positive control – Glibenclamide (2mg /kg),
- Group IV served as aqueous ethanolic extract of *Pulsatilla nuttalliana* – (400mg/kg).

Blood samples were collected by the tail nipping method and glucose level checked by glucometer. After drug Administration blood samples have been collected different time intervals at 30, 60 and 120.

6.9 Oral Glucose Tolerance Test

Groupings were done as follows:

- Group I served as control – Carboxy Methyl Cellulose (CMC) 1% (0.3ml\100g rat),
- Group II served as Positive control – Glibenclamide (2 mg /kg),
- Group III served as aqueous ethanolic extract of *Pulsatilla nuttalliana* – (400mg/kg).

All the groups of animals were fasted for 24h and blood samples were collected before drug or solvent treatment. The drug, extract and solvent, have been administered to different groups and 30mins later all the groups of rats were treated with glucose orally at dose 10gm/kg body weight by using oral feeding needle. Blood samples were collected by the tail vein method and glucose level checked by

glucometer. After drug Administration blood samples have been collected different time intervals at 30, 60 and 120.

6.10 Induction of diabetes to animals

The single dose (100 mg/kg b.w., i.p.) of streptozotocin monohydrate dissolved in sodium citrate buffer was used for the induction of diabetes in rats after overnight fasting. After 1 hr of streptozotocin monohydrate administration, the animals were given feed and libitum and 5% dextrose solution was also given in feeding bottle for a day to overcome early hypoglycaemic phase. The animals were stabilized for a week and animals showing blood glucose level more than 200 mg/dl were selected for the study.

6.11 Experimental design

Four groups of rats six in each groups received the following treatment schedule for 14 days.

Group I - Normal control (normal saline 10 ml /kg, P.O)

Group II - Streptozotocin treated control (100 mg/kg, I.P)

Group III - Streptozotocin (100 mg/kg, I.P) +

Standard drug Glibenclamide (2 mg/kg, P.O).

Group IV - Streptozotocin (100 mg/kg, i.p.) + EEPN. (400 mg/kg, P.O)

Plant leaves extract, standard drug and normal saline were administered with the help of oral feeding needle. Group I serve as normal control which received normal saline for 14 days. Group II to Group IV were diabetic control rats. Group IV (which previously received streptozotocin 100mg/kg) were given fixed doses of ethanol leaves extract (400 mg/kg, P.O) of *Pulsatilla nuttalliana* leaves

extract and group III received standard drug Glibenclamide (2 mg/kg,P.O) for 14 consecutive days.(EEPN-Ethanolic extract of *Pulsatilla nuttalliana*).

6.11.1 Collection of blood samples

Fasting blood samples were drawn from tail vein of rats at weekly intervals till the end of the study 1, 7, and 14 days.

6.11.2 Estimation of biochemical parameters Serum blood glucose

On 1, 7, and 14 days fasting blood samples were collected and analyzed the blood glucose.

6.11.3 Blood glucose level

The blood glucose level test measures the amount of glucose in the blood sample obtained from the animals. The test is usually performed to check for elevated blood glucose levels which can be an indication of diabetes or insulin inhibition.

6.11.4 Statistical analysis

Statistical analysis was done by using GRAPHPAD PRISM 5.0.All the values of Biochemical parameters and body weight were expressed as Mean ± Standard Error Mean (SEM). The values were analyzed for statistical significance using one- way analysis of variance (ANOVA), comparison was done by using Dunnett's t test. P values < 0.05 were considered as significant, P values < 0.01 were considered as very significant, P values < 0.001 were considered as highly significant and P>0.05 were considered as not significant.

7.1 RESULTS

7.1.1 Phytochemical studies

Table No. 2: Results of ethanolic extract of *Pulsatilla nuttalliana*

Class of compounds	Tests performed	Results
Carbohydrates	*Molisch's test*	-
	Fehling's test	-
Phenols	*Phosphomolybdic acid test*	+++
Flavonoids	*Shinoda test*	++
	Lead acetate test	++
Tannins	*Braemer's test*	_
Alkaloids	*Wagner's*	+
	Mayer's	+
	Draggendrof's test	+
Glycosides	*Legal's test*	+
	Brontranger's test	+
Saponins	*Foam test*	+
Sterols	*Salkowski's test*	_
Amino acids	*Ninhydrin test*	_
Terpenoids	*Lieberman Burchardt test*	+

+ In traces

++ Present in moderate amount

+++ More amounts is present

-Absence

The phytochemical studies results revealed that the Molisch's test no characteristic observation indicated the absence of carbohydrates, by phosphomolybdic acid test Blue coloration of the spot indicated the presence of phenols. Shinoda test and Lead acetate test gave pink or red coloration of the solution indicated the presence of flavonoids Flocculent white precipitate also indicated the same. There is no dark blue or greenish grey coloration of the solution indicated the absence of tannins in the drug. No characteristic observation for steroids and dark pink or red coloration of the solution indicated the presence of terpenoids. Orange coloration of the spot indicated the presence of alkaloids. Yellow or reddish brown precipitation precipitation indicated the presence of alkaloids. Pink to red colour solution indicates the presence of glycosides. No layer of foam formation indicates the absence of Saponins. If the response to the test is indicated table-1high it can be noted as++++ or +++ which indicates that the particular group is present as the major class. If the response is average then note it as ++ indicates the presence in moderate quantity and note it as + indicating the presence of only in traces. If no response is then negative.

