JOURNAL ON COMMUNICATIONS

ISSN:1000-436X

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Evaluation of Antioxidant Activity of Metformin - Resveratrol Aldehyde Complex in High Fat Diet Fed - Low Dose Streptozotocin Induced Experimental Type 2 Diabetes in Rats.

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Abstract

The present study is aimed to evaluate the antidiabetic and antioxidant properties of a newly synthesized Metformin–Resveratrol Aldehyde complex (Met-Res-Aldehyde complex) in high-fat diet-fed, low-dose streptozotocin-induced experimental type 2 diabetes mellitus in rats. The effect of oral administration of the Met-Res-Aldehyde complex (5 mg/kg body weight) for a period of 30 days on the levels of biochemical parameters was evaluated in experimental groups of rats. The antidiabetic efficacy of the complex were assessed by measuring a range of biochemical indices, including fasting blood glucose, plasma insulin, haemoglobin, glycosylated haemoglobin, total protein, urea, uric acid, and creatinine. Oxidative stress markers such as TBARS, lipid peroxides, hydroperoxides and protein carbonyls were analyzed in plasma, pancreatic, hepatic, and renal tissues. The status of enzymatic antioxidants, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione S-transferase (GST), gluatathione reductase (GR) as well as non-enzymatic antioxidants such as vitamin E, vitamin C and ceruloplasmin, were also evaluated. Diabetic rats showed significantly increased levels of fasting blood glucose and glycosylated haemoglobin. Oral treatment with the Met-Res-Aldehyde complex resulted in the maintenance of normoglycemia by decreasing oxidative stress markers and improving antioxidant status in diabetic rats. The results of the study indicate that the Met-Res-Aldehyde complex is non-toxic and possesses significant antioxidant properties which in turn responsible for its observed antidiabetic efficacy. These effects are comparable to those of metformin, a standard oral hypoglycemic drug.

Keywords *:* Met-Res-Aldehyde complex; High fat diet fed- Low dose STZ-induced diabetes; antidiabetic; Oxidative stress; Enzymatic and non-enzymatic antioxidants.

1. Introduction

Diabetes mellitus (DM) is a chronic endocrine disorder arises due to the deficiency (Type 1) and/or efficiency of insulin, a hormone secreted by the β-cells of pancreas. DM has been known since antiquity and despite therapeutic advances, it is still remains an incurable chronic disease. Type 2 diabetes represents more than 95% of the total diabetic population in the world and is characterized by metabolic disorders of lipids, carbohydrates, and proteins (Nathan, 1993; Accili et al., 2025). According to the International Diabetes Federation (IDF) report for the year 2025, currently 589 million people aged between 20-79 years were living with DM in 2024, around 11.1% of the world's population in this age group, and it is projected that by the year 2050, based just on future trends in population growth, population aging, and urbanization, diabetes cases will increase to 853 million people; about 45% across the world will be affected by DM (Duncan et al., 2025). In comparison to females, T2DM predominantly affects males with a higher incidence (Stewart and Liolitsa, 1999; Khan et al., 2024), and it is much more prevalent in the countries with low and middle-income groups. Worldwide, the countries with the highest number of diabetic patients are China, India, and USA (Sun et al., 2022). In addition, the demographic pattern of T2DM is shifting from old-onset to young-onset with a more contentious phenotyping enhancing the risk of development of premature diabetes related secondary complications (Kim et al, 2012; Bhatti et al., 2022). A negative correlation between the age of onset of diabetes and diabetes-induced mortality has been observed (Al-Saeed et al., 2016; Zhang et al., 2025). Furthermore, accumulating evidence suggests that a precipitous rise in the prevalence and complications is expected in near

future. This impending epidemic appears to exert a substantial economic and health burden on the individuals and federal governments worldwide.

Free radicals (FR) have gained growing importance in the fields of biology and medicine as they are continuously generated during many diverse endogenous and exogenous processes. Mitochondria are the main source of endogenous reactive oxygen species (ROS) generated at cell level. The overproduction of free radicals can damage macromolecules such as nucleic acids, proteins and lipids. This leads to vital tissue damage in various chronic and degenerative diseases. Antioxidants play a crucial role in the body's defense against free radicals. The manifestations of an oxidative environment due to persistent hyperglycemia represent a formidable clinical challenge characterized by the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, mitochondrial dysfunction, multi-organ damage and dys-regulated intercellular metabolism which eventually leads to the initiation, progression and onset of both primary and secondary complications of DM (Laakso,1993; Jasvinder Singh Bhatti et al., 2022; Lijun Zhao et al., 2026). Chronic hyperglycemia induced excessive generation of free radicals and the development of oxidative stress are correlated with the pathogenesis and progression of DM.

Free radical (FR) may be defined as an atom or diatomic or polyatomic molecule or molecular fragment capable of having independent existence (hence "free"). They are characterized by having an unpaired electron in its outer valency orbit or outermost shell around the atomic nucleus (hence "radical") (Rebeca Gerschman et al., 1954; Wang et al., 2026). Due to their electronic instability, free radicals are intrinsically unstable and extremely reactive. FRs always have a strong propensity to acquire or lose an electron from the bordering molecule through covalent bonding to gain electronic stability and form a stable compound, acting as an oxidizing or reducing agent (Gutowski and Kowalczyk, 2013; Irfan et al.,2026). Free radicals act as oxidants by "stealing" an electron from stable neighboring molecules such as lipids, proteins, or DNA to complete their stability by own electron pair and achieve a lower energy state. Some free radicals, such as the superoxide radical, can act as reducing agents by donating their unpaired electron to another molecule to become a stable non-radical species. However, during the process of accepting or donating an electron, the previously stable molecule to become a new free radical, resulting in a cascade or chain reaction (Pham-Huy et al.,2008; Priyadarsini,2026).

In general pro-oxidants/oxidants are termed as Reactive Oxygen Species/Reactive Nitrogen Species. The most important free radicals generated during normal metabolic reactions are radicals derived from oxygen and hence they are generally termed as "Reactive Oxygen Species" (ROS).The nitrogen derived free radicals are known as "Reactive Nitrogen Species" (RNS).The gift of using oxygen has enabled humans and animals to metabolize fats, proteins and carbohydrates to produce energy in the form of ATP, albeit not without a cost since, paradoxically, the use of oxygen contributes to human aging and illness through the generation of excessive ROS. Since breathing pure oxygen (100%) instead of 20% air is detrimental to aerobic organisms (Fridovich, 1999; Liu et al., 2025). Free radicals are the products of the fractional reduction of molecular oxygen. Four electron reduction of molecular oxygen is an efficient, controlled process in cellular respiration that leads to the production of water without the generation of ROS. Conversely one-electron reduction of molecular oxygen leads to the generation of ROS (Droge, 2002; Liu et al., 2025). It has been reported that nearly 5% of total consumed oxygen forms free radicals in our body (Younes, 1999; Pham-Huy et al.,2008).

Both the ROS and RNS can be broadly classified into two groups namely; radicals and non-radicals. The examples for the radicals include Superoxide ($O_2^{\cdot-}$), Oxygen radical ($O_2^{\cdot}$), Hydroxyl ($OH^{\cdot}$), Alkoxy radical ($RO^{\cdot}$), Peroxyl radical ($ROO^{\cdot}$), Nitric oxide (nitrogen monoxide) ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$) (Beckman and

Koppenol, 1996; Pourova et al., 2010; Mu et al., 2024). The term "reactive" is not always appropriate for radical species; hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet-}$) react promptly with some molecules, while the hydroxyl radical ($HO^{\bullet}$) reacts promptly with almost everything. Species such as peroxyl radicals ($RO_2^{\bullet}$), nitrate radicals ($NO_3^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), hypochlorous acid (HOCl), hypobromous acid (HOBr), carbonate ($CO_3^{\bullet-}$), carbon dioxide radicals ($CO_2^{\bullet-}$), nitrogen dioxide ($NO_2^{\bullet-}$), peroxynitrite ($ONOO^-$), nitrogen dioxide ($NO_2^{\bullet}$) and ozone (O_3) have intermediate reactivities (Jamdade and Bodare, 2022). The high reactivity of these radicals is due to the presence of one unpaired electron which tends to donate it or to obtain another electron to attain stability. Among the FRs, hydroxyl radical is the most reactive ROS known (Sharma et al., 2012; Ren et al., 2026). It is generated by the Fenton- reaction in which H_2O_2 is converted into $OH^{\bullet}$ in the presence of transition metals like (Fe^{+2}, Fe^{+3}) that work as the catalyst (Das and Roy choudhury, 2014; Zárate-Guzmán et al., 2019; Guo et al.,2023). It is also to be noted that copper also behaves in a Fenton-like manner to trigger free radical formation and disease (Winterbourn, 1995; Brewer et al., 2007; Lipinski, 2011; Zhao, 2019; Rynkowska et al., 2020). The Fenton reaction is the oxidation of divalent iron to its trivalent state by hydrogen peroxide, with the resultant formation of hydroxyl radical and hydroxide ion (Di Meo and Veditti, 2020).UV radiation can cause the cleavage of the oxygen–oxygen bond to form hydroxyl radicals.

The highly toxic hydroxyl radical may cleave covalent bonds in proteins and carbohydrates, cause lipid peroxidation and devastate the architecture of cell membranes. It is short-lived (~10^{-9}seconds) but reacts very rapidly with no selectivity and reacts with almost every type of molecule found in living cells including DNA, sugars, amino acids, phospholipids, organic acids and fatty acids (Gutowski & Kowalczyk, 2013; Phaniendra et al., 2015). Since no enzymatic system to scavenge the hydroxyl radical is identified yet, its excessive generation in the cell can lead to cell death (Pinto et al., 2003). $OH^{\bullet}$ are scavenged only through non-enzymatic antioxidants like proline, ascorbate, etc. (Hasanuzzaman et al., 2020).

The non radical species include hydrogen peroxide (H_2O_2), hydrochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium (nitryl) cation (NO_2^+), organic peroxides (ROOH), aldehydes (HCOR) and peroxynitrite (ONOOH) (Halliwell, 2001; Kohen and Nyska, 2002). These non radical species are not free radicals but can easily lead to free radical reactions in living organisms (Genestra, 2007;Mukherjee et al.,2025).The different types of free radicals vary widely in their reactivity; for example, the reactivity of ROS in decreasing order is: $HO^{\bullet} > O_2^{\bullet-} > H_2O_2$ (Halliwell, 2006; Shimokawa, 2020). The chemical reactivity of free radicals is directly associated with their potential to damage biological molecules.

Table 1. List of ROS and RNS produced during metabolism (Genestra, 2007; Mugoni et al ., 2013).

Reactive Oxygen Species (ROS)		
Name Symbol Half-Life (s)	Name Symbol Half-Life (seconds)	Name Symbol Half-Life (sconds)
Radicals		
Superoxide	$O^{\bullet-}$	10^{-6}s
Nitric oxide	NO•s	10^{-10}s
Hydroperoxyl	HO_2•	s

Peroxyl	ROO•	17 s
Alkoxyl	RO•	10^{-6} s
Organic hydroperoxide	ROOH	Stable
Reactive Nitrogen Species (RNS)		
Nitric oxide	NO•	s
Nitrogen dioxide	NO_2•	s
Nitrate radical	NO_3•	s
Non-radicals		
Hydrogen peroxide	H_2O_2	Stable
Ozone	O_3	s
Singlet oxygen	(1O2Dg)	10^{-6}s
Hypochlorous acid	HOCl	Stable (min)
Peroxynitrite	ONOO−	10^{-3}s
Nitrous acid	HNO_2	s
Nitrosonium cation	NO+	s
Nitroxyl anion	NO−	s
Peroxynitrite	ONOO−	10^{-3} s
Dinitrogentrioxide	$N2O_3$	s
Dinitrogen tetroxide	$N2O_4$	s
Peroxynitrous acid	ONOOH	Fair stable
Nitryl chloride	NO_2Cl	s

Free radicals are the products of normal cellular metabolism. Endogenous sources, generated during normal metabolism, include different cell organelles, such as mitochondria, peroxisomes and endoplasmic reticulum, many enzyme activities, fatty acid metabolism and phagocytic cells (Droge, 2002). Exogenous sources include radiation X-rays, -rays, ultraviolet A, visible light in the presence of a sensitizer, chemical reagents such as heavy or transition metals (e.g., Cd, Hg, Pb, As, metal ions such as Fe^{2+} and Cu^{+}), HONOO, ozone, N_2O_2, deoxyosones, ketamine, H_2O_2, HOCl and HOBr, cooking (smoked meat, used cooking oil), high temperatures, environmental pollutants (aromatic hydrocarbons, pesticides, polychlorinated biphenyls, dioxins and many others).

