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ANALYSIS OF ANTIOXIDANT EFFECTS OF QUERCETIN, RUTIN AND PHAGNALON RUPESTRE ON RATS INTOXICATED BY ALUMINIUM

Members of Jury:

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DEDICATION

To my lovely family, the greatest source of my inspiration. Thank you for all the support be it spiritual, moral and financial over the years. It wasn't any easy ride but we finally made it to the finish line.

YIGA HENRY JUNIOR

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LIST OF ABBREVIATIONS

AB : Antioxidative barrier

Al : Aluminium

AlCl3 : Aluminium Chloride

C : Control

CAT : Catalase

D.O : Optical Density

F.control: Female control rats

F.T.plant: Female rats treated with plant

GPx : Glutathione peroxidase

GSH : Glutathione reduced

GSR : Glutathione reductase

GSSG : Glutathione disulphide

GST : Glutathione S-Transferase

H202 : Hydrogen peroxide

HgCl2 : Mercury Chloride.

LPO : Lipid peroxidation

M : Mean

Mg : Milligram

Ml : Millilitre

Mm : Molar mass

Na2SO4 : Sodium Sulphate

NaCl : Sodium Chloride

NAD+ : Nicotine Amide Dinucleotide

NADH : Reduced Nicotinamide Dinucleotide

NADH : Nicotine Amide Dinucleotide

NADPH : Reduced Nicotine Amide Dinucleotide Phosphate

NO : Nitric oxide

NOS : Nitric oxide synthase

O2 : Oxygen

$O2^{\bullet-}$: Superoxide anion

OD : Optical Density

$OH^{\bullet}$: Hydroxyl radical

ONOO- : Peroxynitrate

Q : Quercetin

RNS : Reactive nitrogen species

ROS : Reactive oxygen species

SD : Standard deviation

SOD : Superoxide dismutase

TBARS : Thiobarbituric acid reactive substances

TCA : Tri-chloro Acetic Acid

T.quercetin: Male rats treated with quercetin

T.rutin: Male rats treated with rutin

LIST OF TABLES

LIST OF FIGURES

Abstract

Aluminium (Al) is the third most abundant chemical element found in nature and the most abundant metal in the Earth's crust. The toxicity of different Al forms depends on their physical behaviour and relative solubility in water. Erythrocytes are more prone to oxidative damage due to the presence of high levels of concentrations of oxygen. After delivery to target tissues, elevated Al3+ concentrations induce cytotoxicity which is a consequence of oxidative damage due to Al3+-induced formation of oxygen radicals

Flavonoids include six subclasses: flavonols, flavones, flavanones, catechins, isoflavones and anthocyanidins. Because of their antioxidant activities such as free radical scavenging and inhibition of lipid peroxidation (LPO) they have been assumed to exert advantageous effects on health. Quercetin is one of the best described flavonols and is present in large amounts in berries, onions, apples and broccoli. It is a perfect free radical scavenging antioxidant and reduces oxidative stress. The interplay between the trio of free radicals, antioxidants, and diseases is important in maintaining health, aging and age-related diseases. Life is supported by oxidation; excess oxidation may occur in some situations and powerful innate mechanisms of detoxification have been evolved.

This study was aimed to explore the in vitro analysis of the redox status by determination of (CAT, GST and TBARS) and analysis of the level of protein in both male and female rats intoxicated by Aluminium with specific focus on the heart. I also examined the effect of quercetin and rutin on some biomarkers of oxidative stress induced by aluminium exposure in Wistar rats.

The experiment was carried out on 6 rat groups (Al male intoxicated group, male intoxicated group treated with quercetin, male intoxicated group treated with rutin, Al intoxicated female group, Al intoxicated female group treated with plant (phagnola rupestre) and female Control group.

At the end of the experiment the Aluminium intoxicated group showed an increase in the activity of thiobarbituric reactive substances (TBARS) but a significant decrease in the activity of catalase (CAT), glutathione S transferase assays (GST) compared to the control group. These results indicate that Al-induced oxidative stress in the heart organ of rats. Following administration of quercetin, the levels of the oxidative stress biomarkers were improved significantly, indicating the overall good antioxidant properties of the quercetin.

From my work it has been demonstrated that Quercetin proved to be an effective antioxidant against free radicals produced as a result of aluminium exposure.

Keywords : Aluminium, Redox Status, Oxidative stress, LPO, Quercetin

Résumé

L'aluminium (Al) est le troisième élément chimique le plus abondant dans la nature et le métal le plus abondant dans la croûte terrestre. La toxicité des différentes formes d'aluminium dépend de leur comportement physique et de leur solubilité relative dans l'eau.
Les érythrocytes sont plus plus touches aux dommages oxydatifs en raison de la présence de niveaux élevés de concentrations d'oxygène. Après l'administration, des concentrations élevées d'Al^{3+} induisent une cytotoxicité des tissus cibles qui est une conséquence des dommages oxydatifs dus à la formation de radicaux oxygénés.
Les flavonoïdes contiennent six sous-classes : les flavonols, les flavones, les flavanones, les catéchines, les isoflavones et les anthocyanidines. En raison de leurs activités antioxydantes telles que le piégeage des radicaux libres et l'inhibition de la peroxydation lipidique (LPO), ils ont été supposés exercer des effets avantageux sur la santé. La quercétine est l'un des flavonols les mieux décrits et qui se trouve en grande quantité dans les baies, les oignons, les pommes et le brocoli. C'est un antioxydant parfait qui élimine les radicaux libres et réduit le stress oxydatif. L'interaction entre le trio, les radicaux libres, les antioxydants et les dommages est importante pour maintenir la santé, le vieillissement et les maladies liées à l'âge. La vie est soutenue par l'oxydation ; une oxydation excessive peut se produire dans certaines situations et de puissants mécanismes innés de détoxification ont été développés.

Cette étude vise à explorer l'analyse in vitro du au statut redox par détermination de (CAT, GST et TBARS) et l'analyse du niveau de protéines. Nous avons également examiné l'effet de la quercétine sur ces biomarqueurs du stress oxydatif induit par l'exposition à l'aluminium dans les rats Wistar.

A la fin de l'expérience le groupe intoxiqué à l'aluminium a montré une augmentation de l'activité des substances réactives thiobarbituriques (TBARS) mais une diminution significative de l'activité de la catalase (CAT), la concentration du glutathion-S-transférase (GST) par rapport au groupe témoin. Ces résultats indiquent que le stress oxydatif est induit par Al dans les rats. Après l'administration de la quercétine, les niveaux des biomarqueurs du stress oxydatif ont été significativement améliorés, indiquant les bonnes propriétés antioxydantes globales de la quercétine, avec un effet amélioré généralisé dans la dose plus élevée du flavonoïde.
Dans ce travail, il a été démontré que la quercétine s'est avérée être un antioxydant efficace contre les radicaux libres produits dans les érythrocytes suite à l'exposition à l'aluminium.

Mots clés : Aluminium, Statut redox, Stress oxydatif, Quercétine

TABLE OF CONTENTS

INTRODUCTION

Aluminium (Al) is the most abundant metal in the environment (**Delhaize and Ryan, 1995; Ranjbar *et al.*, 2008; Exley and House, 2011**) occurring naturally in the trivalent state (Al+3) as silicates, oxides and hydroxides, but may combine with other elements such as chlorine, sulphur, fluorine, as well as form complexes with organic matter.

The redox status is known as the balance between oxidants (or pro-oxidants) and antioxidants. Oxidants, including free radicals and other reactive species, are continuously produced in the cell. As it is impossible to completely prevent oxidant production, several antioxidant systems have evolved in the cell. In order to maintain a healthy status, oxidants and antioxidants should be in equilibrium. However, this equilibrium is very difficult to maintain in the cell. When this equilibrium between oxidant and antioxidant is disrupted, tilting the equilibrium toward an oxidized stale, oxidative stress is produced **(Tan *et al.*, 2018).** Oxidative stress is involved in the physiopathology of several diseases, including cardiovascular disease, cancer, diabetes, and many others.

Aluminium (Al) is the most widely distributed metal in the environment although Al ion has no physiological role in metabolic processes **(Exley and House, 2011)**.

Despite being described as 'redox inactive' aluminium is a potent pro-oxidant and may be exerting this activity through the formation of an aluminium superoxide semi-reduced radical cation, $AlO2^{2+}$ **(Exley, 2004)**. The evidence to support both the formation of this complex and its redox activity is burgeoning **(Mujika et al., 2011)** and suggests that its pro-oxidant activity is significant at concentrations of aluminium which are commonly found throughout the body. Toxic effects of Al arise mainly from its pro-oxidant activity which results in oxidative stress, free radical attack and oxidation of cellular proteins and lipids **(Exley, 2013)**.

Quercetin (3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4Hchromen-4-one) has a typical flavonoid structure and contains five hydroxyl groups: it is a dietary flavonoid, which widely exists in plants like caper, black chokeberry, onion, tomato and lettuce **(Bischoff, 2008).** Quercetin has attracted increasing attention due to its anti-obesity, anti-carcinogenic, antiviral**,** antibacterial, anti-inflammatory effects antioxidant properties **(Duenas et al., 2010).** Due to its potential health benefits for human, quercetin has come into the focus of utilization as a nutraceutical ingredient in food and pharmaceutical industries.

The best described property of Quercetin is its ability to act as antioxidant. It seems to be a powerful flavonoids for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or are induced by exogenous damage **(Panche et al., 2016).** It has also shown to be an excellent in vitro antioxidant and a potent scavenger of ROS **(Cushnie and Lamb, 2005),** and RNS **(Heijnen *et al.*, 2001).** These antioxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging **(Heijnen *et al.*, 2002)**. Moreover, quercetin is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity **(Arts *et al.*, 2004).**

Recently, some studies reported that regular intake of flavonoids in foods can decrease the risk of coronary artery disease **(Hooper *et al.*, 2008).** Results from some studies also indicated the effect of quercetin on inhibiting cardiovascular diseases. For hypertensive patients, the intake of quercetin (730 mg/day, 4 weeks) was found to reduce systolic pressure (by 7 mm Hg), diastolic pressure (by 5 mm Hg) and mean arterial pressure (by 5 mm Hg) **(Edwards *et al.*, 2007).** In a similar study, the systolic pressure and atherogenic LDL level were reduced for some obese subjects with metabolic syndrome symptoms after being supplemented with 150 mg quercetin/day for 42 days. However, the supplementation of quercetin scarcely affects the level of TNF-a and C-reactive proteins, even though fasting plasma quercetin concentration was increased from 71 to 269 nmol/L **(Egert *et al.*, 2009).** In order to evaluate the beneficial effect of quercetin supplementation on heart disease and elucidate a potential mechanism for this protective action, some studies were performed using cells and animal models. Treating macrophagocytes with quercetin could decrease the basic expression of inflammatory genes, including TNF-a, IL-6, IL-8, IL-10 and epoxidase-2 (a marker of prostaglandin production) **(Overman *et al.*, 2011**). In human adipose cells, quercetin inhibited the expression of inflammatory genes and reduced the secretion of IL-6, IL-8 and monocyte chemoattractant protein-1. At the same time, it suppressed the NF-kB transcriptional activity induced by TNFa **(Chuang *et al.*, 2010).** In the animal model, Wistar rats were administered orally with quercetin (10 mg/kg) for one week. The results revealed that quercetin protected the rats from myocardial infarction (induced by hypodermic injection of isoprenaline) by reducing the lipid peroxidation products such as lipid hydroperoxides and conjugated dienes in heart and plasma **(Prince and Sathya, 2010).** Cardiovascular disease of human model was also investigated. Cultured human intracutaneous cells incubated with quercetin (0.1%, w/w in diet) were testified to have a lower level of H_2O_2-induced lipid peroxidation and attenuated atherosclerosis by 40% in ApoE*3-Leiden mice **(Kleemann *et al.*, 2011).**

The experimental part involves carrying out assays of certain biochemical and stress parameters (catalase, glutathione-s-transferase, protein). Finally, the last parts of this

manuscript present the results, discussion, general conclusion and future prospects to be pursued.

The objective of this study is to analyse the redox status at the heart level (in-vitro).

CHAPTER 1
LITERATURE REVIEW

1. ALUMINIUM

1.1. General overview

Aluminium (Al) is the most widely distributed metal in the environment **(Exley and House, 2011)** occurring naturally in the trivalent state (Al+3) as silicates, oxides and hydroxides, but may combine with other elements such as Chlorine, Sulphur, fluorine, as well as form complexes with organic matter **(Jones and Bennet, 1986)**. Environmental media may be contaminated by Al from anthropogenic sources and through the weathering of rocks and minerals. Weathering processes on rocks release more Al to the environment than humanrelated activities **(Lantzy and MacKenzie, 1979)**. Exposures to Al occur in occupations associated with mining and processing of ore, scrap metal recycling, deployment and use of Alcontaining compounds and products, and during engagement in Al metal cutting, sawing, filing and welding. Animals and humans living in environments contaminated by industrial wastes may also be exposed to high levels of Al **(Boran *et al.,* 2013)**. Several chemical compounds with Al are in extensive use in various products and processes associated with human activities. These compounds are Al chloride, Al hydroxide (alumina trihyrate), Al nitrate, Al phosphate, Al sulfate (alum), Al potassium (potash alum), Al ammonium sulfate (ammonium alum) and Al silicate **(Lewis, 2001).** The compounds are used in crude oil refining and cracking of petroleum; manufacturing of cooking utensils and foils, parchment paper, printing ink, glass, ceramics, pottery, incandescent filaments, fireworks, explosives, photographic flashlight, electric insulators, cement, paints and varnishes, fumigants and pesticides, lubricants, detergents, cosmetics, pharmaceuticals (drugs), vaccines, as well as in water treatment and purification, treating sewage and fur, tanning leather, waterproofing clothes and concretes, industrial filtration, hemodialysis, measuring radiation exposure, in products as flame retardant and fireproofing, anticorrosion agent, food additives to prevent caking as well as components of baking powders and colorants **(Lewis, 2001; Saiyed and Yokel, 2005).** The Al ion has no physiological role in metabolic processes **(Exley and House, 2011)** but it can be a metallic toxicant to humans and animals **(Becaria *et al.,* 2002)** when there is high body burden of the metal after natural or unnatural exposure **(Exley, 2013).** Al was considered unsafe to humans after the discovery of increased levels of Al in brain tissues of patients with encephalopathy, having been exposed to Al accumulation through dialysis **(Alfrey and Solomons, 1976).**

1.2. Physical and chemical properties of Aluminium

The Al was discovered in 1808 by the English chemist Davy. In 1827 another chemist, Friedrich Wihler, isolated this metal and highlighted its physical and chemical properties. The Al belongs to column IIIB of the periodic classification of Mendeleev with gallium, indium and thallium, its atomic number is Z – 13. The atomic mass of aluminium is 26.98, its atomic number is 13 and its density is 2.7. Its melting point is 660 degrees Celsius and its boiling point is 2467 degrees Celsius. Aluminium, like any element of the boron family, has three valence electrons. It is an amphoteric metal that reacts with acids (hydrochloric, perchloric, sulphuric) and strong bases.3 Aluminium is a silvery, shiny grey metal with interesting physical-chemical properties: low density, great malleability, great ductility, good electrical and chemical conductivity, corrosion resistance, traction. It is three times lighter than steel. It does not alter on contact with air due to the formation of a protective layer of alumina on the surface, which makes it very resistant to corrosion of air but also of water. It's also totally recyclable. **(Gourier-Frery and Frery, 2004).**

Radunovic *et al.*, (1997) showed that 26A1 could be used as a tracer in biological tissues, In the free state, the Al comes in the form of Al3. The Al is a good electric conductor, It is also very resistant to traction as well as corrosion of air and water (this is related to the alumina layer that forms on its surface). Because of its great responsiveness, you never find the Al in its simple form in its natural state. It is still related to other elements, Al in the form of Al hydroxide (Al (OH) 3) and Al or alumina oxide (Al203) in bauxite as well as in the form of silicate in clay and micas. It can also be found in water-soluble forms that are complex with nitrates, sulphates or chlorides in dissolved organic matter. The Al cannot be destroyed in the environment. It can only change its shape or fix or separate from the particles. In general, the solubility and mobility of Al in the soil is more important when the soil is rich in organic matter **(Gourier-Frery and Frery, 2004).**The fate and transport of Al are largely controlled by environmental factors such as pH, salinity and the presence of various species with which it can form complexes. Indeed, the chemical form under which Al is ingested by the body is in the form of Al salts (very often in the combined form, linked to other inorganic molecules such as chlorine, fluorine, sulphate, nitrate...) commonly used for the treatment of water for consumption **(Pilette, 2008).**

1.3. Exposure to aluminium

1.3.1 Aluminium in the air

The largest source of airborne Al-containing particles is the dust from soil and rocks **(Sorenson *et al.*, 1974).** Human activities, such as mining and agriculture, contribute to the dust in winds **(Eisenreich, 1980).** About 13% of atmospheric Al is attributed to anthropogenic emissions **(Lantzy and MacKenzie, 1979).** The major anthropogenic sources of Al-containing particulate matter include coal combustion, Al production, iron and steel foundries, brass and bronze refineries, motor vehicle emissions and other industrial activities such as smelting, filing, sawing, welding of Al metals **(Que Hee *et al.*, 1982).** Cigarette smoke may contribute to the concentration of Al in the air **(Afridi *et al.*, 2015).** The air containing Al particles or droplets becomes the source of Al in inhaled aerosols.

1.3.2 Aluminium in drinking water

Al occurs ubiquitously in natural waters due to weathering of Al-containing rocks and minerals and mobilization from terrestrial to aquatic environment **(Campbell et al., 1992).** This mobilization of Al is often seasonal in nature and is associated with pH depressions (acidification) occurring during the spring snow melt or associated with erosion from specific storm events **(Rosseland *et al.*, 1990).** Al concentrations in surface waters can be increased directly or indirectly by human activities through industrial and municipal discharges, surface run-off, tributary inflow, groundwater seepage, and wet and dry atmospheric deposition **(Eisenreich, 1980).** Industrial release of Al in waste materials into surface waters from processing and manufacturing facilities could be toxic to aquatic life **(Gensemer and Playle, 1999).** Acidic drainage from mines or acid rain may cause an increase in the dissolved Al content of the surrounding water bodies **(Filipek *et al.*, 1987).** The use of Al compounds as coagulating agents in the treatment of water for drinking could increase its Al content **(Cech and Montera, 2000).** In pure water, Al has a minimum solubility in the pH range of 5.5–6.0 and concentrations of dissolved Al increase at higher or lower pH values **(Browne *et al.*, 1990).** The source of water for human and animal consumption and the purification process involved may influence the Al content of drinking water as source of exposure.

1.3.3. Aluminium in food

Al is present in foods naturally or from the use of Al-containing food additives **(Yokel *et al.*, 2008).** The concentrations in foods and beverages vary widely, depending upon the food product, the type of processing used, and the geographical areas in which the food crops are grown **(Sorenson *et al.*, 1974).** The foods highest in Al are those that contain Al additives **(Yokel and Florence, 2006).** The use of Al cookware, utensils and wrappings can increase the amount of Al in food **(Pennington and Schoen, 1995).** The migration of Al from cookware into food increases with the acidity of the food and the duration of exposure **(Lin *et al.* 1997).** Al was also reported to migrate into fish grilled on Al foil and the migration of Al into foods appeared to be dependent on factors such as temperature, duration of cooking, the composition and pH of the food, and the presence of other substances like organic acids and salts **(Ranau *et al.*, 2001).** Foods found to be naturally high in Al include potatoes, spinach and tea **(Pennington and Schoen, 1995).**Processed dairy products and flour may be high in Al if they contain Albased food additives **(Pennington and Schoen, 1995).**

Daily intakes of Al in humans from food range from 3.4 to 9 mg/day **(Yang et al., 2014).** It is unlikely that Al-containing food additives are intentionally added to the diets of livestock and pets yet, Al contamination of some additives used in livestock and pet food is possible **(Burgoin, 1992).** Thus, Al contents of harvested food products, processed foods, and cooked, baked or grilled foods may be sources of Al exposure.

1.4. Aluminium in pharmaceuticals and agrochemicals

The route of intoxications with pharmaceuticals and agrochemical sources may be through inhalation of aerosols, ingestion of medications or by parenteral administration. Humans and animals are exposed to Al-containing medications such as phosphate binders, antacids, buffered analgesics, antidiarrheal and antiulcer drugs **(Krewski *et al.*, 2007).** Various intravenously administered pharmaceutical products were reported to contain 684–5977 µg/g of Al **(Sedman *et al.*, 1985).** Many antacids contain 104–208mg of Al per tablet, capsule or 5 ml of suspension **(Zhou and Yokel, 2005).** The use of other consumer items such as dentifrices, disinfectants, fumigants, pesticides, anti-perspirants and some cosmetics are sources of Al exposure **(Lewis, 2001).** Al hydroxide, Al phosphate, Al potassium sulfate (alum), and Al silicate (zeolite) are used in the preparation of a number of vaccines to adsorb antigenic components and to serve as

adjuvant that enhance immune response **(Issa *et al.*, 2014).** Adjuvant as a source of Al during vaccinations has been receiving attention in research **(Glanz et al., 2015)** and it is presumed that there could be mistakes in adjusting Al content of vaccines to body weights of neonates who stand the risk of Al toxicity from vaccines **(Lyons-Weiler and Ricketson, 2018).** More Al was absorbed into blood by rabbits after intramuscular injection with adjuvant containing Al phosphate compared to Al hydroxide **(Hem, 2002).** It is unlikely that parenteral Al administrations are a major source of Al exposure to livestock or pets **(Issa *et al.*, 2014).**

1.5. Absorption, distribution and elimination of aluminium

The dynamic chain of Al intake, absorption and elimination determines the level of tissue accumulation and development of toxicosis (Figure 1). Inhalation and ingestion (via food and water) are the two main routes through which Al gets into the body **(Jouhanneau *et al.*, 1997).** Following inhalation, Al compounds are deposited in the lungs and the lungs continually receive Al mostly as particles of Al silicates and other poorly soluble compounds **(Thomson *et al.*, 1986).** The concentration of Al in the lungs tends to increase with age and may result in respiratory anomalies where the Al is localized **(Taiwo, 2014).** There is no available evidence in literature that particulate or soluble Al gets into the blood circulation from the lungs to be subsequently distributed to other organs of the body. Gastrointestinal absorption, after ingestion, is the main route through which Al is systemically accumulated in animals and humans, and absorption occurs largely in the duodenum **(Steinhausen *et al.*, 2004).** The absorption of Al is usually low and varied when compared with the amount ingested **(Kawahara *et al.*, 2007).** The uptake of Al through gastrointestinal pathway is complex and is influenced by various factors including individual differences, age, pH, stomach contents and type of Al compound **(Priest *et al.*, 1996).** Al absorption from water intake (about 0.3%) is greater than from food (about 0.1%) **(Zhou *et al.*, 2008).** This was attributed to organic ligands in foods such as phytates and polyphenols that were suggested to form complexes with Al ion and inhibit its absorption **(Reto *et al.*, 2007).** Absorption of Al via the gastrointestinal tract can be enhanced in the presence of citrate, maltol, lactate and fluoride in water or food, and during chronic renal diseases, while the absorption is reduced in individuals with iron overload, or when ingested with phosphate, silicon, polyphenols and sialic acid **(Zhou *et al.*, 2008).** However, there is complete Al uptake from parenteral fluids and vaccines with subsequent distribution to various parts of the body **(Tomljenovic and Shaw, 2011).**

About 90% of the Al circulating in the blood is transported bound to transferrin (iron-transporter protein), while the rest of Al binds to albumin and citrate in the blood **(Hemadi *et al.*, 2003; Chen *et al.*, 2010).** Cellular uptake of Al in tissues is relatively slow and is presumed to be mediated by endocytosis and intracellular transfer of the Al bound to transferrin **(Hemadi *et al.*, 2003).** However, Al-transferrin complex may not bind to the transferrin-receptor **(Sakajiri *et al.*, 2010),** indicating the existence of an alternative mechanism of cellular uptake of Al **(Anon, 2011).** The total body burden of Al in healthy humans has been reported to be approximately 30–50 mg/kg body weight and normal levels of Al in serum are approximately 1–3μg/L **(Krewski *et al.*, 2007).** The mean serum Al level in 44 non-exposed persons who did not use antacids was reported to be 1.6 μg/L **(Valkonen and Aitio, 1997)** and **Chen et al. (2010)** reported that values in hemodialysis patients were ten-fold higher than the values in unexposed individuals. About one-half of the total body Al is in the skeleton, and the levels in human bone tissue range from 5 to 10 mg/kg **(Anon, 2008c).** Al has also been found in human skin, lower gastrointestinal tract, lymph nodes, adrenals, parathyroid glands, and in most soft tissue organs **(Anon, 2008b).**

In rats, accumulation of Al after oral exposure was higher in the spleen, liver, bone, and kidneys than in the brain, muscle, heart, or lungs **(Anon, 2008b).** It has also been reported that Al can reach the placenta and fetus and to some extent distribute to the milk of lactating mothers **(Anon, 2008b).** Al levels increase with age in tissues and organs (bone, muscle, lung, liver, and kidney) of experimental animals **(Krewski *et al.*, 2007).** Moreover, Al has been shown to rapidly enter the brain, extracellular fluid and the cerebrospinal fluid, with smaller concentrations in these organs than in the blood **(Krewski *et al.*, 2007).** The iron status is negatively correlated with Al accumulation in tissues and animal experiments have shown that calcium and magnesium deficiency may contribute to accumulation of Al in the brain and bone **(Anon, 2011).** The Al ion in blood circulation is eliminated primarily by the kidneys (about 95%) in the urine, presumably as Al citrate **(Anon, 2008c).** Tissue accumulation of Al is reduced by citrates and fluorides through renal excretion when the transferrin-Al binding capacity of the blood is exceeded **(Anon, 2008b).** Al is also excreted in the milk, bile, feces, sweat, hairs, nails, sebum and semen **(Greger and Sutherland, 1997).** Urinary excretion of Al is enhanced by chemical chelators such as deferoxamine and malic, malonic, citric, oxalic and succinic acids **(Exley, 2013).** Al accumulation, which is responsible for Al toxicosis, is enhanced by exposure to Al and its continuous intake, as well as increased intestinal absorption and decreased excretion of the metal (Figure 1).

