"Effect of Piperine supplementation on Monensin induced stress using *in vitro* culture method"

A DISSERTATION PROPOSAL

In fulfilment of the requirement for the Award of degree in

Master of Science in Zoology

SUBMITTED BY:

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PUNJAB-144411

INTERNATIONAL JOURNAL OF ADVANCE RESEARCH, IDEAS AND INNOVATIONS IN TECHNOLOGY

ISSN: 2454-132X

Impact factor: 4.295

(Volume 4, Issue 2)

CERTIFICATE

This is to certify that **Fayaz Ahmad Lone** has completed M.Sc dissertation proposal titled **"EFFECT OF PIPERINE SUPLEMENTATION ON MONENSIN INDUCED STRESS"**under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of the dissertation proposal has ever been submitted for any other degree or diploma.

The dissertation proposal is fit for the submission and the fulfilment of the conditions for the award of M.Sc Zoology.

Date: Signature:

DECLARATION

I hereby declare that the dissertation proposal entitled, "**EFFECT OF PIPERINE SUPLEMENTATION ON MONENSIN INDUCED STRESS**" submitted for the M.Sc degree is entirely my original work, all ideas and references have been duly acknowledged. It does not contain any work that has ever been submitted for the award of any other degree or diploma from any other university.

Date: Fayaz Ahmad Lone

Registration no: 11510932

Acknowledgement

With humble sense and intellect of gratitude and thankfulness to Allah (the Almighty), I owe all my accomplishments to his blessings and holy sanctions. I would like to admire the strength and vigor he gave me during the period of hardships and adversities. Otherwise, the completion of the research would have been a mere flight of the imagination and daydream.

It is quiet pleasurable and gratifying to thank my supervisor Dr.Himanshu Agrawal, Assistant professor, School of Biosciences and Bioengineering, Lovely Professional University, Phagwara, Punjab for giving me a virtuous and proficient guidance to complete my research which will definitely prove to be a key march towards a vivid career ahead. I am heartily beholden to his approach and attitude which generated in me the spirit of adventure and quest related to the research and science. It is quiet relevant to mention here that this work would have been incomplete without his consistent encouragement and angelic guidance. I am pleased to acknowledge his advice, contribution, supervision and crucial involvement which run as the supporting thread throughout this study. His involvement with commitment and enthusiasm has prompted in me the intellect of zeal and zest that will promote me for a long time ahead.

I would definitely thank my parents whose heavenly support has never let me fall prey to the hands of distress and prejudice. It is their support which made me to trek the wilderness of uneven challenges of the life with ease and pleasure. I owe all my success and accomplishments to their clemency and blessings. Being bound to the format of this report i cannot appreciate the role they have played the way I should. Their contribution in my success is something that cannot be summarized.

Much of the appreciation goes to the aspiring and encouraging teachers who boosted my zest and quest for science and knowledge. I offer my regards and gratitude to all those who supported me in smoothening the progress of my work. It is high time to mention the support and encouragement that I got from my friends and colleagues. I heartily pay my thankfulness to Mr.M. Amin Wani, Ms Diksha Parihar, Mr.Waseem Ahmad Najar, Mr Tariiq Ahmad Lone and Ms Masrat Lone,Ms Fareeda Gani, Mr Zahoor Habib their prayers , back thumps and blessings paved a route to my success .

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Piperine is one of most and pungent alkaloid present in a popular spice namely black pepper(*Piper nigrum*)and some herbs of leaf moss. It has a long range of biological effects .It and oldest spice used in India due to its various properties now a days it is most used in medicinal purposes to treat certain diseases, including irregular heartbeats, Angina and neurasthenia Piperine has also anti-inflammatory, antiarthritic , antidepression like activity cognitive enhancing effect, and also a blood pressure lowering effect(Wattanathorn, et al., 2008).Besides these effects one of the most and powerful effect of piperine is its Antioxidant in nature which prevents the cells against (ROS) Reactive oxygen Species production or oxidative demage(Hu et, al 2009.,).Antioxidants properties of this alkaloid is due to the presence various secondary metabolites including phenols and Vitamin C A, flavonoids present in spice and leaf moss because they belonging to division plantae which is its own property(Guil-Guerrero et al., 2006).

Flavonoids and phenols posseses the antioxidant property and free radical scavenging activities because of the position of hydroxyl groups and other features in their chemical structures. Antioxidants protects the body against the demage causing reactive oxygen species (ROS).The free radicals are produced due to any strees inducing compounds or biochemical reactions and mostly form due to the unpaired electron which produced during the chain reactions in the cells. The various types of free radicals like peroxides, superoxides, hydroxyl radical and singlet oxygen.ROS production leads to demaging of the lipids, proteins , DNA (Mancuso, et al., 2012)and causes pathogenesis of lifestyle related diseases including hypertension , cancer, diabetes mellitus, aging, atherosclerosisetc. Different methods has been employed to extract these alkaloids from plants especially spice piper nigrum these method uses polar solvents like aqueous, ethyl acetate and acetone . It has been shown that natural antioxidants have wide range biological effects including antioxidant , antimicrobial, antiviral, anti-inflammatory and vasodilator activities(Srinivasan et .al.., 2007) .Natural products have historically and continiually been investigated for promoting new leads in pharmaceuticals development (Bailly et.al., 2009).like in case of bacterial resistance which is one of the main problem at which at which the new new antibiotic production has been produced slowly they are main source with a variety of structural arrangement and properties.From this research article it was

noticed that piperine supplementation decreases the effect of monensin carboxyl ionophore Monensin is one of a carboxyl polyether ionophore which increases Mitochondrial(ROS) Reactive oxygen species production by increases cell cycle arrest apoptosis through regulation of cell cycle and apoptosis.It has been used as as veterinary drug to control the protozoan related diseases especially coccidian parasites mostly demages mitochondrial membrane to much disrupts or stops mitochondrial function there is also breakdown of whole mitochondrial architecture .Mitochondria is most target organ attacked by monensin by increases the induction of the cell cycle disruption and an autophagy like process through a different signalling pathways depends on TgMSH-(Larvine et al., 2012).Monensin is likely to demages ionic gradients throughout cell membranes has been noticed in many animal or plant tissue this monensin interfere with the proper shuttling of protons which ultimately results into the loss of mitochondrial membrane and also cause in interruption of the electron transport chain, oxidative phoshorylation, and ATP production.In case of mammalian fibroblasts cells monensin induced toxity was noticed to develop an ionic improper balance that highly affected the shape size and function of mitochondria.Disruption or demaging of mitochondrial functions leads to generation of reactive oxygen species . Monensin, anionophore is responsible for causing of oxidative stress in prostate cancer cells.Mitochondrial, the most targeted effected by monensin which alters the various changes in mitochondrial functions (Dantasleite et al., 2004).To overcome this problem different types of antioxidant enzymes show various effects to decreases the oxidant stress caused by monsenin a carboxyl polyester ionophore.The various antioxidants enzymes show their their action after the treatment of piperine , an alkaloid obtained from black pepper.Different types of antioxidant enzymes are Catalase, Superoxide Dismutase, Gluthione peroxide, Glutathione , Reduced Glutathione etc.

