POLLEN INDUCED STRESS IN HUMAN LUNG CARCINOMA A549 CELLS

Dissertation Submitted to the Central University of Punjab

For the Award of

Master of Science

In

Biosciences

By

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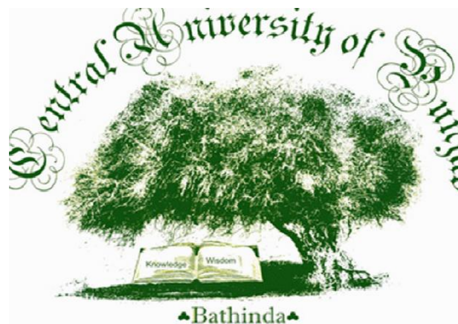

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November 2014

CERTIFICATE

I declare that the dissertation entitled **“Pollen Induced Stress in Human Lung Carcinoma A549 Cells”** has been prepared by me under the guidance of Dr. Anil K. Mantha, Assistant Professor, Centre for Biosciences, School of Basic and Applied Science and Dr. Monisha Dhiman, Assistant Professor, Centre for Genetic Diseases and Molecular Medicine, School of Emerging Life Science Technologies, Central University of Punjab, Bathinda.

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ACKNOWLEDGEMENTS

With utmost modesty, I bow before God Almighty for showering me with blessings and strength. First and foremost, I would like to acknowledge and express my deepest gratitude to my Co-Supervisor Dr. Monisha Dhiman for her utmost guidance, support and patience during the dissertation work. I would like to thank Dr. Anil K. Mantha for his guidance and encouraging me throughout the course of this study. I am grateful to Prof. R. G. Saini, Coordinator, Centre for Biosciences; and Prof. P. Ramarao, Dean Academic Affairs for their generous advice throughout the program. Founder Vice-Chancellor Prof. Jairup Singh and current Vice-Chancellor Prof. R. K. Kohli, Central University of Punjab, Bathinda for providing infrastructure and other technical facilities. The cooperation of all the faculty members of Centre for Biosciences is highly acknowledged.

I am thankful to my seniors Bibekananda Sarkar, Ravi P. Cholia, Nandini Gautam, Shweta Thakur, Shishir Upadhyay and Navrattan Kaur for their endless support and encouragement.

I am also thankful to my labmates Iqbal Kaur and Sukhchain Kaur for their valuable suggestion and immense help. I thank them for questioning me about my ideas, helping me think rationally and even for hearing my problems. I also thankful to my friend Manpreet Kaur always gave a great support in my work. I am thankful to my brother Sanjeev Atri who always supported me in all aspects. I am also thankful to technical staff of CUPB for their help and support.

Last but not the least; I would like to express my deepest thanks to Ankur, Pawandeep, Kirti, Sheetal, Shifali, Shiny, Sonu and Nidhi especially during sampling. I am particularly grateful for the assistance and feedback given by my roommate DeepikaDhiman.

Warmest regards to my family specially my Father for their blessings and valuable suggestions.

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ABSTRACT

Title: Pollen Induced Stress in Human Lung Carcinoma A549 Cells

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Key words: Inflammation, Prickly Poppy Pollen, NADPH oxidase, Nitric Oxide.

The pollen grains are important aeroallergens causing allergic disorders like rhinitis and asthma. Chronic asthma often leads to carcinogenesis in lungs. The various mediators of inflammation like reactive oxygen and nitrogen species (ROS/RNS), cytokines and chemokines form a link between oxidative stress, inflammation and cancer. This study involves induction of oxidative stress by pollen extracts derived from two varieties of prickly poppy in small lung carcinoma A549 cells. To rule out the pathway responsible for pro-inflammatory and oxidative stress enhancer we used the inhibitors of the classical ROS sources such as NADPH oxidase, Xanthine oxidase, mitochondria and general ROS scavenger (NAC). In addition, we also used NF-kB blocking peptide to see whether blocking NF-kB pathway can attenuate the pollen induced stress. It was observed that there was no significant difference in inflammation and/or oxidative stress in the purified pollen antigen versus the crude pollen extract. Lipid peroxidation was significantly increased in pollen extract treated cells whereas no change in MPO activity was observed. The NF-kB levels were significantly reduced in pollen extract treated cells whereas the levels were normalised when treated with NOX inhibitor (DPI) and mitochondria uncoupler (FCCP) suggesting the role of mitochondrial and phagocytic ROS in activating NF-kB. This study signifies the role of pollen as aero-allergens which are very common in Malwa region of Punjab as a key player in enhancing the allergy related complications and chronic lung diseases.

Rekha Atri

Dr. Anil K. Mantha (Supervisor) **Dr. Monisha Dhiman (Co-Supervisor)**

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1.	Allopurinol	Allo
2.	Bovine Serum Albumin	BSA
3.	Concanavalin A	Con A
4.	Cyclo-Oxygenase	COX
5.	Colony Stimulating Factor	CSF
6.	Degree Celsius	°C
7.	Dulbecco's Modified Eagles Medium	DMEM
8.	Dimethyl Sulfoxide	DMSO
9.	Diphenyliodinium	DPI
10.	Enzyme Linked Immunosorbent Assay	ELISA
11.	Fetal Bovine Serum	FBS
12.	Granulocyte Macrophage Colony Stimulating Factor	GM-CSF
13.	Horseradish Peroxidase	HRP
14.	Interleukin	IL
15.	Interferon	IFN
16.	Inducible Nitric Oxide Synthase	iNOS
17.	Microgram	µg
18.	Microlitre	µl
19.	Micro molar	µM
20.	Molar	M
21.	Mililitre	ml
22.	Milimolar	mM

23.	Myeloperoxidase	MPO
24.	(3-(4, 5-dimethylthiazol-2-y1)2, 5-diphenyl tetrazolium bromide)	MTT
25.	Migration Inhibitory Factor	MIF
26.	Malondialdehyde	MDA
27.	Nicotinamide Adenine Dinuleotide Phosphate	NADPH
28.	Nitric Oxide	NO
29.	NADPH Oxidase	NOX
30.	Napthyl Ethylene diamine	NEDD
31.	Non-Small Cell Lung Cancer	NSCLC
32.	N-acetyl Cysteine	NAC
33.	Nuclear Factor Kappa B	NF-kB
34.	Phosphate Buffer Saline	PBS
35.	Phosphate Buffer Saline with Triton-X	PBST
36.	Reactive Nitrogen Species	RNS
37.	Reactive Oxygen Species	ROS
38.	Rel Homology Domain	RHD
39.	Small Cell Lung Cancer	SCLC
40.	Sodium Dodecyl Sulphate	SDS
41.	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis	SDS-PAGE
42.	N,N,N',N'tetra- methylethylenediamine	TEMED
43.	Tri-Chloro Acetic Acid	TCA
44.	Tetra Methyl Benzidine	TMB
46.	Yellow Prickly Poppy	YPP

47.	White Prickly Poppy	WPP
48.	Xanthine Oxidase	XO
49.	Xanthine Oxidoreductase	XOR

Chapter I

Introduction

Cancer is defined as the abnormal or uncontrolled growth of cells and a state in which normal cells are turned into abnormal cells by a process known as carcinogenesis. A total of about 2,468,435 deaths were recorded in the United States in 2010, of which 574,743 (23%) were from cancer. Overall, cancer is the second leading cause of death after heart diseases. The hallmarks or properties of cancer are: (i) sustaining proliferative signaling (Hiramoto *et al.*,2013) processes growth promoting factors (iii) resisting cell death (Dharajiya *et al.*, 2008) enabling replicative immortality (v) having capability to induce angiogenesis and (vi) activating metastasis and invasion. Besides these hallmarks, the role of inflammation and immunity has been proposed as the seventh hallmark of cancer. Inflammation has its role in genetic instability and in escaping the host immune surveillance during cancer (Colotta *et al.*, 2009). Hanahan and Weinberg in their 2011 update of the "Hallmarks of Cancer" explained the host immune escape as an emerging hallmark of cancer and defining tumor promoting inflammation as enabling characteristics. So, inflammation is required for tumor initiation and progression. But at the same time, it also makes the immune system unable to destroy the tumors successfully (Hanahan *et al.*, 2011).

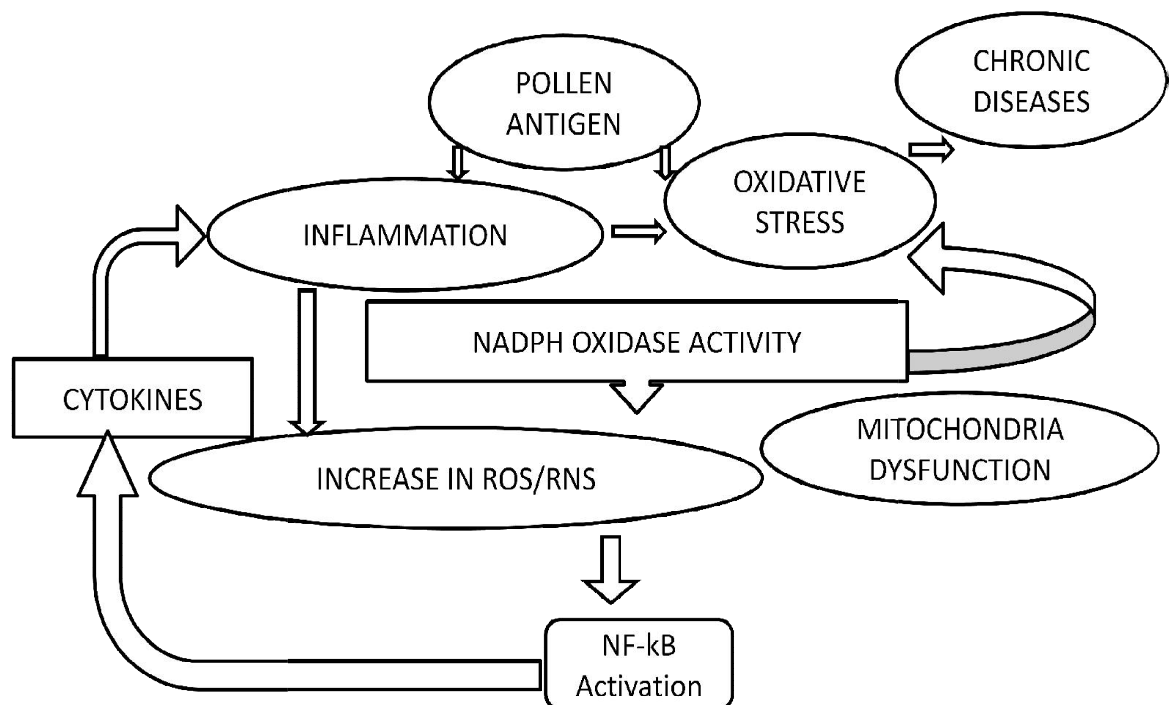
Lung cancer is the uncontrolled growth of abnormal cells in one or both lungs. These cells do not carry out the normal functions and thus do not develop into healthy lung tissue. Lung cancer shows the largest geographic variation due to smoking among different states as compared to prostate cancer and breast cancer (Siegel *et al.*, 2014). There are many risk factors that are responsible for lung cancer first and foremost is consumption of tobacco and cigarette smoking. Cigarette smoking is the number one risk factor for lung cancer and it also increases the risk of mouth cancer. Smoking affects the functioning of the immune system and increases the risk for respiratory and other infections (Sopori, 2002). Workplace exposure to asbestos and other substances like arsenic, chromium and nickel increases the risk of developing lung cancer. Another possible reason of lung cancer is exposure to pollen grains that are considered as one of the most common outdoor allergens (Keskin *et al.*, 2006).

