ASSESSMENT OF ANTIOXIDANT POTENTIAL OF PHYTOCHEMICALS IN HUMAN GLIOBLASTOMA (U-87 MG) CELLS

Dissertation Submitted to the Central University of Punjab

For the Award of

Master of Science

In

Biosciences

By

Manpreet Kaur

Supervisor

Dr. Anil K. Mantha

Centre for Biosciences
School of Basic and Applied Sciences
Central University of Punjab

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DECLARATION

I declare that the dissertation entitled **"Assessment of antioxidant potential of phytochemicals in human Glioblastoma (U-87 MG) cells"** has been prepared by me under the guidance of Dr. Anil Kumar Mantha, Assistant Professor, Centre for Biosciences, Central University of Punjab, Bathinda. No part of this thesis / dissertation has formed the basis for the award of any degree or fellowship previously.

Manpreet Kaur

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CERTIFICATE

I certify that Manpreet Kaur has prepared her thesis/dissertation entitled **"Assessment of antioxidant potential of phytochemicals in human Glioblastoma (U-87 MG) cells"** for the award of Master's degree of the Central University of Punjab, under my guidance. She has carried out this work at the Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab.

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ACKNOWLEDGEMENTS

Completion of this dissertation was only possible with the support of several people. I would like to express my sincere gratitude to all of them.

First and foremost I owe my deepest gratitude to my guide Dr. Anil K. Mantha, for his valuable guidance, scholarly inputs and consistent encouragement that I have received throughout my dissertation. I would like to thank him for his continuous support, scientific advice, insightful suggestions and encouragement that he provided patiently to complete this dissertation.

It gives me immense pleasure to pay my heartful thanks to Dr. Monisha Dhiman who always made herself available to clarify my doubts despite of her busy schedule and I consider it as a great opportunity to complete my research work under her guidance and to learn from her expertise.

I offer my sincere respect to Prof. R.G. Saini, Coordinator, Centre for Biosciences and Prof. P. Ramarao, Dean, Academic Affairs for their valuable suggestions during this study. Prof. (Dr.) Jai Rup Singh, Founder Vice-Chancellor, Central University of Punjab, Bathinda and honourable Vice-Chancellor Prof. R.K. Kohli for providing research facilities to carry out this work.

In my daily work I have been blessed with friendly and cheerful group (lab mates and classmates) for their critical comments. I also thank Ravi P. Cholia, Bibekananda Sarkar, Nandini Gautam, Shishir Upadhyay and Sweta Thakur for their endless support and encouragement. I am particularly grateful for the assistance and feedback given by my lab mate Navrattan Kaur.

My greatest thank goes to my dearest friend Rekha Atri who always gave a great support in my work. I thank her for questioning me about my ideas, helping me think rationally and even for hearing my problems.

I have no words to express my gratitude to my cousin, Nirmal Singh, Chairman Panchyat Sampati, Barnala for supporting me all the way since beginning of my studies. I would like to thank my beloved family, my parents and my younger brother Jaswinder Singh for always believing in me, for their continuous love and their support in my decisions. They always encouraged me with their best wishes. This thesis is dedicated to my family and my friend who has been a great source of motivation and inspiration.

(**Manpreet Kaur**)

ABSTRACT

Title: Assessment of antioxidant potential of phytochemicals in human Glioblastoma (U-87 MG) cells

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Key Words: Antioxidants, Brain Cancer, CuZn-SOD, MnSOD, APE1, Phychemicals

Imbalance between production of reactive oxygen/nitrogen species (ROS/RNS) leads to oxidative stress and has been well documented for mitochondrial dysfunction, a prime cause towards pathogenesis of neurological diseases and cancer. Glioblastoma Multiforme (GBM) is a highly aggressive, invasive and primary brain tumor which shows resistance to chemotherapy and radiotherapy. Superoxide dismutase (SOD) is an antioxidant enzyme that scavenges the production of superoxide radicals and dismutases into H_2O_2 which is further converted into H_2O and O_2 by catalase (CAT) enzyme. Apurinic/Apyrimidinic endonuclease (APE1) is a central enzyme of base excision repair (BER) pathway with two important functions; DNA repair and redox regulation of transcription factors (TFs) responsible for cell survival. In this study, it was seen that oxidative stress induced by endogenously found oxidants H_2O_2 and glucose oxidase (GO) enhanced the activities of both CuZn-SOD and MnSOD in U-87 MG cells. In addition, CuZn-SOD levels were found to be increased in H_2O_2-induced oxidative stress and MnSOD levels were found to be increased in both H_2O_2 and GO-induced oxidative stress. Further, pretreatment with phytochemicals Curcumin and Quercetin modulated the activities and expression of both forms of SOD studied. The BER-pathway enzyme, APE1 level was found to be decreased in mitochondria of oxidative stress induced U-87 MG cells by H_2O_2 and GO, and in contrast APE1 level was found to be increased in cytosol, which indicates that oxidative stress affects the expression level and sub-cellular localization of APE1. Taken together, these results indicate that in GBM it is more likely that activated SOD a key player of antioxidant system and APE1 a key player in BER-pathway might be facilitating cancer cells to survive in oxidative stress environment.

(Manpreet Kaur) **(Anil K. Mantha)**

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Arbitrary Units	a.u
2	Apurinic/Apyrimidinic endonuclease1	APE1
3	Blood Brain Barrier	BBB
4	Bovine Serum Albumin	BSA
5	Base Excision Repair	BER
6	Catalase	CAT
7	Calcium	Ca^{2+}
8	Copper Zinc Superoxide Dismutase	CuZn-SOD
9	Curcumin	Cur
10	Degree Celsius	°C
11	Dimethyl Sulfoxide	DMSO
12	Double Strand Breaks	DSBs
13	Ethylenediaminetetraacetate	EDTA
14	Extracellular Superoxide Dismutase	EC-SOD
15	Glioblastoma Multiforme	GBM
16	Glucose Oxidase	GO
17	Glutathione Peroxidase	GPx

18	**Hour**	**hr**
19	**Hydrogen Peroxide**	**H_2O_2**
20	**Micro Gram**	**µg**
21	**Micro Litre**	**µl**
22	**Micro Molar**	**µM**
23	**Micro Unit**	**µU**
24	**Milli Gram**	**mg**
25	**Milli Litre**	**ml**
26	**Milli Molar**	**mM**
27	**Minute**	**min**
28	**Manganese Superoxide Dismutase**	**MnSOD**
29	**Molar**	**M**
30	**Nanometer**	**mm**
31	**Nicotinamide adenine dinucleotide phosphate oxidase**	**NADPH**
32	**Nitric Oxide**	**NO**
33	**Nitro Blue Tetrazolium**	**NBT**
34	**Optical Density**	**OD**
35	**Phenylmethanesulfonylfluoride**	**PMSF**

36	**Phosphate Buffered Saline**	**PBS**
37	**Quercetin**	**Quer**
38	**Reactive Nitrogen Species**	**RNS**
39	**Reactive Oxygen Species**	**ROS**
40	**Redox Effector Factor-1**	**Ref-1**
41	**Revolutions Per Minute**	**rpm**
42	**Single-Strand Breaks**	**SSBs**
43	**Superoxide Dismutase**	**SOD**
44	**Transcription Factors**	**TFs**

CHAPTER I

INTRODUCTION

Oxidative stress is an imbalance between reactive oxygen and nitrogen species (ROS/RNS) and antioxidant defence mechanism in the cell. Disturbances in normal redox state of cells because of toxic effects with production of peroxides and free radicals that damage the cell including protein, lipids and DNA. Overproduction of ROS/RNS increases the oxidative stress that leads to mitochondrial dysfunction and is well documented for occurrence of neurological diseases and cancer. The increased level of ROS leads to proliferation of cancer cells (Li *et al*., 2013). ROS is produced in our body primarily due to two mechanisms: (i) endogenously with NOx complexes, peroxisomes, oxidative phosphorylation (OXPHOS), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•); and (ii) exogenously with tobacco, smoke, drugs, radiation and xenobiotics (Muller, 2000). Under hypoxic condition, the mitochondrial respiratory chain produces nitric oxide (NO) which can further generate RNS (Poyton *et al*., 2009). Cellular antioxidants eliminate the ROS and RNS by converting these free radicals into H_2O_2 by superoxide dismutase (SOD) and further H_2O_2 is converted into H_2O and O_2 by catalase (CAT) enzyme. For the prevention of cancers, antioxidant enzymes such as SOD, CAT and glutathione peroxidase (GPx) are used because of their potential to eliminate species involved in the initiation of free radical chain reactions and allow the cell to survive.

Cancer is the second most common disease in India responsible for maximum mortality with about 0.3 million deaths per year. India is a growing country playing an important role in the development of the whole world and needs special attention on this issue of cancer that is disturbing the growing economy of the country. Brain tumors are the second most common cancers after hematological malignancies in children between ages of 0 and 14 yr (Das *et al*., 2014). Statistics hint that brain cancer infrequently develops in about 23,000 new people per year with about 13,000 deaths as estimated by the National Cancer Institute (NCI) and American Cancer Society (Ali *et al*., 2011).

Glioblastoma is the deadliest form of malignant brain tumor. Brain tumor is a mass of abnormally grown cells. The skull is very rigid and the brain is enclosed so that abnormal growth of cells inside the brain can cause tumor problems. Brain tumors can be cancerous (malignant) or non-cancerous (benign). World Health Organization (WHO) classified tumors into four grade groups. The grade of a

tumor refers to the way the cells look under a microscope: Grade I and Grade II group of cells look like normal brain cells and grows slowly. Grade III and Grade IV group of cells are abnormal cells that tend to grow quickly. Symptoms of brain tumor include headache, seizures in a person who does not have a history of seizures and cognitive or personality changes ((Freeman *et al*., 1998).

Taking into an account that enhanced oxidative stress exists in brain tumors and the responding elevated antioxidant and DNA repair activities contributes to chemotherapeutic resistance, the present study is focused on understanding the response of antioxidant enzyme SOD and DNA repair enzyme Apurinic/Apyrimidinic endonuclease APE1 (in terms of altered expression pattern at protein and subcellular localization level) against H_2O_2 and GO-induced oxidative stress in the presence of phytochemicals, which may act as potential modulators of SOD or APE1 and thus can have clinical implications for treatment along with chemotherapy for brain tumor.

Objective of the study

The objective of my study is to determine whether oxidative stress in glioblastoma cells affect the expression levels of cellular antioxidant enzyme, SOD and base excision repair (BER) pathway's key DNA repair enzyme, APE1. In this study, biologically occurring oxidants such as H_2O_2 and glucose oxidase (GO) are used as source of oxidative stress in U-87 MG cells. It is also proposed to determine whether oxidative stress in glioblastoma cells affect the activity of SOD and to determine whether phytochemicals Curcumin and Quercetin modulate the expression levels and functions of SOD and APE1 enzymes.

Significance of the study

This study will provide insights into the naturally occurring antioxidants and DNA repair enzyme and their ability towards countering H_2O_2 and GO-induced oxidative stress responses. This study will also allows us to understand whether oxidative stress in U-87 MG cells affects the expression and sub-cellular translocation of DNA repair enzyme APE1; which is currently explored as potential therapeutic agent against various type of cancer. In addition, key role of phytochemicals in modulating the levels and functions of SOD and APE1, the two

key cellular enzymes during oxidative stress exerted in brain tumors. In addition, this study will also elucidate the underlying signalling mechanism(s) that regulates the activities and subcellular localization of APE1; a possible route through which cancer cells are surviving.

CHAPTER II

REVIEW OF LITERATURE

Oxidative stress is a process of overproduction of free radicals which cause damage to biomolecules like lipids, proteins and DNA. The extent of oxidative stress in a cell is determined by the amounts of superoxide, H_2O_2 and hydroxyl radicals that are produced by different cellular mechanisms in response to endogenous and environmental causes/factors (Ma, 2010). Oxidative stress ultimately leads to many chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke, septic shock, aging, neurological disorders and other degenerative disease in humans.

Nervous system has two main cell types namely neurons and Glia. Neurons transmit information throughout the nervous system in the form of electrochemical waves along with axons and dendrites. Glia (neuroglial cell) is non neuronal cells which maintain homeostasis; a process that maintains the stability of human body's internal environment in response to change in external conditions. Astrocytes are polarized glial cells that represent a bridge between blood vessels and neurons. They take up nutrients from the blood, metabolize them and provide them to neurons (Dienel, 2010).

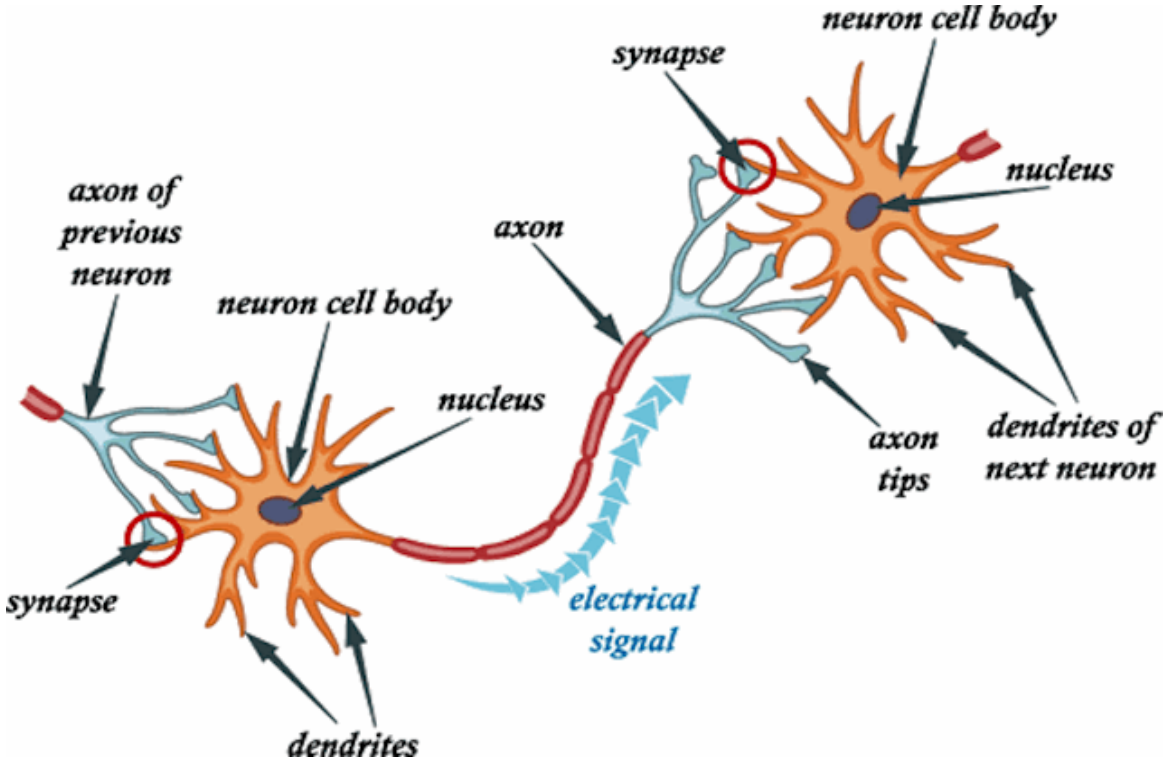

Figure 2.1: Structure of human neuron (adapted from www.studyblue.com).

Research started on Glial cells after definition of Glib coined by Redolf Virchow in 1858. The concept of neuroglia was also introduced by Rudolf Virchow which forms: brain, spinal cord and in higher sensory nerves. First type of Glial cell was identified in 1851 by Heinrich Muller who described retinal Glial cell that now bear his name. In 1857, Karl Bergmann discovered Glial cells of the cerebellum, which known as Bergmann Glial cell. In the 1860s, Otto Dieters found satellite Glial cells in the white and grey matter which to all likelihood were astrocytes (Dieters, 1865). In 1871, Louis-Antoine Rangier named the Myelinating cells of peripheral nervous system (PNS) as Schwann cells (to recognize previous discoveries of Theodor Schwann). The human brain consists of approximately 160 billion cells that mainly represent two types, neurons and Glial cells, each contributing about 50% to the total cell number. Astrocytes are mainly sensitive to pathological changes, and are involved in brain diseases. Activated astrocytes are found at the plaques of Alzheimer's patients. Current research indicates that astrocytes have different physiological properties in different brain regions, at different developmental stages and at different activity levels of the organism (Kandel *et al*., 2000).

Types of Glial cells:

Microglia cells: these are very small cells. These cells protect neurons from bacteria in the central nervous system (CNS).

