A DISSERTATION ON

Elucidation of *In-vitro* Antioxidant and *in-silico* α-amylase Inhibitory Activity of *Phoenix dactylifera* L. (Khudari cultivar) Derived Secondary Metabolites

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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CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Ms. Shraddha Patel** during the months Feb–May, 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 **"Elucidation of In-vitro Antioxidant and in-silico α-amylase Inhibitory Activity of *Phoenix dactylifera* L. (Khudari cultivar) derived secondary metabolites"** 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:**26/June/2022**
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TO WHOM IT MAY CONCERN

This is to certify that the study conducted by **Ms. Shraddha Patel** student of M.Sc. Biotechnology (IV semester), Integral University has completed her four months dissertation work entitled **"Elucidation of In-vitro Antioxidant and in-silico α-amylase Inhibitory Activity of *Phoenix dactylifera* L. (Khudari cultivar) derived secondary metabolites"** successfully. she has completed this work from the Department of Biosciences, Integral University, under the guidance of **Dr. M. Salman Khan.** This dissertation was a compulsory part of her M.Sc. Degree.

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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.

Words will hardly help in expressing my sincerest gratitude to my supervisor **Dr. M. Salman Khan (Associate Professor, Department of Biosciences, Integral University)**, who not only introduced me the fascinating field of Biochemistry, but also helped me to understand the subject matter in all different possible ways**.** He has always been there for me throughout the work and helped me to overcome all odds. He has always taken a keen interest in my welfare.

It gives great gratification to record my earnest thanks to **Dr. Snober S. Mir, Head of the Department, Department of Biosciences, Integral University** for providing me all necessary facilities and excellent research climate in pursuing this study.

Special thanks to **Dr. Sahir Sultan Alvi, Mr. Mohd Waiz and Mr. Parvej Ahmad** for their relentless help and advices. They generously devoted their valuable time for guidance and without their kind efforts my work would not be possible.

I also thank my group mates. I must write about my family members for their unconditional love, support and encouragement. It is equally important to thank my **parents…**but this acknowledgement will never be complete if their name is not there.

Date: **26/June/2022**

Place: **Lucknow** **Ms. Shraddha Patel**

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ABBREVIATIONS

HbA1c	Glycated haemoglobin
HMG-R	β- hydroxy-β-methyl-glutaryl CoA reductase
LDL-C	Low density lipoprotein cholesterol
VLDL-C	Very low density lipoprotein cholesterol
HDL-C	High density lipoprotein cholesterol
TC	Total cholesterol
TG	Triglycerides
ROS	Reactive oxygen species
GST	Glutathione S- transferase
CAT	Catalase
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DNS	Dinitrosalysilic acid
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
GSH	Reduced glutathione

INTRODUCTION

The imbalance between reactive oxygen species (ROS) production and antioxidant defense leads to “oxidative stress.” “Oxidative stress” was first introduced by Helmut Sies in 1985 to describe the disturbance in pro-oxidant- antioxidant balance. It is a situation when steady-state ROS concentration is transiently or chronically enhanced; as a result it disturbs cellular metabolism and its regulation and finally damages cellular constituents (Lushchak 2011).The body takes molecular oxygen and uses it to produce energy via oxidative phosphorylation in mitochondria, and this, and other metabolic reactions generate free radicals imposing oxidative stress on proteins, DNA and lipids which results in altered cellular function, compromised tissue and organ function, and ultimately death. The free radical theory is supported by the “rate of living” hypothesis, which inversely links metabolic rate with the longevity of the organisms and it is also well established that oxidative damage to proteins, DNA and lipids increases with age (Sohal and Weindruch 1996). The formation and removal of free radicals are balanced in a normal cell. However, with more formation of free radicals or when levels of antioxidants are diminished, the cell enters a state called as “oxidative stress, this may cause tissue damage by physical, chemical, psychological factors that lead to tissue injury in human and causes different diseases.” This state if prolonged can cause death. Oxidative stress plays a major role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, and cardiovascular and neurodegenerative diseases.

ROS (Reactive oxygen species) and RNS (Reactive nitrogen species) are the terms collectively describing free radicals and other non-radical reactive derivatives also called oxidants. Radicals are less stable than non-radical species, although their reactivity is generally stronger. ROS/RNS include hydroxyl (OH•), superoxide ($O_2^{\bullet -}$), nitric oxide (NO•), nitrogen dioxide (NO_2•), peroxyl (ROO•) and lipid peroxyl (LOO•). Also, hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$),

dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH), are not free radicals and generally called oxidants, but can easily lead to free radical reactions in living organisms (Genestra M 2007). Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates such as lipids, proteins, DNA. ROS and RNS are generated from either endogenous or exogenous sources. Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, ageing. Exogenous ROS/RNS result from air and water pollution, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat), radiation (Valko M *et al*., 2007). After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals.

In contrast, at high concentrations, ROS can be mediate damage to cell structures, including lipids and membranes, proteins and nucleic acids; this damage is often referred as “oxidative stress” (Poli G *et al.,* 2004). Oxidative stress can arise when cells cannot adequately destroy the excess of free radicals formed. In other words, oxidative stress results from an imbalance between formation and neutralization of ROS/RNS. For example, hydroxyl radical and peroxynitrite in excess can damage cell membranes and lipoproteins by a process called lipid peroxidation. This reaction leads to the formation of malondialdehyde (MDA) and conjugated diene compounds, which are cytotoxic and mutagenic. Lipid peroxidation occurs by a radical chain reaction, i.e. once started, it spreads rapidly and affects a great number of lipid molecules. Proteins may also be damaged by ROS/RNS, leading to structural changes and loss of enzyme activity (Halliwell B, 2007). Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can cause mutations. The body has several mechanisms to counteract these attacks by using DNA repair enzymes and/or antioxidants (Halliwell B, 2007). If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases as well as the aging process and some acute pathologies (trauma, stroke).

Antioxidants are the substances that may protect cells from the oxidative damage caused by free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause. At a time one antioxidant molecule can react with single free radicals and are capable to neutralize free radicals by donating one of their own electrons, ending the carbon-stealing reaction. Antioxidants prevent cell and tissue damage as they act as scavenger. Cell produces defense against excessive free radicals by their preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. 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 non-enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidant includes lipoic acid, glutathione, L-arginine, uric acid, bilirubin etc. While nutrient antioxidant belonging to exogenous antioxidants are compounds which cannot be produced in the body and must be provided through foods such as vitamin E, Vitamin C, carotenoids, trace elements (Se,Cu,Zn,Mn) (Willcox JK *et al*.,2004). Free radicals are highly reactive and are capable of damaging almost all types of biomolecules (Proteins, lipids, carbohydrates & nucleic acid). The fact is that free radicals beget free radicals i.e., generate free radicals from normal compounds which continues as a chain reaction. The body has several mechanisms to counteract these attacks by using DNA repair enzymes and or antioxidants (Pancher P *et al*., 2007). If not regulated properly; oxidative stress can induce a variety of chronic and degenerative diseases such as: cancer, 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) (Subash Vijaykumar *et al*., 2010). Furthermore, oxidative stress has been implicated in neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis, memory loss, depression. Although multiple factors can participate oxidative stress in cells, the neurotransmitter glutamate is the main effecter of this process in the brain, primarily through activation of its ionotrophic receptors. Free radical induced- damage can occur by stimulation of phospholipase A2 and subsequent release of arachdonic acid. These substances and ROS enhance release of glutamate, thereby promoting a vicious cycle. Mitochondrial ROS production and oxidative damage to mitochondrial DNA results in ageing. The most recent review on free radicals and ageing by Barja emphasizes that caloric restriction is the only known experimental manipulation that decreases rate of mammalian ageing (Barja G, 2004). Experimental evidence suggests that destruction of islets of pancreas due to accumulation of free radicals is one of the causes for the pathogenesis of insulin dependent diabetes mellitus. Excess generation of mitochondrial ROS due to hyperglycemia initiates a vicious circle by activating stress-sensitive pathways such as NF- B, p38 MAPK and Jak/STAT, polyol (sorbitol) and hexosamine pathways, PKC and AGEs. Enhanced production of AGEs, sorbitol and proinflammatory cytokines exerts positive feedback on ROS and RNS synthesis and potentiates PKC-mediated vascular dysfunction by altering gene expression as well as vascular function and structure (Johansen JS *et al*., 2005).

Date palm tree (*Phoenix dactylifera L*.) is considered as one of the oldest and main staple and ancient crops in Southwest Asia and North Africa. Besides, dates can be grown in Australia, Mexico, South America, southern Africa, and the United States, especially in southern California, Arizona, and Texas (Chao and Krueger, 2007; Al-Harrasi *et al*., 2014; Hazzouri *et al*., 2015). Date palm tree belongs to Arecaceae family (Angiosperms, monocotyledon) consisting of about 200 genera and more

than 2,500 species. Phoenix (Coryphoideae phoeniceae) is one of the genera with approximately 14 species, which are native to the tropical or subtropical regions of southern Asia or Africa, including *Phoenix dactylifera L.* (Eoin, 2016). The name of the species dactylifera means “finger-bearing” which refers to the fruit clusters produced by this plant. Dactylifera is a grouping of the Greek word dactylus, means “finger,” and the Latin word ferous, mean “bearing” (Ashraf and Hamidi-Esfahani, 2011).

REVIEW OF LITERATURE

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 (Garrido n *et al.,* 2004).

To cope with the oxidative stress elicited by aerobic metabolism, animal and human cells have developed a ubiquitous antioxidant defense system, which consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase together with a number of low molecular-weight antioxidants such as ascorbate, α-tocopherol and glutathione, cysteine, thioredoxin, vitamins, etc. However, this antioxidant defense system may be overwhelmed by various pathological or environmental factors so that a fraction of ROS may escape destruction and form the far more reactive hydroxyl radicals. An increase in ROS- elicited oxidative damage to DNA and other biomolecules may impair normal functions of tissue cells and lead to human aging and disease.

Oxidative stress is critical to the etiology of many "oxidative stress related diseases", especially neurodegenerative diseases and cancers. Inflammation induces ROS and RNS production via respiratory bursts and

inflammatory cytokines, which can activate many oxidant generating enzymes such as inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), myeloperoxidase (MPO) and eosinophil peroxidase (EPO). Respiratory burst oxidase generates superoxide ($O_2^{\bullet -}$) via the one electron-reduction of oxygen by NADPH, with a secondary production of hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), hypochlorous acid (HOCl), and other activated forms of oxygen. In contrast, RNS including nitric oxide (NO) are generated mainly under inflammatory conditions via the expression of iNOS. NO reacts with $O_2^{\bullet -}$ to form highly reactive peroxynitrite (ONOO−).

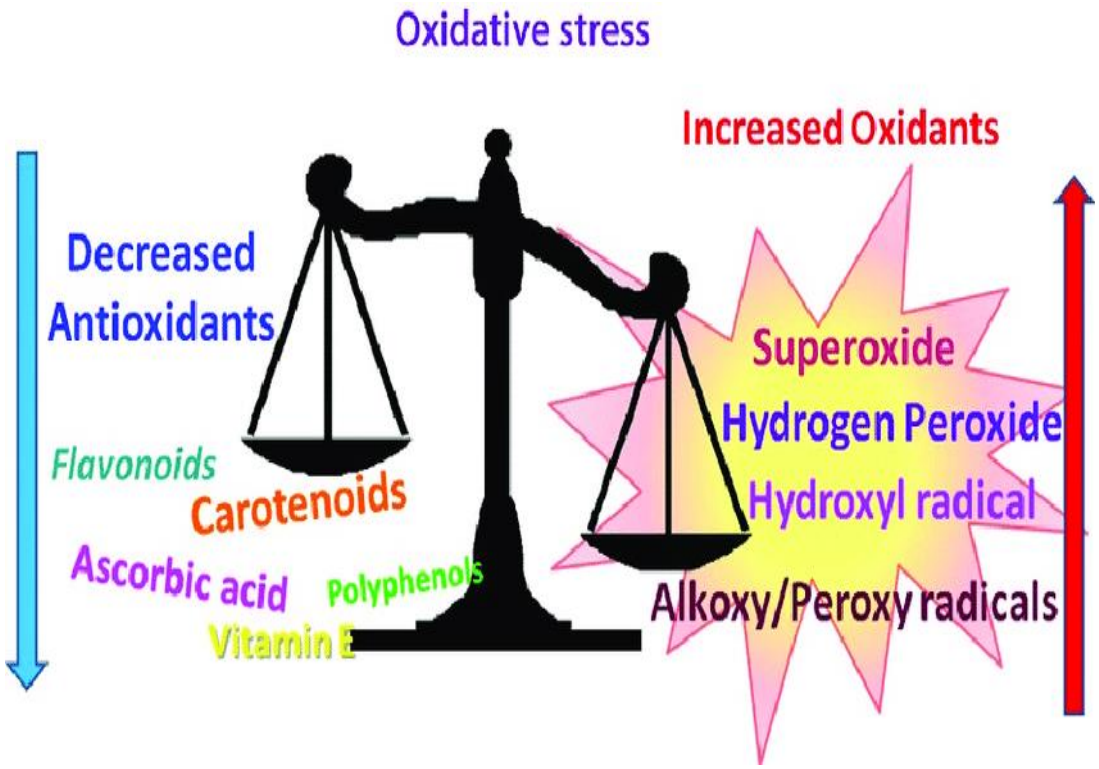

Figure 1: Imbalance between oxidant and antioxidant.

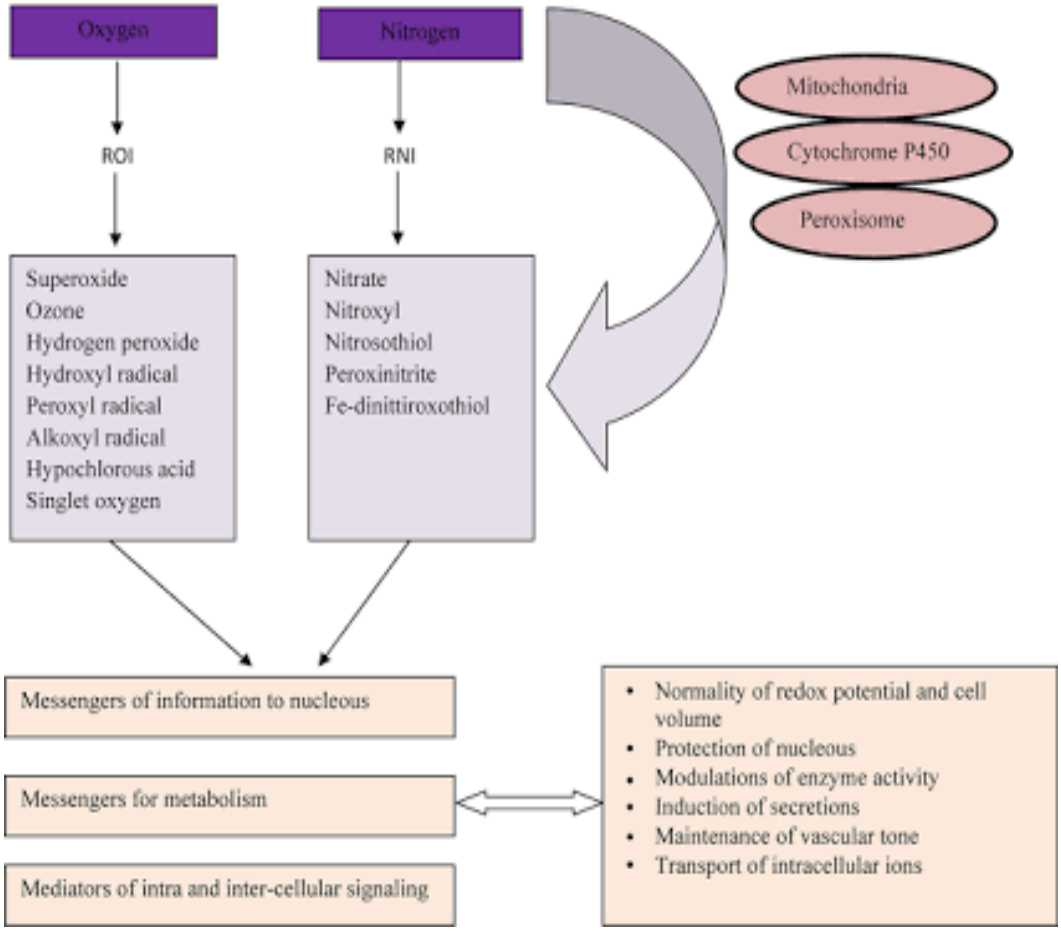

Figure 2: Reactions of ROI and RNI with proteins, carbohydrates and lipids, with consequent alteration both in the intracellular and intercellular homeostasis until possible cell death and regeneration.

