A DISSERTATION ON

In-vitro Evaluation of Phytochemicals, Antioxidant and Antibacterial Activity of Night Blooming Jasmine *(Cestrum nocturnum)*

SUBMITTED TO THE

DEPARTMENT OF BIOSCIENCES

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IN PARTIAL FULFILMENT
FOR THE
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IN MICROBIOLOGY
BY

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CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Ms. Shumaila Parveen** 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 **"In-vitro Evaluation of Phytochemicals, Antioxidant and Antibacterial Activity of Night Blooming Jasmine (*Cestrum nocturnum*)"** therefore, being forwarded for the acceptance in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology, Department of Biosciences, Integral University, Lucknow**.**

Date:**20/June/2022**
Place: Lucknow

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TO WHOM IT MAY CONCERN

This is to certify that the study conducted by **Ms. Shumaila Parveen** student of M.Sc. Microbiology (IV semester), Integral University has completed her Four months dissertation work entitled **"In-vitro Evaluation of Phytochemicals, Antioxidant and Antibacterial Activity of Night Blooming Jasmine (*Cestrum nocturnum*)"** 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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Before I present my work, I would like to gratefully acknowledge the contribution of all those people who have helped in the work described in this Dissertation. I am going to try anyway, and if your name is not listed, it is rest assured that my gratitude is not less than for those listed below.

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

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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: **20/June/2022**

Place: **Lucknow** **Ms. Shumaila Parveen**

CONTENT

ABBREVIATIONS

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
MeOH	Methanol
EtOAc	Ethyl Acetate
DCM	Dichloromethane

INTRODUCTION

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products. Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acid. (McCord, 2000) Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins, and excessive exercise. These injured tissues produce increased radical enzymes (e.g., xanthine oxidase, lipogenase, cyclooxygenase) activation of phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS.

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP (adenosine triphosphate) production by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process. These species play a dual role as both toxic and beneficial compounds. The delicate balance between their two antagonistic effects is clearly an important aspect of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures (Halliwell B et al.,2007,Young I et al.,2001). Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as “free radical scavengers” by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases. The widely accepted theory is the “oxidative stress hypothesis” (Ghezzi et al., 2017)

that advanced and modified the free radical theory of aging. Based on the oxidative stress hypothesis, oxidative damage is not solely triggered by the unrestricted ROS production, but it also caused by other oxidants, such as reactive lipid species and reactive nitrogen species (RNS). The hypothesis of oxidative stress highlights the crucial role of antioxidant defenses as an important component of the overall redox balance of the organism. However, several studies demonstrated that avoiding oxidative stress damage does not increase longevity (Buffenstein et al., 2008; Pérez et al., 2009,ab).

Oxidative stress is considered as an imbalance between pro and antioxidant species, which results in molecular and cellular damage. Mitochondria are major organelles that are accountable for generation of energy through oxidative phosphorylation to generate adenosine triphosphate (ATP), a molecule which is crucial for cellular actions. The electron transport chain consumes up to 90% of total oxygen (O2) taken up by the cells. During this process, ROS are generated as by-products for the partial four-electron reduction of O2 to produce water molecule, which is the last electron acceptor in the ATP generation process. Nearly 0.1–0.5% of inhaled O2 is converted to superoxide (O^{-}_{2}) during the normal physiological states (Servais et al., 2009). In the normal healthy state, the generation and oxidation of ROS occur in a controlled manner. By contrast, the ROS production is increased under high-stress conditions or under disease states. The ROS generated from aerobic respiration caused a cumulative oxidative damage in macromolecules, including lipids, DNA, and proteins, which subsequently lead to cells death, and affect the healthspan of numerous principal organ systems (Dai et al., 2014). Oxidative stress plays a crucial role in the development of age-related diseases including arthritis, diabetes, dementia, cancer, atherosclerosis, vascular diseases, obesity, osteoporosis, and metabolic syndrome. 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 and RNS 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 and RNS include hydroxyl (OH), superoxide (O_2^-), 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, aging. 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 et al.,2007). After penetration into the body by different routes, these exogenous compounds are decomposed or metabolized into free radicals.

Formation of ROS and RNS can occur in the cells by two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions generating free radicals include those involved in the respiratory chain, the phagocytosis, the prostaglandin synthesis and the cytochrome P450 system (Hallliwell B 2007,). For example, the superoxide anion radical (O_2^-) is generated via several cellular oxidase systems such as NADPH oxidase, xanthine oxidase, peroxidases. Once formed, it participates in several reactions yielding various ROS and RNS such

as hydrogen peroxide, hydroxyl radical (OH), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), etc. H2 O2 (a non radical) is produced by the action of several oxidase enzymes, including aminoacid oxidase and xanthine oxidase.

The last one catalyses the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid. Hydroxyl radical (OH), the most reactive free radical in vivo, is formed by the reaction of $O2^-$ with H2 O2 in the presence of Fe2+ or Cu+ (catalyst). This reaction is known as the Fenton reaction. Hypochlorous acid (HOCl) is produced by the neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of H_2O_2.

Nitric oxide radical (NO) is formed in biological tissues from the oxidation of L-arginine to citrulline by nitric oxide synthase. Free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing radiations. The nonenzymatic process can also occur during oxidative phosphorylation (i.e. aerobic respiration) in the mitochondria (Droge W.,2002).

When produced in excess, free radicals and oxidants generate a phenomenon called oxidative stress, a deleterious process that can seriously alter the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acid (DNA). 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 (Frei B.,1997). Proteins may also be damaged by ROS/RNS, leading to structural changes and loss of enzyme activity. 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. 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).

The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in situ (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention.

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.

Antioxidant defense protects biological systems from free radical toxicity and includes both endogenous and exogenous molecules. Endogenous antioxidants include enzymatic and nonenzymatic pathways. The primary antioxidant enzymes are SOD, catalase (CAT), and glutathione peroxidase (GSH-Px). As mentioned above, O2 is converted by SOD to H_2O_2, which is decomposed to water and oxygen by CAT, preventing hydroxyl radicals production. Additionally, GSH-Px converts peroxides and hydroxyl radicals into nontoxic forms by the oxidation of reduced glutathione (GSH) into glutathione disulfide and then reduced to GSH by glutathione reductase. Other antioxidant enzymes are glutathione-S-transferase and glucose-6-phosphate dehydrogenase (Birben E et al.,2012). The non-enzymatic antioxidants are molecules that interact with RONS and terminate the free radical chain reactions: bilirubin, α-tocopherol (vitamin E), and β-carotene are present in

blood while albumin and uric acid account for 85% of antioxidant capacity in plasma.

Exogenous antioxidants include ascorbic acid (vitamin C), which scavenges hydroxyl and superoxide radical anion, α-tocopherol (vitamin E), which is involved against lipid peroxidation of cell membranes, and phenolic antioxidants, which include stilbene derivatives (resveratrol, phenolic acids, and flavonoids), oil lecitinas, selenium, zinc, and drugs such as acetylcysteine.16 Oxidative stress occurs when there is an imbalance between the formation and the removal of RONS because of an overproduction and/or an impaired ability to neutralize them or to repair the resulting damage. (Salisbury D et al.,2015)

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 ($O2^{-}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O2) 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) (Bahorun T et al.,2006). 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 (Willcox JK et al.,2004). 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.

When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized. Therefore, the antioxidant resources must be constantly restored in the body. Thus, while in one particular system an antioxidant is effective against free radicals, in other systems the same antioxidant could become ineffective. Also, in certain circumstances, an antioxidant may even act as a pro-oxidant e.g. it can generate toxic ROS/RNS. The antioxidant process can function in one of two ways: chain-breaking or prevention. For the chain-breaking, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin C, E, carotenoids, etc), or it simply disintegrates into an inoffensive product. The classic example of such a chain reaction is lipid peroxidation. For the preventive way, an antioxidant enzyme like superoxide dismutase, catalase and glutathione peroxidase can prevent oxidation by reducing the rate of chain initiation, e.g., either by scavenging initiating free radicals or by stabilizing transition metal radicals such as copper and iron.

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 4 oxidative stress in cells, the neurotransmitter glutamate is the main effecter of this process in the brain, primarily through activation of its ionotropic receptors. Free radical induced- damage can occur by stimulation of phospholipase A2 and subsequent release of arachidonic 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.

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 et al., 2005).

Night blooming jasmine, botanically known as *Cestrum nocturnum* (C. nocturnum) is an evergreen shrub that grows in tropical and sub-tropical regions throughout the world. *C. nocturnum* is a popular ornamental plant due to its showy and fragrant white flowers. It is also used as a hedge plant and cultivated as a medicinal plant. The flowers volatile compound was identified as phenylacetyl aldehyde and linalool. The leaves of *C. nocturnum* have pharmacological significance in Chinese folks' medicine and have been used for the treatment of burns and swelling.

The medicinal properties of night blooming jasmine include antioxidant, antihyperlipidemic, hepatoprotective, analgesic, antifungal, anticonvulsant, anti-HIV and larvicidal activities (Rokade et al., 2018)

REVIEW OF LITERATURE

Oxidative stress

Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage. Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko IV et al., 2001, Maritim AC et al., 2003). ROS include free radicals such as superoxide (•O2 -), hydroxyl (•OH), peroxyl (•RO2), hydroperoxyl (•HRO2 -) as well as nonradical species such as hydrogen peroxide (H2O2) and hydrochlorous acid (HOCl) (Turko IV et al 2001,Evans JL et al 2002). RNS include free radicals like nitric oxide (•NO) and nitrogen dioxide (•NO2 -), as well as nonradicals such as peroxynitrite (ONOO-), nitrous oxide (HNO2) and alkyl peroxynitrates (RONOO) []. Of these reactive molecules, •O2 - , •NO and ONOO- are the most widely studied species and play important roles in the diabetic cardiovascular complications.

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 ROSelicited 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 7 eosinophil peroxidase (EPO). Respiratory burst oxidase generates superoxide ($O2^{\bullet -}$) via the one electron-reduction of oxygen by NADPH, with a secondary production of hydrogen peroxide (H2O2), 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 $O2^{\bullet -}$ to form highly reactive peroxynitrite (ONOO−).

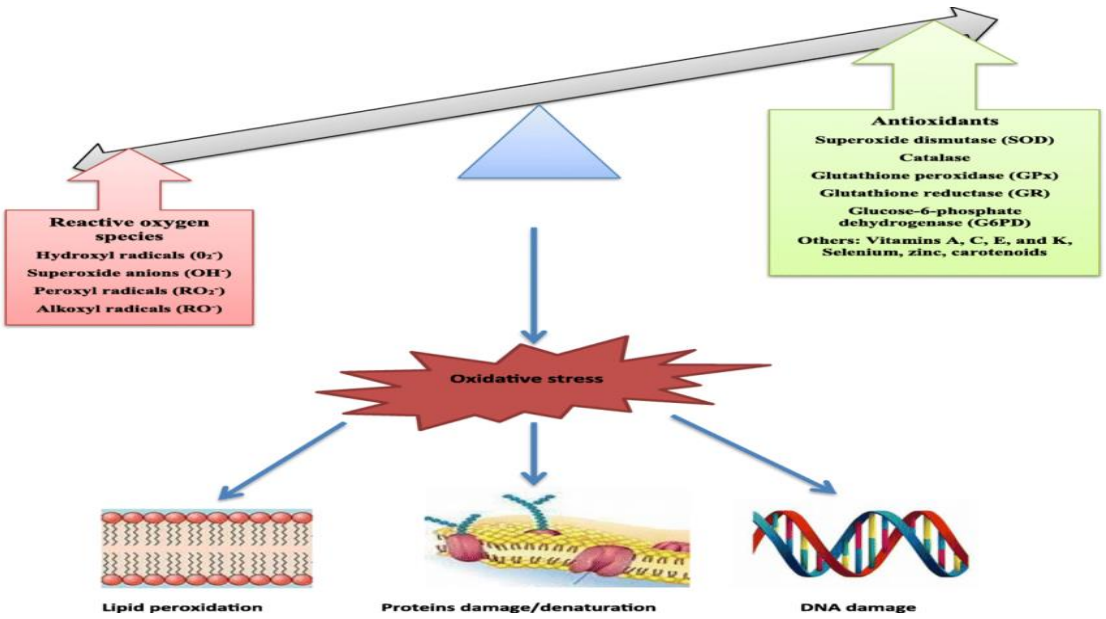

Figure 1: Imbalance between oxidatnt and antioxidants

Oxidants

Oxidants/Free radicals are the products of normal cellular metabolism. A free radical can be defined as an atom or molecule containing one or more unpaired electrons in valency shell or outer orbit and is capable of independent existence. The odd number of electron(s) of a free radical makes it unstable, short lived and highly reactive. Because of their high reactivity, they can abstract electrons from other compounds to attain stability. Thus, the attacked molecule loses its electron and becomes a free radical itself, beginning a chain reaction cascade which finally damages the living cell (Mukherji SM et al., 1986). Both ROS and RNS collectively constitute the free radicals and other non-radical reactive species (Pham-Huy LA et al., 2008). The ROS/RNS play a twofold job as both beneficial and toxic compounds to the living system.