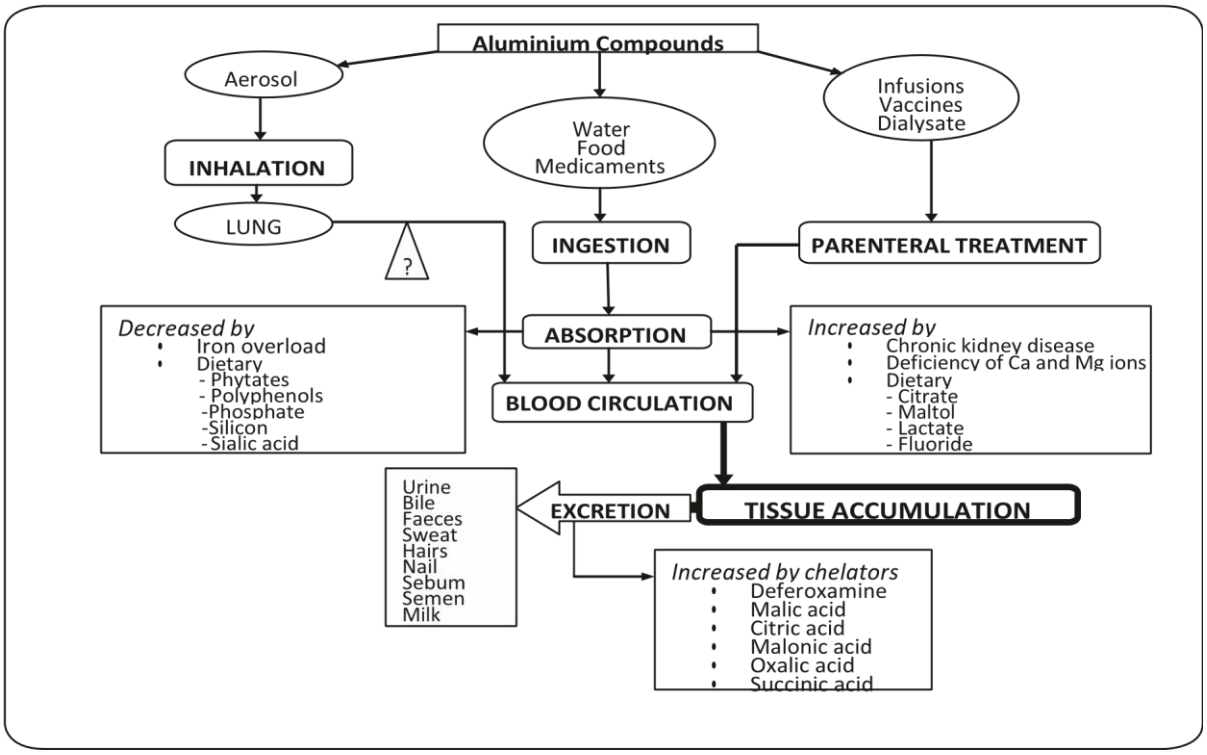


Figure 1: Factors affecting tissue accumulation of aluminium and development of toxicosis **(Igbokwe *et al.*, 2019)**

1.6. Toxic actions of aluminium

The toxic actions of Al responsible for the toxic effects of the toxicities are diverse and capable of causing a multifaceted systemic toxicosis. Toxic effects of Al arise mainly from its prooxidant activity which results in oxidative stress, free radical attack and oxidation of cellular proteins and lipids **(Exley, 2013).** Extracellular surfaces and intracellular ligands may likely associate with Al to induce inhibitory or stimulatory effects **(Exley and Birchall, 1992).** Interaction of Al with metabolic and other enzymes causes inhibition or activation of the enzymes. Al binds to the phosphate groups of nucleotides such as adenosine triphosphate (ATP) and affects energy metabolism **(Kawahara *et al.*, 2007).** Exposure of hepatocytes to Al impedes ATP production, inhibits glycolysis, impairs the function of tricarboxylic acid (Kreb's) cycle and promotes lipid and protein oxidation **(Mailloux *et al.*, 2006).** Al exposure can cause the disruption of iron homeostasis leading to iron overload **(Contini *et al.*, 2007).** Oxidative stress and injury, mediated by iron, seems to be facilitated by Al **(Xiea *et al.*, 1996).** Elevated concentrations of cellular iron can enhance oxidative damage to the cell and are linked to the

pathogenesis of neurodegenerative disorders **(Milton, 2004).** Iron overload due to Al exposure has been shown to result in increased lipid peroxidation, DNA lesions, and apoptosis induced by reactive oxygen species **(Kell, 2009).** Apoptosis of erythrocytes (eryptosis), lymphocytes and osteoblasts is also stimulated by Al ions **(Yu *et al.*, 2019).** The oxidative injury was reported to activate the JNK apoptotic pathway in ostoblasts **(Yang *et al.*, 2018).** In culture, Al induced apoptosis of osteoblasts by inhibiting apoptotic Bcl-2 protein expression and increasing the expression of pro-apoptotic Bax, Bak and Bim proteins **(Xu *et al.*, 2018).** The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) are affected by Al exposure because of oxidative stress **(Campbell *et al.*, 1999).** Abnormal increases in levels of malondealdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were reported along with decreased levels of antioxidants such as GSH, GPx, SOD, and CAT in tissue homogenates of rats exposed to Al **(Zhang *et al.*, 2016; Yu *et al.*, 2019).**

The cellular membrane is vital for the viability of the cell and Al exposure disrupts membrane activity via oxidative stress in various ways. In Alzheimer's disease associated with Al exposure, membrane fluidity increased in platelets and decreased in erythrocytes; and this observation was corroborated by a study where in vitro exposure of membrane suspensions to Al increased fluidity of platelet membranes and decreased the fluidity of erythrocyte membranes **(Van Rensburg *et al.*, 1992)** with consequent effect on the viability of platelets **(Neiva *et al.*, 1997)** and erythrocytes **(Vittori *et al.*, 2002).** Erythrocyte membrane permeability and osmotic fragility are affected by in vivo and in vitro Al exposures **(Igbokwe, 2018).** Erythrocyte osmotic fragility decreased **(Bazzoni *et al.*, 2005)** or increased **(Zhang *et al.*, 2016)** depending on the Al speciation and type of erythrocyte injury. Eryptotic (apoptotic) injury reduces the erythrocyte aggregate size **(Bazzoni *et al.*, 2005)** because of the shrinking effect, thereby increasing osmotic resistance **(Igbokwe, 2016).** On the other hand, eryptotic injury which progresses to oncotic injury may cause swelling of the erythrocyte and increase osmotic fragility. It is therefore presumed that Al may cause either shrinking or swelling effect when the erythrocyte membrane is destabilized by Al exposure because of altered membrane permeability to intracellular and extracellular ions **(Igbokwe, 2016).**

Cell membrane functions which regulate transmembrane transport of ions are expected to be disrupted when ATPase in cell membrane loses some level of activity during Al exposure.

There are reports showing that Al inhibited activities of Na+K+- ATPase, Mg2+-ATPase and Ca2+-ATPase in erythrocytes **(Zhang *et al.*, 2016),**vascular endothelial cells **(Vorbrodt *et al.*, 1994),** testes and ovaries of rats **(Fu *et al.*, 2014)**. The Al-induced change in erythrocyte size

may also be accompanied by change in erythrocyte shape resulting in the formation of echinocytes **(Suwalsky *et al.*, 2004),** acanthocytes and stomatocytes **(Vittori *et al.*, 2002)** in vitro, due to altered membrane morphology **(Lukyanenko *et al.*, 2013).** After long-term oral intake of Al, schistocytes and target cells were observed in stained peripheral blood of rats **(Vittori *et al.*, 1999).** The lipid bilayer of the plasma and mitochondrial membranes was morphologically altered in lymphocytes **(Skarabahatava *et al.*, 2015).** The protein components of membranes are degraded or inadequately expressed during Al exposure, as observed in the loss of band 3 protein of erythrocyte membrane **(Cheng *et al.*, 2018)**

1.6.1. Pulmonary effect

Pulmonary lesions in humans linked to Al exposure during production of Al products include granulomatous pneumonia, pulmonary granulomatosis, pulmonary fibrosis, pulmonary alveolar proteinosis and desquamative interstitial pneumonia **(Taiwo, 2014).** Asthma may be caused by Al exposure **(Burge *et al.*, 2000),** though the asthma among Al workers may be due to other chemical factors like gases and smoke **(Taiwo *et al.*, 2006).** Reactive airways dysfunction syndrome was rarely reported among Al smelter workers **(Wesdock and Arnold, 2014).** Acute-duration oral exposure to Al phosphide has been reported to cause pulmonary edema in persons following accidental or volitional ingestion **(Chopra *et al.*, 1986; Khosla *et al.*, 1988).**The toxicity was probably due to the formation of highly toxic phosphine gas rather than to Al exposure **(Moghadamnia, 2012).Hasseeb *et al.*, (2011)** reported neutrophilic and mononuclear cell infiltrations of lung alveoli of rats administered 37 mg/kg/day of Al chloride in drinking water for 8 weeks. Congested blood vessels in inter-alveolar spaces were reported after administration of different concentrations of Al chloride via gavage for 8 weeks **(Buraimoh and Ojo, 2013).** Pulmonary lesions are rare and inconsistent in experimental animals where Al exposure is not through aerosol vehicles. Under natural conditions, the vehicular substances and the Al speciation may influence the stimulation of chronic pathologic reactions in the lung.

1.6.2. Hematologic effects

Al exposure has been associated with significant inhibition of colony forming units-erythroid (CFU-E) development in the bone marrow of mice exposed to 13 mg Al/ kg as Al citrate or chloride administered via gavage for 5 days/week for 22 weeks **(Garbossa *et al.*, 1996),** rats exposed to 27 mg Al/kg as Al citrate administered via gavage 5 days/week for 15 weeks **(Garbossa *et al.*, 1998),** and rats exposed to 230 mg Al/kg/day as Al citrate in drinking water for 8 months **(Vittori *et al.*, 1999).** The effect of Al on erythroid progenitor cells and

erythrocytes was associated with slow growth and increased degradation of membrane band 3 proteins, respectively **(Vittori *et al.*, 2002).** The genotoxicity from Al exposure in mice resulted in mitodepressive effect in the bone marrow **(D'Souza *et al.*, 2014).** Anaemia caused by Al toxicity is not associated with adequate regenerative activity of the bone marrow and reticulocytosis **(Chmielnicka *et al.*, 1994; Osman *et al.*, 2012).** The additional causes of anaemia appear to be multi-factorial and include defective haemoglobin production due to inhibition of the enzymes of heme synthesis, altered erythrocyte membrane structure and fragility, shortening of red blood cell life span due to eryptotic and oncotic injuries, and inadequate iron utilization **(Lukyanenko *et al.*, 2013; Zhang *et al.*, 2016; Cheng *et al.*, 2018).** Significant decreases in haemoglobin, hematocrit (packed cell volume) and erythrocyte osmotic fragility were reported after Al exposure **(Farina *et al.*, 2005).** The anaemia is characterized by decreases in mean corpuscular volume (microcytosis) and mean corpuscular haemoglobin (hypochromia), but in chronic exposures, the erythrocyte parameters recover with persistence of microcytosis and hypochromia **(Mahieu *et al.*, 2000).** In rats loaded with Al, heme dyshomeostasis was reported with evidence of decreased activity of aminolevulinic acid dehydratase and increased activity of heme oxygenase in the rat liver associated with activation of JNK pathway, indicating an increase in heme degradation **(Lin *et al.*, 2013).** No alterations in haemoglobin, hematocrit and erythrocyte osmotic fragility were reported in a number of experimental Al exposures **(Garbossa *et al.*, 1996).Vittori *et al.*, (1999)** did not find significant alterations in plasma iron levels or total iron binding capacity in rat exposed to 230 mg Al/kg/day as Al citrate in drinking water for 8 months; however, they reported impaired iron uptake and decreased iron incorporation into heme in the bone marrow. **Farina *et al.*, (2005)** found significant decreases in blood iron concentrations and no change in total iron binding capacity in rats exposed to 54.7 mg Al/kg/day as Al sulfate in a sodium citrate solution in drinking water for 18 months. **Florence *et al.*, (1994)** reported decreases in serum iron levels, total iron binding capacity, and transferrin saturation in rats exposed to 75 mg Al/kg/day as Al citrate in the diet for 6 months. Chronic Al exposure in rats disrupted iron homeostasis **(Zhang *et al.*, 2010).**In summary, the hematologic effect of toxicosis consists of anaemia due to erythrocyte and erythroid pathology with suppression of erythropoiesis (Table 1)

Table 1: Summary of hematologic effects of aluminium toxicosis.

Toxic effects	Toxic actions
Depressed erythropoiesis	Inhibition of CFU-E Slow growth of erythroid cells Inhibition of heme synthesis Increased heme degradation Dysregulated erythropoietin receptor function
Anaemia	Reduced erythrocyte life span Erythrocyte apoptosis (eryptosis) Altered erythrocyte fragility Decreased erythrocytemembrane fluidity Inhibition of erythrocyte membrane ATPase Altered erythrocyte shape: echinocytes, acanthocytes, stomatocytes, target cells

1.6.3. Neurologic effects

In humans, Al accumulation in the brain and scalp hairs has been associated with neurodegenerative diseases such as dialysis-associated encephalopathy, Alzheimer's disease, Parkinson's disease (dementia), amyotropic lateral sclerosis, multiple sclerosis and autism **(Arain *et al.*, 2015; Jones *et al.*, 2017; Mold *et al.*, 2018).** There is a role for Al in multiple sclerosis because patients excrete high amounts of Al in urine, facilitated by drinking siliconrich mineral water **(Jones *et al.*, 2017).** Subchronic exposure to Al was associated with reduced population of neural stem cells and hampered cell proliferation and neuroblast differentiation in the brain of mice **(Nam *et al.*, 2014, 2016).** Injection of Al, especially intra-cisternally, induced neurological changes in animal models **(Wisniewski *et al.*, 1980; Anon, 2008c).** Rats orally administered Al (100 mg/ kg/day) for 90 days accumulated more Al in their brains, had increased brain acetyl cholinesterase activity and had decreased brain choline acetyltransferase activity **(BilkeiGorzó, 1993).Oteiza *et al.*, (1993b)** reported that mice fed diets containing 1,000 mg/kg diet of Al (as Al chloride) with sodium citrate accumulated more Al in the brain nuclear fraction and spinal cord, had lower grip strength, and greater startle responsiveness after

5 and 7 weeks. Old (18 months of age) rats exposed to Al (100 mg/kg/day) in drinking water with citrate (356 mg/kg/day of citrate) had decreased numbers of synapses and a greater percentage of perforated synapses than controls, but no changes in behavior **(Colomina *et al.*, 2002).**

In a nutshell, Al exposure promotes oxidative stress and amyloid deposition in the nervous tissue which results in neurodegeneration, neuronal necrosis and dysneurogenesis, which constitute the basis for the neurological diseases associated with Al intoxication.

1.6.4. Musculoskeletal effects

The major myopathy induced by Al exposure is macrophagic myofasciitis (aluminic granuloma) associated with chronic arthromyalgia or myalgia and chronic fatigue syndrome **(Exley *et al.*, 2009; Miller, 2016).** Skeletal muscle necrosis occurred in the diaphragm and abdominal muscles of rats adjacent to the peritoneum after intraperitoneal injection of Al lactate **(Levine *et al.*, 1992).** Smooth muscle contraction induced by K+ ion was inhibited by Al exposure **(Nasu *et al.*, 1998).** Myocardial function may be altered in diabetic individuals by Al exposure, in as much as Al toxicity potentiates the decline in calcium uptake into the sarcoplasmic reticulum of the myocardial fibers of such individuals **(Levine *et al.*, 1990).** There is increased risk of osteoporosis and low bone mineral density during Al exposure **(Sun *et al.*, 2016)** because of disruption of bone formation, and inhibition of osteoblast proliferation, differentiation and mineralization **(Huang *et al.*, 2017).** In individuals with Al overload, undecalcified bone matrix contains Al and bone conditions like exostosis and osteomalacia may occur in circumstances that increase Al uptake and colocalization as observed in celiac disease, hemochromatosis and sickle cell anaemia **(Chappard *et al.*, 2016).** Osteoclast genesis is promoted by low-dose exposure while osteoclast apoptosis is caused by high-dose exposure **(Yang *et al.*, 2018).**

1.6.5. Reproductive and developmental effects

Human reproduction may be affected negatively by Al exposure **(Klein *et al.*, 2014; Mouro *et al.*, 2017).** Human semen and spermatozoa contain Al and patients with oligospermia had higher Al concentration than healthy individuals **(Klein *et al.*, 2014).** At human dietary level of Al and continuous exposure for 60 days, the rat testes accumulated low Al levels of 3.35 µg/g and it was associated with increased oxidative stress and inflammation, decreased daily sperm

production, reduced sperm count and motility and increase in abnormal spermatozoa **(Martinez *et al.*, 2017).** The weights of the testes and epididymis were decreased by Al exposure in rats as serum testosterone levels dropped **(Mouro *et al.*, 2017).** In male rats, testicular development was impaired by Al exposure, associated with reduction in serum levels of testosterone and luteinizing hormone (LH) levels and decrease in androgen receptor protein expression without effect on serum follicle stimulating hormone (FSH) **(Sun *et al.*, 2018).** Exposure to Al during mouse pregnancy resulted in reduced fetal weight and increased frequency of external anomalies in fetuses **(Malekshah *et al.*, 2005)** and fetal micro nucleated erythrocytes **(D'Souza *et al.*, 2014).**

1.6.6. Hepato-renal and pancreatic effects

Al causes oxidative injuries to the kidney and liver leading to tissue degeneration and necrosis, and associated serum biochemical derangements **(Mailloux *et al.*, 2011; Li *et al.*, 2015; Xu *et al.*, 2017).Abdel-Wahab, (2012)** reported a significant increase in the activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and total bilirubin, as well as increased serum urea and creatinine levels after oral administration of 20 mg/kg of Al chloride for 30 days in experimental rats. Ingestion of aluminium phosphide pellets was reported to induce acute pancreatitis in one patient **(Verma *et al.*, 2007).**Rats had moderate pancreatic islet necrosis after intermediate oral exposure (50 mg/kg for 28 days) to Al chloride which was associated with impaired fasting blood glucose and impaired oral glucose tolerance **(Igwenagu, 2017; Igwenagu *et al.*, 2019).** Rats treated intra-peritoneally with Al chloride at 10 mg/kg for 30 days had significantly increased fasting blood glucose, serum insulin level and insulin resistance index on days 10 and 20 of treatment, but as treatment progressed to day 30, serum insulin level had decreased, indicating that pancreatic β-cell function decreased as pancreatic damage occurred with progression of treatment **(Wei *et al.*, 2018).**

1.6.7. Mammary gland or breast effects

Breast cancers and cysts are mammary gland conditions where emerging evidence are suggesting that Al may be involved in their causation **(Darbre, 2016).** Al chloro hydrate in antiperspirant cosmetics and other underarm cosmetic products may be an important source of Al exposure **(Pineau *et al.*, 2014; Linhart *et al.*, 2017).** In a case control study **(Linhart *et al.*, 2017),** the use of underarm cosmetic products containing Al was significantly associated with breast cancer incidence and the Al levels in breast tissues were significantly higher in breast

cancer cases than controls (5.8 versus 3.8 nmol/g). Breast cancer patients had higher levels of Al in breast tissues than in blood serum **(Darbre *et al.*, 2013b).** There were higher levels of Al in nipple aspirates of cancer patients than healthy controls and higher Al levels in breast cyst fluid than serum or milk **(Darbre *et al.*, 2011).** The Al contents of nipple aspirates of breast cancer patients correlated with biomarkers of oxidative stress and inflammation in the breast microenvironment **(Mannello *et al.*, 2013).** The Al accumulating in the breast tissue may influence the biological characteristics of breast epithelial cells and carcinogenesis is considered a probable outcome **(Pineau *et al.*, 2014).** Current evidence suggests that Al can induce DNA damage in human breast epithelial cells and subsequently induce proliferation of the cells **(Darbre *et al.*, 2013a, b).** Thus, Al may increase the risk of breast cancer by acting as a metalloestogen **(Darbre, 2016).**

1.7. Diagnosis and treatment of aluminium intoxication

Al can be measured in the blood, bone, urine, and feces to confirm Al load and association with toxicosis. A variety of analytical methods have been used to measure Al levels in biological materials and they include accelerator mass spectroscopy, graphite furnace atomic absorption spectrometry, flame atomic absorption spectrometry, electro-thermal atomic absorption spectrometry, neutron activation analysis, inductively coupled plasma atomic emission spectrometry, inductively coupled plasma mass spectrometry, and laser microprobe mass spectrometry **(Razniewska and Trzcinka-Ochocka, 2003).**

Treatment of Al intoxication is done with the chelating agent, deferoxamine, which is a colorless crystalline base, produced by the bacterium, Streptomyces pilosus. Deferoxamine is mainly used as an iron-chelating agent to treat iron overload. But due to the chemical similarity between Al and iron, it can also successfully mop-up excess Al from the body, deferoxamine administered intravenously has been shown to reduce the body Al load and to ameliorate injury to the bone and brain in patients receiving hemodialysis and peritoneal dialysis, deferoxamine therapy seems beneficial for those with established Al toxicity **(Klaassen, 1990).**

Malic acid is also a potent chelator of Al used in treatment of Al intoxication **(Domingo *et al.*, 1988).** Treatment with malic acid has been reported to greatly increase the fecal and urinary excretion of Al and reduce the concentration of Al present in various organs and tissues **(AlQayim *et al.*, 2014).** Other chelating agents such as citric, malonic, oxalic, and succinic acids have been used experimentally to reduce aluminium load in rats and mice **(Domingo *et al.*, 1988).** Antioxidants and free radical scavengers such as selenium, melatonin, boric acid and

vitamin C have been employed experimentally to ameliorate the deleterious effects of free radicals produced as a result of Al intoxication **(Omar *et al.*, 2003; Turkez et al., 2011).**

Other researchers have used plant extracts of fenugreek seed, grape seed, ginger, wheat grass powder, black tea, Allium cepa, Caesalpinia crista, Arthrophytum (Hammada scoparia), Moringa oleifera and Celastrus paniculatus to ameliorate the toxicosis caused by Al exposure **(Ravi *et al.*, 2018).**

The neuronal death in the hippocampus of the brain associated with neurodegeneration in rats caused by Al exposure was attenuated by quercetin **(Sharma *et al.*, 2016).** Ginsenoside Rb1 was reported to prevent Al-induced oxidative stress and reverse the osteoblast viability and growth after impairment by Al **(Zhu *et al.*, 2016a).**Chlorogenic acid was effective as a chelating agent and antioxidant in protection against the toxicity of Al **(Wang *et al.*, 2018).** Chenodeoxycholic acid ameliorated the neurotoxic effect of Al by improving insulin sensitivity **(Bazzari *et al.*, 2019).Türkez et al., (2010)** reported that propolis prevented the genetic and hepatic damages induced by Al intoxication. On the whole, the approach to the treatment of Al toxicosis after diagnosis involves strategies that include the following: prevention of Al intake, reduction of Al absorption, increasing Al excretion, maintaining functional kidneys, reducing Al load by chelation with chelating agents and amelioration of toxic effects with antioxidants and other agents that reduce toxicity (Figure 7).