7.1.2 TLC study

Table No: 3, Results of ethanolic extract of *Pulsatilla nuttalliana*

S.No.	Solvents	Concentration	R_f value
1.	Toluene + Ethyl acetate	7:3	0.61
2.	Toluene + Ethyl acetate + Glacial acetic acid	5:5:1	0.75
3.	Petrolium ether+Chloroform	7:3	0.55
4.	Ethyl acetate + Methanol	1:1	0.89
5.	Hexane+Dichloro methane	1:1	0.68
6.	Ethyl acetate + Methanol	3:1	0.60
7.	Dichloro methane +Hexane	3:1	0.73

R_f Value range high-Polar substances present.

R_f value range low-Low polar substance present.

Solubility of compounds depend upon the polarity of solvents.

Obtained R_f values were confirmed by standard R_f values.

R_f values obtained for my extract ranges from 0.56 to 0.88,So my extract may contain compounds like Flavanoids, Glycosides and Alkaloids.

7.1.3 Appearance and percentage yield of EEPN (Ethanolic Extract of Pulsatilla nuttalliana leaves)

EEPN was a semisolid brownish colour extract and the percentage yield was found to be 14.35%

7.1.4 *In-vitro* Antidiabetic Study

Table No:4, α-Amylase Inhibition of Petroleum Ether extract of *Pulsatilla nuttalliana* leaves

Concentration(µg/ml)	IC_{50} %
0	0
25	29
50	33
75	42
100	53
125	56

Figure No. 4: α amylase inhibition of petroleum Ether extract of *Pulsatilla nuttalliana*

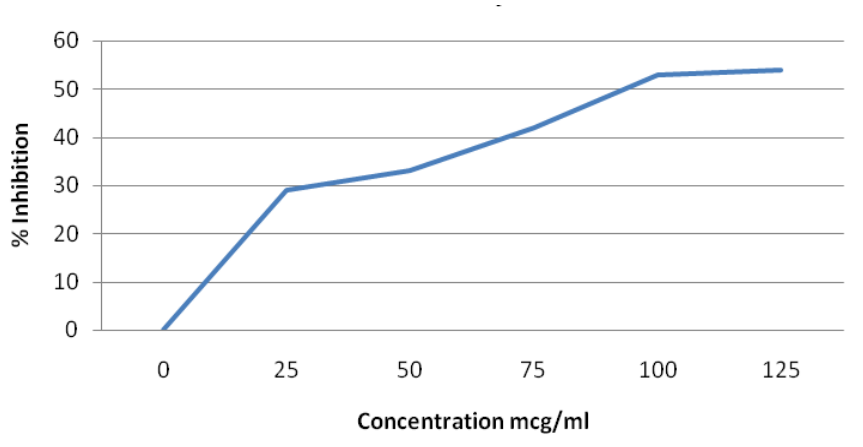


Table No.5: α-Amylase Inhibition of Ethyl acetate Extract of *Pulsatilla nuttalliana* leaves

Concentration(µg/ml)	IC_{50} %
0	0
25	33
50	42
75	49
100	57
125	61

Figure No.5: α amylase inhibition of Ethyl acetate extract of *Pulsatilla nuttalliana*

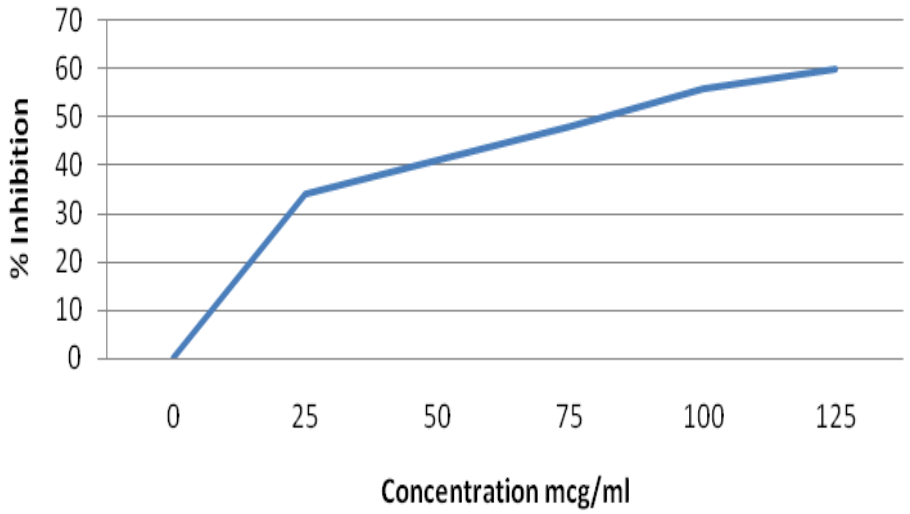


Table No.6:, α-Amylase Inhibition of Ethanolic Extract of *Pulsatilla nuttalliana* leaves

