A DISSERTATION ON

AN *IN-VITRO* ANTI-OXIDANT AND ANTI-DIABETIC STUDY ON SEQUENTIALLY EXTRACTED FRACTIONS OF *CESTRUM NOCTURNUM*

SUBMITTED TO THE

DEPARTMENT OF BIOSCIENCES
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF SCIENCE
IN BIOTECHNOLOGY

BY

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TO WHOM IT MAY CONCERN

This is to certify that **Ms. HUMRA KHAN**, a student of M.Sc. Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled ***"An in-vitro anti-oxidant and anti-diabetic study on sequentially extracted fractions of Cestrum nocturnum"*** successfully. She has completed this work from Department of Biosciences, Integral University, under the guidance of **Dr. M. Salman Khan.**

The dissertation was a compulsory part of her M.Sc. degree. I wish her good luck and future endeavours.

Dr. Snober S. Mir
Head,
Department of Biosciences,
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This is to certify that the study conducted by **Ms. HUMRA KHAN** during the months February–June, 2022 reported in the present thesis was under my guidance and supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is ***"An in-vitro anti-oxidant and anti-diabetic study on sequentially extracted fractions of Cestrum nocturnum"*** therefore, being forwarded for the acceptance in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology, Department of Biosciences, Integral University, Lucknow.

Date:

Place: Lucknow

Dr. M. Salman Khan

(Supervisor)

Associate Professor
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ACKNOWLEDGEMENT

Before I present my work, I would like to gratefully acknowledge the contribution of all those people who have helped in the work described in this Dissertation. I am going to try anyway, and if your name is not listed, it is rest assured that my gratitude is not less than for those listed below.

First, I would like to express my gratitude to **God** for providing me the blessing to complete this work.

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Date: **HUMRA KHAN**

CONTENTS

ABBREVIATIONS

DM	Diabetes Mellitus
T1DM	Type-1 Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
IDDM	Insulin dependent diabetes mellitus
NIDDM	Non-insulin dependent diabetes mellitus
DPPH	2,2-diphenyl-1-picryl hydrazyl
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
EtOAc	Ethyl acetate
MeOH	Methanol
DCM	Dichloromethane
PBS	Phosphate buffered saline
DNS	Dinitrosalicylic acid
CVD	Cardiovascular disease
HbA1C	Glycated Heamoglobin

INTRODUCTION

DM is a chronic disease, characterized by metabolic disorder associated with high blood sugar (Pallavi et al., 2015). It occurs due to the pancreas does not produce enough insulin or when the body cannot use the insulin effectively (Arumugam, et al., 2013). DM can be classified into four types (Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), Gestational diabetes mellitus and non-classical causes of DM) on the basis of the pathogenic process which leads to hyperglycemia (Powers, 2011). T1DM is known as insulin dependent DM. It is a catabolic disorder caused by an auto immune reaction, resulting in selective beta cells destruction which produces severe insulin deficiency due to the inability of β-cells to respond any insulinogenic stimuli. Nowadays children or young adults are suffered very much. T2DM is previously known as non-insulin dependent DM, a heterogeneous group of disorders characterized by insulin resistance, where the cells in the body do not respond to insulin (Mamun-or-Rashid et al., 2014). Gestational diabetes occurs during pregnancy period. This is due to the resistance of insulin by placenta and placental hormones. Non classical causes of DM can be either genetic or acquired. DM is one of the main killers within the next 25 years. The number of people from worldwide multiplies with diabetes. Thus, it is a global concern until the successive treatment is discovered

Diabetes insipidus, characterized by excretion of copious volumes of dilute urine, can be life-threatening if not properly diagnosed and managed. It can be caused by two fundamentally different defects: inadequate or impaired secretion of antidiuretic hormone (ADH) from the posterior pituitary gland (neurogenic or central diabetes insipidus) or impaired or insufficient renal response to ADH (nephrogenic diabetes insipidus). The distinction is essential for effective treatment (Amgard et al 2006).

It is estimated that 366 million people had DM in 2011; by 2030. This would have risen to 552 million (The number of people with T2DM is increasing in every country with 80% of people with DM living in low- and middle-income countries. DM caused 4.6 million deaths in 2011 (Global burden of diabetes,2011). It is estimated that 439 million people Would have T2DM by the year 2030 (Chamnan et al.,2011).

The incidence of T2DM varies substantially from one geographical region to the other as a result of environmental and lifestyle risk factors (Zimmet et al., 2001)

There are a number of major risk factors for DM, especially T2DM. Although some diabetic risks come from genetics, some may be preventable. Diet and lack of exercise are the two common reasons that lead to diabetes. Among older adults, free radical damage and oxidative stress should be given careful consideration. Free radicals may damage lipids, proteins and DNA, given careful consideration. Free radicals may damage lipids, proteins and DNA, which may lead to critical diseases in the aging. Oxidative stress and free radical damage are known to be harmful to many cells and enzymes. By definition, oxidative stress is referred to as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses. Oxidative stress and free radical damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, and rheumatoid arthritis which may lead to critical diseases in the aging. Oxidative stress and free radical damage are known to be harmful to many cells and enzymes. By definition, oxidative stress is referred to as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses. Oxidative stress and free radical damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, and rheumatoid arthritis. The major risk factors for the establishment of DM include genetic defects, environmental exposures, abnormal insulin secretion due to damage/disintegration to B-cells of the islets of Langerhans, insulin resistance, and altered activities of carbohydrate metabolizing enzymes i.e., alpha-amylase and α-glucosidase (Nabi et al., 2019; Nabi et al., 2021; Hashim et al., 2019).Plenty of evidence proposed that persistent and poorly controlled DM initiates diabetic comorbidities in response to the failure in metabolic reply to glycemic index or due to the generation and accumulation of advanced glycation end products (AGEs) (Nabi et al., 2019; 2021).

No cure has yet been found for the disease; However, treatment modalities include lifestyle modifications, treatment of obesity, oral hypoglycemic agents, and insulin sensitizers like metformin, a biguanide that reduces insulin resistance, is still

the recommended first line medication especially for obese patients. Other effective medications include no sulfonylurea secretagogues, thiazolidinediones, alpha glucosidase Inhibitors, and insulin. Recent research into the pathophysiology of type 2 DM has led to the introduction of new medications Like glucagon-like peptide 1 analogoues: dipeptidyl peptidase-IV Inhibitors, inhibitors of the sodium-glucose cotransporter 2 and 11ß-hydroxysteroid dehydrogenase 1, insulin-releasing glucokinase activators and pancreatic-G-protein-coupled fatty-acid-receptor agonists, glucagon-receptor antagonists, metabolic inhibitors of hepatic glucose output and quick-release bromocriptine. Inhaled Insulin was licensed for use in 2006 but has been withdrawn from the market because of low patronage (Abdulfatai et al., 2012). Cardiovascular disease, Neurological diseases, ageing and diabetes. Free radicals can damage DNA and cause mutagenicity and cytotoxicity and thus play a key role carcinogenesis. It is believed that ROS can induce mutations and inhibits DNA repair process that results in inactivation of certain tumor repressor genes, leading to cancer. ROS can also stimulate oxidation of LDL, cholesterol, cholesterol derived species, protein modifications which can lead to foam cell formation and atherosclerotic plaques and vascular thrombosis (Heart attack and Stroke).

The pharmacological regimens in the management of DM include the blockers of dipeptidyl peptidase-4, α-amylase and α-glucosidase, sodium-glucose cotransporter. (SGLT-2) and glucagon-like peptide-1 as well as insulin shots (Nabi et al., 2019; Nabi et al., 2021; Hashim et al., 2013).

There has been enormous interest in natural antioxidants due to their ability to neutralize the effects of ROS that are not only responsible for alleviating the oxidative stress condition in diabetes but are also helpful in managing the postprandial hyperglycemia. The antioxidants may be exogenous or endogenous in nature. The endogenous antioxidants can be classified as enzymatic and non-enzymatic. The antioxidant enzymes include Superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRx).

The needs of diabetic patients are not only limited to adequate glycemic control but also correspond with preventing complications; disability limitation and

rehabilitation. Some of the Indian studies revealed very poor adherence to treatment regimens due to poor attitude towards the disease and poor health literacy among the general public. Glycemic targets in patients with “hypoglycemia unawareness” should be relaxed for prolonged periods, pending the potential reversal of the condition. In patients with severe coexisting conditions that could interfere with implementation of the management strategy, the goal is prevention of clinically significant glycosuria, water and electrolyte loss, infections, and the development of non ketotic hyperosmolar coma. Insulin is indicated for T1DM as well as for T2DM patients with insulinopenia whose hyperglycemia does not respond to diet therapy either alone or combined with oral hypoglycemic drugs. Controlling blood glucose with insulin has the potential to be the most effective blood glucose-lowering therapy. Many patients with type 2 diabetes will eventually require insulin therapy. Since T1DM is associated with insulin resistance, insulin requirements can exceed 1 unit/kg/day.

REVIEW OF LITERATURE

Diabetes mellitus

Diabetes is a lifelong (chronic) disease and is a group of metabolic disorders characterized by high levels of sugar in blood (hyperglycemia) (Unnikrishnan *et al*., 2016). More than 230 million people worldwide are affected, and it is expected to reach 350 million by 2025. Globally the affected people are unaware of the disease and only half receive adequate treatment (da Silva *et al*., 2010). It is caused due to deficiency of insulin or resistance to insulin or both. Insulin is secreted by β-cells of pancreas to control blood sugar levels (Zheng et al., 2018; Ribeiro et.al., 2010). Blurry visions, excess thirst, fatigue, frequent urination, hunger, weight loss is some of the symptoms commonly seen in diabetic patients. It is a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.

Often symptoms are not severe or may be absent, and consequently hyperglycaemia of sufficient degree to cause pathological and functional changes may be present for a long time before the diagnosis is made. The long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction, including sexual dysfunction. People with diabetes are at increased risk of cardiovascular, peripheral vascular, and cerebrovascular disease. Several pathogeneic processes are involved in the development of diabetes.

Classification of diabetes mellitus

Diabetes mellitus, regardless of underlying cause, is subdivided into: insulin requiring for survival (corresponding to the former clinical class of 'Insulin Dependent Diabetes Mellitus—IDDM'), e.g. C-peptide deficient; insulin requiring for control, i.e. metabolic control, rather than for survival, e.g. some endogenous insulin secretion but insufficient to achieve normoglycaemia without added exogenous insulin; and not insulin requiring, i.e. those who may be controlled satisfactorily by non-

pharmacological methods or drugs other than insulin. Together the latter two subdivisions constitute the former class of NIDDM (Solis-Herrera et al., 2018).

Type 1 diabetes mellitus

Type1 DM, also called as the IDDM is a multifactorial autoimmune disease characterized by chronic hyperglycemia and by the development of specific vascular alterations (Punthakee et al., 2018; Solis-Herrera et al., 2018). Autoimmune destruction of β-cell by T-cells, is responsible for T1DM which results in severe insulin depletion. It is also known as juvenile diabetes. It is usually characterized by the presence of anti-GAD, islet cell or insulin antibodies which identify the autoimmune processes that lead to beta-cell destruction (Figure 01). T1DM is mainly triggered by environmental factors (Punthakee et al., 2018; Solis-Herrera et al., 2018).

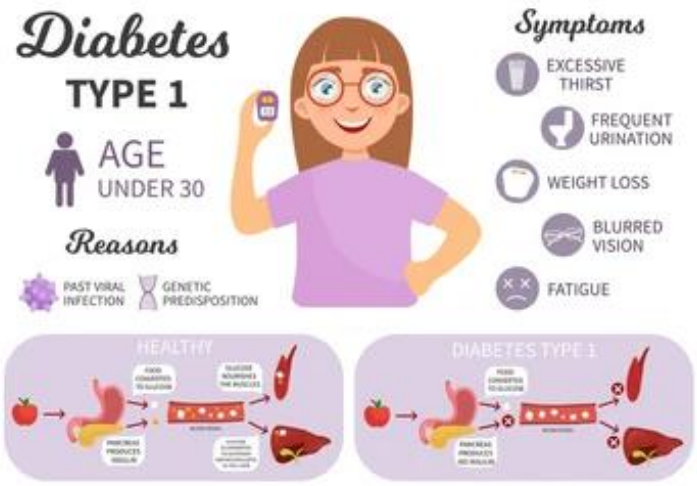

Figure 01: Type 1 diabetes mellitus and its consiquences

Type 2 diabetes mellitus

T2DM, also called as the adult-onset diabetes or non-insulin-dependent diabetes mellitus (NIDDM) among humans is caused by either low levels or absence of insulin or insulin resistance (IR) (Uppu and Parinandi, 2011). It is a chronic disease characterized by insulin resistance, which leads to hyperglycemia. More than 180 million people worldwide have diabetes as estimated by The World Health Organization (WHO). T2DM is expected to reach pandemic levels, rising from 171

million in 2000 to 366 million in 2030. T2DM is the more prevalent form and accounts 90% of all diabetes cases worldwide (Ribeiro et al., 2011; Murgue et al., 2011).

Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is defined as glucose intolerance of varying degrees, which appears, or is first diagnosed, during pregnancy and may or may not persist after delivery (Alejandro et al., 2020).