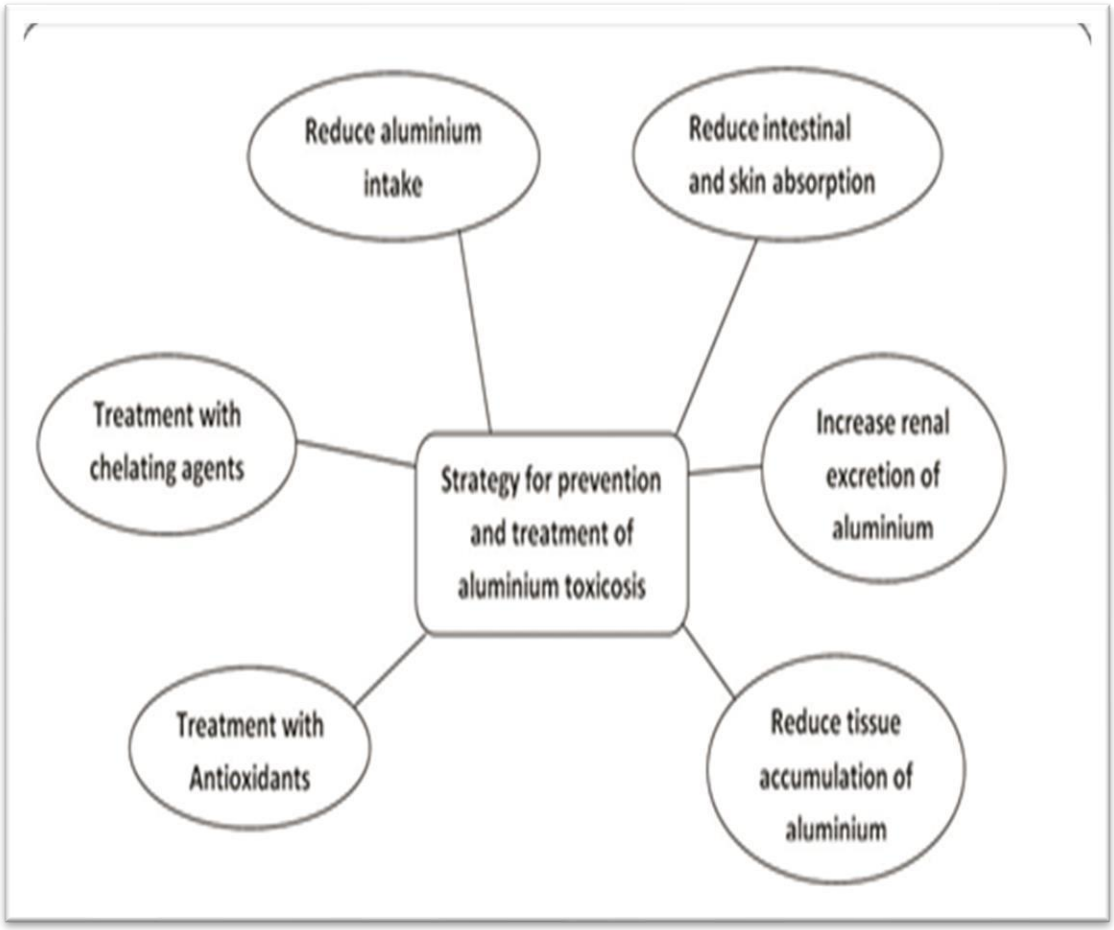


Figure 2: Prevention and treatment of aluminium toxicosis. **(Igbokwe *et al.* 2019).**

2. THE HEART

2.1: STRUCTURE AND FUNCTION

Figure 3: Position of the heart in the human body

The cardiovascular system is divided for descriptive purposes into two main parts.

1. The circulatory system, consisting of the heart, which acts as a pump, and the blood vessels through which the blood circulates.

2. The lymphatic system, consisting of lymph nodes and lymph vessels, through which colourless lymph flows.

The two systems communicate with one another and are intimately associated.

The heart pumps blood into two anatomically separate systems of blood vessels (Fig.5).

• The pulmonary circulation

• The systemic circulation.

The right side of the heart pumps blood to the lungs (the pulmonary circulation) where gas exchange occurs i.e. CO2 leaves the blood and enters the lungs, and O2 leaves the lungs and enters the blood. The left side of theheart pumps blood into the systemic circulation, which supplies the rest of the body. Here, tissue wastes are passed into the blood for excretion, and body cells extract nutrients and O2.The circulatory system ensures a continuous flow of blood to all body cells, and its function is subject to continual physiological adjustments in order to maintain an adequate blood supply. Should the supply of oxygen and nutrients to body cells become inadequate, tissue damage occurs and cell death may follow **(Anne Waugh et Allisson Grant, 2004).** The heart pumps blood into vessels that vary in structure, size and function, and there are several types: arteries, arterioles, capillaries, venules and veins.

Arteries and arterioles

These are the blood vessels that transport blood away from the heart. They vary considerably in size and their walls consist of three layers of tissue

• Tunica adventitia or outer layer of fibrous tissue

• Tunica media or middle layer of smooth muscle and elastic tissue

• Tunica intima or inner lining of squamous epithelium called endothelium.

The amount of muscular and elastic tissue varies in the arteries depending upon their size. In the large arteries, sometimes called elastic arteries, the tunica media consists of more elastic tissue and less smooth muscle.

These proportions gradually change as the arteries branch many times and become smaller until in the arterioles (the smallest arteries) the tunica media consists almost entirely of smooth muscle. Arteries have thicker walls than veins and this enables them to withstand the high pressure of arterial blood.

Veins and venules

The veins are the blood vessels that return blood at low pressure to the heart. The walls of the veins are thinner than those of arteries but have the same three layers of tissue.They are thinner because there is less muscle and elastic tissue in the tunica media. When cut, the veins collapse while the thicker-walled arteries remain open. Some veins possess valves, which prevent backflow of blood, ensuring that it flows towards the heart. The smallest veins are called venules **(Anne Waugh et Allisson Grant, 2004).**

Capillaries and sinusoids

The smallest arterioles break up into a number of minute vessels called capillaries. Capillary walls consist of a single layer of endothelial cells through which water and other small-molecule substances can pass. Blood cells and large-molecule substances such as plasma proteins do not normally pass through capillary walls. The capillaries form a vast network of tiny vessels which link the smallest arterioles to the smallest venules. Their diameter is approximately that of an erythrocyte (7 um). The capillary bed is the site of exchange of substances between the blood and the tissue fluid, which bathes the body cells.

Sinusoids are wider than capillaries and have extremely thin walls separating blood from the neighbouring cells.In some there are distinct spaces between the endothelial cells. Among the endothelial cells there may be many phagocytic macrophages, e.g. Kupffer cells in the liver. Sinusoids are found in bone marrow, endocrine glands, spleen and liver. Because of their larger lumen the blood pressure in sinusoids is lower than in capillaries and there is a slower rate of blood flow **(Anne Waugh et Allisson Grant, 2004).**

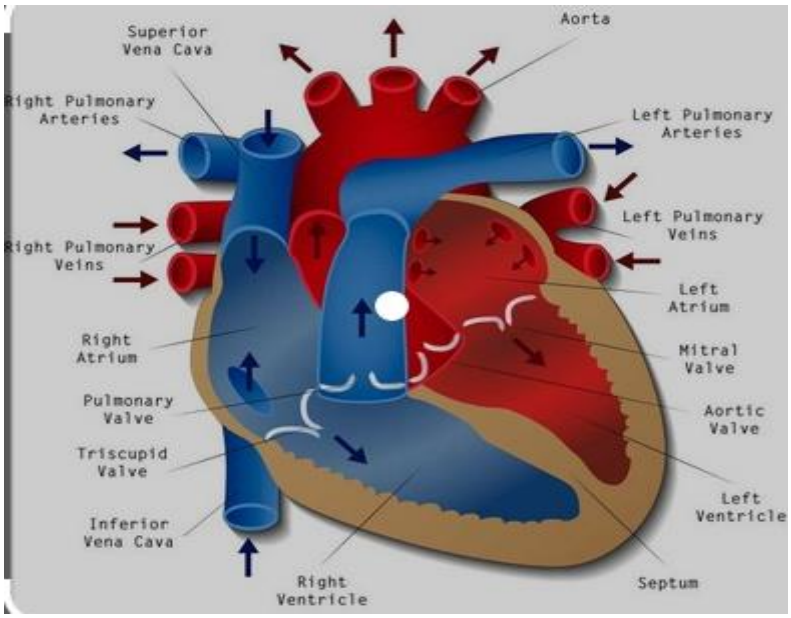


Figure 4: Direction of blood flow inside the heart

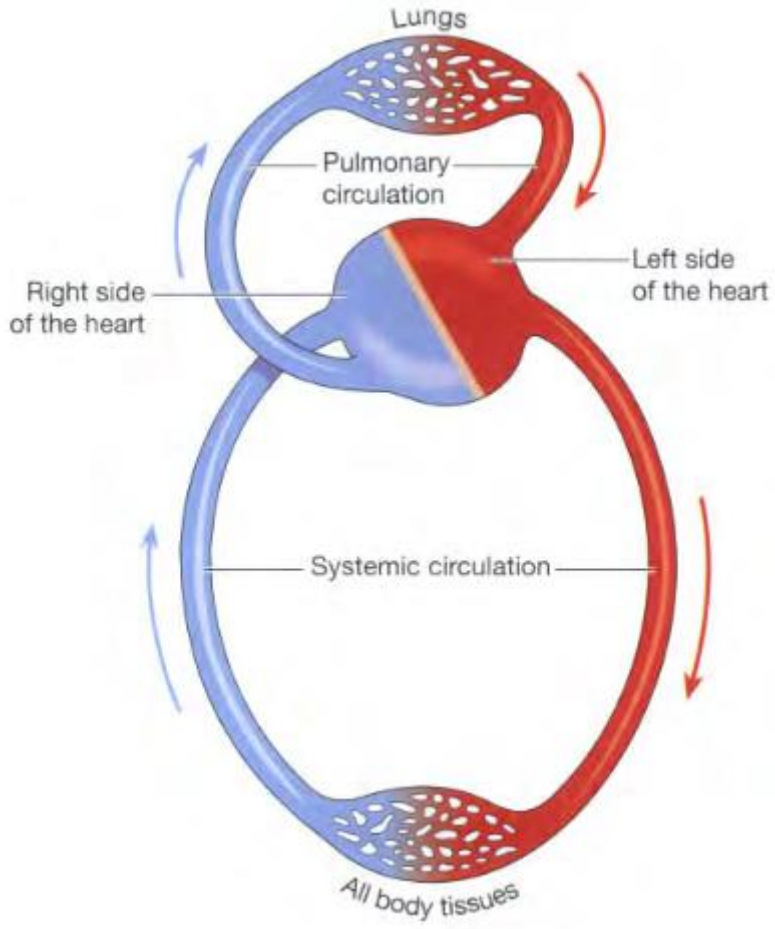


Figure 5: Relationship between pulmonary and systemic circulation

2.2: THE HEART AND ALUMINIUM

Cardiovascular effects of Aluminium

The primary function of the heart is to pump both oxygenated and de-oxygenated blood all over the body wherein transporting oxygen and nourishment. The limitation of transporting oxygen on a regular basis is the resulting exposure to high levels of oxidative stress. In order to attenuate oxidative stress induced damage, the body has a highly complex antioxidant system that encompasses both enzymatic (catalase and glutathione peroxidase) **(Birben *et al.*, 2012)** and non-enzymatic factors (glutathione and ascorbic acid) **(Ighodaro *et al.*, 2017).**

Enzymes in red blood cells and their membranes are ubiquitous. In the cytosol, enzymes such as catalase and glutathione peroxidase minimise the effects of oxidative stress **(Nagababu *et al.*, 2003)** and a protease complex (the 20S proteasome) degrades damaged proteins **(Neelam *et al.*, 2011).**

Figure 6: Blood flow in and out of the heart through vessels

Toxic myocarditis, myocardial hypokinesia, left ventricular thrombosis and myocardial dysfunction were reported in a case of Al phosphide intoxication (Hangouche *et al.*, 2017). Ischemic stroke due to thrombosis in the right middle cerebral artery was reported as the delayed complication of Al phosphide poisoning (Abedini *et al.*, 2014). However, other Al compounds may not cause cardiovascular lesion. Cardiac teratogenessis was reported in embryonic chick heart where defects in ventricular septation and ventricular myocardium were reported (El Mazoudy and Bekhet, 2016). There was significant association between increased maternal hair Al contents and risk of total congenital heart defects in offspring, especially in subtypes such as septal defects, conotruncal defects and right ventricular outflow obstruction in female rats (Wang *et al.*, 2012). No histological changes were observed in the hearts of rats given 70 mg Al/kg/day as Al chloride in drinking water for 30, 60, or 90 days (Dixon *et al.*, 1979). Similarly, no effect on organ weight nor histological changes were found in the hearts of rats that ingested 133 or 284 mg Al/kg/day as Al nitrate in drinking water or base diet for 30 days (Gomez *et al.*, 1986) or 100 days, respectively (Domingo *et al.*, 1987). Organ weight and histological changes were not observed in the hearts of dogs that consumed 75 mg Al/kg/day (Katz *et al.*, 1984) or 88 mg Al/kg/day (Pettersen *et al.*, 1990) as sodium Al phosphate in the diet for 6 months. In summary, cardiovascular effects due to toxicosis are congenital heart defects, inflammation and dysfunction of the myocardium and cardiovascular thrombosis.

3. OXIDATIVE STRESS

3.1. Background

The first radical oxygen was discovered by Linus Pauling in 1930s and it was described superoxide **(Pauling, 1979)** Pauling had no knowledge that this radical could be produced biologically or that it could also be the core of several many disease processes. In the same decade, Mann and Keilin **(Mann and Keilin, 1938)** purified the superoxide dismutase (SOD) protein from bovine blood and liver, as a copper-binding protein of unknown function. The protein was called "erythrocuprein" or "hepatocuprein" or later "cytocuprein." The purification was based solely on copper content. Until late 1960s, the pathophysiological importance of ROS was completely unknown. However, several new findings would dramatically lead to a change of this situation:

1. The discovery of McCord and Fridovich in 1968-1969 described the enzymatic activity of the erythrocyte SOD, which led to eliminate the "Pauling free radical", or superoxide anion (O2•–) terminology, and in the same year it was found that SOD was contained in almost all mammalian cells **(McCord and Fridovich, 1968)** The latter finding suggested that O2•– was a physiological product.

2. In 1969, Knowles et al. showed that the enzyme xanthine oxidase (XO) could indeed produce superoxide **(Knowles *et al*., 1969).**

3. In 1973 Babior *et al*. showed that the bactericide action of the neutrophil was associated with large amounts O2•– generation, thereby linking the inflammation process to ROS generation. It was apparent that some of the tissue damage associated with the inflammatory process could be attributed to neutrophil-generated O2•–, and herein SOD would protect cells and extracellular components from damage **(Salin and McCord, 1975; McCord, 1974).**

4. In 1980, the discovery of the endothelium-derived relaxing factor allowed formulation of a novel concept in the pathogenesis of hypertension **(Furchgott and Zawadzki, 1980).** Nevertheless, it took long seven years to determine the identity of this factor and to accept that it corresponds to NO **(Ignarro *et al*.,1987; Palmer *et al*.,1987).**

5. **Granger *et al.*, (1981)** showed that tissue damage of ischemia/reperfusion in cat intestine was caused by increased ROS generation.

From those years until now, hundreds of further researches were needed to achieve our present knowledge on how oxidative stress is implicated in diverse and seemingly unrelated diseases.

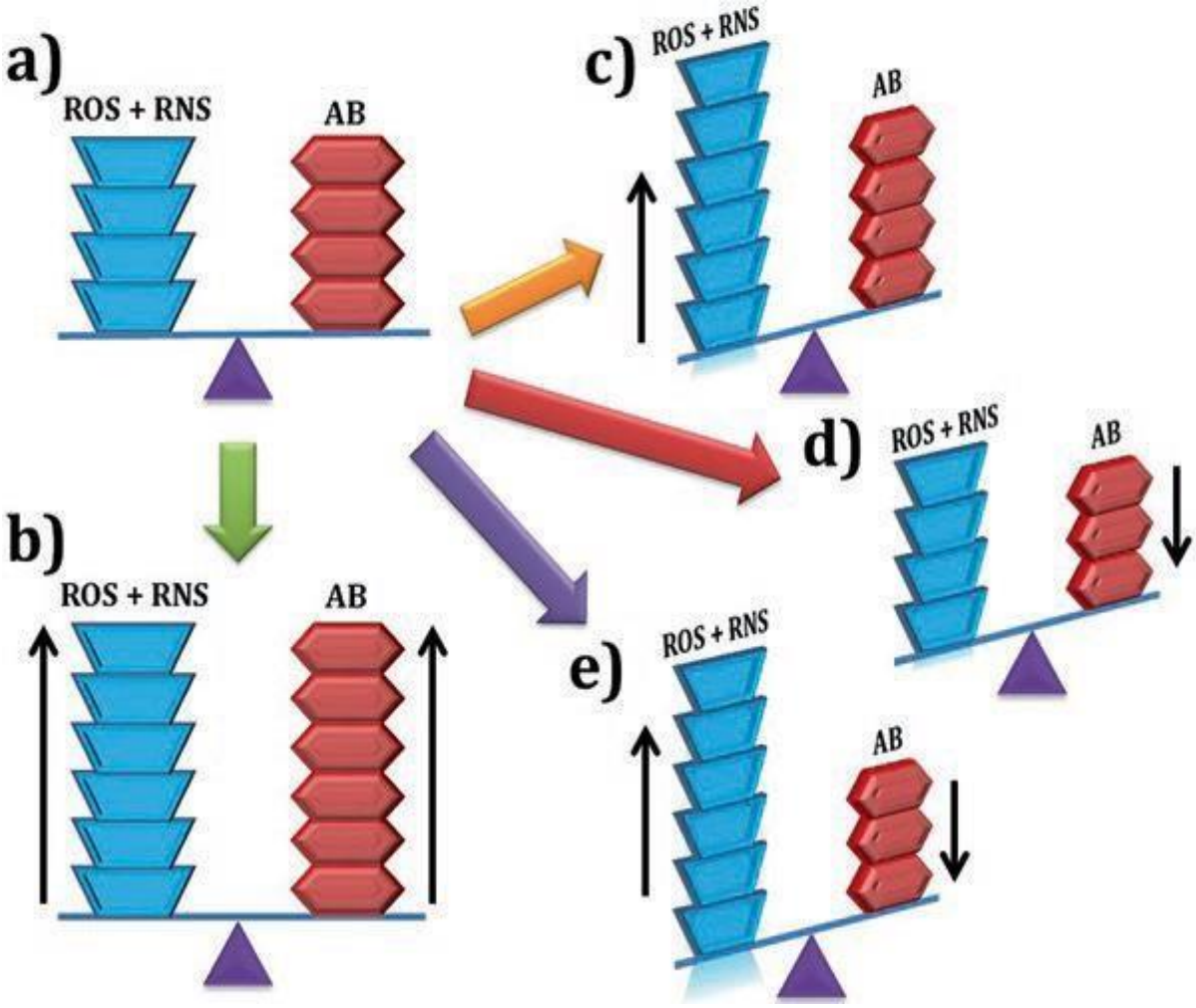


Figure 7: Five hypothetical conditions of pro/antioxidative balance resulting from the mutual relation between sum of ROS and RNS levels and the capacity of the AB.

3.2 The Concept of Oxidative Stress

The definition of oxidative stress as a global concept, in 1985, was **(Sies, 1985**): *A disturbance in the prooxidant-antioxidant balance in favor of the former*. It was an important point, from the beginning, that there is a diversity of prooxidants and a diversity of antioxidants, operating with vastly different chemical and biological reactivities. Likewise, it may be mentioned that there is also a huge diversity of molecular targets: DNA, RNA, proteins, lipids, carbohydrates and other biomolecules.

Prooxidants include free-radical species and non-radical species generated by enzymes or non-enzymatically, and antioxidants include powerful enzymes and also low-molecular mass compounds. A noteworthy insight was the perception that oxidation-reduction (redox) reactions in living cells are utilized in fundamental processes of redox regulation, collectively termed "redox signaling" and "redox control". The concept of oxidative stress was updated in 2007 to include the role of redox signaling **(Sies et Jones, 2007):***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.* The main idea underlying this global concept is that in living biological systems there is a maintenance of a redox balance, that is also called redox homeostasis. This is not an equilibrium as defined in thermodynamics, but it is a steady-state, away from thermodynamic equilibrium, *i.e.*, a non-equilibrium **(Prigogine, 1978).** As for a global definition, specific redox systems are not directly and specifically addressed, but they need to in given cases and conditions. Numerous redox systems of different nature coexist in cells and tissues, and not all of them are directly related or connected, contingent on the presence of redox catalysts, i.e., redox-active enzymes or redox-active compounds.

3.2.1 The Redox Code

These relationships have recently been conceptualized under four principles which, together, make up the *"Redox Code"* **(Jones and Sies, 2015):**

The *first* principle is the use of the reversible electron accepting and donating properties of nicotinamide in NAD and NADP to provide organization of metabolism, operating at nearequilibrium. Substrate oxidations are linked to reduction of NAD+ and NADP+, which in turn are linked to ATP production, catabolism and anabolism, respectively.

The *second* principle is that metabolism is linked to structure through kinetically controlled redox switches in the proteome, which determine tertiary structure, macromolecular interactions and trafficking, activity and function. The abundance of proteins and reactivity of

sulfur switches with oxidants vary over several orders of magnitude to support specificity in biological processes.

The *third* principle is that of redox sensing, in that activation/deactivation cycles of redox metabolism, especially involving H2O2, support spatio-temporal sequencing in the differentiation and life cycles of cells and organisms. The *fourth* principle is that redox networks form an adaptive system to respond to environment from microcompartments through subcellular systems to the levels of cell and tissue organization. This adaptive redox network structure is required to maintain health in a changing environment and, if functionally impaired, contributes to disease and organism failure. The term "Redox Code" applies to the redox organisation of cells, tissues and organisms. It is not confined to mammalian cells, and it extends ultimately to all living matter.