Pollen grains are the important aeroallergens causing allergic disorders like rhinitis and asthma. Chronic asthma often leads to carcinogenesis in lungs. Asthma is

a chronic disease where the airways are narrow and inflamed significantly. Pollen grains and sub-pollen particles can reach lower airways and cause asthma and various allergic diseases (Dharajiya *et al.*, 2008). It is a causative agent of Hay fever/pollen allergy/pollinosis. Hay fever is the ″yearly returning seasonal infection due to allergy to certain airborne pollen grains″ (Räsänen, 2000).

Pollens from *Agremone maxicana* (Yellow prickly poppy) and *Agremone ochroleuca* (White prickly poppy) belongs to Papaveraceae family are used in present work to evaluate their toxicity in A549 lung carcinoma cells and to determine the inflammatory responses activated due to stimulation of pollen as an allergic stimulus to these cells.

<u>Hypothesis of the Study</u>:



Objectives of the Study:

- To test whether purified antigen result in inducing prominent inflammatory or oxidative stress responses.
- To detect the possible source of inflammation and oxidative stress.
- To examine whether the pollen extracts can activate the NF-κB transcription factor and to examine the cross-talk between the NF-κB and inflammation.

Significance of the Study:

- The repeated exposure of pollen leads to chronic inflammatory diseases such as asthma which if not controlled, may further result in lung cancer.
- If the pathway involved in inflammation is ruled out that can take us to some better preventive and therapeutic leads in disease severity.

Chapter II

Review of literature

2.1 <u>Lung Cancer</u>

Lung cancer is considered as one of the most commonly diagnosed cancers as well as the number one cause of cancer deaths (Jemal *et al.*, 2011). Lung cancer is the uncontrolled growth of abnormal cells in one or both lungs. These cells do not carry out the normal functions and do not develop into healthy lung tissue. Based on their origin the lung cancer are of two types- primary lung cancer starts in the lung and remains in the lung till metastasis. Sometimes cancer cells can travel from distant cancer site and metastasize into the lungs. This is known as secondary lung cancer because the lungs act as a secondary site compared to the original primary location of the cancer. Lung cancer is of two types based on their histology-non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC accounts for about 85% of lung cancers and histologically they are of four types- adenocarcinoma, branchio-alveolar carcinoma, squamous cell carcinoma and large cell carcinoma (Ettinger *et al.*, 2010). Several targeted drugs such as Erlotinib and Cetuximab can increase the overall survival of NSCLC patients (Xiao *et al.*, 2014). Small cell lung cancer accounts for the remaining 15% of lung cancers. It results from smoking and grows more rapidly and spreads to other parts of the body faster than non-small cell lung cancer. It is divided into two stages- limited stage (one side of chest, one part of lung and lymph nodes) and extensive stage (spreads to other regions of chest or other parts of body). The reason of separating small cell lung cancer from non-small cell lung cancers is that they spread quickly and are more aggressive than that of non-small cell lung cancers.

There are many causes of lung cancer but first and foremost is consumption of tobacco and smoking. Other reasons are being different occupational exposures to radiotherapy. Another potential reason of lung cancer is exposure to pollen grains that are considered as one of the most common outdoor allergens (Romagnani, 2004).

2.1.1 Pollen Grains as an Aero-allergens

Pollen grains and subpollen particles can reach lower airways and cause asthma and various allergic diseases (Dharajiya *et al.*, 2008). It may also cause hay fever/pollen allergy/pollinosis. Allergy can be defined as a strong and specific reaction of the human body to exogenous molecules that may occur in the normal environment.

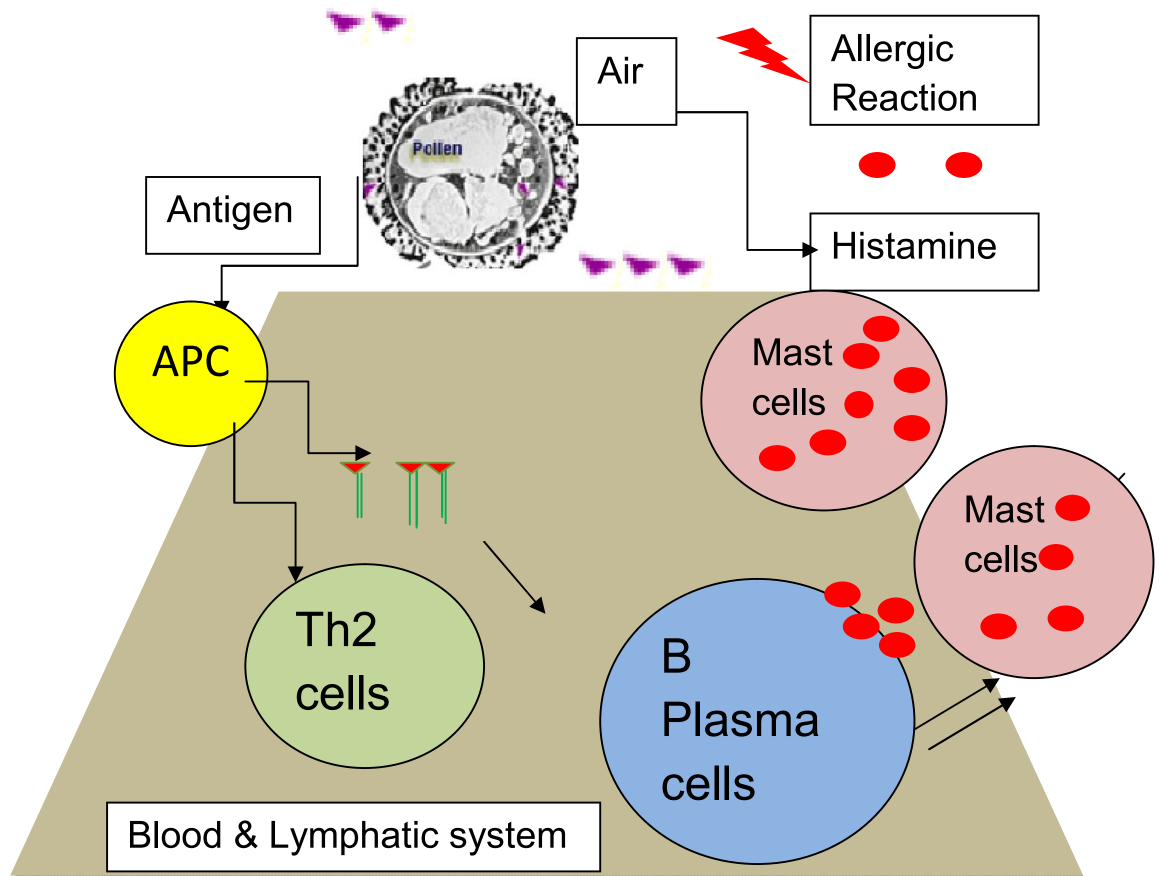


Figure 2.1: Diagram representing mechanism of pollen allergy (Adapted and modified from www.vcbio.science.ru.nl).

Fig 2.1 shows how pollen as an antigen activates B and T cells that in turn results in allergic reaction. It is an important trigger of seasonal rhinitis, conjunctivitis

and allergic asthma. These allergic diseases cause chronic inflammation in respiratory tract and this chronic inflammation in turn act as a mediator of cancer.

2.2 Inflammation

Inflammation is defined as an immune response that enables survival during infection or injury and maintains tissue homeostasis under a variety of noxious conditions like anaphylaxis and septic shock. It is part of complex biological response of tissues to harmful stimuli such as pathogens, radiations, temperature, chemicals and immunological reaction. In other words, it is the body's mechanism of self-protection and initiates the process of healing (Medzhitov, 2010). The four cardinal signs of inflammation are (i) rubor (redness), (Hiramoto *et al.*) calor (heat), (iii) tumor (swelling), and (iv) dolar (pain). These signs correspond to vasodilation, edema and tissue damage. The fifth sign of inflammation is function laesa (loss of function) which was described by Rudolf Virchow in 1858 in his book Cellular Pathologie.

Both innate and adaptive immune systems are involved in inflammation when there is an exogenous or endogenous injury to the cell or tissue. The cells of innate immune system such as macrophages, dendritic cells, mast cells, natural killer cells gets activated by acting as a first line of defense whenever there is such disturbance in tissues homeostasis. Cells of innate immune system initiate the inflammatory response by releasing cytokines, chemokines, reactive oxygen and nitrogen species which lead to the elimination of pathogens and repair of tissue damage. Although neutrophils are mostly related to innate immunity due to their ability to phagocytose bacteria, they are also co-localized with T cells at sites of persistent infections, chronic inflammations or tumors (Arnhold *et al.*, 2010).

2.2.2 Types of Inflammation

There are basically two types of inflammation, intrinsic inflammation and extrinsic inflammation. It is a well-known fact that the hematopoietic inflammatory cells play a very important role in establishing the inflammatory microenvironment.

Immune cells in the tissue may produce and respond to pro-inflammatory mediators that affect the cell's function, proliferation, tissue rearrangement and recruit hematopoietic inflammatory cells (Trinchieri, 2012).

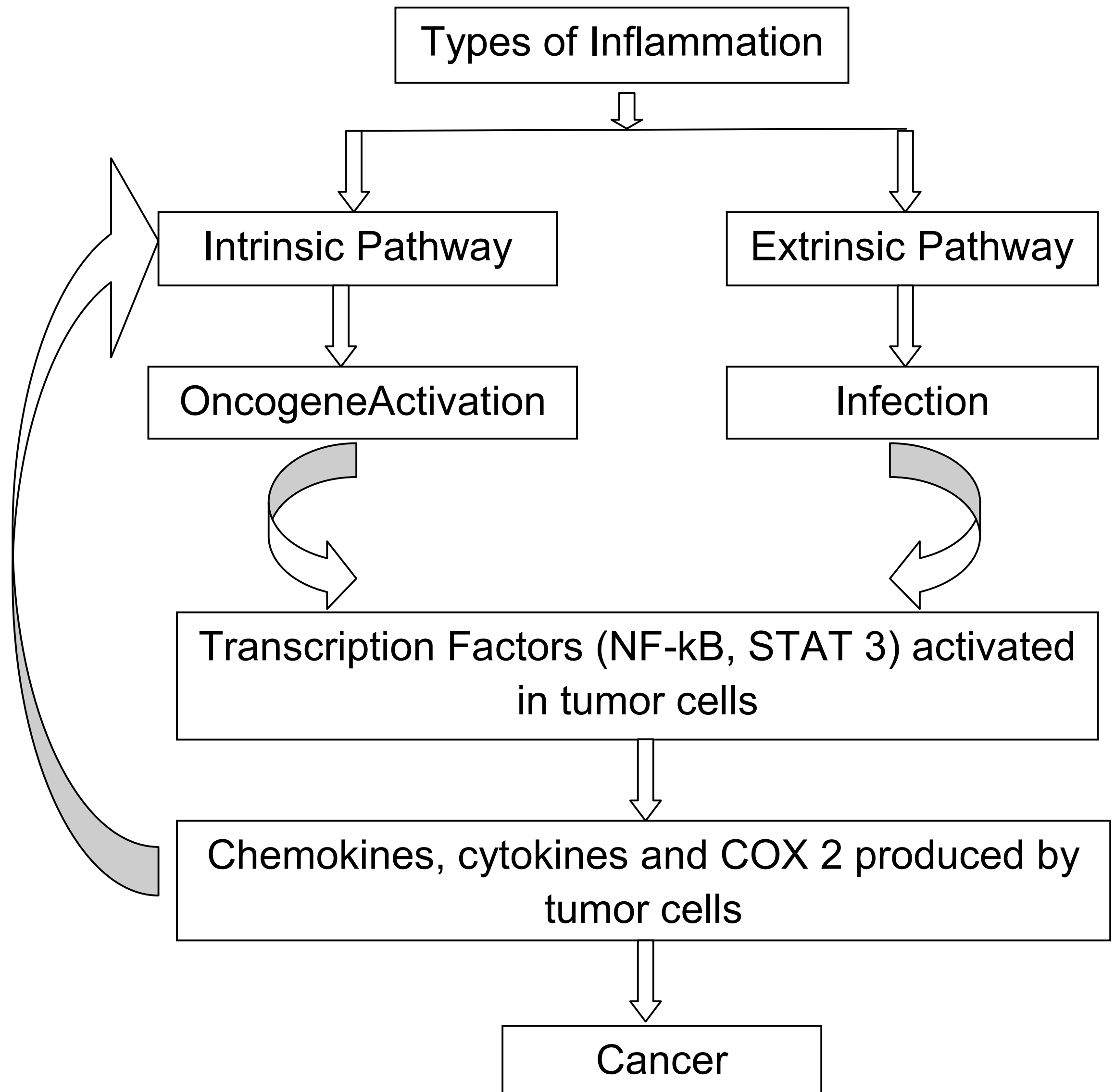


Figure 2.2: Diagram representing link between inflammation and cancer (Adapted and modified from- nature.com).