Table 2. Sources of free radical

Sources of free radical	
Exogenous	**Endogenous**
Air and water pollution,Tobacco smoke,Heavy metals,Transition metals,pesticides,High Temperature, UV radiation,Gamma radiation, Drugs Cooking (smoked meat, cooking oil).	Cells (neutrophills,esionphills), Enzymes (NO synthase, Xanthine oxidase, NADPH oxidase, lipooxygenase), Mitochondrial chain, Endoplasmic Reticulum, Oxidation , Cytochrome p450, Diseases.
Free radicals	
ROS: $O_2^{\cdot-}$,·OH, HO_2· H_2O_2, RO_2.HOCl……	
RNS: NO, NO_2·,$ONOO^-$,HNO_2,RONOO,N_2O_3.....	

Major source of free radicals generation. Either endogenous or exogenous sources generate ROS (O_2•- superoxide anion; ·OH hydroxyl, HO_2• hydroperoxyl; H_2O_2, hydrogen peroxide; RO_2,peroxyl; HOCl, hypochlorous acid); RNS (NO·, nitric oxide; NO_2•, nitrogen dioxide; $ONOO^-$ peroxynitrite;HNO_2, nitrous acid; RONOO, alkylperoxynitrates; N_2O_3, dinitrogen trioxide).

Endogenous free radicals are produced by the activation of immune cells (eosinophils, neutrophils, etc.) to battle against bacteria and other invaders, by the mitochondrial respiratory chain, by enzymatic activity (xanthine oxidase, NADPH

oxidase, lipo-oxygenase, NO synthase, etc.) and by various pathological disorders and diseases. Exogenous free radicals arise from air and water pollution, cigarette smoke, heavy metals or transition, drugs, industrial solvents, radiation and high temperatures.

Mitochondria as a major source of free radicals

Most of the intracellular ROS are derived from mitochondria. Human body cells depend on adenosine tri-phosphate (ATP) to hoard and transport chemical energy. Mitochondria generate more than 90% of ATP by oxidative phosphorylation (Srinivasan and Avadhani, 2012), consuming about 85% of the oxygen requirements of the cell to do so and hence they are dubbed the "Power house of the cell" (Siekevitz,1959).Through cellular respiration, mitochondria convert nutrients into chemical energy, functioning as double-membrane organelles in eukaryotic cells, via oxidative phosphorylation. Most of the oxygen is reduced to water, and a small proportion is converted to free radicals. However, up to 2% of electrons leak along the electron transport chain (ETC) and react directly with oxygen in a one-electron reduction to produce a superoxide (radical anion) instead of a water molecule (Wang et al., 2018). About 5% of the oxygen consumed by living organisms can be converted to superoxide radical by mitochondria under physiological conditions (Valko et al., 2007). Reactive oxygen species generated as by-products of mitochondrial electron transfer mainly include the superoxide radical anion and hydrogen peroxide. The production of superoxide radical in mitochondria is estimated to be approximately 2 to 3 nmol/min per mg of protein (Inoue et al.,2003), confirming its importance as the main source of this radical in living organisms. In eukaryotic organisms, over 90% of ROS are produced by the mitochondrial ETC as a by-product of respiration (Davies, 2004). Quantities of ROS are also produced by the ETC in the plasma (Luthje et al., 2013), nuclear (Vartanian and Gurevich, 1989) and endoplasmic reticulum (Brignac-Huberet al., 2011) membranes. A multi electron reduction of O_2 is carried out by protein complexes in the ETC. The phosphorylation unit combines oxygen and hydrogen to produce H_2O and ATP molecules. The oxidative unit consists mainly of a series of protein complexes in the inner mitochondrial membrane (IMM), known as the respiratory or electron transfer chain (ETC). Hydrogen atoms are established as reducing equivalents. The passage of hydrogen atoms along the respiratory chain is equivalent to the passage of electrons through sequential redox reactions along protein complexes I-IV of the ETC (Rich and Marechal, 2010), where O_2 is reduced to H_2O.

The production of ATP by oxidative phosphorylation associated with the ETC has an energy loss in the form of electrons (Nath, 2016), which determines the production of free radicals. By virtue of its electron configuration (two unpaired electrons in the outer shell), the oxygen molecule is not very reactive (Malanga *et al*., 2014) and consequently tends to accept electrons one at a time. If O_2 accepts a single electron, the electron must enter an antibonding orbital, producing the superoxide radical. A two-electron reduction of O_2, with the addition of $2H^+$, generates hydrogen peroxide (H_2O_2). A one-electron reduction of H_2O_2 forms a hydroxyl radical and a hydroxyl anion. Water is formed after the electron and proton addition to hydroxyl anion. Although the ETC is a highly efficient system, the redox reactions predispose electron vectors to reactions with molecular oxygen. Mitochondria are the most significant intracellular source of superoxide anion. Its concentration 5 to 10 times greater has been estimated in mitochondria than in the nuclear space or the cytosol (Davies, 2004). Ubiquinone links complex I with III and II with III and is regarded as a major player in the formation of super oxide anion. The oxidation of ubiquinone proceeds in a set of reactions known as the Q-cycle, and the unstable semiquinone is responsible for superoxide anion formation (Turrens, 2003). The transfer of electrons from complex I or II dehydrogenase to coenzyme Q or ubiquinone (Q) leads to the formation of a reduced form of coenzyme Q (QH_2) that regenerates coenzyme Q via an unstable intermediate semiquinone anion $Q^{\bullet-}$. The latter transfers electrons to molecular oxygen, leading to the formation of superoxide radical (Turrens, 2003). Since

the generation of superoxide is not enzymic, most ROS production will be linked to the higher metabolic rate. Additionally, mitochondrial superoxide is generated by electron-transfer during fatty acid oxidation, by glycerol-3-phosphate dehydrogenase and other IMM-associated oxidoreductases (Wong et al., 2017).

The superoxide anion serves as a ROS precursor. Most superoxide anion is readily metabolized to non-radical H_2O_2 by superoxide dismutase (SOD) or non-enzyme mechanisms (Zou et al., 2017). The subsequent Haber–Weiss reaction of H_2O_2 and superoxide anion (Leitao, 2017), or Fe^{2+}- (or Cu^{2+})-driven Fenton cleavage of H_2O_2 (Mahaseth and Kuzminov, 2017), may generate the highly reactive hydroxyl radical. The H_2O_2 produced is in its optimum state for respiration, characterized by a high degree of reduction of the electron carriers and a limiting supply of adenosine diphosphate (ADP) (Pollack and Leeuwenburgh, 1999). An additional source of H_2O_2, not related to breathing, is positioned on the external mitochondrial membrane (Hauptmann et al., 1996), where the oxidative deamination of biogenic amines by monoamine oxidases is coupled with the direct two-electron reduction of O_2 to H_2O_2. The hydrogen peroxide produced during the oxidative deamination of catecholamines may be involved in neurodegenerative disorders such as Parkinson's and Alzheimer's diseases, presumably through oxidative damage to the mitochondrial membrane (Fhan, and Cohen, 1992). The factors that control the ETC generation of ROS *in vivo* are not fully established. Conventionally, complex I and complex III, including complex II, are considered the major contributors to ROS production (Quinlan et al., 2013). However, the relative contribution of each site to the total production of superoxide anion and H_2O_2 varies from one organ to another and depends on respiration rate and redox state (Brand, 2016; Turrens, 2003).

The different sites of ROS production have distinct signaling roles and presumably change under different physiological conditions (Quinlan et al., 2013). It is therefore difficult to pinpoint the specific site of ROS production (Andreyev et al., 2015). Up to eleven distinct mitochondrial sites of production of superoxide and/or hydrogen peroxide linked to substrate catabolism, electron transport and oxidative phosphorylation were identified in mammalian mitochondria (Brand, 2016; Andreyev et al., 2015). Sites I (Grivennikova, and Vinogradov, 2013) and III (Turrens, 2003) are considered to produce principally or exclusively superoxide. Site II may generate both superoxide and hydrogen peroxide (Quinlan et al., 2013). These sites may also act as primary sources of mitochondrial redox signal. H_2O_2 is the primary form of ROS utilized for intracellular signaling. Since most ATP is produced by mitochondria, impaired mitochondrial function is implicated in a variety of health chronic conditions and degenerative diseases (Pagano et al., 2014), many of which can be attributed to excessive mitochondrial production of ROS. However, modest levels of ROS stimulate essential biological processes, such as proliferation, differentiation and immunity (Hamanaka and Chandel, 2010). Furthermore, mitohormesis (Ristow and Schmeisser, 2014), a decrease in the net basal metabolism production of ROS, which increases resistance to oxidative stress (Hamanaka and Chandel,2010), may be a way to improve mitochondrial function and resistance to chronic and degenerative diseases. Mitohormesis, a defense mechanism, can therefore promote health and increase longevity through the prevention or delay of diseases (Ristow and Schmeisser, 2014, Scheibye-Knudsen et al., 2015).

Peroxisomes as a source of free radicals

In peroxisomes, the respiratory pathway involves the transfer of electrons from various metabolites to the oxygen leads to H_2O_2 formation (De Duve and Bauduhuin,1966), but is not coupled to oxidative phosphorylation to produce ATP instead free energy is released in the form of heat. Other free radicals produced in peroxisomes include superoxide anions, hydroxyl anions and nitric oxide radicals. The β-oxidation of fatty acids is the major metabolic process producing H_2O_2 in the peroxisomes. However,

different peroxisomal enzymes, such as acyl CoA oxidase, D-amino acid oxidase, L-a-hydroxy oxidase, urate oxidase, xanthine oxidase and D-aspartate oxidase, have been reported to produce different ROS (Schrader and Fahimi, 2006). Peroxisome and electron linked oxidation alterations are involved in many conditions and diseases, such as neurological disorders, and in the development of cancer (Islinger et al., 2018).

Table 3. Reactive Oxygen species producing enzymes in peroxisomes

Enzyme	Substrate	ROS
AcylCoA-oxidases (enzymes of β-oxidation)	Fatty acids	H_2O_2
D-amino acid oxidase	D-proline	H_2O_2
L-α-hydroxy oxidase	Glycolate	H_2O_2
Urate oxidase	Uric acid	H_2O_2
D-aspartate oxidase	D-aspartate	H_2O_2
Xanthine oxidase	Xanthine	$O^{\bullet-}_2$, , H_2O_2

Endoplasmic Reticulum as a source of free radicals

The electron transport chain of the endoplasmic reticulum is the second furthermost source of ROS (Brignac-Huber et al., 2011). Catabolism of cell and foreign chemicals by cytochrome P-450 includes redox steps and is responsible for the production of ROS in the endoplasmic reticulum. The enzymes of the endoplasmic reticulum that contribute to the formation of ROS include cytochrome P-450, b5 enzymes and diamine oxidase (Cheeseman and Slater, 1993). Another important thiol oxidase, Erop1p, catalyzes the transfer of electrons from dithiols to molecular oxygen, resulting in the formation of H_2O_2 (Gross et al., 2006). The other endogenous sources of ROS include prostaglandin synthesis, auto-oxidation of adrenalin, phagocytic cells, reduced riboflavin, $FMNH_2$, $FADH_2$, cytochrome P -450, immune cell activation, inflammation, mental stress, excessive exercise, infection, cancer, aging, ischemia etc. (Gross et al., 2006). On the other hand, ROS are also produced in the biological systems by various exogenous sources shown in Table (Pham-Huy et al., 2008).