Reactive oxygen species (ROS) are the molecules that may either oxidants such as hydrogen peroxide or reductants such as superoxide anion.These molecules imbalance the redox homeostatis of the cell.These are very toxic and produced during the time of reactions in the compound of the cells and develops the oxidative stress within the cells .These molecules mostly develops during the aerobic metabolism of the cell at the time of mitochondrial respiration chain system by the leakage of electrons(.Bouveris et al., 1982) .The reductant type of Reactive oxygen species like superoxide anion is not strongly reactive but can become reactive when strongly reactive with proteins .Whereas the hydrogen peroxide is relatively unreactive but has very deleterious effects when convert into hydroxyl radicals develops at the time oxidation of fatty acids in the peroxisome in the yeast .The main objective of this study

was determine the antioxidant activity in the presence of phytochemical content of various extract of piper nigrum leaves.in this study we will analyze the effects of black pepper which contains a pungent alkaloid piperine against the monensin activity .Monensin is a carboxyl polyether ionophore which causes oxidative injury due to production of ROS by demaging the mitochondrial membrane or disrupt the variors biochemical reactions .Monensin also increases the intracellular calcium ion concentration in the cell membrane and function just like a hydrogen peroxide which also possess this property .Increase in the concentration of calcium ion in the membrane effects the oxidative phosphorylation pathway impaired its function so that mitochondria losses its property to produced ATP molecules in the cell(Graham et al., 1990).The main result which comes after when the monensin is treated or injected by cells leads to oxidative stress or oxidative injury within the cells which demages the cell and causes the death of an organism .Oxidative stress occurs in the cells when cells losses a proper balance of redox regulation .The redox balance is maintained by intracellular level of free radicals and reactive oxygen species (ROS) as soon as the antioxidative capacity of the cell.In this study we explored that monensin induced oxidative stress in the cells by invitro method which transform the cell into oxidative state .Taking of other chemicals cause problems when take as antioxidants during Oxidative inducing stress .So in replace of using a chemical or drug to overcome oxidative stress we use an active compound obtained from spice black pepper called piperine a pungent substance or alkaloid which have a great importance in the medical field to protect against the various diseases caused by different microorganisms .Mostly one of the main property shown by piperine compound is it is antioxidant in nature prevent the body against the formation of free radicals or reactive oxygen species in different cell culture or animal model.It helps in the quenching of the free radicals from the body.So in this study The main objective is the analyse the effect of piperine which may act as antioxidant to prevent the cells against the formation of free radicals which develops in the cells when they are treated with polyether ionophore monensinin i*nvitro* culture method.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE:

Medicinal plants are mostly used having almost positively effect and treat diseases very fast having very less side effect in biological system as compared to other other ones .Piperine is one of the most spice used for various for treatment of diseases .In india and china it is used as a traditional medicine belongings to family piperaceae called as king of spices in india .Piperine[$C_{17}H_{19}NO_3$] a pungent alkaloid obtained from black pepper was first isolated from the fruits of Piper nigrum, (Oersted et al., 2005).Piperine has shown different numerous biological phenomena including anti-inflammatory activity, ehance fertility, antioxidant activity, antitumour, antidepressant, activity, antiplatelet activity(Wattatanathorn et al., 2008).Although piperine supplementation also resulted into toxity in hepatocyte and invitro cultured hippocampal neurons reproductive and immunotoxity , toxicity has also been reported in swiss albino rats.In rat hepatoma cells, piperine also combat the aflatoxin B1 mediated cytotoxicity in a concentration –dependent manner.

2.1 Piperine a bioenhancer: Piperine has shown different numerous biological phenomena including anti- inflammatory activity, enhance fertility, antioxidant activity, antidepreesent activity, antiplatelet activity. Although piperine supplementation also resulted into toxity in hepatocyte and invitro cultured hippocampal neurons. Different biological activities of piperine are reviewed in following section.

2.1.1 Antimicrobial activity of Piperine:

Piperine has shown the antimicrobial activity prevents body against the attack of microbes Khan and siddiqui in 2007 reported that aqueous decoction of Piper nigrum have very high antibacterial activity. They isolated different bacteria from the buccal cavity of atleast two hundred individuals. Aqueous decoction of black pepper shows high activity as antibacterial as compared to aqueous decoction of lourisnobilis and pimpinellaanisum at concentration of 10microlitre/disc. .Kumar et al., 2002in another study, by synthesise silver nanoparticles from stem and the leaf of the Piper nigrum, was count as against agricultural plant pathogens .Silver particles from this show good antibacterial activity against the pathogens that destroys crops .They also concluded that silver nanoparticles isolate from this plant play important role in crop improvement and protection.

2.1.2 Antidepressant activity of piperine

Piperine has also shown antidepressant activity in the mice to reduce the stress in the mice.Antidepressants are the drugs used to control depression helps the body to control stress

Song et al., in 2007 reported the antidepressant effect of piperine in mice suffering from chronic mild stress condition.Continous doses of piperine was given for 14 days 2, 5, 5 and 10mg/kg changes the chronic stress induced type changes in sucrose utilization, plasma corticosterone level etc .Hippocampal progenitor cells proliferation was decreased and the level of brain originated neutrophic factor in the hippocampus of chronic stressed mice was enhanced by piperine supplementation.In another study Wattanathorn et al., (2008) treated wistermale, rats with different concentration piperine i.e. 5, 10, 20mg/kg/day body weight for at least 4 weeks and the various activity like cognitive function , forced swimming test, and behaviour was observed after each 1, 2, 3 4, weeks of treatment. The result noted that piperine dose in all the treatment group decreased the depression activity and increases the cognitive enhanced activity.