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 O *et al.*, 2011). Free radicals are molecules/molecular fragments contain-ing 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 O *et al*., 2011), as well as non-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$)(Valko M *et al.,* 2007).

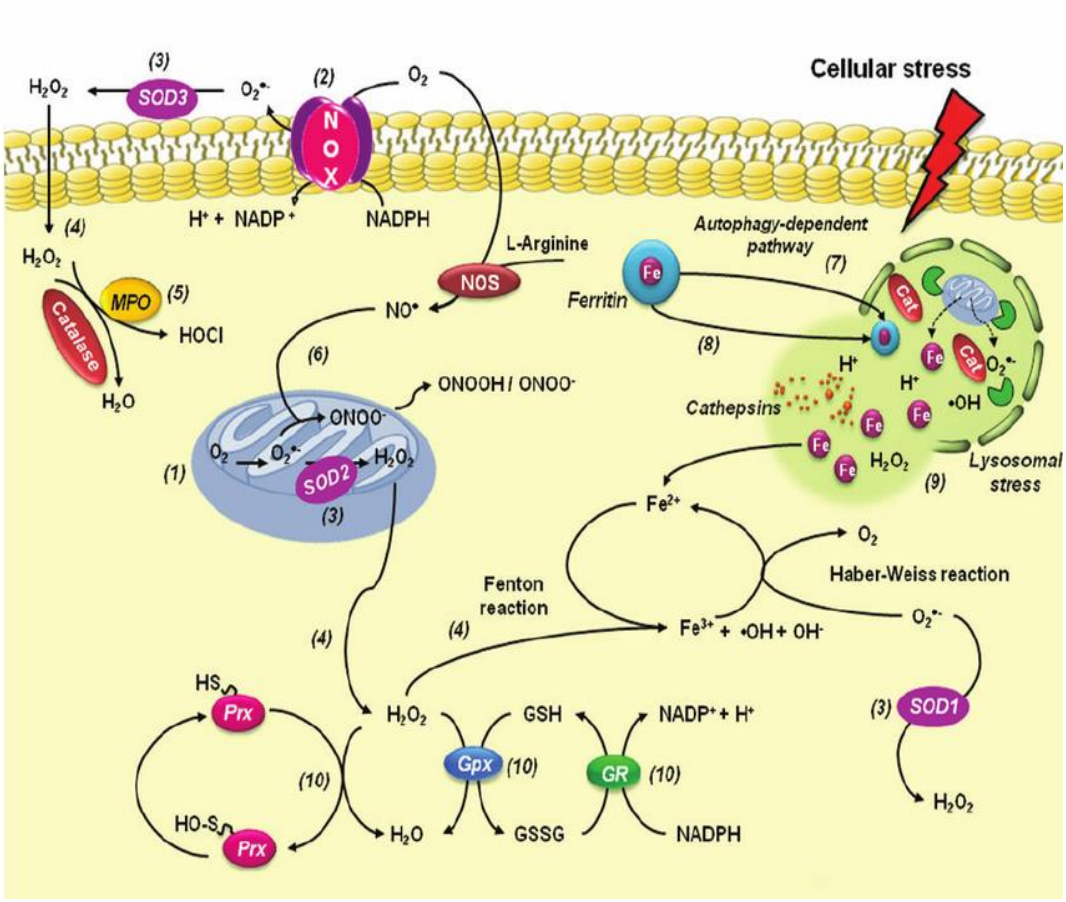

Figure 3: Various Pathways of Reactive Oxygen Species (ROS) Formation and their Transformation.

ROS and RNS are known to play dual roles as species that may be either deleterious or beneficial in living systems (Valko M *et al*., 2007). 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 P *et al.*, 2007). 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. 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 M *et al*., 2007). 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. **Tables 1**: Reactive oxygen Species (ROS)

Radicals	Non-radicals
Superoxide: O_2^-	Hydrogen peroxide: H_2O_2
Hydroxyl: OH^-	Hypochlorus acid: HOCL
Peroxyl: RO_2^-	Hypobrromus acid: HOBr
Alkoxyl: RO^-	Ozone: O_3
Hydroperoxyl: HO_2^-	Singlet oxygen: Δg

Table 2: Reactive Nitrogen Species (RNS)

Radicals	Non-radicals
Nitric oxide: NO^-	Nitrogen dioxide: NO_2
Nitrous acid: HNO_2	Nitrosyl cation: NO
	Nitrosyl anion: $NO^- NO^-$
	Dinitrogen tetroxide: N_2O_4
	Dinitrogen trioxide : N_2O_3
	Peroxynitrite: $ONOO^-$
	Peroxinitrous acid: ONOOH
	Alkylperoxynitrites: ROONO

All these free radicals are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. ROS are generated by a number of pathways. Most of the oxidants produced by cells occur as:

1. A consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.

2. Oxidative burst from phagocytes (white blood cells) as part of the mechanism by which bacteria and viruses are killed, and by which foreign proteins (antigens) are denatured.

3. Xenobiotic metabolism, i.e., detoxification of toxic substances.

4. Consequently, things like vigorous exercise, which accelerates cellular metabolism; chronic inflammation, infections, and other illnesses; exposure to allergens and the presence of “leaky gut” syndrome; and exposure to drugs or toxins such as cigarette smoke, pollution, pesticides, and insecticides may all contribute to an increase in the body’s oxidant load.

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. 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. Traditionally, the impairment caused by increased ROS is thought to result from random damage to proteins, lipids and DNA. In addition to these effects, a rise in ROS levels may also constitute a stress signal that activates specific redox- sensitive signaling pathways. Once activated, these di- verse signaling pathways may have either damaging or potentially protective functions.

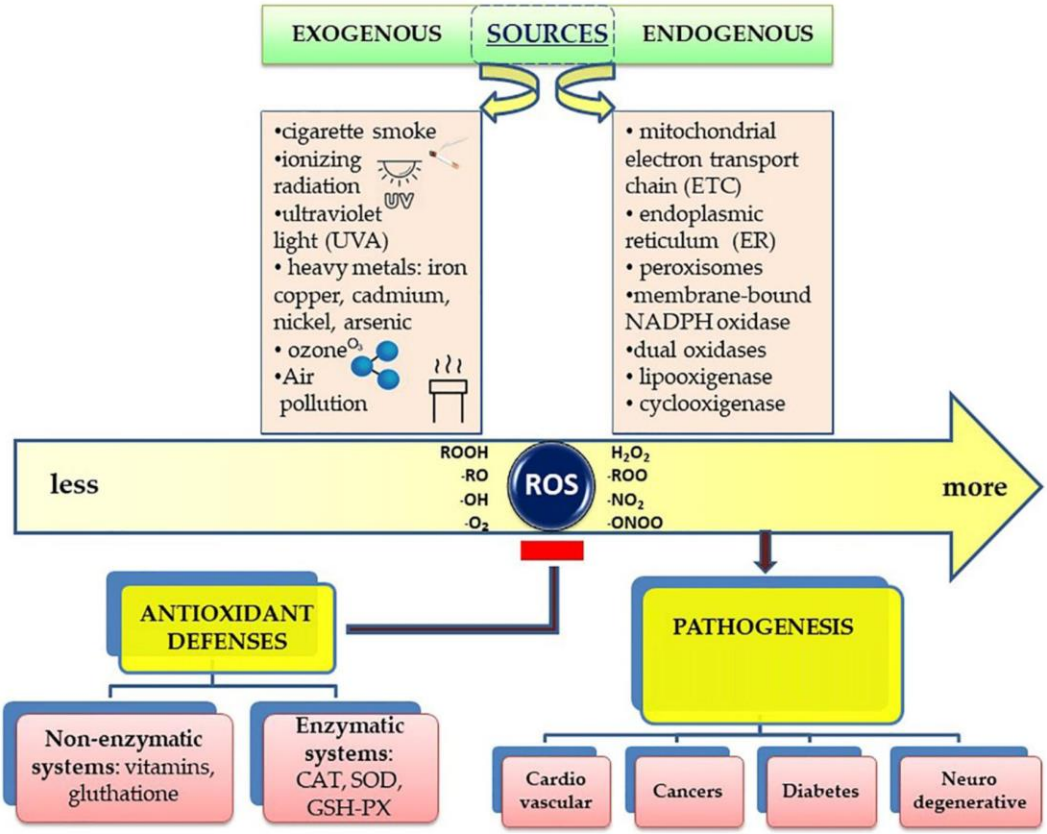

Figure 4: The source and cellular responses to reactive oxygen species (ROS).

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the cytochrome P-450 system. Free radicals can also be formed in nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

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). Hydrogen peroxide, although not a radical species is produced in the mitochondria as is its ROS precursor superoxide. It has been proposed that ubisemiquinone is the main reductant of oxygen in mitochondrial membranes and the generation of superoxide within mitochondria is approximately 2–3 nmol/min per mg of protein, the presence of ubiquitous indicates it to be the most important physiological source of this radical in living organisms (Inoue *et al*., 2003). Since mitochondria are the major site of free radical generation, they contain a variety of antioxidants, which are present on both sides of their membranes in order to minimize ROS induced stress.

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 (Valko *et al.,* 2004). 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. Cytochrome P450 has also been proposed as a source of ROS since on its induction, superoxide anion and hydrogen peroxide production take place following the breakdown or uncoupling of the P450 cycle (Valko *et al.,* 2006). In addition, microsomes and peroxisomes are sources of ROS, and microsomes are responsible for the majority of hydrogen peroxide produced in vivo at hyperoxia sites. Among the very varied endogenous sources, mitochondria, endoplasmic reticulum (ER), and peroxisomes are important cellular organelles which are involved in the ROS production.

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. The mitochondrial electron transport chain is a multicomponent system involved in a series of oxidation-reduction reactions between redox couples and pairs, transfer of electrons from a suitable donor (reductant) to a suitable electron acceptor (oxidant). These oxidation- reduction reactions involve

either the transport of electrons only, as in the case of the cytochromes, or electrons and protons together, as occurs between NADH and FAD. The part of the ETC that actually uses O_2 is the terminal oxidase enzyme, cytochrome oxidase. 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 B *et al*., 2003). Alternatively, ROS can be created during misfolding of protein due to depletion of GSH (Santos CX *et al*., 2009), since after GSH is consumed, thiols are repaired enabling them to interact with ERO-1/ PDI and to be re-oxidized. These steps generate consecutive cycles of disulfide bond formation and breakage, with each cycle producing more ROS as a by-product (Higa A; Chevet E, 2012). 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 JD, Kaufman RJ, 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 phos- phate

pathway. Peroxisomes contain a variety of enzymes that generate H_2O_2 as part of their normal catalytic cycle. These enzymes, which are essentially flavoproteins, include acyl-CoA oxidases, urate oxidase, D-amino acid oxidase, D-aspartate oxidase, L-pipecolic acid oxidase, L-α-hydroxy acid oxidase, polyamine oxidase, and xanthine oxidase (Fransen M *et al.,* 2012). 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. Peroxisomal catalase utilizes H_2O_2 produced by these oxidases to oxidize a variety of other substrates by “peroxidative” reactions. These types of oxidative reactions are particularly important in liver and kidney cells in which peroxisomes detoxify a variety of toxic molecules (including ethanol) that enter the circulation.

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. (Cho AK *et al.*, 2005) have shown that particulate matter (mixture of solid particles and liquid droplets suspended in the air) catalyzes the reduction of oxygen.

Hyperoxia

Hyperoxia refers to conditions of higher oxygen levels than normal partial pressure of oxygen in the lungs or other body tissues. It leads to greater production of reactive oxygen and nitrogen species.

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. In addition, it can generate damaging intermediates through interaction with water, a process termed radiolysis. Since water comprises 55-60% of the human body, the probability of radiolysis is quite high under the presence of ionizing radiation. The outcome is conversion of water into hydroxyl radical (-OH), hydrogen peroxide (H_2O_2), superoxide radical (O_2-) and ultimately oxygen (O_2). Moreover, according to the findings of extant studies, various signal transduction molecules—such as extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38— and transcription factors—such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and p53—are activated under effect of ionizing radiation. This results in the expression of radiation response-related genes. Ultraviolet A (UVA) photons trigger oxidative reactions by excitation of endogenous photosensitizers, such as porphyrins, NADPH oxidase, and riboflavins. 8-Oxo-7,8-dihydroguanine (8-oxoGua) is the main UVA-mediated DNA oxidation product formed by the oxidation of •OH radical, 1-electron oxidants, and singlet oxygen that mainly reacts with guanine (Cadet J *et al*., 2003) The formation of guanine radical cation in isolated DNA has been shown to efficiently occur through the direct effect of ionizing radiation. After exposure to ionizing radiation, intracellular level of glutathione (GSH) decreases for a short term but then increases again.

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.

$$\text{Metal}^{3+} + \cdot O_2 \rightarrow \text{Metal}^{2+} + O_2 \qquad \text{Haber–Weiss}$$
$$\text{Metal}^{2+} + H_2O_2 \rightarrow \text{Metal}^{3+} + OH^- + \cdot OH \qquad \text{Fenton reaction}$$

Besides the Fenton-type and Haber–Weiss-type mechanisms, certain metal ions can react directly with cellular molecules to generate free radicals, such as thiol radicals, or induce cell signaling pathways. These radicals may also react with other thiol molecules to generate $O_2^{\bullet -}$. $O_2^{\bullet -}$ is converted to H_2O_2, which causes additional oxygen radical generation. Some metals, such as arsenite, induce ROS formation indirectly by activation of radical producing systems in cells (Leonard SS *et al*., 2004).