Astrocytes: These are star shaped. These cells help CNS neurons form the blood brain barrier (BBB) that protects CNS from harmful chemicals. They take up chemicals and release it back to axons, enable to send messages in waves. When neurons die they remove waste and controls blood flow to each area of the brain.

Oligodendrocytes: These are the cells that coats axons by specialized membrane, myelin.

Ependymal cells: These cells line the cavities of the CNS and make up the walls of the ventricles. These cells create and secrete cerebrospinal fluid (CSF) and beat their cilia to help circulate that CSF.

Schwann cells: These cells have same function as Oligodendrocytes. These cells provide myelin to axons in the PNS. These cells are essential for nerve development, repair and function.

Satellite cells: These cells regulate the external chemical environment. Like astrocytes, they are also interconnected by gap junction (Kandel *et al*., 2000).

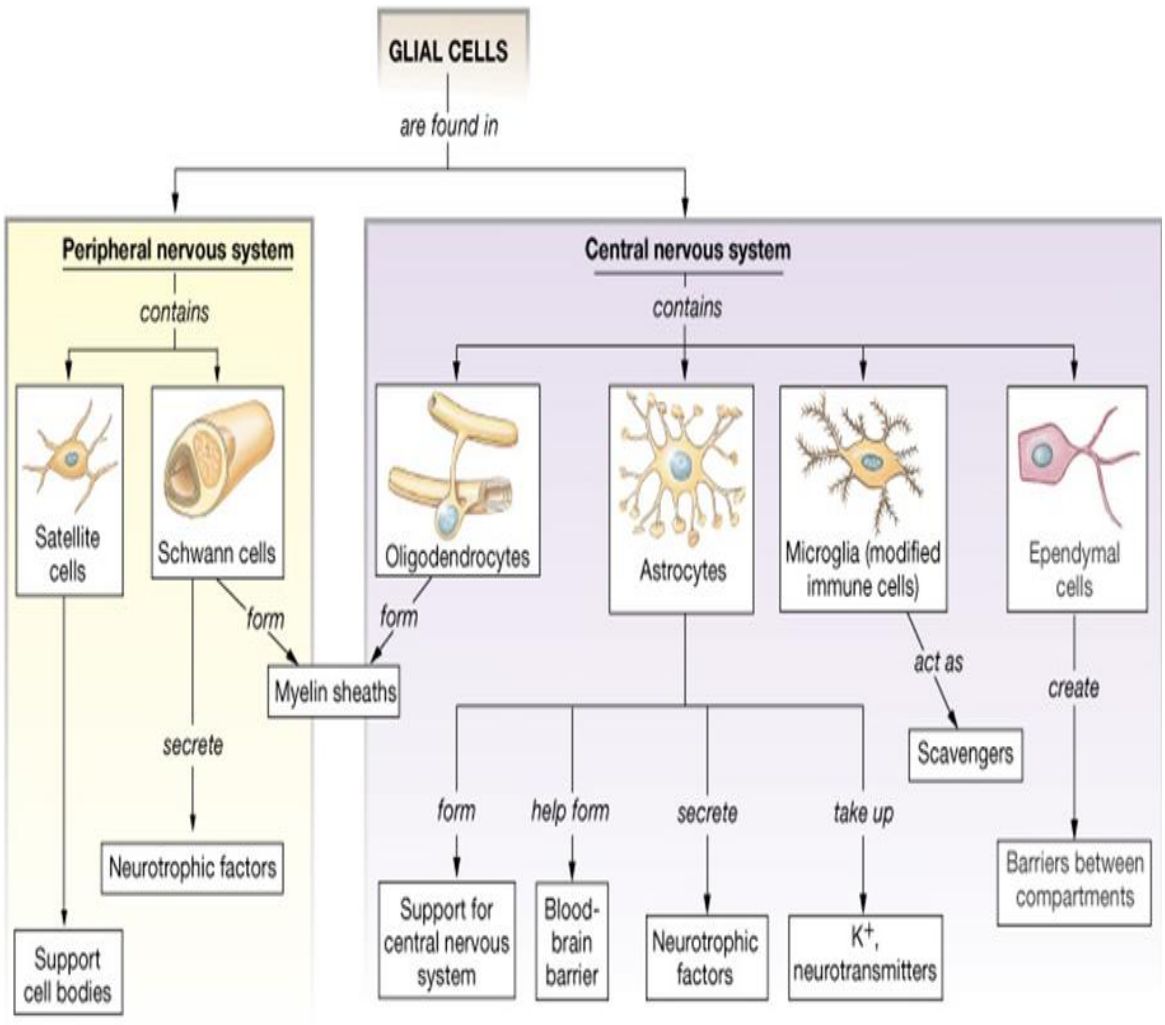

Figure 2.2: Different Types of Glial cells (adapted from www.quizlet.com).

Functions of Glial Cells:

- Provides structure to the brain
- Act like scavengers, remove debris after neural death
- Some glial cells provides myelin, that is an insulated sheath, with covers axons
- Helps in removal of neurotransmitters released by the neurons
- Some glial cells points to the migration of neurons and direct the outgrowth of axons
- Nutritive function for nerve cells

Brain Tumors

In normal condition body cells grow, and then become old, get damaged and die at the end. For replacing old cells, new cell arises in the body. But in case of old and damaged cells do not die, then they make a mass of tissue with abnormal cells. Brain tumors occur when abnormal cells increase irregularly within or around the brain. These are of two types.

Malignant tumors: These tumors are cancerous. These tumors have abnormal cell growth and can spread to other parts of the body.

Benign tumors: These tumors are non cancerous. These have slow growth rate and they grow in a contained area usually encapsulated in a fibrous connective tissue capsule. These are a mass of cells that lacks the ability to invade neighboring tissue.

According to World Health Organization (WHO) tumors are classified into four grade groups. The grade of a tumor refers to the way the cells look under a microscope:

- **Grade I:** The tissue is benign. The cells look nearly like normal brain cells, and they grow slowly.
- **Grade II:** The tissue is malignant. The cells look less like normal cells as compared to the cells in a Grade I tumor.

- **Grade III:** The malignant tissue has cells that look very different from normal cells. The abnormal cells are actively growing (anaplastic).

- **Grade IV:** The malignant tissue has cells that look most abnormal and tend to grow quickly.

Current statistics of Brain tumors in India

A statistical data showed that the number of male and female cancer patients in 2004 were 390809 and 428545 (of total no. 819354) respectively. By 2009, the number of male and female cancer patients increased to 454842 and 507990 (of total no. 962832) respectively. In the same way, 462408 male cancer patients and 517378 female cancer patients were recorded with a total number of 979786 patients in 2010 It is clear that the number of cancer cases has increased gradually with time (Ali *et al*., 2011). Tumors of the CNS are derived from gliomas which are malignant type. A tumor of the CNS accounts for less than 2% of all malignancies about 175,000 cases per year Worldwide. It is also studied to find out the trends in cancers of CNS in both the sexes in five population based cancer registries (Mumbai, Chennai, Bangalore, Delhi and Bhopal) over a period of last two decades under the network of National Cancer Registry Programme (NCRP) of Indian Council of Medical Research (ICMR), New Delhi. From this study it was found that Cancers are ranged in males from 2.53 (Chennai registry) to 4.14 (Delhi registry) while in females it ranged from 1.46 (Bhopal registry) to 2.66 (Delhi registry). The percentage of microscopic verification (MV) between these registries is ranged from 77% to 87% in males and 75% to 89% in females and the percentage of death certification only (DCO) is ranged from 3% to 8%. Whereas incidence of death ratio ranged in between 25% to 50% (NCRP) (Yeole, 2008).

Factors governing brain tumor incidence

The most common malignant brain tumor type is Gliomas because it makes up between 50% to 60% of all brain tumors in both children and adults. Gliomas are cancerous form of Glial cells. Glioblastoma Multiforme (GBM) is a common form of Gliomas. It is estimated that 88% of all GBM patients yield to the disease within 3 years. GBM remains one of the most challenging malignancies Worldwide (Adamson *et al*., 2009). The main role in tumor initiation and survival in animals as

well as in humans is of ROS (Trush *et al*., 1991). ROS oxidize vital cellular components such as lipids, proteins, and DNA. Brain is constantly exposed all the time to excitatory amino acid glutamate, whose metabolism produces ROS. ROS are mostly active in the brain and neuronal tissue as the excitatory amino acid and neurotransmitter, whose metabolism is factory of ROS, Which are unique to the brain and serve as sources of oxidative stress. ROS attack Glial cells and neurons, which are post-mitotic replication deficient cells. As a result, they are particularly sensitive to free radicals, leading to neuronal damage (Suematsu *et al*., 2003)**.** Evidences indicate that mitochondrial dysfunction plays a major role in brain tumor and neurological disorders (Lin *et al*., 2006).

Role of free radicals in oxidative stress

Free radicals are molecules with unpaired electrons in their outer orbit. Free radicals tend to be reactive and participate in chain reactions, in which a single free radical initiation event can be propagated to damage multiple molecules. Oxygen radicals are involved in signal transduction, gene transcription in the cell. NO is an important signalling molecule that essentially regulates the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, platelets aggregation, angiogenesis, thrombosis, vascular tone and hemodynamic. The most common cellular free radicals are hydroxyl (OH·), superoxide (O_2^{-}·) and nitric monoxide (NO·). But H_2O_2 and peroxynitrite ($ONOO^-$) are not free radicals, it is reported that they generate free radicals through various chemical reactions (Uttara *et al*., 2009). It is also studied that free radicals can be generated in chemical and biological systems including the formation of plastics, the ageing of paints, the combustion of fuels and in the human body (Halliwell, 2005). Free radicals are toxic to cells under normal conditions. Cells have efficient regulating system for O_2 and metal ion interaction leading to free radicals and ROS generation.

$$Fe^{3+} + \bullet O_2 \longrightarrow Fe^{2+} + O_2 \text{ (Step I)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \bullet OH \text{ (Step II)}$$

Combining step I & II

$$\bullet O_2^- + H_2O_2 \longrightarrow \bullet OH + HO^- + O_2$$ (Known as Fenton reaction)

Other metals (iron, copper, chromium, cobalt, vanadium, cadmium, arsenic and nickel)are also mediated for the formation of free radicals e.g. by Fenton chemistry (Valko *et al*., 2006). Apart from it, another method of production of ROS *in vivo* is calcium (Ca^{2+}) activation. Ca^{2+} is a signaling molecule which plays a key role in cellular responses and cell to cell communication. But, any disturbance in Ca^{2+} pathway may disrupt the cellular functioning. Metal overload in aged brain leads to oxidative stress and free radical mediated pathological changes in neurons (Simonian *et al*., 1996). It is reported about two faces of ROS are: (i) redox biology, and (ii) oxidative stress. Redox biology involves a small increase in ROS levels that activates signaling pathways to initiate biological processes, while oxidative stress denotes high levels of ROS that result in damage to DNA, protein or lipids and leads to oxidative stress (Schieber *et al*., 2014).

Role of ROS/RNS in oxidative stress

ROS are produced in response to ultraviolet radiation (UV), cigarette smoking, alcohol, nonsteroidal anti-inflammatory drugs, ischemia-reperfusion injury, chronic infections, and inflammatory disorders (Bhattacharyya *et al*., 2014). While RNS includes nitric oxide radical ($NO^{\cdot}$), $ONOO^-$, nitrogen dioxide radical ($NO^{\cdot}_2$) and other oxides of nitrogen arising when NO reacts with O_2, RO and RO_2 (Wiseman *et al*., 1996). ROS molecules are highly reactive due to the presence of unpaired valence shell electrons. ROS are particularly active in the brain and neuronal tissue as the excitatory amino acid glutamate (Glu) which also acts as neurotransmitter, whose metabolism is a factory of ROS production. They are unique to the brain and serve as sources of oxidative stress. ROS attack glial cells and neurons, which are post-mitotic replication deficient cells and they are particularly sensitive to free radicals, leading to neuronal damage. Oxygen-free radicals ROS along with RNS are well recognized for playing a dual role as both deleterious and beneficial species. The “two-faced” character of ROS gives evidence that ROS within cells act as secondary messengers in intracellular

signaling cascades, which induce and maintain the oncogenic phenotype of cancer cells. On the other hand, ROS can also induce cellular senescence and apoptosis and function as anti-tumourigenic species (Valko *et al*., 2006). ROS initiate as a by product of oxygen metabolism in electron transport chain (ETC) within mitochondria. When the rate of production of ROS exceeds the capacity of the antioxidant protection and repair leads to oxidative stress (D'Autréaux *et al*., 2007). A primary ROS is a superoxide which results from the reduction of one electron of molecular oxygen.

$$O_2 + e^- \longrightarrow O_2^{\cdot -} \text{ (Superoxide)}$$

This reaction is catalysed by NADPH oxidase in which electrons are supplied by NADPH.

$$2O_2 + NADPH \longrightarrow 2O_2^{\cdot -} + NADP^+ + H^+$$

Further when the reduction of oxygen occurs, it produces hydrogen peroxide (H_2O_2).

$$2O^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

ROS are also produced during various pathological conditions and play pivotal role in aging, hypertension, atherosclerosis and cancer. The growing production of ROS/RNS through either endogenous or exogenous results oxidative stress that is common for many types of cancer cell that are linked with altered redox regulation of cellular signaling pathways (Valko *et al*., 2006). To manage with this stress some enzymes displaying antioxidant activities like SOD and CAT involved in neutralizing ROS.

Oxidant-mediated TF AP-1 activation enhanced the expression of cyclin D1 and cyclin dependent kinases (cdks) which promotes entry into mitosis and cell division. On the other hand, ROS species function as second messengers involved in activation of another TF, NF-κB by tumor necrosis factor (TNF) and cytokines. But modification of gene expression by ROS has direct effects on cell proliferation and apoptosis through the activation of TFs including MAPK, AP-1, and NF-κB pathways. As a result DNA damage, mutation, and altered gene expression are all play role in the process of carcinogenesis (Waris *et al*., 2006).

Damage induced by free radicals

Free radicals and their products are known to play an important role in the pathogenesis of various human disorders including brain cancer.

Oxidative damage to Lipids

Lipid peroxidation (LPO) is a free radical mediated chain reaction that can inactivate cellular components. Peroxidation involves the direct reaction of oxygen and lipids to form radical intermediates and damage the enzymes, nucleic acids, membranes and proteins. In excess of hydroxyl radical and peroxynitrite can also damage cell membranes and lipoproteins by lipid peroxidation. This reaction leads to the formation of malondialdehyde (MDA) which is cytotoxic and mutagenic. When it spread affects number of lipid molecules (Pham-Huy *et al*., 2008).

Oxidative damage to proteins

Mechanisms of oxidation of proteins result in broad modifications in side chain oxidation. Protein oxidation occur as normal regulatory processes as protection mechanism against oxidative stress and as a harmful process when antioxidant defences overcome. But, proteins play major role for oxidative damage within cells due to their abundance and quick reaction with radicals and singlet oxygen (Barelli *et al*., 2008). Neuronal proteins like histones and tubulin are regulated by acetylation functions in development, stability, plasticity of neurons. Some recent studies have shown, mutations in the genes that regulate DNA and protein methylation or acetylation leads to age-related neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) (Mattson, 2003).

Oxidative damage to DNA

ROS/RNS can attack DNA and causes deletion, insertion and mutations in base pairs in DNA which is observed in oncogene and tumor suppressor genes. Excess amount of ROS overwhelm antioxidant defence. It oxidize DNA, creates DNA modifications like base oxidation (AP sites and 8-oxo-G:C), strand breakers (SSBs and DSBs) in DNA (Bhattacharyya *et al*., 2014). ROS products react with biomolecules produce other by-products that interact with DNA directly and

indirectly and alter purine and pyrimidine bases and damaged the DNA (Valko *et al*., 2006). Damaged DNA participates in neurodegeneration, aging and carcinogenesis (Maynard *et al*., 2009). Formation of RNS with NO and oxygen radicals also cause DNA base alterations and also results in strand breaks (Duncan *et al*., 2005).

Mitochondria dysfunction

Mitochondria generate energy for the cell. Mitochondria are found in every cell of the human body except red blood cells and convert the energy of food molecules into the ATP that powers most of the cell functions. Mitochondrion is main site for neurotoxicity, and also ROS production by ETC (Reed, 1994). Mitochondria link the energy-releasing activities of ETC and proton pumping with the energy conserving process of oxidative phosphorylation (OXPHOS) in the form of ATP (Cadenas *et al*., 2000). Mitochondrial ETC has five main complexes. NADH-ubiquinone oxidoreductase (Complex I) accepts electrons from the Krebs cycle electron carrier nicotinamide adenine dinucleotide (NADH) and passes them to coenzyme Q (ubiquinone) which also receives electrons from succinate dehydrogenase (Complex II), UQ passes electrons to cytochrome-c complex (Complex III), which passes them, to cytochrome-c (cyt c). Cyt-c passes electrons to cytochrome-C oxidase (Complex IV), which uses the electrons and hydrogen ions to reduce molecular oxygen to water (H_2O). Each two electrons through the chain results net transfer of 10 protons across the membranes: 4 at Complex I, 4 at Complex III and 2 at Complex IV (Ide *et al*., 2001). Transformed electrochemical energy then used to drive ATP synthesis by Complex V (ATP synthase).