2.1.3. **Anti inflammatory activity of piperine**

Inflammation is a type of biological response which show by body after the attack of pathogens which is characterised by redness, swelling and pain in the localised area. Sarvesh et al., in 2007 reported that piperine prevents the attachement of neutrophils with endothelial monolayer.Endothelial monolayer cells blocks the tumour necrosis factor –α, which induces the expression of cell adhesion molecules. They also observed that protreatment of endothelial cell with piperine decline the phosphorylation and slow down the IkB-α through by reducting the function of tumour necrosis factor-α induced IkB Kinase activity.Pradeep et al., in 2004 studied that piperine at 2.5, and 10microgram/ml concentration prevents the collagen matrix proliferation of B16F-10 melonoma cells in dose based manner. It also decreases the activity of various pro inflammatory cytokines (IL-6, GM-CSF, TNF-α)

2.1.4 Anti platelet effect of piperine.

Platelets are the cells found in circulatory system of animals helps in blood clotting mechanism.Park et al., in 2007 separated four amides or acrimedes piperine, pipernolanine, piperictadecalide and piperlongunine from the black pepper. They find that these compounds prevent effect on washed rabbit platelet clumps which is influenced by collagen, arachiodonic acid, thrombin and other factor that activates platelets count. Piperine also showed dose dependent inhibitory activitats on platelets clumping together except by thrombin that increase platelet count in normal cases.

2.1.5 Anti thyroid activity of piperine.

Thyroid is a endocrine gland present in the neck region helps to control basal metabolic rate , tissue regeneration growth and development excess or low concentration of thyroid hormones leads to malfunctioning of the gland. Kumar et al., in 2006analyzed the effect of piperine with carbimazole. There was slightly decrease in plasma lipids and lipoprotein concentrations but the concentrations of high density lipoprotein was contionously increase. Continous administration of piperine increase the plasma level of APO, A-1.T3, T4 testosterone and significantly decreasedApo –B and insulin to the normal level. In another study, Pander et al., 2003 provide piperine supplementation for 15 days at 0.25 and 2.5mg/kg/day to mature male swiss albino mice. Piperine at the dose level of 2.50mg/kg decreases the serum level of both the thyroid related hormones T3, T4, Thyroxin, and tri iodothyronine T3 and also reduced the glucose concentration by decrease an enzyme glucose 6-phosphate (G-6-P)level.They also observed that nochanges were found when the animals were treated with 0, 25/kg of piperine except reductance of concentration of T -3 Serum level.

2.1.6. Anti-hypertensive effect of piperine.

Hypertension is increase in the blood flow inside the blood vessels which causes the death of an organism. It is a silent killer disease.Taqviet al., in 2008 studied that intravenous supplementation of piperine 1-10 mg/kg reduce in accurate arterial pressure in anestheized rats, another dose was given 30g /kg did not show in any changes in arterial pressure.In another study, Iinvitro supplementation of piperine on the rabbit cardiac cells decreased the contraction and blood flow towards muscle of heart. In aorta of rabbit, piperine decreased the K^+level up to (80mM) due to blockage of Ca^+channel ions partially.

2.1.7. Anti asthamatic effect of piperine.

Asthama is a type of respiratory disorder in which there is increase in the goblet cells resulting into secretion of more mucous which cause difficulty in breathing. Kin et al., in 2009 in another study supplemented 4.5, 2.5mg/kg of piperine orally 5 times in a week for 8 weeks. Piperine inhibits the eosniohils infiltration and decreased the allergy and lowered the mucous secretion cells i.e goblet cells.Piperine also stops the secretion of various cytokines e.g interlikun-4, Interlikun -5 and immunogloblin E antibody which is responsible during allergic conditions.

2.1.8. Anti apoptosis effect of piperine

Apoptosis is a morphological process to control cellular self distruction of cells.It is important process maintain the population of cells .Choi et al., in 2007 suggested that piperine at the concentrations of (10-100mM)prevents the organ of corti -1 cells of cochlea a hearing organ in ear from cisplantin inducing apoptosis by the increase the enzyme activity of the enzyme oxygenase -1 .the main pathway responsible for this is C-JUN terminal kinase.

2.1.9. Anti-diarrheal activity of piperine

Diarrhea is gastrointestinal disorder in which there increase in bowel moments losing the water balance in the body. Shamkumaret al., in2012 demonstrated that liquid piperine extract at a dose of 75, 150, 300mg/kg showed anti –diarrheal, anti motility and antisecrety property in mice. Castor oil and magnesium sulphate was orally given to mice to induce diarrhea.

2.1.10. Digestive activity of piperine.

Digestive system play important in absorption and assimilation of food particles by killing the microorganisms at the time of digestion.Srinivasan et al., in 2007 demonstrated that dietary piperine increases the digestion physiology by stimulating the activity of pancreatic enzymes and slow down the food transit time in the alimentary canal.Piperine also increases the production of saliva by enzyme salivary amylase .The orally piperine supplementation enhance the function of liver to produce more bile acids which further helps in the emulsification of fats.The oral given compound like piperine, pipene, pipiramine, stimulate the activity of various enzymes of gastrointestinal tract like protease activity, lipase activity and chymotrypsin activity.Dietary piperine20% increases the activity of enzymes piperine also decreases the food transit time.It was observe by stool the piperine at the concentration of 0.2%slighly decreases the food transit time and increases the enzyme activity helps in digestion of foods easily.

2.1.11 Piperine effecsts on metabolism; a bioavailability enhancer.

Metabolism is building and breakdown of materials inside the cells helps in various process that occurs inside the body.Acharyaet al., in 2012 demonstrated that piperine supplementation helps in the absorption of various drugs and nutrient substances from the alimentary canal by different process changes the membrance architecture and increases the penetration level of absorption. Piperine also increases the serum half lives of beta carotene and co enzymes –Q but also reduce the metabolism of different drugs by stops the activity of certain metabolism enzymes e.g cytochrome NADPH(UDP-GDH).Piperine stops the activity of drug inhibitor enzymes and enhances the bioavailabilty of many

antibiotics and nutrients examples like ampicillin, amoxillin, beta carotene , gallic acid various methods so piperine also acts as a bioavailability enhancer.

2.1.12Anticancer activity of piperine.

