Evaluation of *Vitex negundo* L. (Lamiaceae) methanolic leaves extract in diabetes-induced nonalcoholic fatty liver disease rat model

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CERTIFICATE OF APPROVAL

This is to certify that research work embodied in this thesis entitled – **Evaluation of *Vitex negundo* L. (Lamiaceae) methanolic leaves extract in diabetes-induced nonalcoholic fatty liver disease rat model** was carried out by **TATHAGATA KHANRA**, (Exam Roll No. M4PHL24009, Registration No. 163677 of 2022-23 for the partial fulfillment of degree of Master of Pharmacy, to be awarded by Jadavpur University. This research work has been carried out under my guidance and supervision and it is up to my satisfaction.

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DECLARATION

I hereby declare that the Dissertation report entitled – **Evaluation of *Vitex negundo* L. (Lamiaceae) methanolic leaves extract in diabetes-induced nonalcoholic fatty liver disease rat model** is a bonafide and genuine research work carried out by me under the guidance of Prof. (Dr.) Subhash C. Mandal, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032. The work is original and has not been submitted in part or in any form to any other university for the award of any Degree or Diploma or Fellowship. The results reported here are the findings of work carried out by me and plagiarized data have not been incorporated.

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LIST OF ABBREVIATIONS

Sl No.	Abbreviations	Full Form
1	%	Percentage
2	µg	Microgram
3	Ml	Microlitre
4	G	Gram
5	C	Celsius
6	Fig	Figure
7	No.	Number
8	Sl. no.	Serial number
9	Abs.	Absorbance
10	ROS	Reactive oxygen species

11	RNS	Reactive nitrogen species
12	cm	Centimetre (s)
13	DPPH	2,2-diphenyl-1-picrylhydrazyl
14	GAE	Gallic acid equivalent
15	IC_{50}	Half maximal inhibitory concentration
16	kg	Kilogram
17	l	Litre(s)
18	M	Metre
19	M	Molarity
20	mg	Milligram(s)
21	min	Minute(s)
22	mM	Millimolar

23	no.	Number
24	QE	Quercetin equivalent
25	pH	Potential of hydrogen
26	SD	Standard deviation
27	SEM	Standard error of mean
28	UV- Vis	Ultraviolet visible
29	v/v	Volume by volume
30	v/v	Volume by volume
31	Conc.	Concentrated
32	$FeCl_3$	Ferric chloride
33	H_2SO_4	Sulfuric acid
34	STZ	Streptozotocin

35	WHO	World Health Organization
36	nm	Nanometer
37	µl	Microliter
38	i.e.	That is
39	*Et.al*	Et alia
40	w/w	Weight/weight
41	TLC	Thin Layer Chromatography
42	GC-MS	Gas chromatography–mass spectrometry
43	ppt	Precipitate
44	Nacl	Sodium chloride
45	DM	Diabetes Mellitus
46	mmol	Millimolar

47	L.	Linneaus
48	Linn.	Linnaeus
49	h	Hour
50	HCl	Hydrochloric Acid
51	Na	Sodium
52	NaOH	Sodium Hydroxide
53	FCR	Folin-Ciocalteu Reagent
54	TPC	Total Phenolic Content
55	TFC	Total Flavonoid Content
56	w/v	Weight by volume
57	β	Beta
58	α	alpha

59	R_f	Retention factor
60	ANOVA	Analysis of Variance
61	<	Less than
62	±	Plus- minus
63	O_2	superoxide
64	OH	Hydroxyl
65	LD_{50}	Lethal dose
66	e.g.	Exempli gratia (for example)
67	&	And
68	DNS	3,5-Dinitrosalicylic acid
69	*B.*	*Bunge*

Chapter 01

Introduction

1 Introduction

Human societies have been in close contact with their environments since the beginning of their formation and used the ingredients of the environment to obtain food and medicine. Awareness and application of plants to prepare food and medicine have been realized through trial and error, and gradually human became able to meet his needs from his surroundings. Medicinal plants are used as a medical resource in almost all cultures. Ensuring the safety, quality and effectiveness of medicinal plants and herbal drugs very recently became a key issue in industrialized and developing countries. (Jamshidi-Kia, Lorigooini and Amini-Khoei et al., 2018)

Humans have employed herbal medications since long before written records were created. Man-made species and herbs have also reportedly been found to have therapeutic qualities. (Pan et al., 2014)

Written evidence demonstrates that the Sumerians, who produced clay tablets with lists of hundreds of therapeutic plants, were the first people to study herbs more than 5,000 years ago. The numerous records from around the world offer a wealth of important information that has given man the medical expertise he has today. (Petrovska and Biljana Bauer et al., 2012)

Traditional uses of herbal remedies by definition imply extensive historical use, and this is unquestionably true for many of the products marketed as such. Herbal medicine has frequently maintained its popularity for historical and cultural reasons while coexisting with such conventional practices in modern medicine. Commercially speaking, these goods are now more readily accessible, particularly in industrialized nations. Herbs have long been a part of traditional healing practices all across the world. (El-Dahiyat et al., 2020)

Metabolic syndrome (MetS), also variously known as syndrome X, Insulin resistance, etc. in the literature, is really not a single disease but a constellation of cardiovascular disease risk factors and had been defined slightly differently by various organizations. According to WHO as a pathologic condition characterized by abdominal obesity, insulin resistance, hypertension, and hyperlipidaemia. (Saklayen and Mohammad G et al., 2018)

A metabolic condition called diabetes mellitus is characterized by:

1. Hyperglycemia (fasting plasma glucose above or equal to 126 mg/dl and/or above or equal to 200 mg/dl two hours after 75 g of oral glucose).

2. Glycosuria

3. Hyperlipidaemia

4. Negative nitrogen balance

5. Ketonuria

Ancient Egypt and Greece both had a working understanding of diabetic mellitus. The Latin word "Mellitus" means "Sweetened with honey," which relates to thepresence of sugar in urine. The word "DIABETES" is derived from the Greek word "DIAB," meaning to pass through, referring to the cycle of intense thirst and frequent urination (Tripathi, 2008). Ancient Hindu physicians described the condition known as "Madhumeha" as one in which the patient passes delicious urine and displays sweetness in their perspiration, mucous, breath, and blood (Patel et al., 2012).

1.1 Types of Diabetes

Two major types of Diabetes Mellitus are there –

Type 1: Insulin Dependent Diabetes Mellitus (IDDM)/ Juvenile Onset Diabetes Mellitus

The majority of cases of beta cell loss in pancreatic islets are autoimmune (type 1A). Beta cell-destroying antibodies can be detected in blood, some of which are idiopathic (type 1B). Beta cell antibodies are not discovered. Circulating immune levels are always low or very low in type 1 cases, and patients are more likely to go into ketosis. This variety has a lower prevalence and a weaker hereditary tendency.

1.1.1 Type 2: Maturity-Onset Diabetes Mellitus (MODM)/Non-Insulin Dependent Diabetes Mellitus (NIDDM)

There is no loss of beta cell mass or only a mild reduction, insulin levels are low, normal, or even high, anti-beta-cell antibodies cannot be seen, there is a high degree of hereditary predisposition, and the disease typically manifests late in life (after middle age). Over 90 % cases of diabetes are type 2 Diabetes Mellitus. Possible causes include:

1. Abnormalities in beta cells' gluco-receptors.
2. A decrease in the number of insulin receptors, or a decline in the sensitivity (relative resistance) of peripheral tissue to insulin.

3. Obesity and excess hyperglycaemic hormones (glucagon, etc.) lead to a relative insulin deficit.

Other uncommon types of diabetes mellitus include gestational diabetes mellitus (GDM, type 4), other endocrine illnesses, and those caused by unique hereditary diabetes (type 3), such as Maturity Onset Diabetes of Young (MODY). (Toshihiro et al., 2001)

1.2 Mechanism and Present Drug Therapy of Diabetes

The current approach to treating diabetes focuses on managing and bringing blood glucose levels down to a normal range. Western medications and Chinese traditional treatments both work in the same way to decrease blood sugar:

1. To induce the release of insulin from the beta cell of the pancreatic islet
2. To fend against hormones that raises blood sugar.
3. To enhance the amount of insulin receptor sites' sensitivity and affinity to the hormone.
4. To reduce glycogen leading out.
5. To ensure that the tissue and organs use glucose.
6. To reduce free radicals, prevent lipid peroxidation, treat protein and lipid metabolic disorders, and enhance the body's microcirculation. (Siddiqui., 2013; Tripathi 2008)

On the market, there are numerous variations of anti-diabetic medications. These medications can help diabetic people with their blood sugar levels. Orally, these medication classes work well. Through various processes, many drugs are used to treat diabetes and reduce blood sugar levels. By enhancing insulin secretion, sulfonylureas like Tolbutamide, Glibenclamide, Glipizide, and Glimipride, as well as meglitinides like Repaglinide and Nateglinide, can lower blood sugar levels. By reducing insulin resistance, biguanides like metformin and thiazolidinediones like pioglitazone lower blood sugar levels. Acarbose, miglitol, and voglibose are examples of alpha-glucosidase inhibitors that can postpone carbohydrate absorption and reduce blood sugar levels. However, all of these medications have some undesirable side effects, with hypoglycemia being the most common. As additional major side effects, oral anti-diabetic medications can potentially result in hypoglycemia coma, hypersensitivity, hepatotoxicity, and other conditions (Tripathi 2008). Because they are made from natural sources like plants, animals, or mineral parts, herbal formulations are growing in popularity and importance in the market as a way to avoid these complications of allopathic

medications. These formulations are also associated with fewer or almost no side effects and adverse reactions.

1.3 NAFLD (Non-alcoholic fatty liver disease): a brief introduction

Another metabolic condition called Non-alcoholic fatty liver disease (NAFLD) is characterized by:

NAFLD is a condition in which excess fat accumulates in the liver of individuals who drink little to no alcohol. It's one of the most common liver disorders worldwide and is often associated with obesity, insulin resistance, metabolic syndrome, and type 2 diabetes.

Non-alcoholic fatty liver disease is emerging as the most common chronic liver condition in the Western world. It is associated with insulin resistance and frequently occurs with features of the metabolic syndrome. Disease presentation ranges from asymptomatic elevated liver enzyme levels to cirrhosis with complications of liver failure and hepatocellular carcinoma. Current treatment recommendations are limited to weight loss and exercise, although several promising medications are on the horizon. (Pouwels et al., 2022)

1.3.1 Types of NAFLD

Non-Alcoholic Fatty Liver: Also known as steatosis, it's the mildest form of NAFLD and typically doesn't cause serious liver damage.

Non-Alcoholic Steatohepatitis (NASH): In some cases, the fat accumulation in the liver leads to inflammation and liver cell damage, resembling alcoholic liver disease, but occurring in people who drink little to no alcohol. NASH can progress to advanced fibrosis, cirrhosis, and liver failure. (Sattar et al., 2014)

1.3.2 Symptoms of NAFLD

Often asymptomatic in the early stages.

Fatigue

Weakness

Weight loss

Abdominal discomfort or pain in the upper right part of the abdomen

Enlarged liver

Elevated liver enzymes detected in blood tests (Kistler et al., 2011)

1.3.3 Risk Factors of NAFLD

Obesity or overweight

Insulin resistance

Type 2 diabetes

High cholesterol or high triglycerides

Metabolic syndrome

Sedentary lifestyle

Poor diet high in processed foods and sugars. (Souza et al., 2012)

1.3.4 Diagnosis of NAFLD

Blood tests to assess liver enzyme levels and rule out other liver diseases.

Imaging studies like ultrasound, CT scan, or MRI to visualize the liver and assess fat accumulation.

Liver biopsy may be necessary to differentiate between simple fatty liver and NASH and to assess the extent of liver damage. (Festi et al., 2013)

1.3.5 Treatment and Management

Lifestyle modifications are key, including weight loss through a combination of diet and exercise.

Dietary changes, such as reducing sugar intake and consuming a balanced diet rich in fruits, vegetables, whole grains, and lean proteins.

Regular physical activity, aiming for at least 150 minutes of moderate-intensity exercise per week.

Management of associated conditions like diabetes, high blood pressure, and high cholesterol.

Avoidance of alcohol and unnecessary medications that may harm the liver. (Petroni et al., 2021)

1.4 Natural Mechanism from Plant Source Used for Therapy of Diabetes Mellitus

One of the top 12 countries in terms of biodiversity is India. It features a variety of climate zones as well as a flourishing and extensive ethnic medical tradition. Approximately 45,000 plant species and 81,000 animal species have been identified in India to date (Nath et al., 2011). Drugs have always been easily obtained from plants. Several plants have been employed in the treatment of various ailments and as dietary adjuvant. For generations, people have loved plants all around the world. Some herbs are said to have anti-oxidant activity, cholesterol-lowering effects, and the ability to repair the level of liver glycogen in addition to maintaining a normal blood sugar level (Missoun et al., 2018). According to ethnobotanical literature, there are 800 medicinal plants that may have anti-diabetic potential, and more than 1200 plant species have been randomly selected or examined based on ethnopharmacology for activity. The majority of herbal anti-diabetic medications are classified as phytogenetically advanced substances, which include plants, marine algae, and fungi. (Toshihiro et al., 2001)

1.5 Free Radicals and Antioxidants

A free radical is any molecule that has an unpaired electron in an atomic orbital that is capable of supporting itself independently. Since they are unstable, they frequently operate as oxidants or reductants by donating or accepting electrons from other molecules. They damaged the DNA and other components of human cells as a result of their unstable state. One well-known example of a radical is the hydroxyl radical (OH), a compound having one unpaired electron on the oxygen atom. Free radicals and reactive oxygen species (ROS) are common in the environment and are linked to the etiology, progression, and ageing of many diseases. Many of the proteins that free radicals oxidatively alter contain side-chain carbonyl molecules, which can be used as markers for protein oxidation. The side chains of the lysine, arginine, proline, threonine, and glutamic acid residues in proteins react with free radicals such O_2 and H_2O_2 to generate carbonyl derivatives (Moskovitz et al., 2002). A substance that is stable enough to give an unchecked free radical an electron and neutralize it, reducing the radical's potential for harm, is an antioxidant. In addition to neutralizing free radicals, antioxidants also serve as hydrogen donors, electron donors, quenchers of singlet oxygen, decomposers of peroxide, and inhibitors of enzymes, synergists, and metal-chelating agents. Antioxidants, both enzymatic and non-enzymatic, can reduce reactive oxygen species (ROS) in the intracellular and extracellular environment. There have been two suggested primary mechanisms of action for antioxidants. The primary antioxidant initiates the first action, which breaks a chain, by

donating an electron to the system's free radical. In the second method, secondary antioxidants that initiate reactive nitrogen species are removed using a catalyst that starts a quenching chain. Numerous physiological and pathological causes give rise to oxygen free radicals and related oxidants. They are produced by conventional cellular processes, even in little quantities. In addition to intracellular and extracellular sources, exogenous variables such photochemical pollution, ozone, pesticides, xenobiotics, and ionising radiation are known to produce free radicals (Halliwell et al., 1991). There is compelling evidence that damaging free radical reaction. The majority of problems have their origins in the degenerative illnesses that afflict humanity. Some of these conditions include atherosclerosis, cancer, inflammatory joint disease, asthma, diabetes, senile dementia, and degenerative eye disease. Free radicals may potentially play a role in the biological ageing process. (Florence et al., 1995)

Chapter 02

Aims & Objectives

2 Aim & Objective

The present research work was under taken to Evaluation of *Vitex negundo* L. (Lamiaceae) methanolic leaves extract in diabetes-induced non-alcoholic fatty liver disease rat model.

1. Collection, drying and preparation of leaves powder
2. Authentication of the plant specimen
3. Preparation of plant extract with suitable solvent
4. Basic Pharmacognostic study of the leaves of *Vitex negundo* L.
5. Physico-chemical study of the powdered leaves
6. Phytochemical screening of the plant extract
7. TLC of the plant extract
8. Preparation and characterization of tincture
9. Fluorescence characterization of powdered leaves on treatment with various chemical reagents
10. In-vitro antioxidant study
11. In-vitro anti-diabetic study
12. In-vivo diabetic-induced nonalcoholic fatty liver disease study
13. GC-MS analysis of methanolic extract

Chapter 03

Literatures Review

3 Literature Review

The goal of the literature review is to find out different activities reported on *Vitex negundo* L. plant and to check whether it possesses any ant diabetic activity or not.

1. Dharmasiri et al., 2003 Investigated the therapeutic action of *Vitex negundo* L. against the oral anti-inflammatory, analgesic and antihistamine properties of mature fresh leaves (MFL) by orally treating a water extract of the leaves to rats. This study was undertaken to evaluate the anti-inflammatory activity of ethanolic extracts of leaves of *Vitex negundo* L. on carrageenan-induced rat paw oedema, granuloma pouch method, and adjuvant-induced chronic arthritis on experimental animals. This study signifies that *Vitex negundo* L. has significant anti-inflammatory activity.
2. Umamaheswari et al., 2012 evaluated the effect of the various fractions of hydro methanolic extract of the leaves of *Vitex negundo* (Verbenaceae) against ethanol-induced cerebral oxidative stress in rats. The dried powdered leaves of *Vitex negundo* L. were extracted separately by using three different solvents namely – petroleum ether, chloroform, and ethyl acetate. fractions were simultaneously administered with ethanol. Administration of ethanol resulted in a significant elevation in serum biochemical parameters like aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), uric acid, triglycerides and lipoprotein levels. All the fractions prevented the rise in lipid peroxidation and enhanced the antioxidant enzymes. The study signifies that V. negundo offered a significant protection against ethanol toxicity in rat brain.
3. Magdum, Ladda and Naikwade et al., 2011 performed the study on the hepatoprotective activity of ethanolic extract of leaves of *Vitex negundo* Linn. against the injury induced by paracetamol in rats. This study was undertaken to evaluate the ethanolic extract of *Vitex negundo* Linn. leaves were having the hepatoprotective activity which showed promising results in reducing liver damage markers in experimental models.
4. Shrivastava et al., 2018 investigated the hydro-alcoholic extract of *Vitex negundo* Linn. had profound anti-diabetic effects on either sex of Alloxan induced diabetic rats. Extract of (stem) also significantly reduces blood glucose level in alloxan induced diabetic rats and compare with standard glibenclamide. Histopathology study shows that normal control has minute edema, group second have pronounced edema.