At moderate or low levels ROS/RNS have beneficial effects and involve in various physiological functions such as in immune function (i.e. defense against pathogenic microorganisms), in a number of cellular signaling pathways, in mitogenic response and in redox regulation (Valko M et al.,2007, Nordberg J et al.,2001). But at higher concentration, both ROS as well as RNS generate oxidative stress and nitrosative stress, respectively, causing potential damage to the biomolecules. The oxidative stress and nitrosative stress are developed when there is an excess production of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other side. Most importantly, the excess ROS can damage the integrity of various biomolecules including lipids (Yla-Herttuala S.,1999), proteins (Stadtman ER et al.,2000) and DNA (Marnett LJ .,2000) leading to increased oxidative stress in various human diseases such as diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, cataracts, cardiovascular diseases, respiratory diseases as well as in aging process. Among the most important ROS are the hydroxyl radical (•OH), the superoxide radical anion ($O2•^{-}$), nitric oxide (NO•), and peroxyl radicals (ROO•) (Firuzi et al., 2011), as well as non-radical species such as hydrogen peroxide (H2O2), singlet oxygen (O2), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^{-}$) (Valko et al., 2007).

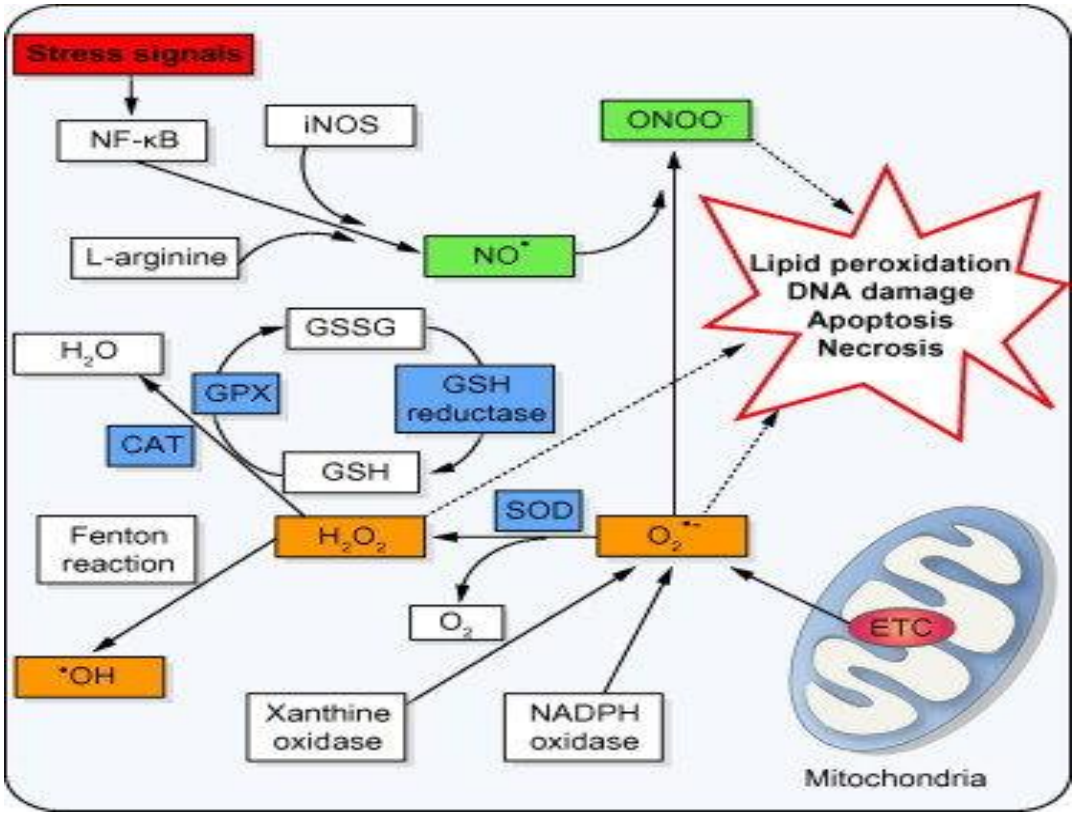

Figure 2: major pathways for the formation of reaction oxygen species and reactive nitrogen species.

ROS and RNS are known to play dual roles as species that may be either deleterious or beneficial in living systems (Valko 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 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.

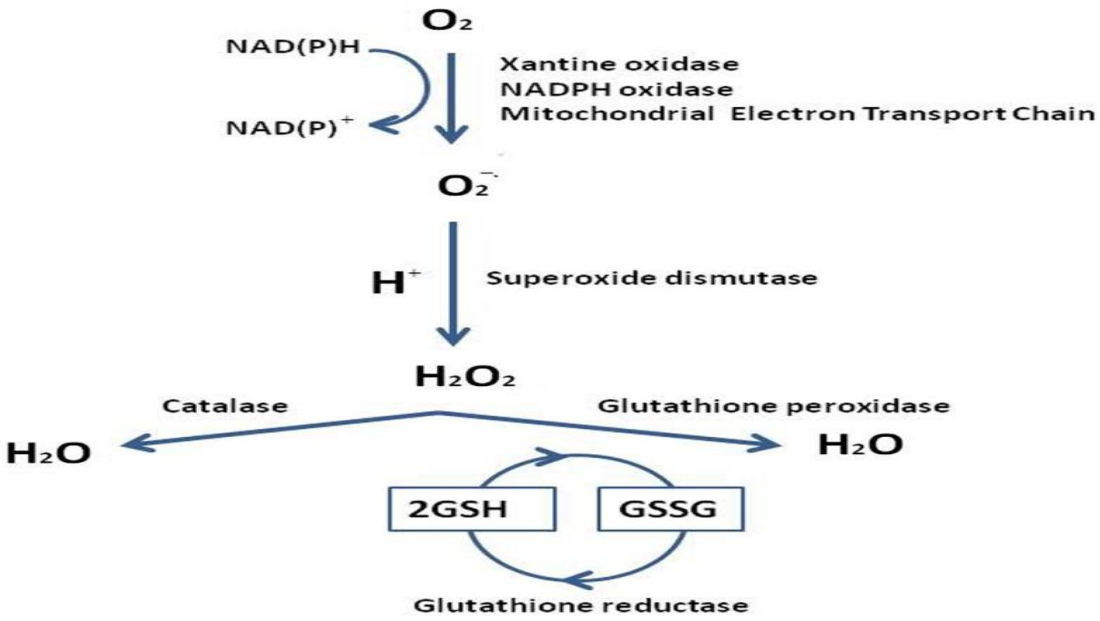

Figure 3: Reactive oxygen species

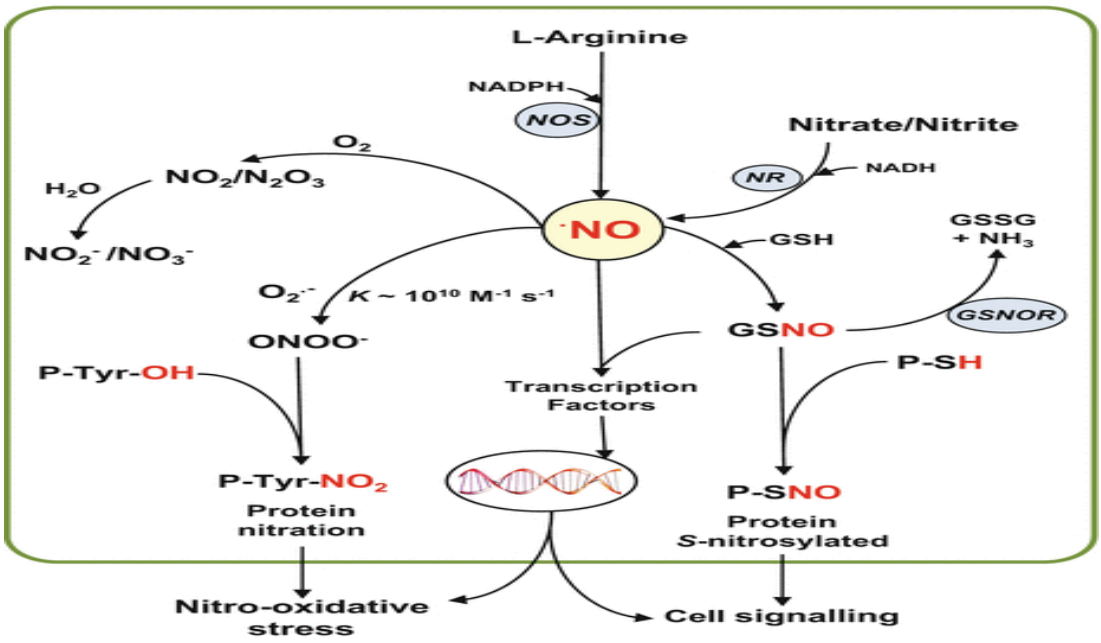

Figure 4: Reactive nitrogen species

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:

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

• 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.

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

• 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 antioxi-dant defense system including catalase (CAT), superoxide dismutase (SOD) and reduced glu-tathione (GSH) counteracts and regulates overall ROS levels to maintain physiological homeostasis. Lowering ROS levels below the homeostatic set point may inter-rupt the physiological role of oxidants in cellular prolif-eration and host defense. Similarly, increased ROS may also be detrimental and lead to cell death or to accelera-tion in ageing and age-related diseases. Traditionally, the im- pairment caused by increased ROS is thought to re-sult 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.