Arsenic is a highly toxic element that produces a variety of ROS, including superoxide ($O_2^{\bullet -}$), singlet oxygen (O_2), peroxyl radical (ROO•), nitric oxide (NO•), hydrogen peroxide (H_2O_2), and dimethylarsinic peroxyl radicals [$(CH3)_2$AsOO•_] (Shi H *et al*., 2004). Arsenic (III) compounds can inhibit antioxidant enzymes, especially the GSH-dependent enzymes, such as glutathione-S-transferases (GSTs), glutathione peroxidase (GSH-Px), and GSH reductase, via binding to their sulfhydryl (–SH) groups. (Waalkes MP *et al.,* 2004)

Lead increases lipid peroxidation. Significant decreases in the activity of tissue SOD and brain GPx have been reported after lead exposure. Replacement of zinc, which serves as a cofactor for many enzymes by lead, leads to inactivation of such enzymes. Lead exposure may cause inhibition of GST by affecting tissue thiols.

ROS generated by metal-catalyzed reactions can modify DNA bases. Three base substitutions, G → C, G → T, and C →T, can occur as a result of oxidative damage by metal ions, such as Fe2+, Cu+, and Ni2+. Reid et al65 showed that G → C was predominantly produced by Fe^{2+} while C → T substitution was by Cu^{2+} and Ni^{2+}.

Enzymatic antioxidants	Nonenzymatic antioxidants
Thioredoxin (Trx)	Vitamins C, E, A
Peroxiredoxins (Prx)	Thiols
Glutaredoxin (Grx)	β-Carotene
Glutathione peroxidase (Gpx)	Polyphenols
Reduced glutathione (GSH)	NAC
Oxidized glutathione (GSSG)	Zinc, selenium
Glutathione reductase (GR)	Glutathione
Extracellular glutathione peroxidase (eGPx)	Uric acid
Catalase	Lycopene
Peroxidase	Allyl sulfide
Superoxide dismutase	Indoles
	Gallic acid
	Hesperitin
	Catechin
	Chrysin

Antioxidants

Endogenous compounds in cells can be classified as enzymatic antioxidants and non-enzymatic antioxidants. The major antioxidant enzymes directly involved in the neutralization of ROS and RNS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). SOD, the first line of defense against free radicals, catalyzes the dismutation of superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid

hydroperoxides while oxidizing glutathione (GSH). The non-enzymatic antioxidants are also divided into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants belonging to endogenous antioxidants, are produced by metabolism in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc. While nutrient antioxidants belonging to exogenous antioxidants, are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc.

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 dismutase catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide.

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 F and Giulivi C, 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 J *et al*., 2004). In higher plants, SOD isozymes have been localized in different cell compartments. Mn-SOD is present in mitochondria and peroxisomes. Fe-SOD has been found mainly in chloroplasts but has also been detected in peroxisomes, and CuZn-SOD has been localized in cytosol, chloroplasts, peroxisomes, and apoplast ((Wuerges J *et al*., 2004; Corpas FJ *et al*., 2006).

In humans (as in all other mammals and most chordates), three forms of superoxide dismutase are present. SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. The first is a dimer (consists of two units), while the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, while SOD2 has manganese in its reactive center (Cao X *et al.,* 2008).

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. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules. All known animals use catalase in every organ, with particularly high concentrations occurring in the liver.

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 selenium cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides. The glutathione S-transferases show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes J *et al.,* 2005).

Nonenzymatic antioxidants

Ascorbic acid

Ascorbic acid or “vitamin C” is a monosaccharide antioxidant found 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 S *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. Due to its high concentration and central role in maintaining the cell’s redox state, glutathione is one of the most important cellular antioxidants. In some organisms, glutathione is replaced by other thiols, such as by mycothiol in the actinomycetes, or by trypanothione in the kinetoplastids.

Melatonin

Melatonin, also known chemically as N-acetyl-5- methoxytryptamine, (Nassar E *et al*., 2007) is a naturally occurring hormone found in animals and in some other living organisms, including algae (caniato R *et al*., 2003). Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier. Unlike other antioxidants, melatonin does not undergo redox cycling, which is the ability of a molecule to undergo repeated reduction and oxidation. Melatonin, once oxidized, cannot be reduced to its former state because it forms several

stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant.

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 MG, Atkinson J, 2007). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidized α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol, or ubiquinol.

Uric acid

Uric acid accounts for roughly half the antioxidant ability of plasma. In fact, uric acid may have substituted for ascorbate in human evolution. However, like ascorbate, uric acid can also mediate the production of active oxygen species.

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. Betacarotene 20-30mg daily in smokers may also increase cardiovascular mortality by 12% to 26%. These adverse effects do not appear to occur in people who eat foods high in beta-carotene content. Beta-carotene is present in many fruits, grains,

oil and vegetables (carrots, green plants, squash, spinach) (Wilcox JK *et al.,*2004).

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 (Dahan K *et al.,* 2008). 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.

Selenium (Se)

Selenium is a trace mineral found in soil, water, vegetables (garlic, onion, grains, nuts, soyabean), sea food, meat, liver, yeast. It forms the active site of several antioxidant enzymes including glutathione peroxidase. At low dose, health benefits of Se are antioxidant, anti-carcinogenic and immunomodulator. Selenium is also necessary for the thyroid function. Exceeding the Tolerable Upper Intake Level of 400 µg Se/day can lead to selenosis which is a selenium poisoning characterized by gastrointestinal disorders, hair and nail loss, cirrhosis, pulmonary edema and death. Selenium deficiency can occur in patients on total parenteral nutrition (TPN) and in patients with gastrointestinal disorders. In certain China areas with Se poor soil, people have developed a fatal cardiomyopathy called Keshan disease which was cured with Se supplement. The role of Se in cancer prevention has been the subject of recent study and debate. Results from clinical and cohort studies about cancer prevention, especially lung, colorectal, and prostate cancers are mixed.

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. They have been reported to prevent or delay a number of chronic and degenerative ailments such as cancer, cardiovascular diseases, arthritis, aging, cataract, memory loss, stroke, Alzheimer ‘s disease, inflammation, infection. Every plant contains a unique combination of flavonoids, which is why different herbs, all rich in these substances, have very different effects on the body. The main natural sources of flavonoids include green tea, grapes (red wine), apple, cocoa (chocolate), ginkgo biloba, soybean, curcuma, berries, onion, broccoli, etc. For example, green tea is a rich source of flavonoids, especially flavonols (catechins) and quercetin. Catechin levels are 4-6 times greater in green tea than in black tea. Many health benefits of green tea reside in its antioxidant, anticarcinogenic, antihypercholesterolemic, antibacterial (dental caries), anti-inflammatory activities.

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. Dietary sources of omega-6 fatty acids (linoleic acid) include vegetable oils, nuts, cereals, eggs, poultry. It is important to maintain an appropriate balance of omega-3s and omega-6s in the diet, as these two substances work together to promote health.

Omega-3 fatty acids help reduce inflammation, and most omega-6 fatty acids tend to promote inflammation. An inappropriate balance of these essential fatty acids contributes to the development of disease while a proper balance helps maintain and even improve health. A healthy diet should consist of about 2-4 times more omega-6s than omega-3s. Omega-3s reduce inflammation and prevent chronic ailments such as heart disease, stroke, memory loss, depression, arthritis, cataract, cancer. Omega-6s improve diabetic neuropathy, eczema, psoriasis, osteoporosis, and aid in cancer treatment. Finally, some endogenous antioxidants such as L-arginine, coenzyme Q-10, melatonin are recently used as supplements for the prevention or treatment of some chronic and degenerative disease.

Enzymatic antioxidants	Nonenzymatic antioxidants
Thioredoxin (Trx)	Vitamins C, E, A
Peroxiredoxins (Prx)	Thiols
Glutaredoxin (Grx)	β-Carotene
Glutathione peroxidase (Gpx)	Polyphenols
Reduced glutathione (GSH)	NAC
Oxidized glutathione (GSSG)	Zinc, selenium
Glutathione reductase (GR)	Glutathione
Extracellular glutathione peroxidase (eGPx)	Uric acid
Catalase	Lycopene
Peroxidase	Allyl sulfide
Superoxide dismutase	Indoles
	Gallic acid
	Hesperitin
	Catechin
	Chrysin

Oxidative stress-induced cellular damage

Oxidative damage to protein

Proteins can be oxidatively modified in three ways: oxidative modification of specific amino acid, free radical mediated peptide cleavage, and formation of protein cross-linkage due to reaction with lipid peroxidation products. Protein containing amino acids such as methionine, cystein, arginine, and histidine seem to be the most vulnerable to oxidation. Free

radical mediated protein modification increases susceptibility to enzyme proteolysis. Oxidative damage to protein products may affect the activity of enzymes, receptors, and membrane transport. Oxidatively damaged protein products may contain very reactive groups that may contribute to damage to membrane and many cellular functions. Peroxyl radical is usually considered to be free radical species for the oxidation of proteins. ROS can damage proteins and produce carbonyls and other amino acids modification including formation of methionine sulfoxide and protein carbonyls and other amino acids modification including formation of methionine sulfoxide and protein peroxide. Protein oxidation affects the alteration of signal transduction mechanism, enzyme activity, heat stability, and proteolysis susceptibility, which leads to aging.

Lipid peroxidation

Oxidative stress and oxidative modification of biomolecules are involved in a number of physiological and pathophysiological processes such as aging, atherosclerosis, inflammation and carcinogenesis, and drug toxicity. Lipid peroxidation is a free radical process involving a source of secondary free radical, which further can act as second messenger or can directly react with other biomolecule, enhancing biochemical lesions. Lipid peroxidation occurs on polysaturated fatty acid located on the cell membranes and it further proceeds with radical chain reaction. Hydroxyl radical is thought to initiate ROS and remove hydrogen atom, thus producing lipid radical and further converted into diene conjugate. Further, by addition of oxygen it forms peroxyl radical; this highly reactive radical attacks another fatty acid forming lipid hydroperoxide (LOOH) and a new radical. Thus, lipid peroxidation is propagated. Due to lipid peroxidation, a number of compounds are formed, for example, alkanes, malanoaldehyde, and isoprotanes. These compounds are used as markers in lipid peroxidation assay and have been verified in many diseases such as neurogenerative diseases, ischemic reperfusion injury, and diabetes.

Oxidative damage to DNA

Many experiments clearly provide evidences that DNA and RNA are susceptible to oxidative damage. It has been reported that especially in aging and cancer, DNA is considered as a major target. Oxidative nucleotide as glycol, dTG, and 8-hydroxy- 2-deoxyguanosine is found to be increased during oxidative damage to DNA under UV radiation or free radical damage. It has been reported that mitochondrial DNA are more susceptible to oxidative damage that have role in many diseases including cancer. It has been suggested that 8-hydroxy-2-deoxyguanosine can be used as biological marker for oxidative stress.

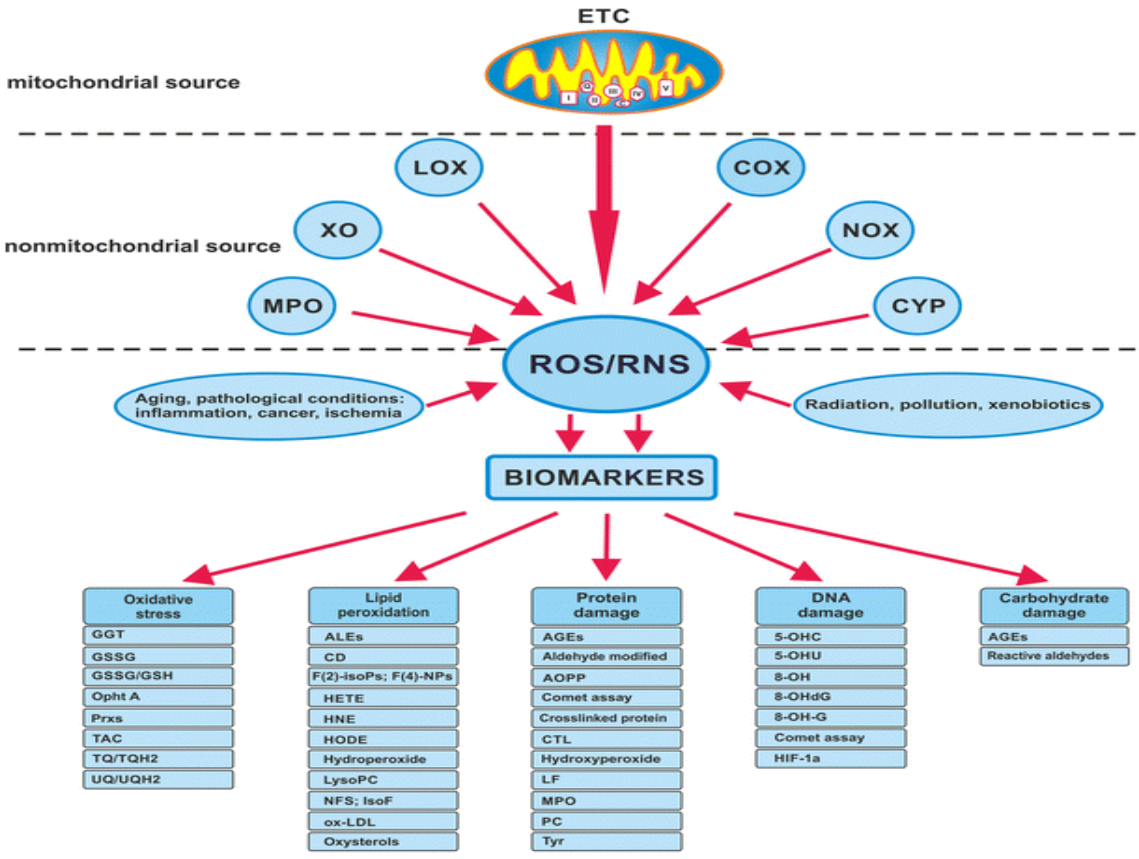

Figure 5: Markers of oxidative damage.

Effects of oxidative stress on signal transduction

Oxidative stress can cause disruption of the GSH/GSSG ratio, leading to activation of redox sensitive transcription factors, such as the nuclear factor of activated T cells (NF-κB) and hypoxia-inducible factor 1

(AP-1). Owing to their involvement in inflammatory responses, these factors can facilitate the transmission of information into the cell. Tyrosine kinase receptors, most of the growth factor receptors—such as epidermal growth factor receptor, vascular endothelial growth factor receptor, and receptor for platelet-derived growth factor—as well as protein tyrosine phosphatases and serine/threonine kinases are targets of ROS . Moreover, oxidants can regulate many of the extracellular signal regulated kinases, such as p38, which are the members of mitogen-activated protein kinase family. As such, they are involved in several processes in the cell, including proliferation, differentiation, and apoptosis. ROS can activate NF-κB by phosphorylating IκBs at serine residues, which frees NF-κB to enter the nucleus to activate gene transcription (Perkins ND, 2007). A number of kinases have been reported to phosphorylate IκBs; these kinases are targets for oxidative signals to activate NF-κB. As a result of NF- κB activation via oxidation, several antioxidant defense- related genes that can participate in immune response are activated. These include IL-1b, IL-6, tumor necrosis factor-α, IL-8, and several adhesion molecules. NF-κB also regulates angiogenesis, proliferation and cell differentiation.