5. Chowdhury, Islam and Khalequzzaman et al., 2011 performed a study that the structure of two compounds extracted from the leaves of *Vitex negundo* L., their repellent activity and toxicity against three strains of *Tribolium castaneum* (Herbst) were elucidated. Two bioactive compounds, 22,23-dihydro-α-spinasterol-β-D -glucoside and salicylic acid were isolated from the methanolic leaf extracts by column chromatography and thin-layer chromatography. Both compounds showed insecticidal activity and the leaf extracts caused 100% repellency in the three tested strains of *T. castaneum*.
6. Tiwari and Tripathi et al., 2007 evaluated the antioxidant activity of the leaves of *Vitex negundo* L. The antioxidant potency of *V. negundo* was investigated by employing various established in vitro systems, such as 2,2′-azino-bis 3-ethyl benzothiazoline-6-sulfuric acid ($ABTS^{*+}$)/Lipid Peroxides (LPO)/Superoxide/Hydroxyl radical scavenging and iron ion chelation. Total antioxidant capacity was determined by the assay based on the preformed radical monocation $ABTS^{*+}$. Lipid peroxidation was assessed in terms of thiobarbituric acid reactive substances by using egg yolk homogenates as lipid rich media.
7. Chitra, Sharma and Kayande et al., 2009 investigated the antitumour activity of the ethanolic extract of leaves of *Vitex negundo* (EVN) has been evaluated against Dalton's ascitic lymphoma (DAL) in Swiss albino mice, The experimental parameters used were tumour volume, tumour cell count, viable tumour cell count, mean survival time and increase in life span to assess antitumour activity. The study revealed that the EVN showed significant antitumour activity in tested animal models. The EVN was found to be cytotoxic to mouse lung fibroblast (L-929) cells in long term chemosensitive cytotoxic assay.
8. Panda, Thatoi and Dutta et al., 2009 evaluated the antimicrobial activity and phytochemicals of the leaves and bark of *Vitex negundo* L. was evaluated against three Gram-positive bacteria viz. Staphylococcus epidermidis, Bacillus subtilis, Staphylococcus aureus and five Gram-negative bacteria viz. Escherchia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Vibrio cholerae and Vibrio alginolyteus. Both polar and nonpolar extracts viz. petroleum ether, chloroform, ethanol, methanol and aqueous extracts were prepared and studied for antibacterial activity using disc diffusion, agar cup and broth dilution methods. The result obtained with ethanol and methanol extracts of leaves; petroleum ether and chloroform extract

of bark exhibited significant antibacterial activity, a property that supports traditional use of the plant in the treatment of some diseases as broad-spectrum antibacterial agents.

9. Kakadia et al., 2019 evaluated the effect of *Vitex negundo* L. in the letrozole-induced polycystic ovarian syndrome. Female Sprague-Dawley rats were divided into six groups, each containing 6 animals. Group I (Control) daily received 1% carboxymethylcellulose (CMC) suspension as a vehicle control. Letrozole (1 mg/kg) was administered per orally (p.o) for a period of 21 days for the induction of PCOS in Group II to VI. PCOS induced animals were treated with aqueous (Group III - 200 mg/kg and IV- 400 mg/kg) and hydro-alcoholic extract (Group V- 200 mg/kg and VI- 400 mg/kg) of *Vitex negundo* up to 66 days using 0.5% w/v CMC as the vehicle. It was able to successfully exert its protective effect by restoring parameters to the normal level and disappearance of cysts in ovaries. This can be attributed to phyto-components present in the extract. The aqueous and hydro-alcoholic extracts of seeds of *Vitex negundo* showed significant amelioration of Letrozole induced PCOS.
10. Kannikaparameswari and Indhumathi et al., 2013 investigated the haematological and cytotoxic effect of the ethanolic extract of *Vitex negundo*. The ethanolic extract of Vitex negundo extract treatment showed decrease in tumor volume, packed cell volume and viable cell count and increases the nonviable cell count and mean survival time (MST), thereby increasing life span of EAC tumor bearing mice. Hematological profile reverted to more (or) less normal levels in extract treated mice. The data presented here clearly encourage the development of *Vitex negundo* for chemopreventive agent.
11. Roosewelt et al., 2011 evaluated the effect of wound healing activity of the methanolic extract of *Vitex Negundo* leaves. Methanolic extract of the shadedried leaves of *Vitex Negundo* was studied for its effect on wound healing in albino Wistar rats, using incision, excision and dead-space wound models, at two different dose levels of 200 and 400 mg/kg. The plant showed a definite, positive effect on wound healing, with a significant increase in the levels of hydroxyproline content, tensile strength and protein content of wounded skin in both incision and dead space wound model. Histopathological observation also supports the use of *Vitex negundo* in the management of wound healing.

Chapter 04

Plan of Work

4 Plan of Work

1. Collection, drying and grinding of leaves
2. Authentication of the plant specimen
3. Pharmacognostic study of the plant *Vitex negundo*
4. Physico-chemical evaluation of the leaves powder
5. Fluorescence Characterization
6. Extraction of the plant leaves with suitable solvent
7. Phytochemical screening of the leaves extract
8. TLC analysis of the extract
9. Preparation and characterization of tincture
10. To carry out In-vitro antioxidant study
    I. Determination of DPPH radical scavenging activity
    II. Determination of Hydrogen peroxide radical scavenging activity
    III. Determination of Total Phenolic content
    IV. Determination of Total Flavonoid content
11. To carry out In-vitro anti-diabetic study
12. To carry out In-vivo diabetic-induced nonalcoholic fatty liver disease study
13. GC-MS analysis of methanolic extract

Chapter 05

Plant Profile

5 Basis of Plant Selection

In West Bengal, the *Vitex negundo* L. plant has been used to treat diabetes for many years. Ethno medicinal evidences support the use of this plant leaves as anti-diabetic agent but its activity has not been fully discovered yet. Therefore, I chose this plant to evaluate whether it has anti-diabetic activity by performing In-Vitro and In-vivo studies.

5.1 Synonym

Vitex negundo L. (Ahuja et al., 2015)

5.2 Vernacular Name:

English: Chinese chaste tree, five-leaved chaste tree

Bengali: Nisinda

Hindi: Bheudi

Sanskrit: Nirgundi (Kalderal et al., 2015)

Figure 1: Plant Photograph

5.3 Taxonomical Classification

Domain: Eukaryota

Kingdom: Plantae

Phylum: Anthophyta

Class: Dicotyledoneae

Sub-class: Asteridae

Order: Lamiales

Family: Lamiaceae

Genus: Vitex

Species: *Vitex negundo* L.

Botanical Name: *Vitex negundo* L. (Ramesh, Arivudainambi, and Rajendran et al., 2017)

5.4 Morphology

Vitex negundo is an erect shrub or small tree growing from 2 to 8 m in height. The bark is reddish brown. Its leaves are digitate, with five lanceolate leaflets, sometimes three. Each leaflet is around 4 to 10 cm in length, with the central leaflet being the largest and possessing a stalk. The leaf edges are toothed or serrated and the bottom surface is covered in hair. The numerous flowers are borne in panicles 10 to 20 cm in length. Each is around 6 to 7 cm long and are white to blue in colour. The petals are of different lengths, with the middle lower lobe being the longest. Both the corolla and calyx are covered in dense hairs. The fruit is a succulent drupe, 4 mm in diameter, rounded to egg-shaped. It is black or purple when ripe. (Salvaña et al., 2019; Du et al 2010; Mani et al., 2020)

5.5 Distribution

Vitex negundo is native to tropical Eastern and South Africa and Asia. It is widely cultivated and naturalized elsewhere. Countries it is indigenous to include Afghanistan, Bangladesh, Bhutan, Cambodia, China, India, Indonesia, Japan, Korea, Kenya, Madagascar, Malaysia, Mozambic, Myanmar, Nepal, Pakistan, the Philippines, Sri Lanka, Taiwan, Tanzania, Thailand, and Vietnam. Due to its medicinal properties and ornamental value, Vitex negundo has been introduced to many other tropical and subtropical regions. It is found in parts of Africa, Australia, and the Americas where it has been cultivated or has escaped from cultivation. (Ladda and Magdum et al., 2012)

5.6 Growth Condition

It prefers a subtropical to tropical climate. It can tolerate a wide range of temperatures but thrives best in warm, humid conditions. It grows well in a variety of soil types, including sandy, loamy, and clay soils. However, it prefers well-draining soil with moderate fertility. It can tolerate slightly acidic to slightly alkaline soils. It is a fast-growing shrub or small tree that can reach heights of 3 to 5 meters (10 to 16 feet). It has aromatic leaves and produces small, fragrant flowers in clusters. (Rani and Nair et al., 2006; Rahman and Bhadra et al., 2011)

5.7 Traditional Uses

V. negundo L. plant is widely planted along the road as a hedge plant and is utilized as traditional medicine to treat a variety of medical issues, some of which have been empirically

verified but its medicinal properties are still yet to be fully discovered. the plant is reported by many studies as an important medicinal plant, specifically in India, where they exhibit multifarious activities, including anti-inflammatory, analgesics, tonic, and antimicrobial properties. In India and Malaysia, the shoot, fruit, and leaf of these plant are used to help women after childbirth where the juice of the shoot and fruit is utilized to increase milk lactation and the leaf was boiled in water for post-partum bath, which helps the mother's recovery. (Vishwanathan and Basavaraju et al., 2010; Bano et al., 2015; Kekuda et al., 2013; Panda et al., 2009)

5.8 Phyto - Chemistry of *Vitex negundo*

It is having these major phytoconstituents alkaloids, flavonoids, terpenoids, phenolic acid, lignan, saponin, sterol. Various alkaloids have been identified in different parts of the plant, including vitricine, vitexicarpine, and others. Flavonoids are abundant in *Vitex negundo* and include compounds such as casticin, isovitexin, luteolin, apigenin, and quercetin. *Vitex negundo* contains several terpenoids, including essential oils such as limonene, sabinene, β-caryophyllene, and α-pinene. Phenolic acids, such as chlorogenic acid and caffeic acid, are present in *Vitex negundo.* (Gautam et al., 2008)

5.9 Pharmacological Activities of *Vitex negundo*

Vitex negundo (VN), commonly known as "chaste tree", is an ethnobotanically important plant with enormous medicinal properties. Different species of Vitex vary in chemical composition, thus producing different phytochemicals. Several bioactive compounds have been extracted from leaves, seeds, roots in form of volatile oils, flavonoids, lignans, iridoids, terpenes, and steroids. These bioactive compounds exhibit anti-inflammatory, antioxidant, antidiabetic, anticancer, antimicrobial. VN is typically known for its role in the modulation of cellular events like apoptosis, cell cycle, motility of sperms, polycystic ovary disease, and menstrual cycle. (Zheng et al., 2015; Singh et al., 2020; Gandhimathi and Aanandhi et al., 2023)

Chapter 06

Methodology

6 Methodology

6.1 Collection and authentication of the plant component

The leaves of the plant *Vitex negundo* L. (Lamiaceae) were collected in the month of November of 2022 from Paschim Medinipur district, West Bengal. A specimen of the plant was deposited in the Central National Herbarium at the Botanical Survey of India, Shibpur, Howrah (CNH/Tech.II/2023/194) for identification and authentication. The leaves were collected, thoroughly washed with water, and then dried for a month under shade. The dried leaves were then mechanically ground into a powder and placed in an airtight container for later usage.

6.2 Morphological and Organoleptic characters

The fresh leaves of the *Vitex negundo* were collected and subjected for evaluation of morphological characteristics like colour, size, shape, and other macroscopic characteristics. Other organoleptic characteristics including taste, odour and texture were also evaluated.

6.3 Microscopic characteristics

Microscopic characterization of the leaves was carried out by cutting thin transverse sections of the lamina of the fresh leaves. The sections were mounted on a glass slide with the help of glycerin and covered with cover slips. The sections were observed under compound microscope with 10X magnifications to study the internal anatomical characteristics of the leaves. In the next step, dried leaves were converted into coarse powder with the help of a mortar and pestle and passed through a sieve to separate the coarse powder from larger fragments. Then a pinch of the powder was placed on a glass slide and mounted with glycerin. The glass slide was covered with cover slip and observed under compound microscope with 10X magnification for powder characteristics. (Khan et al.,2013;)

6.4 Physicochemical assessment

(Kokate et al., 2005; Mukherjee 2002; Mandal et al.,1999; Mandal et al., 2015; Khandelwal 2006)

6.4.1 Loss on Drying (LOD)

2g of the powdered leaves were placed in a shallow, dried weighing bottle. The sample bed's height was kept 10mm or less. The sample was then dried in a hot air oven at 105º C for 30 minutes. Then, the shallow weighing bottle was taken out and cooled in a desiccator, and

weighed at room temperature. The procedure was repeated until the difference between two successive weights was less than 0.5mg. The LOD was determined as percentage of w/w.

6.4.2 Ash Value

Total Ash Value: 2g of the powdered leaf sample was weighed accurately and placed in a silica crucible which had been weighed previously. The crucible was placed in a muffle furnace and incinerated at 450ºC until the sample was free from carbon. After the incineration is complete, the crucible is taken out from the muffle furnace and cooled in a desiccator. Once cooled, the weight of the crucible was recorded at room temperature in a careful way, so that the sample does not absorb moisture from the surrounding air. The total ash was calculated as the percentage (w/w) of ash generated from the leaf sample after incineration.

Water Soluble Ash Value: After obtaining the total ash residue from the crucible, it was carefully placed in a beaker with 25ml distilled water. The beaker was then placed on a water bath and the contents were boiled for 25 minutes. After 25 minutes, the mixture was cooled and filtered using an ash less filter paper (Whatman filter paper 42). Upon completion of the filtration, the residue on the filter paper was thoroughly washed with hot water and placed in a silica crucible which had been weighed previously. The crucible was placed in a muffle furnace and then incinerated at 450ºC till no carbon residue of the filter paper was left in the crucible. The weight of the residue 22 | P a g e in the crucible was carefully recorded and subtracted from the weight of the total ash to calculate the fraction of ash that had been dissolved in water. The value of water-soluble ash was calculated as percentage (w/w) of total ash that got dissolved in distilled water upon boiling.

Acid Insoluble Ash: Total ash from the previous study was placed in a beaker and 25ml of 2N hydrochloric acid was added to the beaker. The contents of the beaker were then boiled on a hot plate for 5 minutes. After boiling, the contents of the beaker were cooled and filtered using ash less filter paper. The residue on the filter paper along with the filter paper was carefully taken in a previously weighed silica crucible and incinerated at 450ºC for 15 minutes. The incinerated residue was weighed carefully to record the amount of ash that remained insoluble in the acid upon boiling. The calculations for the acid insoluble ash percentage (w/w) were made using dried sample data.

6.4.3 Extractive Value

Value of Water-Soluble Extractive: In a closed conical flask, 5g of coarsely powdered leaves were macerated with 100 ml of water for 24 hours. The flask was shaken frequently for the first 6 hours before being left to stand for 18 hours. After 24 hours, the mixture was filtered and 25 ml of the filtrate was then measured, and evaporated in a small porcelain plate. The residue on the porcelain plate was weighed and recorded. With reference to the dried sample, the percentage (w/w) of water-soluble extractive value was calculated.

Alcohol (Ethanol) Soluble Extractive Value: In a closed conical flask, 5g of coarsely powdered leaves were macerated with 100 ml of ethanol for 24 hours. Alcohol soluble extractive value was determined in the same way as water soluble extractive value.

6.4.4 Analysis of Crude Fiber

200ml of 1.25% (v/v) sulfuric acid and 2g of the dried leaf powder were heated together with constant stirring. After heating, the mixture was filtered using a filter paper and the filter paper was washed with hot water for few times. After that, 200ml of a solution containing 1.25% (w/v) sodium hydroxide was added to the filtrate, and it was heated for 30 minutes. The mixture was filtered after being rinsed in hot water and the filtrate was then burned at 110^{o} C to a constant weight. With reference to the dried sample, the crude fiber percentage was computed.

6.4.5 Swelling Index

A 100 ml measuring cylinder was filled with 1g of dried powder and 25ml of water. For an hour, the cylinder was shaken every ten minutes. The measuring cylinder was then kept for 3 hours. Change in volume of the mixture was recorded to calculate swelling index of the leaves.

6.4.6 Foaming Index

1g of the dried sample was added to a conical flask that held 500 ml of boiling water. It was then filtered into a volumetric flask after cooling. Water was added till the content reached 100 ml. Obtained and labeled ten test tubes with stoppers. The drug was added in successive sections of 1, 2ml up to 10 ml into different tubes, and the remaining volume was adjusted with water up to 10ml. The tubes were sealed with stoppers, shaken for 15 seconds, allowed to stand for 15 seconds, and then height of the foam from each tube was measured.

The foaming index is less than 100 if each tub's foam is less than 1cm in height. The dried sample solution decoction volume in this tube (a) is used to calculate the index if the height of

foam of 1 cm is measured in the first through tenth tubes. To get a more accurate result, make an intermediate dilution if this tube is the first or second in a series. The foaming index exceeds 1000 if the height of the foam in the first through tenth tubes is greater than 1 cm.