Exogenous sources include radiation X-rays, ultraviolet A, visible light in the presence of a sensitizer, chemical reagents such as heavy or transition metals (e.g., Cd, Hg, Pb, As, metal ions such as Fe^{2+} and $Cu^{+)}$, HONOO, ozone, N_2O_2, deoxyosones, ketamine, H_2O_2, HOCl and HOBr, cooking (smoked meat, used cooking oil), high temperatures, environmental pollutants(aromatic hydrocarbons, pesticides, polychlorinated biphenyls, dioxins and many others), microbial infections, drugs and their metabolites(Davies,2005;Valko et al.,2007;Pham-Huy et al.,2008).

Table 4. ROS generated from exogenous sources

Air & water pollution	**Ultraviolet light**
Alcohol	Cooking (smoked meat, used oil, fat)
Tobacco smoke	Drugs such as Halothene, Paracetamol,
Transition metals - Cd, Hg, Pb, As.	Bleomycine, Doxorubicin,
Heavy metals - Fe, Cu, Co, Cr	Metrenidazole, Ethanol, CCl_4.
Industrial solvents	
Pesticides	
High temperature	

Hydroperoxyl Radical (HO_2) usually termed hydro peroxyl radical or perhydroxyl radical is the simplest form of a peroxyl radical, produced by the protonation of the superoxide anion radical or by the decomposition of hydroperoxide. Around 0.3%

of superoxide present in the cytosol exists in the protonated form (De Grey, 2002). The hydroperoxyl radical produces H_2O_2, which readily react with active redox metals, including iron and copper, to activate Fenton or Haber–Weiss reactions. The hydroperoxyl radical can also extract hydrogen atoms from NADH or glyceraldehyde-3-phosphate dehydrogenase–NADH, forming H_2O_2 (Bielski et al., 1985). The hydroperoxyl radical plays a vital role in the chemistry of lipid peroxidation. It is a much stronger oxidant than superoxide anion due to its ability to extract hydrogen atoms from linoleic, linolenic and arachidonic fatty acids, evidencing a direct role in the initiation of lipid oxidation (Dizdaroglu and Jaruga, 2012).

Hydrogen peroxide (H_2O_2) can be generated by the dismutation or direct reduction of O_2, and it is mainly produced by enzyme reactions. The presence of oxidases such as urate oxidase, glucose oxidase, D-amino acid oxidase is known to initiate the direct synthesis of hydrogen peroxide by the transfer of two electrons to molecular oxygen. These enzymes are found in microsomes, peroxisomes and mitochondria (Winterbourn, 2013). Hydrogen peroxide is lipid soluble and can therefore easily diffuse through the cell membrane. Being weakly reactive, this non-free-radical cannot readily oxidize most lipids, proteins and nucleic acids. The threat posed by H_2O_2 lies in its conversion to the hydroxyl radical by homolytic fission, induced by UV or by the interaction with transition metal ions (Fenton reaction) (Choe and Min, 2006). Hydrogen peroxide may produce singlet oxygen through a reaction with a superoxide anion or with HOCl or chloramines in living systems (Stief, 2003). The direct action of H_2O_2 involves an attack on the structure of heme proteins with the release of iron, enzyme inactivation and oxidation of DNA, lipids, -SH groups and keto-acids (Kohen and Nyska, 2002).

Singlet oxygen is very reactive because the “spin restriction” is eliminated, allowing the species to react as an electrophilic oxidant (Turrens, 2003) thereby making it a potential aggressor when it is produced inside the cell (Petrou et al., 2017). This is indicated especially by its ability to damage DNA, components of guanine and nucleic acids, leading to toxic and mutagenic effects and tissue damage (Agnez-Lima et al., 2012). It is also involved in the oxidation of cholesterol (Altenhofer et al., 2015) and proteins with high electron density amino acid residues, such as cysteine, methionine, tryptophan, tyrosineand histidine (Davies, 2005). Singlet oxygen can also play a direct role in generating cell signals to modify gene expression (Agnez-Lima et al.,2012) and can be used to fight cancer cells and various pathogens such as microbes and viruses (Stief, 2003).

Ozone was formed from O_2 by the action of high energy electromagnetic radiation and electrical discharges (Malik et al., 2016). It is slightly less reactive than hydroxyl radical and a much stronger oxidizing agent than oxygen (Altenhofer et al., 2015). It can form free radicals by oxidizing biological molecules and causes oxidative damage to lipids (Goldstein et al., 1969), proteins and nucleic acids (Sharma and Graham, 2010). Ozone also plays an important role in inflammatory processes (Lerner and Eschenmoser, 2003).

Hypochlorous acid (HOCl) is a highly reactive species involved in oxidation reactions and chlorination of the protein and lipid components. It is generated by hydrogen peroxide and the chloride anion in a reaction catalyzed by myeloperoxidase in macrophages and neutrophils at sites of inflammation. It can oxidize thiols and other biological molecules, including ascorbate, urate, pyridine nucleotides and tryptophan (Olszewski and McCully, 1993; Winterbourn et al., 2000). HOCl chlorinate compounds such as amines to chloramines, residues of tyrosyl to ring chlorinated products, cholesterol and unsaturated lipids to chlorohydrins and may also chlorinate DNA (Prutz, 1996; Andres et al., 2022).

Carbonate radical anion ($CO3^{\cdot-}$) may be produced by the radiolysis of aqueous solutions of bicarbonate/carbonate (Chen et al., 1973). It can also be formed when -OH

reacts with carbonate or bicarbonate ions. Bicarbonate levels are high (25mM) in blood plasma, facilitating the reaction (Meli et al., 2002). Although carbonate radical anion is not as strong an oxidizing agent as the hydroxyl radical, it is a strong one-electron oxidant that acts by electron transfer and hydrogen abstraction (Augusto, and Miyamoto, 2011). It has a much longer half-life than hydroxyl radical and can therefore spread further and oxidatively modify distant cell targets. A wide variety of biomolecules can be oxidized by carbonate radical anion. Regarded as a major oxidant of proteins and nucleic acids, it oxidizes DNA guanine bases by a one-electron transfer process that leads to the formation of stable guanine oxidation products (Hoffman et al., 2003). The carbonate radical anion has been proposed as a key mediator of oxidative damage derived from peroxynitrite production (Augusto and Miyamoto, 2011; Radi, 2004), xanthine oxidase turnover and superoxide dismutase activity (Liochev and Fridovich, 2004). It is known to play an important role in the modification of selective amino acids in proteins under conditions of oxidative stress, aging and inflammation (Stadtman, 2000). The kinetics of tyrosine nitration in the presence of CO_2 suggests a specific role of carbonate radical anion in Mn-SOD nitration by peroxynitrite (Squadrito and Pryor, 1998; Piacenza et al., 2022). The nitration of tyrosine has been observed in neurodegenerative conditions, cardiovascular disorders and diabetes (Lu et al., 2010; Li et al., 2021).

Nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$) and peroxynitrite ($ONOO^-$), as well as non-radicals such as nitrous acid HNO_2 and N_2O_4 (dinitrogen tetroxide), are included in the combined term reactive nitrogen species (RNS). Nitric oxide or nitrogen monoxide ($NO^{\cdot}$) is a free radical with a single unpaired electron.The chemical reactivity of $NO^{\cdot}$ is rather limited, and therefore its direct toxicity is less than that of ROS. However, it reacts with $O_2^{\cdot-}$, producing peroxynitrite anion ($ONOO^-$), a very destructive species for proteins, lipids and DNA (Douki and Cadet, 1996; Engwa et al., 2022). Nitric oxide also reacts with molecular oxygen and nitrogen to form nitrogen dioxide or dinitrogen trioxide, both toxic oxidizing and nitrosating agents (Radi, 2013). Nitric oxide is generated in biological tissues by specific nitric oxide synthases (Ghafourifar and Cadenas, 2005), through the

Nitrogen dioxide Unlike nitrous oxide (N_2O), nitrogen dioxide ($NO_2^{\cdot}$) can be considered a free radical because the electrons are not paired. It is formed by the reaction of the peroxyl radical and NO in polluted air and smoke (Noguchi et al., 1999). Nitrogen dioxide is a moderately strong oxidant, with reactivity between those of $NO^{\cdot}$ and $ONOO^-$ ·Nitogen dioxide radicals reacts with organic molecules at rates ranging from ~104 to 106 M/s, depending on pH. Two nitrogen dioxideradicals can be dimerized to the highly reactive dinitrogen tetroxide (N_2O_4). Nitrogen dioxide can affect antioxidant mechanisms, causing the oxidation of ascorbic acid, which leads to lipid peroxidation and free radical production (Papas, 1999).

Peroxynitrite ($ONOO^-$) Peroxynitrite ($ONOO^-$) is formed by the reaction of nitric oxide and superoxide anion. It is highly toxic and can react directly with CO_2 to form other highly reactive nitrosoperoxo-carboxylates ($ONOOCO2^-$) or peroxynitrous acid (ONOOH), which may undergo further homolysis to form $^{\cdot}OH$ and $NO_2^{\cdot}$ or rearrange to form NO_3 (Beckman and Koppenol, 1996). Peroxynitrite diffuses readily across cell membranes (Knight, 2000); and therefore can oxidize lipids, methionine residues andtyrosine in proteins and DNA to nitroguanine (Kohen and Nyska, 2002; Douki et al., 1996). It acts as an oxidant in a similar wayto the hydroxyl radical. Nitrotyrosine residues are considered markers of cell damage induced by peroxynitrite and have been associated with tissues aging (Kohen and Nyska, 2002). Peroxynitrite causes tissue injury and oxidizes low-density lipoprotein (LDL); it seems to be generated at sites of inflammation (Papas, 1999).

Role of the Enzyme System in the Formation of Free Radicals

A wide range of oxidative enzymes that are existing in cells can generate free radicals. The prime catalyzing ROS generation include nitric oxide synthases, NADPH oxidase, prostaglandin synthase, xanthine oxidase, lipoxygenases, ribonucleotide reductase, glucose oxidase, myeloperoxidase, cyclooxygenases and cytochrome P450 (Davi et al.,2005; Bonnefont-Rousselot, 2002).In particular, lipoxygenase generates free radicals; it can convert poly unsaturated fatty acids (PUFA) which are present in the cell membrane to hydroperoxides once Fe^{2+} has been oxidized to Fe^{3+}.They can oxidize arachidonic acid, abundantly present in the central nerv ous system, to hydroperoxy eicosatetraenoic acid. Above all, 15-lipoxygenase has been identified in atherosclerotic lesions, evidencing that the enzyme may be involved in the formation of oxidized lipids *in vivo* (Knight, 1999).

High ROS levels are also generated by immune cells (lymphocytes, granulocytes and phagocytes) which defend the body against invading microorganisms (Rosen et al., 1995). Macrophages and neutrophils contain NADPH oxidase complex, which, when activated, generates superoxide radicals and hydrogen peroxide. The latter then interacts with intracellular chloride ions to produce hypochlorite, which destroys the pathogen (Kohchi et al., 2009). The main enzyme expressed by neutrophils is myeloperoxidase. With heme as a cofactor, it produces hypochlorous acid from hydrogen peroxide and chloride anion (Klebanoff, 2005). It also oxidizes tyrosine to the tyrosine radical. Hypochlorous acid and the tyrosine radical are both cytotoxic and are used by neutrophils to kill pathogenic organisms (Heinecke et al., 1993). Cytochrome P450 molecules use O_2 in their biochemical reactions and generate small amounts of ROS. The amount of ROS produced varies depending on the compound degraded and the cytochrome P450 molecule involved. A molecule particularly active in the production of ROS is cytochrome P450 2E1 (Lieber, 1997).