The Fig 2.2, Role of intrinsic and extrinsic pathways in development of cancer. Long term exposure to any type of oxidative stress leads to chronic inflammation. In 19th century Rudolf Virchow, was the first who observed the presence of leukocytes

within tumors and provided the first indication of a possible link between inflammation and cancer (Grivennikov *et al.*, 2010).

2.3 Link between Cancer and Inflammation

Inflammation plays a very important role in tumorgenesis (Medzhitov, 2010). The role of inflammation and immunity has been proposed as the seventh hallmark of cancer. Inflammation has its role in genetic instability and avoidance of immune surveillance in cancer (Colotta *et al.*, 2009) and similarly Hanahan and Weinberg in their 2011 update of the “Hallmarks of Cancer” proposed inflammation as an emerging hallmark of cancer. Inflammation is double edged sword i.e. it is responsible for tumor initiation and early progression and same time also makes the immune system unable to destroy the tumors successfully (Hanahan *et al.*, 2011).

2.3.1 Role of Inflammatory Cells in Tumor Development

The tumor microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, dendritic cells, and natural killer cells) and adaptive immune cells (B and T lymphocytes) in addition to the cancer cells and their surrounding stroma (which consists of fibroblasts, endothelial cells and mesenchymal cells). Macrophages, neutrophils, eosinophils, dendritic cells, mast cells and lymphocytes are also found to be key components in the epithelial originated tumors (Lu *et al.*, 2006).

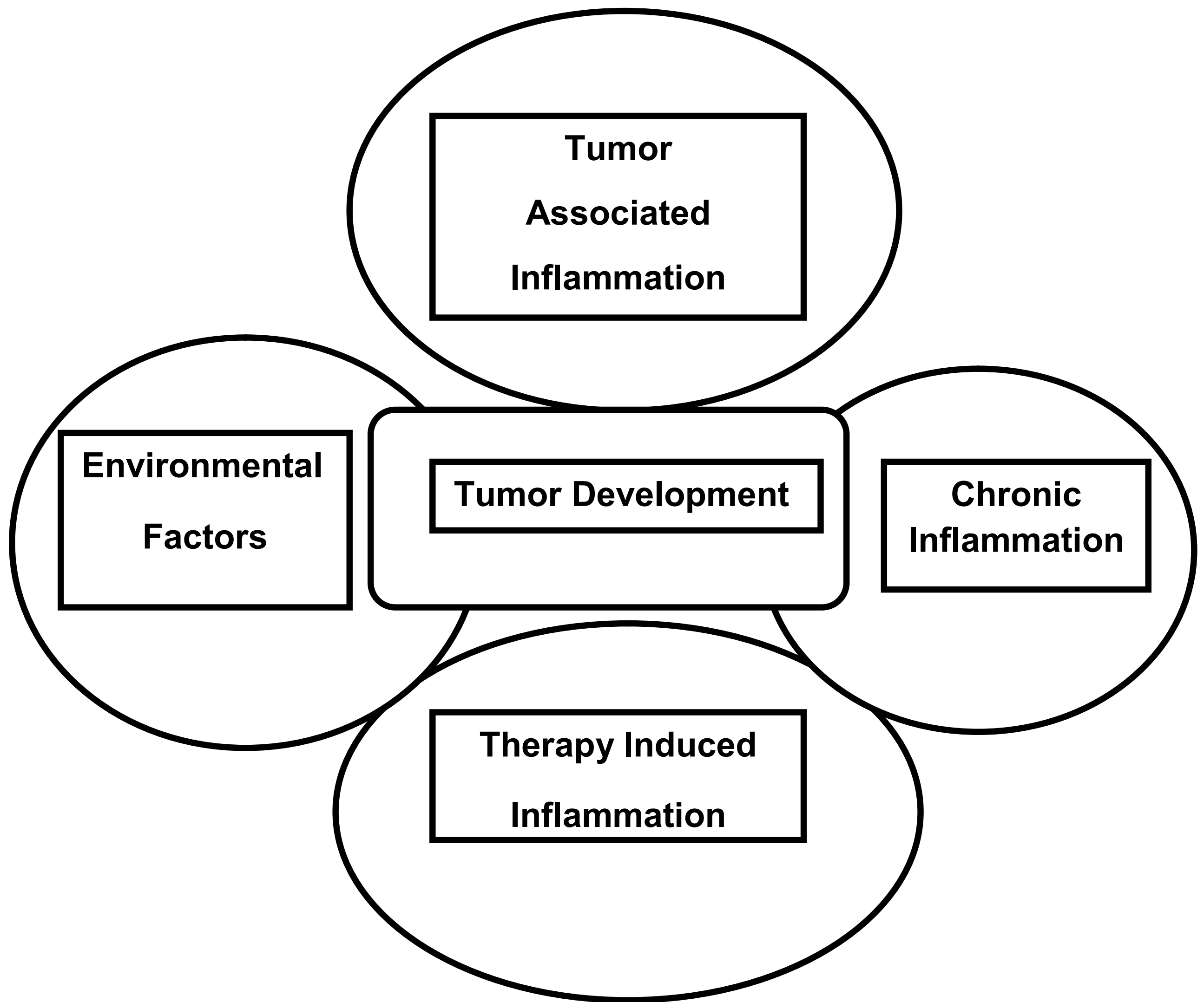


Figure 2.3: Figure representing factors responsible for tumor development (Adapted and modified from Grivennikov *et al*., 2010).

a) <u>Macrophages</u>

Tumor Associated Macrophages (TAM) has been originated from the circulating monocytic precursors. Tumors vary widely in their size of their macrophage (Mantovani, A, 1990). TAMs are the component of the infiltrates in most of the tumors. The colony-stimulating factor (CSF) produced by tumors help in prolonged survival of TAM. TAMs lineage M1 secretes the pro-inflammatory cytokines such as IL-12 and tumor necrosis factor (TNFα) whereas M2 lineages produce anti-inflammatory cytokines such as IL-1, IL-10 and regulate tumor growth and

progression, adaptive immunity, stroma formation and angiogenesis (Mantovani *et al.*, 2002).

b) <u>Dendritic cells</u>

Inadequate presentation of tumor antigens by host professional antigen presenting cells (APCs), including dendritic cells (DCs), is one of the potential mechanism for the escape of tumors from host immune system. They are generally immature and their capacity to activate T- lymphocytes is defective. These are also derived from monocytes in presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF) (Banchereau *et al.*, 1998). Human cancer cell lines release soluble factors that affect DC maturation from precursors without affecting the function of relatively mature DCs. One factor that is responsible for these effects is vascular endothelial growth factor (VEGF). VEGF play a vital role in the pathogenesis of cancer and the blockage of VEGF action improve prospects for immunotherapy (Gabrilovich *et al.*, 1996). They are generally immature and their capacity to activate T- lymphocytes is defective. These are also derived from monocytes in presence of IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF).

2.3.2 <u>Mediators linking Cancer and Inflammation</u>

Inflammation plays a significant role in regulation of promotion and progression of tumor by accelerating the cell cycle progression and proliferation and stimulating neo-vascularization. Cytokines, chemokines, COX-2, prostaglandins, iNOS, NO and NF-κB are mediators of inflammation that contribute to carcinogenesis (Kundu *et al.*, 2008).

I. Cytokines

Cells of the immune system communicate with one another by releasing and responding to chemical messengers known as cytokines. It includes a diverse assortment of interleukins, interferon and growth factors. These can be further classified as pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines that are involved in carcinogenesis are TNFα, IL-1, IL-5,IL-6,IL-8,colony

stimulating factor (CSF) and macrophage migration inhibitory factor (MIF) (Hussain *et al.*, 2007). The anti-inflammatory cytokines (IL-1, IL-6) are immunoregulatory molecules that control pro-inflammatory responses. The cytokines derived from tumor cells and host stromal cells are Fas-ligand, VEGF and transforming growth factor and play a important role in tumor suppression (Lu *et al.*, 2006).

a. Tumor Necrosis Factor α (TNF α)

TNF-α plays a vital role in chronic inflammatory diseases and tumor promoting effects by acting as a mediator of inflammation.TNF-α produced by tumor cells or inflammatory cells in tumor microenvironment can promote tumor cell survival through the induction of genes encoding NF-κB-dependent anti-apoptotic molecules. TNF-α contribute to tumor initiation by stimulating the production of genotoxic molecules, that is, molecules that can lead to DNA damage and mutations, such as NO and ROS (Lin *et al.*, 2007). Genetic polymorphisms that enhance TNF-α production are associated with increased risk of other cancers such as bladder cancer, gastric cancer, and breast cancer. Other actions of TNF-α is that it increases tumor progression, as opposed to tumor initiation, include promotion of angiogenesis and metastasis, as well as impairment of immune surveillance by strongly suppressing many T cell responses and the cytotoxic activity of activated macrophages.

b. Interleukin-1 (IL-1)

IL-1 family members are known to change the host response to an inflammatory and infectious challenge. The four best known members of this family are IL-1α/β, IL-1Ra, and IL-18. IL-1α/β and IL-18 are highly inflammatory cytokines, and any type of dysregulation in them leads to severe complications (Schmitz *et al.*, 2005).

c. Interleukin-6 (IL-6)

IL-6 plays a vital role in carcinogenesis. The expression of genes involved in inhibition of apoptosis, are modulated by IL-6 via JAK-STAT pathway (Kundu *et al.*, 2008).

d. Migration Inhibitory Factor (MIF)

Macrophage migration inhibitory factor (MIF) also known as glycosylation-inhibiting factor (GIF) is a protein encoded by the MIF gene. MIF is an important regulator of innate immunity. The circulating MIF binds to CD74 on other immune cells to trigger an acute immune response. Hence MIF is classified as an inflammatory cytokine and it links inflammation and cancer. It plays a significant role in regulating p53 tumor suppressor gene, angiogenesis, cell cycle and senescence (Hussain *et al.*, 2007).