Foaming index =1000/a

Where, a=the amount of decoction in milliliters that was used to prepare the dilution in the tube where foaming to a height of one centimeter is seen

6.4.7 Fluorescence analysis of the leaf powder

After being treated with various chemical agents, the color of the leaf powder was observed. Observations of the fluorescence properties of the leaf powders treated with various chemicals and reagents were made under both short-wavelength (254 nm) and long-wavelength (366 nm) ultra violet light. (Mandal et al., 1996)

6.4.8 Preparation of extracts

The following solvents, in order of increasing polarity, were used to extract the powdered leaves in a soxhlet extractor; petroleum ether (boiling point 60-80ºC), Ethyl acetate (boiling point 77ºC) and methanol (boiling point 64ºC). Using petroleum ether, the powdered plant material (90g) was first extracted. The extraction procedure was carried out until all the contents had been removed, and this was verified by removing a sample from the soxhlet extractor's syphon tube. It was then evaluated by TLC using the appropriate solvent that had been used for the extraction procedure. The absence of a spot on the TLC plate in the iodine chamber served as a sign that the extraction process has been completed. The complete assembly of the soxhlet extractor has been depicted in Figure 2. The extract was then concentrated by vacuum distillation, dried in the open air, and stored in an airtight container. The marc was dried by air

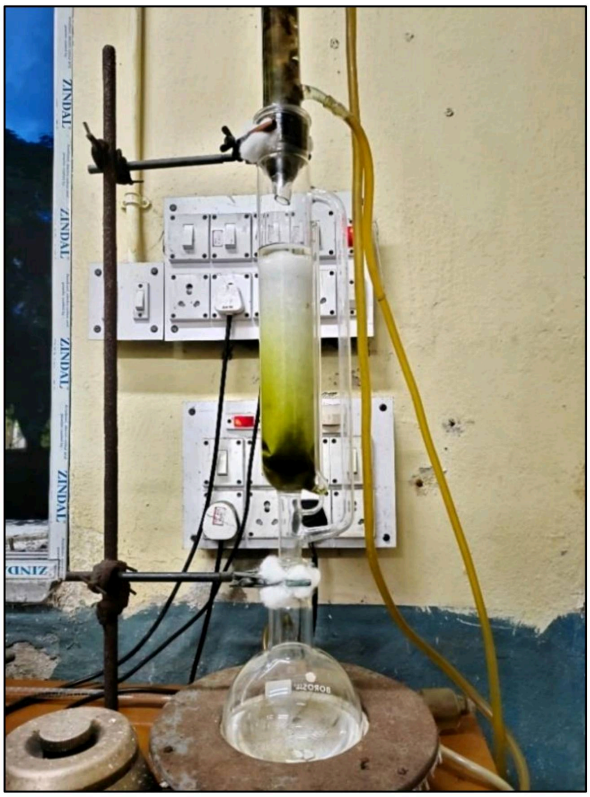


Figure 2: Soxhlet extractor assembly

before undergoing a second methanol extraction process. The extracts that were produced as a result of each extraction procedure were kept in airtight containers.

6.5 Screening for phyto-chemicals in the extract (Mandal et al., 2015; Banerjee et al., 2014)

To identify the presence of various phytocomponents, different chemical analyses were run-on the two drugs extract, petroleum ether extract and methanolic extract.

6.5.1 Test for Carbohydrates

Molisch Test (general): A few drops of the Molisch reagent were applied to 2–3 ml of the extract. Sulphuric acid in the amount of two ml was introduced from the test tube's side. Carbohydrates are present when a purple ring forms at the intersection of two liquids.

6.5.2 Test for Reducing Sugars

Fehling's Test: Boiling for one minute after combining 1ml each of Fehling's solutions A and B, equal volume of the test solution was added. Heated for five to ten minutes in a bath of boiling water. When reducing sugars are present, first yellow, then brick red ppt are seen.

Benedict's test: In a test tube, combine Benedict's reagent and test solution in an equal volume. 5 minutes of heating in a pot of boiling water. The colour of the solution depends on how much reducing sugar is present in the test solution; it might be green, yellow, or red.

6.5.3 Test for Monosaccharides

Barfoed's Test: Combine Barfoed's reagent and test solution in an equal volume; heated in a bath of boiling water for one to two minutes. Red ppt is formed if monosaccharide's is present.

6.5.4 For Non-Reducing Sugar

Test solution does not show positive results for Fehling's and Benedict's test.

6.5.5 Test for Proteins and Amino Acids

Biuret Test (general test): 1 ml of biuret reagent was added to 2ml of test solution (extract). Protein is present when the colour is violet.

Ninhydrin Test (general test): 2ml of test solution and 2 drops of 5% Ninhydrin solution were heated for 10 minutes in a boiling water bath. The test solution turns purple or bluish when amino acids are present.

Millon's Test: Combine 5ml of Millon's reagent with 3ml of test solution. White ppt is formed which turns brick Red after warming if protein is present.

6.5.6 Test for Steroids

Salkowski's Test: Add 2 ml of chloroform and 2 ml of conc. Sulphuric acid to 2 ml of extract and thoroughly shaken. The appearance of a reddish-brown tint in the bottom layer is a sign of steroids.

Liebermann-Burchard Test: Acetic anhydride was used to first treat the extract solution before a few drops of strong sulfuric acid were added along the test tube's side. Green coloration is a sign of the presence of steroids.

6.5.7 Test for terpenoids

Noller's Test: Noller's reagent, which contains0.1% stannic chloride in thionyl chloride, was applied to the extract solution. Terpenoids are present when red hue appears, indicating their existence.

6.5.8 Test for Glycosides

(For cardenolides) Legal's test: 1ml pyridine and 1ml sodium nitroprusside were added to the extract. Glycosides are indicated by the colour pink.

Keller-Killiani test (for deoxy-sugars): Glacial acetic acid, one drop of 5% FeCl3, and sulfuric acid were added to the 2ml of extract. If glycosides are present, a reddish-brown colour occurs at the junction of the two liquid layers, while the upper layer appears bluish green.

6.5.9 Test for anthraquinone glycosides

Borntrager's test: Diluted sulfuric acid was added to 3ml of extract. Filtered and boiled. Chloroform was added in an equal amount to the cold filtrate. Firmly shaken. Make the organic solvent separate. Added ammonia. Anthraquinone glycosides cause the ammonical layer to turn pink or crimson.

Modified Borntrager's test: 5% FeCl3 and 5 ml of diluted HCl were added to 5 ml of extract. Heated in a bath of boiling water for five minutes. Chloroform was added after cooling. Firmly shaken. Poured an equal proportion of diluted ammonia to the separated organic layer. The coloration of the ammoniacal layer is pinkish red.

6.5.10 Test for Saponins

Shake the water and powdered extract vigorously. Saponin is detected when there is persistent froth.

6.5.11 Test for Flavonoids

Shinoda test: 5 ml 95% ethanol and a few drops of conc. HCl were added to the dried extract. 0.5 gm of magnesium turnings was added to it. Flavonoid's give off an orange, pink, red, or purple tint when they are present.

Ferric Chloride Test: Added a few drops of 10% FeCl3 to the extract. It emits a green tint to show flavonoids are present.

Sodium hydroxide Test: Solution of sodium hydroxide was added to the extract. The presence of flavonoids is shown by the appearance of yellow hue, which is destroyed by the addition of acid.

6.5.12 Test for Alkaloids

Mayer's test: Mayer's reagent in a few drops with 2-3ml of filtrate results in a cream-colored ppt.

Dragendorff 's Test: Add a few drops of Dragendorff's reagent to 2 to 3ml of filtrate. Orange and brown ppt. is created

Hager's test: With Hager's reagent, 2-3ml of filtrate produces yellow ppt.

Wagner's test: With Wagner's reagent, 2–3ml of filtrate yields reddish brown ppt.

6.5.13 Test for Tannins and Phenolics

$FeCl_3$ test: Dark green or deep blue colouring after treatment with 5% Fecl3 solution suggests the presence of tannin and phenolics.

Gelatine Test: 10% sodium chloride and 1% gelatin solution were added to the test solution. Precipitate formation suggests the presence of tannins.

10% NaOH test: When 4ml of 10% NaOH solution is added to the extract solution and it is thoroughly agitated, an emulsion forms that shows tannins and phenolics are present.

6.6 Chromatographic analysis of the prepared extracts

Thin layer chromatographic (TLC) analysis was performed on both the extracts prepared from the powdered leaves of the plant. (Kagan et al., 2014)

6.6.1 Preparation of a sample

Dried petroleum ether and methanol extracts, each weighing 1g, were diluted in an adequate quantity of the extraction solvents, petroleum ether and methanol, and then utilized as samples for TLC analysis.

6.6.2 Stationary phase

Pre-coated Silica gel G was utilized as stationary phase for performing TLC analyses of the extracts.

6.6.3 Mobile phase

For methanol extract, the mobile phase was used as hexane: chloroform: methanol (6:2.5:1.5) while for the petroleum ether extract, petroleum ether: acetone (8.5:1.5), was used as mobile phase.

6.6.4 Observation

Retention factor (Rf) value for each of the spots was determined after the spots were observed in an UV chamber at wavelengths of 254 nm and 366 nm.

6.7 Preparation and characterization of tincture (Mandal et al., 1996)

A total of 21 amber-colored bottles with 10 ml capacity were used. To these, 10 ml of alcohol with varied strengths—40, 50, 60, 70, 80, and 90 percent (V/V) and absolute alcohol were added. Each of the twenty-one bottles contained 1g of *Vitex negundo* leaves powder. Each batch was allowed to macerate for a specific number of days: 7, 14, and 21. The materials were filtered through a sintered glass crucible (G4) while under reduced pressure after the required amount of maceration time. Physical characteristics were investigated, including colour, pH, specific gravity, and total solid content.

Figure 3: Tinctures of different alcohol concentrations after maceration for differing time period

6.8 Determination of Total Phenolic content

The total phenolic content of the sample was determined by the Folin-Ciocalteau method (Miliauskas et al., 2004) with slight modifications. About 1 ml of the extract solution was mixed with 5 ml of Folin-Ciocalteau reagent (FCR). After 3 min, 4 ml of sodium carbonate solution was added and the mixture was allowed to stand for 1 h at room temperature followed by measuring the absorbance at 765 nm. From the calibration curve of Gallic acid prepared in different concentrations (15.62, 31.25, 62.5, 125, 250, 500µg/ml). The number of phenolic compounds was determined and expressed as mg Gallic acid equivalent (GAE) of dried or fresh extract exudates. The total phenolic content in the extract expressed in Gallic acid equivalents (GAE) was calculated by the following formula:

T=C X V / M

Where, T- Total phenolic contents in mg/g plant extract, in Gallic Acid Equivalent (GAE)C-Concentration (mg/ml) of Gallic acid obtained from the calibration curve=Volume of extract (ml),

M=Weight (mg) of plant extract.

6.9 Determination of Total Flavonoid content

Total soluble flavonoid content of the fractions was determined with aluminum chloride using quercetin as a standard (Shraim et al., 1985) with slight modifications. To 1ml of each different concentration (15.62, 31.25, 62.5, 125, 250, 500µg/ml) of quercetin, 2ml of methanol was added. Then it was mixed with 0.2 ml of aluminum chloride and 0.2 ml of potassium acetate and finally 5.6 ml of distilled water was added to each concentration. Extract solution of

different concentration was also prepared using the same method. All the samples were incubated for 30 minutes at room temperature and absorbance was measured at 415 nm against control. The total flavonoid content in the fractions was determined as µg quercetin equivalent by using the standard quercetin graph and using the following formula:

T= C X V/ M

Where, T-Total flavonoid content in mg/g plant extract, in Quercetin Equivalent (QE), C=Concentration (mg/ml) of Quercetin obtained from calibration curve, V= Volume of extract (ml),

M= Weight (mg) of plant extract.

6.10 In-vitro antioxidant studies

The word antioxidant simply means "against oxidation". An antioxidant is any substance that retards or prevents the deterioration, damage, or destruction of cells by oxidation (Mandal et al., 2009; Dekkers et al.,1996) It is a classification of a number of organic compounds, including selenium, the carotenoids chemical family, vitamin A (which is made from beta carotene), and the vitamins C and E.

6.11 Determination of DPPH radical scavenging activity

Minor adjustments were made to an earlier approach (Blois 1958) of DPPH activity while using Ascorbic acid as the Standard for comparison. In order to create a series of sample solutions with various pre-set concentrations (15.62, 31.25, 62.5, 125, 250, 500µg/ml) the extract was dissolved in methanol. 2ml of the extract sample solution were combined with 1ml of a 0.3mM DPPH solution in methanol, and the mixture was then incubated for 30min at37°C. A UV-vis spectrophotometer was used to detect absorbance at 517 nm. The same method was applied to standard solutions with comparable concentrations. The ability of the sample/standard to scavenge the DPPH radical was determined as the free radical inhibition percentage using the following formula:

% Inhibition of DPPH scavenging activity =Ac - At/ Ac X 100

Where Ac is the absorbance of the control reaction and At is the absorbance of the test/standard. The extract's antioxidant activity was quantified as IC50. The IC50 values were computed using linear regression of plots, where the ordinate represents the average percent of scavenging capacity and the abscissa indicates the concentration of the studied plant extracts. The IC50

value is defined as the concentration (in µg/ml) of extract that inhibits the formation of DPPH radicals by 50%.

6.12 Determination of hydrogen peroxide radical scavenging activity:

The ability of both M. micrantha extracts to scavenge hydrogen peroxide was determined according to the method of Oktay Munir and others (Oktay et al., 2003) In phosphate buffer with a pH of 7.4, hydrogen peroxide (2m mol/L) was produced as a solution. Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity81 mol/L-1 /cm. Extracts samples (15.62, 31.25, 62.5, 125, 250, 500µg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml). After 10minutes, the absorbance of hydrogen peroxide at 230 nm was measured in comparison to a blank solution made up of phosphate buffer and no hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of both *V. negundo* extract and standard compounds (Ascorbic acid) can be calculated using the following formula-

%H2O2 radical Scavenging activity =Ac At/Ac X 100

Where Ac is the absorbance of the control reaction and at is the absorbance in the presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC50. The IC50 value is defined as the concentration (µg/ml) of dry extract that inhibits the formation of H2O2 radicals by 50%.

6.13 In-vitro anti diabetic activity

6.13.1 Inhibition of α Amylase

1ml of phosphate buffer was added in 5 different test tubes. To these, 0.2 ml alpha amylase solution was added. To the above mixture 0.4 ml of standard drug solution was added. The above mixture from each of the test tubes were incubated at 37º C for 20 minutes. To this incubated mixture of each test tube, 0.4 ml of starch solution was added and the mixture was then again incubated for 30 minutes at a temperature of 37 º C in a water bath. After 30 minutes, 2 ml of DNS solution was added to each test tubes and boiled for 10 minutes. After that the absorbance of the sample was measured at 540nm.The measurement of absorbance of control was conducted using similar method and by replacing standard drug solution with vehicle. Same method was used to measure the absorbance of test sample by using extract solution in place of standard drug solution. The concentration of the plant extract required to scavenge

50% of the radicals (%inhibition) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by-

% Inhibition = Abs Control – Abs Test / Abs Control X 100

The IC50 values were determined from plots of percent inhibition versus log inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was used as the reference alpha amylase inhibitor. All tests were performed in triplicate. All the measurements were done in triplicate and results are expressed in terms of Mean±standard deviation and IC50 values were calculated using MS-Excel software. (Telagari and Hullatti 2015; Velmani and Mandal 2016)

6.13.2 α- glucosidase inhibition assay

1ml of phosphate buffer was added in 5 different test tubes. To these, 0.2 ml alpha glucosidase solution was added. To the above mixture 0.4 ml of standard drug solution was added. The above mixture from each of the test tubes were incubated at 37º C for 20 minutes. To this incubated mixture of each test tube, 0.4 ml of starch solution was added and the mixture was then again incubated for 30 minutes at a temperature of 37 º C in a water bath. After 30 minutes, 2 ml of DNS solution was added to each test tubes and boiled for 10 minutes. After that the absorbance of the sample was measured at 540nm.The measurement of absorbance of control was conducted using similar method and by replacing standard drug solution with vehicle. Same method was used to measure the absorbance of test sample by using extract solution in place of standard drug solution. The concentration of the plant extract required to scavenge 50% of the radicals (%inhibition) was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated by-

% Inhibition = Abs Control – Abs Test / Abs Control X 100

The IC50 values were determined from plots of percent inhibition versus log inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values. Acarbose was used as the reference alpha glucosidase inhibitor. All tests were performed in triplicate. All the measurements were done in triplicate and results are expressed in terms of Mean±standard deviation and IC50 values were calculated using MS-Excel software. (Telagari and Hullatti 2015; Velmani and Mandal 2016)

6.14 Acute toxicity study

The acute toxicity study was already performed by Aiyalu et al., LD50 values of the methanolic extract of *Vitex negundo* L was found to be safe up to 2000mg/kg body weight by oral route (Chattopadhyay et al., 2014)

6.15 In-vivo anti diabetic studies

6.15.1 Preparation of 0.1M sodium citrate buffer solution (4.5pH):

To prepare 100ml of 0.1M citrate buffer mixed 270.278 Mg of Sodium citrate dehydrate and 207.7 mg of citric acid to 80 ml of distilled water. The solution was adjusted to desired p H using HCl or NaOH. The final volume was made up to 100 ml with distilled water. (Gomori 1955)

6.15.2 Preparation of Streptozotocin solution:

A solution of STZ was prepared by dissolving the weighed quantity of streptozotocin in 0.1M freshly prepared ice-cold citrate buffer (pH 4.5) solution.

6.15.3 Preparation of 5% Dextrose solution

5gm of dextrose is weighed and dissolved in 100ml of distilled water.