Concentration(µg/ml)	IC_{50} %
0	0
25	30
50	35
75	51
100	55
125	62

Figure No.6: α amylase inhibition of Ethanolic Extract of *Pulsatilla nuttalliana*

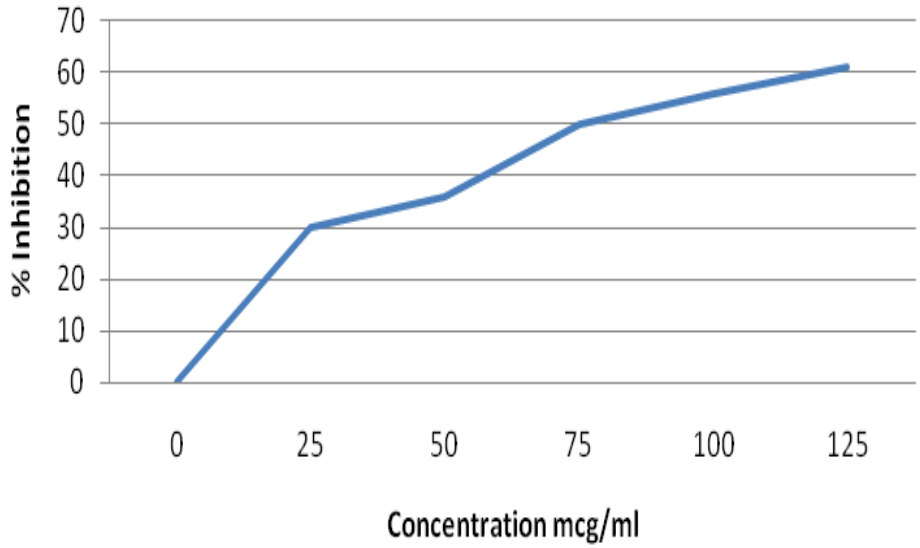
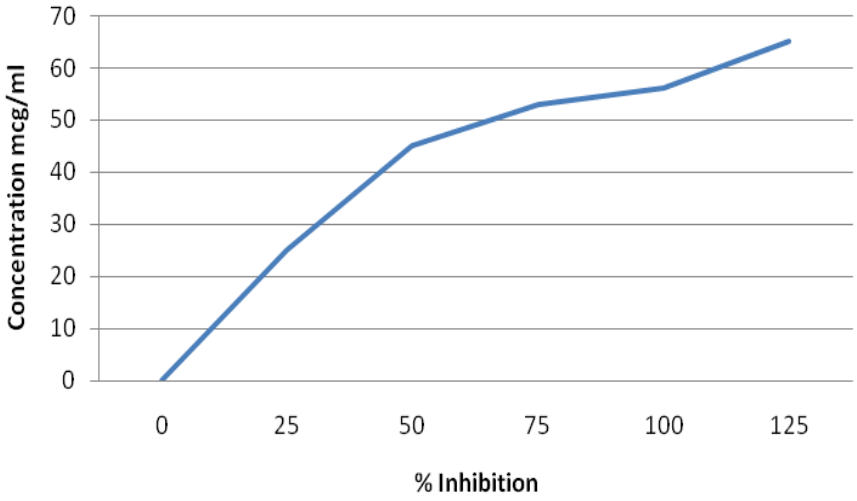


Table No: 7, α-Amylase Inhibition of Acarbose (Positive control)

Concentration(µg/ml)	IC_{50} %
0	0
25	25
50	45
75	53
100	56
125	65

Figure No: 7 α-Amylase Inhibition of Acarbose



7.1.4 Result

- **IC_{50} %** of Petrolium ether extract of *Pulsatilla nuttalliana* leaves = 91 µg/ml
- **IC_{50} %** of Ethyl acetate extract of *Pulsatilla nuttalliana* leaves = 81 µg/ml
- **IC_{50} %** of Ethanolic extract of *Pulsatilla nuttalliana* leaves = 74 µg/ml
- **IC_{50} %** of Acarbose (Positive control) = 62 µg/ml

Minimum **IC_{50} %** was found in Ethanolic extract of *Pulsatilla nuttalliana* leaves which resemblance to **IC_{50} %** of positive control, So Ethanolic extract of *Pulsatilla nuttalliana* contain active constituents of antidiabetic.