Cancer is uncontrolled or abnormal growth of cells which develops into mass like structure called tumour.Samykuttyet al., in 2013 evaluated the anticancer activity of piperine in prostrate cancer cells lines.Piperine induces apoptosis mechanism of cell by the activation of caspase 3-enzyme a family of enzymes which helps in apoptosis mechanism in the cell. These enzymes causes the breakdown of PARP-1 proteins in many prostrate cancer cells like PC-3, DU 145, And LNCAP, Prostrate cancer cells.It was noticed that piperine treatment demaged the androgen receptor expression in LNCAP, prostate cancer cells and cause decrease in the level of prostrate antigen in LNCAP cells. The STAT-3 and other nuclear and transcription factor also reduced in prostate cancer after the treatment.Piperine slightly decrease the androgen dependent and independent growth of tumour in naked mice model. It has been also observed that piperine supplementation in tumour cells blocks the G1/S phase of cell cycle in tumour cells decrease the functions of nuclear and transcription factors of cell.

2.1.13 Piperineas an antioxidant

Antioxidant molecules prevents the oxidation in cells which further leads to development of free radicles. In a previous study, Kumar et al., in 2004 studied the effect of that piperine supplementation in rat feed with diet rich in fats. They demonstrated that regularly supplementation of diet enriched with fats and piperine decrease the level of thiobarbituric acid associated reactive substances conjugated dienes and enhanced the activities the various antioxidant enzymes e.g , superoxide dismutase, , catalase, gluthioneperoxide, gluthione transferaseetc .

In another study Selven diranet al., in 2005 analysed the effect of piperine on DNA demage and quality through detoxifying enzymes, like gluthione- s-transferase, UDP-Gluthanione-transferace in pulmonary cancer animals which is caused by Benzo(a)pyrene.They conclude that administration of piperine (50mg/kg)body/wt increase the working of enzymes and helps in protection the DNA from damage. It also helps in enzymatic detoxification which was analysed by single cell electrophoresis.

2.2Oxidative stress

Oxidative stress is defined as the changes in which there is increase of oxidation process which exceeds the antioxidant system in the body and loss the balance between them .During the process of oxidative stress there is release of free radicals which damages the biomolecules of the cells lipids, proteins , and DNA.It is involved in the pathogenesis of various diseases in the body including hypertension, diabetes mellitus, and atherosclerosis etc.

Atoms made of nucleus and have pairs of electrons orbiting around it.Due to some changes occur which normally happens inside the cell due some chemicals and other changes leads to development of free radicals having odd electrons.Free radicals are unstable and higly reactive and form link with other electrons present in the cell.The various types of free radicals are superoxide, nitric oxide, peroxide, hydroxyl radical, and singlet oxygen.Free radicles mostly attacks the mitochondria of the cell causes their disruption stops the ATP formation and loss of oxidative phosphotylaton pathway.Free radicals reacts with biomoleclar elements of the cells altering the intercellular and intracellular homeostatis.

2.2.1 Types of reactive species

There are two types of reactive species existing in nature ROS Reeactive oxygen species and RNS Reactive Nitrogen species.One of ROS namely supderoxide is produced mostly by electron reduction of oxygen by different oxidases e.g NADPH, Oxidase, Xanthine and mitochondrial electron system during the process of oxidative phosphorylation which helps in the formation of ATP moleclues (Evans et al., 2005).

2.2.2 Sources of ROS generation in yeast cells

Environment is one of main factor which generates the reactive oxygen species production and other reactions of aerobic process.It also play important role in generation of ROS production in yeast.At the time of production of ATP molecules by cells, electrons are transported with protein molecules that having the electron electron transport system to finally receiver , molecular oxygen along with formation of water.Existing of these electrons from the respiratory chain can result in the decrease in the oxygen content generates the ROS in yeast cells.Peroximal fatty acid degradation in case of β-Oxidation pathway and oxidative deamination of amino acids by D-amino acid oxidases the growth conditions also generates ROS production in yeast (Hiltunen et al, 2003).Some exogenous agents like xenobiotics,

carcinogens, and UV and ionizing radiations are the other factors which generates ROS production in yeast(Halliwell 2006).

2.3 Yeast culture and its importance.

Yeast are the unicellular microorganisms belongs to kingdom fungi.They have the cellular organisation as that of same to higher organisms having well developed nucleus which contain the genetic material found inside it this property classifies the yeast into eukaryotic single cell organsism.

2.3.1 Yeast as model organsisms

Yeast(Sachromyces cerevassie) a single cell organism grows very fast. It can be quickly replicated and easy to manipulate genetically. It contain cellular organisation as that of complex organisms including humans.In case of yeast, the doubling time .i.e the time in which yeast cells duplicate and divides its own cell is almost 90 minutes as compared to humans cells which needs 24 hours for growing and divides itself. Different method are available to separate different mutants, cross them with other and map the gene location. These methods help in gene localization and understanding of genome organisation. It is used as model organsism because its cellular structures show relevance to higher organisms which makes it very easy and powerful "model organisms"for study.

2.3.2 Monensin an ionphore

Monensin is a vetenary drug used in veterinary science for different purposes. Monensin produced by streptomyces cinnamonensin is mostly widely used ionophorein vetenary medicine according to food and drug administration mostly used for coccidiosis diseases in poultry birds (Russel et al., 2009). It is carboxylic polyether ionophore obtained from actinobacteria strains of Streptomyces cinnamonensin. It is mostly used to control the protozoa species that causes many diseases especially coccidiosis. Monensin mostly effects microbes by causing the imbalance of ion transport which leads to more intake of sodium ions and loss of potassium ions in cells of microbes (Russel and strobel 1989). Some microbes are unaffected against monensin treatment because they have no potassium in their membranes e.g. some gram positive bacteria (bragget et al., 1977).Monensin is also unaffected against methanogenic bacteria (Russell and strobel, 1989). It has been observed that monensin also decreases the microbial activity of intestine.Monesin early known to kill the T.gondi a protozoan in both invitro and invivo (Frenkel et al., 1982) by rupturing the bradyzoite cysts and causes the

disruption of cell cycle by demaging the mitochondrial membrane of T.gondi (Garrison et al, 1998). Mitochondria is the power house of the cells disruption in the mitochondrial membrane causes the death of an organism .Mitochondrial dysfunction also causes demages in electron transport and oxidative phosphorylation pathways and stops the ATP production (Graham et al, , 1990).Loss of mitochondria function leads to development of reactive oxygen species production which causes oxidative stress in organism .Monensin mostly affects mitochondrial function and is mostly responsible to develop oxidative stress.the main target of monensin was mitochondria.