II. <u>Chemokines</u>

Chemokines “are defined as family of chemoattractant cytokines which are categorized into four categories i.e. CC, CXC, XC and CX_3 depending upon their relative positions of conserved cysteine residues. They are main soluble regulators that control migration of leucocytes at inflammation site”. Angiogenesis during carcinogenesis is regulated by balance between chemokines having pro-angiogenic and anti-angiogenic role. Due to direct migration of tumor cells to specific organ by circulation, chemokines also play important role in invasion and metastasis (Lu *et al.*, 2006). Some chemokines in tumor are: IL-8, IP10, Mig, SDF-1 alpha (Pellegrino *et al.*, 2002).

2.3.3. <u>Role of Cyclooxygenase (COX-2)</u>

COX-2, also known as cyclo-oxygenase, serves as a mediator between inflammation and cancer. It is temporarily activated in certain tissues due to various external stimuli, such as pro-inflammatory cytokines, LPS, UV and ROS which

promotes cellular proliferation, suppression of apoptosis that result in oncogenic functions (Hussain *et al.*, 2007).

Studies supports that COX-2 induction might contribute to the progression of cancers from inflammation and its inhibitor Celecoxib inhibits the COX-2 pathway and delays the developing adenocarcinoma (Lu *et al*., 2006). However, contradiction still exists in regard to the exact role of COX-2 in the development of cancer from inflammation. Another study showed that COX-2expression is independent of the amount of inflammation but related to the premalignant cells that exist in the tissue. These results indicate that COX-2 expression may not be the driving force to cancer but rather play a role in enhancing cancer development through chronic inflammation (Lu *et al*., 2006).

2.3.4. Role of NF-kB

NF-kB has been most widely investigated because of its ubiquitous presence and multiple functions. It controls cell survival, proliferation, and growth, as well as angiogenesis, motility, invasiveness, chemokines and cytokines production (Grivennikov *et al.*, 2010). Improper activation of NF-kB contributes to tumorigenesis by trans-activating several target genes that have inflammatory (COX-2, iNOS, and TNF-α), anti-apoptotic (Bcl-2 and Bcl-3), cell cycle regulatory (cyclin) and pro-angiogenic (VEGF) functions.

NF-kB is expressed in almost all cell types, highly conserved and processes a complex signaling pathway. It consists of five cytosolic transcription factors (p65, RelB, c-Rel, p105 and p100) having common structural motif in their N-terminal regions, the Rel homology domain (RHD), consists of two β-barrel structures that in turn binds to the major groove of DNA. p65, RelB and c-Rel each consist of a RHD followed by a transactivation domain near the C-terminus, there by mediating gene transcription. NF-κB1 and NF-κB2 also known as p105 and p100, respectively are considered as large molecules which contain a RHD followed by a series of ankyrin repeats that block nuclear localization (McMillan, 2013). Both p50 and p52 are formed

due to cleavage of the ankyrin repeats at different stages. But it remains in a dormant stage in cytoplasm but becomes activated and translocate into nucleus when activated by some stimuli like oxidants and cytokines.

NF-kB is a complex transcription factor but despite its complexity, it can generally be divided into two sub-pathways i.e. the canonical and alternative pathways. As seen in Fig 2.4, the canonical pathway usually consists of p65/p50 dimers which are sequestered in the cytoplasm by the inhibitor of NF-kB i.e. IκBs. Any type of pro-inflammatory stimuli (Lipopolysaccharide) and cytokines such as IL-1β and TNF-α, promotes the phosphorylation and degradation of the IκB subunits, which allows the p65/p50 dimers to translocate into the nucleus and regulate expression of their target genes. The non-canonical pathway or alternative pathways, normally consists of RelB/p100 dimers and is activated by cytokines including CD40 ligand (CD40L) and lymphotoxin-β (LT-β). Activation of this pathway promotes the partial degradation of p100 into p52, which allows the RelB/p52 dimers to translocate to the nucleus and regulate their target genes (McMillan, 2013).

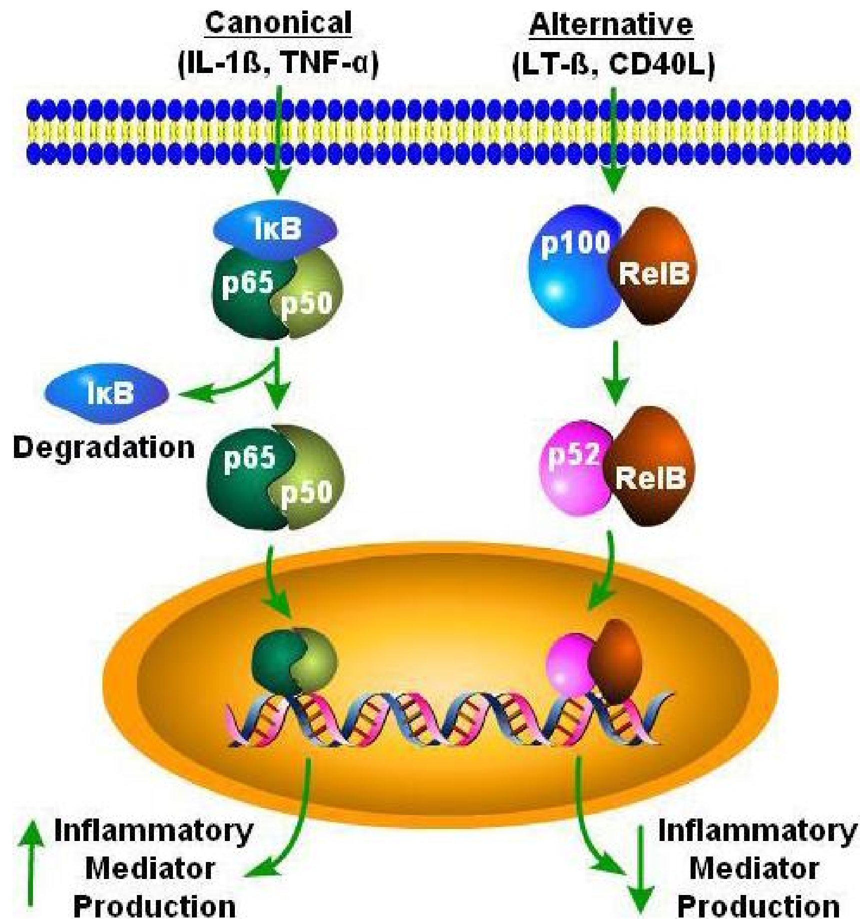


Figure 2.4: Overview of the NF-κB pathway (Adapted from McMillan, 2013).

The Fig 2.4 explains how two pathways are involved in activating NF-kB with different subunits. NF-κB is highly activated at sites of inflammation in diverse diseases and can induce transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, MMPs, COX-2, and inducible nitric oxide (iNOS) (Tak *et al.*, 2001).

2.4 Markers of Inflammation

The NADPH oxidase, iNOS, myeloperoxidase, Nitric Oxide is conventional markers of inflammation.

2.4.1. NADPH oxidase (NOX)

Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NOX) is an enzyme that plays a very important role in physiological and pathological processes like cell signaling and inflammation (Juhasz *et al.*, 2009). It is found in a variety of cells and it is agroup of plasma membrane associated enzymes. It catalyzes the production of superoxide ($O_2^{\cdot -}$) by one electron reduction of oxygen, using NADPH as an electron donor (Babior, 1999).

$$2O_2 + NADP \text{ —(NADPH oxidase)}\longrightarrow 2O_2^{\cdot -} + NADP^+$$

It makes large amounts of superoxides that are found in professional phagocytes like neutrophils, monocytes and macrophages at their different stages. Also, there is a small group of superoxide producing enzymes known as NOX and it plays a vital role in signaling (Babior *et al.*, 2002).

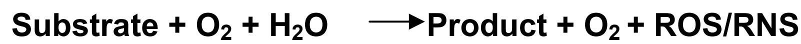
Activation of NADPH Oxidase

Two events that are responsible for NOX activation are: protein phosphorylation and translocation of cytosolic components to the plasma membrane. The oxidative burst is triggered by the binding of a stimulus to a specific receptor such as C5a receptor. This receptor transmits signal through the cytoplasmic membrane via a family of heterotrimeric G proteins i.e. protein binding GTP. G proteins then activate membrane enzymes like phospholipase. Many other protein kinases, such as protein tyrosine kinases, MAP-kinase, and NADPH oxidase are also phosphorylated during neutrophil stimulation. Upon neutrophil activation, $p47^{phox}$ is phosphorylated which is essential in activating NOX (Elbim, 2005).

2.4.2 Xanthine Oxidase

Xanthine Oxidoreductase (XOR) is an evolutionary conserved enzyme that plays a vital role in purine catabolism. It produces radicals such as H_2O_2 and $O_2^{\cdot -}$ while metabolizing hypoxanthine and xanthine to uric acid (Berry and Hare, 2004). However, mutations in XOR results in disorder like xanthinuria i.e. kidney stone formation. The following chemical reactions are catalyzed by xanthine oxidase:

Xanthine Oxidase (XO):



$$\textbf{Substrate} + O_2 + H_2O \longrightarrow \textbf{Product} + O_2 + \textbf{ROS/RNS}$$

Functions of Xanthine Oxidase

Synthesis of both an antioxidant (uric acid) and various free radicals (ROS/RNS) makes XOR an important protective regulator of the cellular redox potential. XOR has an additional physiological function because it synthesizes reactive oxygen/nitrogen species (ROS/RNS) that in turn plays a very vital role in inflammation and host defense making it a conserved component of the innate immune system. Owing to its multifunctional enzymatic activities, it act as potent detoxification enzyme and this detoxification function is not restricted to XOR only but also found generally in the molybdopterin family of enzymes, including aldehyde oxidase (AO) because its amino acid identity and its intron–exon structure is similar to XOR, suggesting that both enzymes evolved through gene duplications. XOR is highly expressed in the liver for purine catabolism and nitrogen elimination as well as for XOR has an important role in phagocytic killing (Vorbach *et al.*, 2003).

2.4.3 Myeleoperoxidase

Myeloperoxidase is a dimeric enzyme with two heavy subunits (55-64 kD) and two light subunits (10-15 kD) (Daugherty *et al.*, 1994). It is mainly expressed in macrophages and neutrophils. It is a major constituent of the cytoplasmic granules and a classical heme peroxidase that uses hydrogen peroxide produced by neutrophils in order to oxidize a variety of aromatic compounds. It is a unique enzyme because it readily oxidizes chloride ions to the strong non-radical oxidant i.e. HOCl which is a bactericidal oxidant. Many species of bacteria are killed readily by a myeloperoxidase/hydrogen peroxide/chloride system (Hampton *et al.*, 1998).

2.4.4 Nitric Oxide

Nitric Oxide is a free radical with an unpaired electron, small highly diffusible gas and a ubiquitous bioactive molecule. It is a tiny lipophillic molecule that has the capability to travel across the biological membrane (Weitzberg *et al.*, 1998). It plays an important role in numerous physiological and patho-physiological conditions e.g. blood pressure regulation, infection and inflammation. It is produced from the amino acidL-arginine by the enzymatic action of nitric oxide synthase (NOS).

NOS isoforms are of three types- neuronal (nNOS or NOS-1), inducible form (iNOS or NOS-2) and the endothelial (eNOS or NOS-3). nNOS and eNOS are constitutively expressed enzymes that are stimulated by increased calcium ion concentrations whereas iNOS expression is induced by inflammatory cytokines and toxins that in turn leads to the production of much higher amounts of nitric oxide as compared to the constitutive enzymes i.e. nNOS and eNOS (Dimmeler *et al.*, 1997).