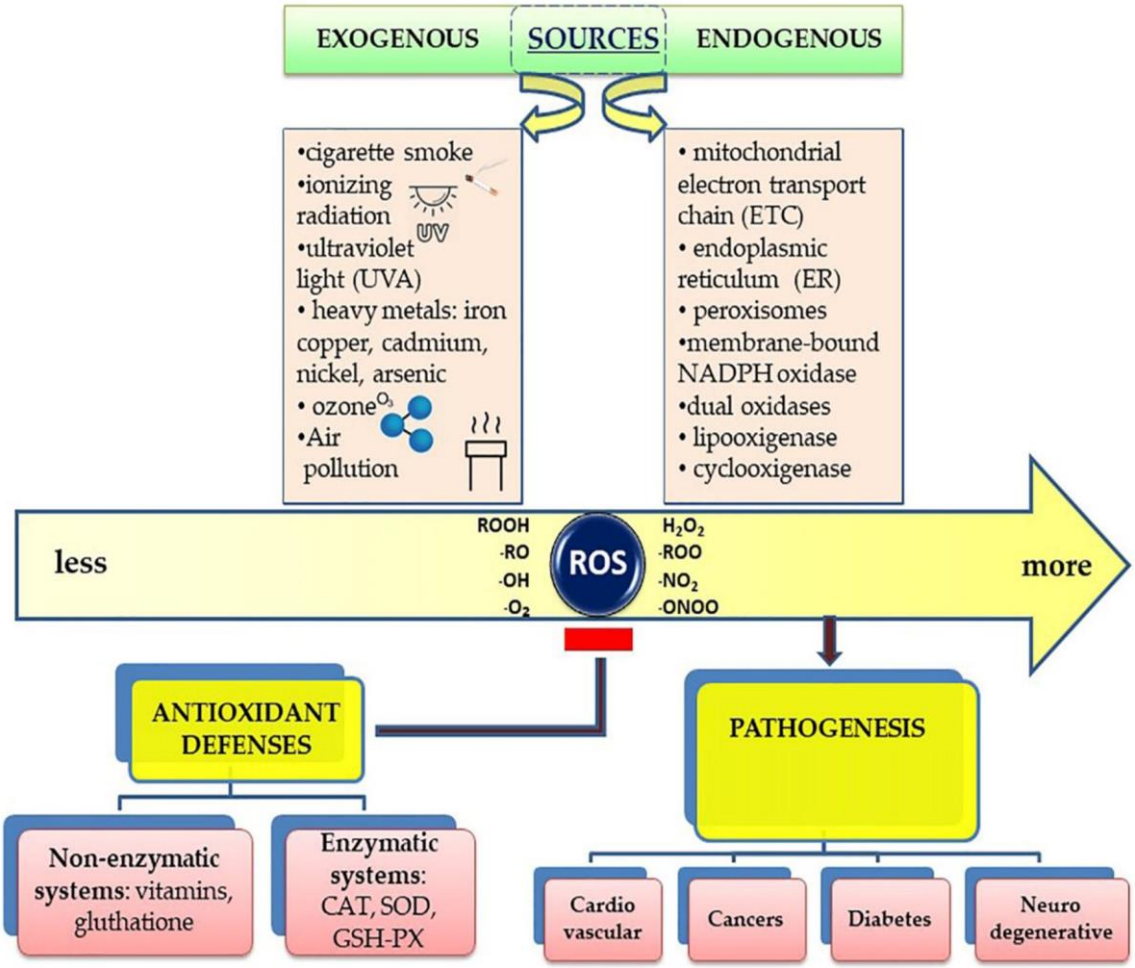

Figure 5: Sources of free radical and their effects on the human body.

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.

Endogenous sources of oxidants

The ROS can be produced from either endogenous or exogenous sources. The endogenous sources of ROS include different cellular organs such as mitochondria, peroxisomes and endoplasmic reticulum, where the oxygen consumption is high.

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, esinophils 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

Most of the intracellular ROS are derived from mitochondria. The superoxide radicals are produced at two major sites in the electron transport chain, namely complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase). The transfer of electrons from complex I or II to coenzyme Q or ubiquinone (Q) results in the formation of reduced form of coenzyme Q (QH2). The reduced form QH2 regenerates coenzyme Q via an unstable intermediate semi quinone anion in the Q-cycle. The formed immediately transfers electrons to molecular oxygen leading to the formation of superoxide radical. The generation of superoxide is non-enzymatic and therefore higher the metabolic rate, the greater is the production of the ROS (Finkel T et al., 2000).

The superoxide anion is converted to hydrogen peroxide by the action of mitochondrial superoxide dismutase (MnSOD). H2O2 can be detoxified by the Catalase (CAT) and glutathione peroxidase (GPx). The other mitochondrial components which contribute to the formation of ROS include monoamino oxidase, aketoglutarae dehydrogenase, glycerol phosphate dehydrogenase and p66shc (Starkov AA., 2008) the p66Shc is an important member of the ShcA protein family, which contains another two more proteins, p46Shc and p52 Shc. The mammalian p66Shc is a 66-kDa isoform of the growth factor adaptor protein involved in apoptosis. It mediates the production of ROS in mitochondria. Most of the p66Shc is located in cytoplasm with a small fraction localized in the mitochondrial intermembrane space. Upon oxidative stress, p66Shc translocates to mitochondrial intermembrane space, where it associates with cytochromec, thus inducing ROS generation (Giorgio M et al., 2005).

ROS production in peroxisomes

In peroxisomes the respiratory pathway involves the transfer of electrons from various metabolites to the oxygen leads to H2O2 formation (De Duve C et al., 1996), but is not coupled to oxidative phosphorylation to produce ATP instead

free energy is released in the form of heat. The other free radicals produced in peroxisomes include H2O2, O2 •- OH• and NO•. The b-oxidation of fatty acids is the major metabolic process producing H2O2 in the peroxisomes. As reviewed elsewhere, the different peroxisomal enzymes such as acyl CoA oxidases, D-amino acid oxidase, L-a-hydroxy oxidase, urate oxidase, xanthine oxidase, D-aspartate oxidase have been shown to produce different ROS (Schrader M et al., 2006). Peroxisomes contain a variety of enzymes that generate H2O2 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 et al., 2011). 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.

ROS production in endoplasmic reticulum

The enzymes of endoplasmic reticulum such as cytochrome p-450 and b5 enzymes and diamine oxidase contribute to the formation of ROS. Another important thiol oxidase enzyme, Erop1p catalyses the transfer of electrons from dithiols to molecular oxygen results in the formation of H2O2 (Gross E et al., 2006). ER is another membrane-bound intracellular organelle, but unlike mitochondria, it is primarily involved in lipid and protein biosynthesis. ER when under stress produces ROS mainly by two mechanisms during disulfide bond formation. First, ROS are produced as a by-product during transfer of electrons from protein thiol to molecular oxygen by endoplasmic reticulum oxidoreductin-1 (ERO-1) and protein disulfide isomerase (PDI) (Bhandary et al., 2003). Alternatively, ROS can be created during misfolding of protein due to depletion of GSH (Santos 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 and Chevet 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 Ca2+ leakage into the cytosol, increasing ROS production in the mitochondria (Malhotra and Kaufman, 2007).

Exogenous sources of oxidants

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

Ionizing radiation acts by converting hydroxyl radicals, superoxides and organic radicals into organic hydroperoxides and hydrogen peroxide. Subsequently, the peroxides react with the metal ions of Fe and Cu at the cellular level through redox reactions with secondary oxidative activity. Several studies have shown that the exposure of fibroblasts to alpha particles has led to an intracellular increase of oxygen and an accelerated production of peroxide at this level (Spitz et al., 2004; Spitz and Hauer-Jensen, 2014).

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 (H2O2), superoxide radical (O2-) and ultimately oxygen (O2). 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 protein1 (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.

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 18 matter (mixture of solid particles and liquid droplets suspended in the air) catalyzes the reduction of oxygen.

Exposure to ozone can affect lung function even in healthy individuals by increasing inflammatory infiltrate in the respiratory epithelium (Wu X. et al., 2019)

Heavy metal ions

Heavy metals play an essential role in the production of free radicals (Sciskalska et al. ´ , 2014). Iron, copper, cadmium, nickel, arsenic, and lead can induce free radicals by Fenton or Haber-Weiss type reactions, but also by direct reactions between metal ions and cellular compounds with similar effects – for example, the production of thiol type radicals. Lead triggers lipid peroxidation and increases glutathione peroxidase concentration in brain tissue. Arsenic induces the production of peroxides, superoxides, nitric oxide and inhibits antioxidant enzymes such as glutathione-transferase, glutathione-peroxidase, and glutathione-reductase by binding to the sulfhydryl group. The free radicals

generated from these reactions can affect DNA, with substitutions of some DNA bases such as guanine with cytosine, guanine with thymine and cytosine with thymine (Jan et al., 2015).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.

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 $O2^{\bullet -}$. $O2^{\bullet -}$ is converted to H2O2, 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 ($O2^{\bullet -}$), singlet oxygen (O2), peroxyl radical (ROO•), nitric oxide (NO•), hydrogen peroxide (H2O2), and dimethylarsinic peroxyl radicals [(CH3)2AsOO•_] (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 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 20 damage by metal ions, such as Fe2+, Cu+, and Ni2+. Reid

et al65 showed that G → C was predominantly produced by Fe2+ while C → T substitution was by Cu2+ and Ni2+ .

Types of oxidants

Oxidants /Free radicals can be classified into following three types:

1. Reactive oxygen species (ROS).
2. Reactive Nitrogen species (RNS)
3. Reactive chlorine species (RCS)

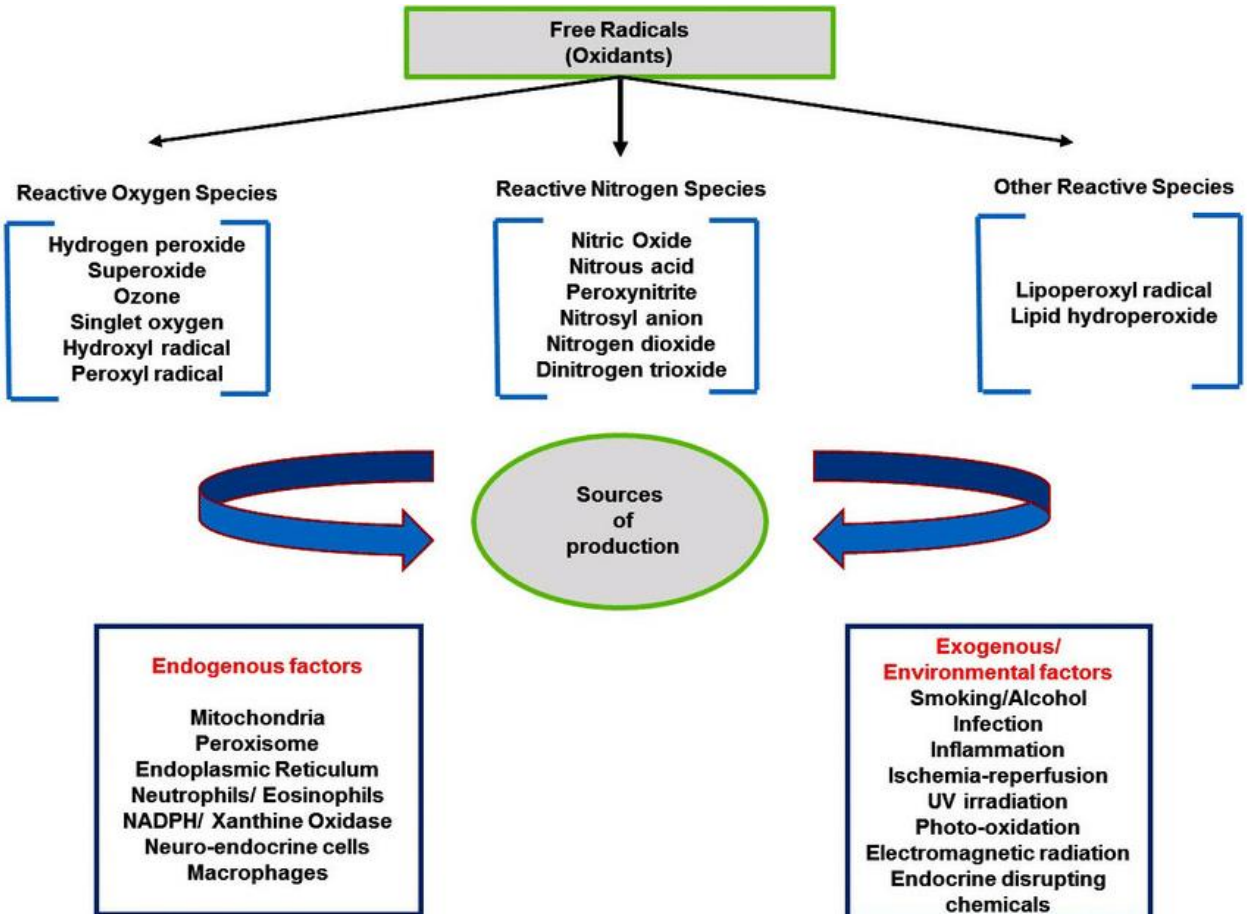

Fig 4: Types of free radical/oxidants and their exogenous and endogenous sources of generation