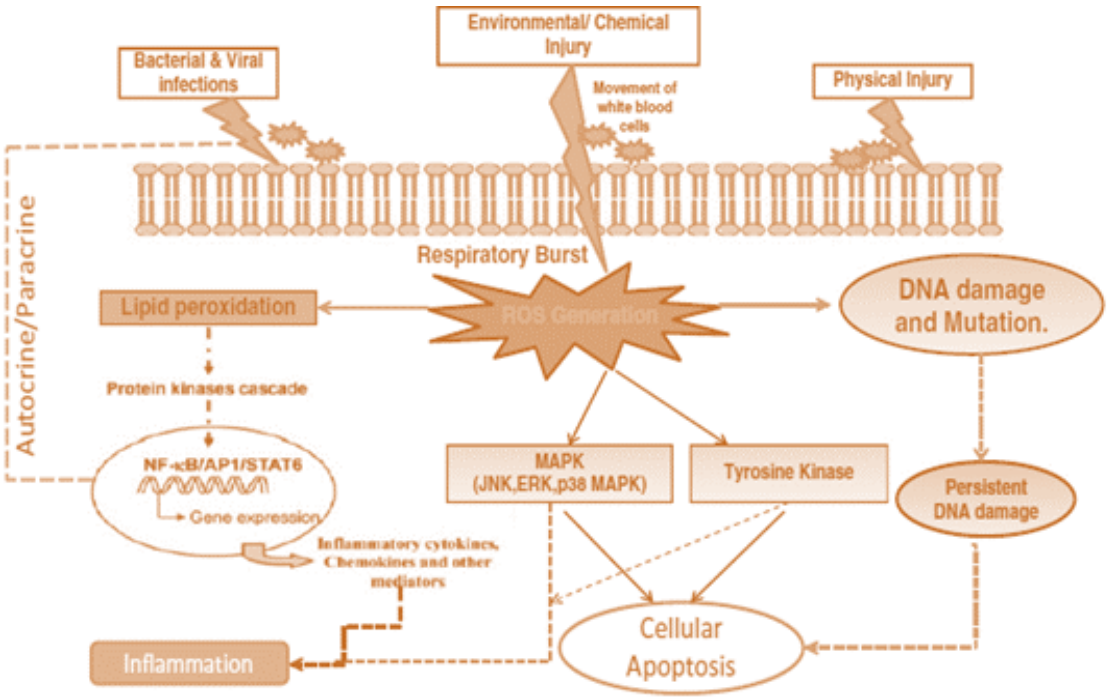

Figure 6: Role of ROS and RNS in tissue damage.

Role of oxidative stress /ROS in diseases

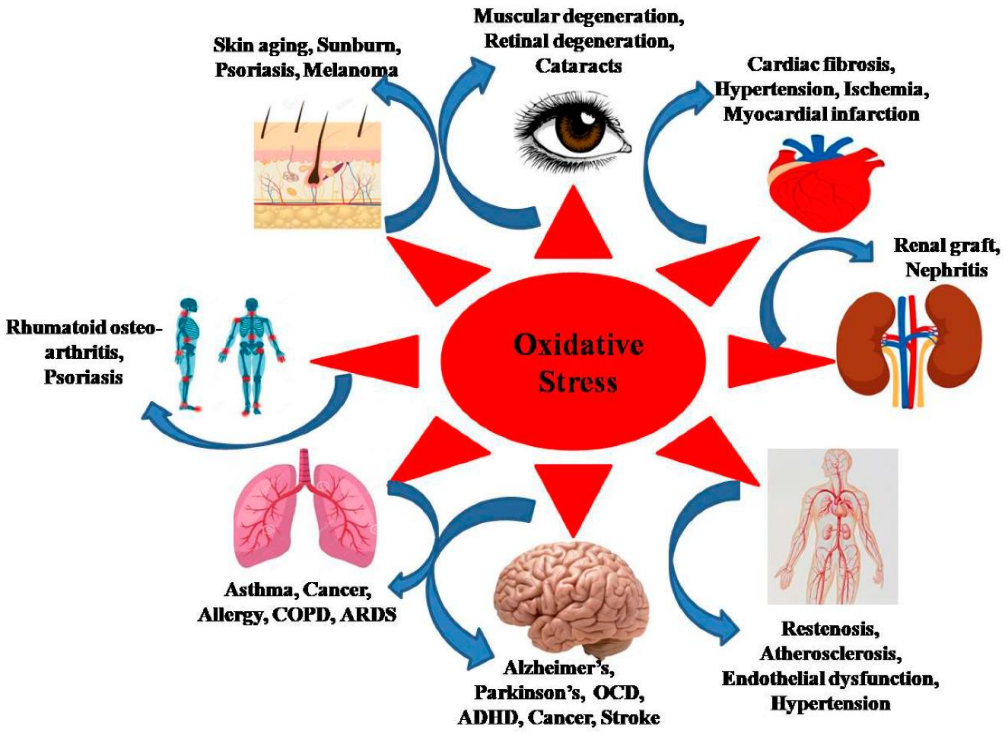

Figure 7: Deleterious effects of oxidative stress on human health.

Cardiovascular disease

The oxidative events may affect cardiovascular diseases therefore; it has potential to provide enormous benefits to the health and lifespan. Poly unsaturated fatty acids occur as a major part of the low-density lipoproteins (LDL) in blood and oxidation of these lipid components in LDL play a vital role in atherosclerosis. The three most important cell types in the vessel wall are endothelial cells; smooth muscle cell and macrophage can release free radical, which affect lipid peroxidation. With continued high level of oxidized lipids, blood vessel damage to the reaction process continues and can lead to generation of foam cells and plaque the symptoms of atherosclerosis. Oxidized LDL is antherogenic and is thought to be important in the formation of anthersclerosis plaques. Furthermore, oxidized LDL is cytotoxic and can directly damage endothelial cells. Antioxidants like B-carotene or vitamin E play a vital role in the prevention of various cardiovascular diseases.

Carcinogenesis

Reactive oxygen and nitrogen species, such as super oxide anion, hydrogen peroxide, hydroxyl radical, and nitric oxide and their biological metabolites also play an important role in carcinogenesis. ROS induce DNA damage, as the reaction of free radicals with DNA includes strand break base modification and DNA protein cross-links. Numerous investigators have proposed participation of free radicals in carcinogenesis, mutation, and transformation; it is clear that their presence in biosystem could lead to mutation, transformation, and ultimately cancer. Induction of mutagenesis, the best known of the biological effect of radiation, occurs mainly through damage of DNA by the HO. Radical and other species are produced by the radiolysis, and also by direct radiation effect on DNA, the reaction effects on DNA. The reaction of HO. Radicals is mainly addition to double bond of pyrimidine bases and abstraction of hydrogen from the sugar moiety resulting in chain reaction of DNA. These effects cause cell mutagenesis and carcinogenesis lipid peroxides are also responsible for the activation of carcinogens.

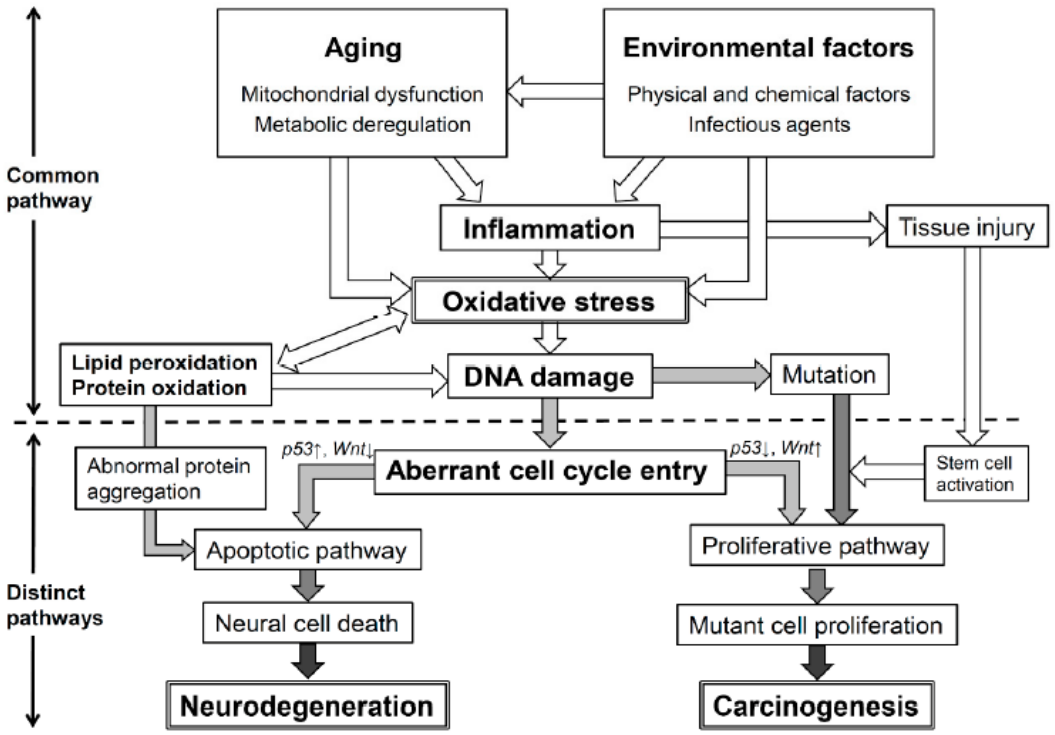

Figure 8: Roles of oxidative stress in neurodegenerative diseases and cancer.

Aging

The human body is in constant battle to keep from aging. Research suggests that free radical damage to cells leads to the pathological changes associated with aging. An increasing number of diseases or disorders, as well as aging process itself, demonstrate link either directly or indirectly to these reactive and potentially destructive molecules. The major mechanism of aging attributes to DNA or the accumulation of cellular and functional damage. Reduction of free radicals or decreasing their rate of production may delay aging. Some of the nutritional antioxidants will retard the aging process and prevent disease. Based on these studies, it appears that increased oxidative stress commonly occurs during the aging process, and antioxidant status may significantly influence the effects of oxidative damage associated with advancing age. Research suggests that free radicals have a significant influence on aging, that free radical damage can be controlled with adequate antioxidant defense, and that optimal intake of antioxidant nutrient may contribute to enhanced quality of life. Recent research indicates that antioxidant may even positively influence life span.

Diabetes

There is increasing evidence that free radical induced damage also plays a significant part in the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, and type 2 diabetes mellitus (Jay *et al.,* 2006). Hyperglycemia can induce oxidative stress, which increases with age, via several mechanisms including glucose auto oxidation, the formation of advanced glycation end-products (AGE), and activation of the polyol pathway. Other circulating factors that are elevated in diabetics such as free fatty acids and leptin also contribute to increased ROS (Jay *et al.,* 2006). There is a significant increase in protein glycation (AGE) with age (Poggioli *et al.,* 2002), which is also increased in diabetics (Wautier and Schimdt 2004). The accumulation of AGE leads to an increase in the micro vascular lesions, which are present in diabetic retinopathy, and is also responsible for cardiovascular complications, which are seen in diabetic patients (Wautier and Schmidt 2004; Jay *et al.,* 2006). The damage caused by ROS has also been implicated in primary open angle glaucoma

(POGA), which is the leading cause of irreversible blindness and the second most common cause of all blindness after cataracts. The incidence of POAG is linked to old age, thus advanced age represents a major risk factor for this disease (Izzotti *et al.,* 2006).

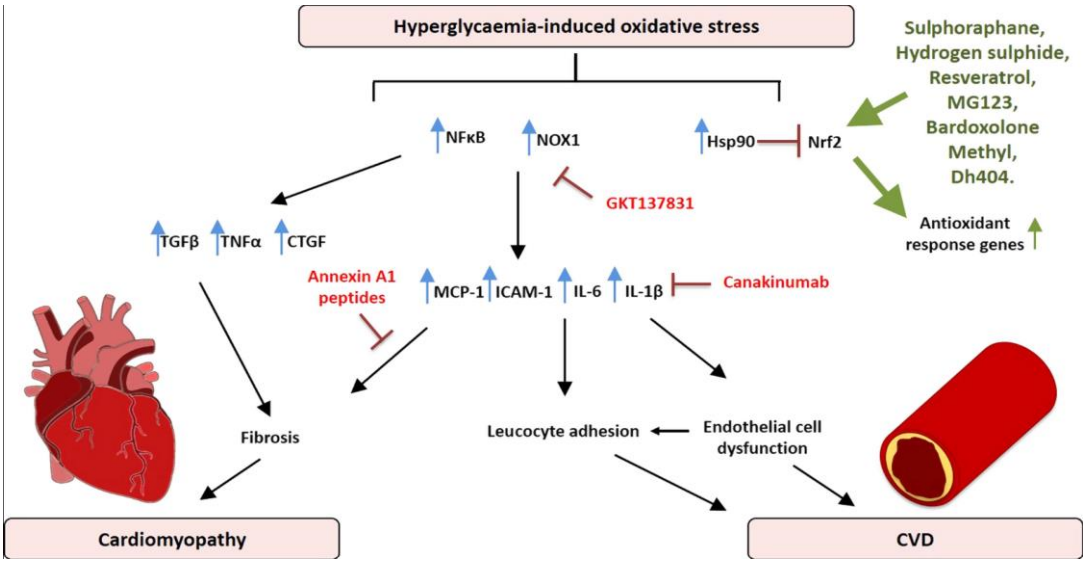

Figure 9: General pathway by which increased oxidative stress may contribute to development of complications in diabetes.

Treatment of diseases with synthetic drugs and its drawbacks

Regardless of the type of diabetes, patients are required to control their blood glucose with medications and/or by adhering to an exercise program and a dietary plan. Insulin therapy by injection is given to those with type 1 DM and also to some patients with type 2 DM when oral hypoglycaemic drugs fail to lower blood glucose (Alam K and Mahpara S 2003). Due to modernization of lifestyle, non-insulin dependent diabetes mellitus is becoming a major health problem in developing countries. Patients with type 2 DM are usually placed on a restricted diet and are instructed to exercise, the purpose of which primarily is weight control. If diet and exercise fail to control blood glucose at the desired level, oral antidiabetic medication is prescribed. Oral antidiabetic agents exert their effects by various mechanisms:

- Stimulation of beta cells in the pancreas to produce more insulin (sulfonylureas and meglitinides).

- Increasing the sensitivity of muscles and other tissues to insulin (thiazolidinediones).
- Decreasing gluconeogenesis by the liver (biguanides).
- Delaying the absorption of carbohydrates from the gastrointestinal tract (alpha- glucosidase inhibitors).

These treatments have their own drawbacks, ranging from the developing of resistance and adverse effects to lack of responsiveness in large segment of patient's population. Sulfonylureas lose effectiveness for 44% of patients within six years.