Nitric oxide acts as a double edged sword in allergic inflammation. On one hand, it acts as a mediator of inflammatory responses to mast cell derived histamine and induce Th2 dependent IgE synthesis. On the other hand, it is shown to inhibit

mast cell activation, mediate inhibitory effects of IFN-γ, or inhibit vascular adhesion molecule expression thus restricting allergic inflammation (Mocellin *et al.*, 2007).

Chapter III

Materials and Methods

3.1 Chemicals

Acrylamide and Bis-acrylamide, Acetic Acid, Ammonium Per Sulphate, Anti-p47^{phox}(E-7) (Santa Cruz), Anti- NF-κB (Abcam), Bovine Serum albumin (BSA), Bromophenol Blue, Coomassie Brilliant Blue G-50, Concanavalin A (ConA), Diethyl Ether, Dithiothreitol, Dulbecco's Modified Eagle Medium (DMEM), Dimethyl sulfoxide (DMSO), Ethanol, Ethylene Diamine Tetra Acetic Acid (EDTA), Ferric Chloride, Foetal Bovine Serum (FBS), Formaldehyde, Glycerol, Glycine, Hydrogen Peroxide (H_2O_2), Magnesium chloride ($MgCl_2$), Methanol, β-2-Mercaptoethanol, 3-(4,5-Dimethythiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), n(-1-Napthyl) Ethylenediamine (NEDD), NADPH Oxidase (NOX), Nitrobluetetrazolium, O-diansidinedihydrochloride, 20% Orthophosphoric Acid, Potassium Chloride, Potassium Hydroxide, Potassium Iodide, Potassium Phosphate, Phosphate Buffer Saline (PBS), Protease Inhibitor, Sodium Carbonate, Sodium Chloride, Sodium Dodecyl Sulphate (SDS), Sodium Nitrite, Sodium Phosphate, Sulphuricacid (H_2So_4), Sulphanilamide, N,N,N',N'tetra-methylethylenediamine (TEMED), Thiobarbituric acid (TBA), Tris HCl (pH 6.8 and 8.8), Trichloroacetic acid, Tris base, 3,3,5,5,Tetra-methylbenzedine Liquid Substrate (TMB), Triton- X.

3.2 Pollen Collection

Prickly poppy got its name from their unique prickly leaves and stems they have except the petals part (Bowers, 1989). Scientifically known as *Agremone maxicana* commonly known as yellow prickly poppy and *Argemone ochroleuca* commonly known as white prickly poppy. Flowers were collected from nearby regions of Bathinda and Barnala in sterile poly bags from March-May, 2014 and dried. Dried anthers were crushed into powder and the extra part of the flower was discarded. Dried pollen powder was sieved by using a sieve having pore size 0.5-1.0 mm (Annexure I).

a) Argemone maxicana **b) *Argemone ochroleuca***

Figure 3.1:a. *Argemone Mexicana* (Yellow prickly poppy) **b.** *Argemone ochroleuca* (White prickly poppy).

Table 3.1: Different locations from where samples were collected

Samples	Location
Yellow Prickly Poppy	Thikriwale village (Barnala)
	Railway track (outside CUPB city campus)
	Chaudari Dwar Army Cantt (Bathinda)
	Green Avenue Colony (Barnala)
White Prickly Poppy	Railway track (Bathinda)
	Bhucho (Bathinda)
	Near Railway Crossing (Bathinda)

3.3 Extract Preparation

In order to study the allergic effects of pollen grains, the protein from the pollen grains was extracted. The pollen grains were defatted with diethyl ether and then extracted in phosphate buffered saline (1xPBS, pH 7.24) by continuous stirring for 24 hours at 4^oC. For some samples, the pollen extract was treated with 30% TCA in order to obtain a pure protein without any contamination of phenols and phenolic

compounds. The samples were centrifuged at 12,500 g for 45 min and the supernatant was sterile filtered by passing through 0.22 μM Millipore filter (Chakraborty *et al.*, 2005). The filtrate was then lyophilized and stored at -20ºC (Sridhara *et al.*, 2011). The protein content of the whole pollen extract and fractions were determined according to Bradford assay using Bovine Serum Albumin (BSA) protein as a standard.

3.4 Bradford Assay

The protein concentration of various samples like dark yellow and light yellow with and without TCA, dark white and light white with and without TCA prickly poppy pollen extract was estimated by using Bradford method. This method involves binding of Coomassie Brilliant Blue (CBB) with protein causing shift in absorption maximum at 595 nm. The color reagent was prepared by dissolving 0.05 gm of Coomassie Brilliant Blue G-50 in 25% Ethanol and 42.5% Orthophosphoric acid and volume was made up to 100 ml with distilled water. The standard curve was prepared using various concentration of 1mg/ml BSA stock. The 20 μl of coloring reagent was added to each well and absorbance was measured after 15 min at 595 nm on multiplate reader (Biotek).

3.5 SDS-PAGE

The method which is useful for separation of proteins and nucleic acids is Gel electrophoresis. SDS-PAGE separates the proteins on the basis of their molecular weight. In SDS-PAGE, there are two sequential gels: the stacking or upper gel having pH 6.8 and the separating or resolving gel having pH 8.8.

Procedure:

Dark yellow and light yellow with and without TCA, dark white with and without TCA, pure white prickly poppy pollen extract (30 μg) boiled with sample buffer (0.2M

Tris HCl, (pH 6.8), 20% glycerol, 0.5% Bromophenol Blue, 10% SDS and 10 mM β-mercaptoethanol) and were resolved on 10% acrylamide gel along with prestained molecular weight markers on a mini-Protean using 0.2 mol/L Tris-HCl anode buffer (pH 8.8) and 0.1 mol/L Tris-Tricine cathode buffer containing 0.1% sodium dodecyl sulfate. The gel was stained with 0.005% Coomassie blue G250 (S.D. Fine-chem limited) and images were acquired using a software image lab 3.0 on Universal Hood II (BioRad).

3.6 Cell Culture

Culturing of A549 cells

The human alveolar epithelial A549 cells were procured from NCCS, Pune. Cells were cultured in Dulbecco's Modified Eagle Medium (Hi-media) containing L-glutamine, 4.5 gms/L glucose, 2gm sodium bicarbonate ($NaHCO_3$) and supplemented with 10% fetal bovine serum (FBS), 1X antibiotic Penicillin/Streptomycin and are maintained at 37°C in 5% CO_2 in humidified incubator.

3.7 Cell Treatments

Lung carcinoma A549 cells ($1X10^5$) were seeded in peteriplates containing complete medium DMEM supplemented with 10% FBS in 37°C in a humidified, 5% CO_2/95% air atmosphere incubator. During treatment the cells were washed twice with 5 ml sterile PBS and all the treatments were given to the cells with 5 ml of serum-free media for 24 hours.

For MTT assay the cells ($1X10^5$) were treated with various concentrations of dark yellow and light yellow with and without TCA, dark white with and without TCA as shown in Table 3.2. Once the optimal concentrations of various extracts was standardized, 5-20 μg of various protein extract were used for further experiments as shown in result section in Table 4.2.

Table 3.2: Table showing the various concentrations of pollen extracts used for MTT assay

Sr.No.	Samples	Concentration (µg/ml)
1.	Dy+TCA	2-30
2.	Dy-TCA	2-30
3.	Ly+TCA	2-30
4.	Dw+TCA	2-30
5.	Dw-TCA	2-30

- **3.7.1 Controls:** A549 cells (1X10^{5}) were counted and maintained in a 24 well plate, for 24 hours along with the test samples to serve as control.
- **3.7.2 Positive control:** A549 cells (1X10^{5}) were counted and treated with 100 µg of Con A and H_2O_2 in a 24 well plate serve as positive controls for inflammation and oxidative stress respectively.
- **3.7.3 Treatment with pollen extracts:** A549 cells (X10^{5}) were counted and treated with various pollen extracts of dark yellow and light yellow with and without TCA and dark white and light white with and without TCA.

- **3.7.4 Treatment with inhibitors:** To rule out the source of ROS along with the pollen extract, cells were treated with Diphenyliodinium (DPI), Allopurinol (Allo), and FCCP, specific inhibitors of NADPH oxidase (NOX), Xanthine oxidase and mitochondrial oxidative phosphorylation respectively. The free radical scavenger N-acetyl cysteine (NAC) and NF-κB blocking peptide were also used in few experiments to see the effect of ROS and NF-κB signaling as shown in Table 3.3. In all experiments the cells treated with 100 µM H_2O_2 and Con A were used as positive controls.

Table 3.3: Treatment plan to detect the source of reactive oxygen species

Sr.No.	Treatment groups	Concentration/well
1.	Dw-TCA	3.4 µg/µl
2.	Concanavalin A (Con A)	10 ng
3.	Hydrogen peroxide (H_2O_2)	10 ng
4.	Allopurinol (Allo)	20 mM
5.	N-acetyl cysteine (NAC)	5 mM
6.	Diphenyliodinium (DPI)	2 mM
7.	FCCP	10 mM
8.	Blocking peptide of NF-kB	100 µg

3.8 MTT Assay

It was first described by Mosmann (1983). This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase enzyme which reduce this into an insoluble, dark purple colored formazan crystal which is largely impermeable to cell membranes thus resulting in its accumulation within healthy cells. The cells are then solubilized with an organic solvent Dimethyl Sulfoxide (DMSO) and read spectrophotometrically. The number of surviving cells is directly proportional to the level of formazan product created. Since reduction of MTT can occur only in metabolically active cells the level of activity is the measure of the viability of the cells.

Procedure:

Cells ($1x10^5$) were plated in 96 well plates and in the FBS free media 20 µl of MTT solution (5 mg MTT dissolved in 1ml sterile PBS) was added then incubated for 4 hours in 37°C in dark sterile atmosphere containing 5% CO_2. Four hours later formazan product was solubilized in acidified DMSO (0.6 ml acetic acid per 100 ml solvent). The optical density (O.D) of each well was measured with test wavelength of 570 nm. The absorbance read is directly proportional to number of living cells. The cell viability of each group was calculated as percentage (%) with the following formula.

% Viability = OD of Treated samples/ OD of Control X100

3.9 Preparation of Cell lysate

For preparing cell lysate, cells were washed with ice cold PBS and 1ml of pre-cooled RIPA buffer was added per 100 mm of petridish. The cells were incubated for 20 min on ice. The lysate was then transferred to micro centrifuge tube and centrifuged at 20,000 g for 10 min. The supernatant was removed and transferred to fresh tube and stored it at 4ºC(Ji, 2010). Before using it for further assays such as MPO, Lipid peroxidation, Griess assay, and ELISA assay the total protein was measured by using Bradford assay and samples were stored in -20°C until further use.