$NADH+H^+ \rightarrow$ Complex I $\rightarrow$ Q $\rightarrow$ Complex III $\rightarrow$ cytochrome-c $\rightarrow$ Complex IV $\rightarrow O_2$

↑

Complex II

↑

Succinate

Reduction of O_2 ($O_2 \rightarrow O_2^{\cdot -} \rightarrow H_2O_2 \rightarrow OH^{\cdot} \rightarrow H_2O$) produces ROS. The primary ROS made by mitochondria is superoxide ($O_2^{\cdot -}$), which is converted to H_2O_2 either by spontaneous dismutation or by the enzyme SOD. H_2O_2 can be further transformed to $OH^{\cdot}$ in the presence of metal ions by Fenton chemistry (Brookes *et al*., 2004). When ROS generation by mitochondria increases, then ROS leads to irreversible damage of mitochondrial DNA, lipids and proteins resulting in mitochondrial dysfunction and ultimately cell death (Kowaltowski *et al*., 1999). Increased level of ROS and oxidative stress plays an important role in neurological diseases like brain tumors, PD and AD associated with mitochondrial dysfunction (Lin & Beal, 2006).

Role of different cellular antioxidants

Antioxidant enzymes such as SOD, CAT and GPx are well studied for their role in eliminating species involved in the initiation of free radical chain reactions. On the other hand small molecule antioxidants such as ascorbate, tocopherols and glutathione can repair oxidizing radicals directly (Slaga, 1995). It has been studied that many of antioxidant compounds possess antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities to greater or lesser extent. In many cases, increased oxidative stress is a widely related in the development and progression of the particular disease and its complications by increased production of free radicals or failure of antioxidant defence (Devasagayam *et al*., 2004; Valko *et al*., 2007). However, it is also studied that the intake of natural antioxidants reduce the risk of cancer, cardiovascular diseases, diabetes and other diseases associated with aging. In addition, studies advocates that antioxidants are needed to scavenge and prevent the formation of ROS and RNS towards countering disease state (Qureshi *et al*., 2007).

Sources of antioxidants

A variety of antioxidants are supplied to human body through diet by both vegetarian as well as non-vegetarian diet (Uttara *et al*., 2009). Vitamin-C and Vitamin-E, β-carotene and coenzyme Q are the most famous antioxidants of our diet. Vitamin E is present in vegetable oils and in wheat germ. It is fat soluble Vitamin absorbed in the gut and carried in the plasma by lipoproteins (Packer,

1991). Plants (fruits, vegetables and medicinal herbs) may contain a large variety of free radical scavenging molecules such as phenolic compounds (Phenolic acids, flavonoids, lignans, tannins etc.), nitrogen compounds (alkaloids, amines, betalains etc.), vitamins, terpenoids, carotenoids and some other endogenous metabolites which are rich in antioxidant activity (Qureshi & Parvez, 2007). Other sources of antioxidants are enzymes like superoxide dismutase CuZn-SOD, MnSOD, CAT and GPx involved in protecting cells from the damaging effects of ROS and non-enzymatic antioxidants [Vitamin-C, Vitamin-E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), flavonoids, selenium and others] are involved in the process of carcinogenesis as well as the antioxidant interactions with various regulatory factors including APE1, NF-κB and AP-1 (Valko *et al*., 2006).

SOD: An antioxidant enzyme

Antioxidant defence mechanisms include removal of O_2, scavenging of ROS/RNS or their precursors, inhibition of ROS formation and binding of metal ions needed for the catalysis of ROS generation and upregulation of endogenous antioxidant defences. Any new antioxidant molecule designed as potential neuroprotective agent for acute or chronic neurological disorders should have the fixed precondition that they can readily cross the Blood Brain Barrier (BBB).

In 1969, McCord and Fridovich discovered the enzyme SOD (McCord and Fridovich, 1969). It is a group of enzymes which are important for removing biologically generated superoxide anion radical (O_2^-). These enzymes function by catalyzing the dismutation of superoxide radicals to H_2O_2 and oxygen, and their action helps to protect cells from oxidation of lipids, proteins and DNA (Powers *et al*., 2008). Three forms of the SOD exist in the humans: SOD1 or CuZn-SOD was the first enzyme to be characterized and is a copper and zinc-containing homodyne that is found almost exclusively in intracellular cytoplasmic spaces. SOD2 or MnSOD exists as a tetramer and is initially synthesized containing a leader peptide, which targets this manganese-containing enzyme totally to the mitochondrial spaces. SOD3 or EC-SOD is the most recently characterized SOD,

exists as a copper and zinc-containing tetramer, and is synthesized containing a signal peptide that directs this enzyme to extracellular spaces (Zelko *et al*., 2002). SOD being a versatile key enzyme that scavenge and regulate levels of superoxide produced within the tumor microenvironment and help the tumor cell to fight and helps in cell survival and progression. To understanding the mechanism of its action and expression during oxidative stress, it advocates for the current study.

APE1: A multifunctional enzyme

APE1 is a multifunctional protein involved in the maintaining the genomic integrity and regulation of gene expression. APE1 is a central enzyme of BER (base excision repair) pathway with two major functions: (i) DNA repair, and (ii) Redox regulation (Thakur *et al*., 2014). DNA glycosylase(s) enzyme recognizes the damage or improper bases and removed by enzymatic hydrolysis of the N-glycosyl bond resulting in the formation of an AP site. APE1 recognizes and incises the DNA backbone immediately 5′ to the AP site via its 5′-endonuclease activity, producing a single-strand break (SSB) with a normal 3′-hydroxyl group and an abnormal 5′-deoxyribose-5-phosphate (dRP) residue and DNA polymerase adds new nucleotides to the break and nick is sealed by DNA ligase. APE1 controls the intracellular redox state of the cell by inhibiting the ROS production (Tell *et al*., 2009). APE1 is constitutively activated in cancer cells and upregulated further in response to certain chemotherapeutics and radiation damage, but is inhibited by dietary agents, such as soy isoflavones, curcumin leading to increased cell killing and tumor growth inhibition (Raffoul *et al*., 2012). Any alteration in APE1 expression and mutations in the *APE1 gene* contribute to the development of a variety of neurodegenerative diseases (Kim *et al*., 2009). APE1 can be used as genetic marker and molecular target. When APE1 is over expressed in cancer cells, it exhibit altered repair and redox functions and helps cancer cells to survive, thus confers resistance against chemotherapy and radiotherapy (Luo *et al*., 2004).

Due to the dual nature, APE1 activates the TFs like NF-κB and HIF-1α which plays role in mechanism of interaction between dietary agents and radiation and chemotherapeutic agents and repair of damage of DNA (Thakur *et al*., 2014). While simultaneously, reducing (Redox) TFs in cancer cells by inhibition of APE1

with dietary agents could decrease the cell survival and further enhance tumors radio and chemo sensitivity (Raffoul *et al*., 2012). This area of research is currently explored by few laboratories Worldwide, including Dr. Mantha's laboratory at CUPB.

Phytochemicals

Phytochemicals are secondary plant products such as phenolic compounds, flavonoids, alkaloids, and isoprene derivatives etc. which possess antioxidant properties and play protective role in various diseases (Gupta *et al*., 2013).

Curcumin

Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl-1,6-heptadiene) is an important constituent of turmeric powder and is obtained from a rhizome, *Curcuma longa*. Curcumin is known to possess anti-inflammatory, anti-oxidative and anti-carcinogenic properties (Campos *et al*., 2013). Curcumin has pleiotropic nature and ability to interact with various molecular targets of inflammation. It down regulates the activity of COX-2, lipoxygenase and iNOS and modulates inflammatory response (Sharma *et al*., 2006). It inhibits the production of various cytokines involved in inflammation such as TNF-α (IL-1,-2,-6,-8 and -12), monocyte chemo attract protein (MCP) and migration inhibiting protein (Julie *et al*., 2009). Curcumin is known to suppress the activation of NF-κB which plays major role in regulation of inflammation (Julie & Jurenka, 2009). Curcumin has been shown to posesses cytotoxic effects on glioma cell lines *in vitro* (Aoki *et al*., 2007). Both mRNA and protein levels of GLI1 signaling and several GLI1-dependent target genes (*CyclinD1, Bcl-2, and Foxm1*) were found to be down regulated. Curcumin treatment prevented GLI1 translocation into the cell nucleus and reduced the concentration of its reporter expression. Curcumin also suppressed cell proliferation, colony formation, migration, and induced apoptosis (Du *et al*., 2013). Intraperitoneal injection of Curcumin *in vivo* reduced tumor volume, GLI1 expression, the number of positively stained cells, and prolonged the survival period as compared with the control group (Du *et al*., 2013). Curcumin has the potential to treat a wide variety of inflammatory diseases including cancer, cardiovascular diseases, arthritis and AD through modulation of numerous

molecular targets. Curcumin has been used for the chemoprevention and treatment of various diseases (Pari *et al*., 2008). Another review focuses on the anti-inflammatory potential of curcumin and role for the prevention and/or treatment of cancer (Basnet *et al*., 2011; Duvoix *et al*., 2005).

Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone) is a common antioxidant found in onion, apples, broccoli, berries and tea (Comalada *et al*., 2005). Quercetin acts as a powerful modulating agent in different biological processes. It has anti-proliferative and pro-apoptotic activities in different cancers. It also has anti-inflammatory activities and ability to prevent against oxidative stress (Nanua *et al*., 2006). Role of Quercetin as a blocker of the STAT 3 activation pathway stimulated by IL-6 with a possible role in the prevention and treatment of glioblastoma, where IL-6 is a cytokine that orchestrates the inflammatory microenvironment surrounding the tumor (Michaud-Levesque *et al*., 2012). Phytochemical, Quercetin will also be studied to see how it modulates the SOD activity. It is already known that Quercetin, a flavonoid, can inhibit proliferation and induce apoptosis in a variety of cancer cells. Recent studies have shown that Quercetin induces apoptotic cell death in human U138 MG glioma cells and decreases cell proliferation (Braganhol *et al*., 2006). Quercetin exerts protective effect against cell damage induced by H_2O_2 in rat glioma cells (Chen *et al*., 2006). Quercetin has therapeutic potential as an anticancer drug. Quercetin also inhibits the growth of hepatoma cells by changing cell cycle sequence (Chang *et al*., 2006) In this study, effect of Quercetin will be studied on the cytosolic and mitochondrial SOD activity in the presence of oxidants, H_2O_2 and GO in glioblastoma cell line, U-87 MG.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals Used: Acrylamide and bis-acrylamide, acetic acid, ammonium per sulphate (APS), bovine serum albumin (BSA), bromophenol blue, coomassie brilliant blue G-50, diethyl ether dulbecco's modified eagle medium (DMEM), dimethylsulfoxide (DMSO), ethanol, ferric chloride ($FeCl_2$), foetal bovine serum (FBS), formaldehyde, glycerol, glycine, hydrogen peroxide (H_2O_2), glucose oxidase (GO), curcumin, quercetin, methanol, β-2-mercaptoethanol, 3-(4, 5-dimethythiazol-2-yl)-2, (N(-1-Napthyl) ethylenediamine (NEDD), nitrobluetetrazolium, o-diansidinedihydrochloride, 20% orthophosphoric acid, potassium chloride (KCl), potassium hydroxide (KOH), potassium iodide (KI), potassium phosphate, sodium carbonate, sodium chloride (NaCl), sucrose, disodium phosphate (Na_2HPO_4) and monosodium phosphate (NaH_2PO_4), digitonin, protease inhibitor cocktail, ethylenediaminetetraacetic acid (EDTA), triton-X, pyrogallol, sodium dodecyl sulphate (SDS), sodium nitrite, sodium phosphate, sulphanilamide, N,N,N',N tetramethylethylenediamine (TEMED), TrisHCl (pH 6.8 and 8.8), potassium cyananide (KCN), tris base, rivoflavin, dithiothreitol (DTT), phosphate buffered saline (PBS), tetramethylbenzidine liquid substrate, SOD 1 and SOD 2 (rabbit Santa Cruz) and Ref-1 (mouse Santa Cruz) and secondary HRP-conjugated anti-rabbit and anti-mouse antibodies.

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3.2 Culturing of Glioblastoma (U-87 MG) Cells: U-87 MG cells (generously gifted by Dr. Gurusharn Kaur, Guru Nanak Dev University, Amritsar) were grown in DMEM medium containing 10% FBS and 100 units/mL penicillin, and 100 µg/mL streptomycin were maintained at 37°C in a 5% CO_2 humidified incubator. When the cells became 70-80% confluent, culture medium was removed and discarded. Then the cells were rinsed with PBS to remove all traces of serum that contains trypsin inhibitor. After that, trypsin-EDTA 0.25% (w/v) solution was added to the flask. It was then allowed to incubate until cells were detached from the surface. DMEM containing 10% FBS medium and penicillin/streptomycin was added afterwards for further culturing of the cells. Appropriate aliquots of the cell suspension was poured onto new culture vessels and maintained as indicated (Mantha *et al*., 2012).

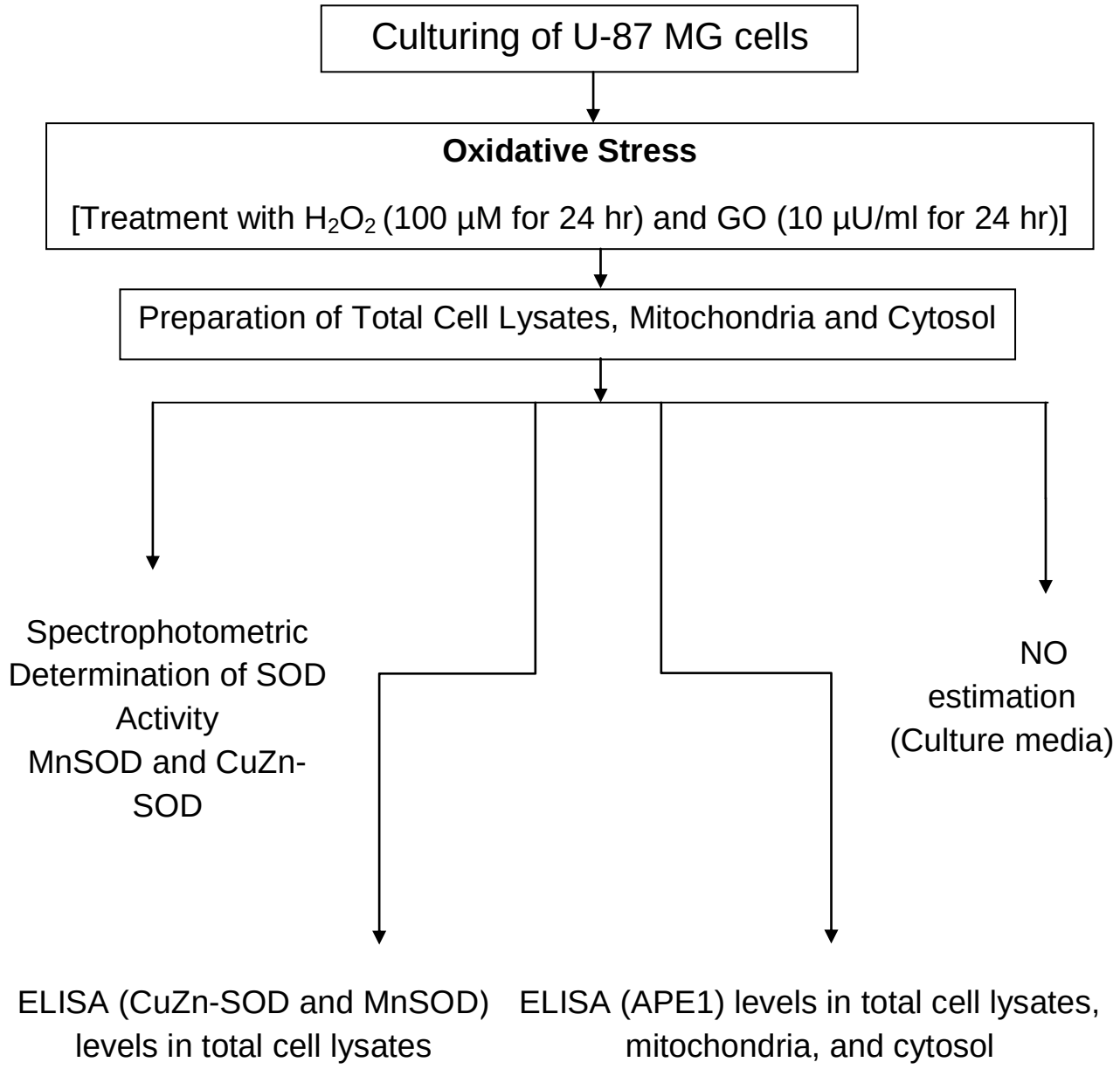

Figure 3.1: Overall Experiment approach of study.