Diagnostic methods of diabetes mellitus

Diabetes mellitus is diagnosed by demonstrating any one of the following methods (American Diabetes Association, 2018)

1. Fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL)
2. Plasma glucose ≥ 11.1 mmol/L (200 mg/dL)
3. Glycated hemoglobin (Hb A1C) ≥ 6.5%
4. Oral glucose tolerance test (OGTT)

Random plasma glucose test

It is the simplest test and doesn't require fasting before taking the test. If 200 or more than 200 mg/dl of blood glucose it probably indicates diabetes but has to be reconfirmed.

Fasting plasma glucose test

There should be eight hours fasting before taking this test. Blood glucose more than 126 mg/dl on two or more tests conducted on different days confirms a diabetes diagnosis People with fasting glucose levels from 100 to 125 mg/dL are considered to have impaired fasting glucose also called as pre-diabetes (Figure 03). Fasting plasma glucose is mostly preferred because of its low cost and is very easy to operate. Diabetes should be confirmed with a second test on a different day (American Diabetes Association, 2018).

Figure 02: Testing blood glucose level using a glucometer

Oral glucose tolerance test (OGTT)

The 2- hour OGTT is a standard test for diagnosing type 2 diabetes but it is expensive and is limited because of its labor-intensive multi-blood draw protocols. When random plasma glucose test is 160-200 mg/dl and thefasting plasma test is 110-125 mg/dl, then this test is conducted. This blood test evaluates body's response to glucose. This test requires fasting at least eight but not more than 16 hrs (American Diabetes Association, 2018; Ye et al., 2012).

Hemoglobin A1c (or Hb A1c or A1c)

It requires only a single point blood draw and is more advantageous because it does not require fasting blood samples and has higher repeatability. HbA1c is an indicator of the average blood glucose concentration over the preceding three months and has been proposed to be a useful alternative test to screen for type 2 diabetes as it overcomes many of the obstacles associated with the OGTT. Glycated hemoglobin is better than fasting glucose for determining risks of cardiovascular disease and death from any cause. The life span of hemoglobin *in vivo* is 90 to120 days. During this time glycated hemoglobin, A forms, being the ketoamine compound formed by combination of hemoglobin A and glucose. Several subfractions of glycated hemoglobin have been isolated.

Complications of diabetes mellitus

Diabetes is root cause for several serious complications such as cardiovascular diseases, cerebrovascular diseases, renal disorders, inflammation and immunity, and

obesity (Papatheodorou et al., 2018). Epidemiological studies of diabetes mellitus have shown that gender, age, and ethnic background are important factors when considering the development of diabetes mellitus and its complications (Stojanović et al., 2018; Yang et al., 2021;).

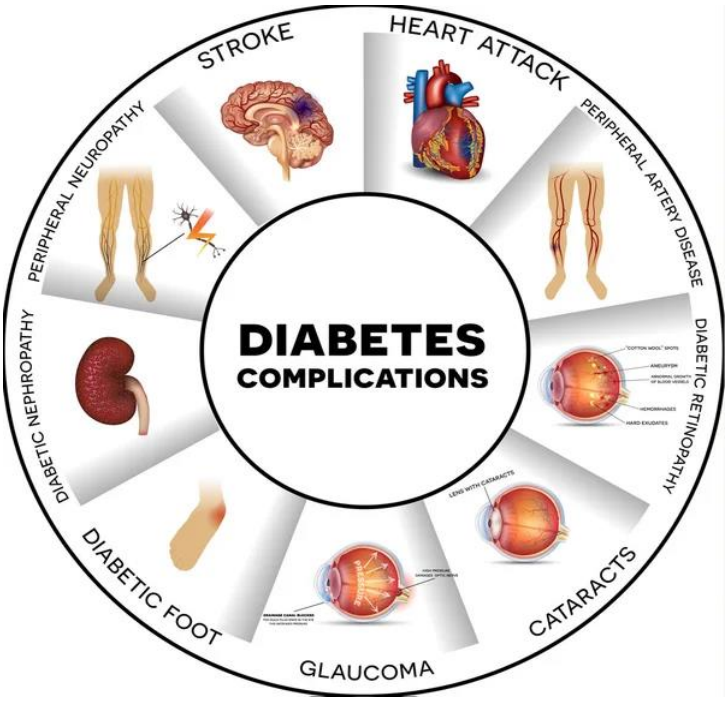

Figure 03: Complications due to diabetes mellitus

Diabetic ketoacidosis

Diabetes leads to increased levels of endothelial micro particles (Mikirova et al., 2011). Diabetic ketoacidosis (DKA) is a serious condition caused by hyperglycemia, if the patient is not treated over a period of days. It is characterized by nausea, vomiting, and a high level of ketones in the blood and urine (Figure 06) (Ali, 2011). DKA is present at T1DM presentation in 15 to 67% of children, its frequency being inversely related to the incidence of T1DM in that area. DKA may also be present in up to 25% of young people presenting with T2DM

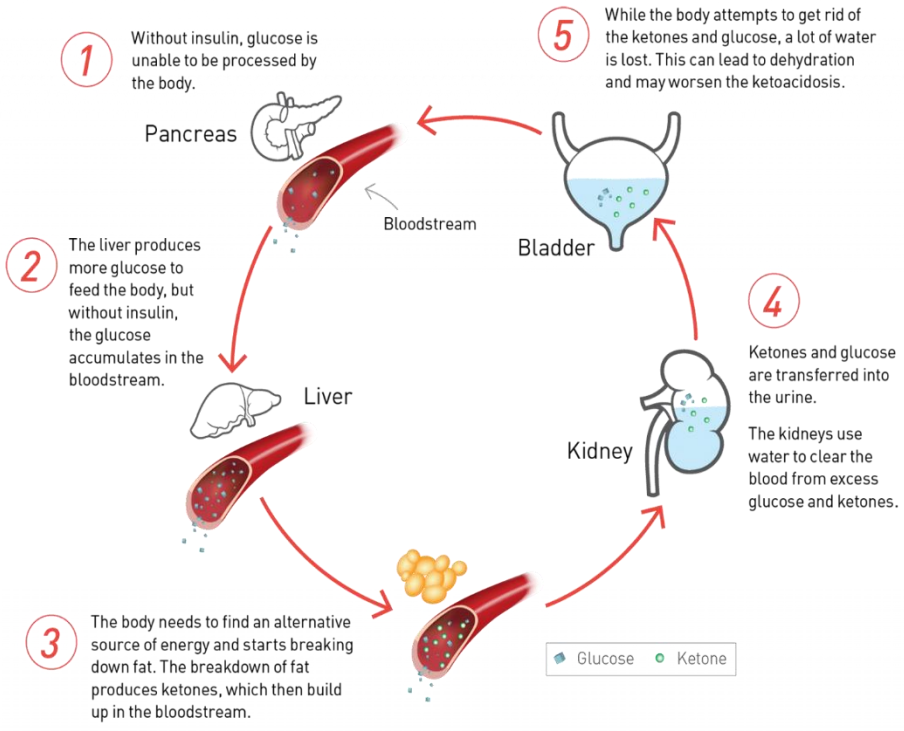

Figure 04: Diabetic ketoacidosis

Chronic Complications – Long-term Side Effects of Diabetes

Microvascular complications

Microvascular complications affect the smaller blood vessels, such as in the eyes (leading to retinopathy), kidneys (leading to nephropathy), and neurons (leading to neuropathy). Individuals with poorly managed blood glucose levels may suffer from one or more of these complications in advanced stages of the disease. Thus, besides monitoring the health of eyes and kidneys, diabetics also require foot care. Interestingly, several large population studies have shown that aggressive management of blood glucose levels (i.e., keeping blood glucose levels within a narrow range) can avoid, or at least delay, the onset of these complications (Nathan et al., 2014). Regular monitoring and management of blood glucose levels is of critical importance in maintaining metabolic balance and avoiding microvascular complications (Viigimaa et al., 2020; Zimmerman, 2016) (Figure 07).

Macrovascular complications

Macrovascular complications affect larger blood vessels, such as those supplying the heart, brain, and extremities. The causes of these complications stem from narrowing of blood vessels due to glycation, inflammation, lipid deposition and other factors. Complications resulting from large vessel damage may lead to cardiomyopathy, stroke, rheumatoid arthritis, osteoporosis, and the degenerative process of aging (Singh et al., 2014). The major concern amongst these complications is myocardial infarction (heart attack). At present, it appears that blood glucose control does not significantly reduce the risks or delay the onset of macrovascular complications. Additional medical management is required (Zimmerman, 2016) (Figure 07).

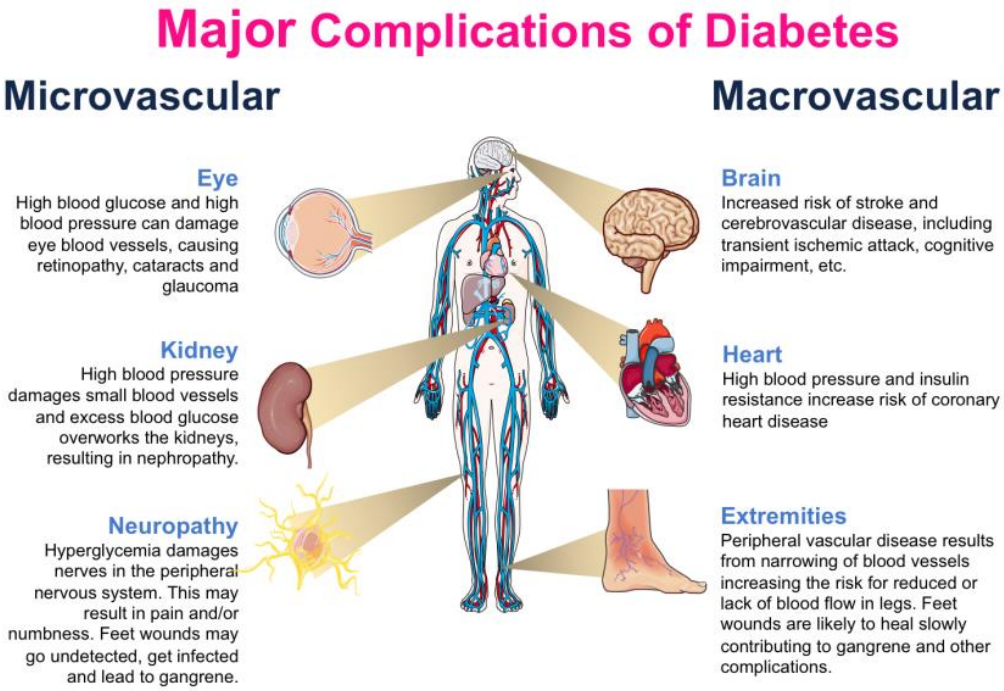

Figure 05: Major microvascular and macrovascular complications associated with diabetes mellitus. Parts of the image were adapted from Servier Medical Art.

Cardiovascular diseases

Cardiovascular disease (CVD) associated with diabetes. CVD is the leading cause of morbidity and mortality in patients with diabetes mellitus. Patients with diabetes mellitus have a 2 to 4 times higher risk of cardiovascular disease and up to 3 times increase in mortality than non-diabetics (Mihai et.al., 2011). Increased body mass index, diabetes, hypercholesterolemia, smoking, male-sex, family history and age are the risk factors for coronary heart disease and atherosclerosis (Guntheroth 2010). Increased pulse pressure causes stiffening of arteries which is an independent risk factor for cardiovascular diseases (Huffman et al., 2011). Use of LXR-alpha ligands may be beneficial for the treatment of diabetes induced CVD (Dave et al., 2011). Some studies confirmed that the risk factor burden tended to be higher among women, with a greater prevalence of obesity and trends toward higher rates of hypertension, diabetes mellitus and home stress (Zarghampour et al., 2011). Atherosclerosis, coronary artery disease myocardial infarction are the commonly associated cardiovascular diseases in diabetic patients (Li and Aronow, 2011). Individuals with T2DM are at higher risk of cardiovascular diseases (CVD) than those without T2DM. Diabetes, dyslipidemia, hypertension and obesity are well-known major and independent cardiovascular risk factors (Taloyan et al., 2010).

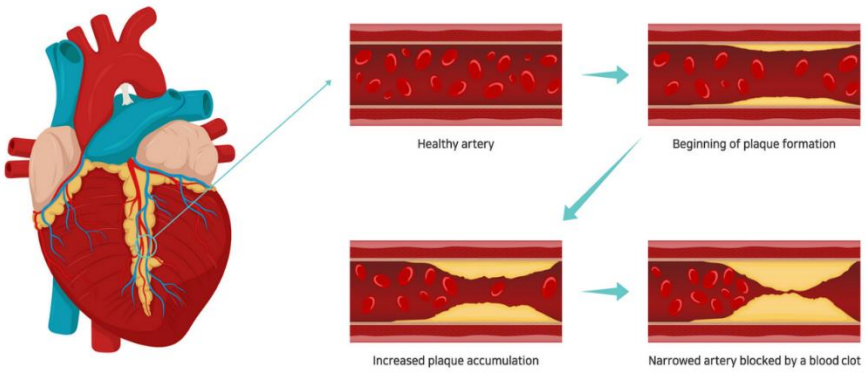

Figure 06: Atherosclerosis – a complication of diabetes mellitus

Diabetic retinopathy

Diabetic retinopathy (DR) is damage to the eye's retina that occurs with long-term diabetes. Diabetic retinopathy is the most common cause of blindness in most of the

countries. It is commonly seen in both type 1(40%) and type 2 DM (20%). There are two types of diabetic retinopathy (Figure 09). They are Non proliferative which develops first, Proliferative is the more advanced and severe form of the disease. In patients with T2DM involvement of fovea by edema and hard exudates or ischemia is the most common cause of visual impairment (Kim et al., 2011). Hyperglycemia and the increased duration of diabetes are the major risk factors for DR. Other risk factors include hypertension, hyperlipidemia, pregnancy, and microalbuminuria (Mohamed and wong, 2007).