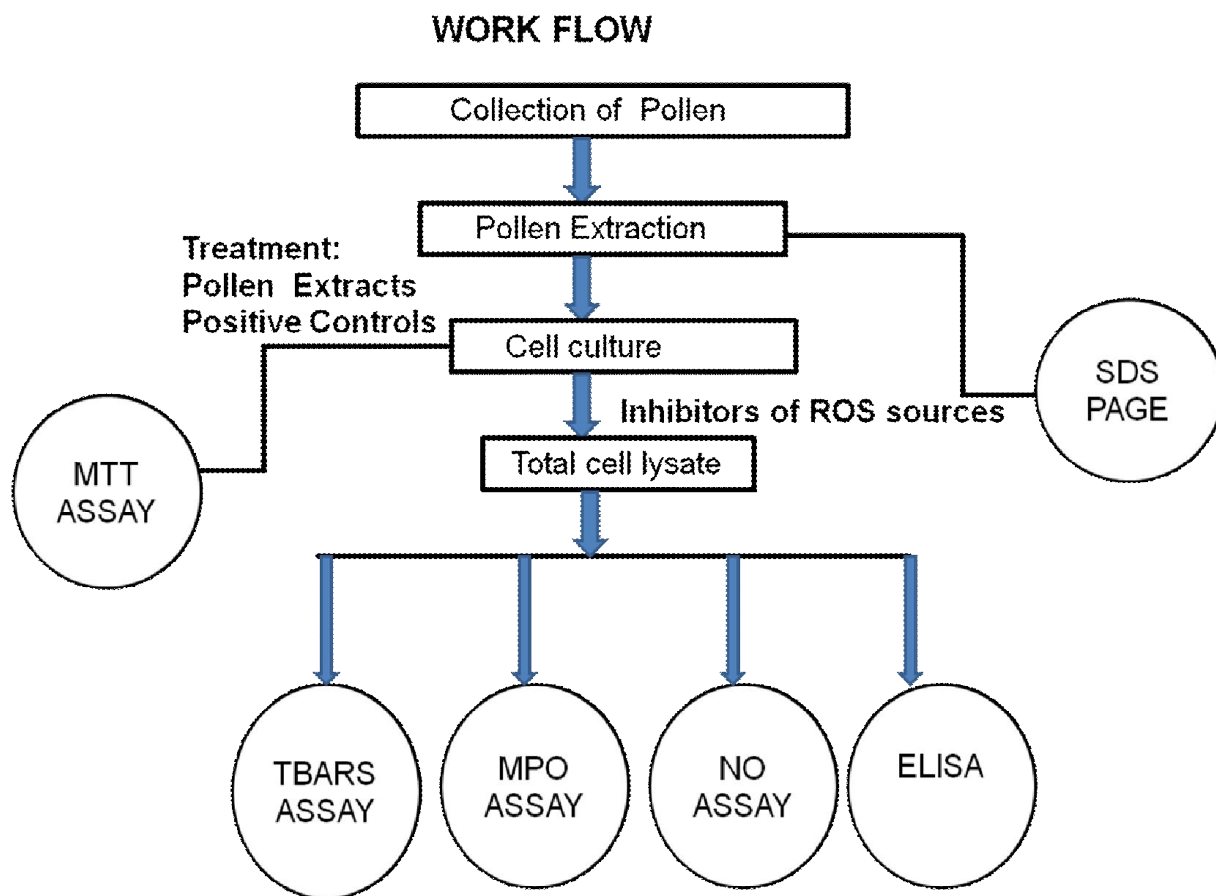


Figure 3.2: Flow Chart for Work Outline.

3.10 Assay for Lipid Peroxidation

Lipid peroxidation is a major mechanism of cellular injury in many biological systems of plant and animal origin. In this mechanism unsaturated lipids are oxidized to form radical species as well as harmful toxic by-products. Polyunsaturated fatty acids are susceptible to this type of damage and can react to form lipid peroxides. Lipid peroxides are themselves unstable, and after additional decomposition to form complex compounds including reactive carbonyl compounds. Polyunsaturated fatty acids peroxides further react to form malondialdehyde (MDA). It is the most widely reported analytes for the purpose of estimating oxidative stress effects in lipids or as a convenient biomarker for lipid peroxidation (Lykkesfeldt, 2007).

Procedure:

Malondialdehyde (MDA), an end product of unsaturated fatty acid peroxidation, can react with thiobarbituric acid (TBA) to form colored complex called thiobarbituric acid-reactive substance (TBARS). The assay was done by using one volume of sample mixed thoroughly with two volumes of a stock solution of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 mol/L HCl. The mixture was then heated for 30 min in a boiling water bath. After cooling, the flocculent precipitate is removed by centrifugation at 1000 rpm for 10 min. The absorbance of a sample was determined at 535 nm and the TBARS concentration is calculated using $0.152X10^5$ M^{-1} cm^{-1} as molar absorption coefficient. The results were expressed as nmoles per mg of protein.

TBARS = Total Volume X O.D/0.152 X Volume X Protein in mg

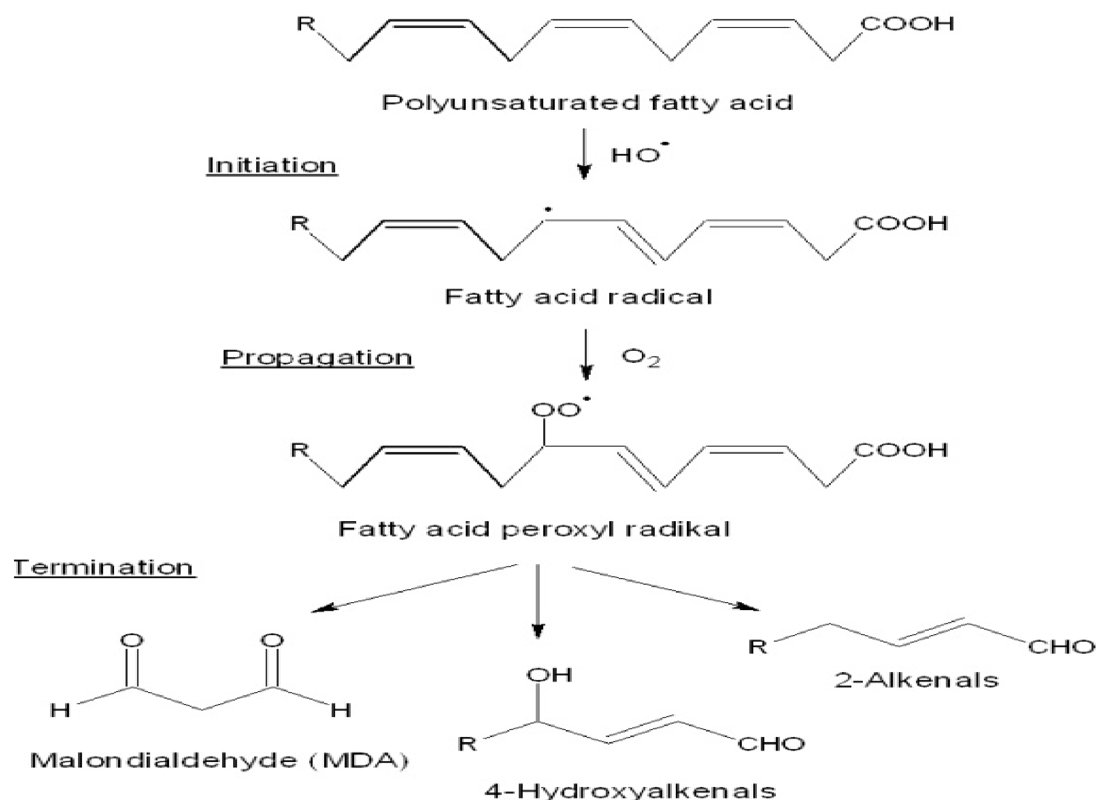


Figure 3.3: Mechanism for Lipid peroxidation (Adapted from www.intechopen.com).

3.11 <u>Assay for Myeloperoxidase Activity</u>

The MPO activity is determined by O-dianisidineH_2O_2 method. This method is based on the enzyme ability to catalyze reaction between chloride and H_2O_2 to form hypochlorous acid using O-dianisidine as a substrate.

Procedure:

In 100 µg sample added 0.53 mM O-dianisidinehydrochloride and 0.15 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 6.0) and incubated for 5 min at room temperature. The change in absorbance was measured at 460 nm (ε = 11,300$M^{-1}cm^{-1}$) in multiplate reader (Biotek). Results are expressed as units of MPO/mg protein, where one unit of MPO was defined as the amount of enzyme degrading 1 µmol H_2O_2 per min at 25°C (Dhiman *et al.*, 2009).

3.12 <u>Estimation of Nitric Oxide</u>

Griess method described by Bryan *et al.*, was used to determine nitrite in A549 total cell lysates (Bryan *et al.*, 2007). The indirect determination of NO involves the spectrophotometric measurement of its stable decomposition products $NO_3^-$and NO_2^-. This method requires NO_3^- first to be reduced to NO_2^- and then NO_2^- isdetermined by the Griess reaction as shown in Figure 3.4. Briefly, 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 ion which is then coupled to *N*-(1-napthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 540 nm.

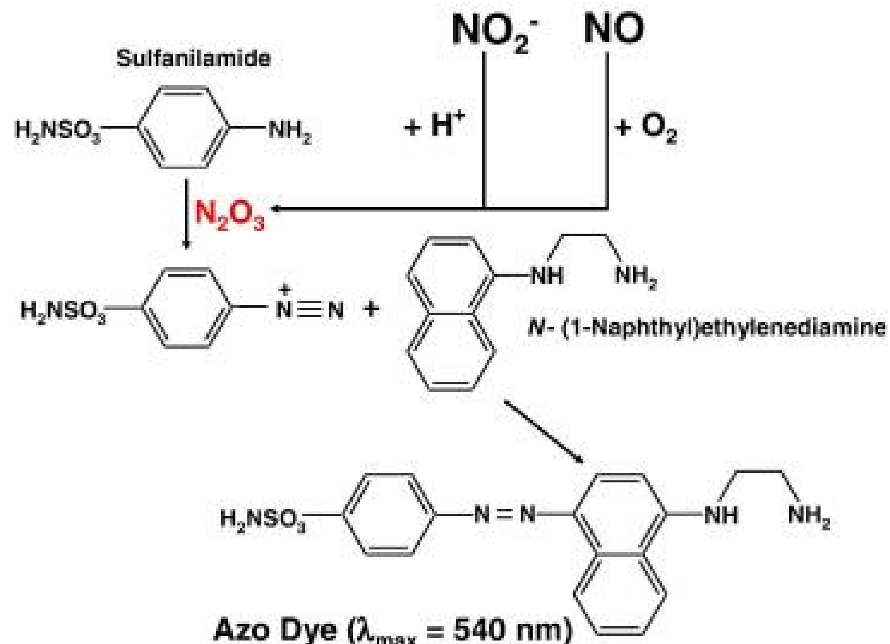


Figure 3.4: Mechanism of Griess assay (Adapted from Bryan *et al.,* 2007).

Procedure:

NO estimation was done by using Griess reagent [1% sulphanilamide and 0.1% N (-1-Napthyl) ethylenediamine (NEDD)] and 100 µg of protein were added in 1:1 ratio and mixed immediately. After 5 min, the absorbance of product formed from above mixture was read at 540 nm on multiplate reader (Biotek). The nitrite content of each sample was evaluated from standard curve obtained after linear regression made with sodium nitrite and was expressed in µM/ml.

3.13 Enzyme Linked Immunosorbent Assay (ELISA)

It 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.

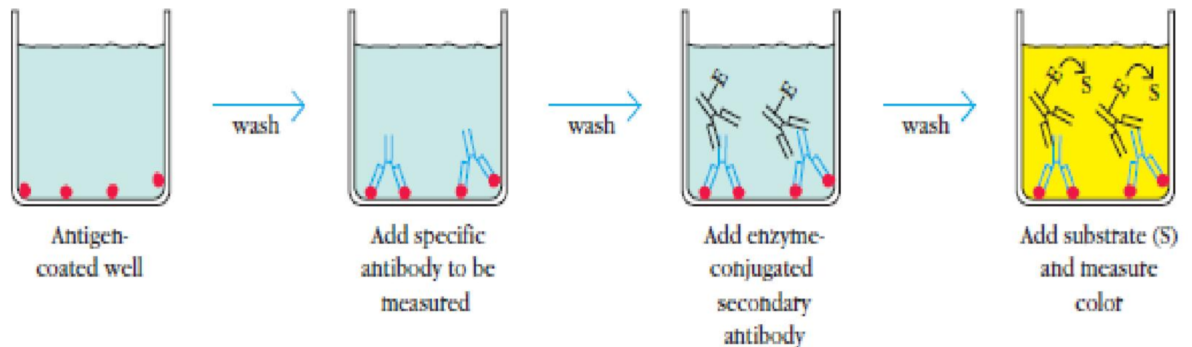


Figure 3.5: Mechanism of ELISA (Adapted from Kuby Immunology 5thedition).