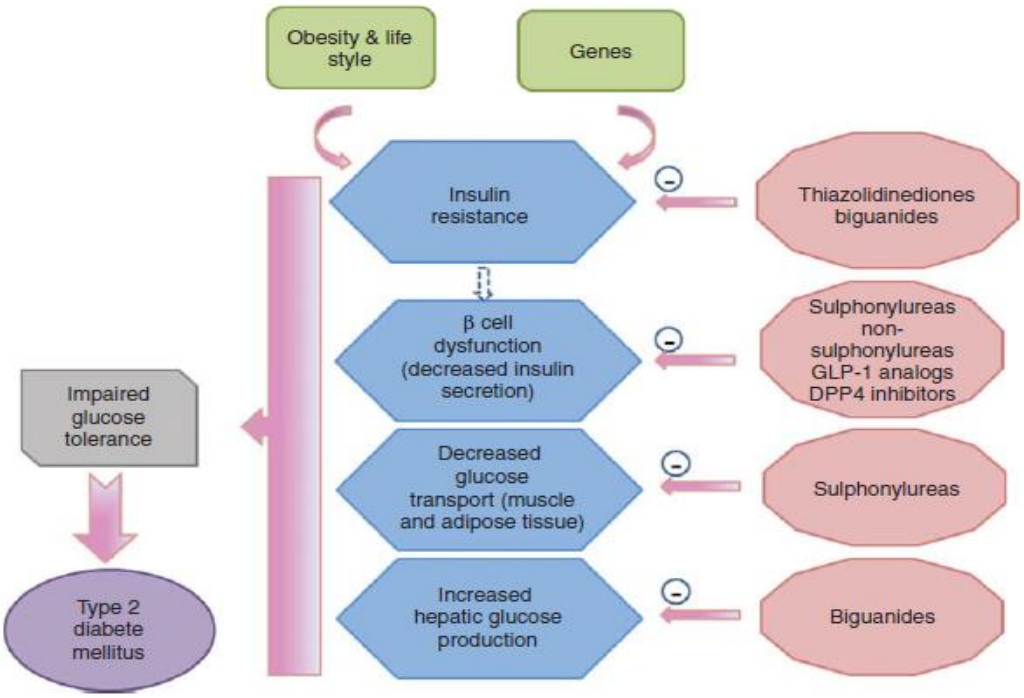

Figure 10: Pathobiology and targets for current drugs in T2DM.

Also, these treatments are associated with side effects or even toxic effects (e.g., thiazolidinediones may cause liver toxicity; sulphonylureas might worsen heart disease, lower the glucose below the normal range and increase the body weight gain; bloating, flatulence, diarrhea and abdominal discomfort and pain are the major complaints with glucosidase inhibitors) (Michael PK *et al*., 2005). According to literature, two-thirds of medications prescribed for use in children have not been proven safe or effective for this patient population (Michael PK *et al.,* 2005). Moreover, none of these

glucose-lowering agents adequately controls the hyperlipidemia that frequently met with the disease. The limitations of currently-available oral anti-diabetic agents either in terms of efficacy/safety coupled with the emergence of the disease into a global epidemy have encouraged a concerted effort to discover drugs that can manage type 2 diabetes more efficiently. Also, with increasing incidence of diabetes mellitus in rural population throughout the world and due to adverse effects of synthetic medicine, there is a clear need for development of indigenous, inexpensive botanical sources for anti-diabetic crude or purified drugs (Venkatesh S *et al*., 2003).

Importance of herbal plants in the treatment of diseases

Nature always stands as a golden mark to exemplify the outstanding phenomena of one race depending on other for food. Natural products from plant, animal and minerals have been the basis of the treatment of human disease from the times immemorial. Today it is estimated that about 80 % of people in developing countries are still depending on traditional medicine based largely on species of plants and animals. Herbal medicines are currently in demand and their necessity is increasing eventually. About 500 plants with medicinal use are mentioned in ancient literature by Theoprastus and 800 plants have been used in indigenous systems of medicinal system (Chatterjee, I *et al.,* 2006). The use of plant medicines is becoming popular due to toxic and side effects of allopathic drugs. This led to sudden increase in the number of herbal drug industries (Chatterjee I *et al.,* 2004).

Benefits of natural products over synthetic drugs

Plants-based products have been popular all over the world for the centuries. In diabetes, some herbal alternatives are proven to provide symptomatic relief and assist in the prevention of the secondary complications of the disease. Some herbs have also been proven to help in the regeneration of ß-cells and in overcoming resistance. In addition to maintaining normal blood sugar level, some herbs are also reported to possess antioxidant activity and cholesterol-lowering action. The

management of type 2 diabetes mellitus (NIDDM) is possible with the drugs that can lower the blood sugar level in one hand and restore the liver glycogen level on the other. One of the major theories about biological ageing is that it depends on oxidation processes. For this reason, there is great interest in the antioxidant capacity of the human diet and of nutrient supplements. So far, most evidence suggests that plant-derived food is protective against age-related diseases, like cardiovascular disease and cancer, rather than ageing itself. Many epidemiological studies have linked diets containing moderate to high proportions of fruit and vegetables to lower mortality and to a reduced risk of developing cardiovascular disease, cancers, cataracts and macular degeneration, cognitive impairment and Alzheimer's disease. Although clear cause and effect relationships are difficult to establish, these protective effects are probably due to combinations of nutrients and also to the non-nutritive substances found in these foods. In cohort studies, a survival advantage can be predicted if the diet contains a variety of food, principally from plant sources.

Mechanism of action of herbal antidiabetics

- The antidiabetic activity of herbs depends upon variety of mechanisms (Jarald *et. al.,* 2008). The mechanism of action of herbal anti-diabetic could be grouped as-
- Adrenomimeticism, pancreatic beta cell potassium channel blocking, cAMP (2nd messenger) stimulation.
- Inhibition in renal glucose reabsorption.
- Stimulation of insulin secretion from beta cells of islets or/and inhibition of insulin degradative processes.
- Reduction in insulin resistance.
- Providing certain necessary elements like calcium, zinc, magnesium, manganese and copper for the beta-cells.
- Regenerating and/or repairing pancreatic beta cells.
- Increasing the size and number of cells in the islets of Langerhans.
- Stimulation of insulin secretion.
- Stimulation of glycogenesis and hepatic glycolysis.

- Protective effect on the destruction of the beta- cells.
- Improvement in digestion along with reduction in blood sugar and urea.
- Prevention of pathological conversion of starch to glucose.
- Inhibition of β -galactosidase and α– glucosidase.
- Cortisol lowering activities.
- Inhibition of alpha-amylase.
- Preventing oxidative stress that is possibly involved in pancreatic ß-cell dysfunction found in diabetes.

Hence, the wide range of plant constituents could have different sites of action within the body, herbs exert different mechanism of actions including the mechanism of actions of synthetic oral hypoglycemic drugs.

***Phoenix dactylifera L.* (Khudari cultivar)**

Date palm

Kingdom:	Plantae
Order:	Arecales
Family:	Arecaceae
Genus:	Phoenix L.
Species	*Phoenix dactylifera L.*

Dates (*Phoenix dactylifera*) are one of the members of the palm family Arecaceae, or Palmae (Zohary D. *et al* 1993). The species name *dactylifera* “date-bearing” originate from two words; one from greek dáktulos “date” (Rahmani, A *et al* 2014) and the stem of the Greek verb ferō (Lewis and Short 1996). The date palm (*Phoenix dactylifera L.*) is one of oldest cultivated plants of

human kind and used as food for 6000 years (Sulieman *et al.*, 2012). There are more than two hundred varieties (Amer 1979) of dates available worldwide.

It is the main crop in Egypt, Saudi Arabia, and Middle Eastern countries. It is thought that the native origin of dates is around the Persian Gulf, and has been cultivated from Mesopotamia to prehistoric Egypt as early as 4000 BCE. Due to the old historical prospective of date, the exact date of origin is very difficult to identify (Chao CT *et al*., 2007). Most likely it originated 4000 BC from the ancient Mesopotamia area (southern Iraq) or western India. Another report regarding the origin of dates is pre-Islamic archaeology; south-eastern Arabia was predicated upon the domestication of the date palm in 2500 BC (Wrigley G. et al, 1995) Over all the origin of dates is very old as per the information from the religious books and literature reports. Another support of the ancient times of the date palm is Egypt's Nile Valley where it was used as the symbol for a year in Egyptian hieroglyphics and its frond as a symbol for a month (Dowson VHW 1982). Earlier report also showed that old background of dates as date cultivation in Mehrgarh around 7000 BCE and in the Indus Valley around 2600 to 1900 BCE (Kenoyer JM *et al*, 2013). The fruits of dates have important place in religion. In Islam dates fruits are used to break the day long fast during the holy month of Ramadan (Al-Shahib *et al*, 2003). The Jews believe the date as one of the seven holy fruits and they celebrate Palm Sunday.

Prophet Muhammed (Peace Be Upon Him) said that the best asset is date palm, dates cure several disorders, and he suggested Muslims to eat the date and have a tendency the date palm (Zaid, A., & De Wet, P. F. 1999). The importance of dates has been documented in the Qur'an in Surah Maryam. One significant role of dates comes as when Mary gave birth to the Prophet Jesus (may peace be upon Him) under a palm tree, she heard a voice telling her: "Shake the trunk of the palm tree towards thee: it will drop fresh, ripe dates upon thee. Eat, then, and drink, and let thine eye be gladdened!" (Qur'an 19: 25-26). Ajwa is a type of dates, cultivated only in Saudi Arabia/Al-Madinah Al-Munawara and have significant value in

diseases cure. The health benefit of Ajwa dates has been documented in hadith as Saud (R.A) narrated that I heard Allah's Apostle saying, "If Somebody takes seven Ajwa dates in the morning, neither magic nor poison will hurt him that day (Al-Bukhari, M. I., & Sahi, A. B. 1976).

Hypothesis

Based on the above literature, *P. dactylifera* contains antioxidant that may provide essential nutrients and potential health benefits to consumer. The paper also reveals that palm date extract can inhibit protein oxidation as well as neutralize superoxide and hydroxyl radicals. However, there is still lack of antioxidant and antidiabetic activity Study on the flesh of *P. dactylifera* (Khudari cultivar).

OBJECTIVES

I. Collection and preparation of Date fruit extract.

II. Solvent based extraction and phytochemical screening of *Phoenix dactylifera L. (*Khudari Cultivar*)* fruit and Estimation of total phenolic content by Follin-Ciocalteu method.

III. *In vitro* antioxidative studies of various fractions of *Phoenix dactylifera L. (Khudari)* fruit by DPPH radical scavenging and FRAP assay.

IV. To perform the LCMS analysis of Khudari date extract.

V. In Silico Antidiabetic studies of LC/MS Predicted compounds of Fruit extract via molecular docking analysis.

MATERIALS AND METHODS

Collection and preparation of date extract

Dates are purchased from the local market of Lucknow, India. Pulp and seeds of dates were separated and shed dried and made in coarse powder, avoiding sun dried due to the Signature modification of the biochemicals.

Processing of plant materials

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

Preparation of plant extracts

Dates are purchased from the local market local market of Lucknow, India. Pulp and seeds of dates were separated and shed dried and made in coarse powder, avoiding sun dried due to the Signature modification of the biochemicals. The dried powder (25 g) of the date was extracted using polar solvents successively with the required amount of each of n-hexane, EtOAc, DCM, MeOH and water solvents in Soxhlet apparatus until it turned colourless. The solvent was removed, filtered, and dried at room temperature and residues were scratched out and stored at −20°C for future use. The percentage yield of different fractions was calculated by using the formula.

$$\%\text{yield} = \frac{\text{Weight of crude extract}}{\text{Weight of raw material}} \times 100$$

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 vapour 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.

Phytochemical screening of plant extract

Phytochemical screening is qualitative assay consists of test for phenols, alkaloids, tannins, flavonoids, saponins and triterpenoids, steroids, cardiac glycosides.

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 amylalcohol were also added. The samples were made up to mark and left to react for 30 min for colour 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 FeCl3. 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 colour 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 triterpenoids: 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 colour indicates triterpenoids.

Test for steriods: Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2S04. The colour 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.

Antioxidant assay

DPPH Radical Scavenging activity

The DPPH radical scavenging capacity of the various extract of *Khudari* 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 colour. DPPH solution (132mM) was prepared in methanol in a dark reagent bottle. 100µl of the fruit extracts from *Khudari* and ascorbic acid 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 colour. Ascorbic acid was used as a reference standard. Percent (%) scavenging of DPPH free radical was measured using the following equation.

$$\%\mathrm{DPPH} = \frac{\Delta\text{Absorbance of control} - \Delta\text{absorbance of test sample}}{\Delta\text{Absorbance of control}} \times 100$$

Further, IC50 value represented the concentration of the extract that caused 50% inhibition of DPPH radicals and was calculated by interpolation of linear regression analysis.

Ferric Reducing Antioxidant Potential

A modified method of Benzie and Strain (1996) was adopted to determine the ferric reducing antioxidant potential (FRAP) of various extracts of *Khudari*. Briefly, the FRAP reagent was freshly prepared by mixing sodium acetate buffer (300mM, pH 3.6), 10mM TPTZ solution (in 40mM HCl) and 20mM Fe (III) chloride solution in a volume ratio of 10:1:1, respectively. 100 µl of the different extracts of *Khudari* was added to 3mL of the FRAP reagent and incubated for 5 minutes at 37ºC.The absorbance was measured after 30min at 593nm. The standard curve was plotted by using various concentration (ranging from 5nmol to 100nmol) of $FeSO_4$ solution, and results were expressed as μmole Fe (II)/g dry weight of plant material.

Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis.

LC-MS analysis according to the protocol of Beer et al., at Sophisticated Analytical Instrument Facility (SAIF), CDRI, Lucknow, India. The LC-MS and MS/MS analyses were conducted on a Waters Alliance E2695/HPLC-TQD Mass Spectrometer, Mass range 100-2000 Da, equipped with Waters Alliance 2695 HPLC pump, in-line degasser, autosampler, and column oven. LC-MS and MS/MS spectra were compared to literature to tentatively identify peaks and bioactive compound as well as the output file weas subjected analyzed the peaks via Mnova software offered by Mestrelab Research, chemistry software solutions.

Computational molecular docking simulation study platform

The computer added drug designing studied were performed on Lenovo Notebook equipped with Intel(R) Core (TM) i5-8265U @1.80GHz powered machine with NVIDIA GeForce MX250 2GB graphics card and 8GB of RAM. For the molecular docking studies AutoDock 4.2 32-bit, (http://autodock.scripps.edu/) Cygwin 32-bit, Discovery studio visualizer, SPDBV, ChemDraw version 20.1, Open Babel GUI, and Notepad++ version 7.9.2 was utilized for the in-silico study.

Retrieval of ligands 3D structure and preparation

The 3D-structures of predicted compound (ligands) were downloaded from the PubChem compound database (http://pubchem.ncbi.nlm.nih.gov), which contains structural and functional information about different organic compounds. Each compound in the database has a unique compound identification number (CID). The selected ligands were downloaded in 3D-SDF file format, with the help of BIOVIA Discovery Studio Visualizer these SDF file further converted in PDB file format; which is used by AutoDock 4.0. Software. Applying the CHARMM force field in the Discovery Studio the molecules were subjected to a single step minimization using the steepest descent method for 500 steps and an RMS gradient of 0.01.

3D structure of the target protein

The 3D structure of the target protein was retrieved from the PDB database (https://www.rcsb.org/search) (Burley et al., 2021). The 3D-structure of the α- amylase was downloaded by taking the proteins ID: 1ACJ. Saved α- amylase structure was investigated and visualized in BIOVIA Discovery Studio Visualizer 2020 (BIOVIA, Dassault Systems; https://discover.3ds.com/discovery-studio-visualizer-download.

The resolution of selected α- amylase crystal structure was 2.80 Å. The protein preparation was done for molecular docking using our earlier well-defined procedure (P. Ahmad et al., 2021; Alvi et al., 2016, 2017). on the other hand, an online tool provided the Play-Molecule https://www.playmolecule.com/ DEEPSITE predict the active site of the α-amylase (Jiménez et al., 2017).