6.15.4 Preparation of Standard (Metformin HCl) solution :(Dose: 40mg/kg)

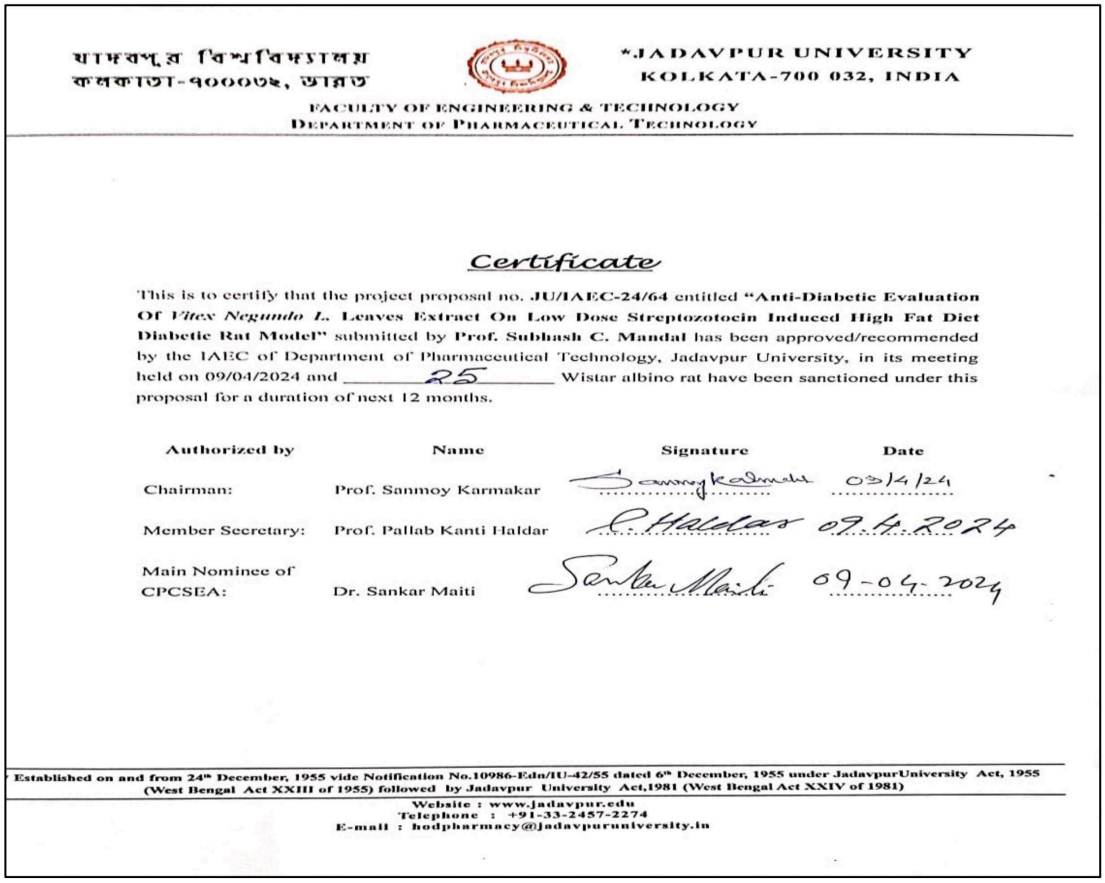
40 mg of Metformin HCl is dissolved in 1ml of distilled water (Mandal et al., 1997; Chakraborty et al., 2018)

6.15.5 Preparation of test solution:

Suspension of methanolic extract of *Vitex negundo* was prepared by using 2% aqueous Tween 80.

6.16 Housing of animals

Wistar albino rats weighing 150-200g used for all experiments studied. Animal experiment protocol (JU/IAEC-22/37) was duly approved by Institutional Animal Ethics Committee (IAEC), Depertment of Pharmaceutical Technology, Jadavpur University; Kolkata-700032.



যাদবপুর বিশ্ববিদ্যালয়
কলকাতা-৭০০০৩২, ভারত

*JADAVPUR UNIVERSITY
KOLKATA-700 032, INDIA

FACULTY OF ENGINEERING & TECHNOLOGY
DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY

Certificate

This is to certify that the project proposal no. **JU/IAEC-24/64** entitled **"Anti-Diabetic Evaluation Of *Vitex Negundo L.* Leaves Extract On Low Dose Streptozotocin Induced High Fat Diet Diabetic Rat Model"** submitted by **Prof. Subhash C. Mandal** has been approved/recommended by the IAEC of Department of Pharmaceutical Technology, Jadavpur University, in its meeting held on 09/04/2024 and 25 Wistar albino rat have been sanctioned under this proposal for a duration of next 12 months.

Authorized by	Name	Signature	Date
Chairman:	Prof. Sanmoy Karmakar		09/4/24
Member Secretary:	Prof. Pallab Kanti Haldar		09.4.2024
Main Nominee of CPCSEA:	Dr. Sankar Maiti		09-04-2024

Established on and from 24th December, 1955 vide Notification No.10986-Edn/IU-42/55 dated 6th December, 1955 under JadavpurUniversity Act, 1955 (West Bengal Act XXIII of 1955) followed by Jadavpur University Act,1981 (West Bengal Act XXIV of 1981)

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Figure 4: Approval certificate from Institutional Animal Ethics Committee

Animals collected randomly from State centre for Laboratory Animal Breeding West Bengal Livestock Development Corporation Limited, Buddhapark, and Kalyani, Nadia, and PIN-741235 separate cage under controlled conditions of temperature (22 ± 2°C). All animals given standard diet (pellet feed) and water add libitum. Animals kept at light/dark cycle of 12 hrs. Animals divided in five groups with six animals in each group. Institutional Animal Ethics Committee (Regd. No 1805/CPCSEA) approved the research protocol and all experimental procedures followed in the study.

6.16.1 Streptozotocin induced hyperglycemia:

The acute experimental hyperglycemia produced in rats by the single dose of streptozotocin (STZ) injection (40 mg/kg b.w.i.p) .The selected animals, weighing between 150-200g fasted overnight were administered with Streptozotocin (40 mg/kg b.w) intraperitoneal (1.0 ml/100 g). Fasting blood sugar levels were determined on 5th day after administering STZ to confirm stable hyperglycemia. The diabetic rats after confirmation of stable hyperglycemia divided into different groups of 6 rats each. That day considered as the 0 th day. Metformin hydrochloride at a dose of 250mg/kg b.w.used as a standard drug for treatment. After seven days, rats with glycemia ≥ 170 mg/dL were selected for further experimentation. Drug and doses administered accordingly as mentioned below:

1. Group1 (Normal control): without any drug treatment received only vehicle/distilled water.
2. Group2(Positive control): Diabetes (STZ-injected rats)
3. Group5 (Standard treated): Diabetes (STZ-injected rats) treated with standard drug– metformin hydrochloride (250 mg/kg b.w.).
4. Group3(Low dose treted): Diabetes (STZ-injected rats) treated with200mg/kg body weight with *Vitex negundo* extract
5. Group4 (High dose treated): Diabetes (STZ-injected rats) treated with 400 mg/kg body weight with *Vitex negundo* extract.

Methanolic extract of *Vitex negundo* in 2% Tween 80 suspension and standard drug metformin was orally administered for 21 days. Body weights and blood glucose levels of overnight-fasted rats were measured weekly. At the end of the experimental period, the animals were sacrificed under the influence of anesthetic and pancreas was collected from histopathologic examination.

6.17 Calculation of Doses: (Mandal et al., 1997; Chakraborty et al., 2018)

6.17.1 For test sample

The LD50 was found from the acute toxicity studies. LD50 values of the Methanolic extract of *Vitex negundo* were found to be safe up to 2000 mg. The in vivo biological studies on serum and tissues of male wistar rats at the doses of 200 mg and 400mg/kg body weight.

For Streptozotocin: 40mg/kgb.w.

For standard (Metformin HCl): 250 mg/kg b.w.

6.18 **Evaluation of hypoglycaemic activity:** (Mandal et al., 1997; Chakraborty et al., 2018)

6.18.1 Fasting Blood Glucose (FBG)analysis

To quantify the levels of blood glucose, blood samples were aseptically taken from the end of rat's tails and placed on glucose test strips. Blood was collected from the tail of overnight fasted rats with the help of a syringe and fasting blood glucose concentration noted down on days 0,7,14 and 21 using ACCU-CHEK Guide Glucometer.

6.18.2 Statistical analysis

The values expressed as Mean±standard Deviation (SD) of the indicated number of experiments/animals. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Sidak comparison test to determine the level of significance.

6.18.3 Estimation of serum lipid profile

Total cholesterol, triglycerides, high density lipoproteins are the main lipids found in serum. Certain pathophysiological conditions, such as hypercholesterolaemia, hyperlipidaemia, hypothyroidism, uncontrolled diabetes, nephrotic syndrome and cirrhosis result increased levels of serum lipid. Malabsorption, malnutrition, hyperthyroidism, anaemias and liver diseases are the causes of lower level of serum lipid. Cholesterol kit uses CHOD/PAP method to determine cholesterol activity in serum or plasma. All the reagents were ready to use.

For cholesterol determination three tubes were prepared and labelled as blank, sample and test. Blank tubes constituted with working reagent and distil water, standard tubes constituted with working reagent and standard and test tubes constituted with serum sample and working reagent. All the contents were mixed properly and incubated at 37°C for 5 min, followed by measuring the absorbance at 505 nm against blank. (Ahmed et al., 2010)

Calculations: Cholesterol in mg/dl = Abs. of test / Abs. of standard * 200 For estimation of triglycerides in serum sample, procedure was same, only standard tubes contained standard triglyceride with working reagent. Triglycerides in (mg/dl) = Abs. of test/ Abs. of Standard × 200

6.18.4 Histopathological studies

Histopathological studies were carried out with the parts of liver which were isolated from the sacrificed rats. The tissues were washed with normal saline and immediately fixed in 10%

formalin for 24 h, dehydrated with alcohol, embedded in paraffin, and then cut into 4-5-µm-thick sections and stained with haematoxylin-eosin dye and oil red o for photo-microscopic observations. (Madic et al., 2021).

6.19 Estimation of Tissue antioxidant parameter

6.19.1 Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) by product of lipid peroxidation in the liver tissue were measured by method (Ohkawa et al., 1979). 0.2 ml of supernatant was mixed with 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA). The total reaction mixture was made up to 4 ml with distil water heated at water bath at 95°C for 60 minutes. All the tubes were incubated and cooled at room temperature and volume of the tube was made up to 5 ml with distilled water. Each tube contained 5.0 ml of n-butanol: pyridine mixture (15: 1) was added and the reaction mixture was vortexed thoroughly for 2 minutes. All the tubes were centrifuged at 30009 for 10 minutes, the upper organic layer was separated and absorbance was measured at 532 nm against an appropriate blank without the tissue sample. TBARS level were expressed as µmoles of malondialdehyde (MDA)/g of liver tissue. (Maharaja et al., 2020)

6.19.2 Reduced Glutathione (GSH)

GSH is predominant in liver tissue, GSH level was determined as per reported method (Ellman, 1959). To 1 ml of protein free supernatant, 2 ml of Tris buffer (pH 8.9) was added. Then 0.05 ml of freshly prepared Eliman's reagent 5,5-dithio bis-2-nitro benzoic acid (CTNB) solution in absolute methanol was added and thoroughly vortexed. After addition of DTINE absorbance was measured at 412 nm after 2-3 min against a reagent blank. Standard reduced glutathione was used as standard. The content of GSH was expressed as µg/g of liver tissue. (Ganie et al., 2011)

6.20 GC-MS analysis of methanolic extract

GC–MS, an analysis was conducted using MSD and GC–MS QP 2024. In GC, 30 m × 0.25 mm × 0.25um thick, fused silica BD-5MS capillary column was used. The temperature of the instrument was initially set at 40°C and maintained for 10 min. The oven temperature was increased to 280°C at the end of the experiment. Mass spectra of the components in the samples were acquired using a detector operating in scan mode between 40 and 650 m/z. MS took 5 min to begin and 51 min to complete, with a solvent break time of approximately 6 min.

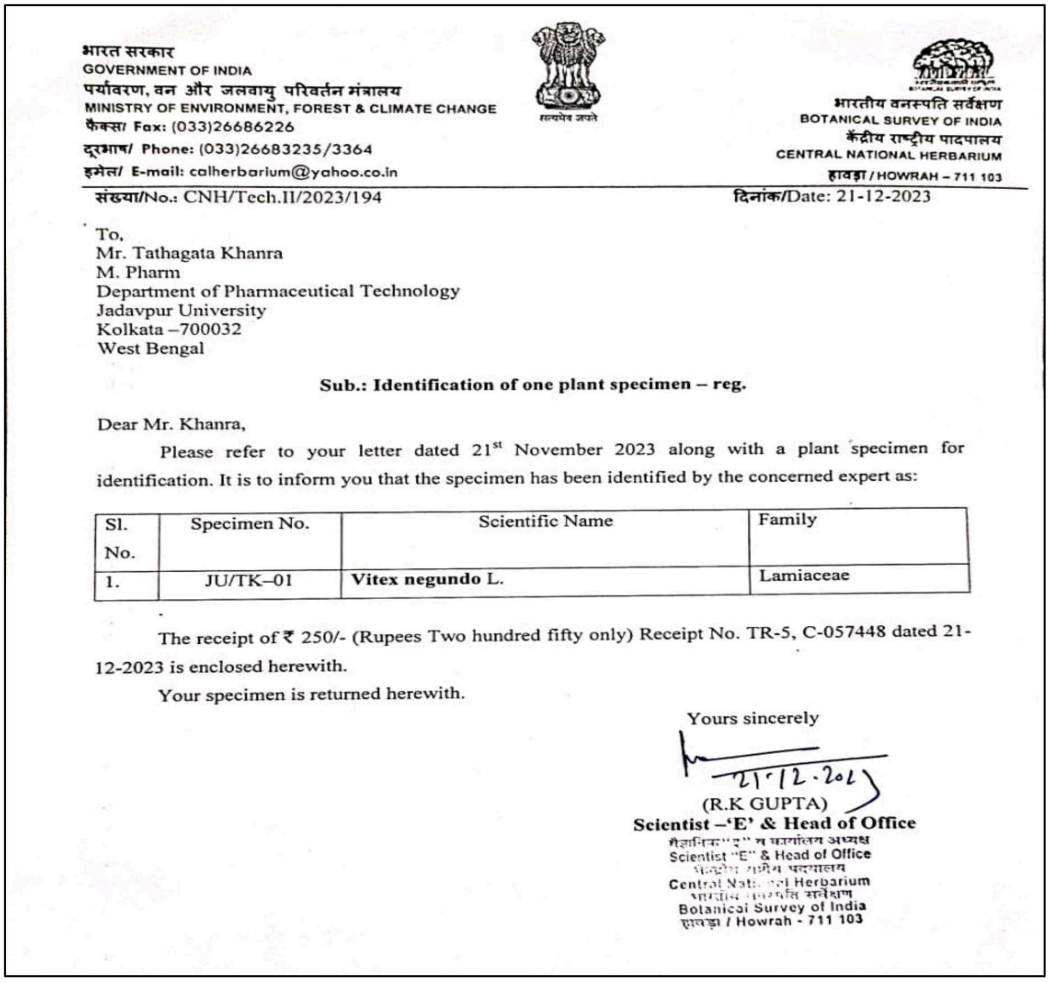
Chapter 07

Results

7 Results

7.1 Authentication of the plant material

A figure of the matured leaves of *Vitex negundo* L. has been provided in figure 4. The authentication certificate from the central national herbarium, Botanical Survey of India is presented in figure 5. The certificate from the CNH confirms the identity of the collected species as *Vitex negundo* L. of Lamiaceae family.



भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
फैक्स/ Fax: (033)26686226
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इमेल/ E-mail: calherbarium@yahoo.co.in

भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA
केंद्रीय राष्ट्रीय पादपालय
CENTRAL NATIONAL HERBARIUM
हावड़ा / HOWRAH – 711 103

संख्या/No.: CNH/Tech.II/2023/194 दिनांक/Date: 21-12-2023

To,
Mr. Tathagata Khanra
M. Pharm
Department of Pharmaceutical Technology
Jadavpur University
Kolkata –700032
West Bengal

Sub.: Identification of one plant specimen – reg.

Dear Mr. Khanra,

Please refer to your letter dated 21st November 2023 along with a plant specimen for identification. It is to inform you that the specimen has been identified by the concerned expert as:

Sl. No.	Specimen No.	Scientific Name	Family
1.	JU/TK–01	**Vitex negundo** L.	Lamiaceae

The receipt of ₹ 250/- (Rupees Two hundred fifty only) Receipt No. TR-5, C-057448 dated 21-12-2023 is enclosed herewith.

Your specimen is returned herewith.

Yours sincerely

21/12/2023

(R.K GUPTA)
Scientist –'E' & Head of Office
वैज्ञानिक "ई" व कार्यालय अध्यक्ष
Scientist "E" & Head of Office
केन्द्रीय राष्ट्रीय पादपालय
Central National Herbarium
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
हावड़ा / Howrah - 711 103

Figure 5: Authentication certificate from Botanical Survey of India

7.2 Morphological characteristics of the species

The morphological characters from the matured leaves of the plant are represented in Table 1. Upon studying the leaves, the colour was found to be light olive green with faint aromatic odour. The heart shaped bitter, pungent leaves had rough texture with a length of 4-10 cm according to their maturity.