When the monensin exposure was given to plants bearing culture cells there was shown the degrowth of plant and decreases the various cellular process of plants. In case of plants, the most target organ of monensin is golgi body , where as in animal cell monensin mostly attack mitochondrial membrane and also Golgi body(Mollenhauer.et, al., , 1990).Monensin reduces the transport of macromolecules inside the cell and also increase the ph of the cell which disrupt or decreases the activity of cell by secrete less secretions and other products .it also decreases the secretion of substances which play important role in various process e.g collagen formation , fibronectin proteins, and proteoglycans, (Morre.et al., 1990).

Park et.al., 2003.demonstrated that monensin have antitumor property by stopping the invasiveness of renal cells by the process called apoptosis in the cells which was cancerous by block the cell cycle in the G or G-2 –M phases. It was also knowing that it also decreases the level of mitosis inducing proteins during cell cycle e.g .CDK2, CDK6, CDC2, Cylin D1 and Cyclin A , CyclinB, Cylin E proteins etc .

2.3.3.Antibacterial activity of monensin.

Monensin due to its ionophoric nature shows very large amount of antimicrobial activity fluctutates the ph balance in the cells by altering the sodium potassium balance within the cells create very treat alterations in the cellular structures which ultimately leads to death of an organism (Russell et.al., 1987). It mostly effects the Gram positive bacteria species of Micrococcus, Baccilus, and Staphylocus (Dorkov et al., 2008). It also possess the antiviral properties due its less or no effect on Gram –ve bacteria it was conclude that the ionophores possess also Antiviral properties.Stop the spreading of semliki forest virus and sendbis virus by inhibit their replication .Schlegal et.al., 1981).Iacoangeli And co-workers in 1995 demonstrated that monensin stops DNA Synthesis , ceases the replication, and increases the strong reduction of firstly viral antigens of murine polyoma virus.

CHAPTER 3

MATERIAL AND METHODS

Material and Method

Saccharomyces cerevisiae(Yeast)stock culture were taken from LPU, Microbiology Department, Phagwara, Punjab. *S.cerevisiae* was revive and used for all the experiments.

3.1 Revival of cultures

The Yeast cells from glycerol stock were first revived on YPD media under specific growth conditions at temperature 30°C for 48 h.

3.2 Monensin and Piperine :

Monensin and piperine was purchased from Himedia labroraties India. Monensin stock and piperine stock solution was prepared using ethanol and methanol . Working stock was prepared from these stock.

3.3. Yeast culture and growth condition:

For antioxidant and MTT assay, cells were grown in YPD media containing 1 % yeast extract, 2 % peptone and 2 % glucose on an orbital shaker at 30 °C and 160 rpm.

Figure 1 : Representative photograph for Yeast culture media

3.4. Agar culture

To check the purity of yeast cells, these cells were grown in YPDA media containing 1 % yeast extract, 2 % peptone, 2 % glucose and 1.5 % agar in a bacteriological incubator at 30°C.

3.5 MTT Assay

To check the effect of monensin and piperine on cell viability, MTT assay was performed. Yeast cells density was adjusted at OD600 of 1.8. These cells were washed once with distilled

water and pH was adjusted to 7.4. 20 µl cells were inoculated in test tube containing 3 ml YPD media supplemented with piperine and monensin and grow till early exponential phase. The different experimental group were control, Monensin (10 µM), Piperine (120 µM) and Monensin (10 µM) + piperine (120 µM). To achieve desired concentration, Piperine and monensin from stock were added in culture media to analyse their effect. 1 ml cells suspension from different group were harvested through centrifugation at 8000 rpm for 10 minutes. Cells were suspended in PBS. 120 µl of cell suspension and 80 µl of MTT was added and incubated for 2-3 hours in a mechanical shaker. Afterwards cells were harvested and resuspended in 100 µl of DMSO followed by incubation for 10 min. lastly, cells were against centrifuge at 8000 rpm for 10 min. Supernatant was collected and O.D. of supernatant was taken at 540 nm.

Figure 2 : Representative figure of Yeast growth in YPDA media

3.7 Estimation of Protein

The basic principle of protein estimation is reduction of phosphomolybdic and phostungustic of folin reagent through aromatic amino acid tyrosine and tryptophan. Biuret reaction of protein with alkaline cupric tartrate also enhanced the colour development and were measured at 660 nm. The different reagent required for this assay are as follows.

- 0.1 M Phosphate buffer
- 2 % Sodium carbonate in 0.1 N NaOH
- 1% Sodmium Potassium tartrate
- 0.5% Copper sulphate
- Follin's reagent 1N
- Homogenate or BSA standard

Lowry reagent was prepared adding 48ml of 2% Sodium carbonate in 0.1 N NaOH + 1 ml of 1% of Sodium Potassium Tartrate + 1 ml of 1% Copper sulphate.

Stock protein standard (BSA) (1mg/ml) was prepared by dissolving 10 mg Bovine serum albumin in 10 ml of water. Working standard (100 µg/ml was prepared by dissolving 1 ml of stock in 9 ml of water. Different concentration of working BSA standard was prepared by as per following table.

Table 1: Different concentration of BSA working standard samples

S. No.	Volume of BSA working standard (ml)	Distilled water (ml)	Concentration (µg/ml)	Optical density
1.	0.2	0.8	20 µg/ml	0.068
2.	0.4	0.6	40 µg/ml	0.137
3.	0.6	0.4	60 µg/ml	0.190
4.	0.8	0.2	80 µg/ml	0.251
5.	1	0.0	100 µg/ml	0.351

5 ml of lowery reagent was added to 1 ml of each standard and mixed thoroughly. Tubes were kept for 5 min in room temperature and thereafter 0.5 ml of 1 N folin reagent was added into each tube. Tubes are incubated for 30 min at RT and then absorbance was taken at 660 nm.

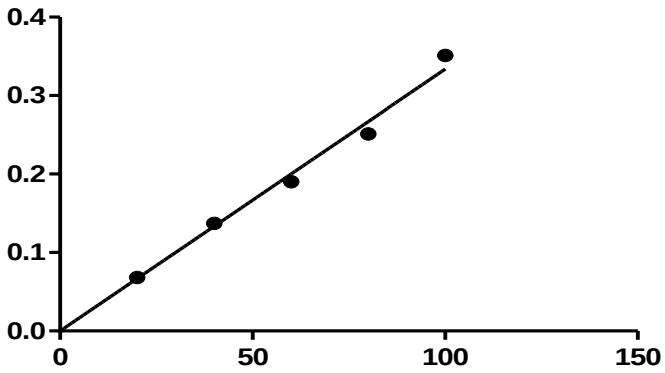

Figure 3 : Standard graph preparation through BSA for protein estimation.