3.2.2 Specific Forms of Oxidative Stress

Given the enormous variety and range of prooxidant and antioxidant enzymes and compounds and of targets, as mentioned above, subforms of oxidative stress were identified **(Sies and Jones, 2007).** Attempts were made to introduce intensity scales ranging from physiological oxidative stress to excessive and toxic oxidative burden **(Lushchak, 2014).** A useful introduction to oxidative stress in biomedical and biological research is available, collecting basic concepts, definitions and currently employed methods used in this field **(Breitenbach and Eckl, 2015).**

3.2.3 Oxidants

However, oxidative stress is used by the immune system to kill the pathogens. Moderate oxidative stress can cause apoptosis and severe oxidation which can result in cell death **(Lennon *et al.*, 1991).** Different oxidants are given in Table 2 Leakage of mitochondrial activated oxygen during oxidative phosphorylation is the main source of reactive oxygen radicals. It has been found that other enzymes contribute to the oxidants in *E. coli* **(Rice-Evans and Gopinathan 1995).** Multiple redox-active flavoproteins may play a vital role in the overall production of oxidants **(Messner and Imlay 2002).** Some enzymes, which can produce superoxides, are xanthine oxidase, cytochrome P450, and NADPH oxidases, while oxidases produce the hydrogen peroxide. Four endogenous sources of oxidants are produced by cells. (a) In aerobic respiration, O2 reduces to produce O2, H2O2, and –OH. (b) Bacteria or virus-infected cells are

destroyed by phagocytosis and produce the nitric oxide (NO), O2-, H2O2, and OCl. (c) Peroxisome produces H2O2 during fatty acid production. (d) Animal cytochrome P450 enzyme may produce some oxidative by-products that can damage DNA. Vitamins C and E, β-carotene, and coenzyme Q are some common antioxidants of diet. Plants may consist of a wide variety of free radical scavenging molecules such as phenolic compounds (phenolic acids, flavonoids, lignans, tannins, quinones, etc.), nitrogen compounds (amines, alkaloids, etc.), vitamins, and terpenoids **(Velioglu *et al.*, 1998).**

Table 2: Different oxidants.

•O_2^-, superoxide anion	It is one-electron reduction state of O_2, formed during electron transport chain and several auto-oxidation reactions. It can form H_2O_2.
H_2O_2, hydrogen peroxide	It is a two-electron reduction state, formed by dismutation of O_2^-, and it is lipid soluble, so it can cross the plasma membrane.
•OH, hydroxyl radical	It is three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. It is extremely reactive.
ROOH, organic hydroperoxide	It is formed by radical reactions with cellular components like lipids and nucelobases.
ONOO-, peroxynitrite	It is formed in the reaction between $•O^-_2$ and NO•

Monitoring of biomarkers like reactive oxygen species and nitrogen species shows that production of these biomarkers may be involved in pathogenesis of the heart.

Alzheimer's disease and schizophrenia, while cumulative oxidative stress with mitochondrial damage and disrupted respiration is related to Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases **(Ramalingam and Kim 2012).** Oxidation of low-density lipoproteins in the vascular endothelium is a precursor to plaque formation, so oxidative stress may link to certain cardiovascular disease. Oxidative stress can cause tissue injury, hyperoxia, and diabetes. It may be involved in age-related development cancer. Reactive species are mutagenic, cause direct or indirect damage to DNA, and may also suppress apoptosis and promote metastasis, invasiveness, and proliferation. High doses of synthetic beta-carotene enhance the rate of lung cancer in smokers. Excess NO combines with tyrosine, which is essential for enzyme ribonucleoside diphosphate. Excess vascular O2 production may cause the hypertension and vasopasm **(Lepoivre *et al.*, 1994).** Reactive oxygen species (ROS) attack glial cells and neurons and lead to neuronal damage **(Gilgun-Sherki *et al.*, 2001).** It has been found that the deleterious effects of ROS on human cells may cause oxidative injury which can lead to apoptosis **(Salganik, 2001).**

3.2.4 Antioxidants

Antioxidant compounds have anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antiviral, and antibacterial activities **(Owen *et al.*, 2000).** Natural antioxidants decrease the risk of cancer, diabetes, and cardiovascular diseases and protect humans from infection, but they can cause oxidative damage and mutation to DNA and cancer. Antioxidant can be exogenous (natural or synthetic) or endogenous; both types of antioxidants can remove the free radicals, scavenge ROS, and bind metal ions which are necessary for catalysis of ROS generation **(Gilgun-Sherki et al. 2001).** Natural antioxidants are classified into two groups, enzymatic and nonenzymatic **(Kurutas, 2016).**Different applications of antioxidants are shown in Fig. 2.3. Enzymatic antioxidants consist of a few proteins such as catalase and glutathione peroxidase. Nonenzymatic antioxidants like ascorbic acid, lipoic acid, polyphenols, carotenoids, etc. comprise directacting antioxidants, which play a vital role in defense against oxygen species. Indirect-acting antioxidants consist of chelating agents. They bind to redox metals and stop the free radical generation **(Gilgun-Sherki *et al.*, 2001).** Activated phagocytes produce reactive oxygen and nitrogen species, used by the immune system to kill the pathogens. It has advantages that it damages almost every part of the target cell and prevents the escaping of the pathogen by mutation of single molecular target **(Rice-Evans and Gopinathan 1995).**

3.3 Generation of Reactive Oxygen and Nitrogen Species

The generation of ROS is a physiological and normal attribute of any kind of aerobic life. In mammalian, under physiological conditions, cells metabolize approximately 95% of the oxygen (O2) to water, without formation of any toxic intermediates. Water if formed according to the following tetravalent reaction:

$$O2 + 4H+ + 4e– \rightarrow 2H2O$$

The first impressions about oxygen as an element were made by the Swedish researcher C.W. Scheele in the XVIII century. However, it was only in XX century when it was demonstrated what Scheele himself had already anticipated that O2 in its pure state at high pressure and concentration is toxic for animals, and herein for several life forms. The later was followed by new interesting discoveries, generating the controversy called until these days as “the oxygen paradox” Several investigations from the last thirty years were needed to agree that, in normal conditions, a minimal 5% of O2 is metabolized through univalent reduction, following four different reactions or stages:

Reaction 1: $O2 + e \rightarrow O2•–$

Reaction 2: $O2•– + e \rightarrow H2O2$

Reaction 3: $H2O2 + e \rightarrow •OH$

Reaction 4: $•OH + e \rightarrow H2O$

Indeed, the final product is still H2O. However, through these four reactions three highly toxic species are formed, two of them being free radicals: O2•– and hydroxyl radical (•OH). Hydrogen peroxide (H2O2) is still a highly reactive compound, but not a radical in strict sense. This four stages model was the first to be discovered, and in fact it explains in general terms the mitochondrial generation of ROS in normal cellular metabolism. The intermediates do not leave the complex before the process is finished, but in some pathophysiological conditions ROS can leave the respiratory burst. On the other hand, once synthesized, NO might follow different pathways:

1. Diffusion to neighbor cells. The presence of an unpaired electron on its molecule allows NO to interact with transition metals, derived from different enzymes, to modulate its activity

(Tamir and Tannenbaum, 1996; Thomas *et al.*, 2001). The diffusion coefficient of this gas depends on lipids and proteins on its microenvironment.

2. Autooxidation: Usually it occurs at severe high concentrations of NO. In the presence of O2 it becomes into dinitrogen trioxide (N2O3) **(Espey *et al.*, 2001; Rafikova *et al.*, 2002).** This reaction increases when it takes place in hydrophobic sites, such as the inside part of lipid membranes or proteic nucleus **(Rafikova *et al.*, 2002; Liu *et al.*,1998).** The molecule of N2O3 is a powerful nitrosant agent, with great affinity for nucleophilic sites **(Thomas *et al.*, 2001).**

3. Reaction with superoxide.The half-life of NO and therefore its biological activity is decisively determined by O2•– concentration **(Gryglewski *et al.*, 1986).** This reaction has a limited diffusion kinetic curve, and thus it is thought that it rules the destination of NO in the presence of O2•– **(Espey, 2002; Daiber *et al.*, 2002).** The final product is ONOO-, a highly oxidant RNS similar to •OH in terms of toxicity. Therefore, ONOO- formation represents a major potential pathway of NO reactivity, depending on the rates of tissue O2•– production. In mammalian cells ROS might be formed through different pathways, either enzymatically or non-enzymatically. For instance, the generation of O2•–, as well as other ROS, requires cell activation involving alteration of the cell membrane structure what in turn activates the generation of lipid peroxidation product molecules. In the context of this chapter, relevant pathways will be described below.

a. Fenton reaction.This reaction has been known since 1894 and is currently one of the most powerful oxidizing reactions available. The reaction involves H2O2 and a ferrous iron catalyst. The peroxide is broken down into a hydroxide ion and a •OH. The latter is the primary oxidizing species and can be used to oxidize and break apart organic molecules.

Similarly, another study identified the presence of translation initiation factors and proteins for cellular defence, as well as the expected protein categories **(Kakhniashvili *et al.*, 2004).** Of note, this study also reported that approximately 24 % of the total observed proteins had unknown functions and could not be categorised **(Kakhniashvili *et al.*, 2004).** The complexity does not end here, with total RNA transcripts of 1019 genes now reported to be present in red blood cells **(Kabanova *et al.*,2009).** These genes were identified as being relevant for many normal cellular processes including metabolism (52 %), programmed cell death (5 %), and even

some genes for translation/transcription of protein (up to 5 %) **(Kabanova *et al.*, 2009).** Red blood cells contain considerably more active cellular components than their primary role would suggest and may be more complex than conventionally described. The complexity of these cells and the presence of unknown molecules calls for significant further investigation.

$$Fe(II) + H2O2 \rightarrow Fe(III) + \bullet OH + -OH)$$ **(Stohs *et al.*, 1995).**

It is well known that organic compounds can be easily oxidized. One primary advantage of the Fenton's Reaction is that it does not produce further organic compounds or inorganic solids such as permanganate and dichromate, since there is no carbon in the peroxide. This makes the Fenton's Reaction more appealing than a biological process, if the goal is removal of organic compounds. The mechanism of reaction with respect to hydrogen peroxide is very complex and may change with conditions of the reaction.

b. Haber-Weiss reaction:

The one-electron reduction of hydrogen peroxide by superoxide has also been invoked as a potential source of •OH:

$$O2\bullet- + H2O2 \rightarrow O2 + \bullet OH + OH-$$

This scheme has been exhaustively investigated and it is now generally accepted that the Haber-Weiss reaction does not occur in the absence of metal catalysis.

This reaction combines a Fenton reaction and the reduction of Fe(III) by O2•–, yielding Fe(II) and O2

$$Fe(III) + O2\bullet- \rightarrow Fe(II) + O2$$ **(Liochev *et al.*, 2002).**

c. Xanthine oxidase: The enzyme xanthine oxidase (XO) catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid, generating O2•-. This enzyme plays an important role in the catabolism of purines in some species, including humans. Under pathological conditions, such as tissue ischemia, xanthine dehydrogenase can be converted to XO. xanthine + $H2O + O2 \rightarrow$ uric acid + O2•-

d. NADPH oxidase: The enzyme NADPH oxidase (Nox) catalyzes the one electron reduction of O2 to generate O2•-, using NADPH as the source of electrons. This enzyme has a complex

function that is most easily understood in the context of the activated neutrophil, wherein it generates large amounts of toxic superoxide anion and other ROS important in bactericidal function. In addition, it is also functional in membranes of vascular endothelial and VSMC, and fibroblasts providing a constitutive source of O2•-. This enzyme consists of several membrane-bound subunits (gp91, Nox, and p22phox) and cytosolic subunits (p47phox, p67phox, p40phox, and Rac2). There appear to be at least three isoforms of NADPH oxidase expressed in the vascular wall.

e. Nitric oxide synthase: NO synthases (NOS) are a family of enzymes that convert the amino acid L-arginine to L-citrulline and NO. All NOS isoforms are homodimeric enzymes that require the same substrate (L-arginine), cosubstrates (molecular oxygen, NADPH) and cofactors such as FMN, FAD, tetrahydrobiopterin (BH4) and hem group.

Three main isoenzymes exist in mammals that are regulated by distinct genes: a constitutive neuronal NOS (nNOS or NOS I), an endotoxin- and cytokine-inducible NOS (iNOS or NOS II) and a constitutive endothelial NOS (eNOS or NOS III). Neuronal NOS performs an important role in intracellular communication. Inducible NOS uses NO to induce oxidative stress on pathogens. Endothelial NOS plays a major role in the regulation of vascular function. For instance, eNOS synthesizes NO by a two-step oxidation of the amino acid L-arginine thereby leading to activation of guanylyl cyclase (sGC). The resulting second messenger cGMP in turn activates the cGMP-dependent kinase, which leads to decrease in intracellular Ca+2 concentrations thereby causing vasorelaxation. However, it has become clear, from studies with the purified enzyme, that eNOS may become uncoupled in the absence of the NOS substrate Larginine or the cofactor tetrahydrobiopterin (BH4). Uncoupled state results in the production of O2•- rather than NO **(Vasquez–Viva *et al*., 1998; Xia and Zweier, 1997).** The key mechanisms causing eNOS uncoupling are attributed to a decrease in intracellular BH4 levels caused either by ONOO--induced BH4 oxidation or by decreased activity of the guanosine triphosphate cyclohydrolase I enzyme and the dihydrofolate reductase, both related to BH4 synthesis **(Schulz *et al*., 2008).**

f. Mieloperoxidase:The mieloperoxidase enzyme (MPO) produces hypochlorous acid (HOCl) from H2O2 and chloride anion (Cl-) during the neutrophil's respiratory burst. It requires heme as a cofactor. In addition, it oxidizes tyrosine to tyrosyl radical using H2O2 as oxidizing

agent (**Heinecke *et al.*, 1993).**Both HOCl and tyrosyl radical are cytotoxic, and used by the neutrophil to kill bacteria and other pathogens.

g. Cytochrome P450:The membrane-bound microsomal monooxygenase is a multienzyme system that generally summarizes as cytochrome P450 (C-P450), as the terminal oxidase and an FAD/FMN-containing NADPH-cytochrome P450 reductase (CPR). The most common reaction catalyzed by the C-P450 is a monooxygenase reaction. This might be, for example, the insertion of one atom of oxygen into an organic substrate (RH) while the other oxygen atom is reduced to H2O.

$$RH + O2 + 2H+ + 2e– \rightarrow ROH + H2O$$

One ROS-generating way is given by ferric P450. Once bounded to the substrate, ferric P450 reduces CPR by accepting its first electron, thereby being reduced. Then, this new ferrous hemoprotein binds an oxygen molecule to form oxycomplex, which is further reduced to give peroxycomplex. The input of protons to this intermediate can result in the heterolytic cleavage of the O–O bond, producing H2O and the 'oxenoid' complex, the latter of which then inserts the heme-bound activated oxygen atom into the substrate molecule. Finally, the decomposition of this final one-electron-reduced ternary complex results in O2•– release. The second ROSproducing branch is the protonation of the peroxycytochrome P450 with the formation of H2O2 **(Davydov *et al*., 2001).**

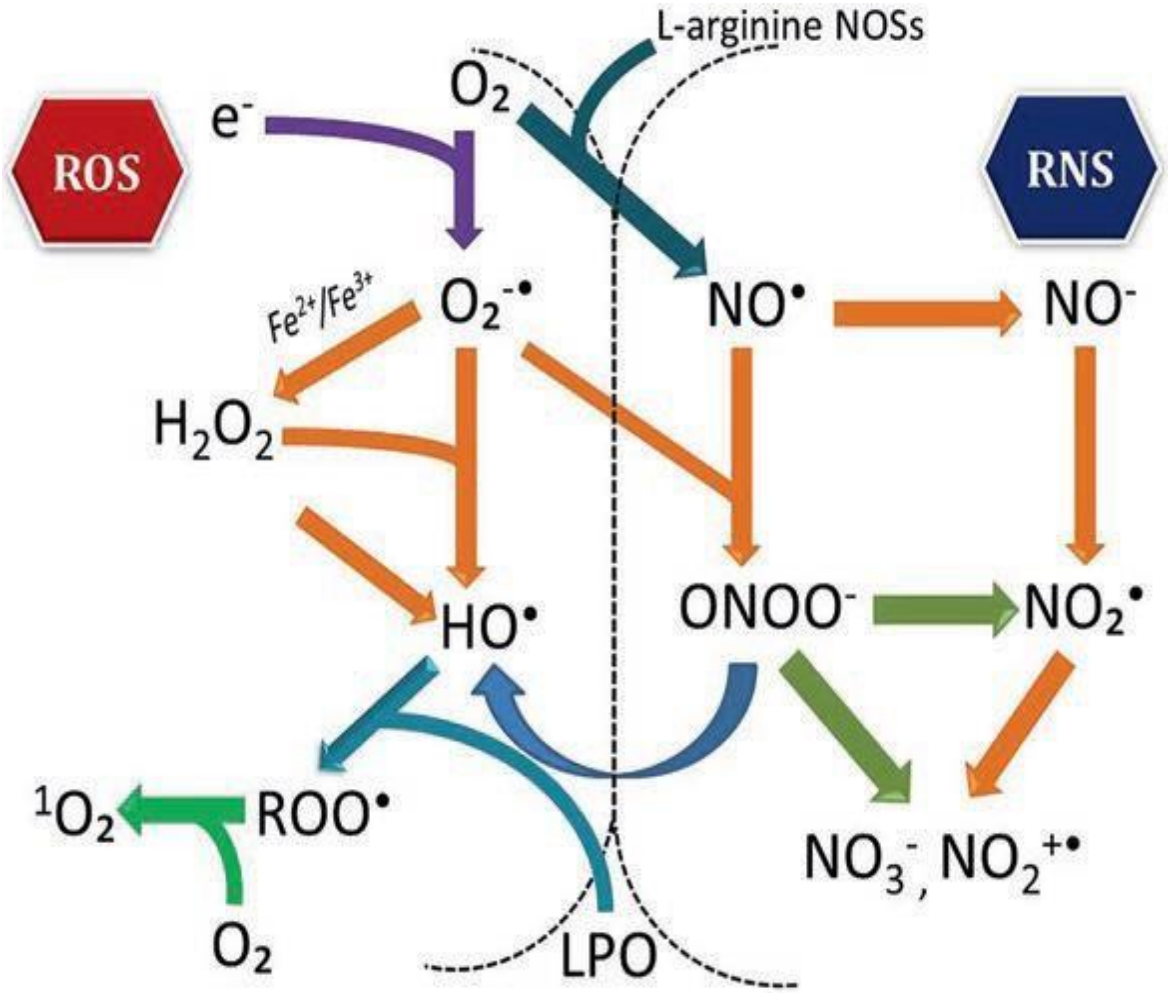


Figure 8: The primary cellular ROS and RNS as well as their biochemical interrelations.

3.3.1. Reactive Oxygen Species as Mediators of Cell Damage

As mentioned previously, ROS have physiological functions that are essential in cells, such as mitochondrial respiration, prostaglandin production pathways and host defense **(Webster and Nunn, 1988).** Moreover, NO plays an important role in antagonizing the vasoconstrictor effects of Angiotensin II (Ang-II), endothelins and ROS **(Pechánová and Simko, 2007).** However, ROS have well known involvement in common-shared pathophysiological models causing cell damage, either directly or through behaving as intermediates in diverse signaling pathways, including DNA damage, protein oxidation and lipid peroxidation resulting, among others, in membrane damage **(Zimmerman, 1995).**

3.3.2. DNA Damage

Oxidative DNA modifications are frequent in mammalian and have been suggested as important contributory factors to the mechanism in carcinogenesis, diabetes and natural aging. The DNA damages are considered as the most serious ROS-induced cellular modifications as DNA is not synthesized *de novo* but copied, perpetuating by this way those modifications and hence inducing mutations and genetic instability. The main responsible ROS of DNA damage is •OH, which reacts with all components of the DNA molecule, damaging both purine and pyrimidine bases and the deoxyribose backbone. This is explained by the diffusion-limited •OH ability to add to double bonds of DNA bases, abstracting a hydrogen atom from the methyl group of thymine and each of the five carbon atoms of 2-deoxyribose **(Dizdaroglu *et al*., 2001).** Further reactions of base and sugar radicals generate a variety of modified bases and sugars, base-free sites, strand breaks and DNA protein cross-links. In addition, RNS such as ONOO- and •NO have also been implicated in DNA damage **(Brown *et al*., 2001).** This can be explained by the following mechanisms:

A. Direct Damage to DNA through Reactive Nitrogen Species:

- Endogenous formation of carcinogenic N-nitrosamine molecules: N-nitrosamines are chemical molecules with known carcinogenic ability, because of their conversion to strong aliquant agents. They are synthesized through the reaction of N2O3 and biogenic amines:

$$\mathbf{R}NH2 + N2O3 \rightarrow \mathbf{R}NHO + NO2–$$
$$\mathbf{R}NHNO \rightarrow \mathbf{R}NNOH \rightarrow ROH + N2$$

Endogenous production of these compounds has been demonstrated in immortalized hepatocytes with the SV 40 apes virus. **(Wiseman and Halliwell, 1996)**

- Bases oxidation:

In cultured cells, protocols on activated macrophages show oxidative and deamination damage of DNA **(Tamir and Tannenbaum, 1996).** Further analysis of the NO final onset revealed that

most part of it was transformed to ONOO- **(Rojas-Walker *et al*., 1995).** The treatment of DNA plasmids with synthetic ONOO-, and its insertion into biological systems for replication and further analysis, confirmed a range of specific mutations, mainly transversions from guanine to thimine, and guanine to cytosine **(Tretyakova *et al*., 2000).** The oxidant power of ONOO- is also enough to directly damage sugar and creates sites with no nitrogenated bases on DNA, as well as oxidizing and modifying bases thus generating hard-reparation class bases **(Tretyakova, Wishnok *et al*., 2000).** The production of DNA damage through this mechanism also occurs mostly in simple chain DNA.

B. Indirect Modifying DNA Sequence by Reactive Nitrogen Species

Some authors have suggested that either deamination, oxidation and DNA chain rupture by RNS requires extremely high concentrations of these species, a situation that would be exceptionally possible in humans. Moreover, *in vivo*, some antioxidants molecules such as ascorbate and reduced glutathione (GSH) are abundant, thus the RNS possibilities of accumulation at enough concentrations to produce direct DNA damage are extraordinarily low **(Halliwel *et al*., 1999).** One of the suggested hypotheses is based on the inhibition of the DNA repairing enzymatic systems, thereby making possible indirect damage. The RNS have a high affinity for the thiol group (-SH) of cysteine **(Jourd'hevil *et al*., 2000)** and it is believed that those enzymes containing critic cysteine for their activity might be inhibited through RNS. Other nucleophilic groups, such as hydroxyl (-OH) from tyrosine **(Fries *et al*., 2003)** and amine (-NH2) of lysine **(Espey, 2002)** are also potentially modifiable. All of the mechanisms exposed before contribute to elucidate from diverse points of view the mutagenic effects of NO. While being on the right position, these mutations could result in the inactivation of suppressor tumor genes, and further activation of oncogenes, thus participating in various stages of carcinogenic process. The most evident example of this is given by the protein for p53 gen, which is mutated on nearly 50% of human tumors **(Oren, 1999).** Previous researches confirmed *in vitro* mutations in p53 gene induced by NO and its methylation **(Murata *et al*., 1997).** Further investigations also verified that there is a significant relation between the RNS activities and the mutations on p53 gene in early staged lung carcinoma **(Fujimoto *et al*., 1998).** Even though in these studies the functionality of the genetic product was not analyzed, the hypothesis on the role of NO is clear enough to consider that NO, in stress conditions such as inflammation, is able to inactivate p53 gene, therefore to create a favorable environment for tumors emerging and development.

3.3.3. Lipid Peroxidation

It is known that ROS attack cellular components involving polyunsaturated fatty acid (PUFA) residues of phospholipids, which are extremely sensitive to oxidation **(Esterbauer *et al.*, 1991).** The overall process of lipid peroxidation consists of three stages: initiation, propagation, and termination **(Pinchuk *et al.*, 1998; Nyska and Kohen, 2002).** Once formed, peroxyl radicals can be rearranged via a cyclization reaction to endoperoxides, being malondialdehyde (MDA) the final product **(Marnett, 1999).** The MDA is a minor lipid peroxidation product generated by heating of endoperoxides derived from arachidonic acid. The main PUFA in tissue is linoleic acid, five times more abundant than arachidonic acid. Linoleic acid generates only traces of MDA **(Pryor *et al.*, 1976),** but it is transformed as easily as arachidonic acid to peroxyl radicals. The F2-isoprostanes are useful to demonstrate the occurrence of non-enzymatic lipid peroxidation processes, nevertheless they are only trace products formed through free radicals catalyzed attack on esterified arachidonate, providing a reliable tool to identify population with enhanced rates of lipid peroxidation **(Patrignani and Tacconelli, 2005).** Lipid peroxidation involves low-density lipoprotein (LDL) as well as highdensity lipoprotein (HDL) oxidation. It is well known that the LDL oxidation is a key process in the pathogenesis of atherosclerosis **(Spiteller, 2003).**The oxidized cholesterol esters are directly incorporated into lipoproteins and transferred to endothelial cells via the LDL where they induce damage and start the sequence of events leading to atherosclerosis.