3.3 Induction of Oxidative Stress: H_2O_2 and GO were chosen as oxidants that have been in use by various studies to produce oxidative stress in U-87 MG cells(Lee *et al*., 2005). The concentrations of H_2O_2 (100 µM) and GO (10 µU) were used for inducing the oxidative stress in $1x10^6$ no. of U-87 MG cells. Then suitable conditions were provided to U-87 MG cells (Deubzer *et al*., 2010).

3.4 Phytochemical Modulation against Induced Oxidative Stress: U-87 MG cells were treated with low dose of two phytochemicals, Curcumin and Quercetin. 10 µM of Curcumin along with 100 µM of H_2O_2 and 10 µU of GO respectively were used in the present study. In another set, 10 µM of Quercetin along with 100 µM of H_2O_2 and 10 µU of GO respectively were used for the treatment of U-87 MG cells.

3.5 Composition of Cell Lysis Buffer: The preparation of the cell lysis buffer contained the following chemicals at a final concentrations dissolved in autoclaved water and adjusted to pH (7.6) are listed as below.

- 75 mM NaCl
- 1 mM NaH_2PO4
- 8 mM Na_2HPO4
- 250 mM Sucrose
- 0.05% Digitonin

Note: Addition of protease inhibitor cocktail was performed to the lysis buffer preparation as and when required at 4 °C (Mantha *et al*., 2012).

3.6 Preparation of Total Cell Lysates: For preparing total cell lysates, the culture medium was separated and stored for NO estimation. The control (untreated) and the oxidative stress induced (H_2O_2 and GO treated as described above) U-87 MG cells were collected separately after washing with ice cold PBS and trypsinization. These cells were transferred to 50 ml falcon tubes separately and centrifuged at 3,000 rpm for 10 min at 4 ºC using table top refrigerated centrifuge. After the centrifugation, the supernatant containing media and PBS was discarded and collected the cell pellet. By adding 0.5 ml of lysis buffer solution with Digitonin (0.05%) to each of the falcon tubes containing cell pellets, the resuspended cells were incubated on ice for 30 min with vortexing at 10 min of intervals. After the

incubation, different treatment groups of cells were centrifuged at 800g for 10 min at 4 ºC to remove cell debris and nuclei. The resultant supernatant (S1) was stored as the total cell lysates for further use at – 80°C in small aliquots.

3.7 Preparation of Cytosolic and Mitochondrial Fractions: A fraction of S1 was further centrifuged at 20,000g for 15 min at 4ºC, the resulting supernatant (S2) was stored as cytosol for further use at –80°C in small aliquots. Then all corresponding group pellets were resuspended in 300 µl of lysis buffer (with 0.6 % Triton-X 100) and 3 µl protease inhibitor cocktail and then incubated on ice for 20 min with vortexing at 5 min of intervals and subjected to centrifugation at 3000g for 10 min at 4 ºC. The resulting supernatant (S3) was stored as mitochondrial extract at -80 ºC till further use (Rhein *et al.*, 2010).

3.8 Protein Estimation by Bradford Method: This assay was used for the estimation of amount of protein present in different samples. At first, calibration curve was determined using BSA as a standard by adding required volume of the sample and by bring the volume up to 800 µl with or PBS. Then added 200 µl of 5x Bradford reagent and incubated at room temperature for 10 min. The absorbance of the samples was measured at 595 nm using Shimadzu double-beam spectrophotometer.

3.9 Superoxide Dismutase (SOD) Assay: The SOD activity measurements were done in control and different treatment groups of total cell lysates, mitochondria and cytosol by the method of (Marklund *et al*., 1974) with few modifications as described (Mantha *et al*., 2006). This method is based on the ability of the SOD enzyme to inhibit auto-oxidation of pyrogallol. The reaction was started by the addition of the extract (total cell lysates, mitochondria and cytosol) containing 20 µg of the protein per ml. The assay mixture in 500 µl contained in a final concentration: 0.1mM sodium phosphate buffer (pH 8.0); 3 mM EDTA and 8.1mMpyrogallol.The change in absorbance was monitored at 420 nm for 3 min against a reference cuvette that contained all the reagents except the enzyme. The SOD enzyme activity is expressed as units per gram of protein per minute. One unit of the enzyme activity is the amount of the enzyme that causes half-maximal inhibition of pyrogallol auto-oxidation.

The formula for the calculation of SOD activity is:

$$\text{SOD Activity} = \frac{[(\Delta OD \text{ Reference} - \Delta OD \text{ Sample}) \times \text{Total Volume}]}{(\Delta OD \text{ Reference}) \times \text{Sample Volume}}{2}$$

3.10 Estimation of Nitric Oxide (NO) Level:

Principle: Griess test detects the presence of organic nitrite compounds. Griess reagent was first described by Peter Griess in 1858.This method for the indirect determination of NO involves the spectrophotometric measurement of its stable decomposition products NO_3^- and NO_2^-. This method requires that NO_3^- first be reduced to NO_2^- and then determination of NO_2^- by the Griess reaction. The Griess reaction is a two-step diazotization reaction in which the NO^- derived nitrosating agent, dinitrogen trioxide (N_2O_3) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO), reacts with sulfanilamide to produce a diazonium salt which develops red pink colour with the addition of azo dye (*N*-1-napthylethylenediamine) to form a chromophoricazo product that absorbs strongly at 540 nm as shown in **Fig.3.2** (Bryan *et al*., 2007).

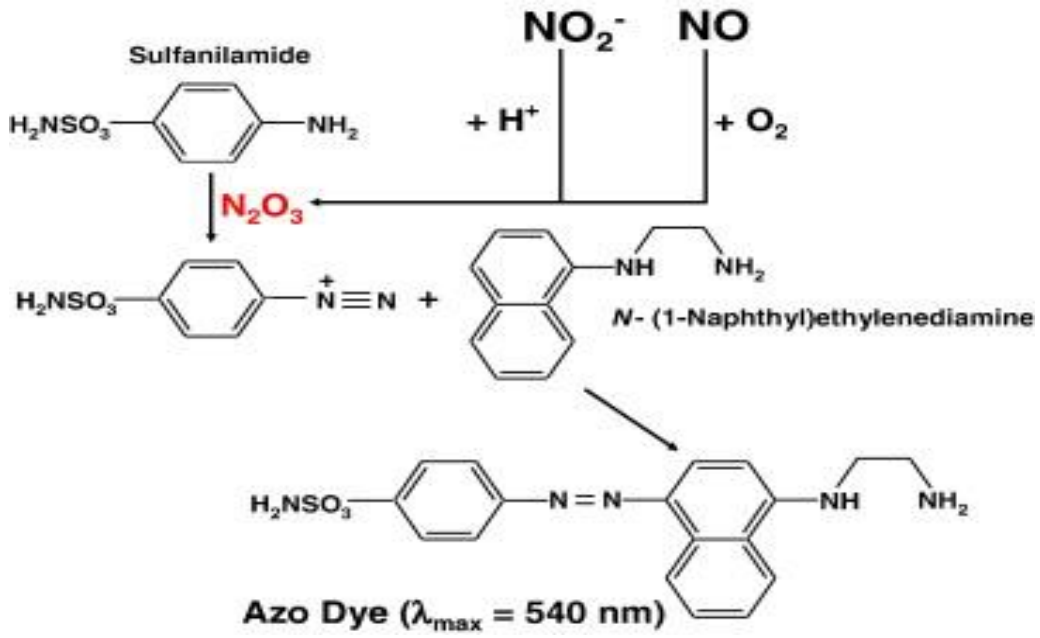

Figure 3.2: Reaction mechanism of NO detection (adapted from Elsevier.com)

3.11 Enzyme Linked Immunosorbent Assay (ELISA): ELISA is a sensitive immunoassay that uses an enzyme linked to an antibody as a marker for the detection of a specific protein, especially an antigen of interest. The fundamental principle of the ELISA is that the target (the antigen) is recognised with high specificity by antibodies, which are proteins produced by the immune system. The immune system of animals produces antibodies in response to the presence of antigens. These antibodies can recognise and bind to the antigens, the labelling of the secondary antibody which is conjugated to an enzyme such as HRP or alkaline phosphatase etc.

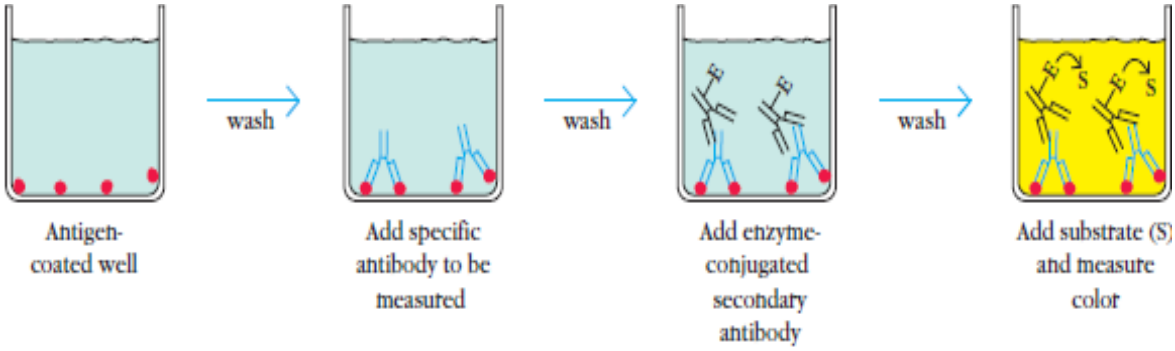

Figure 3.3: Schematic representation of Principle involved in ELISA (adapted from Kuby Immunology).

Procedure: We utilized the ELISA where the total cell lysates, cytosol and mitochondria extracts (antigen, 20µg) were coatedonto the high affinity microtiter plate wells for overnight at -20 °C temperature as described previously (Dhiman *et al*., 2009). All the unbound sites were blocked with 1% BSA to prevent false positive results. The primary antibody for MnSOD and CuZn-SOD (rabbit Santa Cruz) and APE1 (mouse Santa Cruz) was added and incubated for an hour. The HRP conjugated secondary antibody (anti-rabbit and anti-mouse IgG respectively) was added after washing the wells with PBST and PBS. Finally, colour was developed using the tetra methyl benzidine (TMB) substrate for the HRP reaction. The reaction was stopped with 2M H_2SO_4 and final OD was read at 450nm in a microplate reader (Biotek). The OD is directly proportional to the reaction product formed due to interaction between antigen and antibody.

3.12: Data Analysis:

Data was presented as the mean ± the standard deviation for at least 3 independent determinations. The student (t) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *$p \leq 0.05$ and **$p \leq 0.005$ when untreated control cells were compared with H_2O_2, GO, Curcumin, Quercetin treated cells as well as $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.005$ when H_2O_2+Curcumin, H_2O_2+Quercetin, GO+Curcumin, GO+Quercetin treatments were compared with H_2O_2 and GO treatments.

CHAPTER IV

RESULTS

4.1.<u>Protein Estimation in Samples</u>: The amount of protein present in total cell lysates, cytosolic fractions and mitochondrial extracts was estimated by Bradford method by using BSA as a standard (**Figure 4.1**).

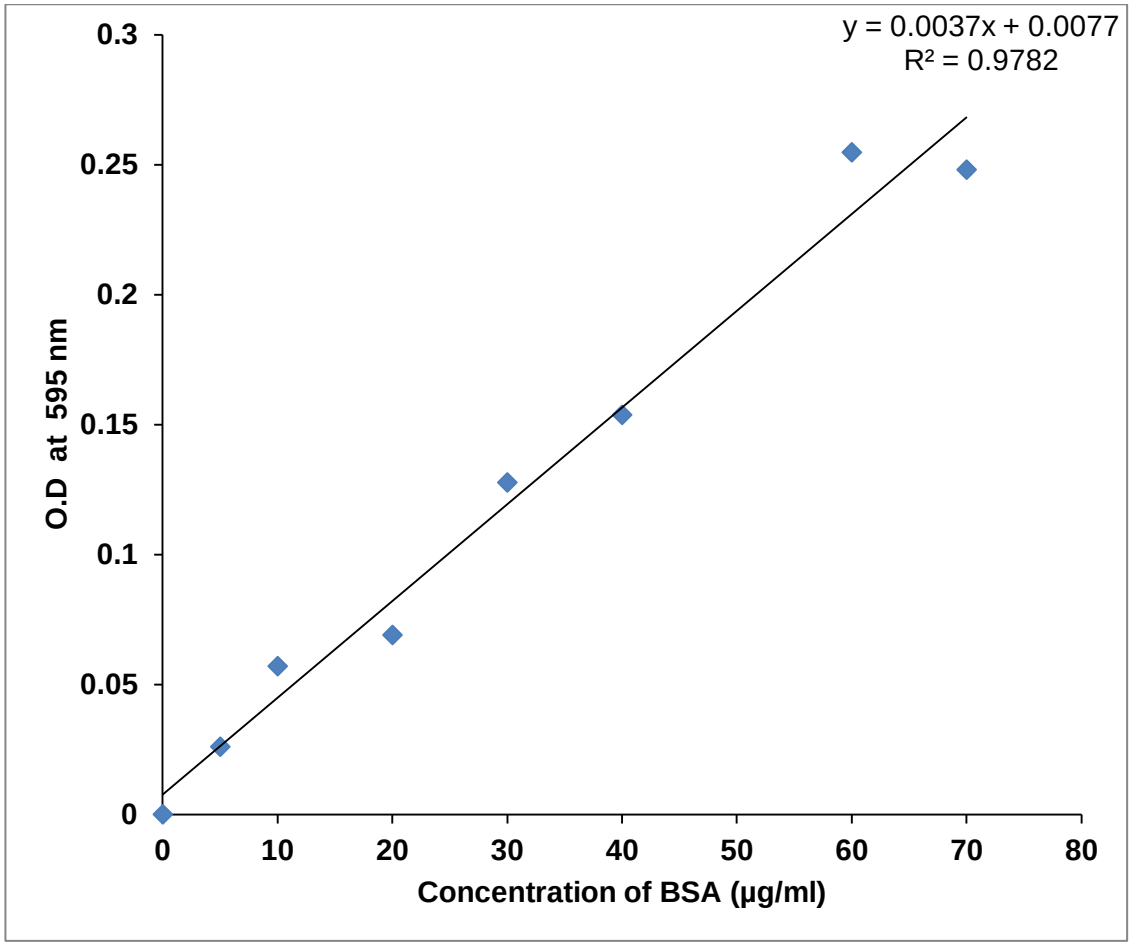

Figure 4.1: Standard curve of BSA for estimation of protein by Bradford method.

Table 4.1: The amount of protein estimated in total cell lysates, cytosolic fractions and mitochondrial extracts made from U-87 MG cells by Bradford method.

Samples	Total cell lysates (µg/ml)	Cytosol (µg/ml)	Mitochondria (µg/ml)
Control	80±29	14±0.9	96±17
H_2O_2	106±12	16±4	119±3
H_2O_2+Curcumin	144±10	31±3	112±15
H_2O_2+Quercetin	138±9	40±6	123±5
GO	108±17	44±7	123±5
GO+Curcumin	132±13	46±11	127±11
GO+Quercetin	140±3	41±10	119±6
Curcumin	84±7	42±10	106±26
Quercetin	ND	26±8	130±9

*ND – not determined.

The results are presented as mean ± standard deviation (n=3).

4.2a.<u>Estimation of extra cellular NO (Griess test)</u> : The Griess test is a chemical analysis test which detects the presence of organic nitrite compounds**,** uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO_2^- in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. The amount of NO is determined by standard curve (**Figure 4.2a**) plotted by using Sodium Nitrite ($NaNO_2$).