3.8 Preparation of cells homogenate:

For this, tissue homogenate buffer was prepared containing 5 ml of 0.5 M EDTA, 5 ml of 0.01 M Tris HCl and 0.5 ml β mercaptoetanol (pH 7.4). 10 ml cell suspension was centrifuge at 8000 rpm for 10 min and cell pellet was lysed in cell homogenate buffer through centrifuging at 3000 rpm for 10 min. Supernatant was collected and further used for the preparation of post nuclear supenatent (PNS) and post mitochondrial supernatant (PMS).

3.8.1 Preparation of post nuclear supernatant (PNS):

The cells supernatant collected in first step was centrifuged at 2500 g for 10 min at 4°C in cold centrifuge to get the nuclear pellet and supernatant. The supernatant (PNS) was removed and stored at -20 °C for future use. Lipid peroxidation and catalase activity was anlyzed using PNS.

3.8.2 Preparation of post mitochindrial supernatant (PMS):

Supernatant obtained after PNS was again centrifuge for 10 min at 10, 000 rpm at 4 °C to get mitochondrial pellet and post mitochondrial supernatant. PMS was also stored at -20°C for future use. Total Glutathione level was measured through PMS.

3.9 Catalase assay:

This method is based on the principle that dichromate dissolve in acetic acid reduced to chromic acetate after reacting with H_2O_2 in hot condition. In this reaction, perchloric acid is produced as unstable intermediate. Chromic acetate was measured in calorimetrically at 610 nm. This reaction was allowed to proceed at time interval to split the H_2O_2. Reaction was stopped at different time interval 0, 30, 45 and 90 second by addition of dichromate / acetic acid mixture. Remain H2O2 was measured at through measuring chromic acetate calorimetrically.

Reagents –

- Hydrogen peroxide 0.5 M
- 0.01 M Phosphate buffer
- Stock Dichromate Acetic Acid reagent – Mix 5% Potassium dichromate with Glacial Acetic acid (1:3)
- Working dichromate/acetic acid solution: To prepare this, stock solution was diluted to 1:5 with the help of distilled water

- 0.2 M H_2O_2

After preparation of the working stocks of chemicals, we taken 0.5ml Hydrogen Peroxide + 1ml buffer + 0.4ml water + 0.2 ml of homogenate in a test tube add mixed thoroughly. After wards 2 ml acetic acid reagent was added after incubation of 0 second 30 second 45 second and 90 second and then tubes were heated for 10 minutes. Control tube was added homogenate after addition of acid reagent and absorbance was read at 610 nm.

3.10. Lipid peroxidation:

Malondialdehyde is the main product of lipid peroxidation which reacts with thiobarbituric acid and produced a red colour and absorb light at 535 nm.

Stock TCA-TBA-HC1 reagent: This reagent was prepared by adding 0.375 w/v thiobarbituric acid, 15% w/v trichloroacetic acid and 0.25 N HC1. To assist the dissolution, solution was heated slightly. To analyse the effect of monensin and piperine, 2 ml of TBA-HCl and 1 ml of PNS was added in a tube. This solution was added for 15 min with the help of a water bath. After cooling, after cooling, precipitate was removed through with centrifugation at 1, 000 g for 10 min. The absorbance was measured at 535nm against blank. Blanks possess all reagent except PNS. Results are expressed as nmoles MDA formed/min/mg protein using an extinction coefficient of the chromophore 1.56 x 10^5 Mol-1 S-1.

3.11Estimation of total reduced Glutathione

This test is based on colour obtained by reaction between reduced glutathione and DTNB reagent as per Bonye and Ellman, 1972.

Regent required:

0.4 M Sodium phosphate Dibasic

0.5 DTNB Reagent - 40 mg of DTNB in 1% sodium citrate

0.6 Standard Glutathione

In a tube 2 ml of PMS, 2ml of sodium phosphate dibasic and 1ml of DTNB was added from each group. Absorbance was read at 412nm within 2 minutes.

3.12 Statistical analysis:

The data were analysed using Graph Pad prism. The differences between means were analyzed by one-way ANOVA followed by tuky test. Differences were considered to be significant at $P < 0.05$.

3.13. Chemical list

- Piperine (molecular weight 285.34)was purchased from Himedia (RM10798-5G)
- Monensin sodium salt was purchased from sigma Aldrich.
- YEPD Broth and Agar meida (GO38-50G) from himedia
- Hydroxylamine hydrochloride
- DMSO
- Phosphate buffer saline
- MTT powder
- Hydrogen peroxide
- Tricloroacetcic acid
- TBA
- Methanol
- Reduced gluthanione
- EDTA
- Cupper sulphate
- Hydroxide pellets
- Sodium potassium tartarate
- Sodium chloride
- Folin reagent.
- DTNB
- Metaphosphoric acid.

CHAPTER 4

OBJECTIVES

OBJECTIVES:

The main objectives of the current investigation are :

- To analyze the effect of Piperine and monensin supplementation on Yeast cell viability.
- To analyse the effect of supplementation of piperine on monensin induced stress as assessed by lipid peroxidation and antioxidant status .

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CHAPTER 5

RESULTS, DISCUSSION AND CONCLUSION

Result

Chapter 5

Result:

5.1 Effect of Monensin and piperine supplementation on cell viability:

MTT is based on reduction of MTT to insoluble purple formazon and widely used to determine the cell viability. Our result show that monensin significantly ($P<0.05$) decreased the cell viability as compared to control and piperine. Piperine also show negative effect on cell viability. Results are summarized in the following figure.