Reactive oxygen species (ROS) are free radicals generated physiologically in human body which having different types mention in following:

- Superoxide (O2),
- Hydrogen peroxide (H2O2)
- Hydroxyl radical (HO)
- Singlet oxygen (1O2)
- Ozone (O3)

Reactive nitrogen species

Reactive Nitrogen species (RNS) are special forms of ROS that contain nitrogen(Doshi SB et al 2012). Similar to ROS, RNS can also include radicals such as nitric oxide (NO) and nitrous acid (HNO2) and non-radicals such as nitrogen dioxide (NO2) and dinitrogen tetraoxide (N2O4) . Nitric oxide synthase (NOS) can convert larginine and oxygen to NO and l-citrulline in the presence of multiple cofactors, including calcium, NADPH, flavin mononucleotide (FMN), calmodulin and flavin adenine dinucleotide (FAD) (Perry JM et al.,1998) . Furthermore, different forms of RNS including nitrosonium cation (NO+), nitroxyl anion (NO−) or peroxynitrite (ONOO−) can be produced as a result of the interactions of NO with various chemicals. Although NO is not reactive enough to cause DNA damage, NO can react with superoxide anion to produce peroxynitrite (ONOO−) (. Pacher P et al.,2007).Reactive nitrogen species (RNS) are free radicals generated physiologically in human body which having different types:

- Nitric oxide (NO)
- Nitrogen dioxide (NO_2)
- Nitrous acid (HNO_2)
- Dinitrogen tetroxide (N_2O_4)
- Dinitrogen trioxide (N_2O_3)
- Peroxynitrite (ONOO)
- Peroxynitrous acid (ONOOH)
- Alkyl peroxynitrites (ROONO)
- Nitronium cation (NO^{2+})

- Nitryl chloride (NO_2Cl)

Reactive chlorine species

Reactive chlorine species (RCS), including the **chlorine radical ($Cl^{\bullet}$) and its derived secondary radicals (e.g., $Cl_2^{\bullet-}$)**, are another type of reactive species with high oxidation rates for organic foulants, comparable to $^{\bullet}OH$.

Reactive chlorine species (RCS) modify several biomolecules, including amino acid side chains, nucleotides, and lipids. Sulfur-containing amino acid side chains, such as cysteine, are particularly susceptible to RCS oxidation and chlorination, reacting ~100-fold faster than other biomolecules (Gray MJ et al.,2013, Sultana S et al.,2020).

Antioxidants

The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in situ (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention.

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 (O2 • ⁻) into hydrogen peroxide (H2 O2) by reduction. The oxidant formed (H2 O2) is transformed into water and oxygen (O2) by catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H2 O2 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)(Bahorun T et al.,2006, Droge W.,2002). 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 (. Droge W.,2002 , Willcox JK et al.,2004). 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.

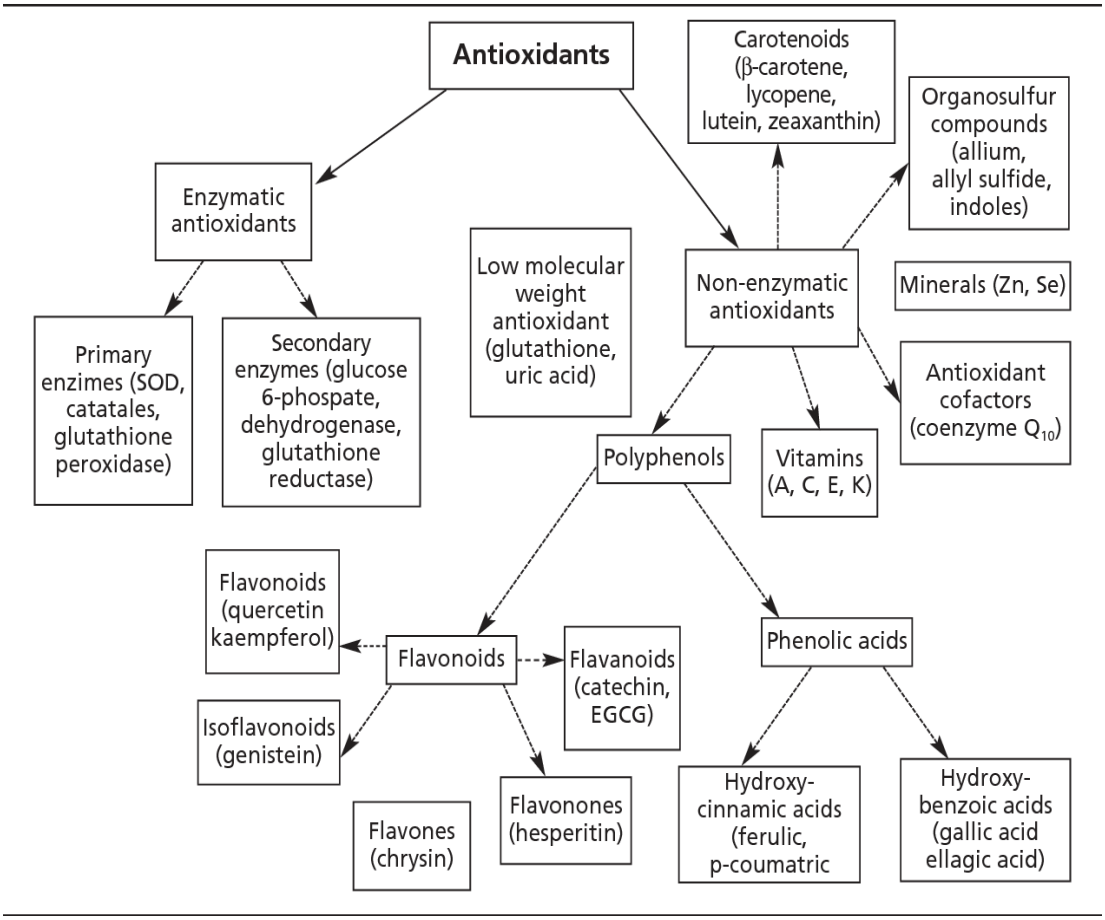

Figure 6:Classification of enzymatic and nonenzymatic antioxidants.

Enzymatic Antioxidants

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes. Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of

multiple enzymes, with superoxide dismutases catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide.

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, 22 peroxisomes, and apoplast ((Wuerges et al., 2004; Corpas 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 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 live.

Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms. Glutathione peroxidase is an enzyme containing four seleniumcofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. 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 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, 24 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- 25 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, anti-hypercholesterolemic, 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.

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 crosslinkage 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, artheroscleosis, 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.

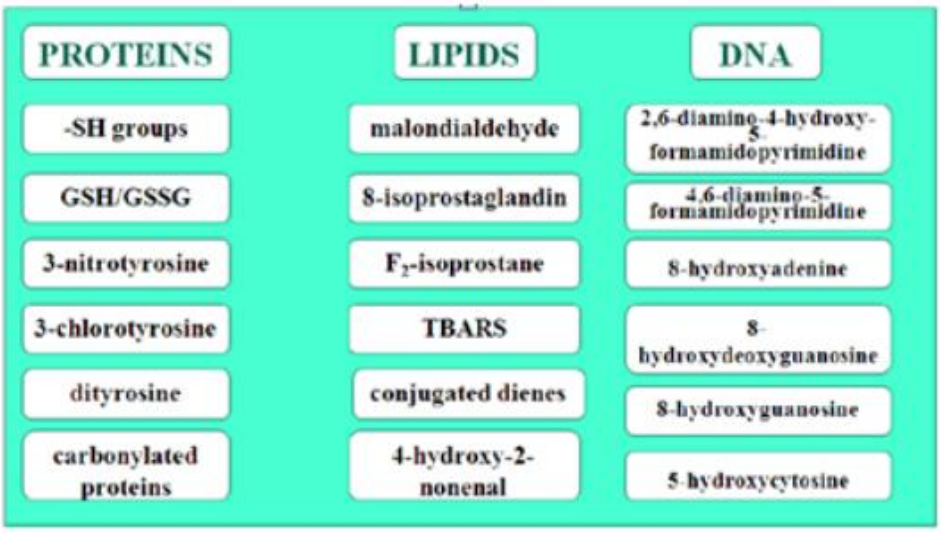

Figure 7: Markers of oxidative damage.

Role of oxidative stress in diseases

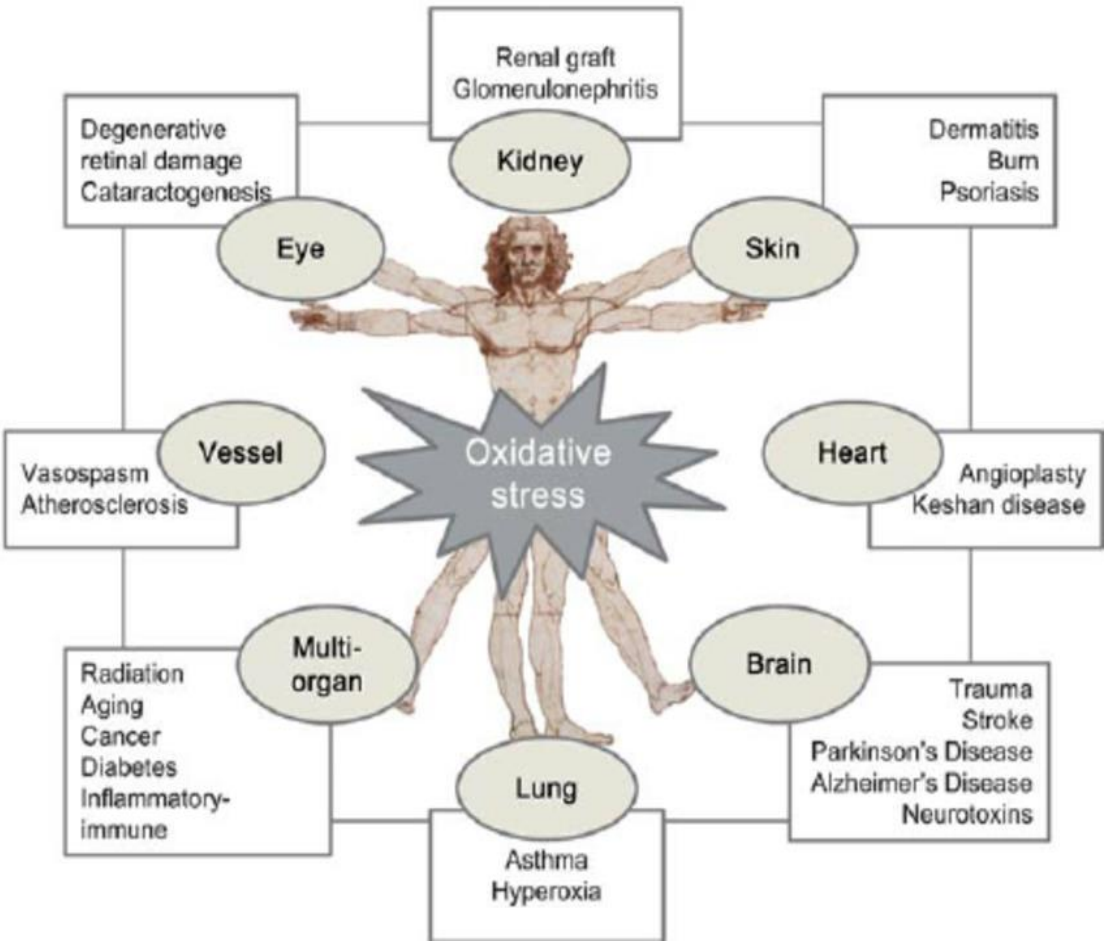

Figure 8: deleterious effect of oxidative stress on human health

Neurodegenrative Diseases

The central nervous system (CNS) is particularly susceptible to the oxidants due to the presence of high lipid content, high consumption of oxygen, and low levels of antioxidant enzymes, for example, SOD is localized primarily in neurons, and GSH and GPx are localized in astrocytes (Pollack M et al.,1999). The lipid peroxidation by ROS leads to progressive loss of membrane fluidity, decreases membrane potential, and increases permeability to ions such as Ca2+. The regions of the brain such as hippocampus, substantia nigra, and the striatum are particularly susceptible to attack by free radicals. The oxidative stress state has been also implicated in several neurodegenerative diseases such as Alzheimer's ,Parkinson's, Huntington's, lateral amyotrophic sclerosis, and multiple Sclerosis .