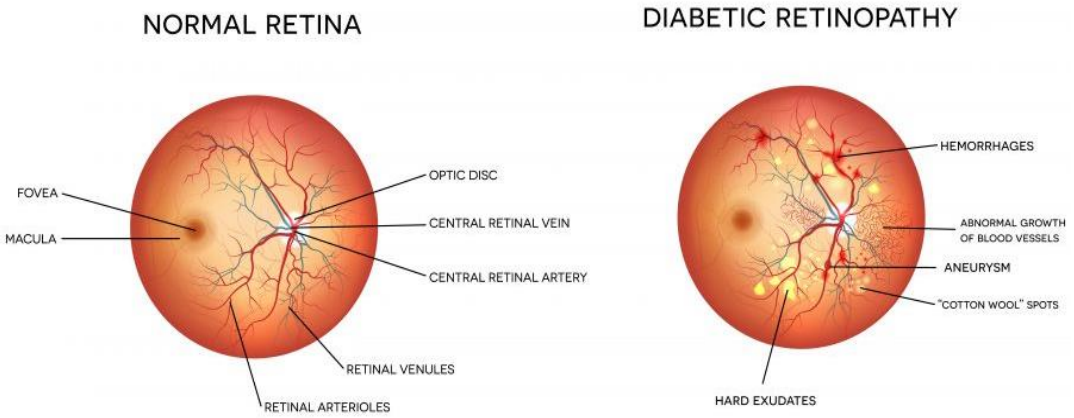

Figure 07: Normal retina and diabetic retinopathy

Cataract

Cataract develops at an earlier age in diabetic patients which is characterized by clouding of the eye lens. In cataract the lens becomes opaque, reducing the amount of light reaching the retina. Connexins (Cx) are a family of proteins that forms hemichannels that communicate the cytoplasm with the extracellular space. Under oxidative stress conditions such as diabetes, it is possible that Cx oxidation may contribute to cataract formation (Kiziltoprak et al., 2019) Neurotrophic corneal ulcers may develop in patients with DM.

Glaucoma

Glaucoma is a condition in which increase in fluid pressure inside the eye leads to optic nerve damage and loss of vision. A person with diabetes is more prone to get glaucoma compared to others (Casson et al., 2012).

Diabetic nephropathy

Diabetic nephropathy is kidney disease or damage that occurs in people with diabetes. Diabetic nephropathy is one of the most important causes (61%) of endstage renal disease that requires renal replacement therapy. In people with diabetes, the nephrons thicken and slowly become scarred over time (Figure 10). The kidneys begin to leak and protein (albumin) passes into the urine. People who have more severe kidney disease may have a poor appetite, feel tired most of the time, and have a general ill feeling. Headache, nausea and vomiting, swelling of the legs, and many other symptoms may also occur. Clinical progression to diabetic nephropathy is not apparently seen in T2DM as it is in T1DM, because of the difficulty in determining the acute onset of diabetes itself.

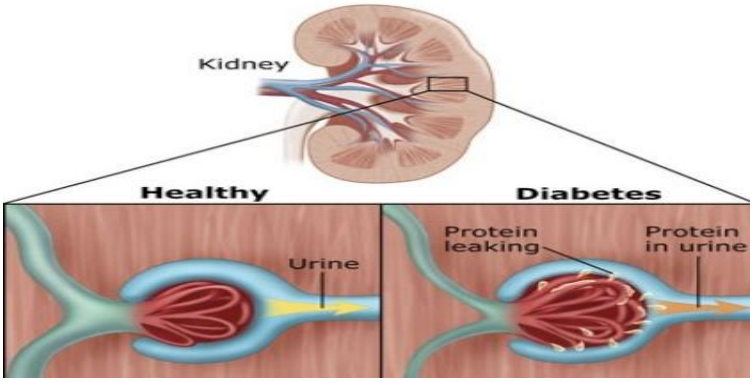

Figure 08: Diabetic nephropathy

Sometimes it is difficult to differentiate minimal change nephritic syndrome (MCNS) and membranous nephropathy (MN) from diabetic nephropathy, especially in middle to advanced aged patients with T2DM because it does not cause hematuria (Soma, 2011) Diabetic nephropathy was the leading cause of end stage renal disease (ESRD) (61%) in patients with Intradialytic hypotension (IDH) (Pavan et al., 2011).

Oxidative stress

Oxidative stress occurs when the generation of free radicals and active intermediates in a system exceeds the system's ability to neutralize and eliminate them. The current concept of "oxidative stress" should also include the pathways related to the "nitrosative stress" and, for their implication in cellular and extracellular metabolic

events, to the “metabolic stress”. Reactive oxygen intermediate (ROI) and reactive nitrogen intermediate (RNI) are constantly produced under physiological conditions, is the crucial event in living organisms. At the moment, the concept of oxidative stress confined to ROI such as hydroxyl and superoxide radicals, and hydrogen peroxide and singlet oxygen has been extended onto RNI such as nitric oxide (NO), per-oxynitrite and, recently, to S-nitrosothiols. Thus, ROI and RNI react with proteins, carbohydrates and lipids, with consequent alteration both in the intracellular and intercellular homeostasis, leading to possible cell death and regeneration (Griendling et al., 2016; Rutkowski et al., 2007).

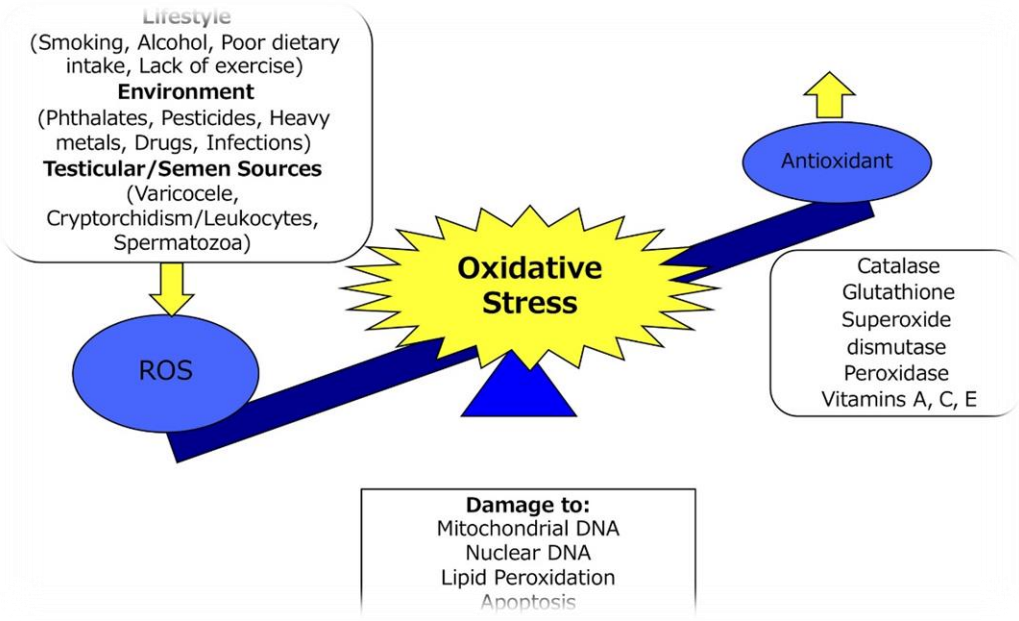

Figure 09: Imbalance between oxidant and antioxidant.

Oxidants

The oxidants/free radicals are species with very short half-life, high reactivity and damaging activity towards macromolecules like proteins, DNA and lipids. In the various fields of biology and medicine, free radicals are more generally known as ROS or reactive nitrogen species (RNS) (Firuzi *et al.*, 2011). Free radicals are molecules/molecular fragments containing one or more unpaired electrons, the presence of which usually makes them highly reactive. Among the most important

ROS are the hydroxyl radical (•OH), the superoxide radical anion ($O_2^{\bullet -}$), nitric oxide (NO•), and peroxyl radicals (ROO•) (Firuzi *et al*., 2011).

The beneficial effects of ROS/RNS tend to occur at low to moderate concentrations and involve their participation in various physiological roles and in numerous cellular signaling pathways (Wu *et al.*, 2017). The harmful effects of free radicals occur in biological systems when there is an overproduction of ROS and/or RNS, on the one hand, and a deficiency of antioxidant enzymes or low molecular weight antioxidants on the other (Figure 13). A sustained and delicate balance between the beneficial and harmful effects of ROS/RNS is an important aspect of healthy organisms, and is achieved by a collection of mechanisms that are described as 'redox regulation' (Valko et al., 2007).

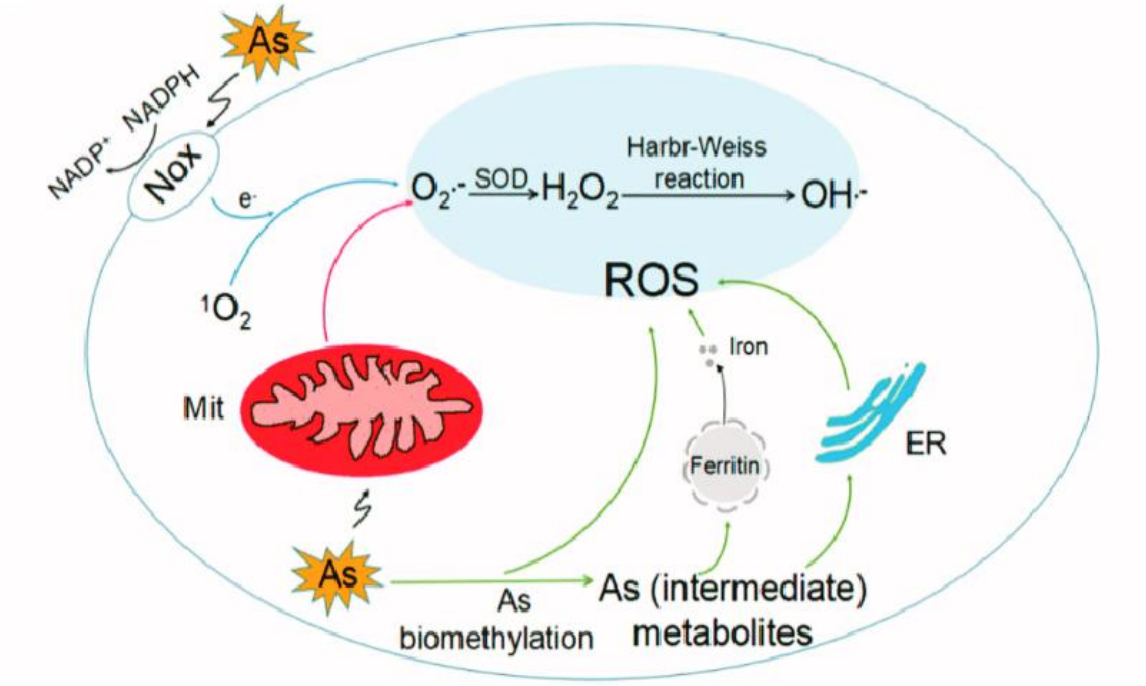

Figure 10: Various Pathways of Reactive Oxygen Species (ROS) Formation and their Transformation.

Oxidative/nitrosative stress results from an imbalance between the formation of ROS/RNS and the impaired ability of an organism to detoxify these reactive intermediates or to repair the damage that they cause. Because alterations in their metabolism and signaling from healthy cells, cancer cells exhibit an increased

formation of ROS/RNS which is counterbalanced by enhanced antioxidant defense mechanisms. Accordingly, cancer cells are able to adapt to an 'initial' redox imbalance by upregulating their antioxidant defense systems (enzymes) which make them insensitive to further stress inducers such as radiation and chemotherapy.

Sources of oxidants

Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition, a number of external agents can trigger ROS production (Figure 14, 15). A sophisticated enzymatic and non-enzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may interrupt the physiological role of oxidants in cellular proliferation and host defense. Similarly, increased ROS may also be detrimental and lead to cell death or to acceleration in ageing and age-related diseases.