Table No: 8, Hypoglycemic Test

Treatment	**Dose mg/kg**	**Blood Glucose Level** (mg/dl)		
		0 min	**0.5hr**	**1 hr**
Control Carboxymethyl Cellulose	1%	67.22±0.15	69.05±0.46	70.93±1.87
Positive Control Glibenclamide	2	68.93±0.54	53.75±1.06**	33.04±1.22***
Aqueous Ethanolic Extract Of *Pulsatilla Nuttalliana*	400	68.64±0.74	54.65±0.42**	35.66±0.58***

The glucose levels were analyzed by using glucometer and each value is the mean ± standard error (n= each group consist of 6 animals) ($p<0.05$)*, ($p<0.001$)**& ($p<0.0001$)*** as compared to control & positive control group evaluated by one way, ANOVA followed by Dunnet 't' test.

Figure No: 8 - Hypoglycemic Test – 30 Minutes

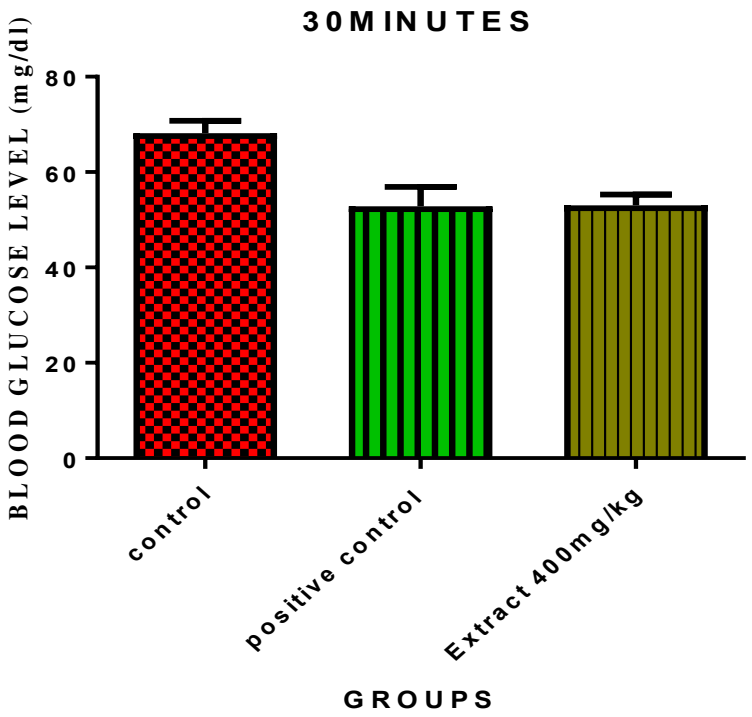
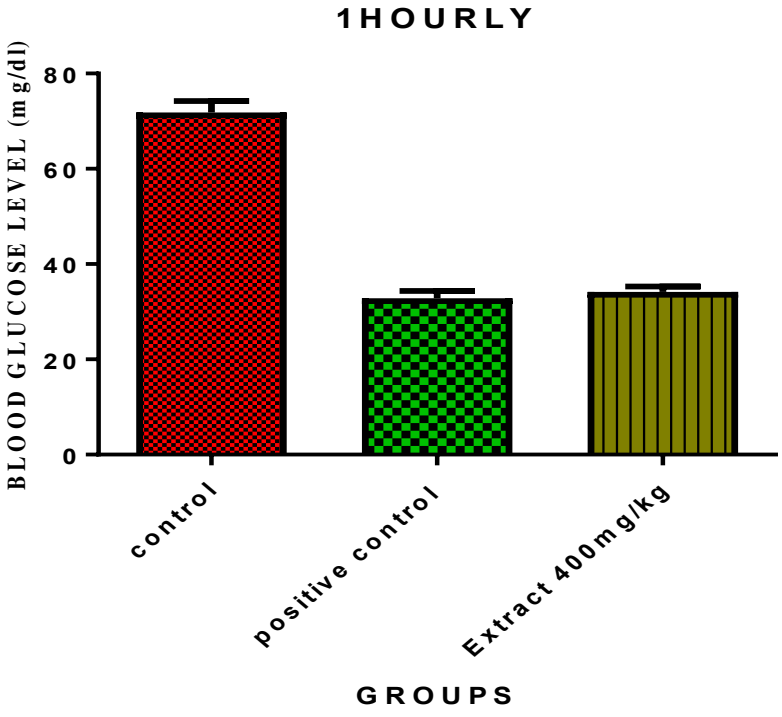


Figure No: 9 - Hypoglycemic Test – 1st hourly



The hypoglycemic test results have shown Table No:8, which indicated aqueous ethanolic extract of *Pulsatilla nuttalliana* treated animals 400 mg/kg , significantly decreased in blood glucose level (18.83±3.879↓) (P<0.05)*, (P<0.001)**& (P<0.0001)*** when compared to control and positive control.

g) *In-vivo* antidiabetic study

Table No: 9, Results of the effects of ethanolic extract on blood Glucose levels

S. No.	Treatment	Blood glucose level (mg/dl) day		
		Day 1	Day 7	Day 14
1	Normal control 10 ml/kg P.O	80.16±1.44	75.7±4.014	77.23± 0.98
2	Negative control 100 mg/kg I.P	266.19±0.46	270.1±2.9	276.32±0.51
3	Positive control (Glibenclamide 2mg/kg) P.O	256.49±0.86	135.63±3.8***	111.92±0.93***
4	EEPN 400 mg/kg P.O	262. 92±1.06	174.04±0.62 ***	163.56±0.58**

EEPN-Ethanoloc extract of *Pulsatilla nuttalliana* leaves

(The values were expressed as Mean ± S.E.M. (n=6 animals in each group).