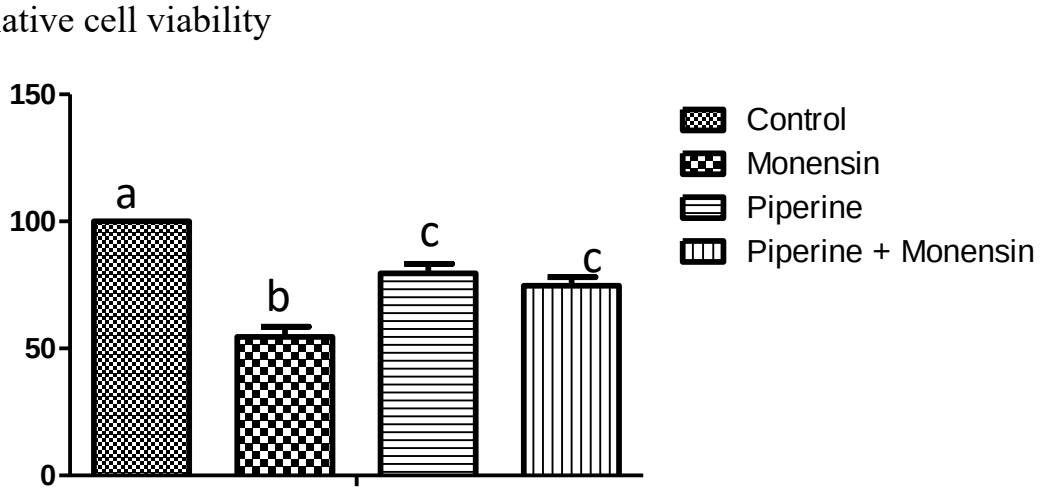

Figure 4: Relative cell viability after the treatment of the yeast cells with monensin, piperine and monensin + piperine. Value are Mean ± SEM. Value with different superscript differ significantly.

Figure 5 : Representative photograph of dissolved formazon crystal used for measuring the absorbance.

5.2. Effect of Monensin and piperine supplementation on lipid peroxidation and antioxidant:

In the present study, effect of monensin and piperine supplementation on yeast cells oxidative stress was analysed through measuring lipid peroxidation and measuring total reduced glutathione and catalase activity.

5.3. Effect of monensin and piperine supplementation on lipid peroxidation:

Effect of monensin, piperine, piperine along with monensin on lipid peroxidation was analysed and presented in the following figure. Following figure shows the trends of lipid peroxidation in different group. There was a significant decrease ($P<0.05$) in lipid peroxidation in all of the group in relation to control**.**

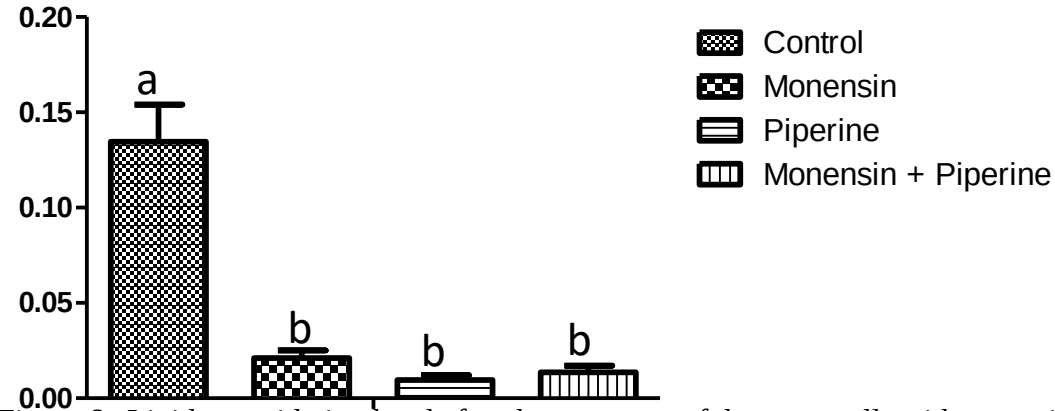

Figure 6 : Lipid peroxidation level after the treatment of the yeast cells with monesin, piperine and monensin + piperine. Value are Mean ± SEM. Value with different superscript differ significantly.

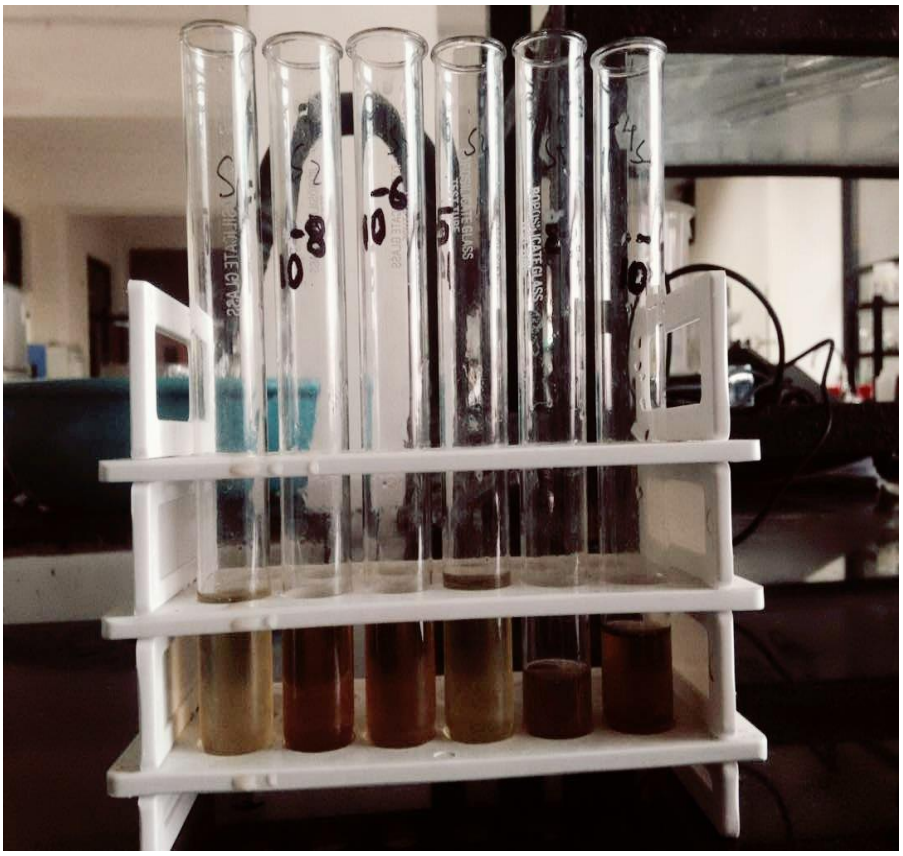

Figure 7: Representative photograph of Lipid peroxidation assay

5.4. Effect of monensin and piperine supplementation on Catalse activity:

Effect of monensin, piperine, piperine along with monensin on catalase activity was analysed and presented in the following figure. Following figure shows the trends of catalase activity in different group. A Significant ($P<0.05$) decrease in catalase was observed in relation to control in all the treatment group. Although in piperine catalase activity are significantly ($P<0.05$) higher than monensin and piperine + monensin.