Role of Metals in the generation of free radicals

The excessive generation of free radicals through reactions mediated by transition metals is well documented (Halliwell, 1996; Min and Ahn, 2005). Nearly all transition metal ions have the aptitude to function in various oxidation states. In the active redox state, these ions may act as catalysts in the autoxidation of many biomolecules. In most cases, the oxidation of biomolecules is initiated by the hydroxyl radical generated in Fenton and Fenton-like reactions between redox active transition metal ions and hydrogen peroxide (Bokare and Choi, 2014). In biological systems, a two-step reaction may occur in the presence of metal ions, especially free iron, leading to the production of hydroxyl radicals. Hydrogen peroxide can produce the hydroxyl radical by removing an electron from the participating metal ion (Halliwell and Gutteridge, 1985). In the second step, the superoxide radical is implicated in regenerating the original metal ions, making them newly available for the reaction with hydrogen peroxide. The two chemical reactions support the role of metals such as iron and copper in creating oxidative stress and cell injury by ROS. The ferrous ion (Fe^{2+}) is a stronger pro-oxidant than the ferric ion (Fe^{3+}) (Halliwell and Gutteridge, 1985). Because of the elemental input of iron to the generation of highly toxic hydroxyl radicals, any increase in cell concentration of free iron promotes the generation of ROS and oxidative stress (Tsukamoto and Lu, 2001)

Beneficial Effects of free radicals

Free radicals play a dual role as both toxic and beneficial compounds. The delicate balance between their two antagonistic effects is clearly an important characteristic feature of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cell structures (Young and Woodside, 2001; Valko et al., 2007). Free radicals, when present in judicious levels, are imperative for good health, acting as essential components in immune defense, cellular signaling, and homeostasis. They function as messengers that regulate vascular tone,

assist in destroying pathogens via phagocytes, and facilitate critical physiological processes like hormone production in the thyroid gland.

Immune System Defense Immune cells, such as macrophages, monocytes and neutrophils, generate free radicals to destroy invading bacteria, viruses, and other pathogenic microorganisms, protecting the body from infections (Droge, 2002; Young and Woodside, 2001).

Cellular Signaling Free radicals act as signaling molecules that regulate vital processes, including cell growth, differentiation, and the removal of damaged cells through programmed cell death ie apoptosis (Genestra,2007; Halliwell, 2007;Pacher et al.,2007).

Regulation of Blood Vessel Function The free radical nitric oxide is crucial for controlling blood flow by dilating blood vessels to prevent cardiac dysfunction and serving as a chemical messenger in the nervous system (Bahorun et al., 2006:Pacher et al., 2007).

Hormone Production Hydrogen peroxide, a free radical produced by the thyroid gland, is essential for synthesizing thyroid hormone.

Mitogenic Response Free radicals are known to play an essential role in promoting the division and proliferation of cells, which is vital for tissue repair and regeneration (Genestra, 2007).

Role of Free Radicals in the Etiology of Diseases

The inferences of free radical processes distress the fields of biology and medicine. Free radicals hoarded due to an imbalance between antioxidants and oxidants that damage macromolecules, such as nucleic acids, proteins and lipids, causing abnormal gene expression, disturbance of receptor activity, cell proliferation, immune perturbation, mutagenesis, tissue damage and various disease conditions (Valko et al.,2007;Kehrer and Klotz,2015). Numerous clinical disorders have been associated to excessive generation free radicals, including diabetes mellitus (Bashan et al.,2009), inflammatory diseases (Li et al.,2013), neurodegenerative diseases (Alzheimer's (Pan et al.,2011), Parkinson's (Sevcsik et al.,2011) and Huntington's disease (Ha and Fung, 2012), lateral amyotrophic (Zhao et al.,2011) and multiple sclerosis (Witherick et al.,2011), cancer (colorectal (Jemal et al.,2011), breast (Brown et al.,2000), prostate (Lim et al.,2005) and lung (Azad,2008), cardiovascular disease (Zhang et al.,2010) (atherosclerosis (Yin et al.,2013) and hypertension (WHO,2013), cataract (Costagliola et al.,1988), rheumatoid arthritis (Vasanthi et al.,2009), asthma (Fujisawa, 2005) and aging (Krisko and Radman,2019). Above all, free radical damage to the plasma membrane occurs primarily through lipid peroxidation, where reactive oxygen species (ROS) attack polyunsaturated fatty acids, causing a chain reaction that breaks down lipid structure. This damage disrupts membrane integrity, increasing rigidity and permeability while damaging structural proteins, which can lead to cellular dysfunction, inflammation, and eventual cell death through necrosis or apoptosis (Hauck and Bernlohr, 2016).

Cardiovascular Disease and Oxidative Stress

During the last years, research data evidencing that oxidative stress should be considered either a primary or secondary cause of many cardiovascular diseases (CVDs) (Dubois-Deruy et al., 2020). Oxidative stress acts mainly as a trigger of atherosclerosis. It is well established that atheromatous plaque formation results from an early endothelial inflammation, which in turn leads to ROS generation by macrophages recruited *in situ.*

Circulating LDL is subsequently oxidized by reactive oxygen species, thus leading to foam cell formation and lipid accumulation. Both in vivo and ex vivo studies provided evidences supporting the role of oxidative stress in atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy, and congestive heart failure (Harrison et al., 2003). Further in vivo and ex vivo studies have provided precious

evidence supporting the role of oxidative stress in a number of CVDs such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure (Bahorun et al., 2006; Ceriello, 2008)

Neurological Disease and Oxidative Stress

Oxidative stress has been linked to the initiation, progression and on-set of several neurological diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, depression, and memory loss.(Halliwell, 2001; Butterfield, 2002; Singh et al.,2004) Beta-amyloid, a toxic peptide often found to be present in the brain tissue of neurological diseases, is produced by free radical action (Christen, 2000; Halliwell, 2001; Butterfield, 2002)

Respiratory Diseases and Oxidative Stress

Several reports are available in the literature evidencing that lung disease such as asthma and chronic obstructive pulmonary disease (COPD), determined by syetemic and local chronic inflammation, are associated to increased oxidative stress (MacNee, 2001; Caramori and Papi, 2004; Guo and Ward, 2007; Hoshino and Mishima, 2008). Oxidants are known to increase inflammation via the activation of several kinases involving pathways and transcript factors such NF-Kappa B and AP-1 (MacNee, 2001; Hoshino and Mishima ,2008).

Rheumatoid Arthritis and Oxidative Stress

Rheumatoid Arthritis (RA) is characterized by macrophages and T cell infiltration (Mahajan and Tandon, 2004; Walston et al., 2006; Valko et al., 2007). Free radicals at the site of inflammation play a relevant role in both initiation and progression of this syndrome, as demonstrated by the enhanced isoprostane and prostaglandin levels in synovial fluid of affected individuals (Mahajan and Tandon, 2004).

Renal Disorders and Oxidative Stress

Oxidative stress has been implicated in the damage of renal apparatus such as glomerulo- and tubule –interstitial nephritis, renal failure, proteinuria, and uremia (Chatterjee et al., 2007; Galle, 2001). Renal tissues are negatively affected by oxidative stress mainly because of the fact that ROS generation induces the recruitment of inflammatory cells and proinflammatory cytokine production, leading to an initial inflammatory stage.

Sexual Maturation and Oxidative Stress

Several authors pointed out that oxidative stress could be primarily responsible for a delayed sexual maturation and puberty onset (Soriano Guillén et al., 2022; Koksal et al., 2023). It is hypothecated that when children in prepubertal age are exposed to cadmium, a well-known responsible for an increase in free radical generation and increased oxidative stress as well as pregnant women are exposed to cadmium.

Cancer and Oxidative Stress

Cancer cells have a hyper metabolism that produces a large amount of ROS compared to normal cells. It has been known that ROS causes DNA strand breaks and oxidative damage to the nucleotides, resulting in mutagenesis and eventually cancer. The susceptible target of ROS in DNA is guanine that causes G→T transvers (Du et al., 1994). In addition, ROS can also cause mutations by oxidative damage to a range of target sites in genetic materials including purines and pyrimidines, alkali labile sites, single strand breaks and disruption of DNA repair processes, leading to genetic instability (Domínguez et al., 1992; Wang et al., 1998). ROS-induced carcinogenesis leads to modification of nucleobases in cancerous and precancerous tissues (Olinski et al., 1998). The initiation of

cancer in humans caused by ROS is evidenced by the presence of oxidative DNA modifications in cancer tissues (Poulsen et al., 1998). Oxidative DNA damage leading to the development of breast cancer has also been reported (Malins and Haimanot, 1991). The role of oxidative stress in the development of hepatocellular carcinoma has also been reported, since ROS caused accumulation of 8-OHdG by oxidative DNA damage in the cells leads to the onset of hepatocellular carcinoma (Li et al., 2023). The association of oxidative DNA damage and carcinogenesis has been found in a variety of other cancers. However, a comparative measurement of distinctively modified DNA bases in tumor tissue and their respective normal tissues is required to provide further insights into the involvement of ROS in carcinogenesis. The highly significant correlation between consumption of fats and death rates from leukemia and breast, ovary, rectum cancers among elderly people may be a reflection of greater lipid peroxidation (Droge, 2002; Genestra, 2007).

Diabetes and Oxidative Stress

Free radicals are key drivers in the development and progression of diabetes mellitus and its complications. Chronic high blood sugar (hyperglycemia) increases ROS production, leading to oxidative stress, which damages pancreatic βcells, reduces insulin secretion, and impairs insulin action. Increase in the levels of oxygen and nitrogen free radicals (ROS/RNS) has been linked with lipid peroxidation, non-enzymatic glycation of proteins and oxidation of glucose which contributes toward diabetes mellitus and its complications. Oxidative damage leads to chronic diabetic complications like retinopathy, nephropathy, and neuropathy. Most of the studies have shown relationship between oxidative stress and diabetes along with their complications related to heart, liver kidney and eye. It concludes that metabolic oxidation is the strongest factor behind insulin dependent and noninsulin dependent diabetes mellitus. Chronic exposure to hyperglycaemia can lead to cellular dysfunction which may become irreversible over time by a process called glucose toxicity (Robertson et al., 2003). Multiple biochemical pathways and mechanisms of action for glucose toxicity include glucose autoxidation, protein kinase C activation, methyl-glyoxal formation and glycation, hexosamine metabolism, sorbitol formation, and oxidative phosphorylation. There are many potential mechanisms whereby excess glucose metabolites travelling along these pathways might cause beta cell damage. However, all of these pathways have in common the formation of ROS that, in excess and over time, cause chronic oxidative stress, which in turn can result in defective insulin gene expression and insulin secretion as well as increased apoptosis (Robertson, 2004).

Role of Antioxidants in health maintenance

To counteract the harmful effects of ROS, endogenous antioxidants of the body or exogenous antioxidants neutralize them and maintain bodily homeostasis. The imbalance between the cellular antioxidant system and ROS production results in oxidative stress, which subsequently results in the development of several diseases. The implication of oxidative stress in the etiology of several chronic and degenerative diseases suggests that antioxidant therapy represents a promising avenue for treatment. In the future, a therapeutic strategy to increase the antioxidant capacity of cells may be used to fortify the long term effective treatment. The body has numerous mechanisms to thwart oxidative stress by producing antioxidants, either naturally generated *in situ* (endogenous antioxidants), or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention. When an antioxidant destroys a free radical, this antioxidant itself becomes oxidized. Therefore, the antioxidant resources must be persistently restored in the body. Thus, while in one particular system an antioxidant is effective against free radicals; in other systems the same antioxidant could become ineffective. Also, in certain conditions, an antioxidant may even act as a pro-oxidant e.g. it can generate toxic ROS/RNS. The antioxidant process can function in one

of two ways: chain-breaking or prevention. For the chain-breaking, when a radical releases or steals an electron, a second radical is formed. The last one exerts the same action on another molecule and continues until either the free radical formed is stabilized by a chain-breaking antioxidant (vitamin C, E, carotenoids, etc), or it simply disintegrates into an inoffensive product.

Endogenous antioxidants in cells can be classified as enzymatic and non-enzymatic antioxidants. The major enzymatic antioxidants unswervingly involved in the lysis or neutralization of ROS and RNS are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) (Stanger and Wonisch, 2011; Bandodkar et al., 2025). SOD, the first line of enzymatic antioxidant defense against toxic free radicals, catalyzes the dismutation of superoxide anion radical ($O2^{\bullet}$) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by the enzyme catalase (CAT) or glutathione peroxidase (GPx). The selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG, with NADPH as a source of reducing power. Besides hydrogen peroxide, GPx also reduces lipid or nonlipid hydroperoxides while oxidizing glutathione (GSH) (Young and Woodside, 2001; Halliwell, 2026).