Target protein and ligands' preparation

The PDB ID: 1ACJ (target protein) was cleaned to remove heteroatoms, polar hydrogen, and Kollmann charges were added using Autodock tools (Forli et al., 2016). The 3D structure of the target protein (α- amylase) was prepared by converting it to PDBQT format. For the preparation of the ligands, 3D-structures were obtained from the PubChem database (Kim et al., 2021) using their respective PubChem IDs and energy minimized using Discovery studio. The energy minimized structures were converted to PDBQT format using AutoDock 4.2 (Forli et al., 2016; Morris et al., 2009).

ADME and drug-likeness studies of selected ligands

The selected ligands were screened for detailed analysis of pharmacokinetics-related ADME indices utilizing the web-based tool as defined previously (Daina et al., 2017). The ligands were also analyzed for their drug-likeness properties on the basis whether they obey Lipinski's rule of five, blood brain barrier (BBB) activity via the Swiss ADME tool (http://www.swissadme.ch).

Predicted toxicity of the selected compounds

Toxicity prediction was done by the ProTox-II (https://tox-new.charite.de/protox_II/index.php?site=compound_input), an online web-based server for the prediction of toxicities of small molecules. It provides the numerous details of the compounds about the toxicity like LD50, Carcinogenicity, Immunotoxicity, Mutagenicity Cytotoxicity as well as most important Hepatotoxicity (Banerjee et al., 2018).

RESULTS

Phytochemical Estimation

Our results show significant presence of carbohydrates, tannins, saponins, alkaloids, flavonoids, glycosides, phenols, terpenoids, cardiac glycosides, flavonoids, and steroids in methanolic as well as in aqueous extract of Khudari (Table 1).

Table 1.

Phytochemicals	n-hexane	EtOAc	DCM	MeOH	Aqueous
Carbohydrates	+	+	++	++	+++
Tannins	+	-	+	+	+
Saponins	-	-	+	-	-
Alkaloids	-	+	-	+	++
Flavonoids	+	+	-	++	+++
Glycosides	+	-	-	+	++
Phenols	+	+	++	++	+++
Terpenoids	-	+	+	+	-
Cardiac glycosides	-	-	-	-	++
Steroids	-	-	-	-	-

Total Antioxidant Activity.

Antioxidant activities of Both methanol and aqueous extracts were assessed by FRAP assay, which is based on their ability to reduce ferric ions to ferrous form. The results illustrated that water extract has significantly higher FRAP values (331.81±4.56μmol Fe(II)/g) as compared to methanol extract (51.57± 1.183 μmol Fe(II)/g) (Fig.11).

Total phenolic content

The total phenolic content of both extracts was determined by the by the standard procedure Folin-Ciocalteu method. The result shows the aqueous extract has higher TPC (17.77±8.21 µg GA/mg Extract) value than MeoH (7.70±0.45 µg GA/mg Extract). Linear relationship between TPC and FRAP was also performed that shows the positive association between them (Fig.12).

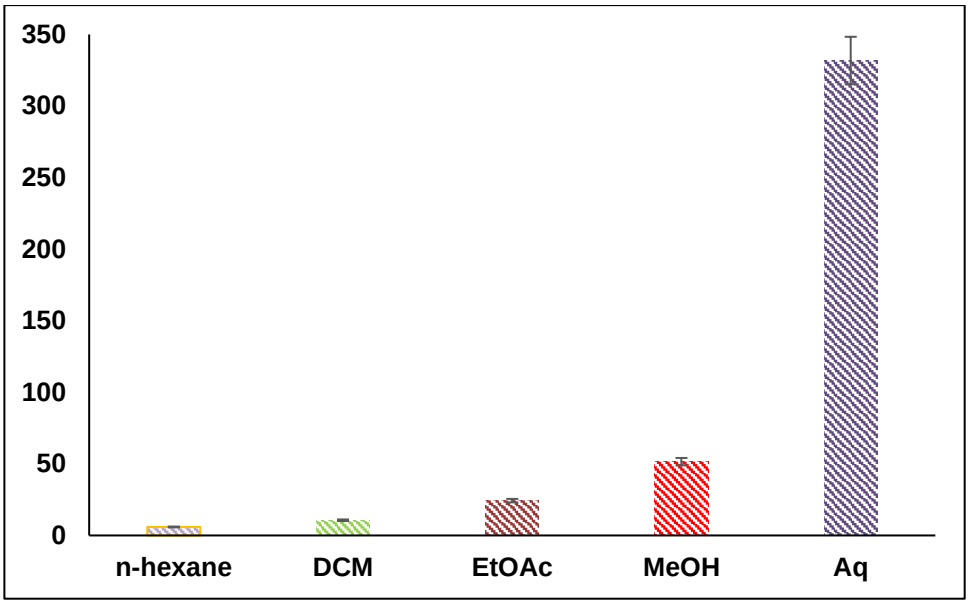

Figure: 11. FRAP Value of various fractions of Khudari. The results are mean ± S.D. of three parallel measurements**.**

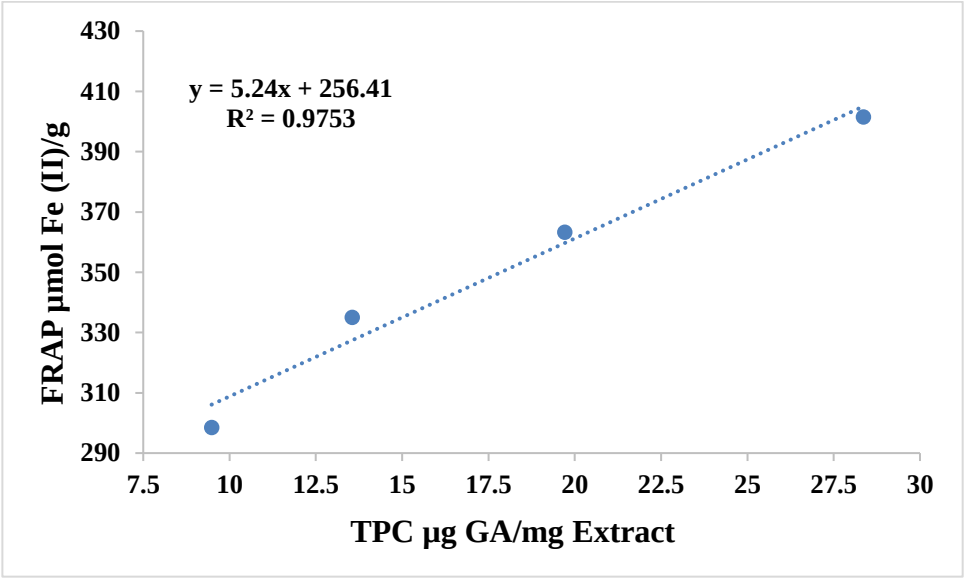

Figure. 12: Linear correlation between the amount of TPC and antioxidant capacity (FRAP) of Khudari in various solvent systems at different concentration.

DPPH Radical Scavenging Activity

The relatively stable DPPH radical is widely used to evaluate the free radical scavenging activity of various natural antioxidants including fruit extract. The data present in Figure 13,14,15 showed the percent inhibition of DPPH radical scavenging activity of different fractions of Khudari dates. The aqueous fraction of Khudari exhibited higher antioxidant activity with an IC_{50} value 235.84 μg/ml. From the data, we observed that DPPH radical scavenging activity was increased as the concentration increased for each individual extract, with marked increase in water extract. The reference standard ascorbic acid was used. The IC_{50} value for standard was 15.58 μg/ml.

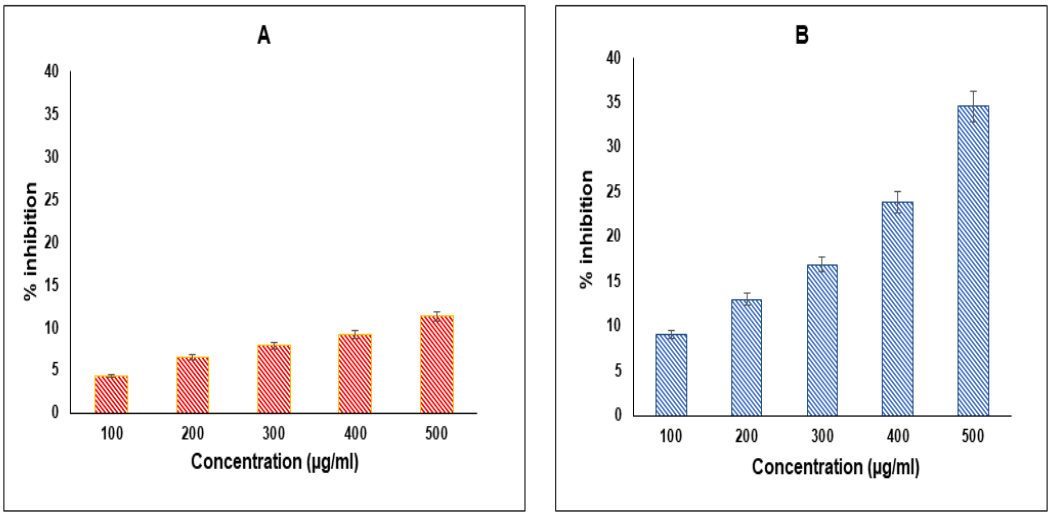

Figure:13. DPPH radical scavenging activity of n-hexane (A) and EtOAc (B) extracts of Khudari. The data represent percent scavenging of DPPH radicals. The results are mean ± S.D. of three parallel measurements.

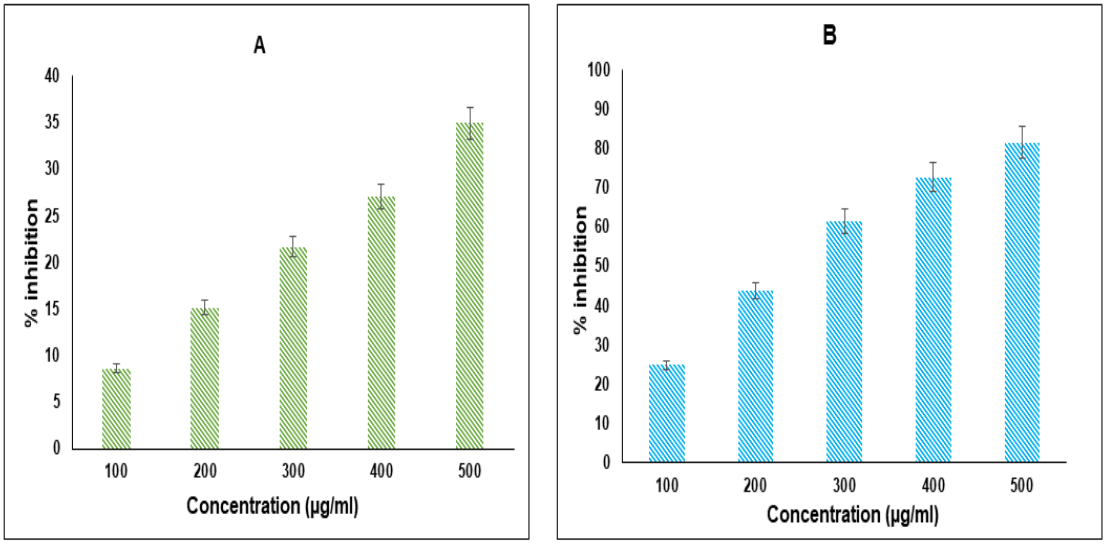

Figure:14. DPPH radical scavenging activity of MeOH (A) and Aqueous (B) standard ascorbic acid. The data represent percent scavenging of DPPH radicals. The results are mean ± S.D. of three parallel measurements.

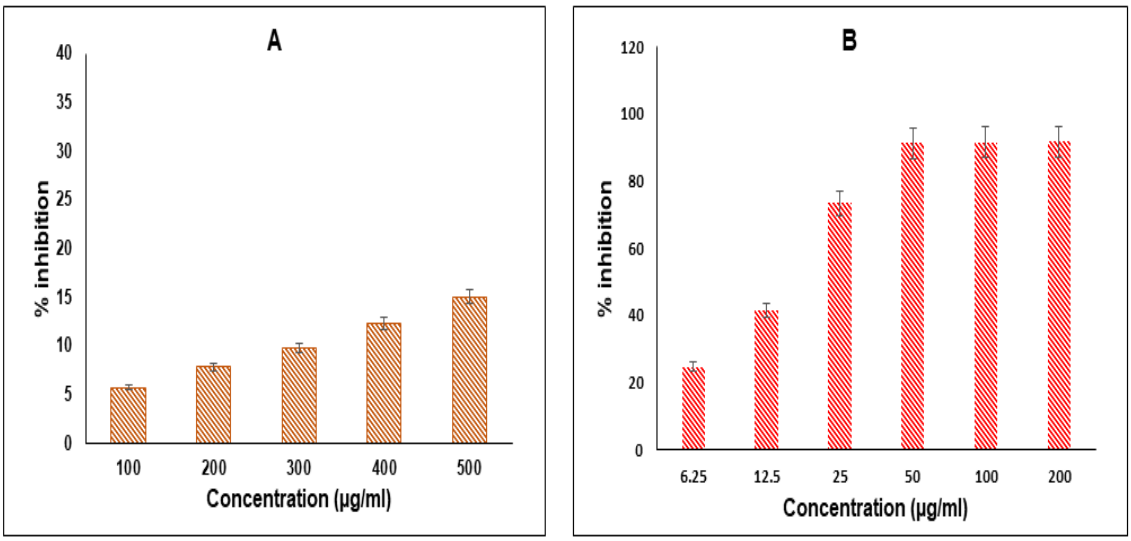

Figure: 15. DPPH radical scavenging activity of DCM (A) and Standard Ascorbic acid (B) standard ascorbic acid. The data represent percent scavenging of DPPH radicals. The results are mean ± S.D. of three parallel measurements.

The LC/MS analysis for identification of phytochemical compounds *Phoenix dactylifera* L. fruits water extract

In the present work, bioactive composition from the aqueous extracts of *Phoenix dactylifera* L. fruit was carried out Waters Alliance E2695/HPLC-TQD Mass Spectrometer in positive as well as negative ionization mode. The output file was analyzed in Mnova software offered by Mestrelab Research, chemistry software solutions and the peaks were matched by using the inbuilt molecular match plugin tool. The reference compounds for the molecular match analysis were downloaded from the PubChem classification browser> spectral information> Mass Spectrometry> LC-MS, sub-category, in which 18804 compound enlisted. According to the Mnova analysis the best matched compound is listed in the **Table 2&3.** The bioactive composition from the aqueous-extract of *Phoenix dactylifera* L. fruit was carried out using LC/MS in positive and negative ionization mode. The method tentatively detected 18 phytochemical compounds **Table 2&3**. The compounds detected in this work were tentatively characterized by means of MS data, together with the interpretation of the observed MS/MS spectra in comparison with particular reference compounds were retrieved from PubChem by Mnova software.