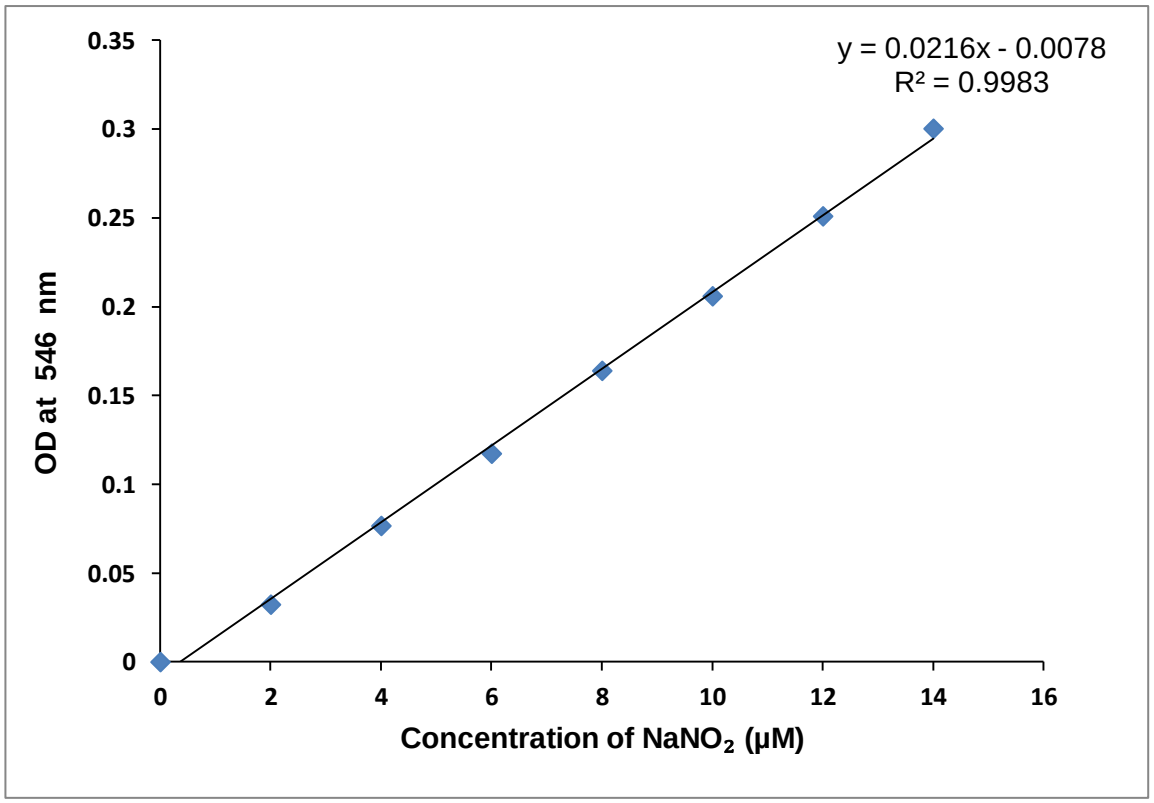

Figure 4.2a: Standard curve of $NaNO_2$ for the Griess test.

The mean of concentration of nitrite (measurement of extra cellular NO produced) has no significant change only by 4% increase when H_2O_2 compared with untreated control cells (122±7 vs. 117±2). H_2O_2+Curcumin and H_2O_2+Quercetin showed decrease in concentrations by 7% and 1% of nitrite when compared with H_2O_2 treated cells (114±7 vs. 122±7), (121±2 vs. 122±7) respectively. GO showed 5% increase in NO production when compared with untreated control cells (123±5* vs. 117±2). GO+Curcumin showed 23% decrease and GO+Quercetin showed 3% increase in NO levels when compared with GO treated cells (96±3$^{\#\#}$ vs. 123±5*), (128±1 vs. 123±5*) respectively. Only Curcumin

and Quercetin treatments showed significant 7% increase in NO levels when compared with untreated control cells (126±2** vs. 117±2) (125±3* vs. 117±2) respectively as shown in **Figure 4.2b** and **Table 4. 2**.

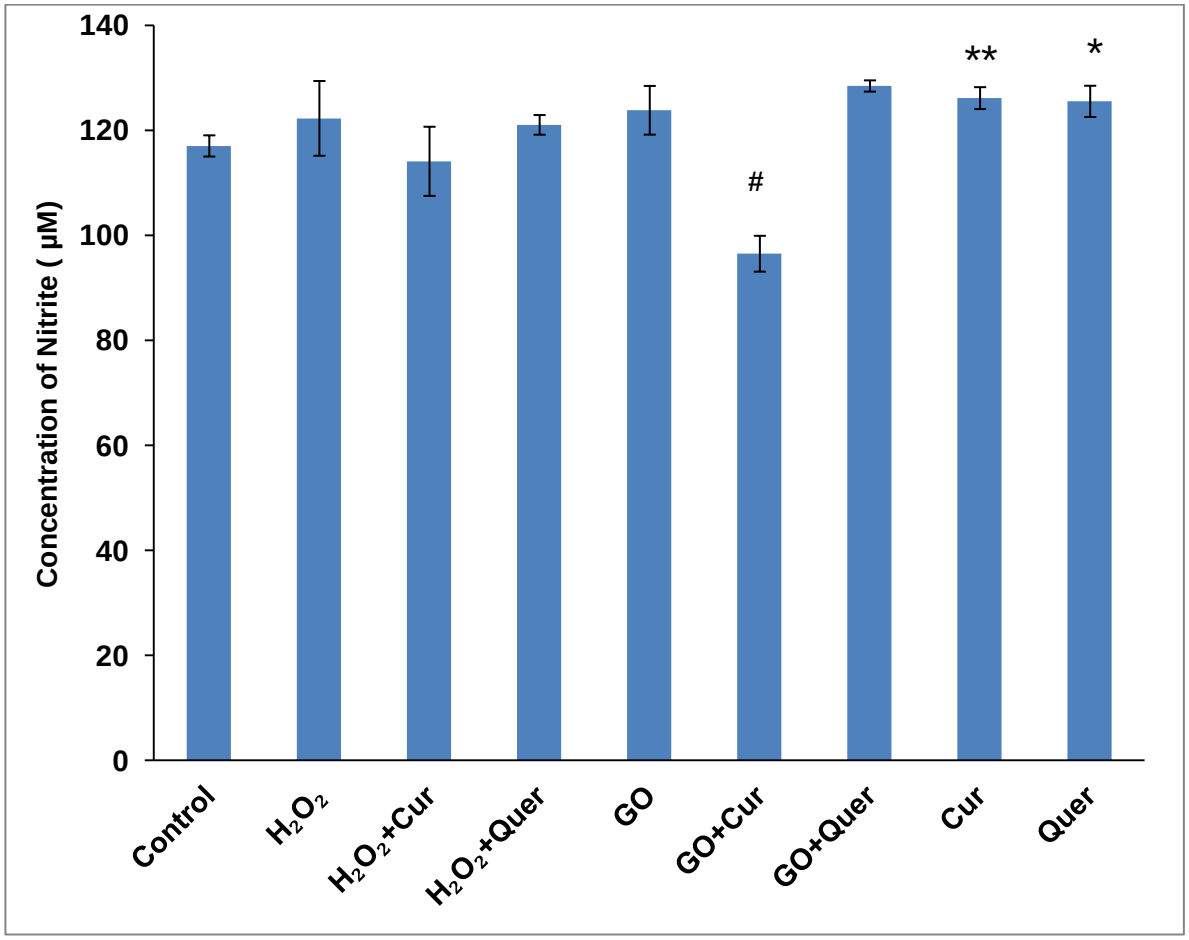

Figure 4.2b: Extracellular NO estimation in cell culture media of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated control cells were compared with H_2O_2 and GO treated cells. $^{\#\#}$p≤0.005 when GO+Curcumin treated cells were compared with only GO treated U-87 MG cells.

Table 4.2: Amount of NO estimated in culture media of U-87 MG cells after 24 hr of treatment with oxidants and phytochemicals by using Griess reagent.

Samples	Amount of NO (µg/ml)
Control	117±2 (100)
H_2O_2	122±7 (104) (100)
H_2O_2+Curcumin	114±7 (93)
H_2O_2+Quercetin	121±2 (99)
GO	123±5 (105) (100)
GO+Curcumin	96±3$^{\#\#}$ (77)
GO+Quercetin	128±1 (103)
Curcumin	126±2** (108)
Quercetin	125±3* (107)

The results are presented as mean ± standard deviation (n=3), and all the values in parenthesis are amount of NO produced in cell culture media when compared with untreated control culture media. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with H_2O_2, GO, Curcumin and Quercetin treated cells. $^{\#\#}$p≤0.005 when GO+Curcumin treated cells were compared with only GO treated U-87 MG cells.

4.3. SOD Activity

The following formula was used to calculate the SOD activity in the total cell lysates, cytosolic and mitochondrial fractions of the control and treated U-87 MG cells.

Where,

$$\% \text{ Inhibition of pyrogallol auto-oxidation} = [1-(\Delta A/\Delta Amax) \times 100]$$

Δ**A** = Absorbance change due to pyrogallol auto-oxidation in the sample

Δ**Amax**= Absorbance change due to pyrogallol auto-oxidation in the blank (without enzyme sample).

One unit of SOD activity was defined as the amount required for inhibition of pyrogallol auto-oxidation per min per mg of protein.

4.3a. <u>SOD activity in total cell lysates</u>: Without KCN treatment, a known inhibitor of SOD, graph showed 12% increase when H_2O_2 treated cells were compared with untreated control U-87 MG cells (747±15 vs.665±48) and 48% and 47% increase in H_2O_2+Quercetin, H_2O_2+Curcumin when compared with H_2O_2 treated cells (1110±37, 1097±26 vs. 747±15) respectively. GO treated showed 16% increase when compared with untreated control cells (778±74 vs. 665±48) and GO+Curcumin 35% and GO+ Quercetin showed 28% increase when compared with GO treated cells (1057±59, 1002±56 vs. 778±74) respectively (Figure 4.3a and Table 4.3a). With KCN, graph showed 30% a significant increase when H_2O_2 treated cells are compared with untreated control cells (467±40 vs. 360±52) and 19% and 25% increase in H_2O_2+Quercetin, H_2O_2+Curcumin when compared with H_2O_2 treated cells (557±1.8 vs. 467±40), (587±18 vs. 467±40) respectively. GO showed 18% increase when compared with untreated control cells (427±16 vs. 360±52) and GO+Curcumin 28% and GO+ Quercetin showed 20% increase when compared with GO treated U-87 MG cells (547±5.4$^{\#}$ vs. 427±16), (511±48 vs. 427±16) respectively (**Figure 4.3a** and **Table 4.3a**).

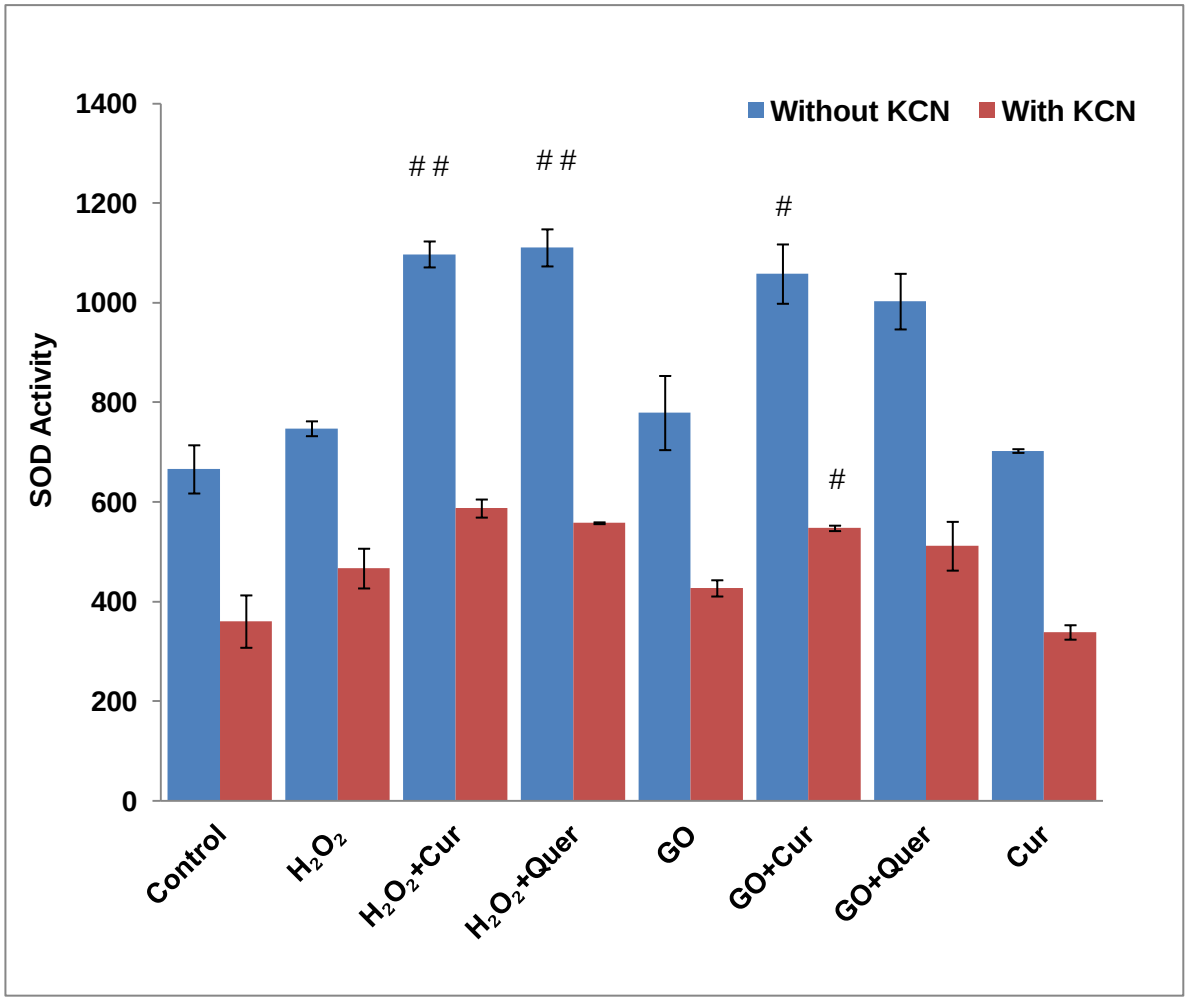

Figure 4.3a: Total SOD activity in total cell lysates with and without KCN, of control and oxidative stress induced U-87 MG cells and modulation by antioxidant phytochemicals Curcumin and Quercetin. The data was considered as statistically significant at $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.005$ when GO+C, H_2O_2+C, H_2O_2+Q treated cells were compared with only GO and H_2O_2 treatments respectively.

Table 4.3a: Total SOD activity in total cell lysates with and without KCN of control and oxidative stress induced (H_2O_2 and GO) U-87 MG cells and modulation by antioxidant phytochemicals Curcumin and Quercetin.

Total Cell Lysates	**SOD Activity**	
	With KCN	**Without KCN**
Control	360 ±52 (100)	665±48 (100)
H_2O_2	467±40 (130) (100)	747±15 (112) (100)
H_2O_2+Curcumin	587±18 (125)	1097±26$^{\#\#}$ (147)
H_2O_2+Quercetin	557±2 (119)	1110±37$^{\#\#}$ (148)
GO	427±16 (118) (100)	778±74 (116) (100)
GO+Curcumin	547±5.4$^{\#}$ (128)	1057±59$^{\#}$ (135)
GO+Quercetin	511±48 (120)	1002±56 (128)
Curcumin	338±14 (93)	1002±56 (105)

The student (*t*) test was performed to evaluate the significance of the results. The data was considered as statistically significant at $^{\#}p\leq0.05$ and $^{\#\#}p\leq0.005$ when GO+C, GO+Q, H_2O_2+C, H_2O_2+Q treated cells were compared with only GO and H_2O_2 respectively. The results are presented as mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with untreated control U-87 MG total cell lysates.

4.3b. <u>Cytosolic SOD Activity (CuZn-SOD)</u>: CuZn-SOD activity was measured in cytosolic samples of U-87 MG cells. When GO, GO+Curcumin, GO+Quercetin and H_2O_2, H_2O_2+Curcumin, H_2O_2+Quercetin treated were compared with untreated control cells it was observed that a significant increase in CuZn-SOD activity in H_2O_2+Curcumin 11% and H_2O_2+Quercetin 9% when compared with that of H_2O_2 treated cells (1935±7, 1910±42 vs. 1740±85). Also, there was 21% change in CuZn-SOD activity in GO treated cells when compared with untreated control U-87 MG cells. Curcumin showed 18% and Quercetin 15% change when compared with that of untreated control cells (2040±14** vs.1685±7), (1980±28** vs. 1685±7), (1930±28 vs. 1685±7) respectively (**Figure 4.3b** and **Table 4.3b**).