The non-enzymatic antioxidants are also classified into metabolic antioxidants and nutrient antioxidants. Metabolic antioxidants belonging to endogenous antioxidants are produced by metabolism in the body, such as lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc (Droge, 2002;Willcox et al.,2004). While nutrient antioxidants belonging to exogenous antioxidants, are compounds which cannot be produced in the body and must be provided through foods or supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, zinc), flavonoids, omega-3 and omega-6 fatty acids, etc.

Oxidative stress markers are quantified by measuring lipid peroxidation (MDA, TBARS), protein oxidation (Protein carbonyls, AOPPs), DNA damage (urinary 8-hydroxydeoxyguanosine) and antioxidant enzyme activity (SOD, GPx, CAT). These biomarkers are commonly quantified in blood, plasma, urine, or saliva using ELISA, Mass spectrometry, or Colorimetric, Spectrophotometric, HPLC, Gas chromatography-Mass Spectroscopy (GC-MS), Fluorescent Probes techniques to assess disease progression and therapeutic efficacy. Antioxidant assay is defined as a analytical methods used to measure the capacity of antioxidants to limit oxidative damage, by neutralizing the free radicals, delaying or preventing oxidative damage which is linked to the initiation and progression of various aging-related diseases.

Common techniques include spectrophotometric methods such as DPPH (2, 2-diphenyl-1-picrylhydrazyl) Assay, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) Assay,FRAP (Ferric Reducing Antioxidant Power) Assay and ORAC (Oxygen Radical Absorbance Capacity) assay that measure color changes, or methods based on hydrogen atom transfer (HAT) and electron transfer (ET).

Metformin (1, 1-dimethyl biguanide hydrochloride) gained the status of “foundation therapy” in T2D patients whose glycemic target is not achieved despite diet and other lifestyle interventions (Clarke and Duncan, 1968; Jacobs and Ellis, 2024). The reason behind this glory is its effective glycemic control, weight neutrality, wide security margin, and low cost (Rojas and Gomes, 2013). Metformin has been the cornerstone of therapy in the management of T2D. With the advent of newer molecules as oral antihyperglycaemic therapies, we do now have a varied therapeutic option; yet, metformin with pleiotropic effects still seems to have an upper hand over other antidiabetic drugs. The insulin-sparing effect of metformin makes it unique along with its diverse intracellular mechanisms. The drug has been found beneficial in a myriad of disorders

including diabetes, obesity, cancers, cardiovascular conditions, neurological conditions, and many more but the majority of the evidence is based on the in vitro and in vivo studies with a supra-therapeutic concentration of the drug. Metformin is an oral antihyperglycemic agent that lowers both basal and post-prandial plasma glucose in T2D. In addition to increasing insulin sensitivity, it acts by decreasing hepatic glucose synthesis and intestinal glucose absorption. It differs from other groups of oral hypoglycemic agents as it does not result in hypoglycemia or hyperinsulinemia.

Metformin alone and in combination with other "glucose-lowering" agents reduces the blood glucose level effectively in T2D (Viollet et al., 2012; McCreight et al., 2016; Lv and Guo, 2020). It was found that combinations of metformin with all noninsulin diabetic drugs result in a similar reduction of HbA1c but with changing weight gain degrees and hypoglycemia risk. In addition to glucose control, metformin is also helpful in diabetes-related co-morbidities. Metformin exhibits significant antioxidant properties beyond its glycemic control, acting as a scavenger of reactive oxygen species (ROS) and activating endogenous antioxidant defenses like superoxide dismutase (SOD) and catalase. It reduces oxidative stress-induced damage in chronic diseases, improves vascular health, and may contribute to its anti-aging and anti-inflammatory effects (Angelika Buczynska et al., 2024).

Resveratrol (RE; (3, 4', 5 trihydoxystilbene)) is a stilbenoid natural polyphenol. Resveratrol was first isolated in 1939 by Takaoka from *Veratrum grandiflorum* (Takaoka, 1939, Hong et al., 2025). Resveratrol is found in over 70 plant species but is highly concentrated in the skin of red grapes. Tea, berries, pomegranates, nuts, blueberries, and dark chocolate are also reported to contain resveratrol at varying concentrations. Resveratrol exists as two isomeric forms (*cis* and *trans*), yet the trans form is the predominant form and it has the most potent therapeutic benefits owing to the lower steric hindrance of its side chains (Cardile et al.,2007;Weiskirchen et al.,2016). Resveratrol was reported to exhibit a plethora of therapeutic benefits, including anti-inflammatory, antioxidant, anti-platelet, anti-hyperlipidemic, immuno-modulator, anti-carcinogenic, cardioprotective, vasorelaxant, and neuroprotective effects (Bejenaru et al., 2024; Baur and Sinclair, 2006). Indeed, resveratrol was reported to be able to maintain or enhance human cerebrovascular functions (Wong et al.,2016), modulate *in vitro* angiogenesis through the expression of vascular endothelial growth factor (VEGF) and the formation of new vascular networks (Wang et al., 2014), stimulate human immune cell functions (Falchetti et al.,2001), promote rat cell viability and proliferation (Ortega et al.,2012), ameliorate mitochondrial respiratory dysfunction, and enhance cellular reprogramming in human fibroblasts derived from patients with a mitochondrial disease (Mizuguchi et al.,2017) a phenomenon potentially mediated by the activation of Sirtuins (Sergi et al.,2019).

Resveratrol has also showed proven cardioprotective (Petrovski et al., 2011, Li et al.,2012), hepatoprotective (Sadi et al., 2015) and neuroprotective activities (Regitz et al.,2016). In particular, this polyphenol seems to alleviate the main risk factors of cardiovascular diseases (CVD) as it can improve endothelial function, scavenge reactive oxygen species (ROS), reduce inflammation, inhibit platelet aggregation, and ameliorate the lipid profile and other main factors that can promote atherosclerosis (Akaberi and Hosseinzadeh, 2016, Magyar et al.,2012). Furthermore, redox-associated mechanisms were implicated as potential pathways via which Resveratrol elicits its cardioprotective effects. These redox-associated mechanisms include preservation of mitochondrial function under hypoxia/reoxygenation-induced oxidative stress (Zhang et al., 2018), upregulation of antioxidant enzymes such as peroxidase and superoxide dismutase (SOD) (Mokni et al., 2013), and modulation of nitric oxide (NO) production (Bradamante et al., 2003). Earlier, we have reported the anti-diabetic, anti-oxidant properties of resveratrol in streptozotocin-nicotinamide induced experimental diabetes in rats (Palsamy and

Subramanian, 2008, Palsamy and Subramanian, 2009; Palsamy and Subramanian, 2010; Palsamy et al.,2010; Palsamy and Subramanian,2011). We have also reported the *in vivo* glucose uptake activity of resveratrol by upregulating the expressions of GLUT4, PI3 kinase and PPAR-gamma in L6 myotubes (Subramanian and Prasath, 2011). Recently, we have synthesised a new metformin-resveratrol aldehyde ligand and evaluated its glucose uptake efficacy using rat L6 muscle cell lines (Subramanian Iyyam Pillai et al., 2011). The *in vitro* molecular docking studies on the effect of the complex against type 2 diabetic target proteins are also reported. More recently we have reported the antidiabetic efficacy of the newly synthesised complex in High fat diet fed- low dose streptozotocin induced experimental diabetes in rats (Rajitha Rajendran et al., 2025).

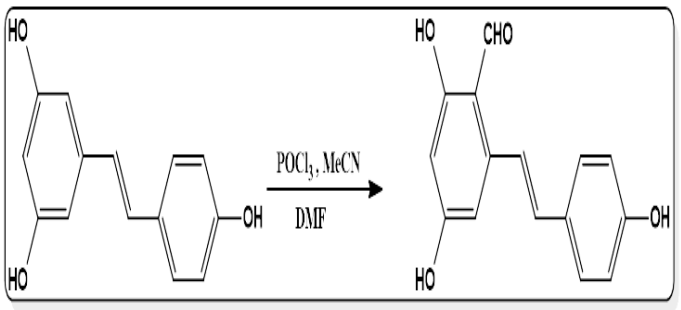
Throughout the years, chemical and synthetic antidiabetic medications have been developed to help repair certain forms of dysfunctions that control the primary and secondary avoid complications (De Souza et al.,2022) Still, their extended use's detrimental effects have diverted many researchers' focus on seeking a new alternative (Mechchate et al., 2020). New studies are now devoted to medicinal plants, natural compounds and synthesized compounds that may have positive effects on diabetes (Chen et al., 2020). In view of the above beneficial and pharmacological aspects of metformin and resveratrol, in the present study an attempt has been to synthesize, characterize a new metformin-resveratrol complex and systematically evaluate its anti-oxidant potential in HFD-Low dose STZ induced experimental diabetes in rats.

2. Materials and Methods

Chemicals such as Resveratrol, Metformin, Streptozotocin, and Insulin were obtained from Sigma Aldrich, USA. Ultra-sensitive ELISA kit for rat insulin assay was purchased from Crystal Chem Inc. Life Technologies, India. All the other chemicals and reagents used were of analytical grade.

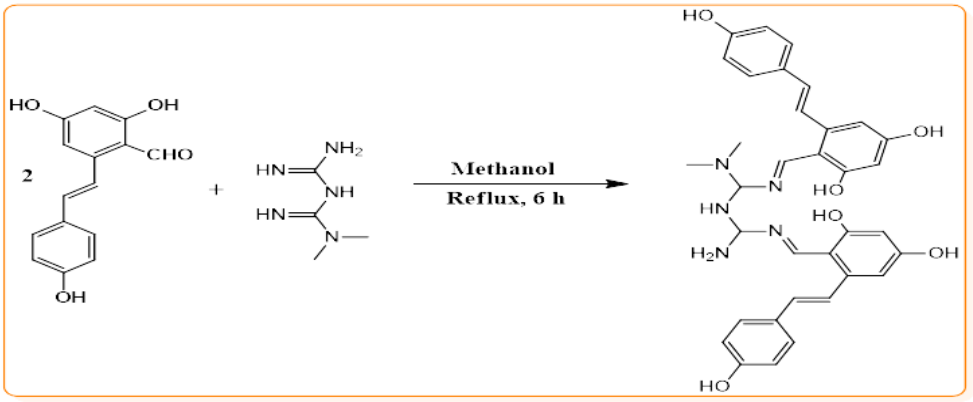
Synthesis of Resveratrol aldehyde

Resveratrol aldehyde (RA) (2, 4-dihydroxy-6-((E)-2- (4- hydroxyphenyl)) benzaldehyde): was synthesized by the methods of (Huang et al., 2008 and Basheer et al., 2015). Resveratrol was treated with Vilsmeier reagent ($POCl_3$, DMF and MeCN) to synthesize Resveratrol aldehyde. Briefly, freshly distilled $POCl_3$ (0.6 ml, 6 mmole) was added drop-wise to a solution of resveratrol (912 mg, 4 mmole) and DMF (464 ml, 6 mmole) in 20 ml of MeCN that was maintained in an ice water bath. The mixture was stirred continuously for 1 hr. at room temperature. Then, the solution was added to a mixture of ice and water, and the yellow mixture was stirred at 40°C in a water bath and extracted with EtOAc (3 × 10 ml and evaporated. The yellow colored crystals of Resveratrol aldehyde were obtained with a yield of 78%. The overall scheme is represented as Scheme 1.



Scheme1: Synthesis of Resveratrol Aldehyde

Synthesis of a mixed ligand from Metformin hydrochloride and Resveratrol Aldehyde (M-RA) Mixed ligand 4-(E)-((amino(E)-(2,4-dihydroxy-6-(4-hydroxyphenethyl)benzylidene) amino) (dimethylamine)methyl) amino) methyl)amino) methyl)-5-(4-hydroxyphenethyl)benzene-1,3- diol was prepared by refluxing the mixture of 1 mmol (0.2 g) of metformin with 2 mmol (0.5 g) of Resveratrol aldehyde in 50 mL methanol for about 6 hours. The pale-yellow colored compound was dried and the yield was 92 mg (72%). The proposed scheme is represented in Scheme 2.