3.3.4. Protein Oxidation

The side chains of all amino acid residues of proteins are susceptible to oxidation by the action of ROS **(Stadtman, 2004).** The protein carbonyl group is generated by ROS through many different mechanisms and its concentration is a good measure of protein oxidation via oxidative stress. The NO reacts rapidly with O2•– to form the highly toxic ONOO- that is able to nitrosate the cysteine sulfhydryl groups of proteins, to nitrate tyrosine and tryptophan residues of proteins and to oxidize methionine residues to methionine sulfoxide **(Valko *et al.*, 2006).** Oxidation of proteins is associated with a number of age-related diseases and aging **(Stadtman, 2001; Levine and Stadtman, 2001).**

3.3.5. Other Damage

Oxidative damage to the mitochondrial membrane can also occur, resulting in membrane depolarization and the uncoupling of oxidative phosphorylation, with altered cellular respiration

(Nathan and Singer, 1999). This can ultimately lead to mitochondrial damage, with release of cytochrome c, activation of caspases and apoptosis **(Macdonald *et al.*, 2003).**

3.3.6. Pathophysiological Conditions

In pathophysiological conditions, the main sources of ROS include the mitochondrial respiratory electron transport chain, XO activation through ischemia–reperfusion, the respiratory burst associated with neutrophil activation, and arachidonic acid (AA) metabolism. Activated neutrophils produce O2•– as a cytotoxic agent as part of the respiratory burst via the action of membrane-bound NADPH oxidase on O2. Neutrophils also synthesize NO that can react with O2•– to produce ONOO-, a powerful oxidant, which may decompose to form •OH. Additionally, in ischemia-reperfusion XO catalyzes the formation of uric acid with the co-production of O2•–. The enhanced O2•– released results in the recruitment and activation of neutrophils and their adherence to endothelial cells, which in turn stimulates the formation of XO in the endothelium, with further O2•– production as a positive feedback model pathway. Accordingly, allopurinol, a XO inhibitor, has been demonstrated that blocks the O2•– production in ischemia–reperfusion settings involving organs such as heart **(Tan *et al.*, 1993),** liver **(Granger, 1988),** kidney **(Terada *et al.*, 1992),** and small intestine **(Grisham *et al.*, 1986).**

3.3.7. Role of Hydrogen Peroxide in Oxidative Stress and Redox Signaling

Hydrogen peroxide, the two-electron reduction product of oxygen, was identified in 1970 as a normal metabolite under aerobic conditions in living cells **(Sies H and Chance, 1970),** occurring at about 10 nM intracellular concentration **(Chance *et al.*, 1979).** A major contributor is the mitochondrial respiratory chain **(Boveris *et al.*, 1972; Yin *et al.*, 2014),** notably Complexes I and III, but also Complex II **(Bleier *et al.*, 2015).** Important further mitochondrial sources of hydrogen peroxide are given by several dehydrogenases, notably 2oxoacid dehydrogenases **(Goncalves *et al.*, 2015; Quinlan *et al.*, 2014),** as reviewed in **(Mailloux, 2015).** In liver, H2O2 is produced at a rate of 50 nmol/min × g of tissue, which is about 2 % of total oxygen uptake in the steady state of physiological conditions **(Oshino *et al.*, 1973).** Metabolically generated H2O2 emerged from recent research as a central hub in redox signaling and oxidative stress **(Sies, 2014).** Hydrogen peroxide is well suited for redox signaling **(Forman *et al.*, 2010),** and the role of peroxiredoxins is of paramount importance **(Rhee and**

Woo 2011).A novel aspect of hydrogen peroxide signaling relates to its diffusion properties. While it has long been assumed that hydrogen peroxide diffusion through lipid containing membranes occurs at a sufficient rate, the discovery of facilitated diffusion through specific aquaporins provides for spatio-temporal control **(Henzler and Steudle, 2000).** This discovery opened an exciting field on membrane transport of hydrogen peroxide by peroxiporins **(Bienert *et al.*, 2006; Bienert *et al.*, 2007; Bienert and Chaumont, 2014)** and its significance for redox signaling **(Hara-Chikuma *et al.*, 2015; Hara-Chikuma *et al.*, 2016; Watanabe *et al.*, 2016).**

3.4. Antioxidant Defense System

All forms of life maintain a reducing environment within the cells. The maintenance of this status is achieved possibly through the antioxidant defense system, which is in action to protect cellular homeostasis against harmful ROS produced during normal cellular metabolism, as well as in the patho-physiological states. The antioxidant system is preserved by antioxidant substances that maintain the reduced state by a constant input of metabolic energy. Antioxidant substances are small molecules that can scavenge free radicals by accepting or donating an electron to eliminate the unpaired condition. **(Lobo *et al.*, 2010).** Typically, this means that the antioxidant molecule becomes a free radical in the process of scavenging a ROS to a more stable and less reactive molecule. In most cases the scavenger molecule provides hydrogen radical that combines with the free radical. Consequently, it is generated a new radical that has an enhanced lifetime compared with the starting one, for instance, due to a conjugated system (**Spiteller, 2003).** The extended lifetime of this radical enables it to react with a second radical by formation of a new molecule and thus one scavenger molecule can eliminate two radicals. Antioxidant molecules can be produced endogenously or provided exogenously through diet or antioxidant supplements. The main endogenous antioxidant enzymes are SOD, catalase (CAT), and glutathione peroxidase (GSH-Px). The SOD converts superoxide anion to H2O2, which is a substrate for CAT and GSH-Px. Catalase metabolizes H2O2 to water and oxygen and GSH-Px reduces both H2O2 and organic hydroperoxides when reacting with GSH **(Andreol, 1991).** Reduced glutathione is present at high concentrations in all mammalian cells, especially in the renal cells, hepatocytes, and erythrocytes **(Sehirli *et al.*, 2003).** This tripeptide protects protein thiol groups from non-enzymatic oxidation or as a co-substrate of GSH-Px **(Meister and Anderson, 1983).**

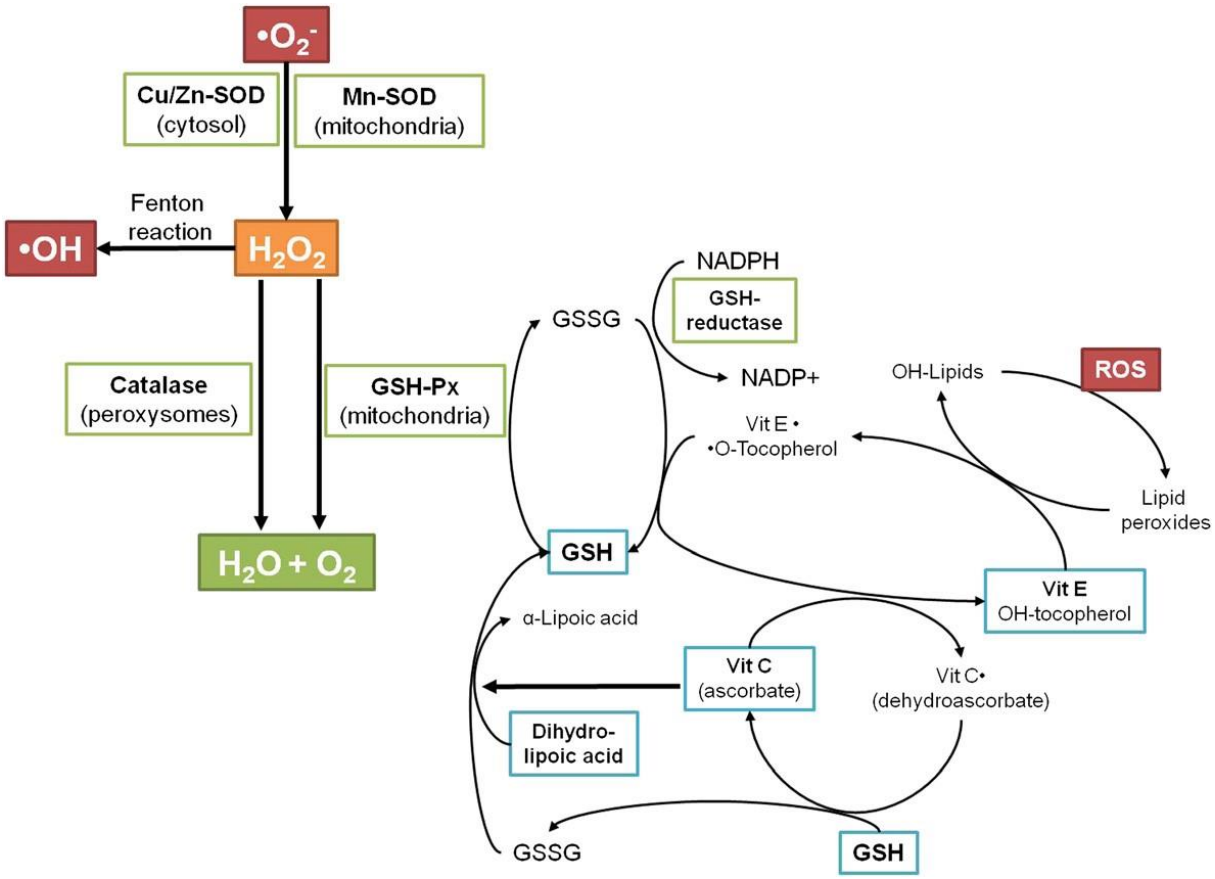


Figure 9: Antioxidant defenses in the organism.

The endogenous antioxidant defense system mainly enzymatic is summarized on Figure 5 Exogenous antioxidants, such as vitamins E and C, exist at a number of locations namely on the cell membrane, intracellular and extracellular. They react with ROS to either remove or inhibit them. The hydrophobic lipid interior of membranes requires a different spectrum of antioxidants. Fat-soluble vitamin E is the most important antioxidant in this environment, which protects against the loss of membrane integrity. Fat-soluble antioxidants are important in preventing membrane polyunsaturated fatty acids (PUFA) from undergoing lipid peroxidation. **(Kurutas, 2016).** Glutathione removes already generated radical, if no radicals are present, the PUFA cannot be attacked. Therefore, they shield the membrane rich in PUFA against ROS **(Spiteller, 2002).** In addition, water-soluble antioxidants including vitamin C play a key role in scavenging ROS in the hydrophilic phase. Other small antioxidant molecules are also naturally present in the plasma, such as uric acid and bilirubin. Recently, it was found that fish, fish oils, and some vegetables contain furan fatty acids that are radical scavengers, partly responsible for the beneficial efficiency of a fish diet **(Spiteller, 2005).**

Table 3: Functional characteristics of the antioxidant enzymes

Antioxidant enzyme	Chemical name	Scavenged oxidant agent	General characteristics
GSH-Px	Glutathione peroxidase	H2O2	It is the major endogenous antioxidant molecule. It catalyzes the conversion of H2O2 and organic peroxides into water or alcohols, respectively.
SOD	Superoxide dismutase	O2•-	It catalyzes the conversion of O2•- to O2 and to less-reactive species like H2O2. Necessary for the release of biologically active NO. It protects NO from inactivation.
CAT	Catalase	H2O2	It catalyzes the breakdown of H2O2 to water and molecular oxygen.

4. POLYPHENOLS, QUERCETIN, RUTIN & PHAGHALON RUPESTRE

4.1.1. General overview

A group of antioxidants that is often suggested to be good candidates for antioxidant therapy due to their potential role in supporting health are the flavonoids. Flavonoids are a class of naturally occurring polyphenolic compounds, ubiquitously present in photosynthesizing cells **(Saito, 1974; Salunkhe *et al.*, 1982).** Over 5000 different naturally occurring flavonoids have already been identified and the list is still growing **(Middleton and Kandaswami, 1993).** Flavonoids are present in fruits, vegetables, nuts and plant-derived beverages such as tea and wine **(Hertog *et al.*, 1993b).** Additionally, many antioxidant supplements and herb-containing medicaments contain high doses of flavonoids.

Most flavonoids share a common three-ring structure, depicted in Figure 7, of which rings A and B are aromatic and ring C is heterocyclic. The variation in the heterocyclic C ring forms the basis of the division of the flavonoids in various subclasses, i.e., the flavones, isoflavones, flavonols, flavanals, flavanones, anthocyanidins and chalcones **(Scalbert and Williamson, 2000).**

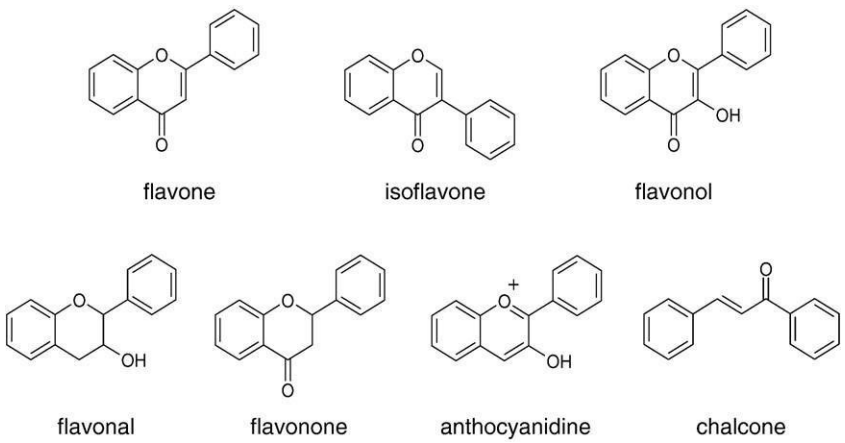


Figure 10: Structures of the major classes of flavonoids

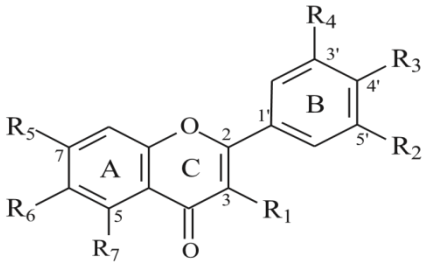
Quercetin (3,5,7-trihydroxy-2-(3,4-dihydroxyphenyl)-4Hchromen-4-one) is a dietary flavonoid, which widely existed in caper, black chokeberry, onion, tomato and lettuce **(Bischoff, 2008).** In plants, quercetin is usually in a bound form with sugars, ethers or phenolic acids and etc. Different forms of quercetin derivatives seem to influence their rate of absorption in the small intestine and stomach **(Mullen *et al.*, 2008).** The content and form of its derivatives play a key role in their absorption **(Rahman *et al.*, 2006).**

Quercetin has attracted increasing attention due to its antioxidant **(Duenas et al., 2010),** antiobesity **(Nabavi *et al.*, 2015),** anti-carcinogenic **(Kumari *et al.*, 2010),** antiviral **(Ganesan *et al.*, 2012),** antibacterial **(Rattanachaikunsopon and Phumkhachorn, 2010)** and anti-inflammatory effects **(Kleemann *et al.*, 2011).** Moreover, quercetin has been reported to have a strong potential in the treatment of cancers. Globally, it is estimated that about 1.68 million new cases of cancer are expected to be diagnosed in 2016 **(Siegel *et al.*, 2016).** As documented, quercetin can inhibit the proliferation of different types of cancer cells (e.g., colorectal cancer cells, prostate cancer cells, liver cancer cells, pancreatic cancer cells and lung cancer cells) by modulating their cellular processes and restraining them from growing **(Lee *et al.*, 2011).** It is also reported that the anticancer function of quercetin is essentially associated to its strong antioxidant capacity **(Conklin, 2000).** Due to its potential health benefits for human, quercetin has come into the focus of utilization as a beneficial ingredient in food and pharmaceutical industries

4.1.2. Chemical structures of quercetin and its derivatives

Quercetin has a typical flavonoid structure and contains five hydroxyl groups. Figure8 displays the structural characteristics of flavonoids: 2 benzene rings (A and B) connected by oxygen containing pyrene ring (C). Quercetin is commonly found in its glycoside form, in which one or more hydroxyl group is replaced by different types of sugar groups. The main groups of quercetin derivatives are quercetin O-glycosides and some other common derivatives. The molecular structure and some physicochemical properties of quercetin and its derivatives are shown in Figure 9. In general, all these compounds have poor solubility in water. Quercetin and its derivatives usually exist in the form of yellow colored powder or crystals. Quercetin Oglycosides are the derivatives with at least one Oglycosidic bond. Many plants and vegetables contain quercetin Oglycosides and the most common glycosylation site is located at the C-3 carbon. The associated monosaccharides may include glucose, galactose and xylose. Quercetin 3-O-glucoside has been found in beans **(Chang and Wong, 2004),** salvia **(Esmaeili and**

Sonboli, 2010) and buckwheat (Kalinova and Vrchotova, 2009). Quercetin 3-O-galactoside is found in lingonberry **(Heyman *et al.*, 2014)** and plum **(Kim *et al.*, 2003),** whereas quercetin 3-O-xyloside is presented in mango fruit **(Masibo and He, 2008).** Quercetin derivatives in the form of disaccharides are also widely present in plants and vegetables. For example, rutin (quercetin 3-O-rhamnosylglucoside) has been found in abundance in cherries **(Goncalves *et al.*, 2004),** spinach **(Kuti and Konuru, 2004),** grapes and prunes **(Gallaher and Gallaher, 2009).** Moreover, three, four or more saccharide groups have also been detected in quercetin 3O-glycoside **(Williams and Grayer, 2004).** Other glycosylation sites in quercetin derivatives can be on the hydroxyl group at C-7 carbon and C-4 carbon. For examples, Quercetin 7-Oglucoside in beans **(Chang and Wong, 2004)** has the glycosylation site at C-7 carbon. The quercetin derivative with glycosylation site at C-4 carbon is only found in onion **(Price *et al.*, 1997).**



Systematic name	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Quercetin	OH	OH	OH	H	OH	H	OH
Quercetin 3-*O*-rhamnoside (quercitrin)	O-Rha	OH	OH	H	OH	H	OH
Quercetin 3-*O*-rhamnozyl-(1→6)-glucoside (rutin)	O-RG	OH	OH	H	OH	H	OH
Quercetin 3-*O*-glucoside (isoquercitrin)	O-Glu	OH	OH	H	OH	H	OH
Quercetin 3-*O*-galactoside (Hyperoside)	O-Gal	OH	OH	H	OH	H	OH
Quercetin 7-*O*-glucoside	OH	OH	OH	H	OH	H	O-Glu
Quercetin 3-*O*-rhamnoside-7-*O*-glucoside	O-Rha	OH	OH	O-Glu	OH	H	OH
Quercetin 6-C- glucoside	OH	OH	OH	H	OH	Glu	OH
Quercetin 3'- methyl ether (isohramnetin)	OH	O-Met	OH	H	OH	H	OH
Quercetin 7- methyl ether (rhamnetin)	OH	OH	OH	H	OH	H	O-Met
Quercetin 4'- methyl ether (tamarixetin)	OH	OH	O-Met	H	OH	H	OH

Gal: galactose; Glu: glucose; Rha: rhamnose; RG: rhamnosyl glucose; Met: methyl

Figure 11: Chemical structures of quercetin and its main derivatives.

4.1.3. Absorption, metabolism and bioavailability

3.1. Absorption and metabolism

The in vivo absorption, distribution, metabolism, and bioavailability of quercetin have been extensively studied in animal models and in human.

In the mouth, quercetin released from the food can interact with salivary proteins, and form soluble quercetin-protein binary aggregates **(Manach *et al.,* 2004).** However, it was reported that the absorption of quercetin hardly changed despite the formation of the binary aggregates **(Cai and Bennick, 2006).**

In the stomach, quercetin is exposed to the strong acidic condition, and may be degraded to phenolic acids (e.g., protocatechuic acid) by bacterial ring fission, leading to the breakdown of the skeleton structure of quercetin **(Weldin *et al.,* 2003).** Furthermore, phenolic acids could also be absorbed in the stomach **(Farrell *et al.,* 2012).**

In the small intestine, there is an efficient glucuronidation of quercetin by the action of uridine diphosphate glucuronosyltransferases and extensive O-methylation of quercetin by the action of catechol-O-methyltransferase. In addition, quercetin glycosides (e.g. quercetin glucosides and quercetin galactoside) can be deglycosylated to quercetin in the small intestine, which is mediated by microbiota-derived b-glucosidase **(Nemeth *et al.,* 2003).** Subsequently, quercetin and quercetin derivatives are transported by the hepatic portal vein to the liver. In the liver, quercetin is further metabolized, including O-methylation, sulfation and glucuronidation **(Murota and Terao, 2005).** The conjugation of quercetin with sulfate is carried out by sulfotransferases. When quercetin is O-methylated, its major product is 30-O-methylquercetin (isorhamnetin) and 40-O-methylquercetin (tamaraxetin) to a lesser extent. The resulted quercetin derivatives and the un-metabolized quercetin are released into blood circulation via the portal vein of liver. Subsequently, quercetin and its derivatives can be conjugated in the liver, resulting in the formation of sulfate or glucuronide **(Boersma *et al.,* 2002).** Moreover, the catechol-O-methyl transferase in the liver and kidney could also take part in further methylation of quercetin and its derivatives **(De Santi *et al.,* 2002).**

The adsorption of quercetin and its conjugates takes place in the large intestine, where colonic microorganisms can disassimilate those compounds. For example, Clostridium orbiscindens plays a key role in executing the fission of the C-ring in quercetin **(Aura, 2008).** The metabolites formed by the colonic microorganisms are absorbed and transported via the portal vein to the liver and undergo the conjugation reactions. A recent study estimates the distribution of quercetin after the intravenous and oral administration in rats. After an oral administration

of quercetin to male Sprague-Dawley rats, about 93% of quercetin was metabolized in the intestine before being absorbed, whereas only 3.1% was metabolized in the liver **(Chen et al., 2005).** The report also revealed that about 59.1% of total quercetin including free and conjugated quercetin as well as its metabolites was adsorbed after an oral administration of a single dose of 10 mg quercetin/kg body weight in rats. A long-term treatment (11 weeks) of rats with quercetin fed in diet (500 mg/ kg BW rat) demonstrated that quercetin and its metabolites were distributed in several organs (e.g., lung, kidney, heart and liver), with the highest level of quercetin in the lung and the lowest level in the brain and spleen. It implies that the intake of quercetin from daily diet can lead to the accumulation of quercetin throughout the body **(de Boer *et al.*, 2005).**

4.1.4. Bio-availability

In human, the total plasma concentration of free and conjugated quercetin as well as its metabolites was in the range of 72 and 193 nmol/L, following the short-term intake of quercetin-rich foods **(Nguyen *et al.*, 2015; Petersen *et al.*, 2016).** This result implies that a short-term treatment of quercetin could not reach the threshold plasma concentration of quercetin that is effective in inhibiting cancer cells **(Dajas, 2012).** However, a long-term supplement of quercetin could be a different situation. **Guo et al., (2014)** interpreted that a daily ingestion of 1095 mg quercetin for 3 days led to a total plasma quercetin concentration of 1430 nmol/L. Similarly, approximately 2317 nmol/L of plasma quercetin concentration is detected after an oral administration of Hypericum perforatum extract for a period of 9 days **(Paulke *et al.*, 2008).** When 600 mg/kg of Ginkgo biloba extract is administered orally to rats, a mean plasma quercetin concentration of 582 nmol/L is detected, whereas a repeated administration of the same dose resulted in a 4.6-fold increase **(Rangel-Ordonez, 2010).** A good deal of literature showed that a repeated quercetin administration obviously increased its bioavailability **(Guo *et al.*, 2014).** However, **Bieger *et al.*, (2008)** reported that the long-term dietary intake of quercetin did not lead to its plasma accumulation.