Cancer

The development of cancer in humans is a complex process including cellular and molecular changes mediated by diverse endogenous and exogenous stimuli. It is well established that oxidative DNA damage is responsiblefor cancer development.Cancer initiation and promotion are associated with chromosomal defects and oncogene activation induced by free radicals. A common form of damage is the formation of hydroxyled bases of DNA, which are considered an important event in chemical carcinogenesis (Valko M et al.,2004 , Halliwell B.,2007). This adduct formation interferes with normal cell growth by causing genetic mutations and altering normal gene transcription. Oxidative DNA damage also produces a multiplicity of modifications in the DNA structure including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites.

For example, tobacco smoking and chronic inflammation resulting from noninfectious diseases like asbestos are sources of oxidative DNA damage that can contribute to the development of lung cancer and other tumors. The highly significant correlation between consumption of fats and death rates from leukemia and breast, ovary, rectum cancers among elderly people may be a reflection of greater lipid peroxidation (Droge W.,2002, Young I et al.,2001).

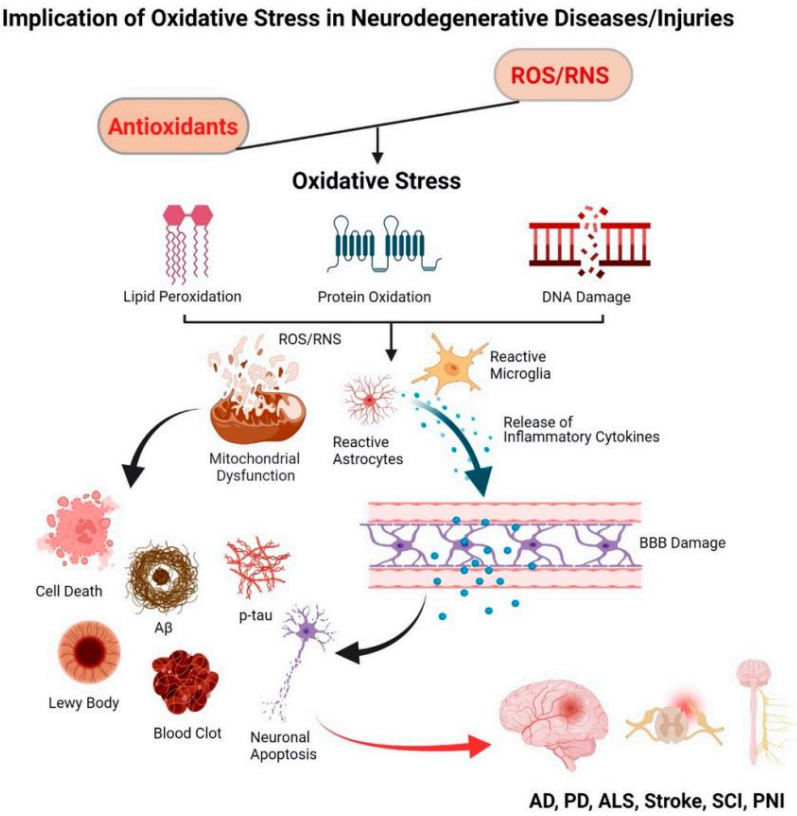

Figure 9: Role of oxidative stress in neurodegenerative disease and cancer

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.

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 (Sastre J et al.,1996). 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.

Bacterial diseases

Tuberculosis

Granuloma formation in TB represents a component of host defence as it results in the containment of infection thus preventing the spread of tubercle bacilli within the same host as well as between the susceptible hosts. However, intracellular bacilli can be released from the granulomas due to cell death/necrosis caused primarily by oxygen free radicals produced by macrophages and other cells. Oxidative stress results in cellular damage due to the oxidation of amino acid residues on proteins, forming protein carbonyls, as well as oxidation of protein (Castegna A et al.,2003) , ultimately resulting in protein fragmentation. On the other hand, both nitrogen intermediates and oxygen radicals may also play an important role in the suppression of infection through mycobacterial inactivation/killing. The pathogenesis of tuberculosis is

finally determined by the balance between various mechanisms, such as the generation of reactive oxygen freeradicals to kill the intracellular bacilli, the antioxidant mechanisms employed by mycobacteria to escape the killing by free radicals in phagocytic cells and antioxidant mechanisms by host cells to prevent the tissue damage. Despite the various host antioxidant mechanisms, the accumulation of free radicals results in cellular and systemic oxidative stress (Palanisamy GS et al.,2011). Various studies have reported the increased levels of markers of oxidant-mediated tissue damage in the peripheral circulation of humans with active tuberculosis.

Oxidative stress has also been implicated in the pathogenesis of lung fibrosis and lung dysfunction in tuberculosis patients, even following antimicrobial therapy. Besides the elevated levels of various by-products of free radical generation, depletion of various antioxidants, e.g. ascorbic acid and glutathione, has also been reported in TB patients, further aggravating the oxidative stress in these patients. These studies clearly show the association between excessive oxidative stress and active TB. Recently, a significant correlation between high oxidant concentration and low concentration of antioxidants with varying bacillary load as well as severity of disease has been shown; it was also suggested that antioxidants supplementation may prove benefi cial as well as may help in fast recovery of TB patients. In another study, it has been demonstrated that the oxidative stress index significantly increased in untreated TB patients and decreased in TB patients on anti-tubercular therapy (ATT) with antioxidant supplementation. Hence, oxidative stress index can be considered as a novel marker in TB patients (Dalvi SM et al.,2012). In vitro, TB has been shown to be susceptible to H_2O_2 induced damage. This reaction often referred to as ‘oxidative burst’ is further aggravated by chlorination in macrophages and neutrophils that increases the toxicity of reactive oxygen species (ROS). At low concentrations, ROS are important for normal cellular functions by acting as signalling molecules in immunological response, blood circulation and endocrine functions. During oxidative stress, the excess of ROS causes cell injury by oxidising various macromolecules including proteins, lipids and DNA, thus assuming an important role in the pathogenesis of various diseases.

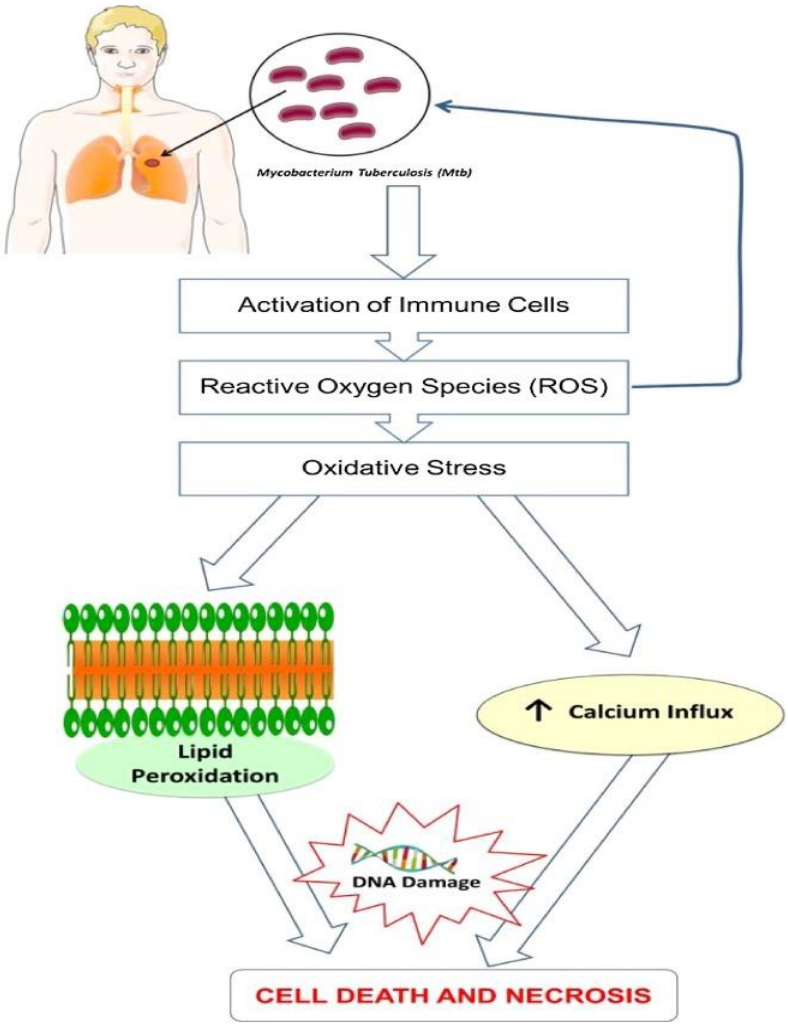

Figure 10: Mycobacterium tuberculosis and oxidative stress complexity

Community-Acquired Pneumonia (CAP)

Oxidative stress plays a crucial role in the development and progression of community-acquired pneumonia, the most common infectious illness. It was revealed that five times higher H2O2 is released in exhaled air of CAP patients than control and the amount decrease with treatment. The authors suggested that the sources of H2O2 were activated leucocytes, monocytes, and macrophages, and development of OS leads to the activation of neutrophils and other effector cells with generation of excess active oxygen forms in the lungs of CAP patients (Majewska et al. 2004). These ROS migrate through the alveolar-capillary membrane in the process of gas exchange and are able to induce the OS development in the erythrocytes. Trefler in their work showed increased levels of TBARS in CAP caused by bacteria compared to control. However, they observed lower TBARS levels and increased glutathione redox

system in viral CAP caused by H1N1 compared to normal. In another study, Vilen et al. showed that there was significant increase in advanced oxidation protein products (AOPP) and MDA in blood plasma of CAP patients compared to control group though there was no change in the concentration of reactive protein carbonyl derivatives. Muravlyova showed that parameters like erythrocyte aggregation, total oxidant status, and OS index increased in IIP patients than controls, whereas some parameters like erythrocyte deformability, PV, and total antioxidant status remain unaltered. It has been found that post influenza Staphylococcus aureus pneumonia leads to extensive lung inflammation even after antibiotic treatment. The chronic granulomatous diseases in humans are linked to X chromosome-linked Nox2 expression. Yuanyuan Chen et al. demonstrated increase in OS and cytokines like TNF-α and IL-6 in the lung and peripheral blood with increase in the severity of CAP. Vitamin C supplementation inhibited ROS, DNA damage, TNF-α, and IL-6 in LPS stimulated macrophages. It also inhibited autophagy in MH-S cells exposed to LPS and H2O2 (Chen et al. 2014). Rodrigo et al. observed lower FRAP and the GSH/ GSSG ratio and increased lipid peroxidation in both plasma and erythrocytes in CAP than control values. Thus, the antioxidant status alterations are correlated with clinical severity.