Procedure:

We utilized the ELISA where the total cell lysate and nuclear extract (antigen, 20µg and 30µg respectively) were coated onto the high affinity microtiter plate wells overnight. All the unbound sites were blocked with 1% BSA to prevent false positive results. The primary antibody for p47^{phox} (mouse anti p47^{phox} from Santa Cruz) and NF-kB (rabbit anti-NF-kB from Abcam) was added and incubated for an hour. The HRP conjugated secondary antibody (e.g. anti-mouse and anti-rabbit IgG for p47^{phox} and NF-kB respectively) was added after washing the wells with PBST and PBS. Finally color was developed using the Tetra Methyl Benzidine (TMB) substrate for HRP reaction. The reaction was stopped with 2 M H_2SO_4 and final optical density (Oboh *et al.,* 2012) was read at 450 nm in a multiplate reader (Biotek). The O.D is directly proportional to the reaction product formed due to interaction between antigen and antibody (Dhiman *et al.*, 2009).

3.14. <u>Statistical Analysis</u>

Data was presented as the mean ± standard deviation for at least 3 independent determinations. The Student's *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared

with untreated controls and $^{\#\#}p \leq 0.01$ when Dw-TCA with various inhibitors like Allopurinol, NAC, DPI, FCCP and NF-κB were compared with only Dw-TCA treated samples.

Chapter IV

Results

4.1 Pollen Extract on SDS-PAGE

Pollen extracts (30 µg of protein) were resolved on 10% acrylamide gel and were subjected to Coomassie Brilliant Blue as described in material and methods. When gels were stained with Coomassie Brilliant Blue, faint bands were observed in yellow prickly poppy and white prickly poppy extracts with and without TCA treated as shown in Figure 4.1.

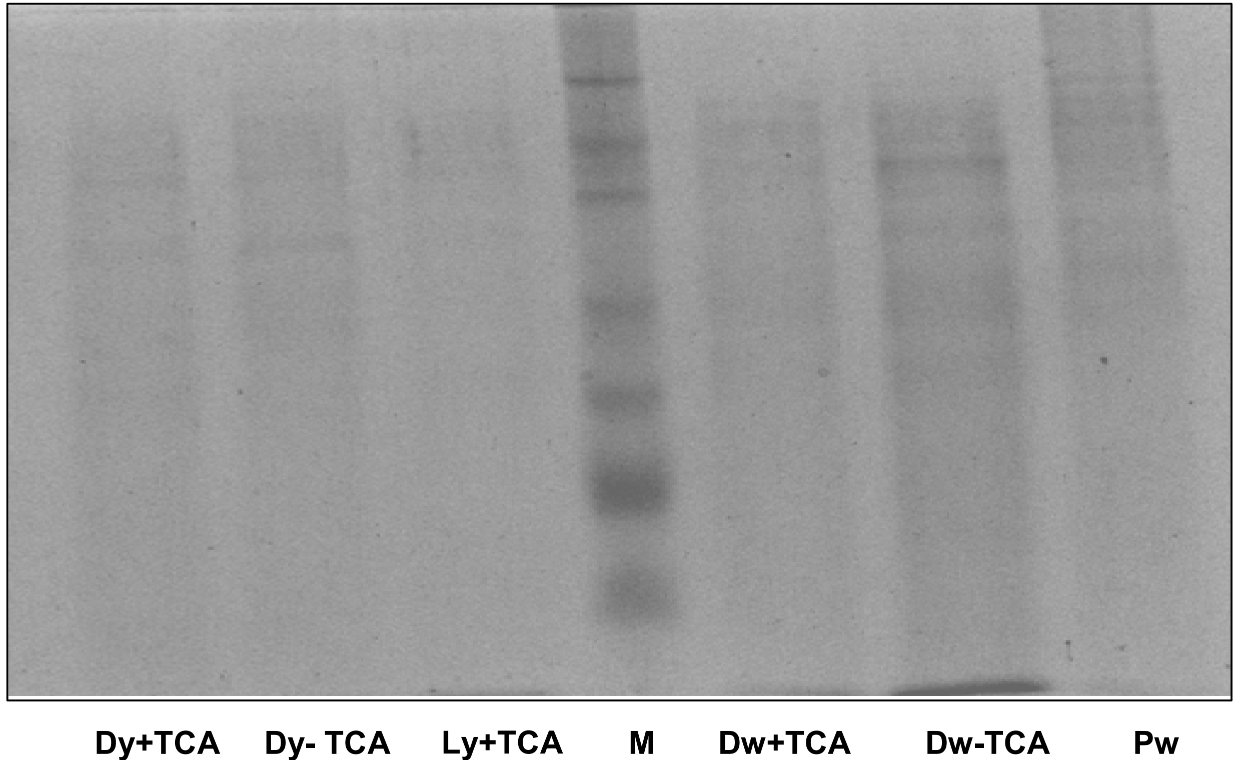


Figure 4.1: Coomassie Stained SDS-PAGE Gel.

Dy±TCA: Dark yellow prickly poppy pollen extract with and without TCA treatment, **Ly+TCA:** Light yellow prickly poppy pollen extract with TCA treatment, **Dw±TCA:** Dark white prickly poppy extract with and without TCA, **Pw:** Pure white pollen extract, **M:** Marker.

4.2 Protein Estimation of Pollen Extracts and Cell Lysates

The protein of pollen extracts and various cell lysates were estimated by Bradford assay using BSA (Bovine Serum Albumin) as a standard and protein concentration was determined as shown in Figure 4.2.

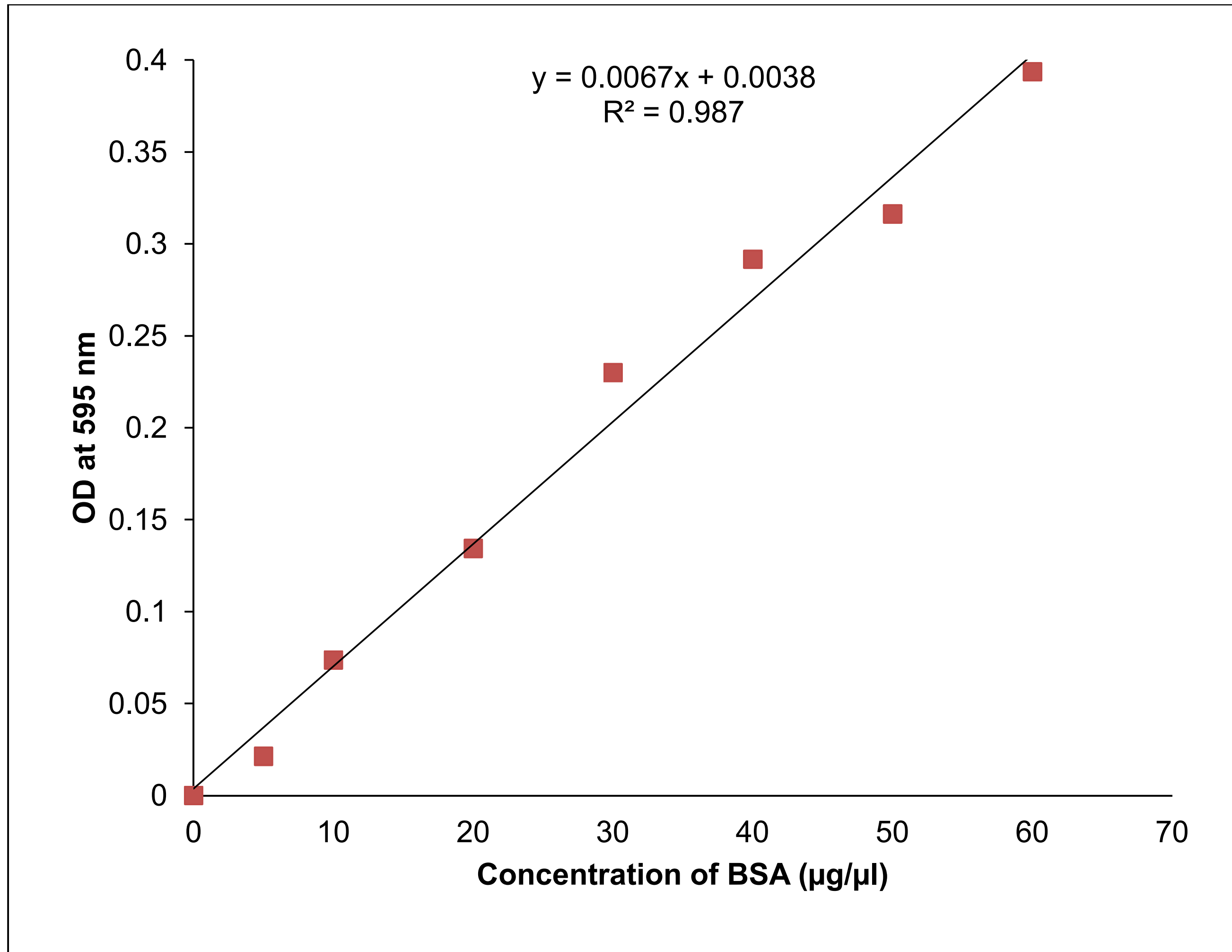


Figure 4.2: Standard curve of BSA used for Bradford assay.

4.3 Pollen Extracts Induced Cytotoxicity

A549 cells were treated with different concentrations of pollen extract dark yellow with TCA (Dy+TCA) in order to see their toxicity on cells. The cells treated with 2 µg of Dy+TCA showed 17% decrease in cell viability as compared to untreated cells that served as control (0.61±0.06 vs. 0.73±0.01). The cells when treated with 5 µg and 10 µg showed no significant change as compared to control (0.70±0.05 vs. 0.73±0.01, 0.76±0.11 vs. 0.73±0.01) respectively. A non-significant decrease of about 29% in cells viability was seen in cells treated with 20 µg ofDy+TCA as compared to untreated control cells and this concentration is toxic to cells (0.52±0.14 vs. 0.73±0.01) as shown in Figure 4.3a and Table 4.1.