Following a single-dose administration of 10 mg quercetin/70 kg of body weight dissolved in three beverages, i.e., vegetable homogenate, grape juice and white wine, the subsequent serum quercetin concentrations were 10.8, 25.3 and 12.7 ng/L, respectively **(Goldberg *et al.*, 2003).** Supplementation of the capsule containing 22 mg quercetin resulted in 109 nmol/L plasma concentration of quercetin **(Petersen et al., 2016).** Therefore, food matrix also seems to play an important role in the bioavailability of quercetin.

Normally, human quercetin plasma concentrations are in the low nanomolar range, but upon quercetin supplementation they may increase to the high nanomolar or low micromolar range **(Hollman *et al.*, 1997).** Recently, a study regarding the tissue distribution in rats and pigs has shown that, upon quercetin supplementation, the highest accumulation of the flavonoid and its metabolites is found in (rat) lungs and (pig) liver and kidney. Unfortunately, pig lungs were not analysed **(de Boer *et al.*, 2005).** It has been shown that the half-lives of the quercetin metabolites are rather high, i.e. 11 to 28 h. This indicates that, upon repeated quercetin supplementation, they could attain a considerable plasma level **(Manach *et al.*, 2004).**

4.1.5. Excretion

The absorbed quercetin and its derivatives are excreted in urine **(Nishijima *et al.*, 2015)** or excreted into the bile and eliminated in the excrement **(Shi and Williamson, 2015).** In the other case, quercetin undergoes bacterial ring fission and decomposed into phenolic acids and CO_2, which is then excreted through feces and breath **(Guo and Bruno, 2015).** In human experiments following an oral administration, absorbed quercetin is excreted via CO_2, urine or stool as glucuronide or sulfate conjugates, and accounted for 52.1%, 4.6% and 1.9%, respectively **(Walle *et al.*, 2001).** Although quercetin underwent extensive metabolism and was mostly recovered in the form of metabolic products.

Moon *et al.*, (2008) demonstrated that a trace level of unchanged quercetin (varied from 0.25 to 18 mg within 10 healthy subjects) also existed in the urine after the ingestion of 500 mg Quercetin 500-Pluscapsules.

4.1.6. Biological activities of quercetin

4.1.6 Beneficial effects (Antioxidant in vitro studies)

Quercetin has been shown to be an excellent in vitro antioxidant. Within the flavonoid family, quercetin is the most potent scavenger of ROS, including $OUU2^{-}$ **(Cushnie and Lamb, 2005),** and RNS like NO and $ONOO^{-}$ **(Heijnen *et al.*, 2001).** These antioxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e., the catechol group in the B ring and the OH group at position 3 of the AC ring **(Heijnen *et al.*, 2002)**. Moreover, quercetin is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity which is 6.24 times higher than the reference antioxidant trolox, whereas for example the contribution of both vitamin C and uric acid virtually equals that of trolox **(Arts *et al.*, 2004).**

4.1.7. Anti-oxidative action

The best described property of Quercetin is its ability to act as antioxidant. Quercetin seems to be the most powerful flavonoids for protecting the body against reactive oxygen species, produced during the normal oxygen metabolism or are induced by exogenous damage **(De Groot, 1994; Grace, 1994).**

One of the most important mechanisms and the sequence of events by which free radicals interfere with the cellular functions seem to be the lipid peroxidation leading eventually the cell death. To protect this cellular death to happen from reactive oxygen species, living organisms have developed antioxidant line of defense systems **(Halliwell, 1995).** These include enzymatic and non-enzymatic antioxidants that keep in check ROS/RNS level and repair oxidative cellular damage. The major enzymes, constituting the first line of defence, directly involved in the neutralization of ROS/RNS are: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The second line of defence is represented by radical scavenging antioxidants such as vitamin C, vitamin A and plant phytochemicals including quercetin that inhibit the oxidation chain initiation and prevent chain propagation. This may also include the termination of a chain by the reaction of two radicals. The repair and de novo enzymes act as the third line of defence by repairing damage and reconstituting membranes. These include lipases, proteases, DNA repair enzymes and transferases **(Bahorun *et al.*, 2006)**

4.1.8. Direct radical scavenging action

Free radical production in animal cells can either be accidental or deliberate. With the increasing acceptance of free radicals as common place and important biochemical intermediates, they have been implicated in a large number of human diseases **(Wegener and Fintelmann, 1999; Ares and Outt, 1998).** Quercetin acting as free radical scavengers was shown to exert a protective effect in reperfusion ischemic tissue damage **(Halliwell, 1994; Fraga *et al.*, 1987).** Quercetin prevents free radical induced tissue injury by various ways. One way is the direct scavenging of free radicals. By scavenging free radicals, Flavonoid; particularly Quercetin can inhibit LDL oxidation in vitro **(Kerry and Abbey, 1997).**This action protects against atherosclerosis.

4.1.9. Inducible nitric oxide syntheses Inhibitory action

Quercetin results in a reduction in ischemia – reperfusion injury by interfering with inducible nitric oxide synthase activity **(Shoskes, 1998).** Nitric oxide is produced by several different types of cells including endothelial cells and macrophages. Although the early release of nitric oxide through the activity of constitutive nitric oxide synthase is important in maintaining the dilatation of blood vessels **(Huk *et al.*,1998),** the much higher concentration of nitric oxide produced by inducible nitric oxide synthase in macrophages can result in oxidative damage. In these circumstances the activated macrophages greatly increase their simultaneous production of both nitric oxide and superoxide anions. Nitric oxide reacts with free radicals, thereby producing high damaging peroxynitrite. Peroxynitrite can directly oxidize LDLs resulting in irreversible damage to cell membranes. Quercetin causes scavenging of free radicals; therefore, can no longer react with nitric oxide, resulting in less damage **(Shutenko *et al.*, 1990).** Nitric oxide interestingly can be viewed as radical itself and can directly be scavenged by Flavonoids **(Van-Acker *et al.*, 1995).**

4.1.10. Xanthine oxidase inhibitory action

The xanthine oxidase pathway has been implicated as an important route in the oxidative injury to the tissues especially after ischemia reperfusion **(Santrueza *et al.*, 1992).** Both xanthine dehydrogenase and xanthine oxidase are involved in the metabolism of xanthine to uric acid. Xanthine dehydrogenase is the form of the enzyme present under physiological condition but its configuration changed to xanthine oxidase during oxidative stress and ischemic conditions. Quercetin seems to inhibit xanthine oxidase activity thereby resulting in decreased oxidative injury **(Shoskes, 1998; Chang *et al.*, 1993; Lio *et al.*, 1986)**

4.1.11. Beneficial effects: anti-inflammatory

Quercetin is known to possess strong anti-inflammatory capacities **(Orsolic *et al.*, 2004).** Several in vitro studies using different cellines have shown that the flavonoid is capable of inhibiting LPS induced cytokine production. For instance, quercetin inhibits LPS induced TNFα production in macrophages **(Manjeet and Ghosh, 1999)** and LPS-induced IL8 production in lung cells (A549) **(Geraets *et al.*, 2007).** Moreover, in glial cells it was even shown that quercetin can inhibit LPS-induced mRNA levels of two cytokines, i.e., TNFα and IL-1α **(Bureau *et al.*, 2008).** In a microglial–neuronal coculture, this effect of the flavonoid

resulted in a diminished apoptotic neuronal cell death induced by microglial activation **(Bureau *et al.*, 2008).**

A possible explanation for these anti-inflammatory effects of quercetin may be found in the interplay between oxidative stress and inflammation. ROS are not only involved in the occurrence of oxidative stress, but also in the promotion of inflammatory processes via activation of transcription factors such as NF-κB and activator protein (AP)-1 which induce the production of cytokines like TNFα **(MacNee, 2001; Rahman, 2002).** Consequently, scavenging ROS would not only prevent the occurrence of oxidative stress but also help mitigate inflammation. Indeed, it has already been shown that quercetin can inhibit the production as well as the gene expression of TNFα via modulation of NF-κB in human peripheral blood mononuclear cells **(Nair *et al.*, 2006).** A possible mechanism behind this modulation was reported to be the inhibition of the degradation of the inhibitory part (IκBα) of this transcription factor **(Peet and Li, 1999).**

4.1.12. Anticancer activity

Quercetin has been proven to be a strong anticancer agent from in vitro studies in various cancer cells, e.g., U138MG, Hep-2 cells and A549 lung cancer cells, and also from in vivo tests **(Dajas, 2012; Gibellini *et al.*, 2011).** Quercetin can prevent cancer induced by oxidative stress due to its antioxidant activity and suppression of many kinases involved in the growth of cancer cells, proliferation and metastasis **(Baghel *et al.*, 2012; Gibellini *et al.*, 2011).** In terms of human breast carcinoma cells, such as SK-Br3 and MDA-MB cells, a low dose of quercetin inhibited their proliferation **(Jeong *et al.*, 2009).** Quercetin was also found to induce the death receptormediated apoptosis in ascite cells of Dalton's lymphoma-bearing rats **(Li *et al.*, 2016).** Moreover, quercetin restrained the activity of protein kinase C, which contributed to cancer progression **(Maurya and Vinayak, 2015).**

4.1.13. Beneficial effects: miscellaneous

Furthermore, it has been shown in vitro that quercetin also possesses anti-fibrotic **(Lee *et al.*, 2003),** anti-coagulative **(Bucki *et al.*, 2003),** anti-bacterial **(Cushnie and Lamb, 2005),** antiatherogenic, anti-hypertensive **(Perez-Vizcaino *et al.*, 2006)** and anti-proliferative properties **(Orsolic *et al.*, 2004; Gulati *et al.*, 2006).** Furthermore, quercetin is reported to directly modulate the gene expression of enzymes involved in biotransformation **(Schwarz *et al.*, 2005; Moon *et al.*, 2006)** and to inhibit cell proliferation by interacting with estrogen binding sites **(Caltagirone *et al.*, 1997).** Altogether, these studies indicate that quercetin may

exert healthbeneficial capacities via various damage modulating effects. However, most of these studies have been performed with immortalized or cultured cell lines only and are thus not easy to extrapolate to the in vivo human situation.

4.1.14. Toxic effects

Many reports showed that the oxidation products such as semiquinone and quinones displayed several toxic effects, because the oxidated products could alter redox homeostasis and deplete cellular protein-SH by arylation **(Russo et al., 2012).** By reacting with free radical of human body, quercetin can form toxic oxidation products, namely quercetin-quinine which is highly reactive with thiols and GSH might be the principal reactant **(Boots *et al.*, 2003).Boots *et al.*,(2008)**has proven that if the GSH concentration was high enough, it could trap quercetin-quinine as GSQ. However, at low concentration of GSH, it might be ineffective to trap quercetin quinine which could react with other sulfhydryl compounds such as protein-SH. Once it happened, it might produce toxic effects, e.g., causing cell injury by destroying the integrity of cell membrane and proteins **(Wagner *et al.*, 2010),** or destroying the function of enzymes containing sulfhydryl structure **(Kalyanaraman *et al.*, 1987).** In a model system of isolated mice liver nuclei, quercetin decreased the nuclear GSH content in a dose-dependent manner and might lead to DNA damage **(Sahu and Gray, 1996). Ramos and Aller, (2008)** used quercetin cooperating with arsenic to induce apoptosis in human leukemia cell lines (THP-1, HL-60) and found that GSH content was decreased during the process. Considering that arsenic is highly reactive towards GSH, reduction of GSH may increase the free arsenic concentration, and hence resulted in DNA and cellular damage.

There are also numerous reports about the mutagenic/genotoxic effect of quercetin. In vitro, quercetin was tested positively for mutagenic effects in bacteria **(Joseph and Priya, 2011).** It could also induce reverse mutations **(Resende *et al.*,2012),** and prevent DNA strand breakage **(Ozyurt *et al.*, 2014).** The mutagenicity of quercetin was observed in hamster ovary cell, at concentrations ranging from 0.2 mM to 1 mM **(Engen *et al.*, 2015).** Quercetin induced significant frequencies of sister chromatid exchange in ovary cells compared to spontaneous occurrences. However, the mutagenicity/genotoxicity effect of quercetin has not been full confirmed in vivo. Supplementation of quercetin consistently resulted in no significant change in several genotoxicity endpoints (e.g., micronuclei and chromosomal aberrations) in bone marrow cells of rats **(Cierniak *et al.*, 2004).** However, it is recently reported that female rats intraperitoneally supplemented with quercetin suggested a protective effect of quercetin against

genotoxic damage induced by Cr (VI) **(Da-Silva *et al.,* 2002).** While positive results were also obtained in some in vivo mutagenicity/genotoxicity assays, following up a treatment with quercetin **(Da-Silva *et al.,* 2002).** Because of the discrepancy in different studies, further research work is required to better understand the potential risk or safety of dietary quercetin.

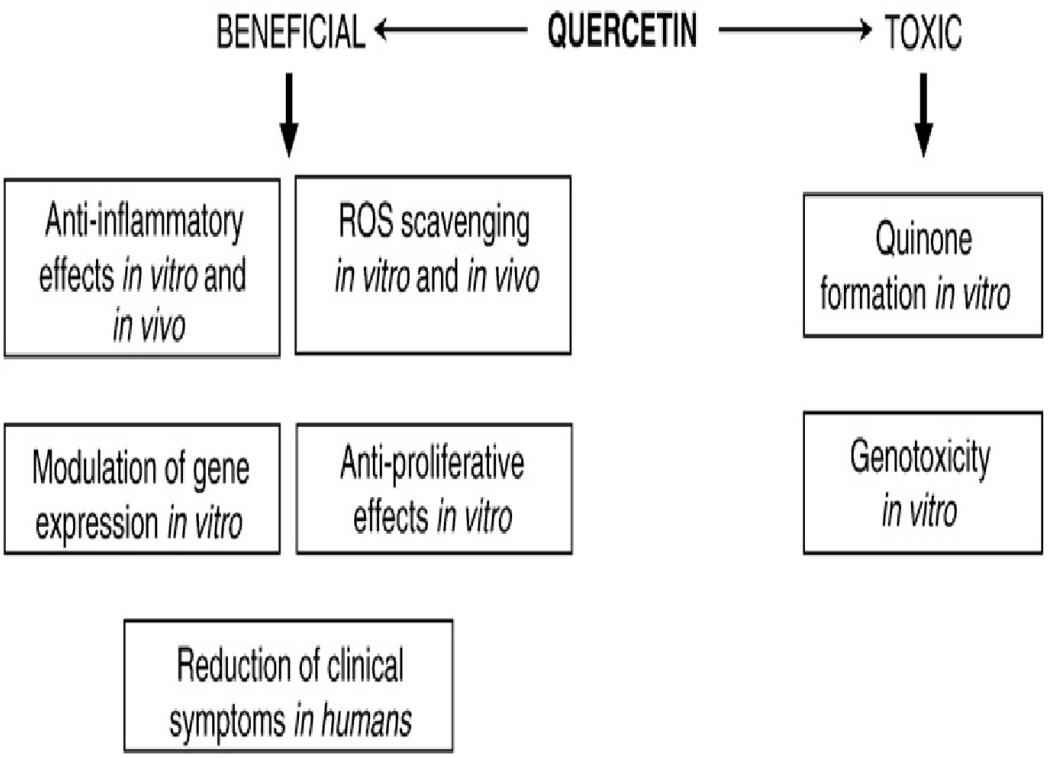


Figure 12: Schematic overview of the beneficial and toxic effects of quercetin in vitro and in vivo **(Igbokwe *et al.*,2019)**

4.2. PHAGHALON RUPESTRE

The genus Phagnalon belongs to the tribe Gnaphalieae (Asteraceae) and is distributed from Macaronesia in the west to the Himalayas in the east, from Southern Europe in the north to Ethiopia in the south; it comprises about 36 species (Qaiser and Abid, 2003). Phagnalon species are suffruticose shrubs or subshrubs and grow in a variety of habitats ranging from rocky crevices in high mountains to sandy soils in coastal plains (Montes Moreno et al., 2013). Many hybrids have been described (Quézel and Santa, 1963) to possess genetic stability, which reinforces their taxonomic values.

In Algeria this genus includes four species: Phagnalon sordidum (L.) Rchb., Phagnalon garamantum Maire, Phagnalon saxatile (L.) Cass., **Phagnalon rupestre (L.)** DC. (Quézel and Santa, 1963). The Phagnalon species are known locally as “Foddia” or “Arfedj”, and have been used in folk medicine for treating asthma, headache and as anesthetic for toothache (Ali-Shtayeh et al., 1998). The literature logs their antimicrobial and skin clearing activities, anti-allergic, anti-inflammatory and anticholinesterase properties (Ali-Shtayeh et al., 1998; Conforti and Rigano, 2010; Erdogan Orhan et al., 2013; Góngora et al., 2002; Hausen and Schulz, 1977; Olmoset al., 2005).

Algeria with its large surface and therefore its immense biodiversity provides a great potential for the discovery of new flavors and products. However, there is still a lack of chemical and biological activities information associated with plant species due to modest support in research work in this field.This thesis is also evaluating comparing and contrasting the efficacy of quercetin, retinin and phagnalon rupestre in chelating AL free radicals and other reactive oxygen species that have long been implicated in oxidative damage inflicted on biomolecules, e.g.proteins, amino acids, lipids or DNA (Krumova and Cosa, 2016).

Their overproduction is associated with numerous disorders (Namiki, 1990). Oxidative stress caused by the imbalance between excessive formation of free radicals and limited antioxidant defenses is connected to many pathologies including age-related disorders, diabetes, atherosclerosis, dyslipidemia, cancer, inflammatory, and neurodegenerative diseases (Lobo et al., 2010; Santo et al., 2016). Under such conditions supplementation with exogenous antioxidants is required to regain a balance between free radicals and antioxidants.

4.3. RUTIN

Rutin also known as vitamin P or rutoside is a bioflavonoid, or plant pigment, that is found in certain vegetables and fruits. Apples are full of rutin. Buckwheat, most citrus, figs, and both black and green tea also contain rutin.

Rutin has powerful antioxidant properties. It also helps your body produce collagen and use vitamin C. You can add rutin to your diet by eating foods that contain it or taking it in supplement form. Traditionally, rutin has long been used to aid circulation. It's thought that rutin can help strengthen and increase flexibility in blood vessels, such as your arteries and capillaries. Research shows that rutin can prevent the formation of blood clots in certain animals. This suggests rutin may reduce the risk of blood clots. Preventing blood clots can help lower your chances of developing life-threatening conditions such as:

- heart attacks
- stroke
- pulmonary embolisms
- deep vein thrombosis

Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonol, abundantly found in plants, such as passion flower, buckwheat, tea, and apple. It is a vital nutritional component of food stuff (Harborne, 1986). Rutin also called as rutoside, quercetin-3 rutinoside, and sophorin is a citrus flavonoid glycoside found in buckwheat (Kreft et al., 1997). The name 'rutin' comes from the plant Ruta graveolens, which also contains rutin. Chemically it is a glycoside comprising of flavonolic aglycone quercetin along with disaccharide rutinose. It has demonstrated a number of pharmacological activities,

including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective activities.

CHAPTER2 MATERIAL AND METHOD

1. EXPERIMENTAL PROTOCOLE

1.1 Animals and tissue preparation

In this study Wistar rats both female and male weighing (200g & 300-900g) respectively were used. The rats were housed starting on 26 December 2021 under normal conditions with free access to food, aluminium intoxication and water (12hours light/dark, Temperature 22 ± 2°C). The study protocol was approved by the University's Scientific Committee. Sacrifice was later made on 20 January 2022 and in my case extracted the heart organs and stored them in a deep freezer (congelateur).

1.2 Preparation of the aluminium chloride solution (AlCl3)

Aluminium chloride molecular weight of 241,43g/mol solution was prepared with 60mg/L of AlCl3 into 1000ml of distilled water. We administered 60mg/Kg of body weight.

1.3 Preparation of quercetin and Rutin solutions

Solutions of quercetin and rutin were prepared: 15mg/L by weighing 15mg of powdered quercetin and rutin by an electronic scale, we then poured this amount into a beaker containing 1000ml of distilled water, stirred it to homogenize the solution, and lastly closed with paraffin paper.

1.4 Preparation of Phosphate Buffer

Phosphate-buffered saline (PBS) is a buffer solution (pH= 7.4) commonly used in biological research. It is a water-based salt solution containing disodium hydrogen phosphate, sodium chloride and in some formulations potassium chloride and potassium dihydrogen phosphate. The buffer helps to maintain a constant pH.

Preparation

* Phosphate hydrogen phosphate (KH2PO4) ……9.08g/L

* Sodium hydrogen phosphate (Na2HPO4) ……9.47g/L

To 322ml of Na2HPO4 we added 78ml of KH2PO4 to have 400ml of phosphate buffer. To the buffer we added 1.36g (0.45mM) of mannitol and 0.02M of Kcl and 0.02M of MgCl2.

2. BIOCHEMICAL ASSAYS FOR OXIDATIVE STRESS

2.1. PROTEIN ASSAY

The Lowry Assay Protein **(Lowry *et al.*, 1951)** has been the most widely used method to estimate the quantity of proteins (already in solution or easily-soluble in dilute alkali) in biological samples.

Principle of the Assay

The principle of Lowry et al. (1951) is based on the biuret reaction with additional steps and reagents to increase the sensitivity of detection. In the biuret reaction, copper interacts with four nitrogen atoms of peptides to form a cuprous complex. Lowry adds phosphomolybdic/phosphotungstic acid also known as Folin-Ciocalteu reagent. This reagent interacts with the cuprous ions and the side chains of tyrosine, tryptophan, and cysteine to produce a blue-green color that can be detected between 650 nm and 750 nm.The end product of this reaction has a blue color. The quantity of proteins in the sample can be estimated via reading the absorbance (at 750 nm) of the end product of the Folin reaction against a standard curve of a selected standard protein solution.

$$Cu+ + (F\text{-}C)_{ox} \longrightarrow Cu^{2+} + (F\text{-}C)_{red}.$$

- **Assay procedure**

To 100 µl of sample add 500 µl of solution C (Lowry's reagent). After vortexing, incubate for 10 min, and then add 50 µl of solution D (Folin-Ciocalteu reagent). Vortex again and incubate in the dark for 30 min. Take the reading at 650 nm.

Preparation of the Standard Curve

A standard solution of known protein concentration BSA [bovine serum albumin] (0.2mg/ml) is diluted with a standard dilution factor in my case df = 50. Distilled Water is then added to dilute BSA to obtain equal volumes. Then we add the copper reagent and vortex the mixture and incubate for 10 minutes. After incubation we then add the reagent Folin-Ciocalteu to each test tube, vortex the tubes once more and incubate them for 10-45minutes at room temperature. Then we use the blank to calibrate the spectrophotometer and after we get the absorbance of the rest of the tubes at 550nm. Lastly we plot a standard curve from which we can extrapolate the unknown.

Cmg/ml	DO
0.0625	0.076
0.25	0.366
0.5	0.655
1	1.036

Figure13: D.O values of the BSA standard solution

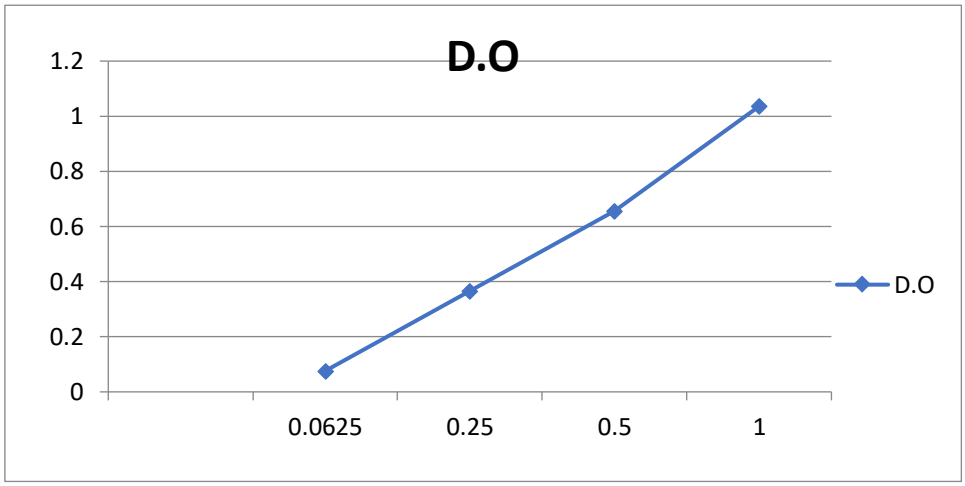


Figure 14: standard BSA curve (mg/ml)

2.2. THIOBARBITURIC REACTIVE ACID REACTIVE SUBSTANCES (TBARS ASSAY)

Oxidizing agents can alter lipid structure, creating lipid peroxides that result in the formation of malondialdehyde (MDA), which can be measured as Thiobarbituric Acid Reactive Substances (TBARS), First used in 1978, the measure of TBARS is still a commonly used and convenient method of determining the relative lipid peroxide content of sample sets, including serum, plasma, urine, cell lysates and cell culture supernates **(Okhawa *et al.*, 1978)**

Principle of the Assay

Thiobarbituric acid reactive substance (TBARS) assay is another method to detect lipid oxidation. This assay measures malondialdehyde (MDA), which is a split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of lipid substrates.