Scheme 2: Synthesis of Metformin- Resveratrol Aldehyde mixed ligand.

The physico-chemical parameters of Resveratrol aldehyde and Metformin - Resveratrol aldehyde mixed ligand was recorded. The resveratrol aldehyde and the mixed ligand were subjected to spectral characterization by FT-IR, 1H NMR, 13C NMR and Mass spectral analysis (Subramanian Iyyam Pillai et al., 2021).

Acute Toxicity and Dosage Fixation Studies

Acute toxicity studies were performed as per OECD guidelines for testing of chemicals in normal rats. Graded doses (2.5 mg, 5 mg, 7.5 mg, and 10 mg/kg b.w./rat/day) of Met-Res-Aldehyde complex dissolved in 5% DMSO were orally administered to rats using gavages. All observations were systematically recorded, with individual records being maintained for each animal. Cage side observations included the evaluation of skin and fur, eyes, respiratory effects, lethargy, autonomic effects such as salivation, diarrhea, and urination, and central nervous system effects including tremors and convulsions, changes in the level of activity, gait, and posture. The changes in food consumption, fluid intake, and body weight were continuously monitored for a period of 30 days. Macroscopic examinations were also performed on vital organs. The suitable optimum dosage of the drug was assessed.

Experimental Animals

Male Albino rats of Wistar weighing around 160 to 180 g were procured from the Tamilnadu Veterinary and Animal Sciences University, Chennai, and were housed in the Biomedical Research Unit and Lab Animal Centre, Saveetha Dental College and Hospitals, Chennai, under standard husbandry conditions (12 ± 1 h light and dark cycle, relative humidity 55% ± 10%). The animals were fed a balanced diet (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. The rat pellet diet is composed of 55% nitrogen-free extract, 21% protein, 5% fat, and 4% fiber (w/w) with sufficient levels of vitamins and minerals. The experimental design was strictly conducted according to the ethical norms approved by the Ministry of Social Justices and Empowerment, Government of India, and Institutional Animal Ethics Committee Guidelines (Approval number - BRULAC/SDCH/SIMATS/IAEC/08- 2022/137) for the examination of experimental pain in conscious animals.

The High Fat Diet (HFD) was prepared indigenously by using a normal pellet diet, raw cholesterol, and a mixture of Vanaspati ghee and pure coconut oil (2:1). Briefly, the normal rat pellet diet was powdered by grinding and mixed with 2.5% cholesterol and a mixture of Vanaspati ghee and coconut oil (5%). The mixture was made into pellet form and orally fed to rats to induce metabolic syndrome (Suman et al., 2016; Manzano et al., 2022). The rats were divided into two dietary regimens by feeding either normal or HFD for the initial period of 2 weeks. After 2 weeks of dietary management to develop insulin resistance, the groups of rats fed with HFD were intraperitoneally injected with a freshly prepared low dose of STZ (35 mg/kg b.w.) dissolved in 0.1M ice-cold citrate buffer, pH 4.5 (Rakitan, 1963; Ghasemi and Jeddi, 2023). Rats having the fasting blood glucose levels ≥300 mg/dL on the 3rd day after STZ injection were considered diabetic and subjected to further studies. The rats were allowed to continue to feed on their respective diets until the end of the experimental period.

Experimental Design:

The rats were allocated into two dietary regimens by feeding either a normal pellet diet (NPD) or a high-fat diet (HFD) for 2 weeks of dietary manipulation. HFD contains powdered NPD, 365 g/kg; lard, 310 g/kg; casein, 250 g/kg; cholesterol, 10 g/kg; vitamin and mineral mix, 60 g/kg; DL-methionine, 3 g/kg; yeast powder, 1 g/kg; and NaCl, 1 g/kg. After 2 weeks of HFD supplementation, Group II, Group III, and Group IV rats were injected with a single dose of STZ (35 mg/kg b.w./rat); control rats (Group I) fed with NPD were injected intraperitoneally with the same volume of freshly prepared cold citrate buffer (pH 4.5, 0.1 mol/L) only (Rakieten,1963). After one week of STZ injection, rats having fasting blood glucose levels ≥ 300 mg/dL were considered diabetic rats and chosen for further studies

The animals were divided into four groups, each comprising six rats as follows:

Group 1: Control.

Group 2: HFD+STZ (i.p. 35 mg/kg b.w.) induced diabetic rats.

Group 3: HFD+STZ induced diabetic rats treated with Metformin Resveratrol Aldehyde Complex (5mg/kg b.w./rat/day) for 30 days.

Group 4: HFD+STZ induced diabetic rats treated with Metformin 500mg/kg b.w./rat/day) for 30 days.

Biochemical Parameters

At the end of the experimental period, overnight fasted rats were anaesthetized, using ketamine (80 mg/kg b.w./rat.) and sacrificed by cervical decapitation.Blood samples were collected with and without anticoagulant for separation of plasma and serum, respectively. The basic biochemical parameters such as fasting blood glucose (Trinder, 1969), glycosylated hemoglobin (Nayak and Pattabiraman,1981), plasma protein (Lowry et al.,1951), blood urea (Natelson et al.,1951), and serum creatinine (Brod and Sirota,1948) levels were estimated. Urine strips were used to detect the presence of glucose in urine. The levels of plasma insulin was assayed by ELISA using a rat insulin assay kit (Linco Research, St. Charles, MO, USA).

The levels of lipid peroxides, hydroperoxides and protein carbonyls were determined in plasma and tissue homogenate (Ohkawa et al., 1979; Jiang and Chen,1992). The activities of enzymatic antioxidants such as SOD, (Misra and Fridovich, 1972) Catalase, (Takahara et al.,1960) GPx,(Rotruck et al.,1973) GST (Habig et al.,1974) were assayed in the pancreatic, hepatic and renal tissue homogenate of control and experimental groups of rats. The levels of non-enzymatic antioxidants, vitamin C (Omaye et al., 1979) vitamin E(Desai, 1984) ceruloplasmin (Ravin,1961)and GSH (Sedlak and Lindsay,1968) were determined.

Statistical Analysis

Statistical Analysis the values are expressed as mean values of six rats in each group ± SEM. Data analysis was done with SPSS software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed with least significance difference (LSD). The value of P <0.05 was considered to indicate statistical significance.

3. RESULTS

The effect of oral administration of Met-Res-Aldehyde complex at a concentration of 5mg/kg/bw/ rat/day for 30 days on the levels of fasting blood glucose, hemoglobulin, glycosylated hemoglobulin (HbA1c) and plasma insulin in experimental type 2 diabetic rats is presented in Table 5. In the diabetic group of rats, the levels of fasting blood glucose and HbA1c were significantly increased with a concomitant decrease in the levels of plasma insulin and hemoglobin. Treatment with Met-Res - Aldehyde complex as well as metformin brought the altered levels of the above biochemical indices to physiological values. The urine sugar which was present in the diabetic group of rats was absent in experimental groups of rats treated with Met-Res - Aldehyde complex.

Table 5. The levels of fasting blood glucose, Hemoglobin, glycosylated hemoglobin (HbA1c), plasma insulin and urine sugar in control and experimental groups of rats.

Groups	Fasting Blood Glcose	Hemoglobin	Glycosylated Hemoglobin	Insulin	Urine sugar
Control	77.5 ± 1.38^{a}	13.51 ± 0.26^{a}	4.58 ± 0.21^{a}	13.14 ± 0.30^{a}	Nil
Diabetic	231.66 ± 6.66^{b}	8.35 ± 0.09^{b}	7.84 ± 0.12^{b}	7.56 ± 0.31^{b}	+++
Diabetic+ Met-Res-Aldehyde complex	124.33 ± 3.01^{c}	11.53 ± 0.34^{c}	5.67 ± 0.15^{c}	11.28 ± 0.33^{c}	Nil
Diabetic + Metformin	112.5 ± 3.81^{c}	12.54 ± 0.27^{ac}	5.16 ± 0.14^{ac}	12.37 ± 0.38^{ac}	Nil

Units are expressed as: mg/dL for blood glucose, g/dL for hemoglobin, % hemoglobin for HbA1c, µU/mL for plasma insulin, +++ indicates more than 2% sugar.

Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 6 depicts the levels of plasma protein, blood urea, serum uric acid and serum creatinine in control and experimental groups of rats. The decreased levels of plasma protein and increased levels of blood urea, serum uric acid and serum creatinine observed

in the diabetic group of rats were reverted to near normalcy after treatment with Met-Res - Aldehyde complex as well as metformin.

Table 6. Effect of Met-Res-Aldehyde complex on the levels of plasma protein, blood urea, serum uric acid and serum creatinine in control and experimental groups of rats.

Groups	Protein	Urea	Uric Acid	Creatinine
Control	8.16 ± 0.26^{a}	25.28 ± 1.79^{a}	4.01 ± 0.15^{a}	0.90 ± 0.13^{a}
Diabetic	5.46 ± 0.17^{b}	47.47 ± 0.67^{b}	8.16 ± 0.07^{b}	2.04 ± 0.16^{b}
Diabetic + Met - Res - Aldehyde complex	7.11 ± 0.29^{c}	26.63 ± 0.47^{a}	5.26 ± 0.18^{c}	1.25 ± 0.19^{a}
Diabetic + Metformin	7.82 ± 0.06^{ac}	25.10 ± 0.41^{a}	4.97 ± 0.20^{c}	1.02 ± 0.27^{a}

Units are expressed as: g/dL for plasma protein, mg/dL for blood urea, serum uric acid and serum creatinine. Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 7. The levels of TBARS in the plasma, pancreas and liver of control as well as experimental group of rats are presented in Table 7. STZ induced diabetic rats showed marked increase in the levels of TBARS when compared to control rats. Treatment of Met-Res - Aldehyde complex to diabetic rats showed a significant decrease in the levels of TBARS.

Table 7. Effect of Met-Res-Aldehyde complex treatment on the levels of TBARS in plasma, pancreas and liver of experimental groups of rats.

Groups	TBARS - Plasma	TBARS - Pancreas	TBARS - Liver
Control	4.68 ± 0.86^{a}	40.56 ± 1.79^{a}	1.67 ± 0.13^{a}
Diabetic	8.35 ± 0.09^{b}	78.33 ± 1.25^{b}	4.43 ± 0.16^{b}
Diabetic+Met-Res-Aldehyde complex.	5.38 ± 0.21^{a}	58.43 ± 1.86^{c}	2.51 ± 0.01^{c}
Diabetic + Metformin	5.04 ± 0.20^{a}	55.11 ± 0.43^{c}	2.76 ± 0.04^{c}

Units are expressed as: nmol/mL in plasma. nmol/g of tissues.Values are given as mean ± SD for groups of six rats in each.Means with different superscript letters within the same column differ significantly at $p < 0.05$.

The significantly increased levels of lipid peroxides, hydroperoxides and protein carbonyls in plasma, pancreatic, hepatic and kidney tissues of HFD-STZ diabetic rats were declined near normal values by the treatment of Metformin Resveratrol Aldehyde Complex as well as metformin to diabetic groups of rats (Tables 8,9,10,11) respectively.

Table 8. Effect of oral treatment on the levels of lipid peroxides, hydroperoxides and protein carbonyls in plasma of experimental groups of rats after 30 days of experimental period.

Groups	Lipid peroxides	Hydroperoxides	Protein carbonyls
Control	3.65 ± 0.39^{a}	9.49 ± 0.10^{a}	6.49 ± 0.10^{a}
Diabetic	10.02 ± 1.15^{b}	27.69 ± 0.13^{b}	26.69 ± 0.13^{b}
Diabetic + Met-Res - Aldehyde complex	4.68 ± 0.16^{a}	17.58 ± 0.10^{c}	14.85 ± 0.05^{c}
Diabetic + Metformin	4.42 ± 0.13^{a}	15.85 ± 0.05^{d}	14.58 ± 0.10^{c}

Units are expressed as: nmol/mL for lipid peroxides and hydroperoxides; nmol/mg of protein for protein carbonyls. Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 9. Effects of Met-Res - Aldehyde complex on the levels of lipid peroxides, hydroperoxides, and protein carbonyls in pancreatic tissues of experimental groups of rats.