Many studies have showed that administration of antioxidants such as Vitamins E and C have positive effects on oxidative biomarkers in *in-vivo* and *in-vitro* models of CAP. There are controversies regarding the use of vitamins in CAP though (Merchant et al. 2004).

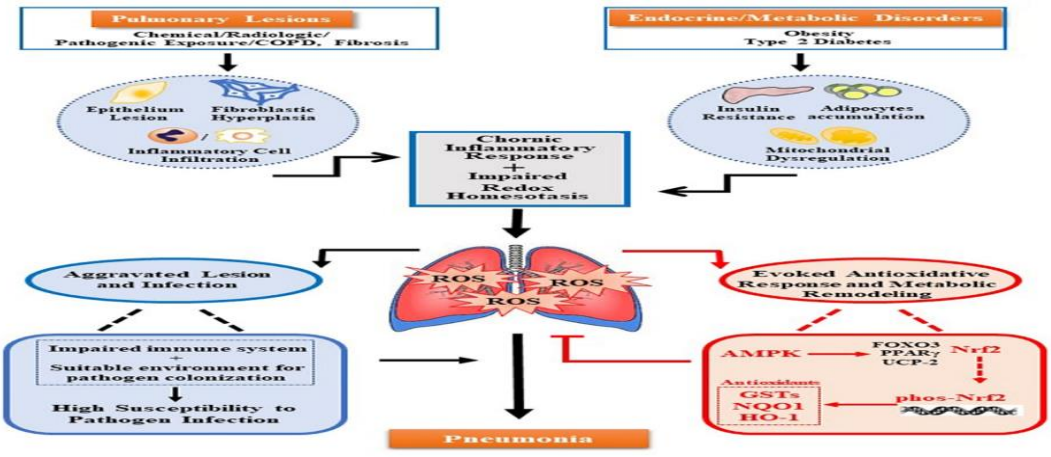

Figure 11: Pathogenesis of Pneumonia

Leprosy

Leprosy is a chronic granulomatous disease caused by Mycobacterium leprae. Although the prevalence of leprosy has decreased over past 20 years, the incidence of newly detected cases is still high (Sunil D et al.,2013) India records the highest number of new leprosy cases in the world. Depending on host resistance, leprosy may present as tuberculoid or lepromatous type with a spectrum of intermediate stages appearing between the two.

The pathogenesis of leprosy has been found to be influenced by a number of factors including oxidative stress (OS). The major defense against microbial infection is the macrophage system. The infected macrophages show increased phagocytosis and oxygen consumption known as “respiratory burst,” associated with production of free radicals like reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radicals These free radicals apart from killing the bacteria, also damage host tissues mainly lipids, proteins and nucleic acids. Cells have the “antioxidant defense” system that comprises enzymatic antioxidants like superoxide dismutase (SOD), catalase, peroxidase and non-enzymatic antioxidants like Vitamins A, E, C and glutathione (Yu BP 1994) Under normal conditions, the production of ROS is

presumed to be in balance with antioxidant defenses. OS is a severe disruption of balance in favor of ROS. In leprosy, this delicately maintained physiological balance is shifted in favor of ROS from phagocytes. The drugs used in multidrug therapy (MDT) also generate ROS and may further increase the damage to host tissues. In addition to increased production of ROS, decreased anti-oxidants contribute to OS in leprosy. Malnutrition co-exists with the depletion of micronutrients and antioxidant vitamins in leprosy and aggravates infection, while infection with intracellular M. leprae adversely affects the nutritional status. Prime targets of peroxidation by ROS are polyunsaturated fatty acids (PUFA) in membrane lipids, which are degraded to malondialdehyde (MDA). The level of MDA in serum serves as a marker of cellular damage due to free radicals. Another way of studying OS is to investigate the serum antioxidants. SOD is an enzymatic antioxidant that catalyzes the dismutation of superoxide ion into oxygen and hydrogen peroxide. The ratio of MDA/SOD in serum may be considered as an index of OS (Abdel-Hafez HZ et al.,2010).

Cestrum nocturnum

Kingdom	**Plantae**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Order	**Solanales**
Family	**Solanaceae**
Genus	**Cestrum**
Species	**C.nocturnum**

There are more than 300 species contain genus Cestrum mostly in Asia, Europe, Africa as well as most of them are grown in warm ‘subtropical’ as well as ‘tropical regions’. *C. nocturnum* is a member of the family Solanaceae. It is a strongly scented flower that blooms at night thus alternatively known as ‘lady of the night’, ‘Queen of the night’ and ‘night blooming Jasmine’. Jasmines generally grow in all types of soils. However, they are better adapted to rich

loamy or dry sandy and irrigated soil. n soil with more clay, the vegetative growth is vigorous but flower production is lest in amount, while in soil with gravel, the plants exhibit stunted growth. *C. nocturnum* is used as a remedy for different health disorders.

This sprawling shrub has glossy simple leaves, vine like stems, greenish-creamy white tubular flowers and fleshy berries. The berries are marfil white or aubergine in colour. The species name 'nocturnum' refers to the species' habit of opening its small, heavily-scented flowers at night. The flowers release powerful sweet perfume at night. It is made into a rare attar (raat ki rani) which is used in Indian and Middle East perfumery. It is said to be the world's strongest smelling plant. Indeed, the scent can reach up to 165 feet away from the location of plant (Ratsch and Christian 1998; Sarkar et al., 2016). According to WHO, more than 80% of developing country's population depends on plant-based medicines for their health care needs. From the time immemorial, this shrub is used as a traditional medicine. In India the Malasar people use its juice for cataracts. It contains secondary metabolites such as saponins, flavonoids, cardiac glycosides, alkaloids, steroids and tannins which have biological activity, kindling scientific interest. Optimal growth occurs at about 80˚F. Though night blooming jasmine blooms in night, it requires at least 6 hours of sunlight and partial shade every day to bloom.

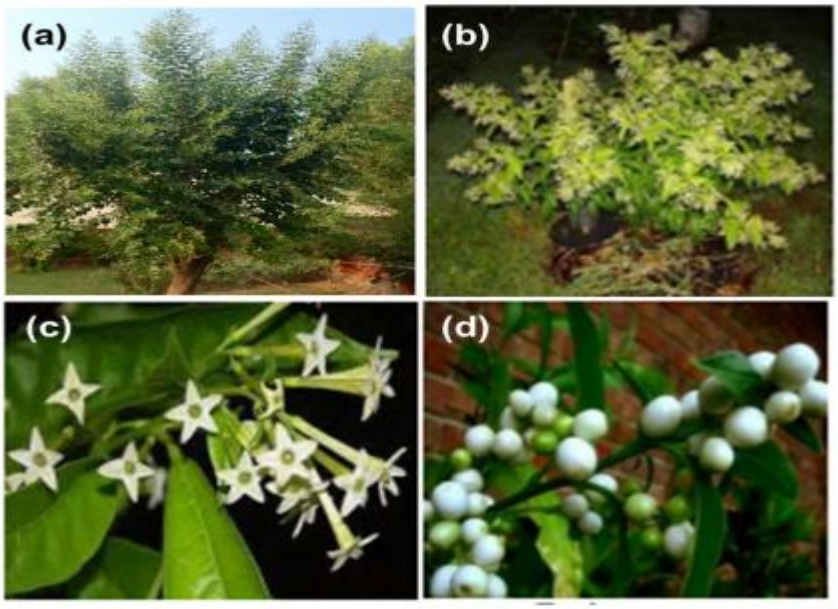

Figure 14: Cestrum nocturnum A: Whole Tree, B: Leaf C: Flower, D: Fruits

Traditional uses

CN flowers are presented as offerings to Shiva and Ganesh in Kathmandu. Nepalese shamans create a ritual incense from the leaves and fresh flowers, eat the fresh flowers and smoke them when dried to increase the spiritual healing energies. The plant is also used as a stupefying charm medicine in West Indies. The Yucatec Maya use CN leaves and flowers in hot baths as a treatment for night sweats. The plant is occasionally added to liquor in Kalinchok, a region north of Kathmandu. CN has long been used in traditional Chinese medicine (TCM) to treat digestive diseases for centuries (Illustrated Handbook of Chinese Advanced Plant). Numerous studies have identified that CN has a great deal of pharmacological actions, including analgesic action, central inhibitory action, and antidiabetic activity (Kamboj et al., 2013).

Medicinal uses

In traditional medicine, leaves of *Cestrum nocturnum* have been used for their pharmacological significance in burns and swellings. It is also used for treating epilepsy (He´ctor and Marı´a 2008). Pharmacological studies on the plant proved that the leaves have significant analgesic and bactericidal activity (Zeng et al., 2003; Avijit et al., 2013). The volatile oil is known to be mosquito-repellent and hence *C. nocturnum* is used to prevent malaria in several African Nations (Jawale at al., 2010). Local anaesthetic effect, inhibitory effect on central nervous system and cardiac arrthymic effect of plant are also documented. Zhong et al., in 2008 reported that n-butanol and polysaccharide extracts from C. nocturnum has tumor inhibition ability. respectively. *C. nocturnum* possesses many pharmacological properties such as anti-inflammatory, analgesic (Mazumder et al. 2010), antimicrobial (Khan et al. 2011), antiepileptic (Perez-Saad and Buznego, 2008), anti-cancer (Zhong et al. 2008) and insecticidal (Savchenko et al. 2000) activities. Plant is also being used as a local anesthetic and CNS depressant agent (Zeng et al. 2002). Important phytoconstituents including many flavonoids, alkaloids and phenols have been reported in C. nocturnum (Prasad et al. 2013). Most of these flavonoids have hepatoprotective activity (Ali et al. 2013).

Anti-cancerous activity of *C. nocturnum*

Lu et al. performed the n-butanol part isolated from the flowers of *C. nocturnum* produced an inhibitory effect on the proliferation of human hepatocellular carcinoma Bel-7404, human gastric carcinoma SGC-7901, and cervical cancer HeLa cells in a dose-dependent manner (Lu et al., 2010). However, the fractions responsible for the antiproliferation effect of n-butanol part from *C. nocturnum* flowers and related mechanisms remain unknown. The fraction C4 and C5 extracted from the n-butanol part of *C. nocturnum* flowers showed significant cytotoxic potential towards a wide range of human malignant cell lines with low cytotoxicity to immunocytes and exhibited strong antitumor activities against Bel-7404 cells. These antitumor activities include attenuation of cancer cell proliferation as well as induction of apoptosis at the G0/G1 and G2/M phases through enhancement of DNA damage and inhibition of topoisomerase II relaxation activity (Wu et al., 2017).

Hypothesis

Plants are widely known for their antioxidant, anti-inflammatory, wound healing potential. Various traditional plant and their fruits possess anti-hypercholesterolemic, anti-diabetic, anti-cancerous, antibacterial, antifungal activity. Oxidative stress involved in progression of number of bacterial diseases. On the basis of above literature survey, we hypothesized that *C. nocturnum* may possess antioxidant and antibacterial activity.

OBJECTIVES

I. Collection, and preparation of Plant material.

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

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

IV. Assessment of antibacterial activity by agar well diffusion Method.

V. To Determine the Minimal Inhibitory Concentration (MIC).

MATERIAL AND METHODS

Collection of plant material

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

Processing of plant materials

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

Preparation of plant extracts

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

Instruments

Soxhlet Apparatus

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

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

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

Chemicals

All the solvents, antibiotic and synthetic compounds used in the study were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA). Mueller-Hinton agar was provided by Hi-media (Mumbai, India).