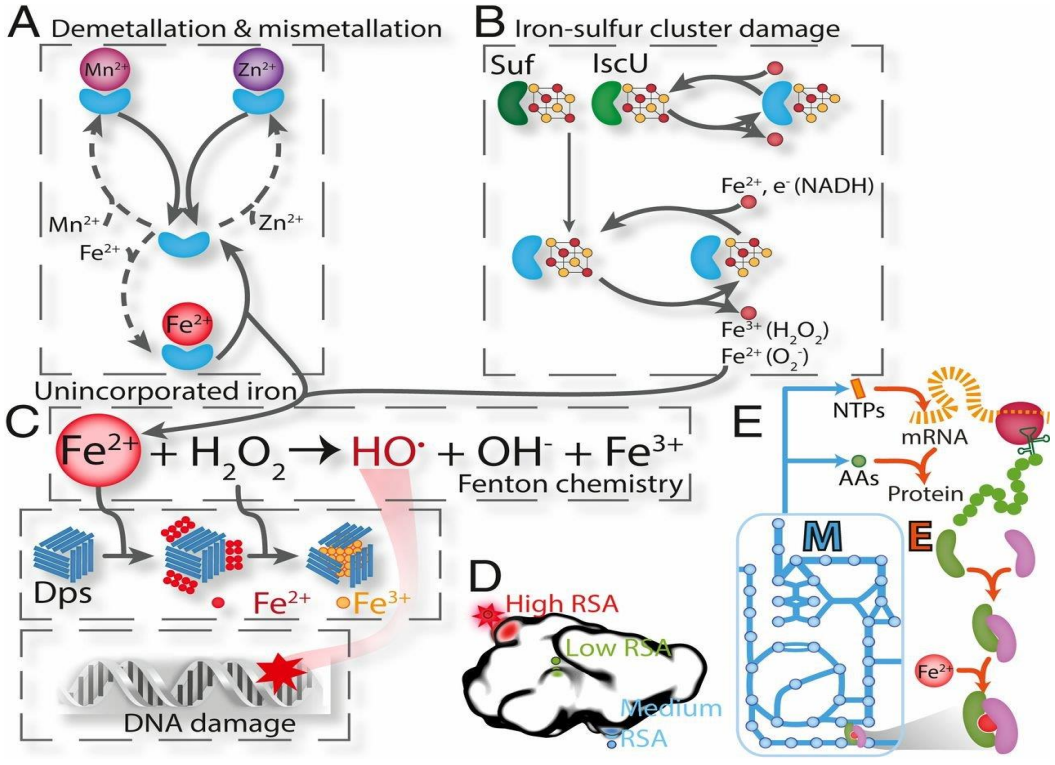

Figure 11 : The source and cellular responses to reactive oxygen species (ROS).

Some internally generated sources of free radicals are:

- Mitochondria
- Xanthine oxidase
- Peroxisomes
- Inflammation
- Phagocytosis
- Arachidonate pathways
- Exercise
- Ischemia/reperfusion injury

Some externally generated sources of free radicals are:

- Cigarette smoke
- Environmental pollutants
- Radiation
- Certain drugs, pesticides
- Industrial solvents
- Ozone

Endogenous sources of oxidants

The amount of free radical production is determined by the balance of many factors, and ROS are produced both endogenously and exogenously. The endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation (Inoue *et al*., 2003). There are also other cellular sources of superoxide radicals present such as the enzyme xanthine oxidase, which catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion followed by the generation of hydrogen peroxide (Hayyan *et al.,* 2016). Additional endogenous sources of cellular ROS are neutrophils, eosinophils and macrophages. On activation, macrophages initiate an increase in oxygen uptake giving rise to a variety of ROS, including superoxide anion, nitric oxide and hydrogen peroxide.

ROS Production in Mitochondria

The reduction of oxygen to water in the mitochondria for ATP production occurs through the donation of four electrons to oxygen to produce water. Mitochondrial electron transport chain (ETC) reduces 95 % of O_2 by tetravalent reduction to H_2O without any free radical intermediates. However, the remaining 5% of oxygen is reduced via the univalent pathway in which free radicals are produced. Mitochondria from different tissues

may vary conspicuously in their capacity to produce ROS using different substrates, and this capacity may be related to membrane composition, animal species, and age. During the process of ROS production, several major oxygen derivatives are formed, and considerable quantities of superoxide and hydrogen peroxide (H_2O_2) are formed. Cytochrome oxidase releases no detectable oxygen radicals into free solution. However, during the transfer of electrons through earlier components of the transport chain, a few electrons do leak out directly on to O2, resulting in the generation of $\bullet O_2^-$.

ROS Production in Endoplasmic Reticulum

ER is another membrane-bound intracellular organelle, but unlike mitochondria, it is primarily involved in lipid and protein biosynthesis. ER when under stress produces ROS mainly by two mechanisms during disulfide bond formation. First, ROS are produced as a by-product during transfer of electrons from protein thiol to molecular oxygen by endoplasmic reticulum oxidoreductin-1 (ERO-1) and protein disulfide-isomerase (PDI) (Bhandary *et al*., 2003). The second mechanism presumes ROS are generated by unfolded proteins, independent of the formation of disulfide bonds. Accordingly, accumulation of unfolded proteins in the ER elicits Ca^{2+} leakage into the cytosol, increasing ROS production in the mitochondria (Malhotra and Kaufman 2007).

ROS production in peroxisomes

Peroxisomes participate in fatty acid oxidation and contain peroxide-producing enzymes. Peroxisomes are an important source of total cellular H_2 O_2 production. Peroxisomes in mammals play an important role in a variety of metabolic pathways such as fatty acid α- and β-oxidation, ether phospholipid biosynthesis, glyoxylate metabolism, amino acid catabolism, polyamine oxidation, and oxidative part of the pentose phosphate pathway. As peroxisomes contain a large number of ROS-producing enzymes, hence using all the above-mentioned metabolic pathways, different types of ROS such as hydrogen peroxide, superoxide, nitric oxide radicals, hydroxyl radical, and peroxynitrites are produced. Catalase is also a peroxisomal enzyme which metabolizes the hydrogen peroxide formed in these organelles.

Exogenous sources of oxidants

Cigarette Smoke

Cigarette smoke contains many oxidants and free radicals and organic compounds, such as superoxide and nitric oxide. In addition, inhalation of cigarette smoke into the lung also activates some endogenous mechanisms, such as accumulation of neutrophils and macrophages, which further increase the oxidant injury.

Ozone Exposure

Ozone exposure can cause lipid peroxidation and induce influx of neutrophils into the airway epithelium. Short-term exposure to ozone also causes the release of inflammatory mediators, such as MPO, eosinophil cationic proteins and also lactate dehydrogenase and albumin. Even in healthy subjects, ozone exposure causes a reduction in pulmonary functions (Paulin et al., 2020; Rich *et al.*, 2020) have shown that particulate matter (mixture of solid particles and liquid droplets suspended in the air) catalyzes the reduction of oxygen.

Ionizing radiation

In the presence of O_2, ionizing radiation converts hydroxyl radical, superoxide, and organic radicals to hydrogen peroxide and organic hydroperoxide, which can react with redox active metal ions, such as Fe and Cu, via Fenton reactions, and thus induce oxidative stress (Borek, 2004). In addition, it can generate damaging intermediates through interaction with water, a process termed radiolysis.

Heavy metal ions

Heavy metal ions, such as iron, copper, cadmium, mercury, nickel, lead, and arsenic, can induce generation of reactive radicals and cause cellular damage via depletion of enzyme activities through lipid peroxidation and reaction with nuclear proteins and DNA. One of the most important mechanisms of metal-mediated free radical generation is via a Fenton-type reaction. Superoxide ion and hydrogen peroxide can interact with transition metals,

such as iron and copper, via the metal catalyzed Haber–Weiss/Fenton reaction to form OH radicals (Das et al., 2020).

Antioxidants

Enzymatic Antioxidants

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes. Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide (Firuzi, 2011).

Superoxide dismutase

Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide. SOD enzymes are present in almost all aerobic cells and in extracellular fluids (Johnson and Giulivi, 2005). There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and finally the Ni type which binds nickel (Wuerges *et al*., 2004).

Catalase

Catalase is a common enzyme found in nearly all living organisms, which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani P *et al.,* 2004). Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other, less dangerous substances.

Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms. Glutathione peroxidase is an enzyme containing four seleniumcofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals (Hayes *et al.,* 2005).

Nonenzymatic antioxidants

Ascorbic acid

Ascorbic acid or “vitamin C” is a monosaccharide antioxidantfound in both animals and plants. As it cannot be synthesized in humans and must be obtained from the diet, it is a vitamin. Most other animals are able to produce this compound in their bodies and do not require it in their diets. In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins. Ascorbic acid is a reducing agent and can reduce and thereby neutralize ROS such as hydrogen peroxide (Padayatty *et al*., 2003). In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants.

Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants.

Melatonin

Melatonin, also known chemically as N-acetyl-5- methoxytryptamine, (Nassar *et al*., 2007) is a naturally occurring hormone found in animals and in some other living organisms, including algae (Caniato *et al*., 2003). Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier.

Tocopherols and tocotrienols (Vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties one of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolizing this form. It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from

oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber and Atkinson 2007).

Beta-carotene

Beta-carotene is a fat-soluble member of the carotenoids which are considered provitamins because they can be converted to active vitamin A. Betacarotene is converted to retinol, which is essential for vision. It is a strong antioxidant and is the best quencher of singlet oxygen. However, beta-carotene supplement in doses of 20mg daily for 5-8 years has been associated with an increased risk of lung and prostate cancer and increased total mortality in cigarette smokers (Radwan et al., 2017; Muller *et al.,* 2011).

Lycopene

Lycopene, a carotenoid, possesses antioxidant and antiproliferative properties in animal and in vitro studies on breast, prostate and lung cell lines, although anticancer activity in humans remains controversial (Imran et al., 2020; Radwan *et al.,* 2017; Alvi et al., 2016). Lycopene has been found to be very protective, particularly for prostate cancer. Several prospective cohort studies have found associations between high intake of lycopene and reduced incidence of prostate cancer, though not all studies have produced consistent results. The major dietary source of lycopene is tomatoes, with the lycopene in cooked tomatoes, tomato juice and tomato sauce included, being more bioavailable than that in raw tomatoes (Imran et al., 2020; Radwan *et al.,* 2017; Alvi et al., 2016).

Flavonoids

Flavonoids are polyphenolic compounds which are present in most plants. According to chemical structure, over 4000 flavonoids have been identified and classified into flavanols, flavanones, flavones, isoflavones, catechins, anthocyanins, proanthocyanidins. Beneficial effects of flavonoids on human health mainly reside in their potent antioxidant activity.

Omega-3 and omega-6 fatty acids

They are essential long-chain polyunsaturated fatty acids because the human body cannot synthesize them. Therefore, they are only derived from food. Omega-3 fatty acids

can be found in fat fish (salmon, tuna, halibut, sardines, pollock), krill, algae, walnut, nut oils and flaxseed. However, certain big fishes like tilefish, shark, swordfish are to be avoided because of their high mercury levels. There are three major dietary types of omega-3 fatty acids: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA). EPA and DHA are abundant in fish and are directly used by the body; while ALA is found in nuts and has to be converted to DHA and EPA by the body.

Treatment approaches against diabetes

Medical nutrition therapy

Medical nutrition therapy, an important component of healthy lifestyle, remains a cornerstone of diabetes prevention and management. Medical nutrition therapy has been shown to accrue sustained reduction in hemoglobin A1c in diabetic patients (Funnell et al., 2010;) and also improvement in lipid profile and blood pressure in non-diabetic individuals (Van Horn 2008).

Oral hypoglycemic agents

The choice of antiglycemic agents in T2DM should be guided by medical needs of the patient and treatment goals, potency of the agent in achieving optimum glycemic control, tolerability and side effect profile, ease of administration and convenience, cost-effectiveness, and other beneficial extraglycemic effects.

Insulin therapy

Patients with T2DM have insulin resistance and progressive pancreatic β-cell failure, which results in deficient insulin secretion and consequent hyperglycemia and elevated free fatty acid level. The resulting glucotoxicity and lipotoxicity initiate a vicious cycle that further compromise's ability of the β-cell to secrete insulin in response to hyperglycemia or oral hypoglycemic agents. Furthermore, the inexorable decline of pancreatic β-cell functions in T2DM results in therapeutic failure of oral agents over time (Klein et al., 2004). Thus, most patients with T2DM will ultimately require insulin therapy to achieve and maintain adequate glycemic control. Insulin is also indicated in the critically ill and

hospitalized diabetic patient to maintain adequate glycemic control. Although early initiation of insulin therapy has been shown to be beneficial in inducing long-term glycemic control in newly diagnosed type 2 diabetic patients with severe hyperglycemia (Li et al., 2004), about 50% of general practitioners delay initiation of insulin because of barriers (Peyrot et al., 2005).

Multiple approaches of phytomedicines in combating diabetic disorders

Progress in understanding the metabolic staging of diabetes over the past few years has led to significant advances in regimen for treatment of this devastating disease. The most challenging goal in the management of patients of diabetes mellitus is to achieve blood glucose level as close to normal as possible (Mooradian.et al.,1999).

Glucose absorption

It is envisaged therefore, that there are several approaches to retard glucose uptake in the small intestine:

- By inhibiting digestive enzymes,
- by inhienzyme active transport of glucose across intestinal brush border membrane, and
- by delaying the gastric emptying ratan of gastrointestinal content. The water-soluble dietary fibres, guar gum, pectin (Nelson et al.,1989) polysaccharides contained in plants (Yuan et al.,1998) have been reported to increase the viscosity of gastrointestinal content, thereby decreasing the gastric emptying rate and suppressing/delaying the digestion and absorption of carbohydrates.