The experimental results have indicated on Table No:9. The negative control group glucose levels were significantly increased when compared to each other groups. All the groups of animals were affected in diabetes, which indicated blood glucose levels were slight changes in the blood glucose level (4.13±1.207↓& 1.±0.93↑) for normal control group at 7^{th} and 14^{th} days. On day 7^{th} glucose levels

were significantly decreased glibenclamide 2mg/kg treated group (121.2±1.414↓& 23±1↓) (P<0.05)*, (P<0.001) **& (P<0.0001) *** when compared with control group at 7th and 14th days. The ethanolic leaves extract of *Pulsatilla nuttalliana* treated group 400 mg/kg were dose dependent manner decreased (P<0.0001)*** (90±1.67↓) when compared with control group but positive control have more anti diabetic activity at 7th day. The aqueous ethanolic leaves extract of *Pulsatilla nuttalliana* at the dose level 400mg/kg have equipotent activity (120.2±1.414↓)when compared with positive control at 7th day. The ethanolic leaves extract of *Pulsatilla nuttalliana* 400 mg/kg have been expressed dose dependent anti diabetic action (P<0.0001) *** when compared to control and positive control. On day 14th, ethanolic leaves extract of *Pulsatilla nuttalliana* treated animals 400 mg/kg significantly decreased and maintain the blood glucose level (5. 11±1.08↓), (P<0.0001) *** when compared to control and positive control.

Table No:10 Oral Glucose Tolerance Test

Treatment	Dose mg/kg	BLOOD GLUCOSE LEVEL (mg/dl)						
		0 min	**0.5hr**	**1 hr**	**1.5hr**	**2 hr**	**2.5hr**	**3hr**
Control Carboxymethyl Cellulose(Cmc)	1 %	69.12±0.56	141.91±0.23	188.11±0.32	173.21±0.32	158.03±0.62	154.20±0.76	131.21±0.03
Positive Control Gliben clamide	2	68.90±0.45	105.31±0.64**	111.54±0.43***	94.01±0.43***	82.41±0.71***	78.23±0.71***	75.62±0.21***
Aqueous Methanolic Extract of *Pulsatilla Nuttalliana*	400	68.53±0.13	116.23±1.32**	120.21±0.12**	104.11±0.04***	94.11±0.41***	87.21±0.31***	84.30±1.92***

The glucose levels were analyzed by using glucometer and all values are expressed as Mean±SEM (n=6), Group 2 was compared with group 1, Groups —3,4 were compared with group 2; *p<0.05, **p<0.01,***p<0.001 evaluated by one way, ANOVA followed by Dunnet 't' test.