Groups	Lipid peroxides	Hydroperoxides	Protein carbonyls
Control	37.73 ± 0.59^{a}	14.13 ± 0.52^{a}	5.13 ± 0.21^{a}
Diabetic	65.91 ± 1.06^{b}	32.26 ± 0.40^{b}	20.26 ± 0.24^{b}
Diabetic+Met-Res - Aldehyde complex	40.43 ± 0.47^{a}	17.93 ± 0.42^{c}	11.1 ± 0.27^{c}
Diabetic + Metformin	38.08 ± 0.55^{a}	15.2 ± 0.61^{a}	10.53 ± 0.92^{c}

Units are expressed as: nmol/g of wet tissue for lipid peroxides and hydroperoxides; nmol/mg of protein for protein carbonyls. Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table10. Effect of Met-Res - Aldehyde complex treatment on the levels of lipid peroxides, hydroperoxides and protein carbonyls in hepatic tissues of experimental groups of rats.

Groups	Lipid peroxides	Hydroperoxides	Protein carbonyls
Control	1.12 ± 0.08^{a}	47.66 ± 0.99^{a}	4.21 ± 0.14^{a}
Diabetic	4.3 ± 0.53^{b}	129.26 ± 3.87^{b}	14.35 ± 0.54^{b}
Diabetic+Met-Res - Aldehyde complex	2.23 ± 0.22^{ac}	82.51 ± 3.49^{c}	7.15 ± 0.18^{c}
Diabetic + Metformin	2.88 ± 0.30^{c}	71.15 ± 1.83^{d}	7.55 ± 0.30^{c}

Units are expressed as: nmol/g of wet tissue for lipid peroxides and hydroperoxides; nmol/mg of protein for protein carbonyls. Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 11. Effect of oral treatment with Met- Res - Aldehyde complex on the levels of lipid peroxides, hydroperoxides and protein carbonyls in renal tissues of experimental groups of rats.

Groups	Lipid peroxides	Hydroperoxides	Protein carbonyls
Control	1.17 ± 0.14^{a}	50.67 ± 0.71^{a}	3.32 ± 0.16^{a}
Diabetic	3.28 ± 0.17^{b}	84.4 ± 1.25^{b}	17.21 ± 0.54^{b}

Diabetic+ Met-Res-Aldehyde complex	2.29 ± 0.15^{c}	58.9 ± 3.88^{ac}	9.19 ± 0.76^{c}
Diabetic + Metformin	1.81 ± 0.03^{c}	60.81 ± 1.03^{c}	7.33 ± 0.37^{c}

Units are expressed as: nmol/g of wet tissue for lipid peroxides and hydroperoxides; nmol/mg of protein for protein carbonyls. Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Tables: 12,13,14 depict the activities of enzymatic antioxidants such as SOD, catalase, Gpx and GST in pancreatic, hepatic and renal tissues of control and experimental groups of rats. The diminished activities of the above enzymes in all the three tissues of the diabetic group of rats were improved after oral treatment with Met-Res-Aldehyde complex. The observed decrease in the levels of plasma non-enzymatic antioxidants such as vitamin C, vitamin E and ceruloplasmin (Table 15) and pancreatic, hepatic and renal GSH (Table 16) in the experimental diabetic group of rats were improved to the physiological range after treatment with Met-Res-Aldehyde complex as well as metformin.

Table 12. Effect of oral treatment with the Met-Res-Aldehyde complex on the activities of Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx) and Glutathione-S-transferase (GST) in pancreatic tissues of control and experimental groups of rats.

Groups	SOD	Catalase	GPx	GST
Control	5.36 ± 0.16^{a}	25.16 ± 0.06^{a}	6.86 ± 0.01^{a}	5.86 ± 0.01^{a}
Diabetic	3.32 ± 0.06^{b}	6.50 ± 0.07^{b}	3.32 ± 0.16^{b}	2.20 ± 0.14^{b}
Diabetic+Met-Res-Aldehyde complex	4.39 ± 0.05^{c}	13.28 ± 0.03^{c}	5.41 ± 0.14^{c}	4.5 ± 0.12^{c}
Diabetic + Metformin	4.96 ± 0.16^{a}	15.76 ± 0.05^{d}	6.09 ± 0.04^{d}	4.90 ± 0.01^{d}

Activities of enzymes are expressed as: 50% of inhibition of epinephrine autoxidation/min for SOD; µmol of hydrogen peroxide decomposed/min/mg of protein for catalase; µmol of glutathione oxidized/min/mg of protein for GPx; U/mg of protein for GST.

Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 13. Effect of newly synthesized Met-Res-Aldehyde complex on the activities of Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx), Glutathione-S-transferase (GST) and GR in hepatic tissues of experimental groups of rats.

Groups	SOD	Catalase	GPx	GST	GR
Control	11.61 ± 0.20^{a}	81.16 ± 0.18^{a}	10.61 ± 0.10^{a}	8.48 ± 0.09^{a}	29.36 ± 0.47^{a}

Diabetic	4.43 ± 0.16^{b}	38.50 ± 0.15^{b}	4.37 ± 0.10^{b}	3.45 ± 0.13^{b}	13.03 ± 0.43^{b}
Diabetic+Met-Res -Aldehyde complex	7.64 ± 0.07^{c}	$67.4\ 9 \pm 0.13^{c}$	7.34 ± 0.07^{c}	6.27 ± 0.03^{c}	22.31 ± 0.57^{c}
Diabetic + Metformin	7.26 ± 0.07^{c}	70.62 ± 0.11^{d}	8.42 ± 0.10^{d}	7.37 ± 0.08^{d}	24.32 ± 0.50^{d}

Activities of enzymes are expressed as: 50% of inhibition of epinephrine autoxidation/min for SOD; µmol of hydrogen peroxide decomposed/min/mg of protein for catalase; µmol of glutathione oxidized/min/mg of protein for GPx; U/mg of protein for GST; µM of DTNB-GSH conjugating formed/min/mg of protein for GR.

Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 14. Effect of treatment with the newly synthesized Met-Res-Aldehyde complex on the activities of Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx), Glutathione-S-transferase (GST) and GR in renal tissues of experimental groups of rats.

Groups	SOD	Catalase	GPx	GST	GR
Control	17.16 ± 0.70^{a}	44.23 ± 1.98^{a}	8.08 ± 0.49^{a}	6.7 ± 0.69^{a}	33.14 ± 0.88^{a}
Diabetic	8.16 ± 0.60^{b}	17.96 ± 1.49^{b}	3.47 ± 0.26^{b}	2.29 ± 0.37^{b}	11.32 ± 0.36^{b}
Diabetic+Met - Res - Aldehyde complex	14.5 ± 0.34^{c}	29.67 ± 1.84^{c}	6.53 ± 0.18^{c}	4.01 ± 0.52^{bc}	26.62 ± 0.72^{c}
Diabetic + Metformin	13.66 ± 0.66^{c}	30.98 ± 0.50^{c}	7.16 ± 0.51^{ac}	5.10 ± 0.75^{ac}	27.29 ± 0.48^{c}

Activities of enzymes are expressed as: 50% of inhibition of epinephrine autoxidation/min for SOD; µmol of hydrogen peroxide decomposed/min/mg of protein for catalase; µmol of glutathione oxidized/min/mg of protein for GPx; U/mg of protein for GST;µM of DTNB-GSH conjugating formed/min/mg of protein for GR.

Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 15. Effect of newly synthesized Met-Res -Aldehyde complex on the levels of Vitamin E, Vitamin C, Ceruloplasmin and reduced glutathione in plasma of experimental groups of rats.

Groups	Vitamin E	Vitamin C	Ceruloplasmin	GSH

Control	1.033 ± 0.08^{a}	1.52 ± 0.22^{a}	14.09 ± 1.10^{a}	39.42 ± 2.00^{a}
Diabetic	0.41 ± 0.081^{b}	0.45 ± 0.01^{b}	5.57 ± 0.29^{b}	17.77 ± 1.57^{b}
Diabetic + Met -Res - Aldehyde complex	0.81 ± 0.03^{ac}	0.92 ± 0.02^{bc}	10.07 ± 0.27^{c}	27.99 ± 0.31^{c}
Diabetic + Metformin	0.85 ± 0.01^{ac}	1.18 ± 0.16^{ac}	11.68 ± 0.26^{c}	29.95 ± 0.48^{c}

Units are expressed as: mg/dL for Vitamin E, C and Ceruloplasmin; µmol/L for GSH Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

Table 16. Effect of Met- Res-Aldehyde complex treatment on the level of reduced glutathione in pancreas, liver and kidney tissues of experimental groups of rats.

GROUPS	Reduced Glutathione		
	Pancreas	Liver	Kidney
Control	22.32 ± 0.56^{a}	48.73 ± 1.38^{a}	37.54 ± 1.05^{a}
Diabetic	9.90 ± 0.57^{b}	23.39 ± 1.26^{b}	21.67 ± 0.40^{b}
Diabetic+Met-Res-Aldehyde complex	14.15 ± 0.85^{c}	38.25 ± 1.38^{c}	27.45 ± 1.64^{c}
Diabetic + Metformin	18.06 ± 0.86^{d}	40.56 ± 1.09^{c}	30.65 ± 0.75^{c}

Units are expressed as: µmol/g of wet tissue.Results are expressed as mean ± S.E.M (n=6). One-way ANOVA followed by post hoc test LSD. Means with different superscript letters within the same column differ significantly at $p < 0.05$.

4. DISCUSSION

Though drugs having diverse mechanism of action are plenty for the treatment of diabetes and its secondary complications, none is found to be ultimate due to the development of drug resistance and undesirable side effects after extended use (Jain and Lai , 2024: Lijun Zaho et al., 2026). This scenario necessitates the search for newer drugs with maximum therapeutic efficacy and without side effects. Natural products offer perpetual source of compounds to aid in the design of pharmacologically active New Chemical Entities (NCE). In this time of shifting paradigms in health care, it is no wonder that the people living in developed countries are receiving complementary alternative medicine (CAM). As CAM practices and therapies are proven safe and efficacious, they are considered as the mainstream health care practices. In fact, the National Institute of Health (NIH) funds multiple centers for CAM research. In fact, more than one third of Food and Drug Administration (FDA) approved drugs over the past twenty years are originally identified from the medicinal plants and more than 40% of the commercially available drugs for the treatment of chronic diseases are originally isolated from the medicinal plants (Balunas and Kinghorn,2005; Alipour et al., 2022).

Several reports are available in the literature to show that when the phytochemicals are combined together, they interact synergistically with each other and (i) may enhance the therapeutic efficacy of the drug, (ii) minimizing the dosage but increasing or maintaining the same efficacy. Likewise, the decreased dosage could lead to reduction in toxicity, (iii) minimizing or slowing down the development of drug resistance, (iv) providing selective synergism against target versus host and, (v) enhancing the

pharmacokinetic-pharmacodynamic properties such as bio-availability and clearance (Maria Maisto et al., 2025). The recent adoption of good manufacturing practices (GMP) resulted in increased use of plant based combinatorial medicines and the data obtained for several clinical trials are submitted for FDA approval (Hongting Wang et al., 2023).