The activity of catalase was expressed as umoles of H_2O_2 decomposed / min / mg protein.

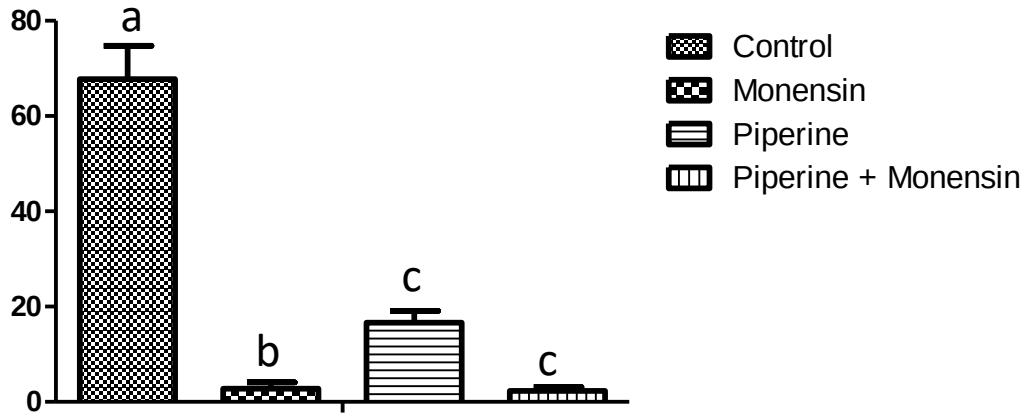

Figure 8: Catalase activity after the treatment of the yeast cells with monensin, piperine and monensin + piperine. Value are Mean ± SEM. Value with different superscript differ significantly.

Figure 9 : Representative photograph of catalse assay

5.5. Effect of monesin and piperine supplementation on reduced glutathione level:

Effect of monensin, piperine, piperine along with monensin on total reduced glutathione level was analysed and presented in the following figure. Following figure shows the trends of reduced glutathione in different group. A Significant ($P<0.05$) decrease in total reduced glutathione was observed in relation to control in all the treatment group. The amount of glutathione is expressed as uM / mg protein.

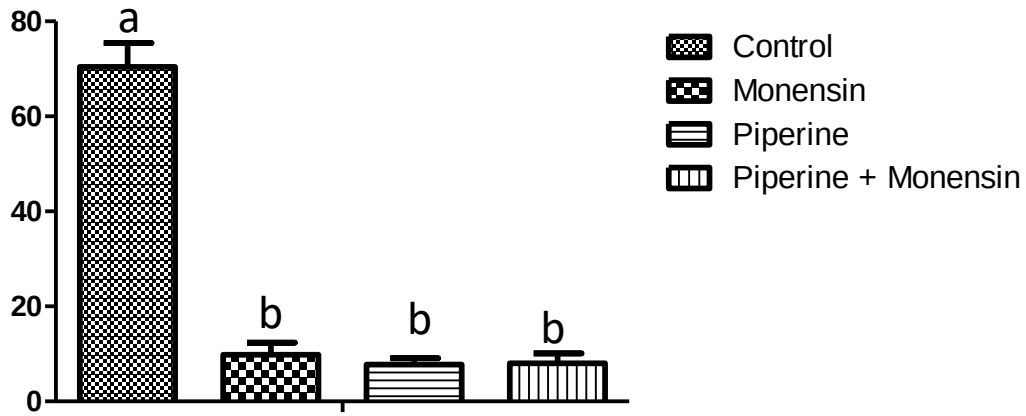

Figure 10: Total reduced glutathione level after the treatment of the yeast cells with monensin, piperine and monensin + piperine. Value are Mean ± SEM. Value with different superscript differ significantly.

Figure 11 : Representative photograph of total reduced glutathione assay

Discussion and conclusion:

Monensin is one of a carboxyl polyether ionophore which increases Mitochondrial (ROS) Reactive oxygen species production by increases cell cycle arrest apoptosis through regulation of cell cycle and apoptosis.It has been used as as veterinary drug to control the protozoan related diseases especially coccidian parasites mostly demages mitochondrial membrane to much disrupts or stops mitochondrial function there is also breakdown of whole mitochondrial architecture.

Piperine has shown different numerous biological phenomena including anti-inflammatory activity, ehance fertility, antioxidantactivity, antitumour, antidepressant, activity, antiplatelet activity (Wattatanathorn et al., 2008). Although piperine supplementation also resulted into toxity in hepatocyte and invitro cultured hippocampal neurons. In animal studies, regularly supplementation of diet enriched with fats and piperine decrease the level of thiobarbituric acid associated reactive substances and enhanced the activities the various antioxidant enzymes e.g , superoxide dismutase, , catalase, gluthione peroxide, gluthione transferaseetc .

The present results demonstrated that monensin and piperine both severely affected the cell growth and induces oxidative stress in yeast cells after 24 h of treatment as indicated by cell viability test and different antioxidant assay. These finding are not in concurrence with the previous studies which demonstrate that administration of piperine (50mg/kg)body/wt increase the working of antioxidant enzymes and helps in protection the DNA from damage. Our result are in agreement with the previous report which shown that Piper nigrum have very high antibacterial activity and protect body against the attack of microbes. Thus Piperine can have very high fungicide activity. ROS are generally concerned in toxicity in several cell cultures system or even in different animal system. Monensin and piperine toxicity also reduced the level of reduced glutathione and protein-bound sulfhydryl groups. Which ultimately might resulted into significant increase into production of several ROS like superoxide ion, hydrogen peroxide, and hydroxyl radicals. This increase level of ROS may be due to decrease reduced glutathione and and ultimately resulted into severe DNA damage.

GSH is required to maintain the thiol redox state of the sulfhydryl group present in proteins. Additionally, GSH works as an important antioxidant in cells. Data from many studies has demonstrated that reduced level of GSH can be assigned as initiation of apoptosis. GSH not only involve in the scavenging the oxygen free radicals. GSH also play important role in the

myeloperoxidas oxidase mediated reaction to reduce the ROS generation.MDA works as lipid peroxide and produced during the process of peroxidation of polyunsaturated fatty acid and increase the cell damage. During the oxidative damage, ROS and side chain of polyunsaturated fatty acid react to produce the lipid peroxidation. Thus MDA level in cells imitate the degree of lipid peroxidation and reactive oxidative damage of cells.

CHAPTER 6

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