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 amyl alcohol were also added. The samples were made up to mark and left to react for 30 min for color development.

Test for tannins: 10 mg sample was boiled in 50 mL of distilled water and then filtered. A quantity (5 mL) of test solution was added into a test tube followed

some drop of $FeCl_3$. Formation of brownish green or blue-black coloration indicates presence of tannins.

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

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

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

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

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

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

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

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

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

Antioxidant assay

DPPH Radical Scavenging activity

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

$$\%\ \mathrm{DPPH} = \frac{\mathrm{Absorbance\ of\ Control} - \mathrm{Absorbance\ of\ test\ sample}}{\mathrm{Absorbance\ of\ Control}} \times 100$$

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

ABTS radical scavenging assay

Free radical scavenging activity of plant samples was determined by ABTS radical cation decolorization assay (Pellegrini et al., 1999). ABTS·+ cation radical was produced by the reaction between 7 mM ABTS in water and 2.45

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

Bacterial Strains and Growth Conditions

Gram-negative strains; *Salmonella Abony* (ATCC BAA-2162) and Gram-positive *Bacillus pumilus* (ATCC 7061) were adopted to assess the antibacterial activity of different fractions of *C. nocturum*. Fresh inoculum for each bacterial strain was prepared in Luria–Bertani (LB) broth and incubated at 37 °C for 20 h. Before antibacterial activity, the turbidity of the culture was adjusted to the 0.5 McFarland standard, equivalent to 1.5 × 108 CFU/mL, using LB broth.

Assessment of Antibacterial Activity

Agar Well Diffusion Method

The antimicrobial action of pure levofloxacin and *C. nocturnum* fractions was determined using the agar well diffusion method. A 100 µL fraction of microbial inoculum was obtained using a micropipette to ensure a uniform lawn of cells onto the agar plates. The agar plates were inoculated by evenly swabbing across the whole surface of the plate three times and rotating the Petri plates at a 60° angle after each application. Following that, a hole (6 mm in diameter) was aseptically punched with a sterile cork tip, and 100 µL of different fractions of *C. nocturnum* and pure levofloxacin were poured into the wells, however, phosphate buffer saline (PBS) was used as a control. The agar plates were then incubated under appropriate conditions overnight at 37 °C. Post-incubation, the Petri plates were examined for the zone of inhibition, which was quantified in millimetres using a millimetre scale. To prevent the error, the

experiments were performed three times. The inhibitory zone was determined as mean ± standard deviation.

Determination of Minimal Inhibitory Concentration (MIC)

The broth microdilution method was adopted to estimate the MICs of different fractions of *C. nocturnum* against a gram positive and gram-negative bacterium as described previously Briefly, in a 96-well plate, aliquots of 10 µL of each bacterial inoculum (1 × 105 CFU/mL) were inoculated to each well. Subsequently, serial dilutions of the extract of *C. nocturnum*, in sterilized double distilled water, within the concentration range of 200 µg/mL were then added to each well. Additionally, aliquots of free levofloxacin (100µg/mL) were added to each well for comparison. After adding the extract and levofloxacin, the plates were further incubated at 37 °C for 24 h. Afterwards, cell viability was assessed using an ELISA plate reader at 625nm. The MIC was the lowest concentration of extract or free levofloxacin that efficiently suppressed the bacterial growth after overnight incubation. PBS was added as a negative control, and the results obtained represented the mean ± SD of three independent experiments.

RESULTS

Table 1: %Yield of phytochemicals in various extracts of *C. nocturnum* leaf

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

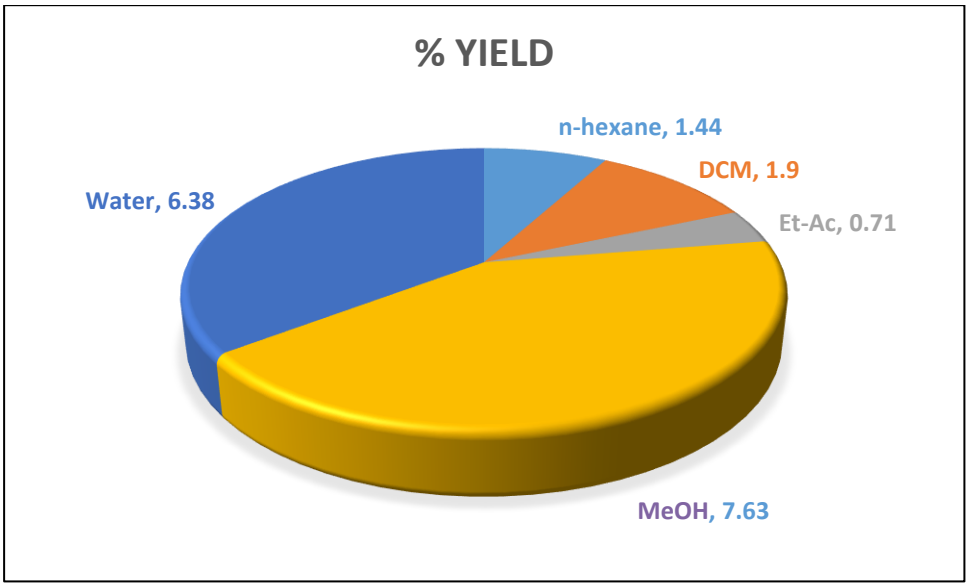

Figure 15: Pie chart representation of %yield leaf extract of *C. nocturnum*

Table 2: Phytochemical profiling of different extracts of *C. nocturnum*

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

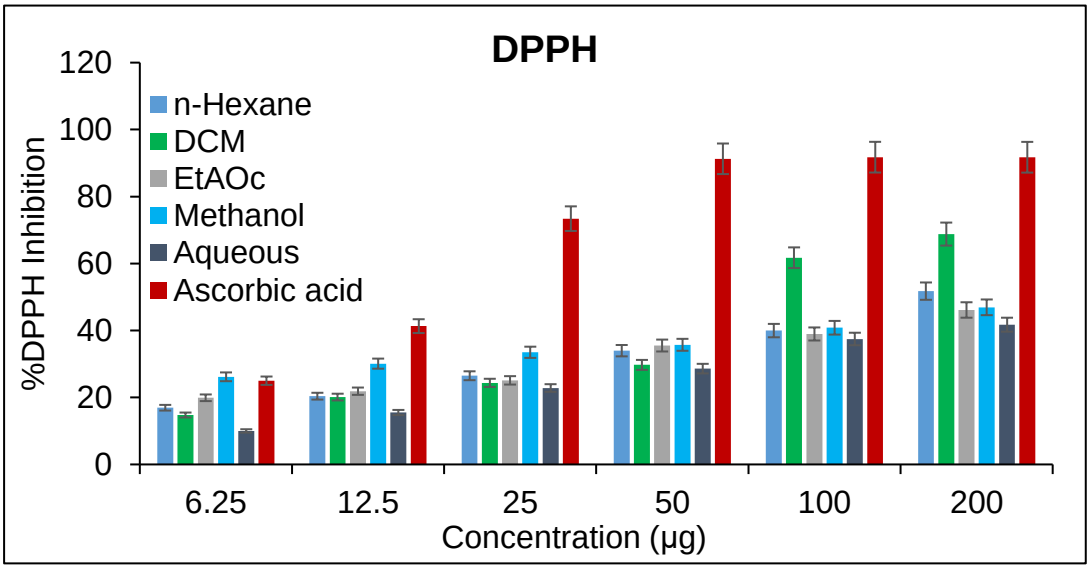

Figure 16: Percent DPPH radical scavenging activity different leaf extract of *C. nocturnum* and standard ascorbic acid. The results are mean ± S.D. of three parallel measurements.

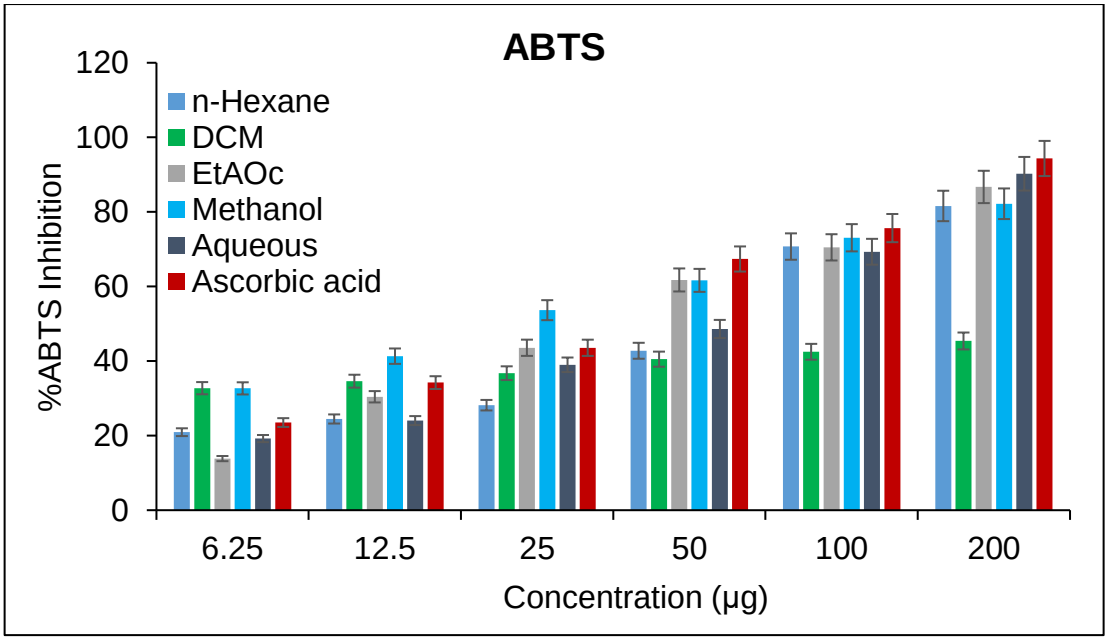

Figure 17: Percent ABTS radical scavenging activity of different leaf extract of *C. nocturnum* and standard ascorbic acid. The results are mean ± S.D. of three parallel measurements.