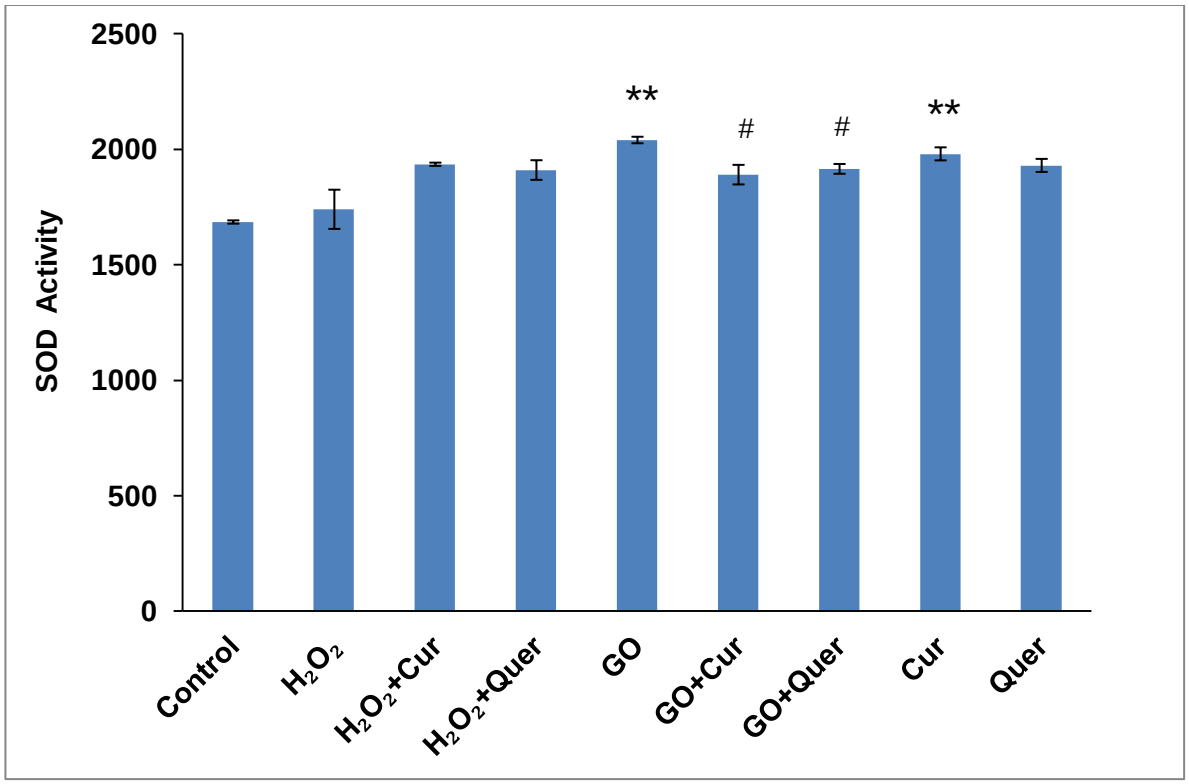

Figure 4.3b: The CuZn-SOD activity in cytosol of U-87 MG cells treated with H_2O_2, GO, Curcumin, Quercetin and their combinations for oxidative stress and phytochemical modulation for the inhibition of oxidative stress. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated control cytosolic samples were compared with Curcumin and GO treated; and $^{\#}$p≤0.05 when GO+C, GO+Q, H_2O_2+C H_2O_2+Q treated cytosolic fractions were compared with only GO and H_2O_2 treated samples respectively.

Table 4.3b: The CuZn-SOD activity in cytosolic fractions of U-87 MG cells.

Cytosolic fractions	CuZn-SOD Activity
Control	1685±7 (100)
H_2O_2	1740±85 (103) (100)
H_2O_2+Curcumin	1935±7 (111)
H_2O_2+Quercetin	1910±42 (109)
GO	2040±14** (121) (100)
GO+Curcumin	1890±42$^{\#}$ (93)
GO+Quercetin	1915±21$^{\#}$ (94)
Curcumin	1980±28** (118)
Quercetin	1930±28 (115)

The student (*t*) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 and **p≤0.005 when untreated cells were compared with H_2O_2 and GO treated cells; and $^{\#}$p≤0.05 when GO+C, GO+Q, H_2O_2+C, H_2O_2+Q treated cells were compared with only GO and H_2O_2 respectively. The results are presented as mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with that of untreated control U-87 MG cytosolic fractions.

4.3c. <u>Mitochondrial SOD Activity (MnSOD)</u>: The mitochondrial MnSOD activity was determined in isolated mitochondria after the treatment of oxidants and phytochemical modulation in U-87 MG cells. There was a significant increase by 80% in MnSOD activity was observed when H_2O_2 treated cells were compared with normal untreated cells (2700±325 vs. 1495±49). A 76% increase in MnSOD activity was observed when GO treatment was compared with normal untreated cells (2640±240 vs. 1495±49). Curcumin and Quercetin showed significant increase by 66% and 98% in MnSOD when compared with untreated control cells (2495±176* vs. 1495±49) (2965±346* vs. 1495±49) respectively (**Figure 4.3c** and **Table 4.3c**).

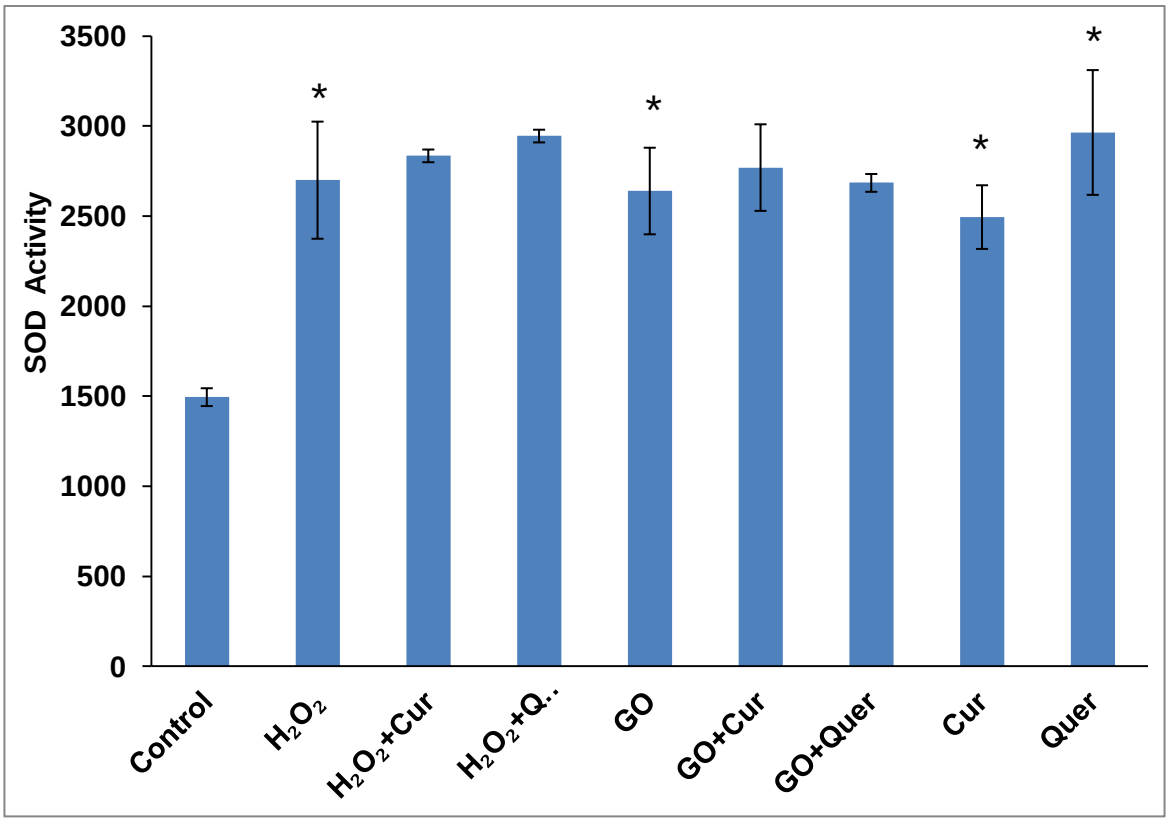

Figure 4.3c: MnSOD activity in mitochondrial samples made from U-87 MG cells. These samples were treated with H_2O_2 and GO; and only Curcumin and only Quercetin for oxidative stress induction and Curcumin and Quercetin as phytochemical modulation for inhibition in MnSOD activity by H_2O_2 and GO. The data was considered as statistically significant at *p≤0.05 when mitochondria of untreated cells were compared with H_2O_2 and GO, Curcumin and Quercetin treated cells.

Table 4.3c: MnSOD activity in mitochondrial samples of U-87 MG cells.

Mitochondria	MnSOD Activity
Control	1495±49 (100)
H_2O_2	2700±325* (180) (100)
H_2O_2+Curcumin	2835±35 (105)
H_2O_2+Quercetin	2945±35 (109)
GO	2640±240* (176) (100)
GO+Curcumin	2770±240 (105)
GO+Quercetin	2685±49 (102)
Curcumin	2495±176* (166)
Quercetin	2965±346* (198)

The student (*t*) test was performed to evaluate the significance of the results. The data was considered as statistically significant at *p≤0.05 when mitochondria of untreated cells were compared with H_2O_2 and GO, Curcumin and Quercetin treated U-87 MG cells. The results are presented as mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with that of untreated control mitochondria of U-87 MG cells.

4.4 Estimation of Antioxidant SOD Levels by ELISA

4.4a.<u>CuZn-SOD levels in total cell lysates</u>: SOD1 levels were estimated by using specific antibody for CuZn-SOD enzyme in treated and untreated total cell lysates of U-87 MG cells showed 26% increase when H_2O_2 treatment was compared with untreated control cell lysates (0.45±0.05 vs. 0.36±0.007). Whereas H_2O_2+Curcumin treatment showed 13% increase in levels of CuZn-SOD when compared with that of H_2O_2 treated total cell lysates (0.51±0.014 vs. 0.45±0.05). GO treatment did not alter any change in levels of CuZn-SOD. While Curcumin treatment decreased levels of CuZn-SOD by 78% when compared with untreated control total cell lysates (0.08±0.02** vs. 0.36±0.007) as shown in **Figure 4.4a** and **Table 4.4a**.

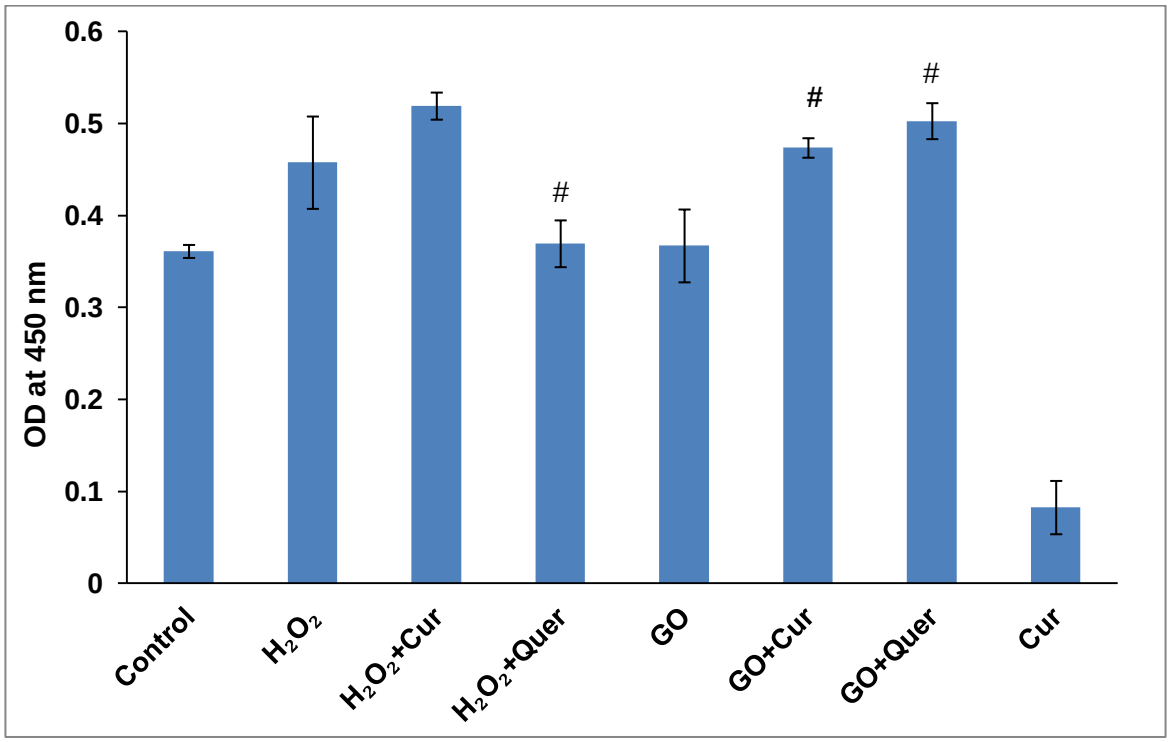

Figure 4.4a: CuZn-SOD levels measured by ELISA in total cell lysates of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at **p≤0.005 when untreated cells were compared with Curcumin treated cells; and $^{\#}$p≤0.05 when GO+C, GO+Q, H_2O_2, H_2O_2+Q treated cells were compared with only GO and H_2O_2 respectively.

Table 4.4a: CuZn-SOD levels measured by ELISA in total cell lysates of U-87 MG cells after induction of oxidative stress and modulation with phytochemical.

Total Cell Lysates	Levels of CuZn-SOD
Control	0.36±0.007 (100)
H_2O_2	0.45±0.05 (126) (100)
H_2O_2+Curcumin	0.51±0.014 (113)
H_2O_2+Quercetin	0.37±0.025$^{\#}$ (81)
GO	0.36±0.039 (101) (100)
GO+Curcumin	0.47±0.01$^{\#}$ (129)
GO+Quercetin	0.50±0.019$^{\#}$ (136)
Curcumin	0.08±0.02** (22)

The data was considered as statistically significant at **p≤0.005 when untreated cells were compared with Curcumin treated cells; and $^{\#}$p≤0.05 when GO+C, GO+Q, H_2O_2+C, H_2O_2+Q treated cells are compared with only GO and H_2O_2 respectively. The results are presented as showed in mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with the untreated control total cell lysates.

4.4b. <u>MnSOD levels in total cell lysates</u>: SOD2 levels using specific antibody for MnSOD estimated levels of the enzyme in treated and untreated total cell lysates of U-87 MG cells showed 537% and 785% significant increase in levels of MnSOD when H_2O_2+C and H_2O_2+Q was compared with that of H_2O_2 treated total cell lysates (0.39±0.039 vs. 0.06±0.0007) and (0.54±0.036** vs. 0.06±0.0007). GO treatment did not alter any change in levels of MnSOD. While GO+C, GO+Q and Curcumin treatments significantly increased the levels of MnSOD by 552%, 1023%, 447% when compared with GO treated total cell lysates (0.31±0.012** vs. 0.04±0.003), (0.52±0.032** vs. 0.04±0.003) and (0.30±0.021** vs. 0.04±0.003) respectively as shown in **Figure 4.4b** and **Table 4.4b**.