In the presence of heat and acid, MDA reacts with TBA to produce a colored end (pink chromogen) product that absorbs light at 530-540 nm. The intensity of the color at 532 nm corresponds to the level of lipid peroxidation in the sample. Unknown samples are compared to the standard curve.

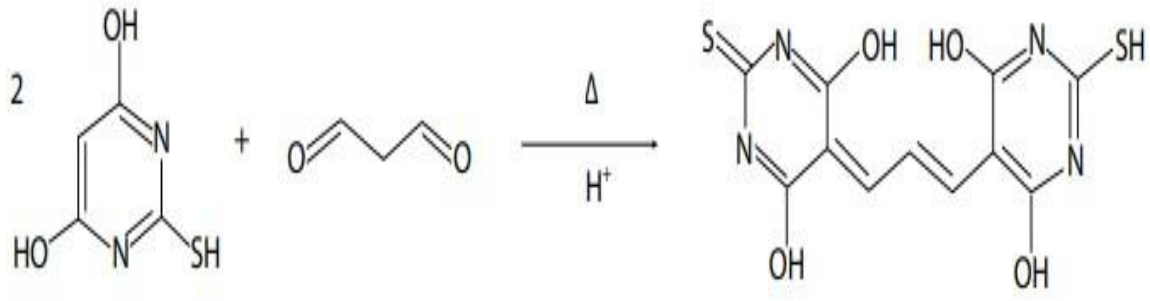


Figure 15: In the presence of acid and heat two molecules of 2-thiobarbituric acid (TBA) react with MDA to produce a colored end product that can be easily quantified. **(Du and Bramlage, 1992)**

Assay Procedure

Take 100 µl of the homogenate in glass tubes, Add 100 µl of Sodium Dodecyl Sulfate (SDS 8.1%), then 1.5 ml of the acetic acid solution (20%; pH = 3.5) and 1.5 ml of TBA (0.8%). Then make up the volume to 4 ml with distilled water. Vortex and incubate in a water bath at a temperature of 95 ° C for 60 min. Cool in an ice bath for 5 min and add 500 µl of distilled H2O and 2 ml of solvent (n-butanol). Centrifuge at 4000 rpm for 10 min.

Reading of the organic phase greater than λ = 532nm.

Calculation:

TBARS (nmol / mg protein) = OD / Ɛ MDA

- **OD**: Optical density read at 532 nm
- **Ɛ MDA** : Molecular extinction coefficient

2.3. CATALASE ASSAY

Catalase is present in the peroxisomes of nearly all aerobic cells and serves to protect the cell from the toxic effects of hydrogen peroxide by catalyzing the decomposition of H_2O_2 (**Deisseroth and Dounce, 1970)** Catalase causes rapid decomposition of hydrogen peroxide to water and oxygen; the mechanism of catalysis is not fully elucidated, but the overall reaction is as follows: $2H_2O_2 \longrightarrow 2H_20 + O_2$

Catalase has one of the highest catalytic activities reported, near the diffusion-controlled limit. Catalase activity can be directly monitored in the ultraviolet region; however, the UV assay is subject to interference due to absorption by protein and other components in biological samples. **(Wakimoto et al., 1998).**

Principle of Assay

The method is based on the fact that dichromate in acetic acid reduces to chromic acetate when heated in the presence of H_2O_2 with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced is measured calorimetrically at 610 nm. Since dichromate has no absorbance in this region, the presence of the compound in the assay mixture does not interfere with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at specific time intervals by the addition of dichromate / acetic mixture and the remaining H_2O_2 is determined by measuring chromic acetate calorimetrically after heating the reaction. **(Sinha, 1972).**

Assay Procedure

Mix 0.1 ml of homogenate with 1 ml of phosphate buffer (0.001 M, ph = 7.4) and 0.4 ml of H2O2 solution, vortex then incubate at 37 ° C for 10 min. Add 2ml of Potassium dichromate solution (K2Cr2OH) at 5% / Acetic acid (1 V / 3V).

The reaction is monitored by a spectrophotometer reading at a wavelength of 620 nm and is expressed in (µmol / mg protein).

2.4. GLUTATHIONE-S-TRANSFERASE (GST) ASSAY

Glutathione S Transferase (GST) is an enzyme involved in detoxification of a wide range of compounds and is involved in reducing free radical damage. **(Mannervik *et al.*, 1988)**

Principle of Assay

GST catalyzes the conjugation of L-glutathione to CDNB through the thiol group of the glutathione.

GST

GSH + CDNB GS-DNB Conjugate + HCl

The reaction product, GS-DNB Conjugate, absorbs at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

The reaction is measured by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). This is done by watching an increase in absorbance at 340nm. One unit of enzyme will conjugate 10.0 nmol of CDNB with reduced glutathione per minute at 25oC. **(Habig *et al.*, 1974)**

Assay Procedure

Solution C was prepared using 20.26mg CDNB and 153.65mg of GSH and diluted into 1ml of ethanol and 100ml of PBS. The solution was mixed afterwards. Place 100µl of Solution into the Eliza plate and add 600 µl of sample also into the Eliza plate and to the blank add 100 µl of distilled water. Measure absorbance at 340nm each min for five mins.

GST Assay Procedure

PBS Buffer (0.1M pH= 6)

A. Na2HPO4
   35.8g => 1L of distilled water
B. NaH2P04
   13.8g => 1L of ditilled water

Solution CDNB 1mM /GSH 5mM

20.26mg CDNB + 153.65mg GSH diluted in 1ml of ethanol then we add 100ul of PBS.

Protocol

Blank:

To 1.2ml of CDNB & GSH mixture add 200ul of distilled water.

Specimen:

To 1.2ml of CDNB & GSH mixture add 200ul of supernatant.

Calculation:

The specific activity of GST = ΔDO / e × Vt / Vs / mg of protein
(mmol/ min / mg of protein)

- **Δ DO**: slope of the regression line obtained after hydrolysis of the substrate.
- **e**: Molar extinction coefficient of CDNB = 9.6 mM-1 cm-1.
- **Vt**: Total volume in the tank = 1.4 ml [0.2 ml supernatant + 1.2 ml CDNB / GSH mixture].
- **Vs**: Volume of the supernatant in the tank = 0.2 ml.
- **mg of protein**: Quantity of protein expressed in mg.

2.5. SUPEROXIDE DISMUTASE (SOD) ASSAY

SOD is known to catalyze the dismutation of superoxide ($O2^{-}$) to hydrogen peroxide (H_2O_2) and O_2. SOD is present in most aerobic organisms and is assumed to play a central role in providing defense against oxidative stress (Boguszewska et al., 2010). The chemical moiety of SOD contains some metal ions such as Cu^{+2}, Zn^{+2}, Mn^{+2}, and Fe^{+2} in the active site, which influences and mediates the dismutation process. On the basis of

these metallic cofactors, SOD can be classified into three distinct types viz, Cu/Zn-SOD, Mn-SOD, and Fe-SOD. The sensitivity of these three isozymes to hydrogen peroxide (H_2O_2) and potassium cyanide are different.

SOD Assay Procedure

This is a typical kinetic reaction. Enzyme kinetics is the study of the rates of enzyme-catalysed chemical reactions. In enzyme kinetics the reaction rate is measured and the effects of varying the conditions of the reaction are investigated.

Products:

- Tris-Hcl (Trisma-hydrochloric) = 50mM, pH=8.2, Mm=121.14g/mol, V=20ml
- We adjust the PH using a Ph meter using NaOH (1N) and Hcl (1N)
- EDTA-Na2 (Mm=372.24g/mol) 1mM
- Pyrogallol (Mm=126.11g/mol) Cm=12mM V=20ml

  Formula => m = Cm*V*Mm
- Hcl = 1mM 2.0ul of Hcl ----------> 25ml 0f distilled water

Protocol

950ul (Tris-Hcl + EDTA), 20ul of specimen, 50ul of pyrogallol

We read absorbance in the spectrophotometer every after a minute for 5 minutes at 420nm.

2.6. GLUTATHIONE REDUCED AND GLUTATHIONE PEROXIDASE (GSH & GPx) ASSAY

Glutathione synthesis increases in response to pro-oxidants. Glutathione in its reduced form (GSH) is a critical cofactor for several antioxidant pathways, including thiol-disulfide exchange reactions and glutathione peroxidase. Glutathione peroxidase has a higher affinity for hydrogen peroxide than catalase, and it disposes of lipid peroxides, free radicals, and electrophilic metabolites. GSH is also a cofactor for conjugation reactions catalyzed by the glutathione-S-transferases involved with phase3 transport of metabolites into bile.

GSH Assay Protocol

1. **TCA at 50%**

   50g ========➔ 100ml of distilled water

2. **Tris-Hcl Buffer**

   PH = 8.9, V= 0.4mol/l, Mm=121.14g/mol, 100ml = 0.04moles, mass = n*Mm

3. **DTNB** (0.01mol/l) ==========➔ 0.3963g in 100ml 0f Distilled Water

- To 500ul of supernatant of each specimen we add 400ul of iced water then 100ul of TCA, we then vortex the tubes for 10 minutes and do centrifugation for each tube at 2400tr/min for 15minutes.
- 400ul of supernatant is then added to 800ul of Tris-Hcl Buffer and lastly 20ul of DTNB
- Optical Density for each specimen is read at 412nm.

GPx Assay Protocol

Tris-Hcl Buffer => 0.4mol, PH=7, Mm= 121g/mol==========➔2.42g in 50ml of distilled water.

Sodium Azide==========➔at 10Mm, 0.032g in 50ml of distilled wate

GSH =========➔ 0.0061g in 200ml of distilled water

Hydrogen Peroxide (0.02mM) 175ul in 100ml of distilled water

TCA at 10%

Ellman's Reagent

1.38mg of DTNB + 100ml of sodium Nitrate at 0.1%

- To 100ul of Tris-Hcl add 50ul of Sodium Azide then 100ul of supernatant for each specimen then 100ul of GSH then 50ul of H202. Mix and incubate at body temperature for 10minutes then add 200ul of TCA. Centrifugation at 2400tr/5min. Get the new supernatant and add 200ul of Ellman's Reagent to it. Read the Optical density of each specimen.

2.7. LIPIDS (CHOLESTEROL & TRIGLYCERIDES)

In this Assay I used only male heart specimen divided in 3 categories of Al intoxicated, intoxicated and treated with quercetin and lastly intoxicated and treated with retinin. To 0.5g of each specimen add 10ml of 2v chloroform & 1v of methanol. Incubation was done for a night and then filtration into flacons. The flacons with and without the lipids are weighed and then add 3ml of n-Xexane to the mixture. By using the BIOLABO Cholesterol and Triglyceride Assay Kit we find the Optical Density of each specimen.

Assay Protocol for both Cholesterol & Triglyceride

- To 10ul of each specimen we add 1ml of reagent from the kit. For the blank we use 1ml of the kit reagent and 10ul of a standard solution whose concentration is (200mg/dl) from the kit

Calculation

- The formula to get the concentration of both Cholesterol & Triglyceride is:

Concentration = **(Absorbance of specimen/Absorbance of standard solution) * Standard Solution concentration (200mg/dl)**

CHAPTER 3
RESULTS

1.3. Biochemical assays for oxidative stress

1.3.1. Determination of total proteins

	PROTEIN	D.O VALUES				
	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
rat1	0.142	0.226	0.183	0.183	0.195	0.112
rat2	0.136	0.152	0.166	0.094	0.129	0.094
rat3	0.129	0.172	0.156	0.097	0.154	0.225

Table 4: Protein Optical Density Values for different samples

	Concentration of proteins in mg/ml					
	Intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
rat1	6.314478833	10.0498043	8.13767343	8.137673426	8.67129136	4.98043401
rat2	6.047669868	6.75916044	7.38171469	4.180007115	5.73639274	4.18000711
rat3	5.736392743	7.64852366	6.93703308	4.313411597	6.84809676	10.0053362

Table 5: Protein Concentration Values for different Samples

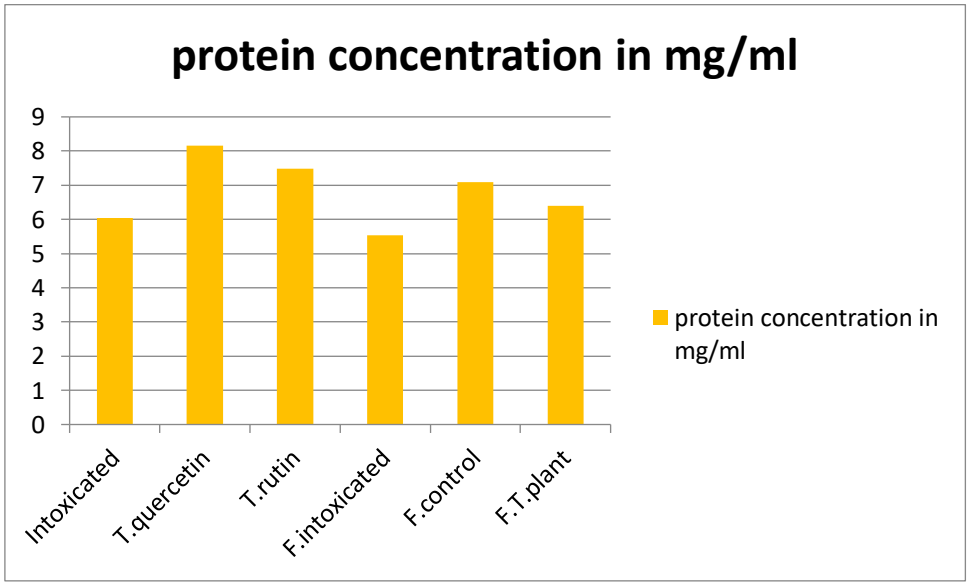


Figure 16: **Graph of Variation of concentration of protein activity in the specimen samples**.

Values are represented as mean ± SD for each group**. (Annex 01)**

We noticed a significant difference of (P<0 .05) between the intoxicated (Al) group compared to the control group.

Protein content showed a decrease in both intoxicated male and female groups.

The group intoxicated with Aluminium and treated with Quercetin showed a slight increase compared to the control group, group treated with rutin and group treated with plant.

1.3.2. Determination of Catalase assay

	CATALASE	D.O VALUES				
	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
rat1	0.013	0.073	0.051	0.349	0.469	0.542
rat2	0.016	0.054	0.031	0.37	0.578	0.568
rat3	0.013	0.109	0.019	0.399	0.59	0.225

Table 6: Catalase Optical Density Values for different samples

	Concentration of catalase in mmol/min/mg of protein					
	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
rat1	2.573450704	9.07977876	7.83393443	53.60868852	67.6081538	136.032321
rat2	3.307058824	9.98644737	5.24945783	110.6457447	125.950233	169.85617
rat3	2.832790698	17.8138953	3.42365385	115.627732	107.694156	28.11

Table 7: Catalase Concentration Values for different Samples

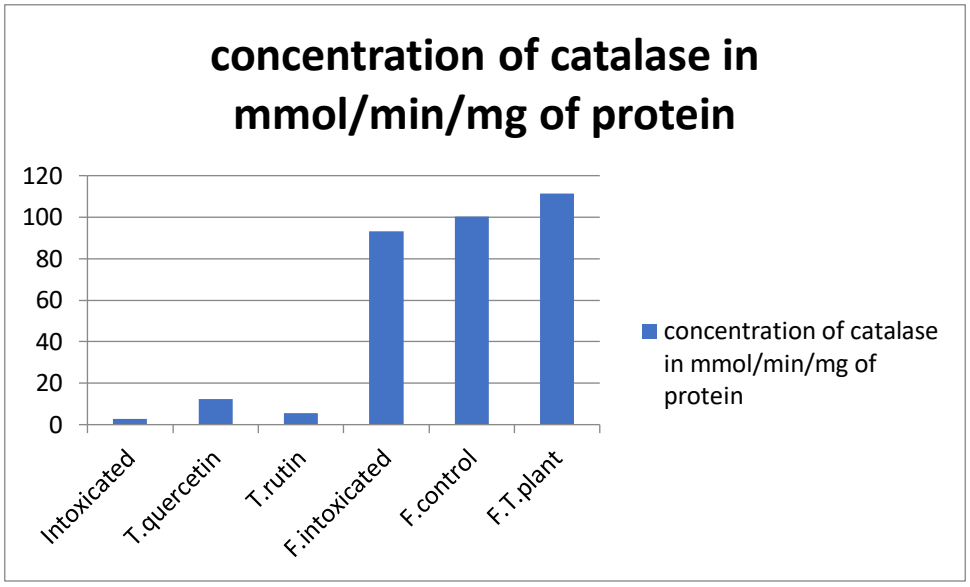


Figure 17: **Graph of Variation of Catalase activity in the specimen samples**.

Values are represented as mean ± SD for each group. (**Annex 02)**

.

Catalase activity reflected a significant decrease in the groups intoxicated by aluminium compared to the control group, group treated with quecertin, group treated with plant and group treated with plant.

1.3.3. **Determination** of TBARS assay

Specimen D.O	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
rat1	0.196	0.11	0.245	0.173	0.122	0.162
rat2	0.154	0.163	0.137	0.276	0.167	0.297
rat3	0.16	0.102	0.246	0.218	0.205	0.19

Table 8: TBARS activity Optical Density Values for different samples

Column1	Concentration in mmol/mg of protein	Column2	Column3	Column4	Column5	Column6
	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
rat1	10.01283053	3.53080217	9.71189847	6.857789529	4.53852109	10.4926728
rat2	8.2143074	7.77916808	5.98689468	21.29954701	9.39108777	22.9201647
rat3	8.997449362	4.30190548	11.4393052	16.30323911	9.65655635	6.12576344

Table 9: TBARS activity Concentration Values for different samples

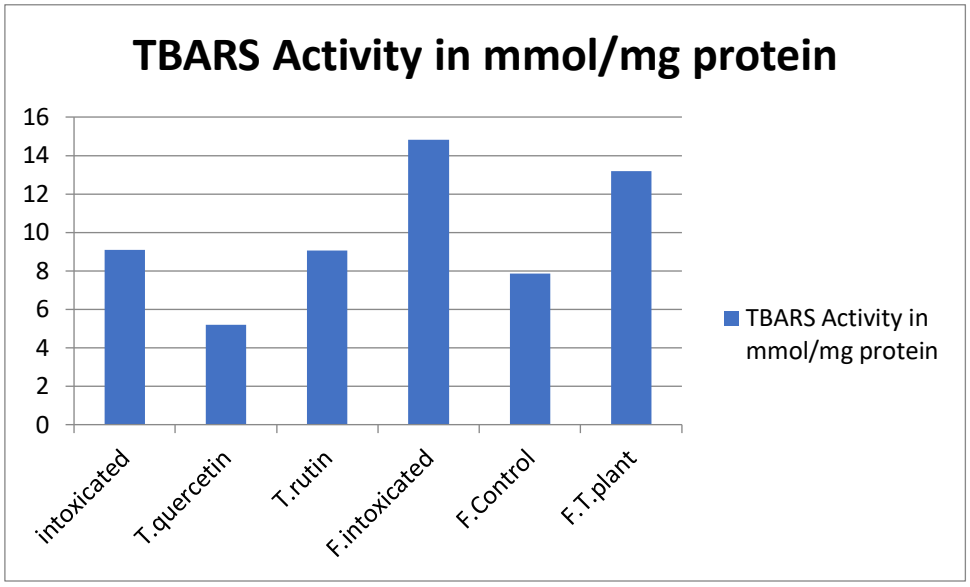


Figure 18: **Graph of Variation of TBARS activity in the specimen samples**

Values are represented as mean ± SD for each group. **(Annex 03)**

TBARS activity revealed a significant increase in both male and female Al intoxicated samples compared to the control group; group treated with quercetin has the lowest TBARS activity followed by group treated by rutin and lastly group treated with the medicinal plant.

1.3.4. Determination of GST assay.

These results show a significant decline in GST levels in Al-exposed rats. This can be attributed to the direct binding of GST to pro-oxidants in this case Aluminium.

	GST activity in mmol/min/mg protein					
	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.control	F.T.plant
rat1	7.423415493	16.5840708	21.760929	8.00034153	22.5240385	15.6863839
rat2	12.05698529	13.0995066	17.2865211	11.21409574	24.5145349	74.137633
rat3	12.25726744	14.0731589	15.3914263	15.69716495	10.6477273	23.6852778

Table 10: GST activity concentration values for different specimen samples

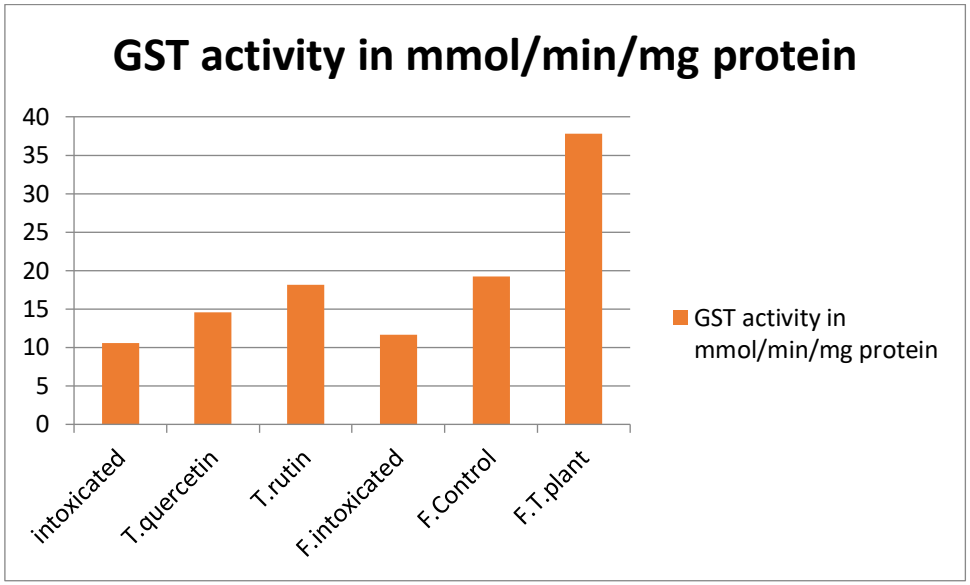


Figure 19: **Graph of Variation of GST activity in the specimen samples**

Values are represented as mean ± SD for each group. **(Annex 04)**

GST activity showed a significant decrease in all intoxicated samples compared to the control group, quercetin group, rutin group and group treated with plant.