Antioxidant defense

The antioxidant defence system represents a complex network with interactions, synergy and specific tasks for a given antioxidan. studies (Polidori et al.,2000) show that majority of the plasma antioxidants are depleted in Type 2 diabetes patients. The depletion of antioxidants in the diabetic patients was independent of body mass index and dietary intake and this depletion is a major cause of diabetes-related complications and onset of

other disease conditions like atherosclerosis and coronary heart disease (Price et al.,2001)Antioxidant activities of the majority of compounds mentioned in the text have been reviewed recently (Tiwari et al.,2001) and their benefits in oxidative stress and related disease conditions have been widely described.

Conventional drugs used for treatment of diabetes and associated side-effects

Despite the fact that insulin treatment and oral hypoglycemic drugs are the main mode of treatment for the diabetes mellitus for type I and type II respectively, they have their limitations, and these agents don't amend the course of diabetic complications. Human insulin is a polypeptide and it is main stay of treatment for type I diabetes.

Evolution Of Herbs

The utilization and delivery of herbal prescription in treatment and forestalling of disease has a long history which began with the consumption of mesopotamia in 2600 B.C. Around 21,000 plants have been recorded by the World Health Organization (WHO), which are utilized for therapeutic rationale of various diseases around the world (Paramanick et al., 2017).

Clinical studies related to anti diabetic herbs

Allium cepa (Onion)

Allium cepa often recognized as onion has been used medicinally for centuries. Concentrates of onion have been appeared to have hypoglycemic potential by effecting the activities of liver hexokinase, HMG coenzyme-A reductase and glucose 6-phosphatase (Akash et al.,2014). According to a clinical trial, hypoglycemic action of Allium cepa was investigated in type 1 and type 2 diabetic patients. Total 42 diabetic patients were divided into two groups i.e., 21 in each group. The results demonstrated that intake of crude fresh slices of Allium cepa (100 g) administered orally to the patients caused a significant decrease in fasting blood glucose levels by around 89 mg/dl.

Azadirachta indica (Neem)

A trial was conducted to assess the glucose lowering potential 70% alcoholic neem root bark extract (NRE) in diabetic patients. The results demonstrated that neem extract significantly improves blood glucose levels at 800 mg/kg dose in comparison with the standard medication glibenclamide at the dosage of 200,400 and 800 mg/kg (Choi et al.,2013).

Eugenia jambolana (Jamun)

Eugenia jambolana Lam., generally called black plum or “jamun” is an important therapeutic plant in herbal medicine. It is helpful in the management of diabetes mellitus, swelling, ulcers and stomach problems, attributed to saponins, glycosides, and flavonoids present (Swami et al., 2012).

Ocimum sanctum (Basil)

Tulsi is among the most popular herbs used in South East Asia. The leaves of the Tulsi contain fundamental oils including carvacrol, ursolic acid, eugenol and the seeds contain fixed oils, including oinoleic acid, oleic acid, palmitic acid, and stearic acid. In Asian continent, fresh leaves of this plant are most commonly used for the treatment of cough, cold, abdominal pain, skin diseases, arthritis, painful eye diseases, measles, and diarrhea (Krawinkel et al.,2018).

Cestrum nocturnum

Kingdom	Plantae
Phylum	Spermatophyte
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Solanales
Family	Solanaceae
Genus	Cestrum
Species	Cestrum nocturnum

Taxonomical classification of Cestrum nocturnum

Cestrum nocturnum is a garden shrub from the family Solanaceae, commonly known as “lady of the night” which is used as a remedy for different health disorders. This sprawling shrub has glossy simple leaves, vine like stems, greenish-creamy white tubular flowers and fleshy berries. The berries are marfil white or aubergine in colour. The species name ‘nocturnum’ refers to the species’ habit of opening its small, heavily-scented flowers at night. The flowers release powerful sweet perfume at night. It is made into a rare attar (raat ki rani) which is used in Indian and Middle East perfumery. It is said to be the world’s strongest smelling plant. Indeed, the scent can reach up to 165 feet away from the location of plant (Ratsch et al.,1998).

Cultivation

Climate: *C. nocturnum* thrives in moist or wet forests, dense lowland forests and is commonly cultivated in gardens (ISSG,2014). It does not tolerate frost and drought. Optimal growth occurs at about 80˚F. Though night blooming jasmine blooms in night, It requires at least 6 hours of sunlight and partial shade every day to bloom. However excessive sun exposure causes leaves to wilt.

Soil: It grows best in average to moist soil that is light and sandy with a neutral pH of 6.6 to 7.5, and hardy to hardiness zone 8. It can adapt to a variety of soil types and conditions, but has low salt and waterlogging tolerance. *C. nocturnum* can be fertilized biweekly with a weak dilution of seaweed and fish emulsion fertilizer.

Night blooming jasmine Flowers Fruits

Anti-cancerous activity of C. nocturnum

Lu et al. performed the n-butanol part isolated from the flowers of C. nocturnum produced an inhibitory effect on the proliferation of human hepatocellular carcinoma Bel-7404, human gastric carcinoma SGC-7901, and cervical cancer HeLa cells in a dose-dependent manner (Lu et al., 2010). However, the fractions responsible for the antiproliferation effect of n-butanol part from C. nocturnum flowers and related mechanisms remain unknown. The fraction C4 and C5 extracted from the n-butanol part of C. nocturnum flowers showed significant cytotoxic potential towards a wide range of human malignant cell lines with low cytotoxicity to immunocytes and exhibited strong antitumor activities against Bel-7404 cells. These antitumor activities include attenuation of cancer cell proliferation as well as induction of apoptosis at the G0/G1and G2/M phases through enhancement of DNA damage and inhibition of topoisomerase II relaxation activity (Wu et al., 2017).

Anti-Bacterial and anti-fungal activity of C. nocturnum

The crude MeOH extract of plant of C. nocturnum (Solanaceae) and its subsequent fractions were tested against various bacterial and antifungal strains with the exception of Pseudomonas aeruginosa, Staphylococcus aurous, Bacillus subtilis, Escherichia coli and Shigella flexenari. The zone of inhibition ranged from 19 to 280 µg/ml. The crude extract and fractions were also susceptible to Candida species and Asper species. The zone of inhibition for various fungi ranged from 170 to 290 µg/ml (Rokade et al., 2016).

Medicinal uses

- In traditional medicine, leaves of Cestrum nocturnum have been used for their pharmacological significance in burns and swellings.
- It is also used for treating epilepsy (Pe´rez-Saad et al.,2008)
- Pharmacological studies on the plant proved that the leaves have significant analgesic and bactericidal activity (ZENG Jing et al.,2003,05).
- The volatile oil is known to be mosquito-repellent and hence C. nocturnum is used to prevent malaria in several African Nations (Jawale et al., 2010)
- Local anaesthetic effect, inhibitory effect on central nervous system and cardiac arrthymic effect of plant are also documented.

OBJECTIVES

I. Collection, identification and preparation of Plant material.

II. Solvent based extraction and phytochemical screening of *C. nocturnum* leaves.

III. *In-vitro* antioxidative studies of different leaf extracts of *C. nocturnum* by DPPH radical scavenging assay.

IV. *In vitro* antioxidative studies of different leaf extracts of *C. nocturnum* by ABTS assay.

V. *In vitro* Antidiabetic studies of different leaf extracts of *C. nocturnum* via targeting α-amylase.

VI. Enzyme kinetics study of different leaf extracts of *C. nocturnum.*to determine the mode of inhibition of α-amylase inhibition.

MATERIALS AND METHODS

Collection of plant material

C. nocturnum leaves, were collected from the local area around Integral University, Lucknow, India the month of February 2022.

Processing of plant materials

Fresh leaves of the plant were shed dried at room temperature (25-35ºC) for 4-6 days. The dried leaves were coarse powered in a grinder, avoiding sun-dried due to the significant modification of the biochemicals and weighed before extraction for calculating the yield.

Preparation of plant extracts

The dried powder (30g) of leaves of *C. nocturnum* was extracted using nonpolar, partially polar, and polar solvents successively with the required amount of each of n-hexane Dichloromethane (DCM), Ethyl acetate (EtOAc), methanol (MeOH), and aqueous solvents in Soxhlet apparatus until it turned colorless. The solvent was removed, filtered, and dried at room temperature, and residues were scratched out and was collected in an Eppendorf tube, weighed, and used for further phytochemical screening.

Instruments

Soxhlet Apparatus

A Soxhlet extractor is a type of laboratory glassware invented in 1879 by Franz Von Soxhlet. It was originally designed for the extraction of lipid from a solid test material, but can be used whenever it is difficult to extract any compound from a solid. The key advantage of this type of extraction; only clean warm solvent is used to extract the solid in the thimble. This increases the efficiency of the extraction when compared with simply heating up the solid in a flask with the solvent. In the Soxhlet extractor, there are five main components. The components are condenser, extraction chamber, thimble, siphon arm and round boiling flask. *Condenser*- It is placed at the top of the Soxhlet extractor body. It is converted a vapor into a liquid that trickles into the extraction chamber containing the sample. *Extraction chamber*- It allows the sample of solvent that used during the extraction process. The solvent which condenses at the condenser drips down through

the extraction chamber. *Extraction thimble*- Cellulose and glass microfiber extraction thimbles are known for their purity and consistent high quality. The thimbles are widely used in Soxhlet extraction units providing a safe, convenient and efficient method of solvent extraction of solids and semi-solids. Cellulose extraction thimbles are produced from high quality alpha cellulose cotton linter and have excellent mechanical strength and retention. *Round Bottom Flask*- It contains a solvent that was used in the extraction. The capacity is 500 ml. Percentage yield of sequentially extracted plants in different solvent system Percentage yield of sequentially extracted plants in different solvent system was calculated by using the formula.

$$\%Yield = \frac{Weight\ of\ crude\ extract}{weight\ of\ raw\ materia} \times 100$$

Phytochemical screening of plant extract

Phytochemical screening is qualitative assay consists of test for phenols, alkaloids, tannins, flavonoids, saponins and triterpenoids, steroids, cardiac glycosides, Coumarins, Anthraquinones, Phlobatannins, and Anthracyanine (Roghini and Vijayalakshmi, 2018).

Test for phenols: The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark and left to react for 30 min for color development.

Test for tannins: 10 mg sample was boiled in 50 mL of distilled water and then filtered. A quantity (5 mL) of test solution was added into a test tube followed some drop of $FeCl_3$. Formation of brownish green or blue-black coloration indicates presence of tannins.

Test for flavonoids: 10 mg sample was mixed with 10 mL of distilled water. The mixture was heated for 5 minutes and filtered. The filtrate was mixed with Mg powder, 1 mL of strong HCl and 1 mL of amyl alcohol. Formation of color in amyl alcohol layer indicates flavonoids.

Test for saponins: 10 mg sample was added into test tube and 10 mL of boiling water was added and then cooled. The mixture was agitated vertically for 10 seconds. For 10 minutes formation of foam indicates saponins.

Test for terpenoids: 10 mg sample was mixed with 5 mL of ether solution and evaporated. Test solution was mixed with anhydrate acetate acid and strong H_2SO4 (2:1). Formation of red-green color indicates triterpenoids.

Test for steroids: Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2S04. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for cardiac glycosides (Keller-Killani test): Five ml of each extract was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer (Roghini and Vijayalakshmi, 2018).

Test for Coumarins: 1 ml of 10% sodium hydroxide was added to 1ml of the extract. Formation of yellow colour indicates the presence of coumarins.

Anthraquinones: To 1 ml of fruit extract few drops of 10% ammonia solution was added, appearance of pink color precipitate indicates the presence of anthraquinones.

Test for Phlobatannins: Few drops of 2% hydrochloric acid were added to 1ml of the extract. Appearance of red color precipitate indicates the presence of phlobatannins.

Anthracyanine: To 1 ml of the extract was added 1 mL 2N sodium hydroxide and heated for 5 min at 100 °C. Formation of bluish green color indicates the presence of anthocyanin

Antioxidant assay

DPPH Radical Scavenging activity

The DPPH radical scavenging capacity of the various extract of *C. nocturnum* was determined by the method of Brand-Williams *et al. (*1995). Briefly the free radical scavenging activity based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical. DPPH molecule determines with the occurrence of a purple color. DPPH solution (132mM) was prepared in MeOH in a dark reagent bottle. 100µl of the leaf, stem, and fruit extracts from *C. nocturnum* and ascorbic acid (Concentration ranging from 7.81 to 1000µg/ml) was added to 2ml of DPPH solution and the reaction mixture was incubated for 15 minutes at 27ºC in a water bath and absorbance was measured at 517 nm. The reduced form of DPPH was generated, accompanied by the disappearance of the violet color. Ascorbic acid was used as a reference standard. Percent (%) scavenging of DPPH free radical was measured using the following equation.

$$\%\ DPPH\ radical\ scavenging = \text{Absorbance of Control} - \text{Absorbance of test sample} \times 100$$

Further, the IC_{50} value represented the concentration of the extract that caused 50% inhibition of DPPH radicals and was calculated by interpolation of linear regression analysis.