Table 1: Macroscopic characteristics of leaves

Sl No.	Characteristics	Leaves part
1	Color	Light olive Green
2	Odour	Faintly aromatic
3	Taste	Bitter, pungent
4	Texture	Rough
5	Shape	Heart-shaped
6	Size	4-10cmlong

7.3 Microscopical characteristics of the species

The microscopic characters from the transverse section of the leaves of the plant are represented in Figure 6. The section shows the presence of various anatomical characters of the leaves including epidermal cells, vascular bundle and diagnostic character like multicellular covering trichomes. On Peeling of lower epidermis presence of another diagnostic characteristic in the form anisocytic stomata is represented in Figure 8. Powder characterization of the leaves revealed presence of anisocytic stomata along with large fibres and annular xylem vessels as shown in Figure 7.

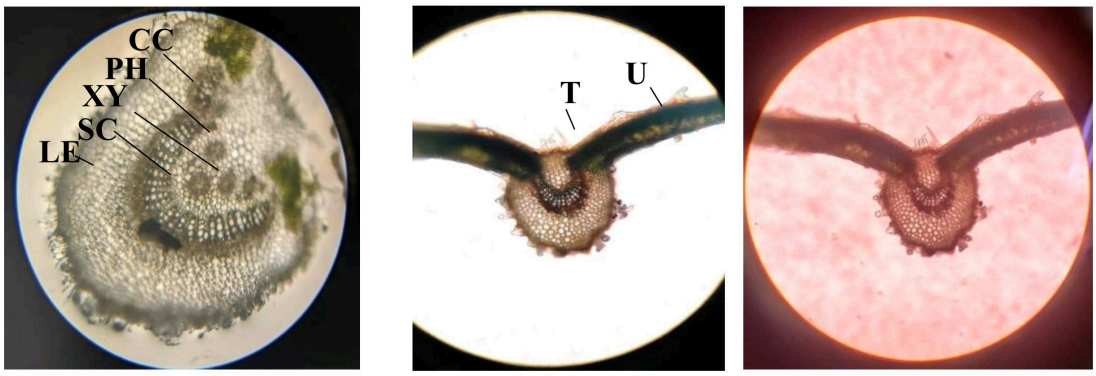


Figure 6: Transverse section of leaves Figure (CC = cortical cells; LE= lower epidermis; PH= phloem; PX= proto xylem; SC= sclerenchyma tous cells; T= trichome; UE= upper epidermis; XY= xylem)

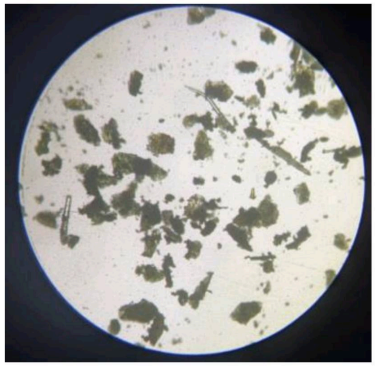


Figure 7: Powder characteristic of *Vitex negundo* L. leaves

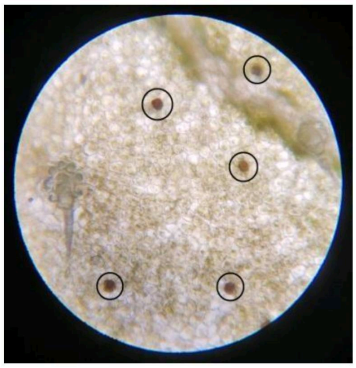


Figure 8: Stomata present in the lower epidermis

7.4 Physicochemical Characteristics of Leaves Powder

The leaves powder was used for the determination of different physicochemical parameters which helps in identification of the plants have been evaluated and they are represented in Table 2.

Table 2: Physicochemical Characteristics of Leaves Powder

Sl No.	Physico-Chemical Parameters	Results, Mean±SD(n=3)
1	Loss On Drying	9.33±0.036
2	Total Ash	5.23±0.035
3	Acid Insoluble Ash	4.77±0.064
4	Water Soluble Ash	5.11±0.050
5	Water Soluble Extractive	25±0.090
6	Alcohol (Ethanol)soluble extractive	15±0.082
7	Crude fiber Content	7.5±0.07
8	Swelling Index	2.5ml/g
9	Foaming Index	100

7.5 Fluorescence analysis of the leaf powder

The fluorescence characteristics of leaves powder on treatment with various chemicals and reagents on normal light is shown in Table 5 and under both short (254nm) and long (366nm) wavelength of UV light is shown in table 4.

Table 3: Colour of the powdered leaves on treatment with various chemical reagents

Sl No.	Reagents	Colour of powder
1	Powder+ Picric acid	Sacramento Green
2	Powder+ HNO3	Amber Orange
3	Powder+ HCl	Forest Green
4	Powder+ H2SO4	Lime Green
5	Powder+ Glacial acetic acid	Olive Green
6	Powder+ NaOH (5Naq.solution)	Olive Green
7	Powder+ Iodine(aq.Solution)	Leguna Yellow
8	Powder+ FeCl3(5%aq. Solution)	Olive Green
9	Powder+ antimony trichloride (alcoholic solution)	Moss Green
10	Powder+ methanol	Hunter Green
11	Powder+70%ethanol	Army Green
12	Powder+ Petroleum ether	Sacramento Green
13	Powder+50%H2SO4	Lime Green
14	Powder+1N NaOH(aq.solution)	Corn Yellow
15	Powder+1N NaOH (alcoholic solution)	Olive Green
16	Powder+50% HNO3	Fire Orange
17	Powder+5% KOH	Lime Green
18	Powder+ Ammonia	Olive Green

Table 4: Fluorescence analyses of powdered leaves on treatment with different chemical reagents under UV light

Sl No.	Reagents	Fluorescence under UV	
		Short wavelength(254nm)	Long wavelength(366nm)

1	Powder+ Picric acid	Army green	Hunter green
2	Powder+ HNO3	Chocolate brown	Mahogany red
3	Powder+ HCl	Sacramento green	Fern green
4	Powder+ H_2SO_4	No change	Kalley green
5	Powder+ Glacial acetic acid	Crimson red	Kalley green
6	Powder+ NaOH (aq. solution)	Forest green	Kalley green
7	Powder+ Iodine (aq. solution)	Sacramento green	Hunter green
8	Powder+ $FeCl_3$(5%aq. Solution)	Shadow green	Hunter green
9	Powder + antimony trichloride (alcoholic solution)	Bronze orange	Forest green
10	Powder+ methanol	Charcoal grey	Sacramento green
11	Powder+70% ethanol	Shadow grey	Forest green
12	Powder+ Petroleum ether	Mauve violet	Fern green
13	Powder+50% H2SO4	Byzantine violet	Kalley green
14	Powder+1N NaOH (aq. solution)	Sacramento green	Fern green

15	Powder+1N NaOH (alcoholic solution)	Hunter green	Kalley orange
16	Powder+ 50% HNO3	Kalley green	Kalley green
17	Powder+ 5% KOH	Forest green	Kalley green
18	Powder+ Ammonia	No change	Kalley green

7.6 Phytochemical screening of the extracts

The results of phytochemical screening of both petroleum ether and methanol extracts of leaves were represented in table 5. The phytochemical studies have revealed the presence of carbohydrates, monosaccharides, steroids, terpenoids, saponins and phenolics in the petroleum ether extract. Methanol extract on the other hand has revealed the occurrence of carbohydrates, reducing sugar, steroids, terpenoids, glycosides, and flavonoids through phytochemical analysis.

Table 5: Phytochemical Screening of the petroleum ether and methanol extracts of the leaves

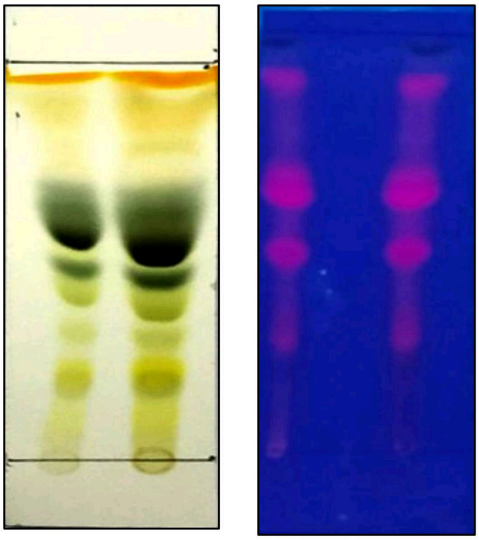
Chemical Constituents	Chemical Test	Ethyl acetate	Methanol extract
Carbohydrates	Molisch Test	-	-
Reducing Sugars	Fehling's Test	+	+
	Benedict's Test	_	-
Monosaccharides	Barfoed's Test	-	-
	Biuret Test	-	-

Proteins and Amino Acids	**Ninhydrin Test**	_	-
	Millon's Test	_	-
Steroids	**Salkowski reaction**	+	+
	Liebermann-Burchard Test	+	+
Terpenoids	**Noller's Test**	+	+
Glycosides (Cardiac Glycosides)	**Legal's test**	-	+
	Keller–killianitest	-	+
Anthraquinone glycosides	**Borntrager's test**	-	+
	Modified Borntrager's test	-	+
Saponins	**Foam Test**	+	-
Flavonoids	**Shinoda Test**	-	-
	Ferric Chloride Test	-	+
	Sodium hydroxide Test	-	+
Alkaloids	**Mayer's test**	+	+
	Dragendorff's test	-	+
	Hager's test	-	+
	Wagner's test	-	+
Tannins and Phenolics	**Ferric Chloride Test**	+	+

	Gelatin Test	+	+
	10% NaOH test	-	-

7.7 TLC Profiling

For the methanol extract (a), hexane: chloroform: methanol (6:2.5:1.5) was used as mobile phase while for the petroleum ether extract (b), petroleum ether: acetone (8.5:1.5) was used as mobile phase. The ethyl acetate extract showed 6 distinct spots while the methanolic extract showed 5 spots as presented in Figure 9. The TLC characteristics in the form of retention factor (Rf) of both petroleum ether and methanol extract of *Vitex negundo* leaves powder were shown in table 6.



Ethyl acetate **Methanolic**

Figure 9: Illustrating distribution of compounds of different polarities after separation on a TLC plate

Sl. No.	Rf Value	
	Ethyl acetate extract	Methanol extract
1	0.2	0.16
2	0.25	0.31
3	0.2	0.59
4	0.38	0.65
5	0.45	0.71
6	0.7	

Table 6: TLC Profiling of extracts

7.8 Characterization of tincture

The different characteristics of the prepared tincture were shown in table 7, 8 and 9. The pH of the prepared tincture at different strengths of alcohol is represented in table 7. The study shows that greater alcohol concentration in the tincture increases acidity of the tincture while storage for longer period of time also slightly increases acidic property. Solid content of the tincture also decreases with time as seen from table 8, while greater alcohol concentration also imparts diminished solid content. Finally, as seen from table 9, specific gravity the prepared tinctures do not vary substantially with time.

Table 7: pH of the tincture

Alcohol Strength	pH		
	7 Days	14 Days	21 Days
40%	6.05	5.85	5.73
50%	5.93	5.81	5.64
60%	5.84	5.64	5.60
70%	5.73	5.59	5.54
80%	5.71	5.63	5.59
90%	5.39	5.56	5.58
Absolute (100%)	5.34	5.24	5.07

Table 8: Total Solid Content of Tincture

Alcohol Strength	7 Days	14 Days	21 Days
40%	19.09	17.2	14
50%	17.18	15.47	12.83
60%	16.95	15.38	12.5
70%	16.69	14.81	10.33
80%	15.96	13.57	10.71
90%	12.13	13.28	10.16
Absolute (100%)	9.93	7.17	5.53

Table 9: Specific Gravity of Tincture

Alcohol Strength	7 Days	14 Days	21 Days
40%	0.952	0.950	0.954
50%	0.932	0.941	0.946

60%	0.900	0.921	0.949
70%	0.887	0.895	0.902
80%	0.865	0.899	0.912
90%	0.841	0.900	0.929
Absolute (100%)	0.797	0.799	0.801

7.9 Total Phenolic Content Assay

The absorbance of the Standard and test solution was represented in table no.10. The total phenolic content in terms of mg Gallic Acid Equivalent (GAE) of Ethyl acetate extract was found to be 35.149 mg/g and methanolic extract was found to be 28.82 mg/g. These results suggest that higher the presence of phenolic components was responsible for the levels of antioxidant activity. The Standard curve of Gallic acid was shown in figure 10.

Table 10. Observation of absorbance in Total phenolic estimation

Sl No.		Concentration(µg/ml)	Absorbance
1	Standard (Gallic acid)	3.125	0.0559
2		6.25	0.1201
3		12.5	0.1295
4		25	0.686
5		50	0.308
6		100	0.5794
1	Sample (Ethyl acetate)	100	0.3665
	Methanolic extract	100	0.3115

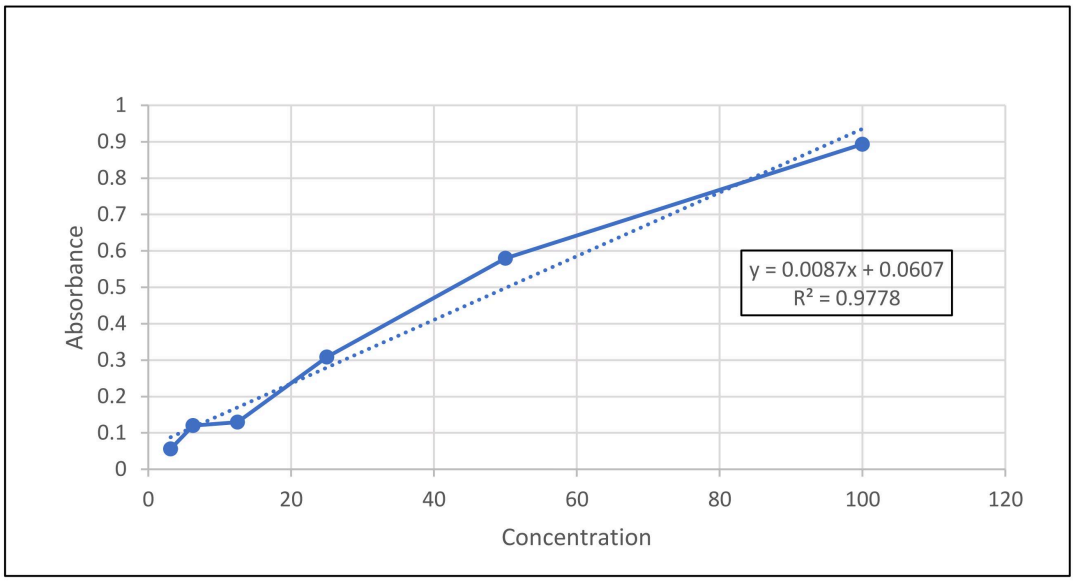


Figure 10: Calibration curve of Gallic acid

7.10 Total Flavonoid Content Assay

The content of flavonoid compound in both Ethyl acetate and methanolic extract of *Vitex negundo* leaves was measured by aluminium chloride reagent in terms of quercetin equivalent and was found to be 43.005 mg/g and 85.372 mg/g. The absorbance of the Standard and test solution was represented in table no. 11. It is well known that flavonoids have significant antioxidant activity and have a positive impact on human nutrition and health. The methanolic extract of Milania micrantha leaves contains a substantial quantity of flavonoids which may contribute considerable function to the antioxidant activity of the plant. The Standard curve of Quercetin is shown in figure 11.

Table 11: Observation of absorbance in total flavonoid estimation

Sl No.		Concentration (µg/ml)	Absorbance
1	Standard (Quercetin)	20	0.1428
2		40	0.250
3		60	0.358

4		80	0.454
5		100	0.547
1	Sample (Ethyl acetate)	100	0.265333
	Methanolic extract	100	0.482

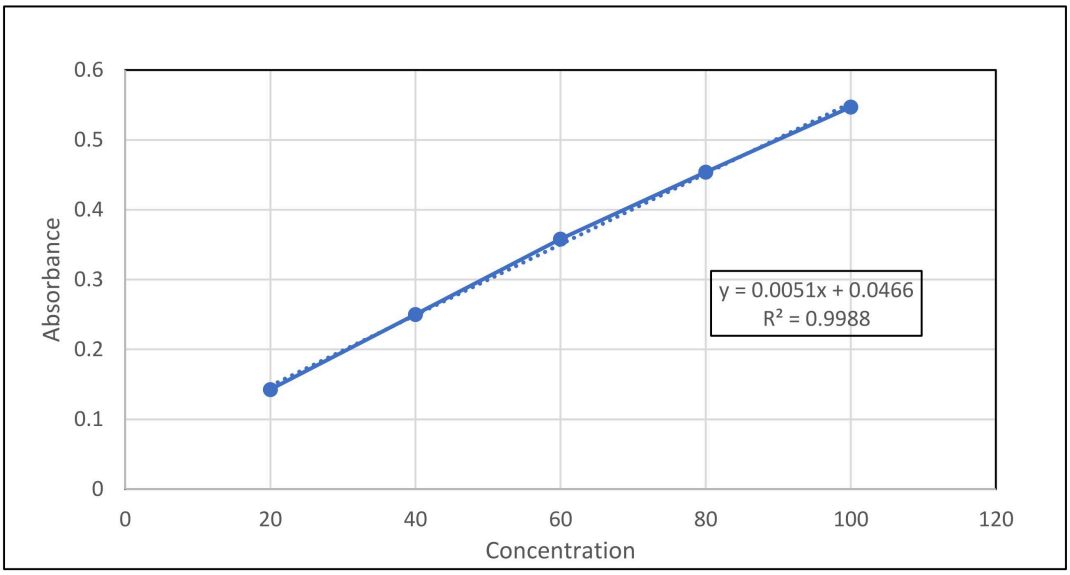


Figure 11: Calibration curve of Quercetin

7.11 Antioxidant Assay

7.11.1 7.11.1: Inhibition of DPPH radicals:

The drop in DPPH's absorbance at 517 nm, which is brought on by antioxidants, was used to assess its capacity for reduction. The extract showed maximum hydrogen donating ability in the presence of DPPH free radicals at high concentrations. The ethyl acetate and methanolic extract showed antioxidant activity with an IC50 value of 688.14 µg/ml and 653.28 µg/ml. However, the known antioxidant ascorbic acid exhibited an IC50 value of 197.77 µg/ml on DPPH radical. *Vitex negundo* had significant scavenging effects with increasing concentration when compared with that of Ascorbic acid. The results of the DPPH scavenging activity were shown below in the tableFigure12 represents DPPH radical scavenging assay of standard and test sample.