Assessment of Antibacterial Activity

Agar well diffusion method

The antibacterial abilities of different fractions of *C. nocturnum*, pure levofloxacin was validated by testing them against Gram-negative (*salmonella Abony*) and Gram-positive (*Bacillus pumilus*) bacterial strains. Following the experiment, it was noted that pure levofloxacin and extracts of *C. nocturnum* diffused into the agar and only DCM fraction suppressed bacterial growth (as shown in Table 3). It is noteworthy that the concentration of the extraction was only 100 µg/well in comparison to the concentration of pure levofloxacin i.e., 20 µg/well. (**Fig.18 &19).**

Determination of Minimal Inhibitory Concentration of DCM fraction of *C. nocturnum*

MIC50 values of standard Levofloxacin and DCM fraction represent the concentrations that inhibit 50% of the population of tested bacterial strains. The quantified MIC50 values were 27 µg/mL (levofloxacin) and 98.28µg/ml (DCM fraction of *C. nocturnum*) for *salmonella Abony*, respectively, as represented in **(Fig.20 A&B).**

Table 3. Zone of inhibition in mm

Drug	Salmonella Abony	Bacillus Pumilus
Levofloxacin	22 ± 0.5 mm	25.8 ± 1.5 mm
DCM	16.6 ± 1.7 mm	NA

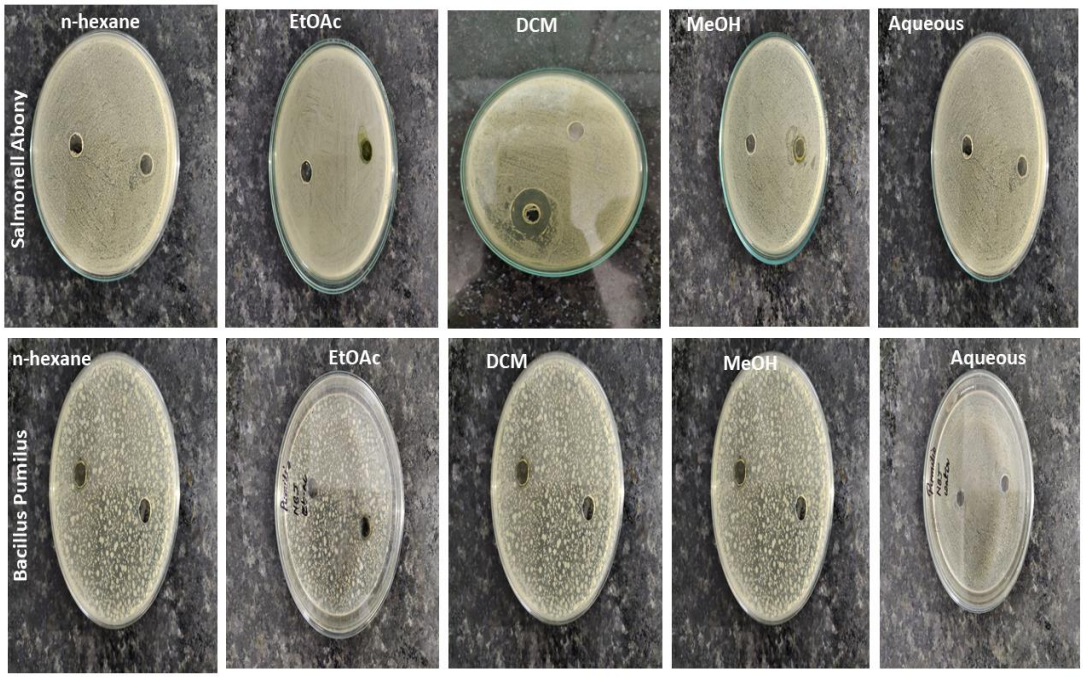

Figure 18: Antibacterial activity of Different extracts of ***C. nocturnum***

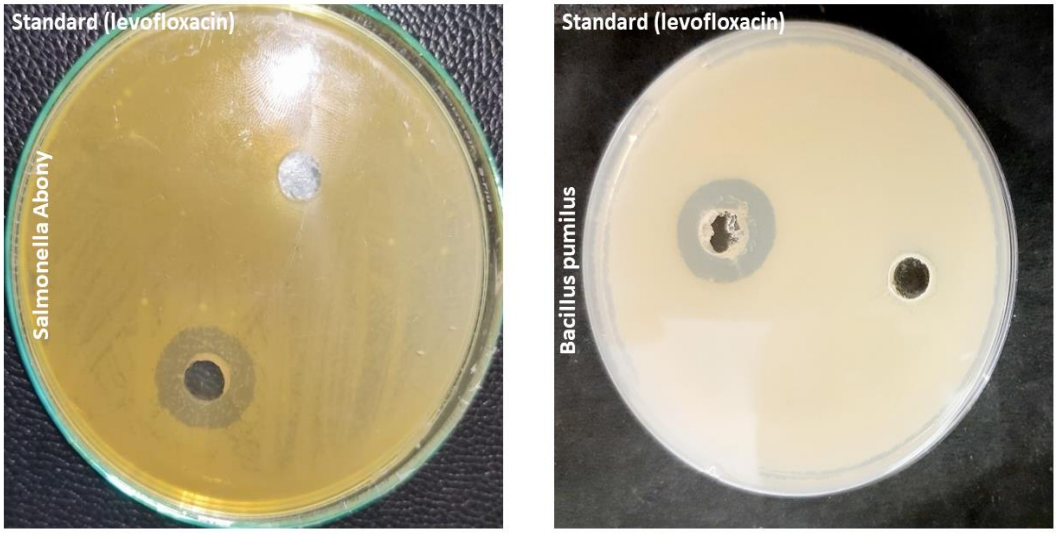

Figure 19: Antibacterial activity of levofloxacin against gram-positive and gram-negative bacterial strain.

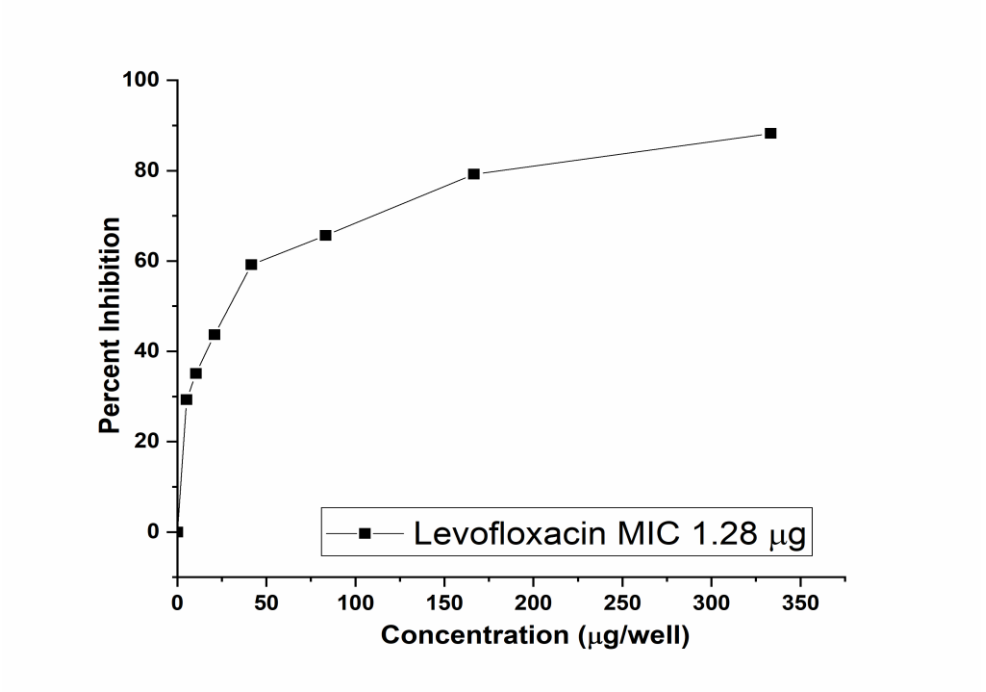

Figure 20.A: MIC graph of Standard (Levofloxacin)

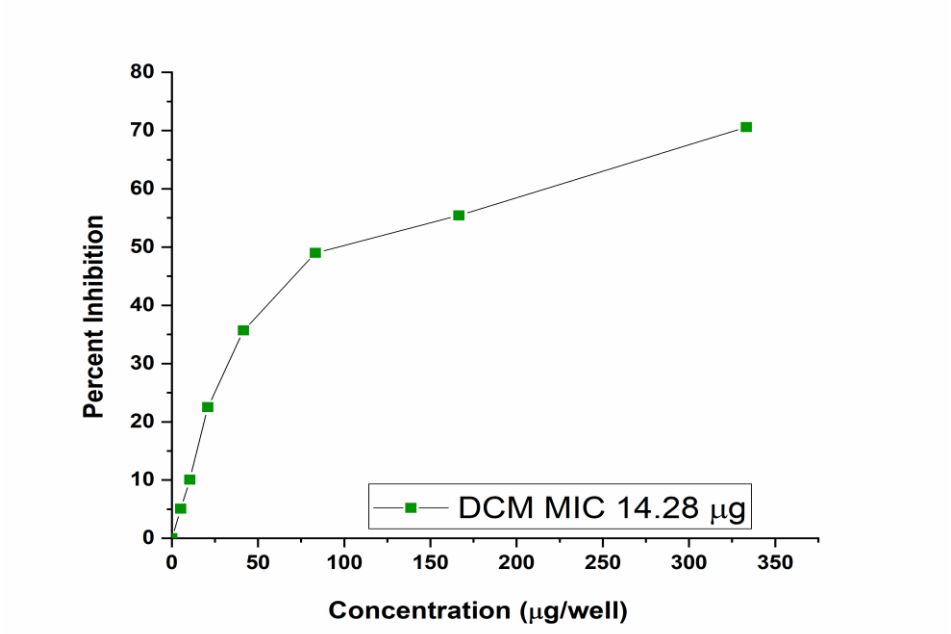

Figure.20.B: MIC graph of DCM fraction of *C. nocturnum*

DISCUSSUION

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). These free radicals include reactive oxygen free radical species (ROS), reactive hydroxyl radicals (OH−), superoxide anion radical (O2−), hydrogen peroxides (H2o), and peroxyl (ROO−).

Viral, bacterial, and parasitic infections comprise a vast group of etiological agents that cause acute or chronic diseases. According to WHO, they represent one of the major causes of human morbidity and mortality. AIDS, lower respiratory tract infections, and diarrheal diseases underlie up to 5 million deaths each year, especially in the middle- and low-income countries. In bacterial infections oxidative stress arises, at least in part, from altered metabolic pathways and has also been implicated in organ damage and the development of malignancies. *Helicobacter pylori*, for example, induces ROS-generating enzymes such as spermine oxidase and upregulates proinflammatory and pro-cancerogenic redox-regulated genes like cyclooxygenase 2. Despite the overwhelming evidence of the role of oxidative stress in acute and chronic infection and the associated diseases, the impact of the majority of infectious agents on the host redox systems is not sufficiently characterized, with published data plagued by the controversies. There are number of papers published that showed that plants have antioxidant and antibacterial activity (DS arora et al. 2007; EA palombo 2001; A sokmen et. Al. 1999). We hypothesized that *C. nocturnum.* (Night blooming jasmine) may possess the antioxidant and antibacterial activity. The only fraction which showed the efficient antibacterial activity was DCM against gram negative bacterial strain (*Salmonella Abony*). Plants have various phytochemicals such as flavonoid, phenols, tannins, coumarins, anthocyanins that involved in their antioxidant defense system. Bacterial infection and inflammation increase the oxidative stress in the body. Naturally occurring drug is needed to overcome bacterial infection and their complications, because the antibiotics possess various side effects such as hepatotoxicity, nephrotoxicity, and antibiotic resistance is a very major drawback of synthetically available antibiotics.

CONCLUSION

In conclusion, our results demonstrated the phytochemical screening, antioxidant and antibacterial activity of sequentially extracted *C. nocturnum* extract. We found that all the extracts of *C. nocturnum* were able to reduce the DPPH radicals with maximum DPPH radical scavenging in DCM, whereas, n-hexane showed minimum DPPH radical scavenging activity among all the *c. nocturnum* extract. The antioxidant potential of our extracts was also confirmed by ABTS assay. In which All the extracts from *c. nocturnum* showed some extent of reducing power but the aqueous extract showed the highest ABTS value. Thus, it is a good approach to manage oxidative stress caused by the bacterial infection as a whole with these extracts, which showed good antioxidant activities. MIC50 values of standard Levofloxacin and DCM fraction represent the concentrations that inhibit 50% of the population of tested bacterial strains. The quantified MIC50 values were 27 µg/mL (levofloxacin) and 98.28 µg/ml (DCM fraction of *C. nocturnum*) for *salmonella Abony*, respectively. Only DCM fraction of *C. nocturnum* showed the antibiotic activity. All other fractions were not able to inhibit the bacterial growth. Further, a thorough and full-fledged *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 bacterial infection and oxidative stress induced by bacterial infection.

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