ABTS radical scavenging assay

Free radical scavenging activity of plant samples was determined by ABTS radical cation decolorization assay (Re et al., 1999). ABTS·+ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 12-16 h before use. ABTS·+ solution was then diluted with MeOH to obtain an absorbance of 0.700 at 734 nm. After the addition of 5 µl of plant extract to 3.995 ml of diluted ABTS·+ solution, the absorbance was measured at 30 min after the initial mixing. An appropriate solvent blank was run in each assay. All the measurements were carried out at least three times. Percent inhibition of absorbance at 734 nm was calculated using the formula, ABTS·+ scavenging effect (%) = ((AB–AA)/ AB) ×100 (2), where, AB is absorbance of ABTS radical + MeOH; AA is absorbance of ABTS radical + sample extract/standard. Trolox was used as standard substance.

α-Amylase Inhibitory Assay

To determine the in vitro α- amylase inhibition by various extracts of *C. nocturnum*, the standard procedure (Bernfeld, 1955) was adopted with slight modification. Briefly, porcine pancreatic α-amylase was dissolved in ice-cold phosphate buffer (20mM), pH 6.7, containing sodium chloride (6.7mM) to give a concentration of 0.15 Units/mL for each assay samples were taken in triplicate including blank. In each test tube, 250 μL of the enzyme preparation was mixed with 100 μL of each of the extracts except the blank. The mixtures were stirred in a vortex and preincubated in a water bath at 37◦C for 20 minutes. After incubation, 250 μL of the substrate preparation (0.5% w/v starch in 20mM phosphate buffer; pH 6.7) was transferred into each test tube to start the reaction. The mixture was vortexed and then incubated at 37◦C for 15 minutes. 2 mL of DNS color reagent (Containing DNS 40mM, K-Na tartrate 1M and sodium hydroxide 0.4M) was added, vortexed and boiled in a water bath at 100◦C for 10 minutes. Thereafter, the mixture was cooled down, and the absorbance was taken at 540 nm. Acarbose was used as standard inhibitor of this enzyme. Inhibition rates were calculated as percentage controls using the formula:

% inhibition= 100 − % reaction,

where % reaction = (mean product in sample/mean product in control) × 100.

Further, IC_{50} value represented the concentration of the extract that caused 50% inhibition of α-amylase and was calculated by interpolation of linear regression analysis.

Determination of Mode of Action (Kinetics of α-Amylase)

Mode of inhibition of *N. arbortristis* leaf and stem extracts against α-amylase was determined by the method of Mogale et al., (2012). For the assay, two sets (A and B) of 6 duplicate test tubes were prepared to determine the enzyme activity in the presence [set A] and absence [set B] of an inhibitor (leaf and stem extracts/standard acarbose). In set A, 100 μL of the inhibitor (plant extract or acarbose, 1mg/mL) solution was added in each test tube except the blank; this was followed by the addition of 100 μL of the enzyme porcine α-amylase (0.15units/mL). In set B, 100 μL of phosphate buffer (20mM), pH 6.7, containing sodium chloride (6.7mM) was added in each test tube followed by 100 μL of the enzyme solution.

Both sets of test tubes were thoroughly mixed in a vortex mixer and preincubated in a water bath at 37ºC for 20 minutes. 100 µl of serial dilutions of the substrate solution were added in both sets of test tubes with concentration ranging between 5 mg/mL and 0.156 mg/mL. All the tubes were then incubated at 37ºC for 15 minutes, followed by the addition of 2mL of DNS color reagent and the mixtures were boiled for 10 minutes. Absorbance of the colored solution was read at 540nm. Double reciprocal curve (1/V v/s1/[S]) for both sets was plotted to determine the effect of the plant extract/acarbose on Vmax and Km of the enzyme, where V and [S] are, respectively, the velocity of the reaction and substrate concentration (Ahmad et al., 2021).

RESULTS

Table 2: %Yield of phytochemicals in various extracts of *C. nocturnum* leaves

Extract	%Yield leaf extract
n-Hexane	1.44
Dichloromethane	1.9
Ethyl acetate	0.71
MeOH	7.63
Aqueous	6.38

Table 3: Phytochemical profiling of different extracts of *C. nocturnum* leaves

	n-Hexane	EtOAc	DCM	MeOH	Aqueous
Cardiac glycosides	-	+++	-	+	-
Steroids	-	-	-	-	-
Phenols	++	+++	-	-	-
Flavonoids	+++	-	-	+	+
Tannins	++	+++	-	-	-
Saponins	-	-	-	+++	++
Terpenoids	-	-	-	-	-
Quinone	++	+++	-	+++	-
Coumarins	+++	-	-	+	+
Phlobatannins	-	-	-	-	-
Anthocynin	-	-	-	-	-

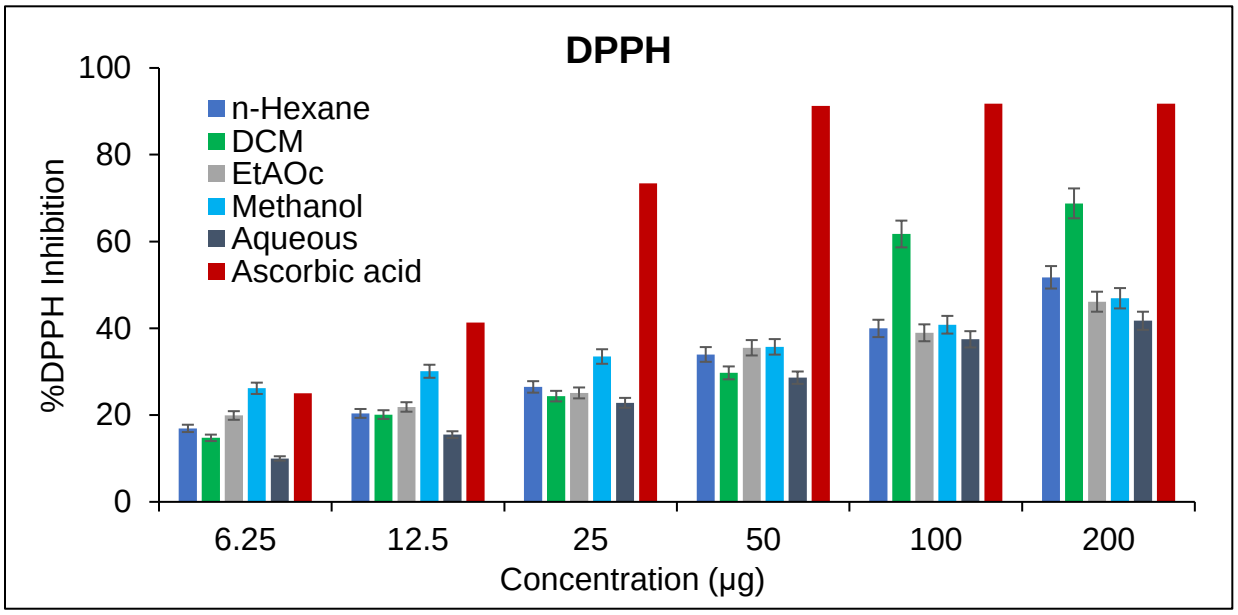

Figure 12: Percent DPPH radical scavenging activity different leaf extract of *C. nocturnum* and standard ascorbic acid. The results are mean ± S.D. of three parallel measurements.

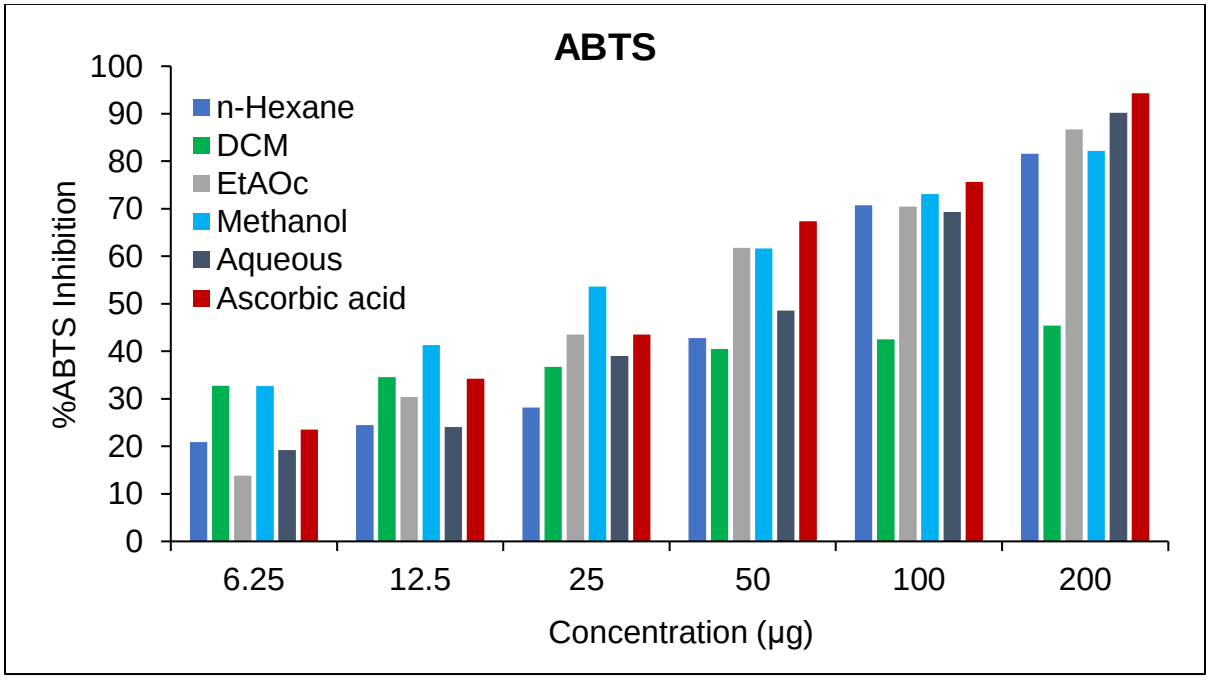

Figure 13: Percent ABTS radical scavenging activity of different leaf extract of *C. nocturnum* and standard ascorbic acid. The results are mean ± S.D. of three parallel measurements

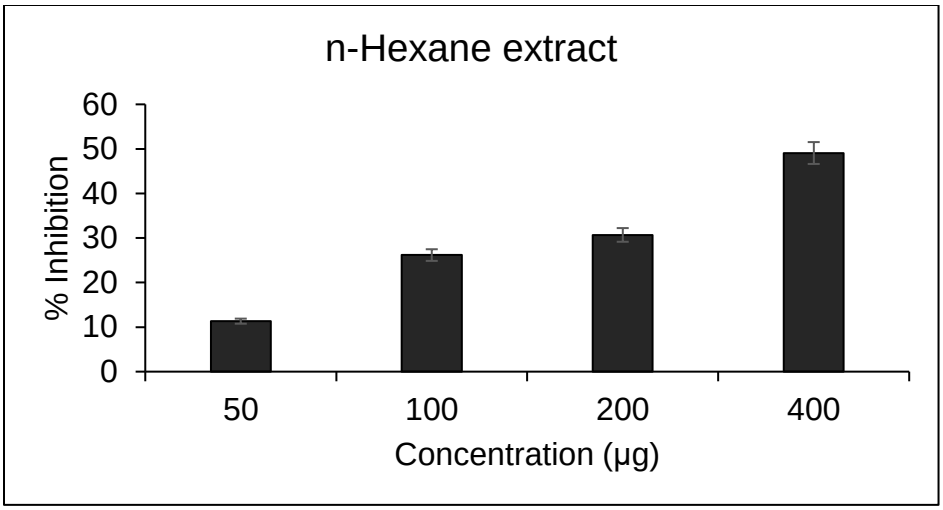

Figure 14: In-vitro α-Amylase inhibitory activity of *C. nocturnum* leaves extracts of n-Hexane. The results are mean ± S.D. of three parallel measurements.

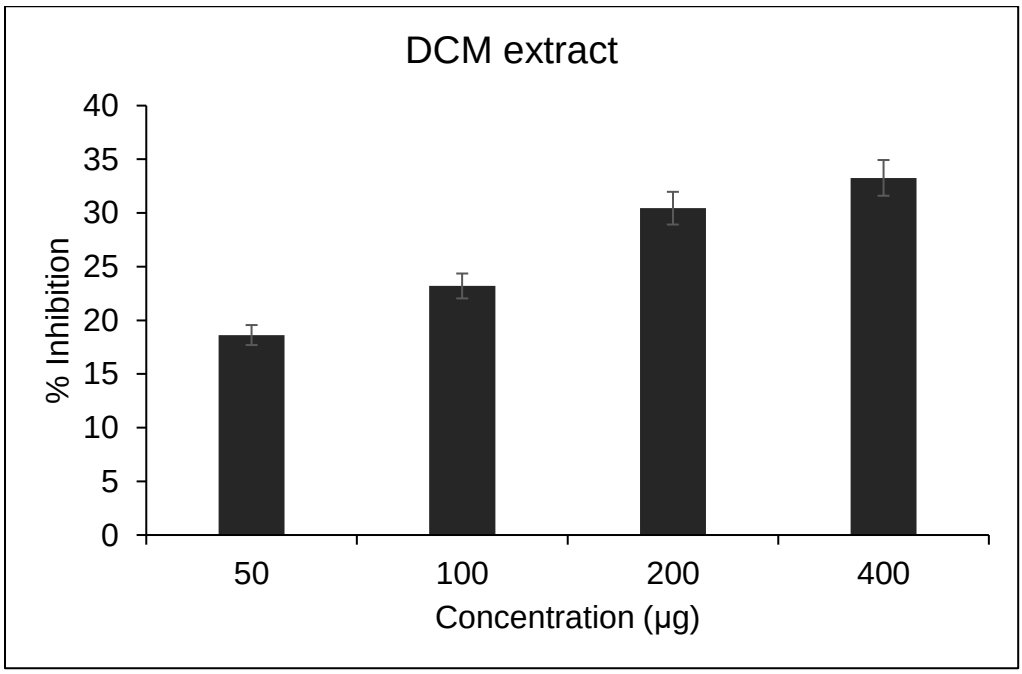

Figure 15: *In-vitro* α-Amylase inhibitory activity of *C. nocturnum* leaves extracts of Dichloromethane. The results are mean ± S.D. of three parallel measurements.