1.3.5. Determination of SOD assay.

Column1	Column2	Column3	Column4	Column5	Column6	Column7
	%inhibition SOD/mg of protein					
	intoxicated	T.quercetin	T.rutin	F.intoxicatedF.	F.control	F.T.plant
rat1	1437.758257	308.463717	430.098361	485.3967213	530.486154	471.846429
rat2	1380.697059	495.623684	1219.22892	2858.846809	1821.70233	1734.44681
rat3	566.5581395	1281.29302	540.576923	1472.152577	649.815584	189.898667

Table 11: SOD % inhibition concentration values for different specimen samples

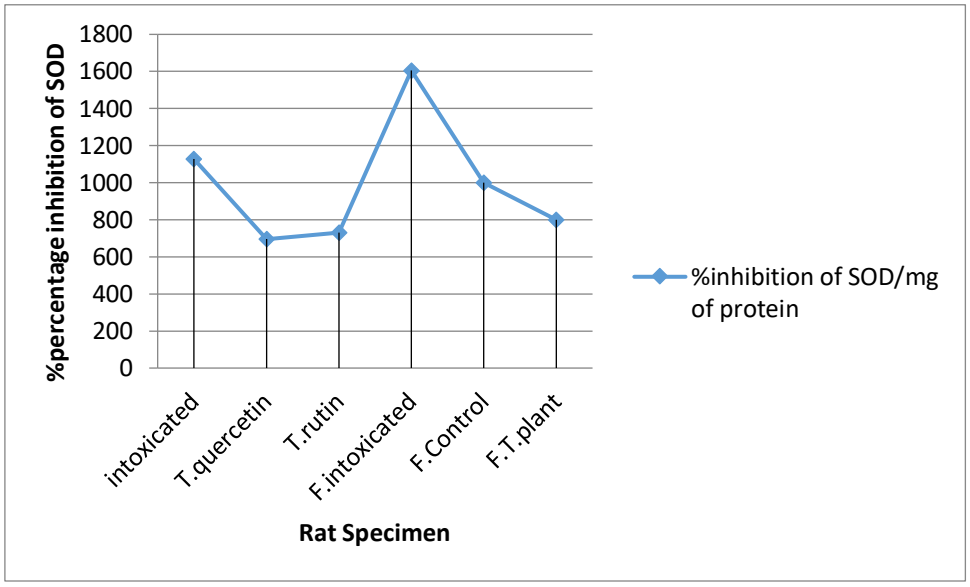


Figure 20: **Graph of Variation of SOD %inhibition in the specimen samples**

Values are represented as mean ± SD for each group. **(Annex 05)**

SOD % inhibition was most significant in both male and female Al intoxicated groups and more pronounced in females compared to the control group, quercetin group, rutin group and group treated with plant.

1.3.6. Determination of GSH & GPx assay.

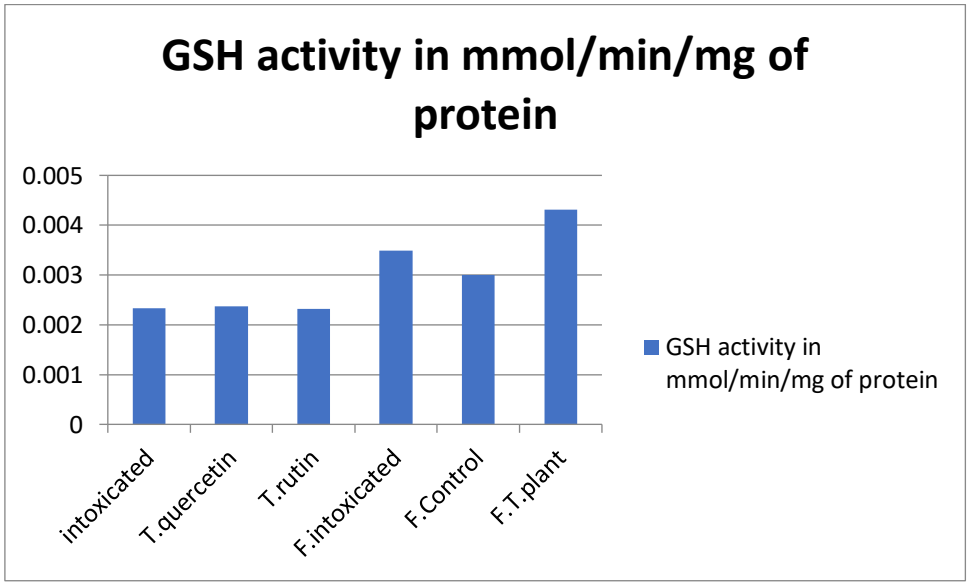


Figure 21: **Graph of Variation of GSH activity in the specimen samples**

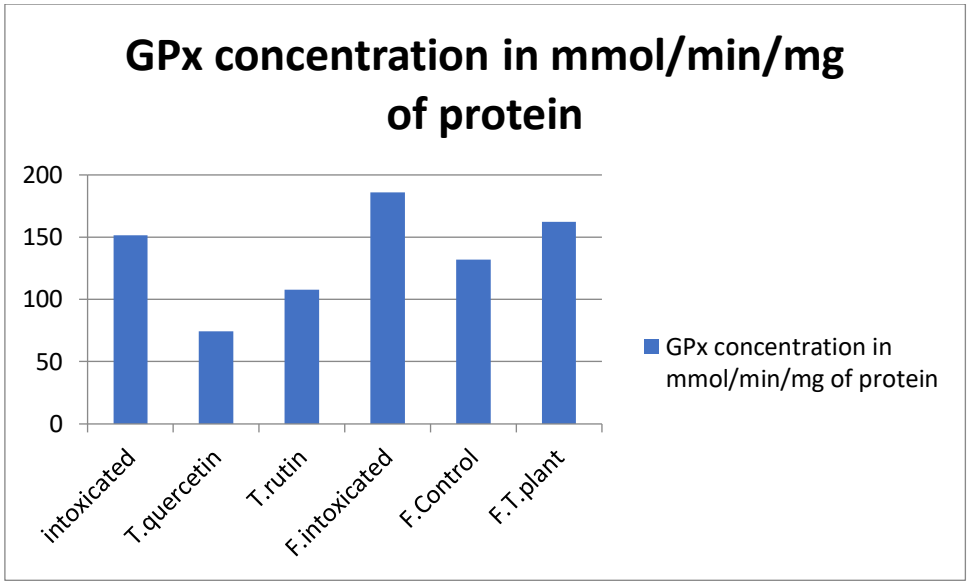


Figure 22: **Graph of Variation of GPx activity in the specimen samples**

From the results GSH increased drastically with initial acute intoxication but decreased after continuous chronic intoxication while GPx showed an overall increase in activity with increase in intoxication.

1.3.7. Determination of Lipids (Cholesterol & Triglycerides)

	CHOLESTEROL		
	Intoxicated	T.quercetin	T.rutin
RAT1	0.27	0.287	0.292
RAT2	0.295	0.324	

Table 12: Cholesterol Optical Density values for different samples

	CHOLESTEROL		
	Intoxicated	T.quercetin	T.rutin
RAT1	91.8367347	97.61905	99.3197279
RAT2	100.340136	110.2041	

Table 13: Cholesterol concentration values for different samples

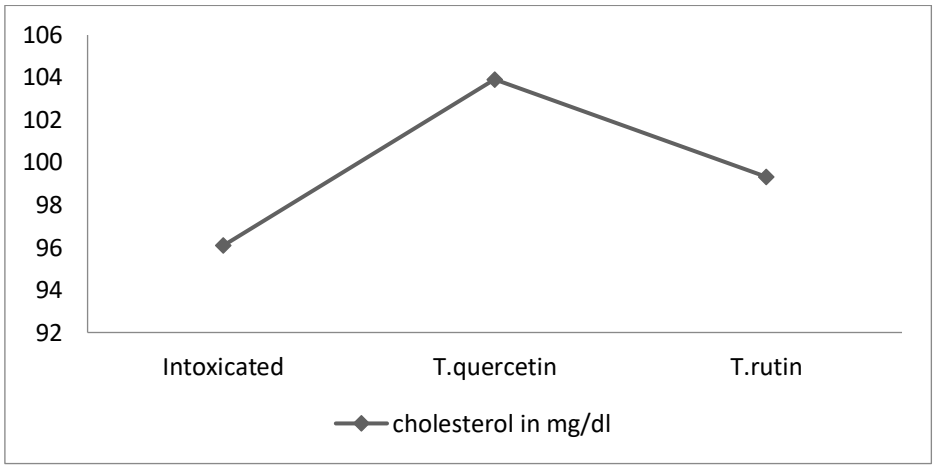


Figure 23: **Graph of Variation of cholesterol concentration in the specimen samples**

From the results obtained it is evident that both Cholesterol and Triglycerides count decreased in Al intoxicated specimen samples and the sample that received treatment with quercetin and retinin showed the higher concentrations in lipids.

	TRIGLYCERIDES		
	Intoxicated	T.quercetin	T.rutin
RAT1	0.224	0.263	0.325
RAT2	0.274	0.34	

Table 14: Triglycerides Optical Density values for different samples

	TRIGLYCERIDES		
	Intoxicated	T.quercetin	T.rutin
RAT1	74.916388	87.95987	108.695652
RAT2	91.638796	113.7124	

Table 15: Triglycerides concentration values for different samples

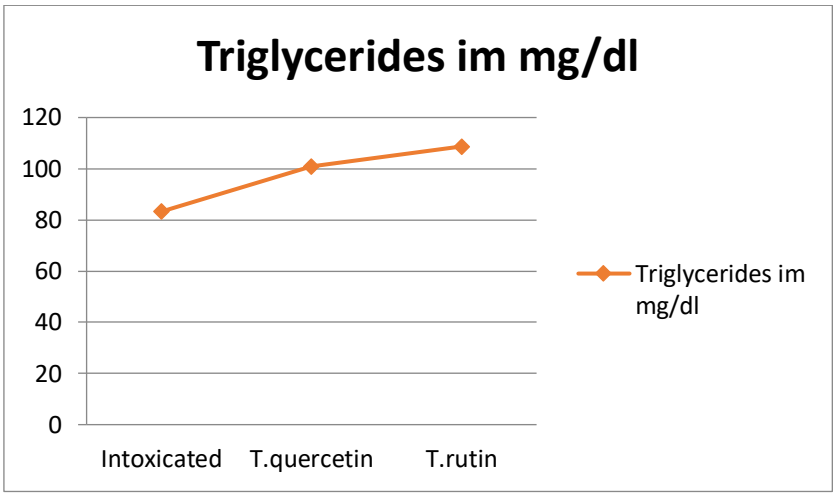


Figure 24: **Graph of Variation of cholesterol concentration in the specimen samples**

CHAPTER 4
DISCUSSION

4.1. EFFECTS OF ALUMINIUM INTOXICATION ON THE HEART

4.1.1 Heart

Mikstacka et al., (2010), showed that Quercetin protects membranes against lipid peroxidation (IC50 value = 64±8.7 μM). The cell membrane is prone to lipid peroxidation under oxidative stress that involves cleavage of polyunsaturated fatty acids at their double bonds leading to the formation of MDA **(Ayala *et al.*, 2014).**

Quercetin shows its anti-oxidant actions, such as protection of cardiovascular cells associated with a decrease in triacylglycerol concentration and an increase in HDL-cholesterol concentration, endothelium-dependent vasodilation due to an increased NO production, and prevention of endothelial cell apoptosis. **(Pfeuffer et al., 2013; Dayoub et al., 2013).** Thus, quercetin to some extent had a protective effect in vitro lipid peroxidation, which also agrees with **Bustos *et al.*, (2016).**

During this work i found out that the relative weight of rats and heart organs intoxicated with Aluminium and treated with antioxidants was significantly elevated as compared to the control group. Enhanced TBARS production was also observed which correlates MDA production and lipid peroxidation. CAT, GSH, GST, Protein, Lipids were determined from heart homogenates as indicators of Oxidative Stress. Aluminium intoxication induces production of free radicals (prooxidants) which destroy the intergrity of enzyme cell membranes thus reducing enzyme activity.

4.2. EFFECTS OF ALUMINIUM INTOXICATION ON BIOMARKERS OF

OXIDATIVE STRESS

4.2.1. Proteins

This study shows a general decrease in the number of proteins across all rats male and female intoxicated with Aluminium as compared to the control group and groups treated with quercetin, rutin and the medicinal plant.

Davies, (2016) demonstrated that ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation; which could explain a reduction in the number of proteins compared to the control group.

From the review of **Hassan *et al.*, (2017),** under extreme conditions, metal ions profoundly affect cellular protein homeostasis by interfering with their folding process and stimulate aggregation of nascent or non-native proteins. Also, toxic metal ions at cellular level evoke oxidative stress by generating reactive oxygen species **(Li *et al.*, 2016a).** They promote DNA damage and/or impair DNA repair mechanisms, impede membrane functional integrity, nutrient homeostasis and perturb protein function and activity **(Tamás *et al.*, 2014**). This overall could explain the reduction in the number of proteins in the intoxicated groups in comparison to the control group.

A significant increase in protein concentration observed in the heart organs of rats treated with Quercetin agrees with **Marunaka *et al.*, (2017)** on the antioxidant effects on quercetin in the body.

Hahn *et al.*, (2020), demonstrated that Quercetin shows anti-oxidant actions via radical scavenging ability and by interacting with anti-oxidant enzymes, such as heme oxygenase-1 (HO-1), which protects oxidative stress H2O2-induced apoptosis, and reduces intracellular ROS production and mitochondria dysfunction.

4.2.2. Catalase

Under normal conditions the body contains sufficient levels of scavenger enzymes such as Cu, Zn-SOD, CAT, and selenium-dependent GSH-Px to protect against free radical injury. Cu, Zn-SOD catalyses the dismutation of superoxide (O2.–) to H2O2, which is then independently converted to water by CAT or by GSH-Px **(Kurutas, 2016)**.

Hydrogen peroxide itself is a harmful co-product of many general metabolic processes. To ensure that damage to cells and tissues is avoided, the produced hydrogen peroxide must be immediately converted into other, less-reactive substances. For this purpose, CAT is often used by cells to quickly catalyse the degradation of hydrogen peroxide into less-reactive oxygen and water molecules **(Di Marzo et al., 2018)**.

My work however showed significant decrease in the concentration of catalase in both male and female aluminium intoxicated rats compared to the control group.

From **Lei *et al.*, (2016),** antioxidant defence enzymes normally act in concert, thus superoxide dismutase protects catalase and peroxidase against inhibition by O2-, while catalase and peroxidase provide protection for superoxide dismutase against inactivation by hydrogen peroxide.

A general decrease in vitro in the activity of catalase over time is a result of increased concentration of O2- which gradually inactivates catalase as shown by the work of **(Bauer, 2015).**

However, variations in the results shown by the Quercetin treated group showed to some extent the antioxidant effect of quercetin.

Ademosun *et al.*, (2016) demonstrated the antioxidant effect of quercetin by showing that the –OH groups on the side phenyl ring of quercetin are bound to important amino acid residues at the active site of two enzymes. In this way, it has a stronger inhibitory effect against key enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are associated with oxidative properties. My findings may also explain in agreement with **Chen *et al.*, (2017)** that reported that pre-treatment with quercetin significantly enhanced the expression levels of endogenous antioxidant enzymes such as Cu/Zn SOD, Mn SOD, catalase (CAT), and GSH peroxidase.

4.2.3. Glutathione S- Transferases

Glutathione S- Transferases are ubiquitously distributed in nature, being found in organisms as diverse as microbes, insects, plants, fish, birds and mammals **(Gullner et al., 2018).** The transferases possess various activities and participate in several different types of reaction. Most of these enzymes can catalyse the conjugation of reduced glutathione (GSH) with compounds that contain an electrophilic centre through the formation of a thioether bond between the sulphur atom of the GSH and the substrate **(Cooper and Hanigan, 2018).** In addition to conjugation reactions, a number of GST isoenzymes exhibit other GSH-dependent catalytic activities including the reduction of organic hydroperoxides **(Allocati *et al.*, 2018)**.

These enzymes also have several non-catalytic functions that relate to the sequestering of carcinogens, intracellular transport of a wide spectrum of hydrophobic ligands, and modulation of signal transduction pathways **(Cho *et al.*, 2001).**

Many endogenous GST substrates are formed as a consequence of modification of macromolecules by ROS and the transferases are therefore considered to serve an antioxidant function **(Singhal *et al.*, 2015).**

Among numerous defence mechanisms against oxidative injury, glutathione S-transferase (GST) plays a crucial role. The GST family, which comprises a relatively high amount of total cytosolic protein, is responsible for the high-capacity metabolic inactivation of electrophilic compounds and toxic substrates **(Wu *et al.*, 2004; Townsend *et al.*, 2005).**

My work showed a significant decrease in GST concentrations in the aluminium intoxicated groups both male and female compared to the group treated with quercetin, retinin, plant and the control group. In a similar study by **Roth *et al.*, (2011),** this may be due to the inhibition of the activity of GST in vitro by the aluminium; as seen by a significant reduction in concentration across all intoxicated groups (aluminium intoxicated only, and treated with Quercetin) with respect to the control group. Also the fact that GST conjugates with toxins and eliminates them through bile, urine and faeces.

Moreover, treatment with quercetin caused a slight increase in concentration in comparison with the aluminium intoxicated only group.

This agrees with a similar work by **Hader *et al.*, 2016** which showed the ameliorative effects of quercetin in antioxidant defence.

4.2.4. Thiobarbituric Acid Reactive Substances (TBARS)

MDA has been widely used in biomedical research as a marker of lipid peroxidation due to its facile reaction with thiobarbituric acid (TBA). The reaction leads to the formation of MDATBA2, a conjugate that absorbs in the visible spectrum at 532 nm and produces a red-pink colour (**Ohkawa *et al.*, 1978)**.

In all groups both male and female the aluminium intoxicated groups revealed a significant increase in TBARS activity compared to the control group. My work agrees with (**Tsikas, 2017)**, who showed lipid peroxidation as a process in which free radicals, such as

ROS and RNS, attack carbon-carbon double bonds in lipids, a process that involves the abstraction of a hydrogen from a carbon and insertion of an oxygen molecule; which leads to a mixture of complex products including, lipid peroxyl radicals, and hydroperoxides as the primary products, as well as malondialdehyde (MDA) and 4-hydroxynonenal as predominant secondary products.

We see that the increase in concentration of MDA in the Aluminium intoxicated group compared to the control group caused its significant rise in concentration. This also agrees with abnormal increases in levels of malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) in tissue homogenates of rats exposed to Al **(Zhang et al., 2016; Yu et al., 2019).**

With the group treated with Quercetin, we observed a significant decrease in TBARS concentration compared to the Aluminium intoxicated groups, control group and group treated with plant. This may prove that ameliorative effect of quercetin in this assay in other words quercetin being a better antioxidant than rutin and Phagnola Rupestre.

4.2.5. SUPEROXIDE DISMUTASE (SOD)

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are three major antioxidant enzymes in animals. Superoxide dismutase (SOD), the first defense line against oxygen-derived free radicals, catalyzes the dismutation of the superoxide anion ($O2-\cdot$) into hydrogen peroxide (H2O2), which is then transformed into H2O and O2 by catalase. Cu/Zn-SODs, Fe-SODs, and Mn-SODs are sensitive to different inhibitors which include Co^{2+}, Hg^{2+}, K^{+} and, Al^{3+} and other metal ions also show their inhibition effects on

SOD activity. In my case SOD activity was inhibited by Aluminium. The group treated with quercetin, rutin, plant and control group showed lower levels of SOD inhibition proving the antioxidant properties of polyphenols.

4.2.6. GSH & GPx

GSH is a cosubstrate for GPx. The initial increase in GSH activity can be explained by the enzyme being the first line of defense against prooxidants and its ability as a coenzyme but with increase in intoxication the enzyme is inhibited. GPx increased in intoxicated samples probably due to its high affinity for H2O2 as compared to catalase.

4.2.7. LIPIDS (CHOLESTEROL & TRIGLYCERIDES)

Enhanced TBARS production is an indication of Lipid Peroxidation. Total Cholesterol and Triglycerides levels decreased significantly in the specimen samples intoxicated with Al compared to the control group, group treated with plant, quercetin and rutin antioxidants. The reduction in lipids is attributed to lipid peroxidation, phosphine toxicity and reduced lipase activity due to Al toxicosis. It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids.

CHAPTER 5
CONCLUSION

It can be considered that two opposite mechanisms are involved in the redox status molecular mechanism; the mechanisms leading to the production of oxidants and the mechanism of antioxidants.

Maintaining this status quo (homeostasis) proves to be a delicate task; cells are constantly overwhelmed with a barrage of free radicals from both exogenous and endogenous sources. Organs like the heart maintain this balance in vivo by constant production of more cells to mop out excess free radicals through various defence mechanisms including apoptosis.

My study shows that the maintenance of this balance is limited as with time and increase in free radicals; organisms lose this battle against free radicals in my case generated by Aluminium intoxication.

Anti-oxidants like Vitamins, medicinal plants with antioxidant properties and polyphenols in my case Quercetin and Rutin proved to be a relief to combat the danger of oxidative stress.

In the battle of prooxidants (free radicals) and antioxidants, whatever help needed by antioxidants to prevent oxidative stress is welcomed by the organism. This reinforcement already adds up to the inbuilt enzyme defence system already employed by organisms through their various enzymes (CAT, GST, GSH, SOD and GPX).

My study shows that Quercetin is a better antioxidant than Rutin against Al intoxication. The protective properties of Quercetin were even better than the medicinal plant (Phagnola Rupestre). The results also show that the plant had some protective abilities against the free radicals. The study also shows that female rats were more affected by the toxicosis than male rats.

Perspectives for the future will be to

Analyse the effects of Aluminium on lipid membrane phospholipids.

Find out why female rats where more affected by Al intoxication than male rats.

Analyse the effect of intoxication on other key enzymes such as cytochrome oxidase.

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ANNEX

ANNEX 01

Table 7: Total protein content on average and standard deviation in the male group intoxicated with aluminium, male group intoxicated and treated with quercetin, male group intoxicated and treated with rutin, female group intoxicated with Al, female control group and finally female intoxicated group treated with plant.

	Intoxicated	T.quercetin	T.rutin	F.intoxicated	F.control	F.T.plant
Average	6.032847148	8.152496146	7.48547373	5.543697379	7.08526029	6.38859243
Standard Deviation	0.289327957	1.702226636	0.60700804	2.247439206	1.48175314	3.15765693

ANNEX 02

Table 8: Average Catalase content and standard deviation in the male group intoxicated with aluminium, male group intoxicated and treated with quercetin, male group intoxicated and treated with rutin, female group intoxicated with Al, female control group and finally female intoxicated group treated with plant.

	Intoxicated	T.quercetin	T.rutin	F.intoxicated	F.control	F.T.plant
Average	2.904433408	12.29337383	5.5023487	93.29405505	100.417514	111.332831
Standard Deviation	0.372014417	4.802356755	2.2159894	113.1367383	29.8439554	74.0306884

ANNEX 03

Table 9: TBARS content and standard deviation in the male group intoxicated with aluminium, male group intoxicated and treated with quercetin, male group intoxicated and treated with rutin, female group intoxicated with Al, female control group and finally female intoxicated group treated with plant.

	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
Average	9.074862431	5.203958576	9.04603278	14.82019188	7.86205507	13.1795337
Standard Deviation	0.901757146	2.26327817	2.78652615	7.334211724	2.88132382	8.71363268

ANNEX 04

Table 10: GST average content and standard deviation in the male group intoxicated with aluminium, male group intoxicated and treated with quercetin, male group intoxicated and treated with rutin, female group intoxicated with Al, female control group and finally female intoxicated group treated with plant.

	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
Average	10.57922274	14.58557876	18.1462921	11.63720074	19.2287669	37.8364316
Standard Deviation	2.734843283	1.797909209	3.27063385	3.865816351	7.49774629	31.6911422

ANNEX 05

Table 11: SOD % inhibition average and standard deviation in the male group intoxicated with aluminium, male group intoxicated and treated with quercetin, male group intoxicated and

treated with rutin, female group intoxicated with Al, female control group and finally female intoxicated group treated with plant.

	intoxicated	T.quercetin	T.rutin	F.intoxicated	F.Control	F.T.plant
Average	1128.337819	695.1268081	729.968066	1605.465369	1000.66802	798.730635
Standard Deviation	487.3513116	516.1882769	427.29792	1192.327788	713.535476	822.524896

ANNEX 06

Table 12: The different components of the phosphate buffer

Salts (g/l)	
0.005g	NaH_2PO_4
0.02g	Kcl
0.02g	$CaCl_2$
0.8g	NaCl
0.01g	$MgCl_2$
0.1g	Na_2CO_3