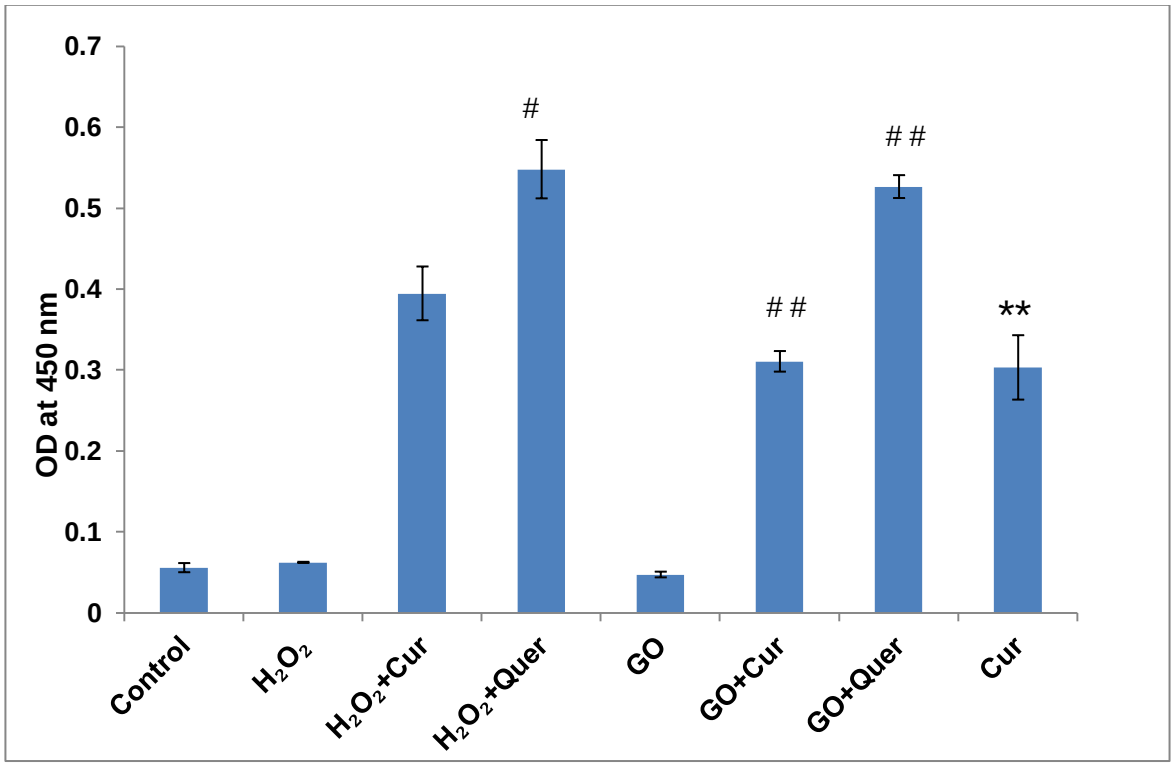

Figure 4.4b: MnSOD levels measured by ELISA in total cell lysates of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at $^{\#\#}p \leq 0.005$ when H_2O_2+Q, GO+C, GO+Q compared with H_2O_2, GO treated cells; and $^{*}p \leq 0.05$ when curcumin treated cells were compared with untreated control cells.

Table 4.4b: MnSOD levels measured by ELISA in total cell lysates of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals.

Total Cell Lysates	Levels of MnSOD
Control	0.05±0.005 (100)
H_2O_2	0.06±0.0007 (111) (100)
H_2O_2+Curcumin	0.39±0.039 (637)
H_2O_2+Quercetin	0.54±0.036$^{\#\#}$ (885)
GO	0.04±0.003 (84) (100)
GO+Curcumin	0.31±0.012$^{\#\#}$ (662)
GO+Quercetin	0.52±0.032$^{\#\#}$ (1123)
Curcumin	0.30±0.021** (547)

The data was considered as statistically significant at $^{\#\#}p \leq 0.005$ when H_2O_2+Q, GO+C, GO+Q treated cells were compared with H_2O_2 and GO treated cells; and $^{**}p \leq 0.005$ when Curcumin treated cells are compared with untreated control total cell lysates respectively. The results are presented as showed in mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with the untreated control total cell lysates.

4.4c.<u>MnSOD levels in Mitochondria</u>: SOD2 levels using specific antibody for MnSOD estimated levels of the enzyme in treated and untreated cells in mitochondria of U-87 MG cells showed 58% significant increase in levels of MnSOD when H_2O_2 treatment was compared with untreated control cell lysates (0.535±0.035* vs. 0.338±0.039). whereas H_2O_2+Curcumin and H_2O_2+Quercetin showed 44% and 37% decrease in levels of MnSOD when compared with that of H_2O_2 treated total cell lysate (0.301±0.020** vs. 0.535±0.035*), (0.338±0.053* vs. 0.535±0.035*).While GO, Curcumin and Quercetin showed only 19%, 12% and 14% increase in MnSOD levels when treated with untreated control cell lysates (0.402±0.044 vs. 0.338±0.039) and (0.379±0.036 vs. 0.338±0.039), (0.386±0.049 vs. 0.338±0.039) respectively as shown in the **Figure 4.4c** and **Table 4.4c**.

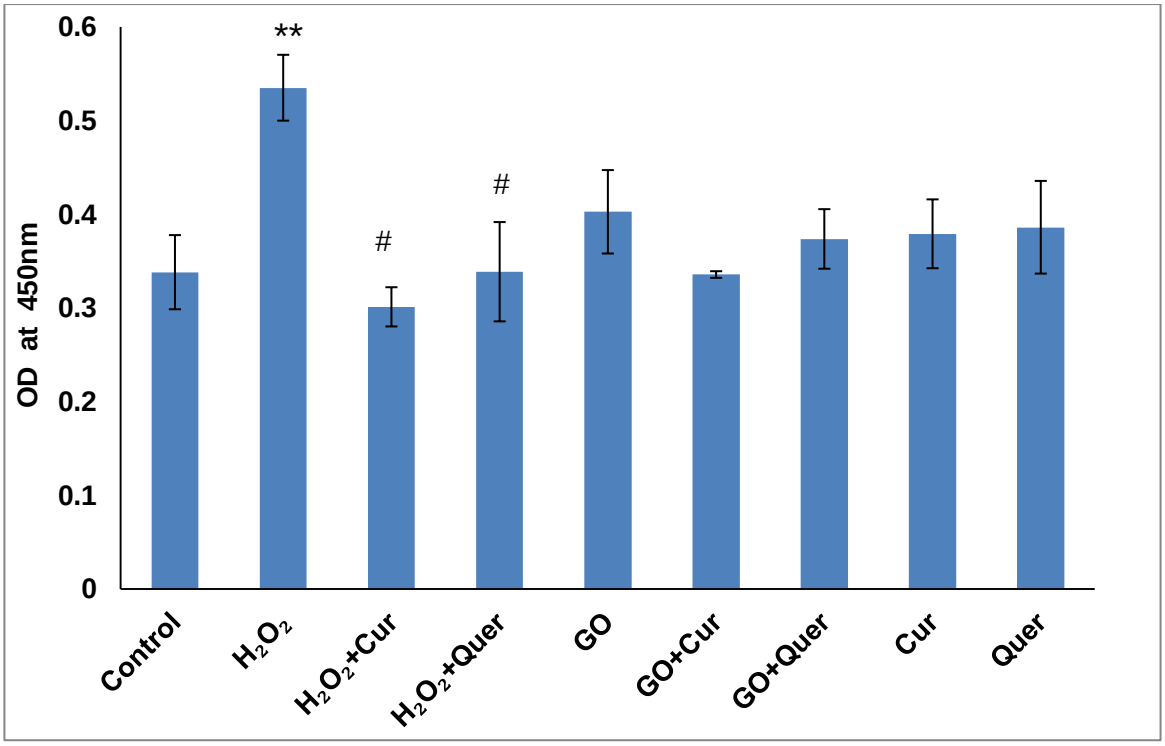

Figure 4.4c: MnSOD levels measured by ELISA in mitochondria of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at $^{\#\,\#}p\leq0.005$ when H_2O_2+C, H_2O_2+Q compared with H_2O_2, GO treated cells; and $^{*}p\leq0.05$ when H_2O_2 treated cells were compared with untreated control cells.

Table 4.4c: MnSOD levels measured by ELISA in mitochondria of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals.

Mitochondria	Levels of MnSOD
Control	0.338±0.039 (100)
H_2O_2	0.535±0.035** (158) (100)
H_2O_2+Curcumin	0.301±0.020$^{\#\#}$ (56)
H_2O_2+Quercetin	0.338±0.053$^{\#}$ (63)
GO	0.402±0.044 (119) (100)
GO+Curcumin	0.335±0.003 (83)
GO+Quercetin	0.373±0.031 (92)
Curcumin	0.379±0.036 (112)
Quercetin	0.386±0.049 (114)

The data was considered as statistically significant at **p≤0.005 when untreated cells were compared with H_2O_2 treated cells and $^{\#}$p≤0.05 and $^{\#\#}$p≤0.005 when H_2O_2+Curcumin, H_2O_2+Quercetin treated cells were compared with only H_2O_2 treated cells. The results are presented as showed in mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with the untreated control mitochondria.

4.5. Estimation of DNA Repair Enzyme APE1 levels by ELISA

4.5a. <u>APE1 levels in total cell lysates:</u> APE1 levels using specific antibody estimated levels of the enzyme in treated and untreated in total cell lysates of U-87 MG cells showed 2% increase when H_2O_2 treatment was compared with untreated control cell lysates (0.018±0.002 vs. 0.017±0.007). Whereas H_2O_2+Curcumin treatment and H_2O_2+Quercetin showed 163% increase and significantly 58% increased in levels of APE1 when compared with that of H_2O_2 treated total cell lysates (0.047±0.016 vs. 0.018±0.002) and (0.028±0.0007$^{\#}$ vs. 0.018±0.002). while GO and Curcumin treatment showed 73% and 97% increase in levels of APE1 when compared with untreated control total cell lysates (0.030±0.010 vs. 0.017±0.007) and (0.034±0.006 vs. 0.017±0.007) respectively as shown in the **Figure 4.5a** and **Table 4.5a**.

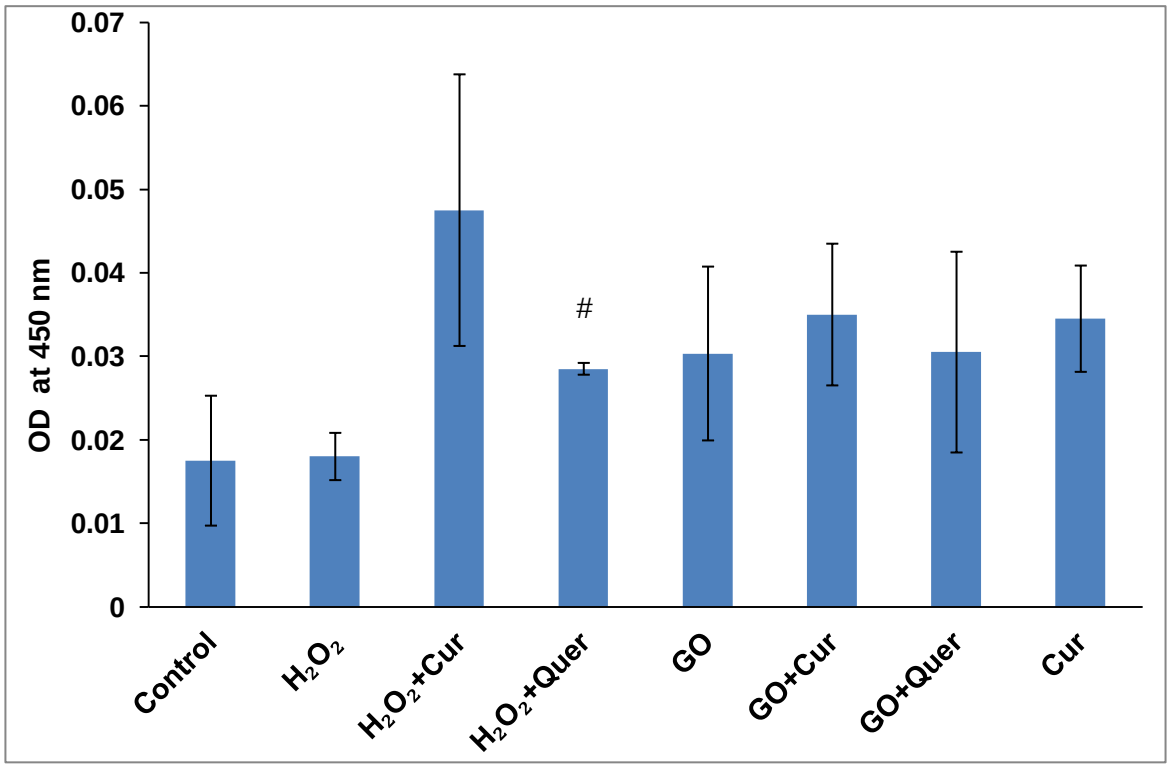

Figure 4.5a: APE1 levels measured by ELISA in total cell lysates of U-87 MG cells with induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at $^{\#}p\leq0.05$ when H_2O_2+Quercetin treated cells were compared with H_2O_2 treated cells.

Table 4.5a: APE1 levels measured by ELISA in total cell lysates of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals.

Total Cell lysates	Levels of APE1
Control	0.017±0.007 (100)
H_2O_2	0.018±0.002 (102) (100)
H_2O_2+Curcumin	0.047±0.016 (263)
H_2O_2+Quercetin	0.028±0.0007$^{\#}$ (158)
GO	0.030±0.010 (173) (100)
GO+Curcumin	0.035±0.008 (115)
GO+Quercetin	0.030±0.012 (100)
Curcumin	0.034±0.006 (197)

The data was considered as statistically significant at $^{\#}p \leq 0.05$ when H_2O_2 Quercetin treated cells were compared with only H_2O_2 treated cells. The results are presented as showed in mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with the untreated control total cell lysates.

4.5b. <u>APE1 levels in Cytosol</u>: APE1 levels in cytosolic fraction were measured using specific antibody. The enzyme in treated and untreated of U-87 MG cells showed 146% significant increase in APE1 levels when H_2O_2 treatment was compared with untreated control cytosolic fractions (0.020±0.0002* vs. 0.008 ±0.002). GO treatment showed 128% significant increase, Curcumin and Quercetin treatments increased by 222% and 76% when compared with untreated cytosolic fractions (0.019±0.004 vs. 0.008 ±0.002) and (0.027±0.002* vs. 0.008 ±0.002) and (0.015±0.0005* vs. 0.008 ±0.002) respectively as shown in the **Figure 4.5b** and **Table 4.5b**.

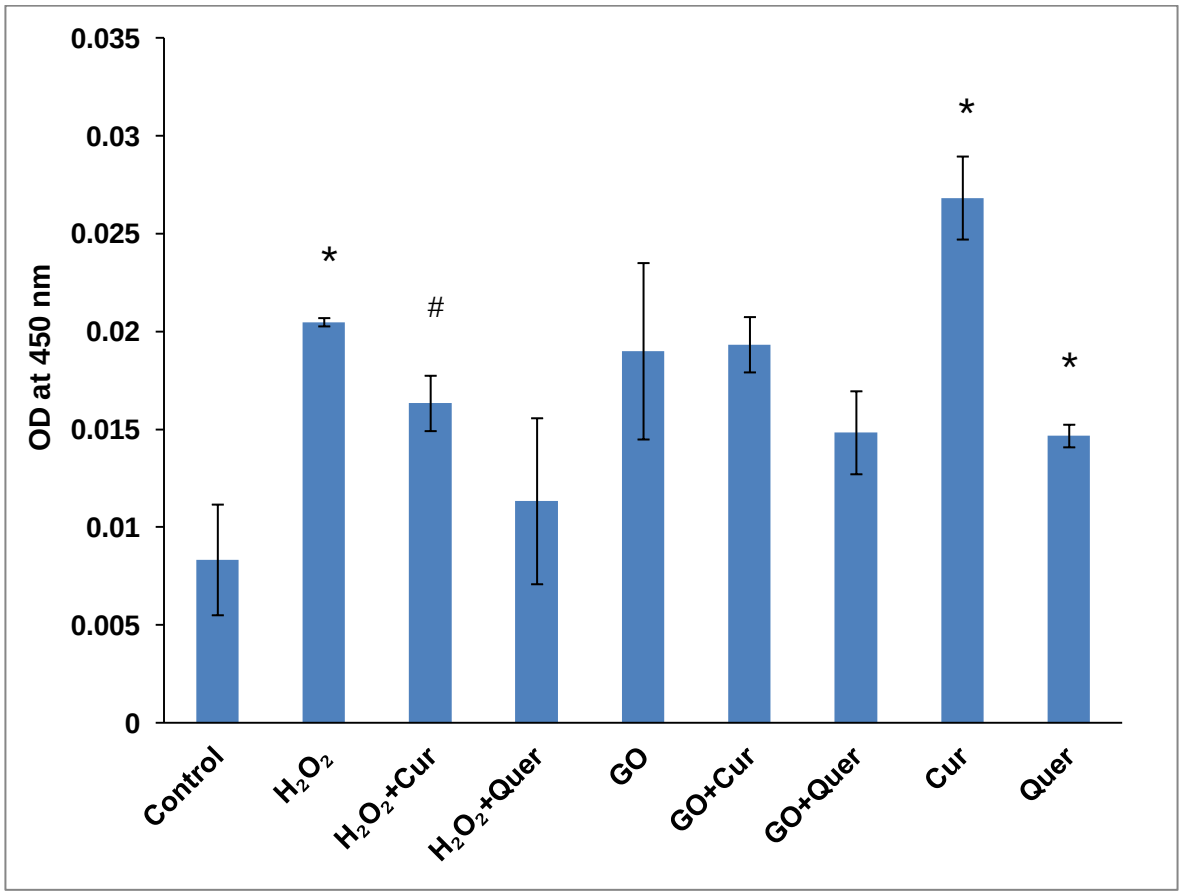

Figure 4.5b: APE1 levels measured by ELISA in cytosolic fractions of U-87 MG cells with induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at $^{\#}p \leq 0.05$ when H_2O_2+Curcumin treated cells were compared with H_2O_2 treated cells and $^{*}p \leq 0.05$ when H_2O_2, Curcumin and Quercetin treated cells were compared with untreated control cytosolic fractions.