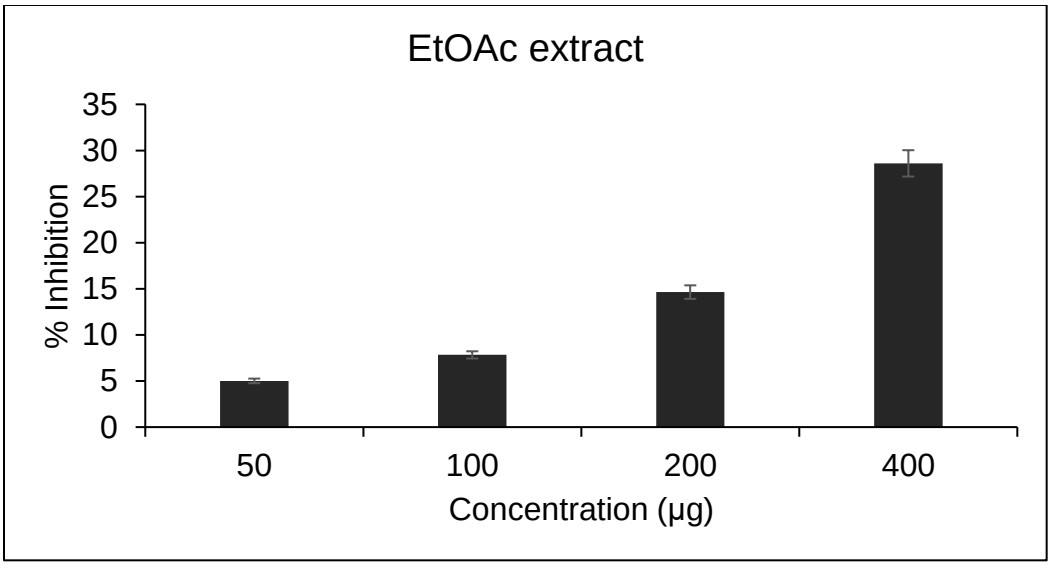

Figure 16: *In-vitro* α-Amylase inhibitory activity of *C. nocturnum* leaves extracts of Ethyl acetate. The results are mean ± S.D. of three parallel measurements.

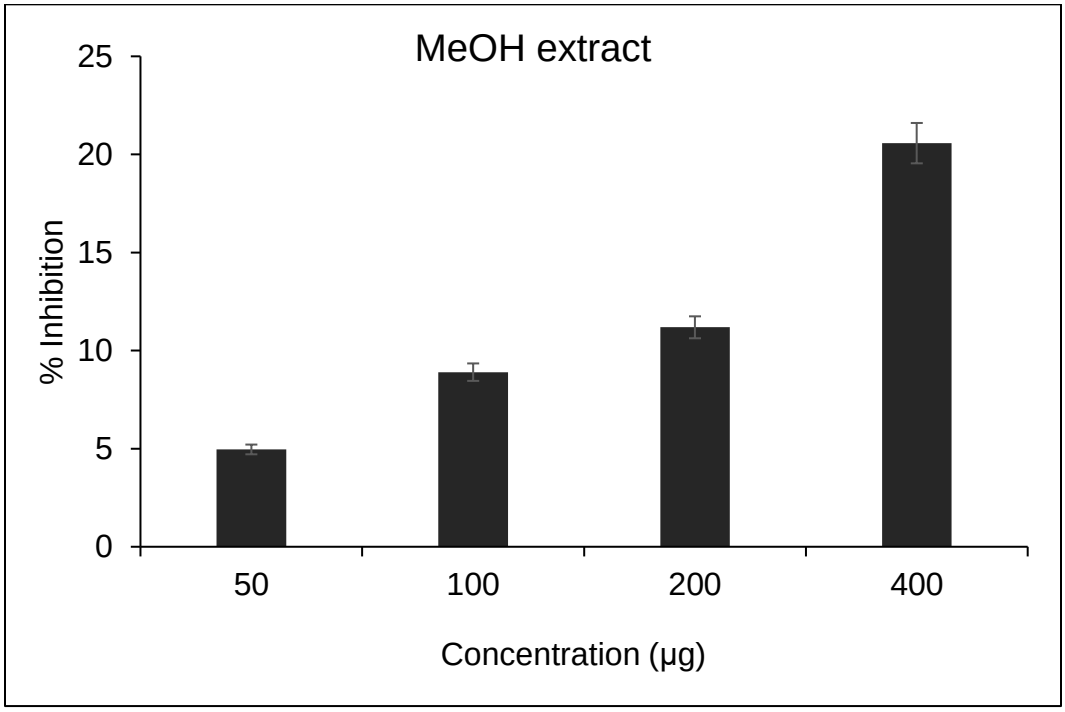

Figure 17: *In-vitro* α-Amylase inhibitory activity of *C. nocturnum* leaves extracts of MeOH. The results are mean ± S.D. of three parallel measurements.

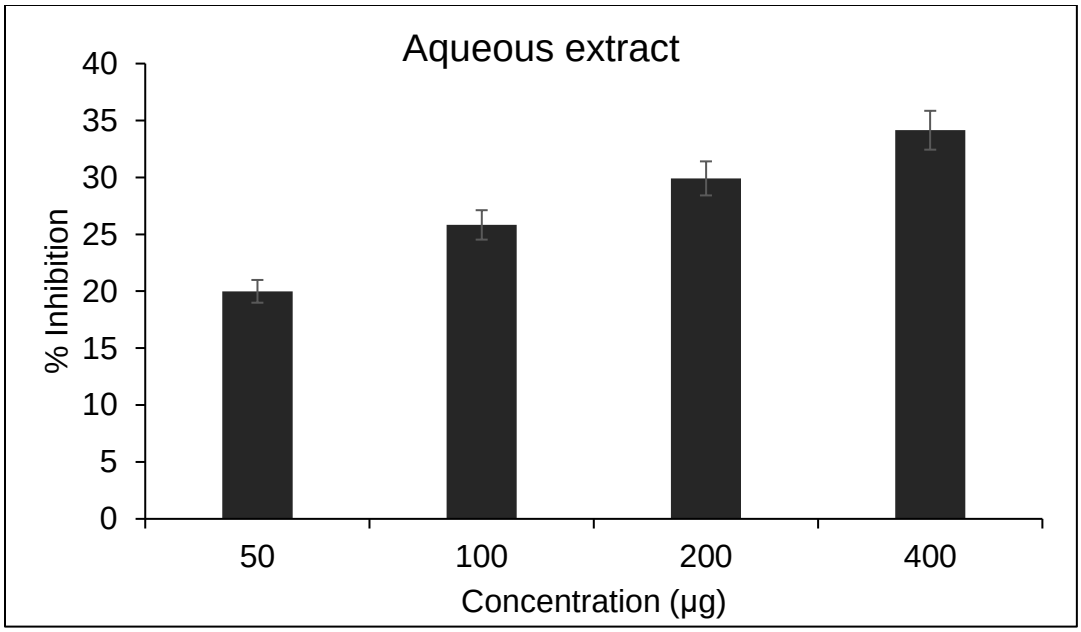

Figure 18: *In-vitro* α-Amylase inhibitory activity of *C. nocturnum* leaves extracts of Water. The results are mean ± S.D. of three parallel measurements.

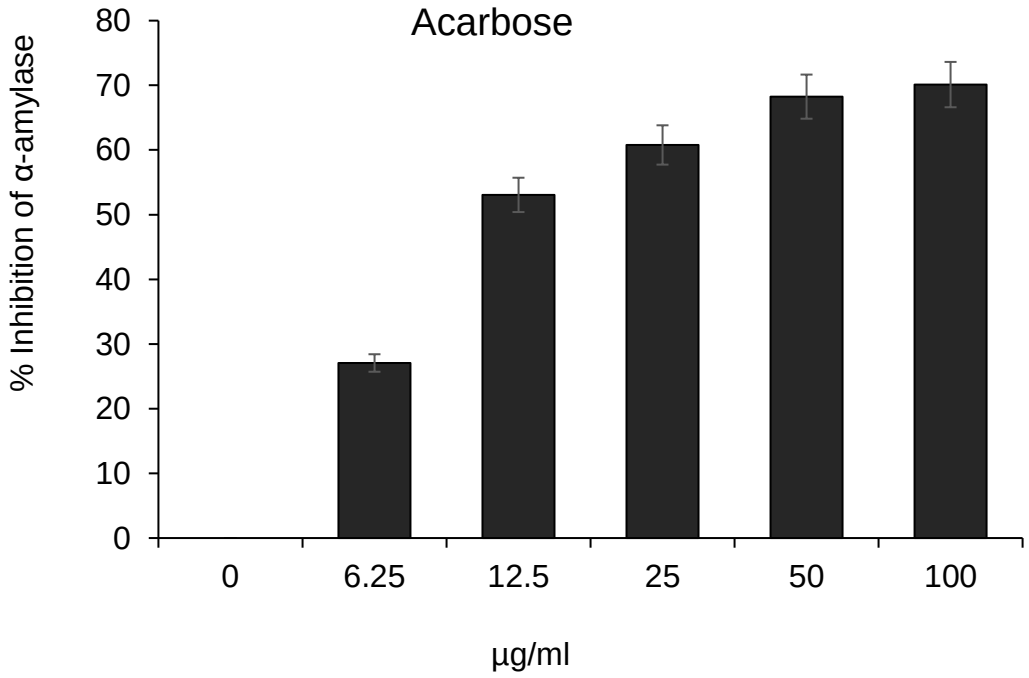

Figure 19. In-vitro α-Amylase inhibitory activity of acarbose leaves extracts of Water. The results are mean ± S.D. of three parallel measurements.

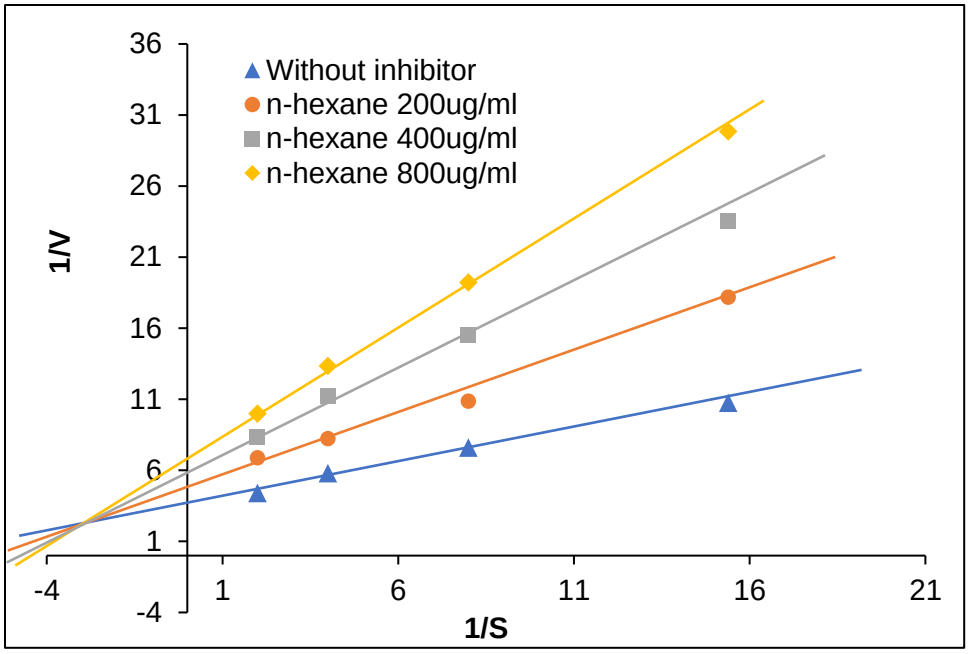

Figure 20: Lineweaver- Burk double reciprocal plot of 1/v versus 1/s of *C. nocturnum* leaves n–Hexane extracts against porcine pancreatic α-Amylase. n-Hexane extract of leaves showed non-competitive mode of α-Amylase inhibition.