Table12: DPPH Radical Scavenging Assay of Standard sample and Test Sample

Sl No.	Concentration(µg/ml)	Percentage Inhibition (Mean±SEM)		
		Standard (Ascorbic acid)	Test Sample VN-ME	Test Sample VN-EA
1	15.62	54.04±0.28	4.33±0.33	7.75069±0
2	31.25	60.27±0.13	11.5722±0.08	13.1493±0.21
3	62.5	75.95±0	15.8189±0.5	23.688±01
4	125	85.85±0.1	30.2331±0.8	14.947±0
5	250	86.04±0.21	33.1934±0.09	36.6973±0.13
6	500	85.80±0	43.9798±0	53.2644±0.28

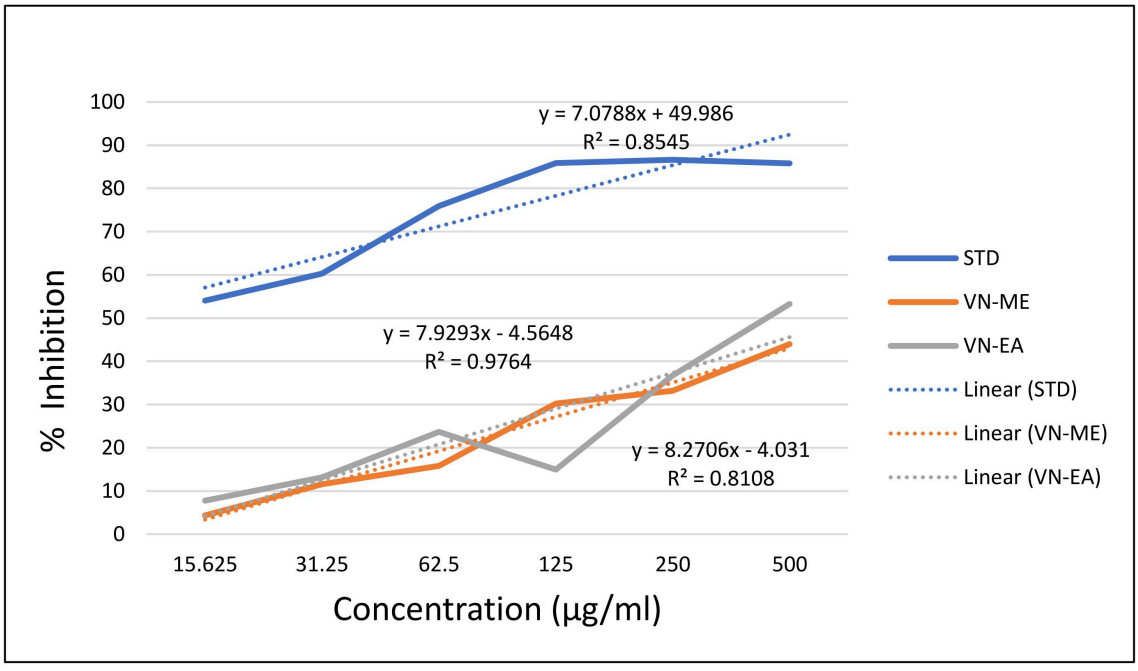


Figure 12: Percent inhibition of extract in DPPH antioxidant

7.11.2 Inhibition of hydrogen peroxide radicals:

In addition to being highly reactive, hydrogen peroxide itself can occasionally be hazardous to cells due to the hydroxyl radical it produces inside them. Thus, removing H_2O_2, as well as O_2 is very important for the protection of food systems. Here hydrogen peroxide scavenging activity of the extract is compared with ascorbic acid. The table below displays the outcomes. The IC50 value of ethyl acetate extract and methanolic extract were 484.44µg/ml and 360.85µg/ml respectively. The IC50 value of extract and ascorbic acid was 405.20µg/ml. The results showed that the test sample was effective in scavenging hydrogen peroxide in a dose- dependent manner. The outcome is comparable to that of the ascorbic acid reference standard. Figure 13represents hydrogen peroxide radical scavenging assay of standard and test sample.

Table 13: H_2O_2 radical scavenging activity of extract and standard

Sl. No.	Concentration (µg/ml)	Percentage inhibition (Mean ± S.E.M)		
		Standard (Ascorbic acid)	Test Sample VN-ME	Test Sample VN-EA
1	15.625	8.85±0.11	8.25±0.10	24.9±0.61
2	31.25	41.29±1.23	25.50±0.62	10.9±.02
3	62.50	57.08±1.15	29.70±1.14	32.3±0.72
4	125	89.39±0.67	70.20±0.72	30.2±1.14
5	250	85.14±.011	85.10±.02	62.2±0.62
6	500	78.00±2.58	71.30±0.61	68.5±0.10

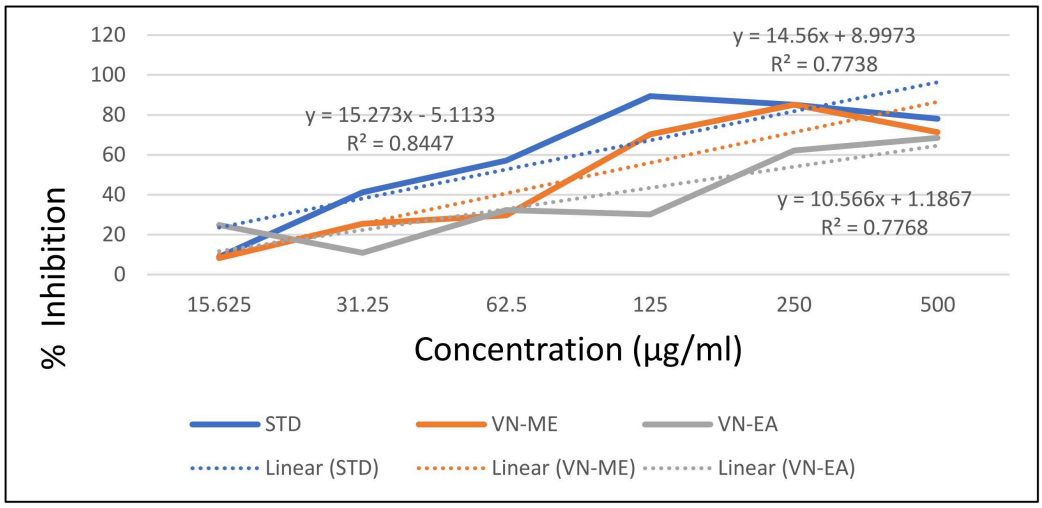


Figure 13: Percent inhibition of extract in H_2O_2 antioxidant assay

7.12 Determination of in-vitro Anti diabetic activity

7.12.1 α-amylase inhibition assay

α-amylase is one of the enzymes that hydrolyses the α-1,4 glycosidic linkages in starch to produce glucose and maltose. α-amylase hydrolyses complex polysaccharides into oligosaccharides and disaccharides and then hydrolyses by α-glucosidase to monosaccharides. The in vitro antidiabetic activity was evaluated by using α amylase inhibitory assay. The methanolic extract of *Vitex negundo* produces some inhibitory effects on this enzyme. Acarbose was used as a standard which shows marked inhibitory effect of the enzyme (my thesis ref). The IC50 of Acarbose, Ethyl acetate and Methanolic extract was calculated and found to be 156.17µg/ml 222.7µg/ml and 458.89µg/ml respectively. Table 14 shows the α-amylase inhibitory activity of Standard and test sample. Figure 14represents the graph of α-amylase inhibition assay of Acarbose and test sample.

Table 14: α -amylase inhibition activity of extract and standard

Sl. No.	Concentration (µg/ml)	% Inhibition of Acarbose, mean±SD(n=3)	% Inhibition of Methanolic extract of mean±SD	% Inhibition of Ethyl acetate extract of mean±SD
1	15.625	77.65143±0.447	62.7512±0.615	48.30866±0.517
2	31.25	79.70607±0.510	63.24144±0.520	49.49646±0.708

3	62.50	81.37292±0.585	64.05635±0.605	50.88393±0.585
4	125	84.00397±0.708	65.19524±0.796	53.02153±0.510
5	250	86.47107±0.517	65.40442±0.818	54.37496±0.447
6	500	89.54087±0.617	66.70958±0.918	56.35915±0.617

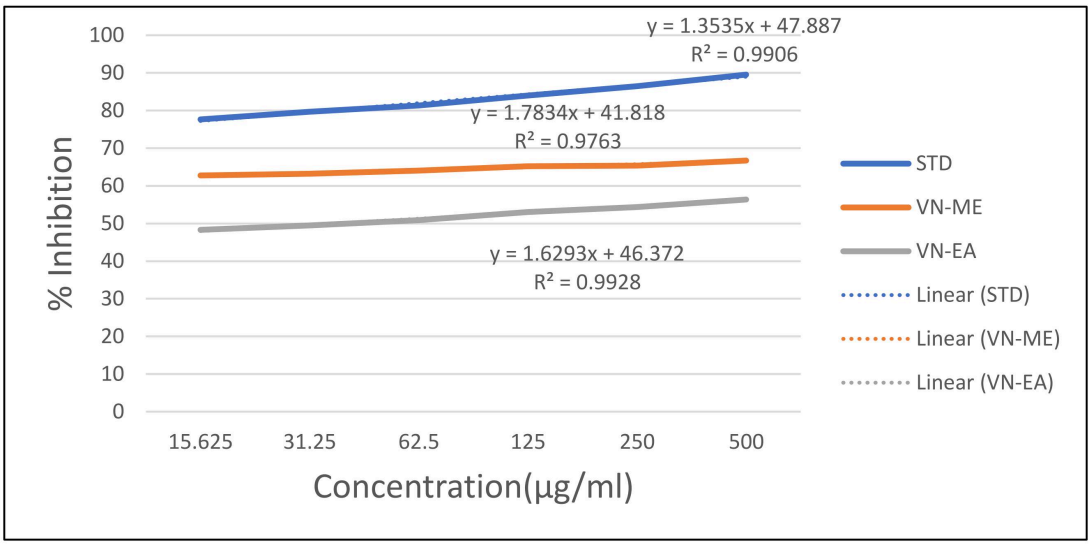


Figure 14: α-amylase inhibition activity of Extract and Standard

7.12.2 α- glucosidase inhibition assay

α-glucosidase is an enzyme that plays a crucial role in carbohydrate metabolism. It completes the digestion of starch by breaking down the products of amylase activity into glucose, the simplest form of carbohydrate, which can be easily absorbed into the blood. The in vitro antidiabetic activity was evaluated by using α-glucosidase inhibitory assay. The methanolic extract of *Vitex negundo* produces some inhibitory effects on this enzyme. Acarbose was used as a standard which shows marked inhibitory effect of the enzyme (my thesis ref). The IC50 of Acarbose, Ethyl acetate and Methanolic extract was calculated and found to be 789.98µg/ml 236.31µg/ml and 778.77µg/ml respectively. Table 15 shows the α-amylase inhibitory activity of Standard and test sample. Figure 16represents the graph of α-glucosidase inhibition assay of Acarbose and test sample.

Table 15: α - glucosidase inhibition activity of extract and standard

Sl. No.	Concentration (µg/ml)	% Inhibition of Acarbose, mean±SD(n=3)	% Inhibition of Methanolic extract of mean±SD	% Inhibition of Ethyl acetate extract of mean±SD
1	15.625	82.3419±0.447	48.1223±0.615	42.6836±0.818
2	31.25	82.8848±0.510	49.662±0.520	42.823±0.796
3	62.50	83.3642±0.585	49.7209±0.605	44.1065±0.605
4	125	86.9888±0.708	52.5813±0.796	44.1111±0.520
5	250	86.9888±0.517	55.4706±0.818	46.099±0.615
6	500	87.3605±0.617	59.0266±0.918	49.1632±0.918

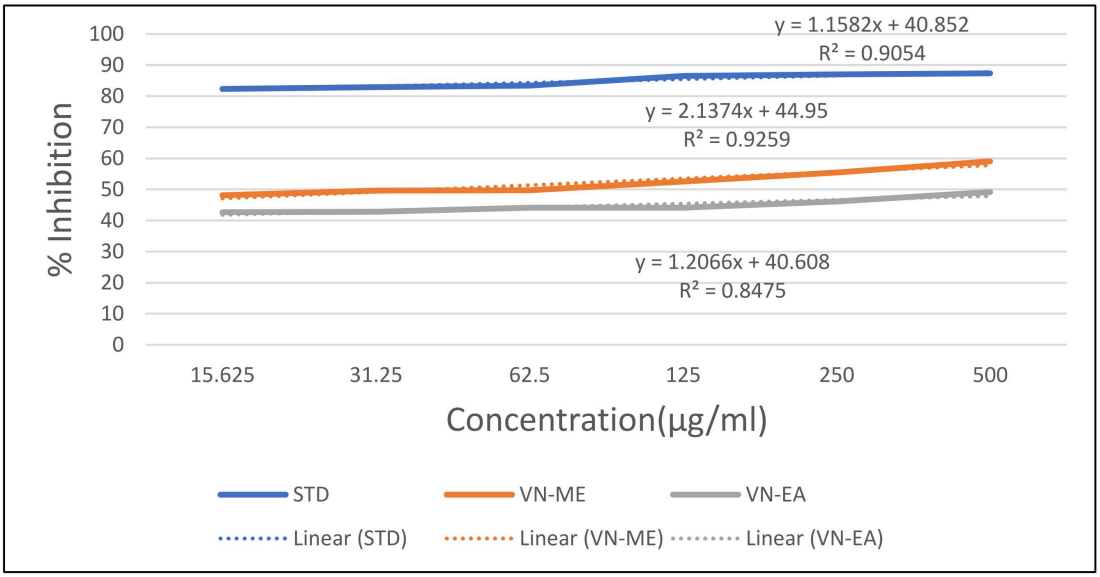


Figure 15: α-glucosidase inhibition activity of Extract and

7.13 Determination of in-vivo Anti diabetic Activity

7.13.1 Estimation of Body weight in experimental rats

Table16: Effects of *Vitex negundo* extract on the changes of body weight in normal control and experimental rats observed at weekly interval

Groups	Body weight (g)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	148.60±2.966	153.00 ±1.581	164.20±3.493	173.20±3.493	195.20±2.588
Disease control	152.20±3.421	135.40±3.975	119.40±6.348	106.40±4.037	101.40±3.507
Standard control	154.60±2.702	142.40±6.656	173.00±3.162	185.60±4.037	209.20±4.919
Low dose treated	149.80±3.033	134.80±4.817	174.20±3.701	186.40±1.949	212.80±4.970
High dose treated	154.20±2.588	143.80±3.701	178.80±3.033	192.00±3.317	220.40±6.189

All values are expressed as Mean±SD, (n=5), ANOVA followed by Sidak test was performed for significance data, comparing with group 1. *p<0.05 considered as statistically significant;

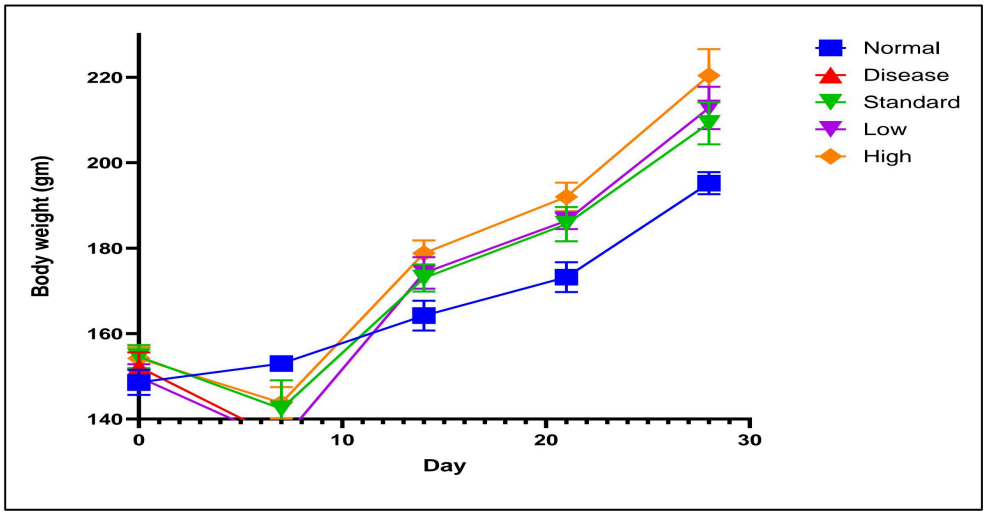


Figure 16: Illustrative representation of changes in Body Weight from Table no.16