Table.2: Positive ion mode $[M-H]^+$

Compound ID	Match score	RT	Adduct /Loss	Predicted m/z	Matched m/z
73053139	0.905	1.71	H+/-	260.2373	260.5172
99359	0.944	2.67	H+/-	238.1662	238.4553
90675402	0.917	5.24	H+/-	228.1383	228.2916
8182	0.859	9.18	H+/-	171.2107	171.5068
10241527	0.950	11.63	H+/-	187.0502	187.3247
139583859	0.851	12.50	H+/-	279.2319	279.4917
139586920	0.943	14.45	H+/-	425.3778	425.6685
132579590	0.864	19.14	H+/-	339.1551	338.8782
10474528	0.855	21.09	H+/-	375.0711	374.8500

Table 3. Positive ion mode [M-H]$^-$

Compound ID	Match score	RT	Adduct/Loss	Predicted m/z	Matched m/z
45359339	0.989	1.66	-/H$^+$	486.2486	486.477
8292	0.874	1.66	-/H$^+$	307.0222	306.9764
43655	0.872	1.70	-/H$^+$	342.1004	342.3123
42611990	0.859	2.66	-/H$^+$	453.3727	453.3151
222284	0.943	3.53	-/H$^+$	413.3778	413.3541
3883	0.971	20.58	-/H$^+$	368.0675	368.2130
3783	0.967	20.58	-/H$^+$	300.1594	300.2700
3126	0.966	20.58	-/H$^+$	300.2897	300.2700
9817839	0.954	20.66	-/H$^+$	300.1131	299.8538

Figure 16

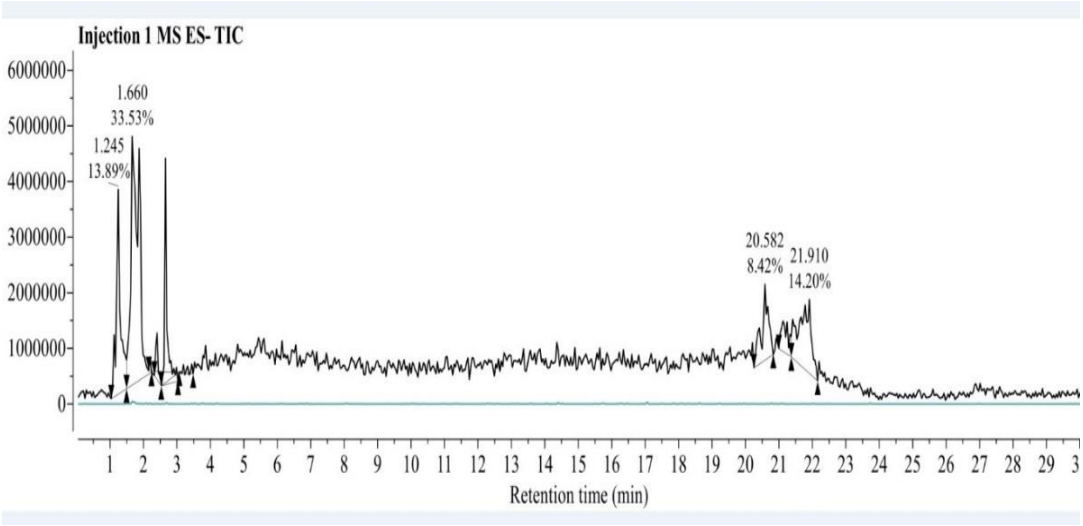

Figure:16. gram of positive ion [M-H]$^+$

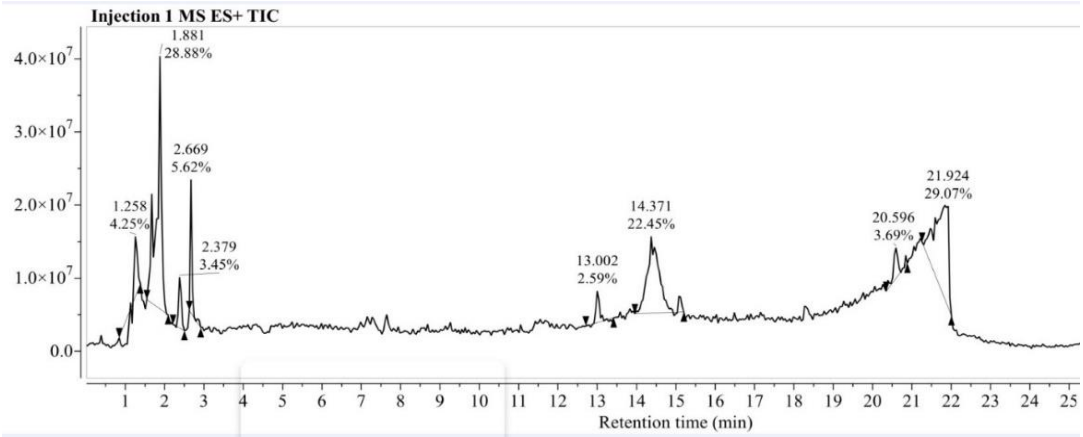

Figure 17. Chromatogram of negative ion [M-H]$^-$

Our study utilized negative ion mode $[M-H]^-$ as well as positive ion mode (Farag et al., 2014). The identification of phytochemical compounds was based on five different parameters: retention time, molecular weight of each compound, m/z $[M-H]^-$, m/z $[M-H]^+$, molecular formula and fragmentation pattern. Representative chromatograms of LC-MS data from one extraction sample of *Phoenix dactylifera* L. in the present study were shown in **Figure 16**. The positive ion $[M-H]^+$ chromatograms showed identification of 9 compounds i.e., 5-(7-Methyloctyl)-1,2,3,4-Tetrahydroquinoline (MS peak at *m/z* 260.5, and retention time *Rt* at 1.71 min; Argvalin, (m/z 238.4, and *Rt* 2.67 min; 2-{5-[(1E)-3-methylbuta-1,3-dien-1-yl]-1H-indol-3-yl}ethanol (*m/z* 228.2, and *Rt* 5.24); Dodecane (*m/z* 171.5, *Rt* 9.18); Pyrocoll (*m/z* 187.3 and *Rt* 11.63); Methyl Hydroxysterpurate Ethylidene Acetal (*m/z* 279, and *Rt* 12.50); 3β-Hydroxy-4β-methylfusida-17(20)(16,21-cis),24-diene(3β-hydroxy-protosta 7(20)(16,21-cis),24 diene) (*m/z* 425.6 and *Rt* 14.45); Terezine E (*m/z* 338.8 and *Rt* 19.14); and Paeciloquinone D (*m/z* 374.8 and *Rt* 21.09) **Figure 17**. while in the negative ion mode $[M-H]^-$ chromatograms showed identification of 9 compounds i.e., 4-[(2R,3S,7R,8R,8aS)-2,3,4'-trihydroxy-4,4,7,8a-tetramethyl-6'-oxospiro[2,3,4a,5,6,7-hexahydro-1H-naphthalene-8,2'-3,8-dihydrofuro[2,3-e]isoindole]-7'-yl]butanoic acid (*m/z* 486.4, and *Rt* 1.66); Fensulfothion (*m/z* 1.66 and *Rt* 306.9); 8-Hydroxyloxapine (*m/z* 343.3 *Rt* 1.70); Gilvsin A (*m/z* 453.3 and *Rt* 2.66); Sitosterol (*m/z* 413.3 and *Rt* 3.53); Lansoprazole (m/z 368.2 and *Rt* 20.58); Isoxsuprine (*m/z* 300.2 and *Rt* 20.58); 2-Aminooctadecane (*m/z* 300.2 and *Rt* 20.58) and Dehydroevodiamine (*m/z* 299.8 and *Rt* 20.66).

As our target is α- amylase, which are primarily expressed in digestive system of mammals, so that the test compound must penetrate the pass the gastrointestinal absorption test. The prediction of distinct pharmacological measures along with the GI absorption through AI-based *in-silico* approach α- amylase is the most fundamental and persuasive step in the screening of chemical libraries for further drug discovery (Schneider et al., 2020a). The SWISS ADME indicate the BBB prediction in form of the boiled egg graph. The white region is for high probability of passive

absorption by the gastrointestinal tract, and the yellow region (yolk) is for high probability of brain penetration. According to the SWISS ADME, there are five compounds (2-Aminooctadecane, Isoxsuprine, 8-Hydroxyloxapine 2-{5-[(1E)-3-methylbuta-1,3-dien-1-yl]-1H-indol-3-yl} ethanol (MDIE), and Methyl Hydroxysterpurate Ethylidene Acetal (MHEA) that lies in the yellow region of the graph. That means all these five compounds cross the BBB penetration. According to the preADME tools the BBB analysis was done with score ranging from 0.49 to 7.46. The small molecules with BBB score >2.0, between 2.0–0.1 and <0.1 show high, moderate and low absorption across BBB, respectively (Leão et al., 2020; Ma et al., 2005). Therefore, these five compounds were further carried out for ADMET analysis.

Our ADME analysis depicted that all the selected five compounds exert considerable aq. solubility ranging from 2.32 to 629.45 mg/L. Further, the assessment of Caco-2 and MDCK cell's permeability has been established as the crucial measure of the drug development process (Bittermann & Goss, 2017; Leão et al., 2020; Volpe, 2008). The Caco-2 and MDCK cell permeability was predicted through preADME. Our results from preADME analysis showed that the selected five compounds have a Caco-2 permeability range of 15.32–57.68 nm/Sec while the MDCK permeability score ranged from 0.23 to 231.82 nm/sec, which signifies that OSCs have a significant ability to permeate across Caco-2 and MDCK cells as the permeability range <4, >4–70 and >70 (nm/sec) are predictive of low, middle and high permeability, respectively, across these cellular models (Yamashita et al., 2000). In addition to the BBB, Caco-2, and MDCK cell permeability, it is very important to assess the skin permeability in case of transdermal drug penetration and the accidental exposure of distinct chemical entities (Lundborg et al., 2018).

The skin permeability (Log Kp) of five compounds were found to be in the range of -0.77 to -3.75 cm/h which falls under the acceptable range of Log Kp for drug candidates (Chen et al., 2018). The distribution of drugs from plasma to target tissues can be affected by a number of factors, such as high molecular weight, but perhaps the most important is plasma protein binding (PPB). Compounds that are extensively bound to plasma proteins will have a low volume of distribution (Vdss), can have long plasma half-

lives (T1/2), and have low hepatic and renal clearance. High PPB may also have an impact on efficacy since it is usually the free fraction of drugs that is responsible for the pharmacological action (Gurevich, 2013; Roberts et al., 2013). Our selected OSCs showed distinct PPB efficiencies (76.91-100.00%) with the highest PPB of 2-Aminooctadecane and Methyl Hydroxysterpurate Ethylidene Acetal (MHEA) (100% for each). It is well established that lower PPB efficiency is associated with higher diffusion and transport throughout the body to achieve their respective targets, whereas, the drugs showing higher PPB efficiency (>90%) are less effective toward their molecular targets. Here, in this case, all OSCs showed acceptable PPB efficiencies except four OSCs (PPB > 90%) which makes these OSCs suitable for trans-membrane diffusion and transport.

Human intestinal absorption (HIA), a key determinant of drug suitability in the current drug discovery program, is derived from bioavailability, absorption and excretion of the drugs (Zhao et al., 2001). the predicted HIA for the selected five compounds in the current study ranged from 90.24 to 100.00%. This high HIA signifies the enhanced bioavailability of these OSCs which might be responsible for their potent pharmacological actions.

Similarly, the cytochrome P450 2D6, also reckoned as CYP2D6, is responsible for almost one-fourth of the metabolism and excretion of the drugs in the body (Wang et al., 2009; Zanger & Schwab, 2013). The rate of CYP2D6-mediated drug metabolism varies in different subjects as some of them are ultra-metabolizers while others are poor metabolizers. Ultra-metabolizers face the challenge of diminished efficacy of drugs while poor metabolizers may face cytotoxic issues (Teh & Bertilsson, 2012; Zanger & Schwab, 2013), hence, CYP2D6-mediated metabolism is a key determinant in dose adjustment (Walko & McLeod, 2012). Our results depicted that two compounds 2-Aminooctadecane, and Isoxsuprine showed CYT. p450 2D6 inhibitory activity while the rest showed non inhibitor. This CYT. p450 2D6 inhibition by these compounds reflects their persistent bioavailability and delayed clearance from the circulation, which ultimately may result in the enhanced therapeutic efficacy of these compounds. On the other hand, three compounds 2-Aminooctadecane, Isoxsuprine, and 8-Hydroxyloxapine showed as CYT. p450 2D6 substrate

that signifies their comparatively faster metabolism, instant therapeutic actions as well as clearance from the body.

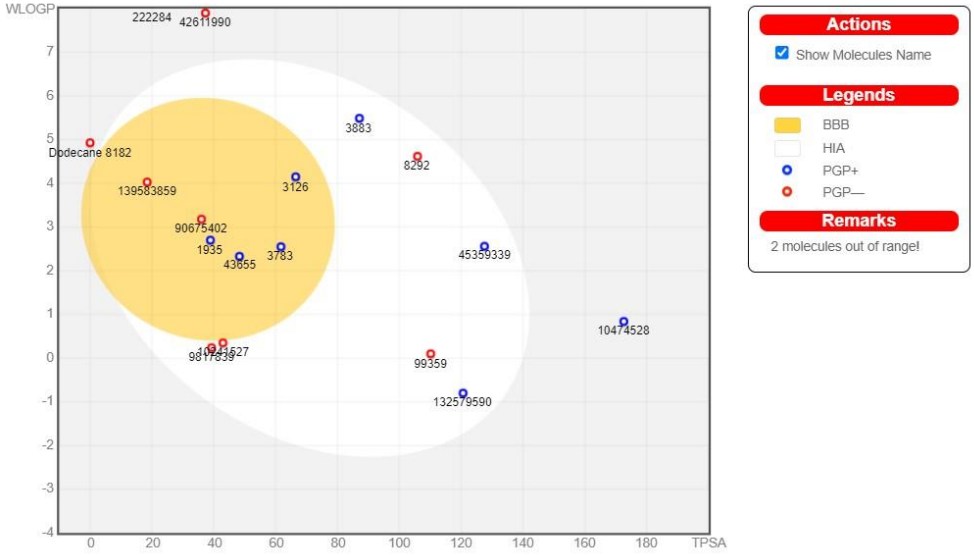

Figure: 18. The boiled egg representation of selected compounds to evaluate of passive gastrointestinal absorption (HIA) and BBB. The white area indicates for high probability of passive absorption by the gastrointestinal tract, and the yellow region (yolk) is for high probability of brain penetration.