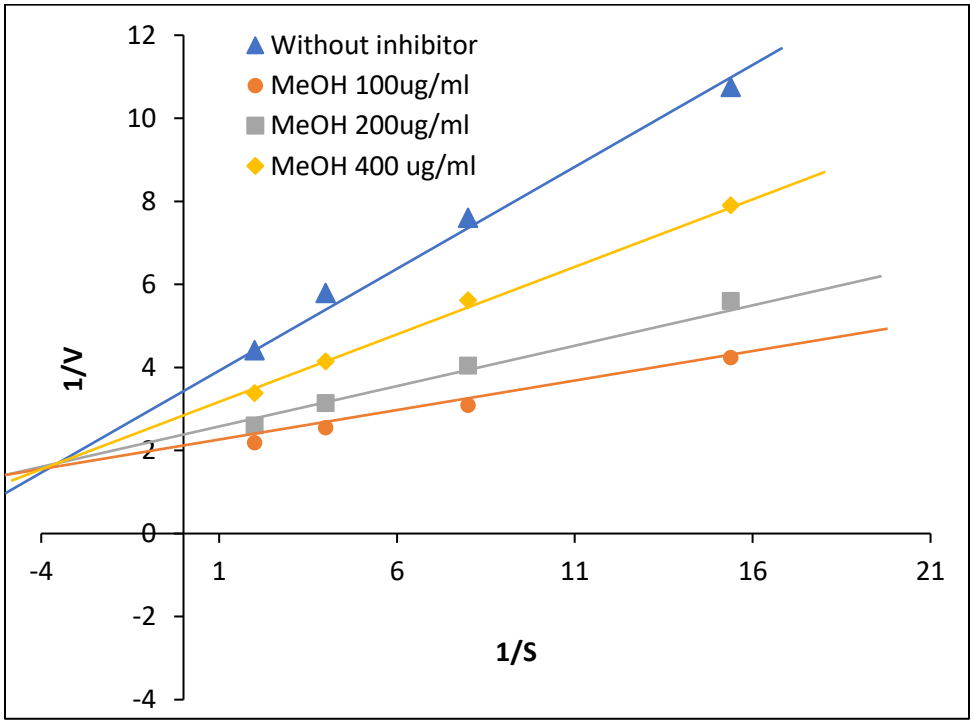

Figure 21: Lineweaver- Burk double reciprocal plot of 1/v versus 1/s of *C. nocturnum* leaves MeOH extracts against porcine pancreatic α-Amylase. MeOH extract of leaves showed non-competitive mode of α-Amylase inhibition.

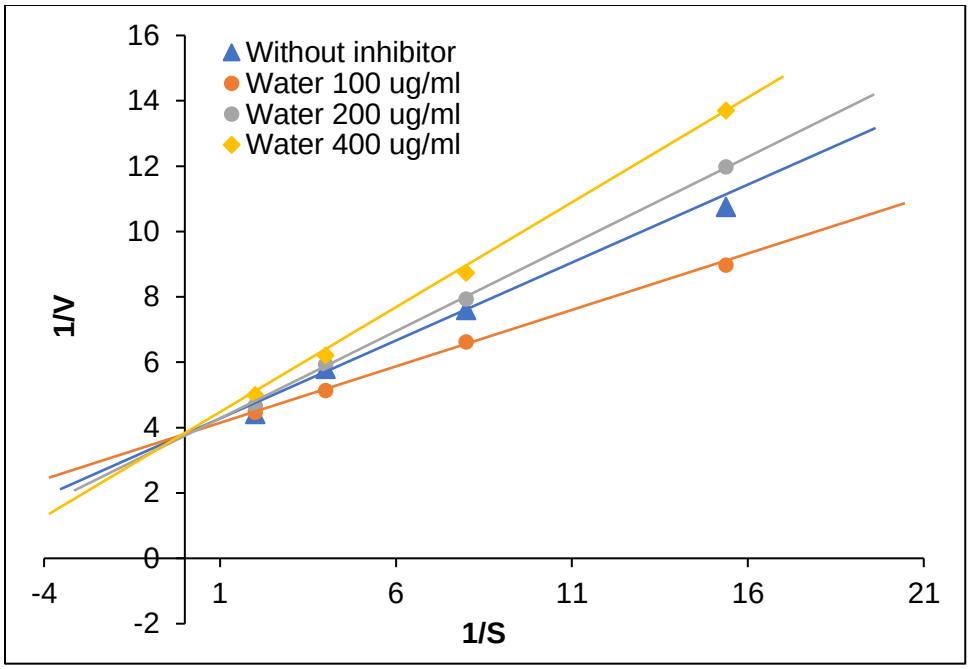

Figure 22: Lineweaver- Burk double reciprocal plot of 1/v versus 1/s of *C. nocturnum* leaves aqueous extracts against porcine pancreatic α-Amylase. aqueous extract of leaves showed competitive mode of α-Amylase inhibition.

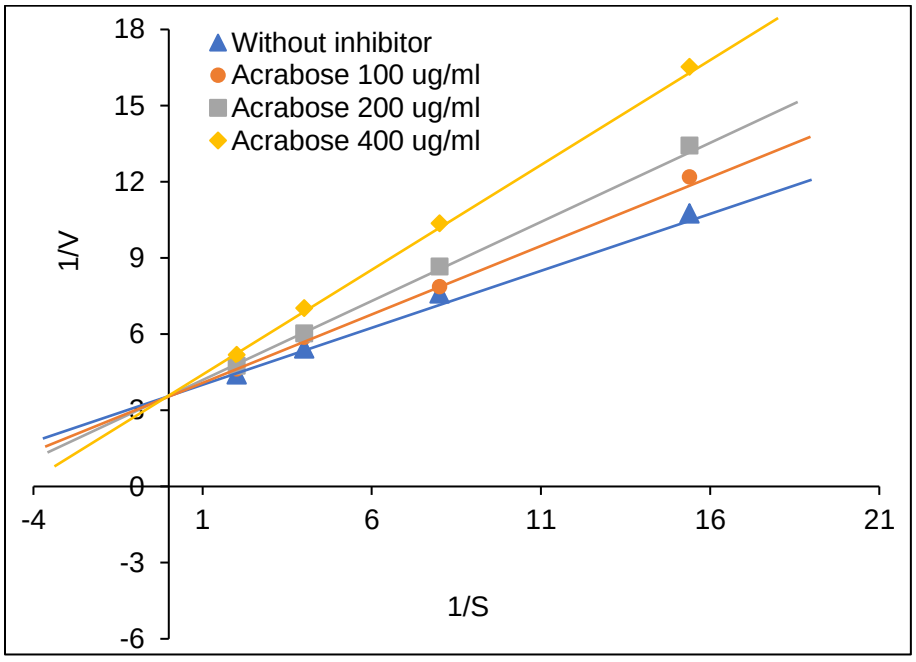

Figure 23: Lineweaver- Burk double reciprocal plot of 1/v versus 1/s of standard drug acarbose against porcine pancreatic α-Amylase. acarbose showed competitive mode of α-Amylase inhibition.

DISCUSSION

Oxidative stress induced by reactive oxygen species (ROS) can cause cell membrane disintegration, protein, lipid, and deoxyribose nucleic acid (DNA) damage which can further initiate or propagate the development of many chronic and degenerative diseases (Vikram et al., 2014; Burton and Jauniaux, 2011). When there is imbalance between ROS generation and antioxidant protection mechanism, it leads to cellular dysfunction causing various diseases inducing diabetes mellitus (DM). Diabetes is an important metabolic syndrome affecting about 200 million people worldwide. The critical effect of diabetes is postprandial hyperglycemia and reduction in antioxidant defense mechanism. So, the management of type 2DM could be done both by reducing oxidative stress as well as by delaying the absorption of glucose through the inhibition of any one of the carbohydrates-hydrolyzing enzymes, α-glucosidase, and α-amylase that are responsible for the breakdown of oligosaccharides and disaccharides into monosaccharides suitable for absorption (Ahmad et al., 2021; Gong et al., 2020; Li et al., 2022). There has been enormous interest in natural antioxidants due to their ability to neutralize the effects of ROS that are not only responsible for alleviating the oxidative stress condition in diabetes but are also helpful in managing the postprandial hyperglycemia. The growing interest to combat the side effect of the drugs available for diabetes leads to the development of green medicines due to their higher stability, higher antioxidant potential, low cost, and low cytotoxicity. Plants are rich sources of phytochemicals, which possess a variety of biological activities including antioxidant and antidiabetic potential both *in vitro* and *in vivo* (Kumar *et al.*, 2012).

Plants are the vital source to combat the severe diseases in the world (Thirumurugan et al., 2010). World health organization (WHO) reported that the more than 80 % of the world population used the remedies based on plants for their primary health care need (Rajasekaran, 2008; Kalaivani *et al.,* 2012). The plants are the source for the new drugs, in which the majority are still unexplored. Among the 25 000 000 to 50 000 000 plant species - several percentages of the plants are investigated for their phytochemical and biological screening. India is known for the thousands of species for its medicinal value and the use of the different parts of the plant to cure specific alignment.

The leaves of *C. nocturnum* have pharmacological significance in Chinese folk medicine and have been used for the treatment of burns and swellings (Murad et al., 2011). The leaves of the plant have shown significant analgesic and bactericidal activity (Huang et al., 2006; Chatterjee and Bhattacharjee, 2007). Local anesthetic effect, inhibitory effect on central nerve system and cardiac arrhythmic effect of plant is also documented (Zeng *et al.,* 2002, 2003, 2003a)

In the current study, we observed a significant % yield of the phytochemicals in all the extracts of *C. nocturnum* with the maximum % yield was reported in MeOH and water extract i.e., 7.63% and 6.38 respectively. Furthermore, we also analyzed the presence of various phytochemicals i.e., phenols, cardiac glycosides, steroids, flavonoids, tannins, saponins, terpenoids, Quinone, Coumarins, Phlobatannins and Anthocynin in this attempt, we found that Cardiac glycosides majorly present in EtOAc extract of *C. nocturnum* while low amount of cardiac glycosides was observed in MeOH as well as not observed in n-heaxne, DCM and water extract. However, steroids, terpenoids, phlobatnnins and anthocynin were not observed in the above mentioned five extracts of *C. nocturnum.* High concentration of Flavonoids and Coumarins was observed in n-Hexane. The MeOH and aqueous extracts showed positive results but at the lower concentration of Flavonoids and Coumarins while in EtOAc and DCM showed negative result for Flavonoids and Coumarins. On the other hands, Phenols and Tannins only observed in n-Hexane and EtOAc. Similarly, the Saponins only observed in MeOH and aqueous extracts. Rich amount of Quinone was observed in EtOAc, MeOH, and n-hexane extract but it was not observed in DCM and aqueous extract of *C. nocturnum*.

The current study also evident the antioxidant potential of all extracts of *C. nocturnum*. We found that all the extracts showed significant inhibition of DPPH radicals with maximum DPPH radical scavenging (68.79 ± 5.32 %) in DCM extract, while the standard ascorbic acid (91.74 ± 9.53 %) inhibition. the antioxidant potential of our extracts was also confirmed by ABTA assay. We found that ABTS value was highest in aqueous extract (90.23%) which is comparable to the standard compound ascorbic acid (94.3%). Similarly, n-Hexane EtOAc, and MeOH extract were also showed the maximum precent ABTS inhibition i.e., 81.60 ± 8.22, 86.69 ± 6.25, and 82.19 ± 10.14 % respectively. The DCM extract of *C. nocturnum.* was observed very low percent ABTS inhibition. These

therapeutic potential of all the extracts of *C. nocturnum* encouraged us to proceed further in order to evaluate the antidiabetic potential of these extracts via targeting α-amylase, the major carbohydrate metabolizing enzyme.

In this attempt, we found that our extracts showed significant inhibitory activity against pancreatic amylase. All the extracts from *C. nocturnum* showed marked inhibition of pancreatic amylase with maximum inhibition in n-Hexane and aqueous extracts, i.e., 49.08 ± 4.24 % and 34.14 ± 2.11 %, respectively. Additionally, reference drug acarbose also showed significant inhibition of α-amylase much higher (75.59 ± 4.32%) than *C. nocturnum* extracts. The α-amylase inhibitory potential our plant might be due the presence of antioxidant, phytochemicals in all the extracts which might occupy the active pocket of α-amylase. To further confirm our results, we performed enzyme kinetics studies which confirmed the mode of enzyme inhibition by our test extracts from *C. nocturnum* leaves. In this order, we found that n-Hexane and MeOH extracts showed non-competitive type inhibition of α-amylase, whereas, aqueous extracts showed competitive inhibition of α-amylase.

CONCLUSION

In conclusion, the results for the first time demonstrated a strong antioxidant and α-amylase inhibitory activity of sequentially extracted *C. nocturnum* fractions. We found that all the extracts showed significant inhibition of DPPH radicals with maximum DPPH radical scavenging in DCM extract, whereas, the antioxidant potential of our extracts was also confirmed by ABTS assay. All the extracts of *C. nocturnum* showed marked inhibition of pancreatic amylase with maximum inhibition in n-Haxane and aqueous extracts, in which the aqueous extract showed the competitive mode of inhibition. These beneficial effects of our plant might be due the presence of antioxidant in all the extracts which might occupy the active pocket of α-amylase. Thus, it is a good approach to manage type 2DM as a whole with these extracts, which showed good enzyme inhibitory and antioxidant activities. Further, a thorough and full-fledged *in-vitro* (LC/MS, GC/MS analysis for identification of particular compound and their assessment of amylase inhibitory potential will be needed) as well as *in-vivo* study is also needed to explore the role of these extracts and also their bioactive compounds in order to establish a better treatment approach to get rid of diabetes and other oxidative stress consequences

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