Figure No: 10 - Oral Glucose Tolerance Test- Initial Zero minutes

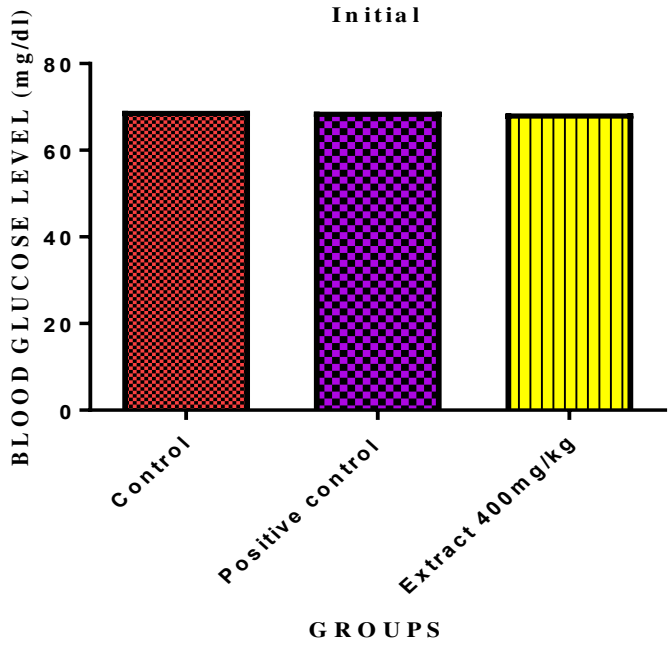


Figure No:11 - Oral Glucose Tolerance Test- 30 minutes

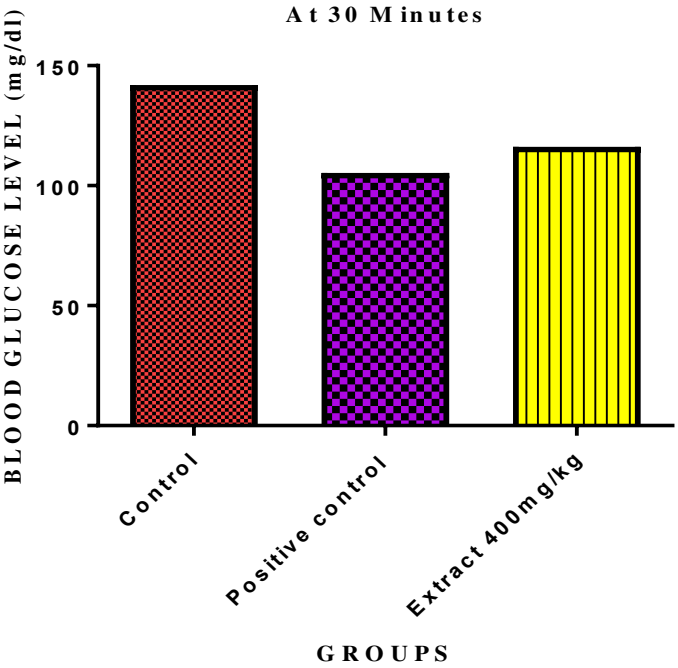


Figure No:12 - Oral Glucose Tolerance Test- At 1st hourly

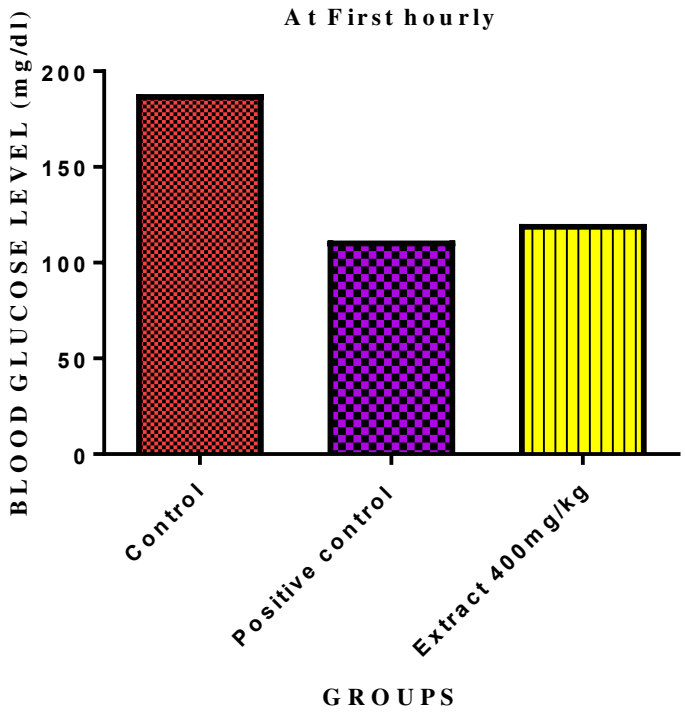