Metformin is as a rule considered as a standard, first-line medication for the treatment of type 2 diabetes due to its high efficacy, low risk of hypoglycemia, and minim al side effects. It is weight-neutral and often very affordable. Gastrointestinal issues such diarrhea and nausea) are the main side effects but are usually temporary and managed by taking it with food or using the extended-release version. It reduces oxidative stress-induced damage in chronic diseases, improves vascular health, and may contribute to its anti-aging and anti-inflammatory effects (Angelika Buczynska et al., 2024). Noel et al., (1979) reported that an increase in the dosage of metformin is accompanied by decrease in its absorption in the gastrointestinal tract. However, prolonged treatment with metformin may cause lactic acidosis in some diabetic patients. Metformin carries a black box warning for lactic acidosis, an infrequent yet severe adverse effect with an incidence rate of approximately 1 in 30,000 patients (Wang et al., 2017). Lactic acidosis occurs due to lactate accumulation in the body, which cannot be eliminated quickly, leading to metabolic acidosis. This decrease in blood pH can result in nonspecific signs and symptoms such as malaise, respiratory distress, elevated lactate levels, and anion gap acidosis. Several risk factors contribute to developing lactic acidosis, including hepatic or renal impairment, advanced age, surgery, hypoxia, and alcoholism (Hsu et al., 2018).These risk factors either lower the blood's pH or hinder proper lactate elimination.

In order to circumvent the toxicity as well as development drug resistant and lactic acidosis associated with high levels of metformin, combination therapy is considered for the development of new drug molecules. Metformin alone and in combination with other “glucose-lowering” agents reduces the blood glucose level effectively in T2D (Viollet et al., 2012; McCreight et al., 2016; Lv and Gu, 2020). It was found that combinations of metformin with all noninsulin diabetic drugs result in a similar reduction of HbA1c but with changing weight gain degrees and hypoglycemia jeopardy.

Extensive research has established that resveratrol (RVT) and its constituents possess significant pharmacological properties (Rajitha et al., 2025). One of the most precious findings has been the antioxidant nature of resveratrol. Because of its capacity to stimulate the activity of a wide range of antioxidant enzymes, resveratrol is both a free radical scavenger and a powerful antioxidant. The competence of polyphenolic compounds to behave as antioxidants is dependent on the redox characteristics of their phenolic hydroxy groups and the prospect for electron delocalization across the chemical structure (Banskota et al., 2006), highlighting the significance of resveratrol as a natural antioxidant, proposing three possible antioxidant mechanisms: (i) competing with coenzyme Q and decreasing the oxidative chain complex, which is the location of ROS formation, (ii) scavenging O_2 free radicals generated in the mitochondria, and (iii) suppression of LP (lipid peroxidation) induced by Fenton reaction products. Several reports have evidenced that resveratrol may scavenge both super oxide and hydroxyl free radicals (Burkitt and Duncan, 2000; Chouikh et al., 2025).

Resveratrol can maintain the concentration of intracellular antioxidants in biological systems stable. Stilbene, for example, protects the glutathione concentration in peripheral blood mononuclear cells against oxidative damage produced by 2-deoxy-D-ribose (Losa, 2003). Reserveratrol significantly reduced the oxidation of protein thiol groups in human blood platelets (Kursvietiene et al., 2016). Likewise, resveratrol increased glutathione levels in human lymphocytes stimulated with H_2O_2 in a concentration-dependent way. In another research it was found that resveratrol increased the levels of many antioxidant enzymes, including glutathione peroxidase, glutathione

Stransferase, and glutathione reductase (Truong et al., 2008). Resveratrol's antioxidant capability for the protection of polyunsaturated fatty acids (PUFA) has been described by (Wang et al., 2020) . Earlier, we have reported the anti-diabetic, anti-oxidant properties of resveratrol in streptozotocin-nicotinamide induced experimental diabetes in rats (Palsamy and Subramanian, 2008; Palsamy and Subramanian, 2009; Palsamy and Subramanian,2010; Palsamy et al.,2010; Palsamy and Subramanian, 2011) . We have also reported the *in vivo* glucose uptake activity of resveratrol by upregulating the expressions of GLUT 4, PI3 kinase and PPAR-gamma in L6 myotubes (Subramanian and Prasath, 2011). Recently, we have synthesised a new metformin-resveratrol aldehyde ligand and evaluated its glucose uptake efficacy using rat L6 muscle cell lines (Subramanian Iyyam Pillai et al., 2011). The *in vitro* molecular docking studies on the effect of the complex against type 2 diabetic target proteins are also reported. More recently we have reported the antidiabetic efficacy of the newly synthesised complex in High fat diet fed- low dose streptozotocin induced experimental diabetes in rats (Rajitha Rajendran et al., 2025).

In the present study an earnest attempt has been made to evaluate the hypoglycemic and anti-oxidant properties of a newly synthesized metformin- resveratrol aldehyde complex in high fat diet fed- low dose streptozotocin induced experimental diabetes in rats. Among the various animal models used for the induction of experimental type 2 diabetes in animal model, high fat diet fed-low dose streptozotocin induced experimental type 2 diabetes in male rats of different strains is more effective in terms of clinical and pathological features such as insulin resistance and insufficiency which are very similar to human type 2 diabetes mellitus (Subramanian et al., 2025).

STZ is a nitrosourea analogue, preferably taken by the pancreatic β-cells-cells via glucose transporters-2 which are expressed abundantly in the pancreatic β-cells-cells and cause DNA alkylation followed by the activation of poly ADP ribosylation leading to the depletion of cytosolic concentration of NAD^+ and ATP. Enhanced ATP dephosphorylation after STZ treatment provides substrate for xanthine oxidase resulting in the excessive generation of free radicals such as superoxide radicals and nitric oxide radicals (Szkudelski et al., 2001). It was observed that STZ administration primarily abolished the beta β-cells-response to blood glucose stimulation. Though, temporary return to glucose responsiveness appears, it is followed by permanent loss of β-cells-cell mass and its functions (West et al., 1996). The dose of STZ itself obviously has a significant impact on the phenotype of HFD-fed rats (Subramanian et al., 2025). Further. Pancreatic β-cells -cells are vulnerable to oxidative stress because of their relatively diminished activities of free radical quenching enzymatic antioxidants. Chronic hyperglycemia induced oxidative stress in pancreatic β-cells leads to defective insulin gene expression accompanied by a marked decreased decrease in insulin secretion and early apoptosis (Poitout and Robertson, 2008). Excessive generation of free radicals can potentially damage the mitochondria and obviously blunt insulin secretion.

Liver is an insulin-sensitive tissue and plays a vital role in maintaining the glucose homeostasis in addition to detoxification. Therefore, STZ induced damage to liver is of prime importance in the progression and development of diabetes and its secondary complications. The abundant existence of long chain polyunsaturated fatty acids in the renal tissues makes it as another important organ vulnerable to extensive damage caused by the free radicals. In the present study, HFD-STZ- induced diabetic rats showed a significant increase in fasting blood glucose and decreased levels of insulin indicating that insulin resistance has been established in HFD-STZ-induced rats. Hyperglycemia is the essential feature of diabetes mellitus resulting in oxidative stress mediated tissue damage contributing to the onset of diabetes and its associated complications (Zhang et al., 2010).Met-Res-Aldehyde complex treated rats in significantly decreased the levels of fasting blood glucose and glycosylated Hb and increase in insulin levels. The absence of sugar in the urine samples in Met-Res-Aldehyde

complex treated rats evidenced the maintenanace of normoglycemia and its reno protective effect. We have recently reported the insulin stimulatory as well as insulin mimetic actions of the newly synthesized complex (Rajitha et al., 2026).

Irrespective of the etiologies, chronic hypeglycemia can provoke the excessive generation of free radicals through different metabolic processes such as glyceraldehydes auto-oxidation during anaerobic glycolysis, increased flux of glucose through polyol pathway causing sorbitol and fructose accumulation, increased formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC) Chronic hyperglycemia induced oxidative stress leads to increased oxidation of lipids as evident by the increased lipid peroxide levels in experimentally induced type 2 diabetic rats.(Montilla et al.,1998; Baynes and Thorpe,1999,).The increased concentration of lipid peroxides may further propagate oxidative damage by increasing peroxy radicals and hydroxyl radicals.

The significant increase observed in the levels of lipid peroxides, hydroperoxides and protein carbonyls in plasma, pancreatic and hepatic and renal tissues of experimental type 2 diabetic rats were declined to near normalcy by the treatment with the newly synthesized complex and the efficacy was more pronounced when compared to metformin treated diabetic group of rats, indicating an oxyradical - scavenging action of the complex. The increased levels of lipid peroxides, hydroperoxides and protein carbonyls are the markers of oxidative stress that are increased as a result of the toxic effect of free radicals generated during chronic hyperglycemia (Evans et al., 2002). The increased levels of lipid peroxides may further propagate oxidative damage by increasing the generation of peroxy radicals and hydroxyl radicals. In diabetic condition, increased lipid peroxidation leads to many secondary complications such as retinopathy, atherosclerosis, neural disorders and myocardial infarction through the activation of NADPH oxidase. Further, increase in lipid peroxidation impairs membrane functions by diminishing membrane fluidity and changing the activities of membrane bound enzymes and receptors. Hydroperoxides elicit toxic effects on cells both directly and through degradation by higly toxic hydroxyl radicals and also react with transtition metals like iron and copper to form stable aldehydes such as malondialdehyde (MDA), that extensively cause damage to cell membranes (Halliwell and Chirico, 1993). The product of lipid peroxidation, MDA has the ability to interact with and bind to cellular proteins and make them non-functional. MDA induces oxidative stress by targeting mitochondrial complexes I and II and thereby disrupting proper flow of electrons the ETC (Long et al., 2009).

Chronic oxidative stress due to excessive generation of free radicals is associated with a decrease in the antioxidant competence, which can further increase the deleterious effect of free radicals. All the living cells in the system contain an array of antioxidant machinery, which averts the excessive generation of free radicals to maintain redox balance (Atli et al., 2004). Enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase constitute the first line of defense against the free radical induced oxidative stress in the system. They are known to play an essential role in scavenging the toxic intermediate of incomplete oxidation (Mates and Sanchez-Jimenez, 1999; Davi et al., 2005; Droge, 2002, Bekris and Shephard, 2005).The diminished activity of enzymatic antioxidants observed in diabetic rats is the result of excessive accumulation of ROS in cellular organelles as well as to glycation of the enzymes under supraphysiological glucose levels (Raha and Robinson, 2000; Liu et al., 2008). In the present study treatment of diabetic rats with the Met-Res-Aldehyde complex increased the activity of these antioxidant enzymes.

The SOD, GSH and GPx are the important antioxidant enzymes present in our body which prevents our body from oxidative damage. Reduction of the superoxide radical into hydrogen peroxide is done by SOD enzymes. Hydrogen peroxide formed is

further converted into water molecule by other mechanism. Similarly, the GSH prevents the free radical intermediated lipid peroxidation and GPx plays an important role in the reduction of preoxidative stress (Matcovis et al., 1982; Meghana et al., 2007). MDA is a breakdown product on oxidation of polyunsaturated fatty acid in the cells. Serum with high MDA concentration indicated that higher lipid peroxidation concentration, causing high oxidative stress and leads to development of diabetes (Ceriello, 2000).

In addition to enzymatic antioxidants the endogenous non enzymatic antioxidants GSH and cerulopalsmin and the dietary antioxidants GSH and ceruloplasmin and the dietary antioxidants vitamin C and vitamin E play a significant role in the maintenance of antioxidants status. In the diabetic condition because of hyperglycemia mediated increases in oxidative stress these antioxidants are depleted (Garg and Bansal, 2000 Sharma et al., 2000; Aldini et al., 2007). However, oral administration of the Met-Res-Aldehyde complex significantly improved levels of vitamin C, vitamin E, ceruloplasmin and GSH which may be due to the sparing action of Met-Res-Aldehyde complex in the scavenging of radicals.

Conclusion

In conclusion, the results of the present study provide scientific evidence that oral administration of Met-Res-Aldehyde complex regulates the carbohydrate metabolism in experimental type 2 diabetic rats by decreasing the insulin resistance and enhancing insulin secretion from the remnant pancreatic β-cells. The observed decrease in the oxidative stress markers and a significant increase in the levels of both enzymatic and non-enzymatic anti-oxidants in the complex treated rats signify the potential antioxidant efficacy of the Met-Res-Aldehyde complex. The data obtained further suggests that the considerable antidiabetic properties of the newly synthesized complex could be due to its crucial antioxidant properties. Thus, the newly synthesized complex may be considered as a potential candidate for detailed investigation for therapeutic purpose to treat diabetes and its secondary complications.

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