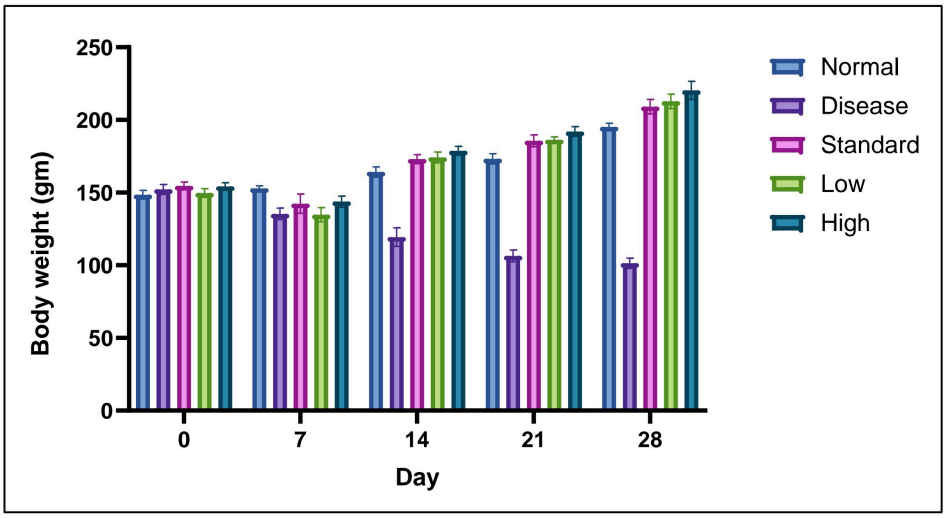


Figure 16: Illustrative representation of changes in Body Weight from Table no.16

7.13.2 In-vivo blood glucose determination

Table 17: Blood Glucose Level (mg/dl)

Groups	Fasting Blood Glucose level (mg/dl)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	98.40±11.781	116.20±4.494	128.00±2.915	130.60±2.074	132.40±4.669
Disease control	104.00±6.782*	193.00±13.266*	203.40±19.087*	227.20±14.789*	236.60±21.173*
Standard control	103.40±9.370	119.60±3.050	128.60±4.615	132.00±3.808	125.20±5.404
Low dose treated	104.40±8.620	171.40±11.929	159.20±6.723	155.60±12.779	151.40±12.621
High dose treated	109.00±8.803	218.20±16.223	134.60±4.669	131.40±4.159	121.80±3.701

All values are expressed as Mean±SD, (n=5), ANOVA followed by Sidak test was performed for significance data, comparing with group 1. *p<0.05 considered as statistically significant;

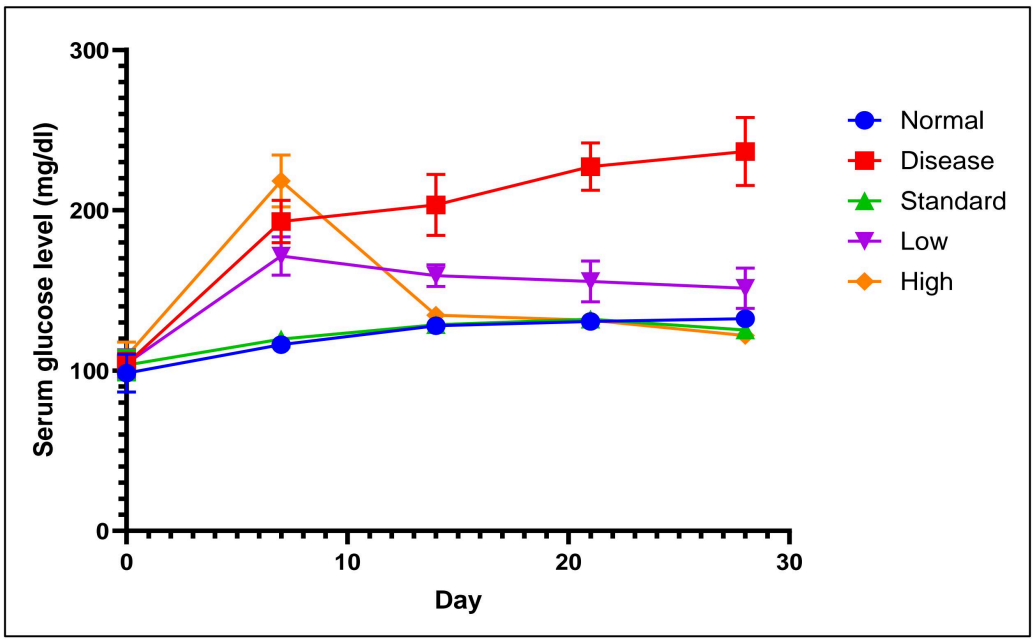


Figure17: Illustrative representation of changes in Blood Glucose level from Table no.17

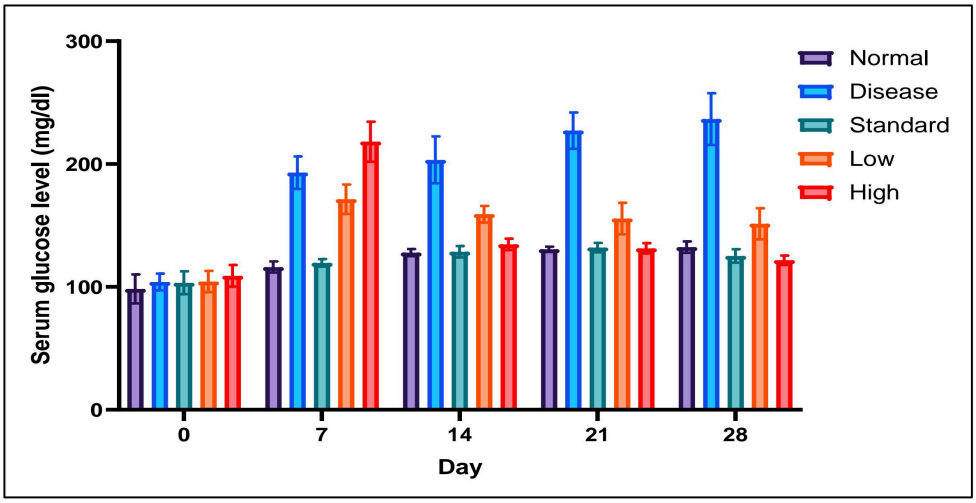


Figure 18: Illustrative representation of changes in Blood Glucose from Table no.16

7.13.3 Estimation of SGOT(AST)

SGOT(AST) level in STZ-induced diabetic rats were significantly ($p< 0.05$) elevated compared to normal control group. Treatment with VNME at the doses of 200 and 400 mg/kg significantly ($p< 0.05$) reduced the SGOT level and when compared to the diabetic control group (Table 18)

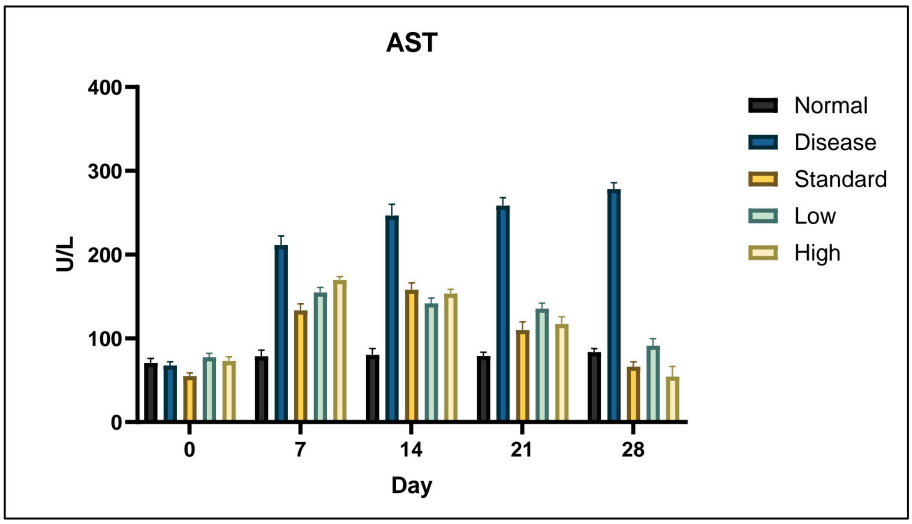


Figure 19: Illustrative representation of AST from Table no.18

7.13.4 Estimation of SGPT(ALT)

SGPT(ALT) level in STZ-induced diabetic rats were significantly ($p< 0.05$) elevated compared to normal control group. Treatment with VNME at the doses of 200 and 400 mg/kg significantly ($p< 0.05$) reduced the SGPT level and when compared to the diabetic control group (Table 19)

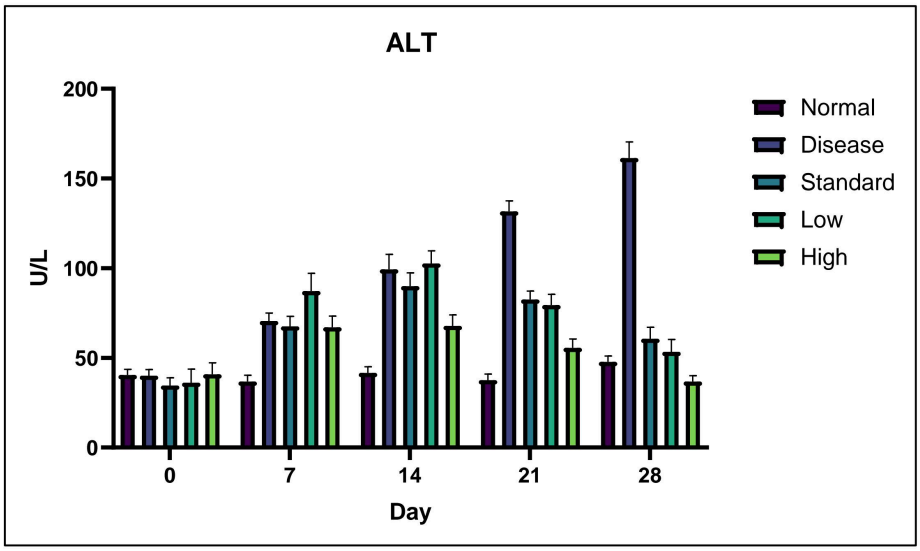


Figure 20: Illustrative representation of ALT from Table no.19

Table 18: Estimation of SGOT (U/L)

Groups	SGOT/AST(U/L)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	70.60±5.549	78.6,0±7.436	80.00±7.713	78.80±4.604	83.40±4.505
Disease control	67.40±4.505**	211.40±10.807**	246.40±13.649**	258.40±9.528**	278.20±7.596**
Standard control	54.80±3.768	133.20±7.981	158.00±8.124	109.80±9.833	66.20±5.630
Low dose treated	77.40±4.775	154.60±6.107	141.80±6.221	135.40±6.656	91.20±8.526
High dose treated	72.80±5.263	169.60±4.037	153.20±5.404	117.00±8.718	54.40±11.929

Table 19: Estimation of SGPT (U/L)

Groups	SGPT/ALT(U/L)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control	40.40±3.209	36.80±3.564	41.60±3.435	37.60±3.435	47.80±3.194
Disease control	40.00±3.391**	70.60±4.393***	99.40±8.264**	131.60±5.941**	161.40±8.989**
Standard control	34.60±4.278	67.60±5.505	90.00±7.450	82.60±4.615	60.60±6.387
Low dose treated	36.20±7.497	87.20±9.884	102.60±7.057	79.40±5.983	53.40±6.804
High dose treated	40.80±6.419	67.00±6.285	67.80±6.140	55.60±4.930	36.80±3.271

7.13.5 Estimation of serum Lipid profile

Serum lipid profiles like total cholesterol, triglyceride in STZ-induced diabetic rats were significantly ($p< 0.05$) elevated compared to normal control group. Treatment with VNME at the doses of 200 and 400 mg/kg significantly ($p<0.05$) reduced the total cholesterol, triglyceride level and when compared to the diabetic control group (Figure 21, 22)

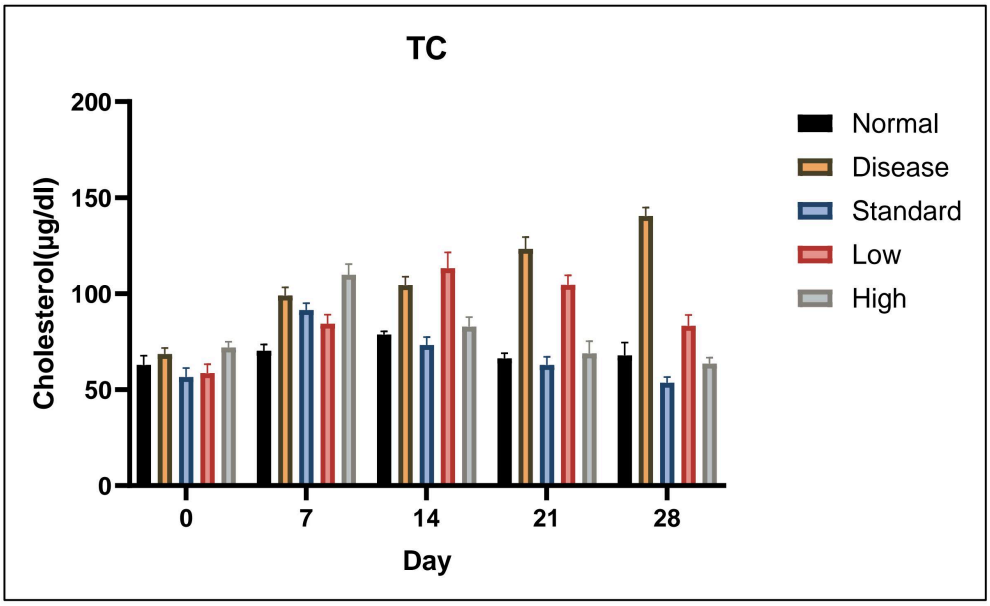


Figure 21: Illustrative representation of Total Cholesterol

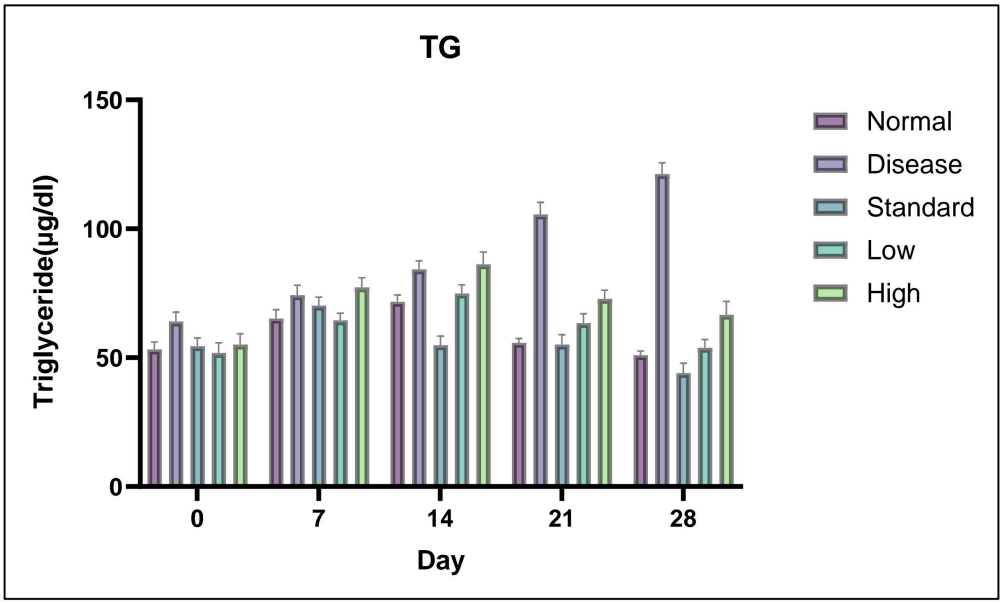
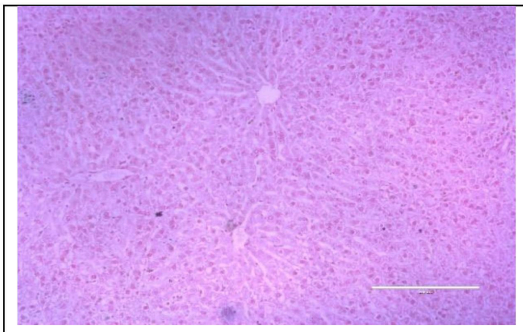
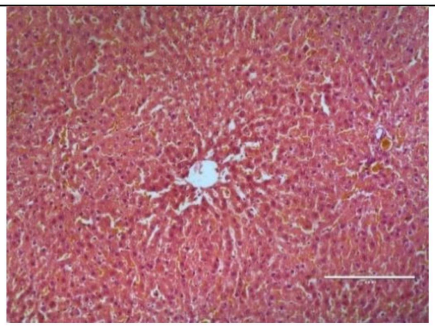
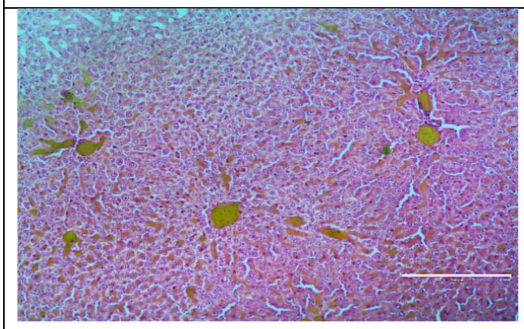
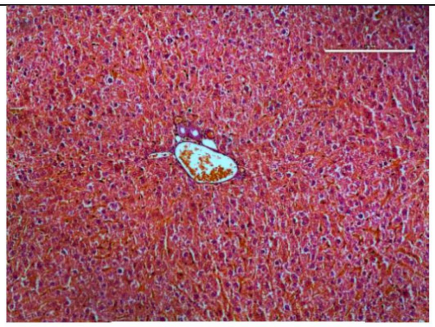
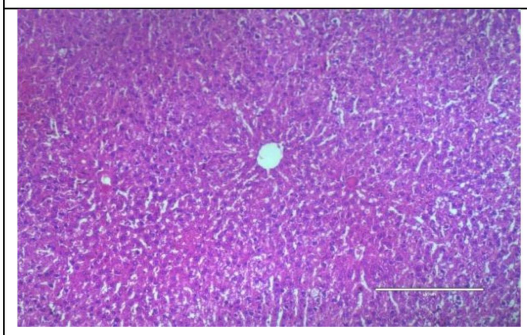
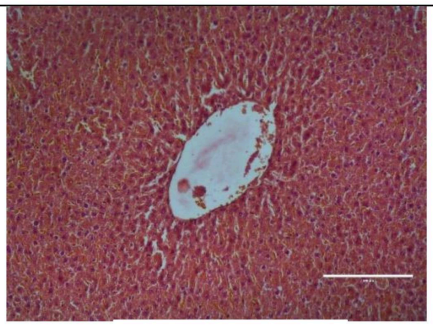
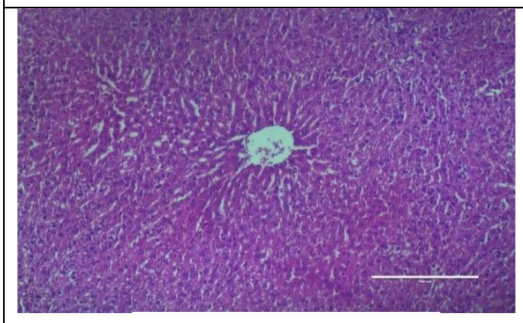
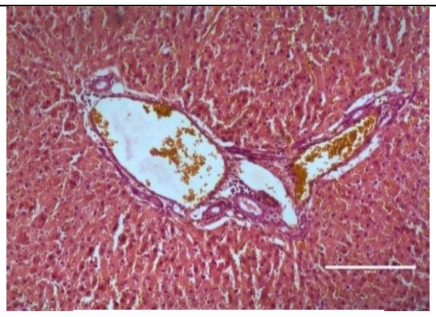
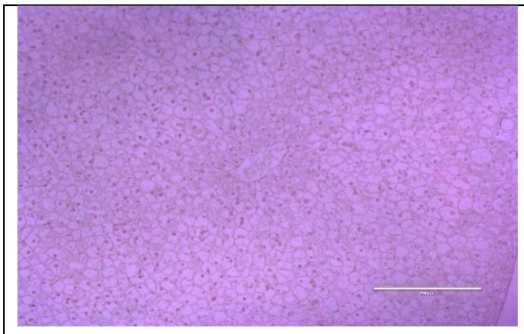