Figure No:13 - Oral Glucose Tolerance Test- At 1.5 hourly

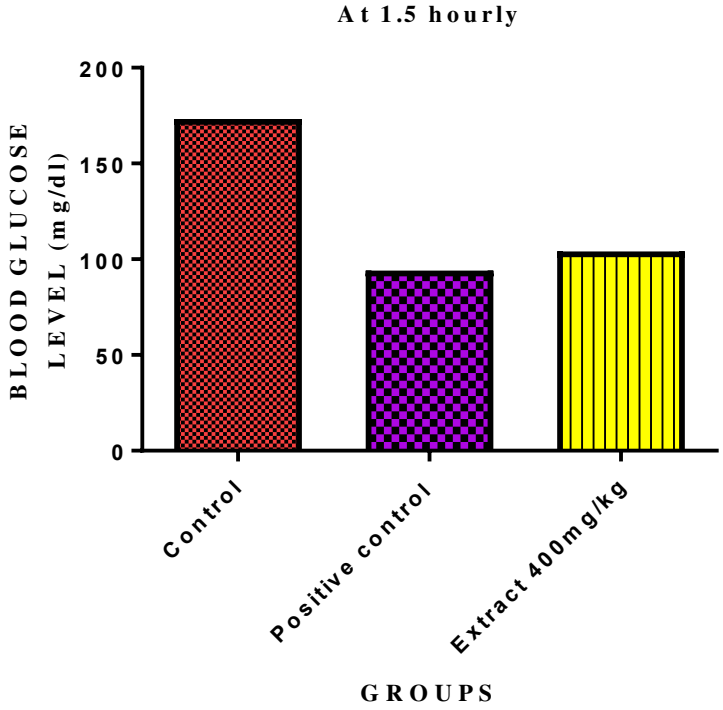


Figure No:14 - Oral Glucose Tolerance Test- At 2nd hourly

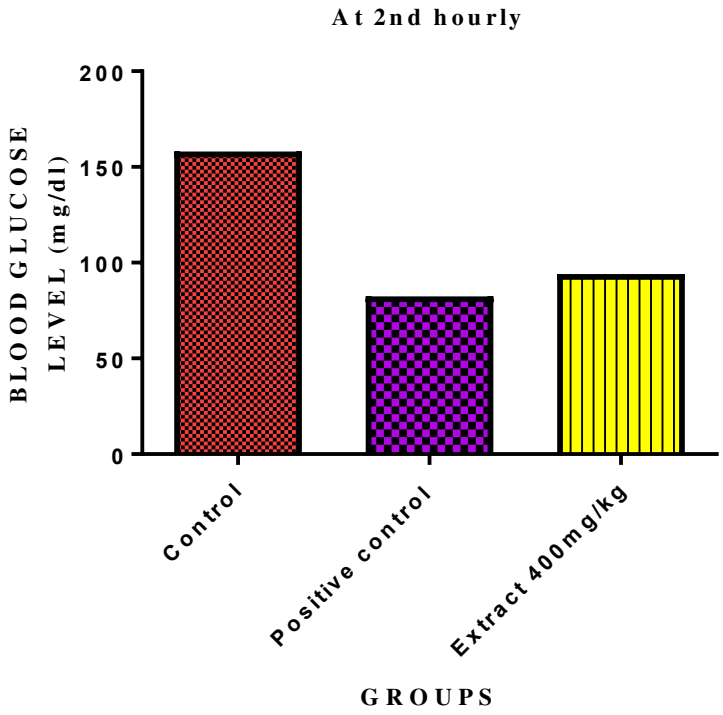


Figure No:15 - Oral Glucose Tolerance Test- At 2.5 hourly

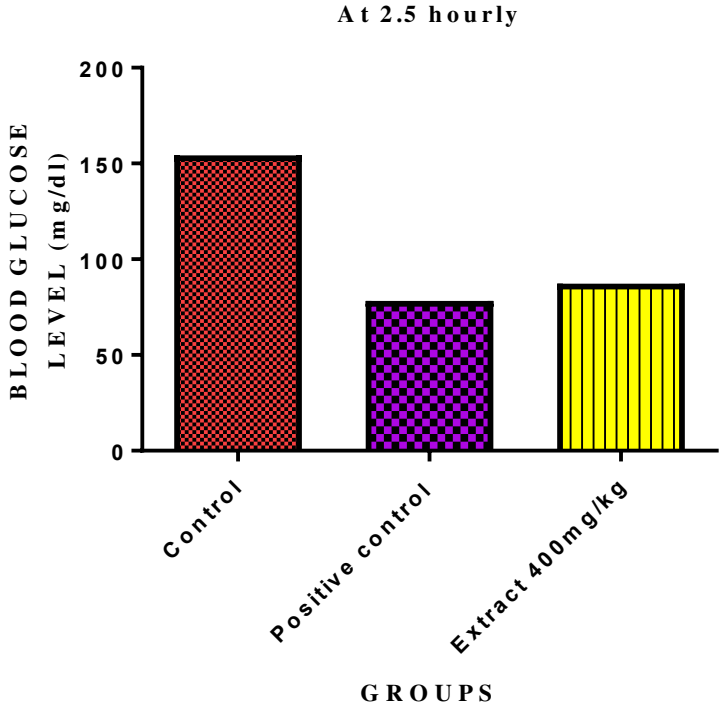
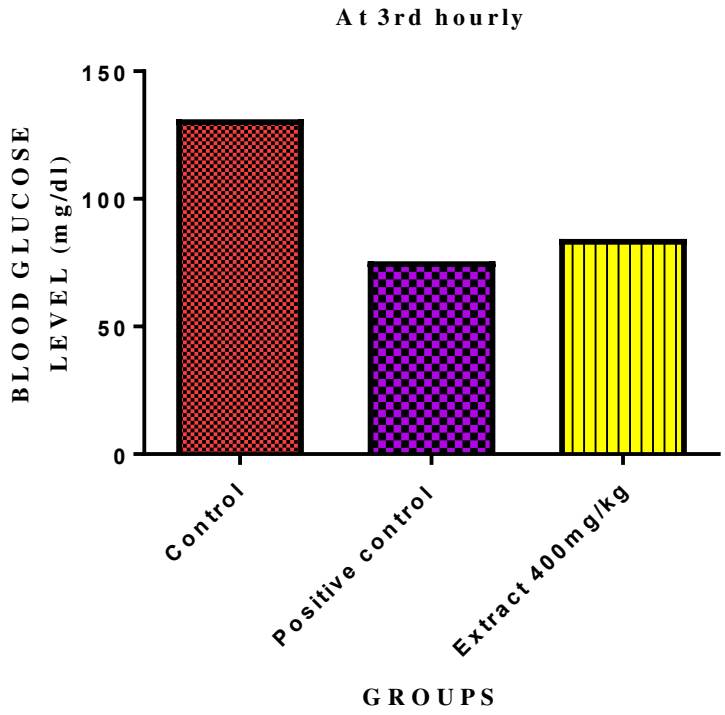