Table 4.5b: APE1 levels measured by ELISA in cytosol of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals.

Cytosol	Levels of APE1
Control	0.008 ±0.002 (100)
H_2O_2	0.020±0.0002* (246) (100)
H_2O_2+Curcumin	0.016±0.001# (79)
H_2O_2+Quercetin	0.011±0.004 (55)
GO	0.019±0.004 (228) (100)
GO+Curcumin	0.019±0.001 (101)
GO+Quercetin	0.014±0.002 (78)
Curcumin	0.027±0.002* (322)
Quercetin	0.015±0.0005* (176)

The data was considered as statistically significant at #p≤0.05 when H_2O_{2+}Curcumin treated cells were compared with only H_2O_2 treated cells and *p≤0.05 when H_2O_2, Curcumin and Quercetin treated cells were compared with untreated control cytosolic fractions. The results are presented as showed in mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with the untreated control cytosol.

4.5c. <u>APE1 levels in Mitochondria</u>: APE1 levels in mitochondria were estimated in treated and untreated of U-87 MG cells. Which showed 56% significant decrease in levels of APE1 when H_2O_2 are compared with untreated control mitochondria (0.033±0.003** vs. 0.074±0.011). Whereas H_2O_2+Curcumin treatment showed 97% significant increase in levels of APE1 when compared with that of H_2O_2 treated mitochondria (0.65±0.013$^{\#}$ vs. 0.033±0.003**). while GO, Curcumin and Quercetin showed 53%, 60% and 41% significant decrease in level of APE1 when compared with untreated control mitochondria (0.035±0.007*vs. 0.074±0.011.) and (0.03±0.003** vs. 0.074±0.011), (0.044±0.007* vs. 0.074±0.011) respectively as shown in **Figure 4.5c** and **Table 4.5c**.

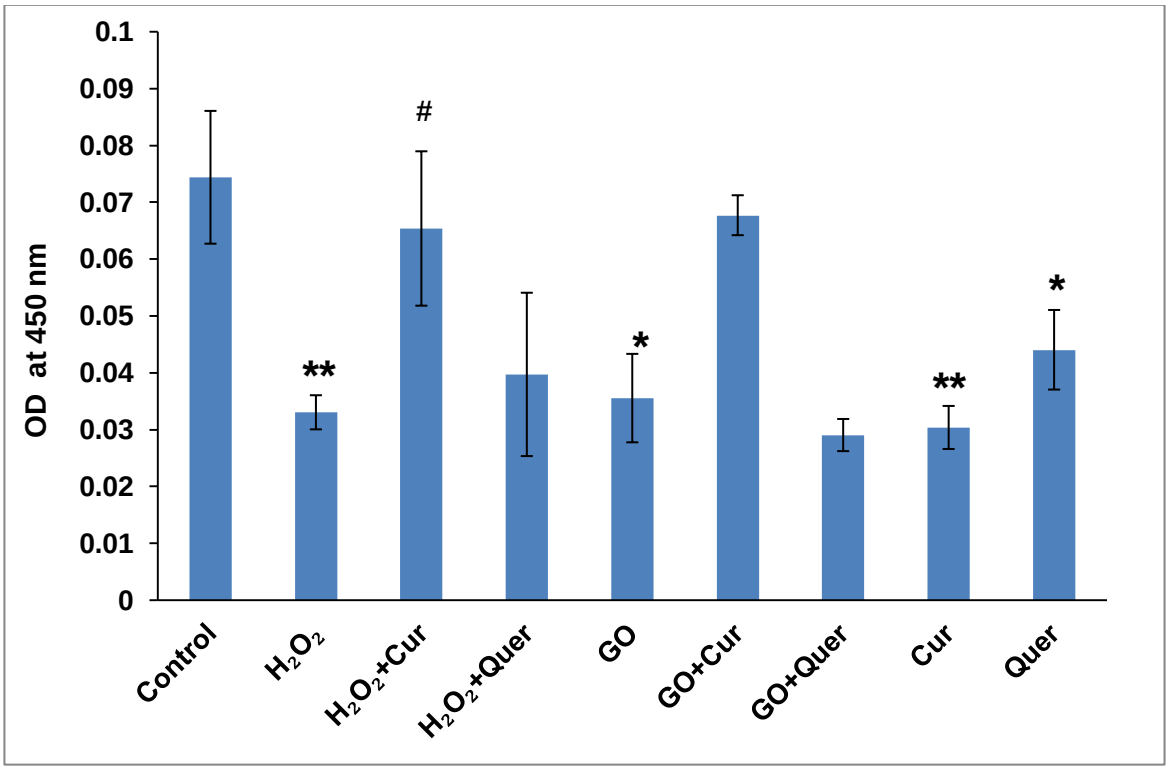

Figure 4.5c: APE1 levels measured by ELISA in mitochondria of U-87 MG cells with induction of oxidative stress and modulation with phytochemicals. The data was considered as statistically significant at $^{\#}p\leq0.05$ when mitochondria of H_2O_2+Curcumin treated cells were compared with H_2O_2 treated cells and $^{*}p\leq0.05$ when H_2O_2, GO, Curcumin and Quercetin treated cells were compared with untreated control cells of mitochondria.

Table 4.5c: APE1 levels measured by ELISA in mitochondria of U-87 MG cells after induction of oxidative stress and modulation with phytochemicals.

Mitochondria	Levels of APE1
Control	0.074±0.011 (100)
H_2O_2	0.033±0.003** (44) (100)
H_2O_2+Curcumin	0.65±0.013$^{\#}$ (197)
H_2O_2+Quercetin	0.039±0.014 (120)
GO	0.035±0.007* (47) (100)
GO+Curcumin	0.067±0.003 (190)
GO+Quercetin	0.029±0.002 (81)
Curcumin	0.03±0.003** (40)
Quercetin	0.044±0.007* (59)

The data was considered as statistically significant at $^{\#}p\leq0.05$ when mitochondria of H_2O_2+Curcumin treated cells compared with only H_2O_2 treated cells and $^{*}p\leq0.05$ when H_2O_2, GO, Curcumin and Quercetin treated cells were compared with untreated control Cells. The results are presented as showed in mean ± standard deviation (n=3), and all the values in parenthesis are percent change when compared with untreated control mitochondria.

CHAPTER V

DISCUSSION

ROS are mostly active in the brain and neuronal tissue and attack glial cells and neurons. Overproduction of ROS results in oxidative stress, which leads to mitochondrial dysfunction. Evidences indicate that mitochondrial dysfunction plays a key role in brain tumors and neurological disorders. In addition, ROS mediated oxidative stress results in overexpression of TFs such as AP-1 and NF-κB, which increases the survival of gliomas. The most common malignant brain tumor type is Glioma because it makes up 50% to 60% of all brain tumors in both children and adults (Adamson *et al*., 2009; Freeman *et al*., 1998).

Recent studies indicate that ROS plays a major role in tumor development (Storz, 2005; Waris & Ahsan, 2006). SOD is considered as the first line of defence against ROS generated in the cells. SOD scavenges and regulates level of superoxide produced within the tumor microenvironment and helps the tumor cell to fight and this helps in cell survival and progression. Superoxide anion radical that is produced during mitochondrial respiration is also involved in the production of several potentially damaging ROS. Different studies suggest that mitochondrial superoxide production and mitochondrial dysfunction play key role in neuronal apoptosis (Lin & Beal, 2006; Mattson *et al*., 2008; Sastre *et al*., 2000).

In the current study, it was hypothesized that how oxidative stress in glioblastoma can affect the ROS scavenging ability of the antioxidant enzymes. This study was planned to understand and determine whether oxidative stress in glioblastoma cells affect the expression levels of cellular antioxidant enzyme, SOD and BER-pathway's key DNA repair enzyme APE1.In addition, whether oxidative stress in glioblastoma cells affects the activity of SOD, another aspect of this study was to see whether phytochemicals such as Curcumin and Quercetin can modulate the expression levels and functions of two key cellular enzymes SOD and APE1.

In order to induce oxidative stress in U-87 MG cells, endogenously produced oxidants H_2O_2 and GO (10 µU of GO and 100 µM of H_2O_2) were used. But, before inducing oxidant stress with GO and H_2O_2, cells are pretreated with phytochemicals, Curcumin and Quercetin for 3 hr. SOD (CuZn-SOD in cytoplasm and MnSOD in mitochondria) activity was measured in order to see the superoxide

scavenging ability of SOD in response to the superoxides produced in the U-87 MG cells.

In this study, it was seen that no significant change in extra cellular NO levels occurred upon oxidative stress induced by H_2O_2 and GO treated cells but significant decreased level of extracellular NO was found in case of GO with Curcumin treated U-87 MG cells.

Total SOD activity (CuZn-SOD and MnSOD) was increased under the influence of oxidants (H_2O_2 and GO) in all cellular lysates (total cell lysates, cytosolic lysates and mitochondrial lysates) of U-87 MG cells in the presence and absence of KCN in all treatments groups. H_2O_2 and GO caused oxidative stress which lead to an increase in the activity of antioxidant enzyme SOD in total cellular lysates of U-87 MG cells. Our results are in accordance with the previously performed study in the laboratory, current study is an extension of that work (Kaur *et al*., 2013). Phytochemicals play an important role in the enhancement of cellular defence system as observed in various diseases. Under oxidative stress Curcumin and Quercetin, enhanced the activity of SOD to tackle the generated oxidative stress due to H_2O_2 and GO in U-87 MG cells. KCN is an inhibitor of CuZn-SOD activity, presence of KCN inhibited the activity of CuZn-SOD only in Curcumin treated, but not in Quercetin treated U-87 MG cell lysates. This indicates that Quercetin effect may be hindered by the other cellular active components.

Presence of phytochemicals (Curcumin and Quercetin) in H_2O_2 and GO treated U-87 MG cells; there was a further increase in the total SOD activity as compared with that of the treated groups of U-87 MG cells. A similar trend of increase was also observed in the cytosolic CuZn-SOD activity and MnSOD activity in total cell lysates of U-87 MG cells. The present result also indicates that phytochemicals enhance the antioxidant activity of SOD under oxidative stress by inhibiting the production of superoxide radicals.

This study found that the activity of SOD increased upon oxidative stress in the total cell lysates, cytosolic fractions and mitochondrial fractions of U-87 MG cells. To confirm further, whether the oxidative stress also affects the level of antioxidant enzyme SOD, ELISA was performed. Levels of CuZn-SOD in total cell lysates was found to be increased in H_2O_2 treated cells as compared to untreated

control, but comparable levels in GO treated cells. Treatment of phytochemicals, Curcumin and Quercetin in GO treated cells enhanced the levels of CuZn-SOD in total cell lysate as compared with untreated control and GO treated control total cell lysates. While, the pre-treatment of Quercetin the H_2O_2 treated cells showed comparable levels with untreated control and H_2O_2 control. Another phytochemical, Curcumin pretreatment to the H_2O_2 treated cells enhanced the level of CuZn-SOD in total cell lysates compared with H_2O_2 treated and untreated controls. There was no significant change in H_2O_2 and GO treated cells when analyzed for the level of MnSOD in total cell lysates. But, the pretreatment of phytochemicals, Curcumin and Quercetin significantly enhanced the level of MnSOD in total cell lysates. Further in mitochondria, MnSOD level upon treatment of H_2O_2 and GO enhanced in similar trend. An increase was also observed in the presence of Curcumin and Quercetin; but, pretreatment of Curcumin and Quercetin in H_2O_2 treated cells showed a decrease in levels of MnSOD in mitochondrial lysates as compared with untreated control. In GO treated mitochondrial lysates, Quercetin showed increase and comparable levels of MnSOD in mitochondrial lysates as compared with untreated control, but decreased level when compared with that of treated controls.

Recent studies by Mantha *et al*., (unpublished data) identified that the DNA repair enzyme APE1 is involved in regulation of intracellular redox state of the neuronal cells by inhibiting excess ROS generation produced upon oxidative stress. For determining the levels of APE1 in oxidative stress conditions that resulted in increased superoxide production and enhanced SOD activity advocates for these studies. In this study, the level of DNA repair enzyme APE1 was estimated using specific antibody with ELISA in total cell lysates, mitochondrial and cytosolic fractions after the oxidative stress induced by oxidants, H_2O_2 and GO in U-87 MG cells. Results indicate that APE1 level has been found to be increased in all treatment groups in the presence or absence of phytochemicals Curcumin and Quercetin which is in accordance with the published studies with other phytochemicals (Kelley *et al*., 2008). Other theory of literature suggested that APE1 and (NF-κB), two signaling molecules involved in survival pathways. Soy isoflavones decreased APE1 expression *in vitro*, whereas radiation up-regulated it. Pretreatment with soy isoflavones followed by radiation inhibited APE1 expression

(Raffoul *et al*., 2007). But H_2O_2 treatment showed presence of comparable levels of APE1 in total cell lysates of U-87 MG cells. APE1 level in cytosolic fraction was increased in all treatment groups, including phytochemicals Curcumin and Quercetin as compared with untreated cells. Presence of Curcumin and Quercetin in oxidants treated cells showed decreased levels of APE1 as compared with treated controls. The levels of APE1 recorded level in mitochondrial fractions found to be opposite in the treatment groups as compared with that of cytosolic fractions. There is a decrease in the level of APE1 in all treatment groups. Presence of Curcumin and Quercetin showed enhanced levels of APE1 in H_2O_2 treated cells as compared with that of H_2O_2 treated control. But in GO treated cells, Curcumin caused an enhanced level of APE1 and Quercetin caused a decreased level of APE1 in mitochondrial lysates as compared with that of GO treated cells. Phytochemicals, Curcumin and Quercetin were found to be potential antioxidants modulating levels and activities of both SOD and APE1 enzymes studied.

Some studies highlights that significant fraction of APE1 is cytosolic, and oxidative stress induces its nuclear and mitochondrial translocation (Mitra *et al*., 2007). Another studies state that APE1 subcellular localization is mainly nuclear, but cytoplasmic staining has also been reported, the latter being associated with mitochondria and/or presence within the endoplasmic reticulum. both expression and subcellular localization are altered in tumors and aging (Tell *et al*., 2005).

SUMMARY AND CONCLUSION

Oxidative stress is the central causative factor in various diseases including GBM, which is the deadliest form of brain tumors. Patients suffering with GBM have less than 15 months of survival period even after chemotherapy. ROS/RNS generated intra-cellularly cause oxidative stress and leads to mitochondrial dysfunction resulting in various neurological disorders. This study explored the possible role of intracellular oxidants such as H_2O_2 and GO in generating oxidative stress in human glioblastoma U-87 MG cells, further effect of generated oxidative stress on the activity and subcellular levels of antioxidant enzyme SOD.

In this study, it was seen that oxidative stress induced by H_2O_2 and GO in U-87 MG cells enhanced the activity of SOD (Cu-Zn SOD and MnSOD) in U-87 MG cells. CuZn-SOD levels were increased in U-87 MG cells treated with H_2O_2. Furthermore, MnSOD levels were increased in U-87 MG cells treated with H_2O_2 and GO.

This study also explored and observed that the modulatory role of phytochemicals, Curcumin and Quercetin on the SOD (total SOD, CuZn-SOD and MnSOD) activity and levels during oxidative stress environment in U-87 MG cells. In addition, the levels of DNA repair enzyme APE1 in U-87 MG cells under the influence of oxidative stress generated *in vivo* by H_2O_2 and GO was also determined. The key BER-pathway enzyme APE1 levels were found to be decreased in mitochondria, and found to be increased in cytosol in H_2O_2 and GO treated U-87 MG cells. Further, treatment with phytochemicals, Curcumin and Quercetin modulated subcellular levels of APE1.

This study provides target for further studies to find the expression levels and activities of other antioxidant enzymes to gain more insight. In addition, this study also advocates for further studies which can elucidate the underlying signalling mechanism(s) that regulates the expression pattern and sub-cellular localization of APE1; that may also be a route through which cancer cells survive.

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