Figure 22: Illustrative representation of Triglyceride

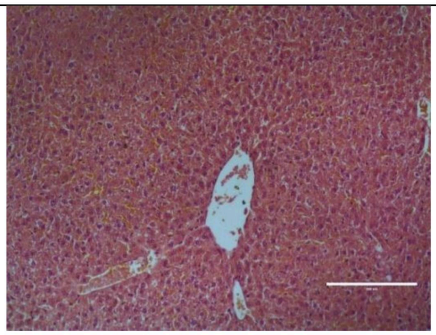
7.13.6 Histopathology:

 **Normal control**	 **Normal control**
 **Disease control**	 **Disease control**
 **Standard drug**	 **Standard drug**
 **200 mg/kg of VNME**	 **200 mg/kg of VNME**



400 mg/kg of VNME

Figure 25: Histopathological sections of the Liver of different group of rats with H&E (Haematoxylin &Eosin) Stains



400 mg/kg of VNME

Figure 26: Histopathological sections of the Liver of different group of rats with ORO (Oil Red O) Stains

7.14 Estimation of Tissue antioxidant parameter

Lipid peroxidation (LPO) results in the formation of ROS species and subsequently elevates the level of malondialdehyde (MDA) in liver tissue of STZ-induced diabetic rats. In the present study the MDA level was significantly ($p< 0.05$) increased in HFD/STZ-induced diabetic rats compared to normal control group. Interestingly, treatment with VNME at the doses of 200 and 400 mg/kg significantly ($p< 0.05$) reduced the MDA levels compared to diabetic control group (Table 20) The levels of reduced GSH was significantly ($p< 0.05$) increased, antioxidant enzyme levels in the liver of HFD/STZ-induced diabetic rats compared to the diabetic control group (Figure 23, 24)

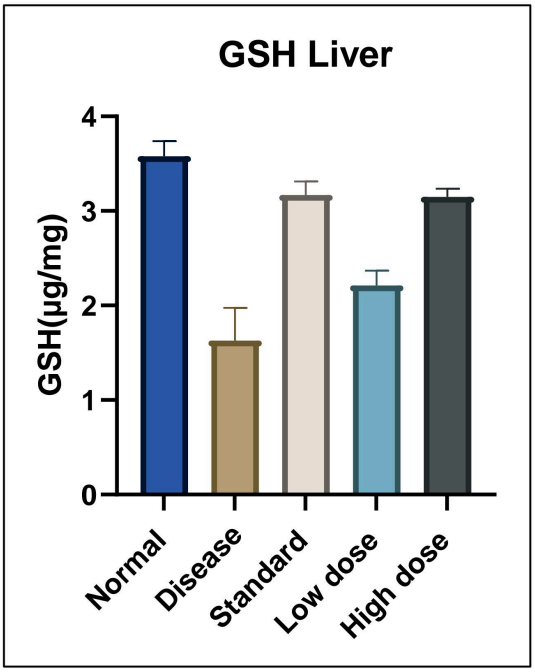


Figure 23: Illustrative representation of GSH Liver

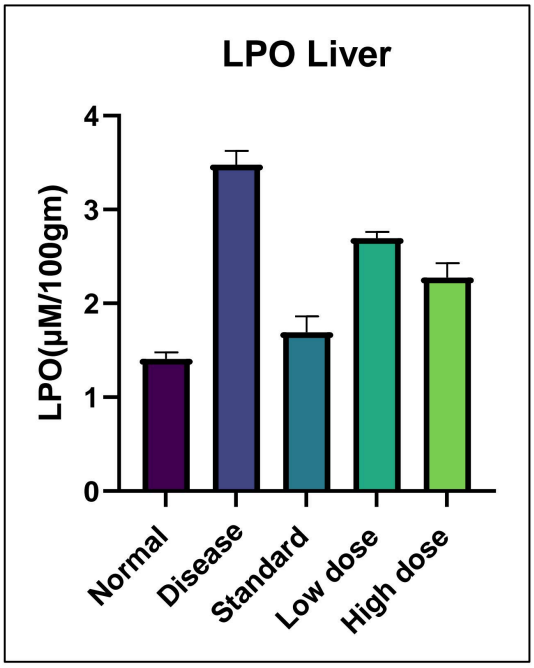


Figure 24: Illustrative representation of LPO Liver

7.15 GC-MS analysis of methanolic extract

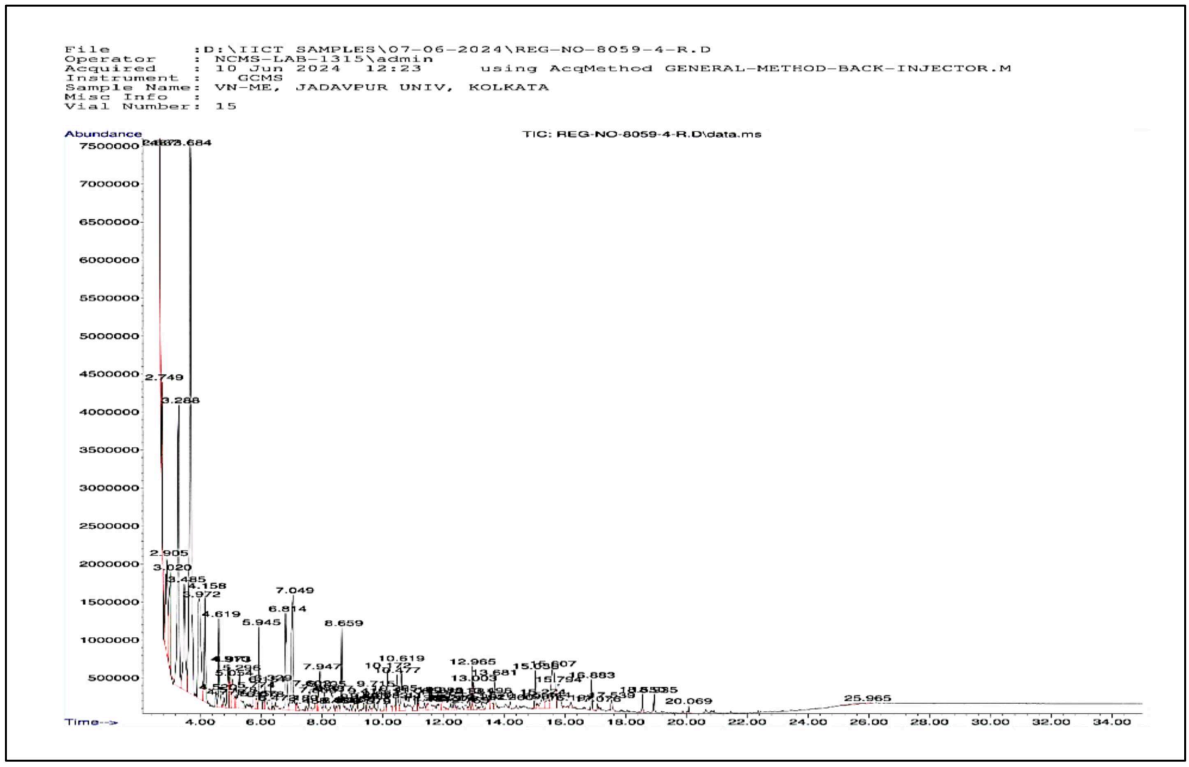


Figure 25: Chromatogram of V. negundo leaves by GC-MS

Sample_VN-ME_07062024			
Sr.No.	**Compound Name**	**Retention Time**	**Area%**
1	Hexanoic acid, 3-hydroxy-, methylester	5.020	0.78
2	Caryophyllene	18.787	6.21
3	Cyclohexanemethanol,4-ethenyl-.alpha.,.alpha.,4-trimethyl-3-(1-methylethenyl)-,[1R-(1.alpha.,3. alpha.,4. beta.)]-	22.122	2.26
4	Epiglobulol	15.83	12.41
5	Hexanoic acid, 2-ethylhexyl ester	24.212	10.23
6	(S)-Ethyl 3-methyl-5-((4aS,8aS)-2,5,5,8atetramethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)pentanoate	29.435	16.78
7	Cycloheptane, 4-methylene-1-methyl-2- (2-methyl-1-propen-1-yl)-1-vinyl	29.899	2.19
8	.gamma.-Elemene	30.126	7.49
9	Hexadecanoic acid, methyl ester	30.537	1.80
10	1,3,6,10-Cyclotetradecatetraene, 3,7,11- trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	31.324	2.13
11	n-Hexadecanoic acid	31.427	2.50
12	Kolavenol acetate	32.640	2.32
13	9,12-Octadecadienoyl chloride, (Z,Z)-	34.295	4.72
14	Phytol	34.591	25.17
15	Tetrahydroedulan	39.966	3.00
These are the list of prominent compounds present in the sample			

Table 20: Identified bioactive compounds using methanol extraction of V. negundo employing GC–MS

Chapter 08

Discussion

8 Discussion

In the area, *Vitex negundo* is one of the most prevalent plants. During the characterization of powders, pharmacognostic research has discovered microscopic diagnostic features such as multicellular covering trichomes and anisocytic stomata. The findings indicate that the main phytoconstituents of *Vitex negundo* include carbohydrates, steroids, flavonoids, glycosides, and tannins. According to TLC profiling, petroleum ether extract had a maximum of 7 spots while methanol extract had 5. The tincture's pH gradually drops with time, and on the seventh day, the tincture with a 40% (w/v) alcohol concentration showed the highest pH of 6.05. The highest solid content was found in the tincture with a 40% (w/v) alcoholic strength on the 7^{th}, 14^{th},21^{th} and 28^{st} days. Since each drug has a different fluorescence and colour, the leaf powder's features can be used to identify the original medication. The primary authentication of the genuine medicine from the contaminated one is made easier by these discoveries. Though a thoroughly investigations by various high through put instruments like GC-MS, HPTLC, MS, LC-MS/MS analysis of methanolic extract of the leaves also reveals the presence of many terpenoids, phenolic and flavonoids as major Phytoconstituents such as Gallic acid, Epiglobulol, Phytol, Quercetin, Rutin, Ellagic acid, Kaempferol, etc. These flavonoids are considered to be responsible for the antioxidant and antidiabetic potential and as well as effective against non-alcoholic fatty liver disease.

The in vitro antioxidant investigations of the methanolic extract of *Vitex negundo* leaves were carried out with great care. When compared to other commonantioxidants, the methanolic extract of *Vitex negundo* leaves shown potent anti- oxidant activity by suppressing DPPH radical scavenging activities. As a result, it may be inferred that the plant *Vitex negundo* can be employed as a significant and accessible source of natural antioxidants with subsequent health advantages. These *in vitro* studies demonstrate that this plant extract is a significant natural source of antioxidants, which may be helpful in reversing the effects of a variety of oxidative stresses. It was seen that there was a dose-dependent increase in percentage inhibitory activity against alpha-amylase enzyme while performing the *in vitro* anti diabetic evaluation. Evaluation for non-alcoholic fatty liver disease was also performed.

Inhibitory activity against alpha-amylase enzyme while performing the *in vitro* anti diabetic evaluation. Evaluation for non-alcoholic fatty liver disease was also performed.

The acute toxicity study of *Vitex negundo* leaf extract was already performed. LD_{50} values of the *Vitex negundo* extract were found to be safe up to 2000mg.

Since diabetes caused a significant lessening in body weight due to increased musclewasting and loss of tissue proteins which attributed to gluconeogenesis i.e., catabolism of proteins and fats. An increase in body weight in rats treated with optimized *Vitex negundo* extract was observed as compared to the STZ-induceddiabetic control rats (Group 2) which might relate to the rise in insulin levels and fallin glucose levels, which prevent muscle atrophy. The hypoglycaemic activity of *Vitex negundo* extract treated (Group 4&5) were comparatively significant with that of standard metformin-treated (Group 3). The ability of the extract to augment insulin secretion from pancreatic beta cells or sensitizing insulin receptors may be attributed to the potential mechanism. Thus, it can be concluded that the methanolic extract of *Vitex negundo* leaf exhibited significant *in vitro* antioxidant and anti-diabetic activity and also effective against non-alcoholic fatty liver disease. The protective activity of this plant could be attributed due to the presence of tannins, flavonoids, phenols, etc. as confirmed by the preliminary phytochemical screening. Further studies are required in knowing the exact mechanism of action, particularly in terms of reducing blood glucose levels. The De Ritis ratio, which is the ratio of aspartate aminotransferase (AST) to alanine aminotransferase (ALT), is often used to help distinguish between different types of liver disease. This ratio can provide insights into the underlying pathology of liver conditions, including non-alcoholic fatty liver disease (NAFLD).

Chapter 09

Conclusion

9 Conclusion

Diabetes is a condition affecting the metabolism of proteins, fats, and carbohydrates that is brought on by either an increase in insulin resistance or a reduction in insulin production. Patients with diabetic retinopathy, diabetic peripheral neuropathy, insulin-dependent and non-dependent diabetes, and other diabetes-related disorders have been treated using herbal remedies. Numerous plant species found in India have been used to confirm the efficacy of botanicals in lowering blood sugar levels. This thesis is related to scientific studies on methanolic extract of *Vitex negundo* leaves against non-alcoholic fatty liver disease. The introductory part of the thesis is designated by the concept of diabetes. The mature leaves of VN were collected from West Medinipur, West Bengal, India. After collection, leaves were cleaned, Shade dried and grind into a coarse powder. The powdered plant material was extracted using Petroleum ether followed by methanol by Soxhlet extraction. The extract was filtered and evaporated to dryness to get the dry extract that was used in the study. Qualitative phytochemical analysis of Petroleum ether extract revealed the presence of terpenoids, alkaloids, tannins and phenolics compounds while the methanolic extract revealed the presence of carbohydrate, flavonoids, alkaloids, tannins phenolics, saponins and glycosides.

Though a thoroughly investigations by various high through put instruments like GC-MS, HPTLC, LC-MS/MS are required, in future, to confirm the existence of such Phytochemicals. Science GC-MS analysis have shown several picks of unknown compounds it is necessary to isolate such phytochemicals through various isolation techniques which include preparative Column chromatograph preparative thin layer chromatography and characterizing them by the help of mass spectrometry.

NMR spectrometry. The study shows that greater alcohol concentration in the tincture increases acidity of the tincture while storage for longer period of time also slightly increases acidic property. Solid content of the tincture also increases with time while greater alcohol 97 concentration also imparts diminished solid content. It also shows that specific gravity of the prepared tinctures does not vary substantially with time.

In vitro antioxidant and antidiabetic studies of *Vitex negundo* methanolic extract (VNME) showed significant Free radical scavenging and alpha-amylase inhibitory activity. Total Phenolic and Flavonoid content of the extract was found 28.82mg/g and 85.372mg/g respectively. The extract showed antioxidant activity with an IC50 value of 165.27 µg/ml.

The in vivo antidiabetic activity of VNME was assessed by using high fat diet and low dose streptozotocin induced diabetic rat model. VNME were administered orally in a dose of 200

mg/kg and 400 mg/kg, respectively for consecutive 28 days. After 28 days of study rats were sacrificed and all biochemical parameters, tissue antioxidant parameters, immune histopathology were performed at the end of the study.

VNME was showing significant blood glucose lowering effect in fasting blood glucose. VNME also exhibited its antidiabetic effect such as glycemic control, total serum lipid profile, where increased dose conveys more effect on these biochemical parameters. *Vitex negundo* methanolic extract also exhibited significant tissue antioxidant activity with increased dose and of pancreas reveals that VNME have beta cell protective property during diabetic condition. From the present study it can be concluded that VNME have in-vitro and in vivo antioxidant antidiabetic and NAFLD activity against STZ- induced high fat diet diabetic rat model.

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