Figure No:16 - Oral Glucose Tolerance Test- At 3rd hourly



The Oral Glucose Tolerance Test (OGTT) results have been expressed on Table Half hour after the glucose treatment, all the groups of animal blood glucose levels were significantly increased (74.5±3.863↑, 35.2±6.6905↑, 59.5±3.764↑ & 65.58±3.762↑). The blood glucose levels were significantly decreased for , aqueous ethanolic extract of *Pulsatilla nuttalliana* 400 mg/kg (65.58±3.762↓) ↓), (P<0.0001) ** when compared to control and positive control at 1hour and each and every ½ hour blood glucose levels(400 mg/kg:17.9±1.422,9.97±0,14.7±1.093&5±0.696) (P<0.05)*, (P<0.001)**& (P<0.0001)*** the extract treated group of animals compared to control and positive control, the EEPN 400mg/kg produce the equipotent activity.

7.2 DISCUSSION

The α-amylase, enzyme that plays a role in digestion of starch and glycogen are considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes. Pancreatic α-amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller. Sequential extraction was done according to increasing polarity order (Petrolium ether,Ethyl acetate and Ethanol). Each extracts were tested for α-amylase inhibition to get the extraction with minimum IC_{50} value. As per the above mechanism all the extract have concentration dependent affinity towards the inhibition of α-amylase. Finally ethanolic extract was observed as more active extract.. In this present study Acute toxicity study was carried out in rats. The procedure was followed by using OECD 423 (Acute Toxic Class Method). The acute toxic class method is a step wise procedure with three animals of a single sex per step. Average two to three steps may be necessary. The method used to defined doses (2000, 1000, 500, 50, 5 mg/kg body weight, Up-and-Down Procedure). The starting dose level of EEPN was 2000 mg/kg body weight p.o as most of the crude extracts posses LD_{50} value more than 2000 mg/kg p.o and also found to be the maximum safe dose. Observe for signs for toxicity and were noted for 14 days. The onset of toxicity and signs of toxicity also noted. Hence 1/5th (400mg/kg) of this dose were selected for further study. The principle involved in the alloxan induced diabetes mellitus in rats, STZ, a beta cytotoxic, diabetes induced chemical but wide variety of animal species by damaging the insulin secreting cells of the pancreas. Literature sources indicate that the STZ induced rats are hyperglycaemic. The treatment of lower doses of STZ (100mg/kg b.w.) produced partial destruction of pancreatic β-cells even though the animals become permanently diabetic. Thus these animals have surviving β-cells and regeneration is possible. It is well known that the sulfonylurea's act by directly stimulating the β-cells of the islets of Langerhans to release more insulin

and these compounds are active in mild alloxan induced diabetes. *Invivo* anti diabetic screening was performed for the confirmation of above mechanism of action was undergone the ethanolic extract of *Pulsatilla nuttalliana* in biological system (Which was already resulted for α-amylase inhibitory activity by *Invitro* methods). At the end of the ethanolic extract of *Pulsatilla nuttalliana.*(400 mg/kg p.o.) showed statistically significant decrease in blood glucose levels. So the ethanolic extract of *Pulsatilla nuttalliana* showed antidiabetic activity. This work will be useful for further diabetes mellitus and it's related diseases research worker to develop new entity for the treatment of diabetes mellitus.

8.0 SUMMARY AND CONCLUSION

The leaves extraction has been performed by sequential extraction method The leaves *of Pulsatilla nuttalliana using* the solvent with increasing polarity order (petroleum ether, ethyl acetate and ethanol) and the active extract was tested by *invitro* antidiabetic screening method. The *invitro* antidiabetic study have been performed based on the α-amylase inhibition assay. Each extracts were tested for α-amylase inhibition and the extract with minimum IC_{50} have been undergone phyto chemical screening. The preliminary phytochemical tests was performed to identify the active phytochemicals present in the ethanolic extract of *Pulsatilla nuttalliana* and showed the presence of Phenols, Flavanoids, Alkaloids, Glycosides, Saponins and Terpenoids. The *in-vivo* antidiabetic activity of Ethanolic extract of *Pulsatilla nuttalliana* leaf was tested by using STZ induced diabetic rat. Acute toxicity study was carried out in rats. The procedure was followed by OECD 423 (Acute toxicity class method). $1/5^{th}$ (400mg/kg) of the maximum safe dose (2000mg/kg) were selected for further study. Fasting blood sample were drawn from tail vein of rats at weekly intervals till the end of the study 1,7 and 14 days. On these days fasting blood glucose were collected and analysed for glucose. At the end of the study (14^{th} day)the ethanolic extract of *Pulsatilla nuttalliana* leaf (400 mg/kg p.o) treated diabetic groups showed statistically significant decrease in blood glucose similar to the standard drug glibenclamide (2mg/kg), which indicated block the alfa amyalase activity. The present study suggested that the isolation of active constituents from ethanolic extract of *Pulsatilla nuttalliana* leaf and characterize the compounds by using preliminary phytochemical studies.

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