Compound name	2-Aminooctadecane	Isoxsuprine	8-Hydroxyloxapine	2-{5-[(1E)-3-methylbuta-1,3-dien-1-yl]-1H-indol-3-yl} ethanol	Methyl_Hydroxysterpurate Ethylidene_Acetal
ID	**3126**	**3783**	**43655**	**90675402**	**139583859**
BBB	5.73	2.28	0.49	7.46	1.96
Buffer solubility mg/L	6.46	541.43	54.65	8.46	66.19
Pure water solubility mg/L	2.32	629.45	21.31	25.39	22.11
MDCK	77.07	260.92	97.93	199.83	280.41
Caco2	20.60	15.32	42.70	26.95	57.68

Skin Permeability	-0.77	-3.17	-3.75	-3.07	-2.66
Plasma Protein Binding	100.00	84.22	76.91	83.53	100.00
HIA	87.18	90.24	96.12	91.77	100.00
CYP_2D6_inhibition	Inhibitor	Inhibitor	Non	Non	Non
CYP_2D6_substrate	Substrate	Substrate	Substrate	Non	Non

Predicted compound have drug-like properties

The drug discovery program involves the assessment of a set of distinct factors to identify the drug-likeness of a chemical entity. To date, various artificial intelligence (AI)-based strategies i.e., absorption, distribution, metabolism, excretion, and toxicology (ADMET) are currently being exploited to avoid unnecessary wasting of time, money, and manpower (Hessler & Baringhaus, 2018; Schneider et al., 2020b). In the same context, we also implied AI-based approach i.e., analysis of Lipinski's rule of five, physiochemical property to assess the drug-like properties of our selected compounds. Lipinski's rule of five includes molecular weight (<500 Da), H-bond donors (HBD) <5, H-bond acceptors (HBA) not more than 10 and octanol-water partition coefficient Log P not exceeding 5. The initial drug likeness analysis depicted that all selected OSCs fall under the acceptable scores of Lipinski's rules of five except one compound i.e., 2-Aminooctadecane which violated one rules of Lipinski (LogP >5) however, this violation was not significant enough to rule out these compounds from this study **(Table 4**). On the other hand, considering the potential of hydrophobicity in drug distribution pattern, analysis of Log P has widely been used to assess the permeability of drug candidate and topological polar surface area (TPSA) is used as the measure of the polarity and trans-membrane transport of compounds (Hitchcock & Pennington, 2006; Pajouhesh & Lenz, 2005). The results of our Log P and TPSA analysis showed that all the OSCs have desirable Log P value (Log P ≤ 5) except one compound, as well as TPSA thresholds as established by previous studies (Prasanna & Doerksen, 2008). These properties are signifying the fact that these selected compounds qualify all the tests for drug-likeness and can further be assessed for other pharmacological effects.

Table 4: Physicochemical property of the predicted compounds

Compound	miLogP	M. Wt.	TPSA	Hydrogen Bond Acceptor	Hydrogen Bond Donor	No. of violations	No. of rotatable bond	volume
3126	5.04	301.51	66.48	3	4	1	16	342.20
3783	2.77	301.39	61.72	4	3	0	7	293.28
43655	3.75	343.81	52.74	5	1	0	1	296.90
90675402	2.98	213.28	36.02	2	2	0	4	209.79
139583859	4.22	278.44	18.47	2	0	0	0	287.92
1935*	3.05	198.27	38.91	2	2	0	0	191.53

Toxicity assessment of the selected compounds

The toxicity effect of the selected compound was done by the Protox II, an online web server for the prediction of toxicities of small molecules. The prediction of compound toxicities is an important part of the drug design development process. Computational toxicity estimations are not only faster than the determination of toxic doses in animals, but can also help to reduce the amount of animal experiments. ProTox-II server predicted the hepatotoxicity, carcinogenicity, immunogenicity, mutagenicity as well as cytotoxicityalong with LD_{50}. The output results were tabulated in **Table 5**. Toxicity classes are defined according to the globally harmonized system of classification of labelling of chemicals (GHS). LD_{50} values are given in [mg/kg]: Class I: fatal if swallowed ($LD_{50} \leq 5$), Class II: fatal if swallowed ($5 < LD_{50} \leq 50$), Class III: toxic if swallowed ($50 < LD_{50} \leq 300$), Class IV: harmful if swallowed ($300 < LD_{50} \leq 2000$), Class V: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$), Class VI: non-toxic ($LD_{50} > 5000$). The

output result indicates that 2-Aminooctadecane and MHEA have successfully qualify all the parameter of toxicity while MDIE indicate suitable LD_{50} but it showed active immunotoxin. On the other hand, Isoxsuprine and 8-Hydroxyloxapine showed low predicted LD_{50} i.e., class III and class II LD_{50}, respectively, in which the Isoxsuprine does not poses any other toxicity but 8-Hydroxyloxapine indicates the immunotoxicity.

Table 5: Toxicity assessment of selected five compounds

PubChem CID	Compound Name	predicted LD_{50} mg/kg	Predicted toxicity class	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
3126	2-Aminooctadecane	3500	5	Inactive	Inactive	Inactive	Inactive	Inactive
3783	Isoxsuprine	200	3	Inactive	Inactive	Inactive	Inactive	Inactive
43655	8-Hydroxyloxapine	40	2	Inactive	Inactive	Active	Inactive	Inactive
90675402	2-{5-[(1E)-3-methylbuta-1,3-dien-1-yl]-1H-indol-3-yl}ethanol	1680	4	Inactive	Inactive	Active	Inactive	Inactive
139583859	MethylHydroxysterpurate Ethylidene Acetal	7800	6	Inactive	Inactive	Inactive	Inactive	Inactive

Selected five compounds strongly bind to the active pocket of α-amylase

In this attempt, we found that all the selected five compounds strongly occupied the active pocket of α- amylase crystal structure with binding energy (ΔG) values ranging from -5.4 to -8.0 kcal/mol. Among these, two compounds i.e., 8-Hydroxyloxapine and Methyl Hydroxysterpurate Ethylidene Acetal showed the highest affinity for α-amylase with ΔG values -8.0, and -7.6 kcal/mol, respectively. The residues of α-amylase stabilizing the interaction with the above-mentioned ligands lied from Arg195, to His 299 of the active pockets. The interaction of 8-Hydroxyloxapine with α-amylase was stabilized by 19 amino acid residues (Arg195, Asp197, Thr163, Leu165, Gln63, Tyr62, Trp59, Trp58, Asp356, His305, Asp300, His 299. While, Methyl Hydroxysterpurate Ethylidene Acetal occupied the

α- amylase active pocket with 17 interacting amino acid residues (Thr163, Leu162, Leu165, His101, Gln63, Trp59, Trp58, Tyr62, Asp300, His 299, Arg195, Asp197), (Figure 17). Similarly, other LC/MS predicted compounds interacted with α- amylase (Figure 17). The interactions of all the selected compounds with the active pocket of α- amylase including the above-mentioned residues in this study are in accordance with a previously published report which also reported similar binding patterns of different natural compounds within the active pocket of α- amylase (S. S. Ahmad et al., 2021; Chlebek et al., 2019; Ibrar et al., 2018; Rehman et al., 2022).

In contrast, the standard acarbose also occupied the active pocket of Alpha amylase (ΔG: -8.25) with almost common residues (i.e., Glu233, Asp197, Ala,198, His101, Tyr,62, Trp59, Trp58, Thr163, Aa106, Asp300, His 299). These *in-silico* findings clearly depicted that *Phoenix dactylifera* derived fruit water extracted compounds may have potent inhibitors of α-amylase activity as they compete with acarbose for the active pocket of this enzyme **(Figure 19,20,21**). Hence, these compounds may be promoted for further drug development steps as well as to introduce natural alternative therapeutic options in the management of diabetes, considering the adverse effects associated with synthetic inhibitors of α-amylase.

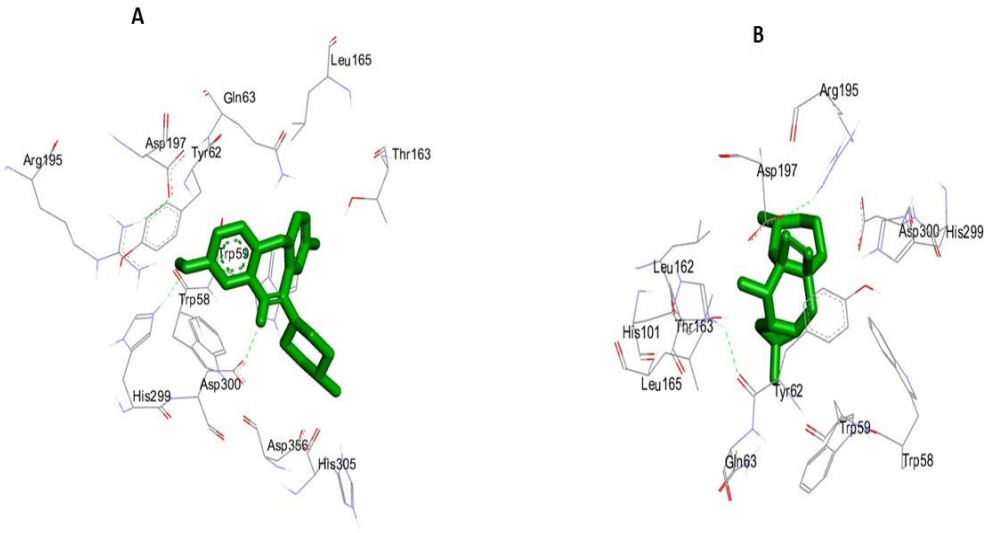

Figure 19: *In-silico* binding patterns of 8-Hydroxyloxapine, Isoxsuprine, MDIE, MHEA, and 2-Aminooctadecaneand against the active pocket of α-amylase (PDB ID: 1B2Y). (A) 2D-Interactions of 8-Hydroxyloxapine within the active pocket of α-amylase and MHEA (B).

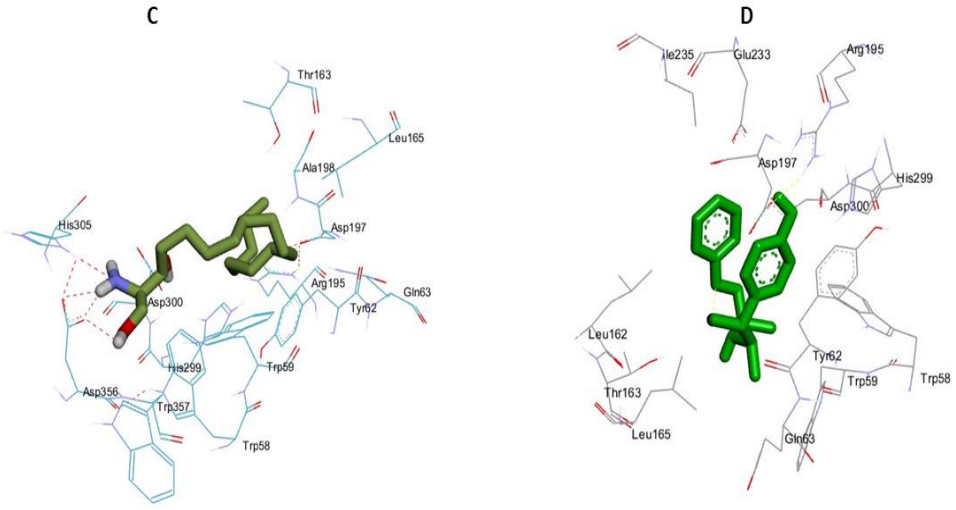

Figure 20: *In-silico* binding patterns of C) 2-Aminooctadecane; (D) Isoxsuprine against the active pocket of α- amylase (PDB ID: 1B2Y).

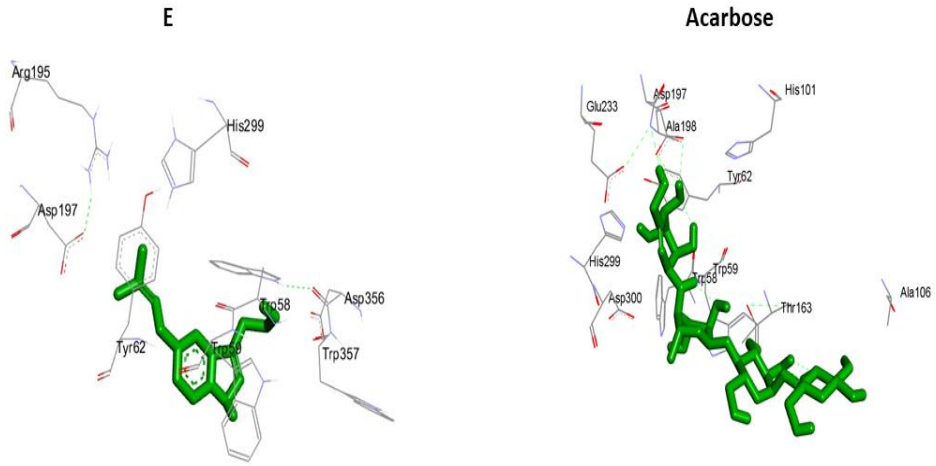

Figure 21: *In-silico* binding patterns of MDIE (E) and standard drug Acarbose (PubChem ID: 41774) against the active pocket of α- amylase (PDB ID: 1B2Y).

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 (Styskal J *et al*., 2012.). When there is imbalance between ROS generation and antioxidant protection mechanism, it leads to cellular dysfunction causing various diseases such cancer, diabetes, cardiovascular disease neurodegenerative disease (Moreno P.R., & Fuster V 2004).

Free radicals are highly reactive molecules or chemical species capable of independent existence. Generation of highly reactive oxygen species (ROS) is an integral feature normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, ovulation and fertilization. The production however, multiplies several folds during pathological conditions. The release of oxygen free radicals has also been reported during the recovery phases from many pathological noxious stimuli to the cerebral tissues (Halliwell B 1989).

Oxidative stress has been identified as critical in most of the key steps in the pathophysiology of atherosclerosis and acute thrombotic events, including dyslipidemia leading to atheroma formation, the oxidation of LDL, endothelial dysfunction, plaque rupture, myocardial ischemic injury, and recurrent thrombosis (i.e., the secondary or subsequent clot that often occurs after initial thrombolysis). The role of oxidative stress in the connection between the various coronary disease risk factors such as elevated blood pressure, diabetes and cigarette smoking, and the clinical sequelae of disease associated with vasoconstriction, thrombosis, plaque rupture, and vascular remodeling has been recognized.

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 S *et al*., 2012). Phoenix dactylifera is very commonly consumed in many parts of the world and is a vital component of the diet in most of the Arabian countries. There are different cultivars (cv.) of *P. dactylifera* that exhibit different characteristics and benefits. According to Subash et al. (2015), date palm fruits may represent protective strategies to minimize the risk of developing various disease by their antioxidant defense system. Zehra *et al*. (2015) describe that antioxidant have always helped in preventing the damage done to cells by free radicals that are released during normal metabolic process of oxidation. These free radicals include reactive oxygen free radical species (ROS), reactive hydroxyl radicals (OH−), superoxide anion radical (O2−), hydrogen peroxides (H2o), and peroxyl (ROO−). It is well known that P. dactylifera is a rich source for natural phytochemical antioxidants including vitamins (ascorbic acid, Vitamin A, and a-tocopherols), carotenoids, and phenolic compounds (Allaith 2008). According to Tang et al. (2013),

CONCLUSION

In conclusion, our results demonstrated the phytochemical screening, antioxidant and total phenolic content and their correlation with ferric reducing antioxidant potential of sequentially extracted Phoenix dactylifera L. (Khudari cultivar) extract. We found that all the extracts of Date palm (Khudari) showed significant inhibition of DPPH radicals with maximum DPPH radical scavenging in Aqueous extract, whereas, n-hexane showed minimum DPPH radical scavenging activity among all the Khudari extract. The antioxidant potential of our extracts was also confirmed by FRAP assay. Which is based on their ability to reduce ferric ions to ferrous form. In which All the extracts from Khudari showed some extent of ferrous reducing power but the aqueous extract showed the highest FRAP value. Thus, it is a good approach to manage oxidative stress as a whole with these extracts, which showed good antioxidant activities. We also performed the LCMS analysis to find out the Khudari derived secondary metabolites. Further, we explore the antidiabetic activity of the selected 5 compound via molecular docking analysis. Apart from the *in-silico* study, a thorough and full-fledged *In-vitro* and *in-vivo* study is 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 oxidative stress consequences.

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