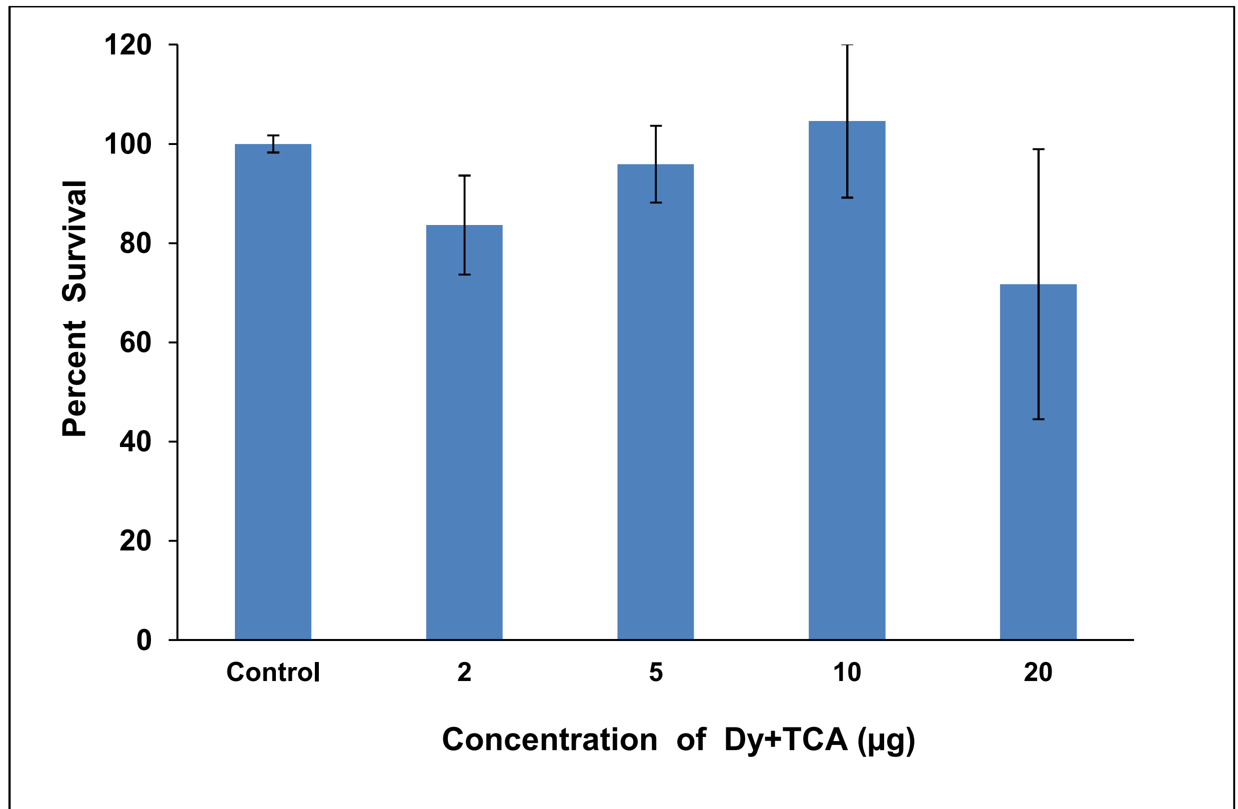


Figure 4.3a: The percent viability of A549 cells treated with Dy+TCA pollen extract. The results are expressed as mean ± standard deviation.

A549 cells were treated with different concentrations of pollen extract dark yellow without TCA (Dy-TCA) to see their cytotoxicity. The cells treated with 2 µg of Dy+TCA showed 15% decrease in cell viability as compared to untreated cells that served as control (0.62±0.11 vs. 0.73±0.01). The cells treated with 5 µg and 10 µg showed 10% and 18% decrease as compared to control (0.66±0.01* vs. 0.73±0.01,0.60±0.09 vs. 0.73±0.01) respectively, thus this concentration is slightly cytotoxic. A significant decrease of about 44% in cell viability was seen in cells treated with 20 µg of Dy+TCA as compared to untreated control cells (0.41±0.02** vs. 0.73±0.01). Thus, these concentrations are significantly toxic to the cells as shown in Figure 4.3b and Table 4.1.

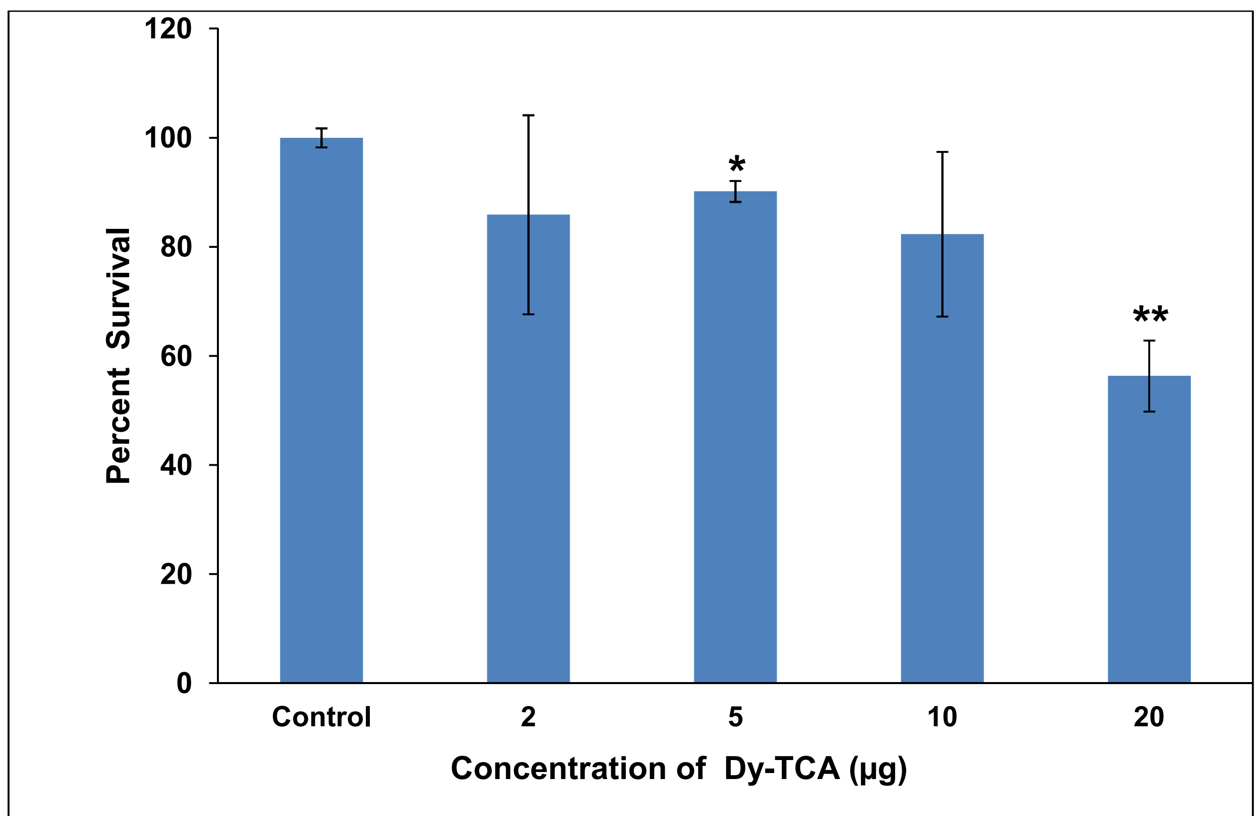


Figure 4.3b: The percent viability of A549 cells treated with Dy-TCA pollen extract. The results are expressed as mean ± standard deviation. Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls.

A549 cells were treated with different concentrations of pollen extract light yellow with TCA (Ly+TCA) to check their cytotoxicity. The cells treated with 2 µg of Ly+TCA showed 14% decrease in cell viability as compared to untreated cells that served as control (0.61±0.11 vs. 0.73±0.01). The cells treated with 5 µg showed no significant change as compared to control (0.73±0.12 vs. 0.73±0.01). The cells treated with 10 µg and 20 µg showed 34% and 13% increase results in proliferation as compared to control (0.98±0.13 vs. 0.73±0.01) (0.82±0.20 vs. 0.73±0.01) respectively. Thus, none of these concentrations are toxic to the cells as shown in Figure 4.3c and Table 4.1.

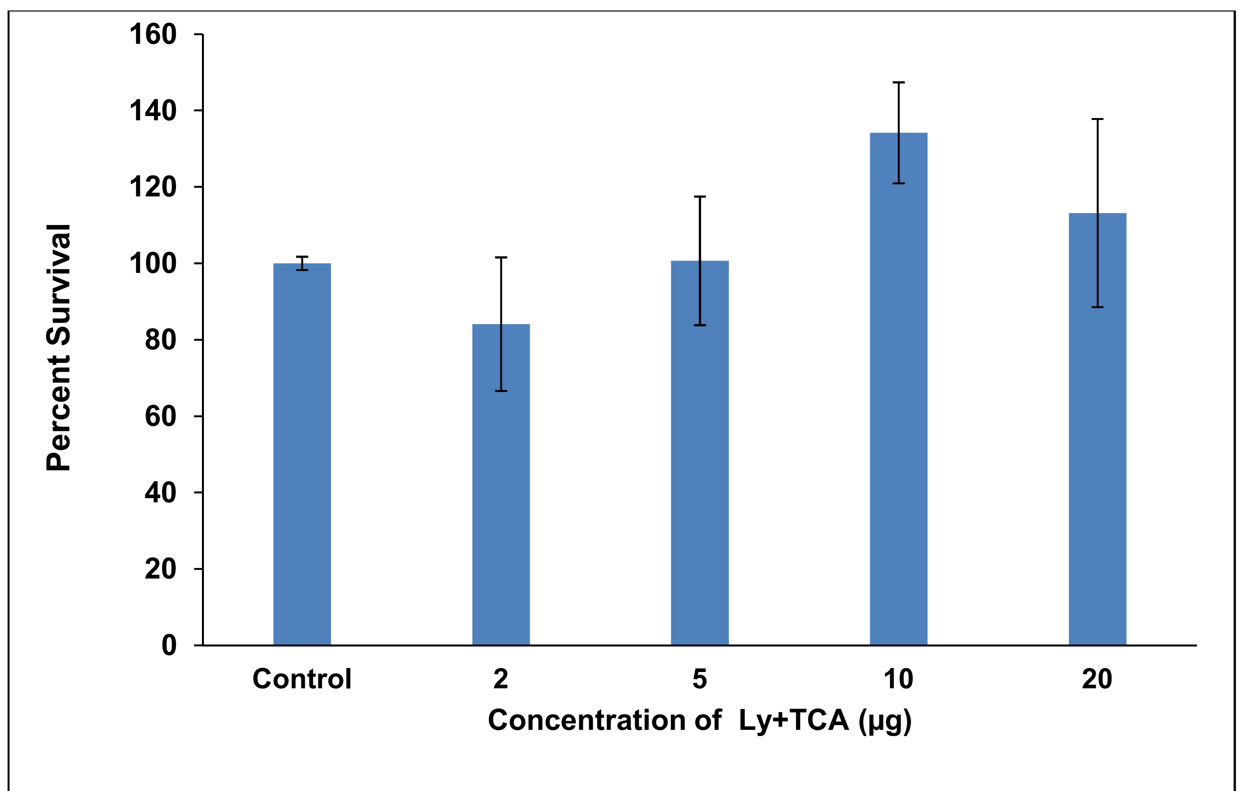


Figure 4.3c: The percent viability of A549 cells treated with Ly+TCA pollen extract. The results are expressed as mean ± standard deviation.

A549 cells were treated with different concentrations of pollen extract dark white with TCA (Dw+TCA) in order to check cytotoxicity. The cells treated with 2 µg of Dw+TCA showed significant 27% decrease in cell viability as compared to untreated cells that served as control (0.54±0.05* vs. 0.73±0.01). The cells treated with 5 µg

showed 12% decrease as compared to control (0.64±0.09 vs. 0.73±0.01).The cells treated with 10 µg showed 8% increase results in proliferation as compared to control (0.79±0.12 vs. 0.73±0.01). The cells treated with 20 µg showed 19% decrease as compared to control (0.59±0.07 vs. 0.73±0.0) as shown in Figure 4.3d and Table 4.1.

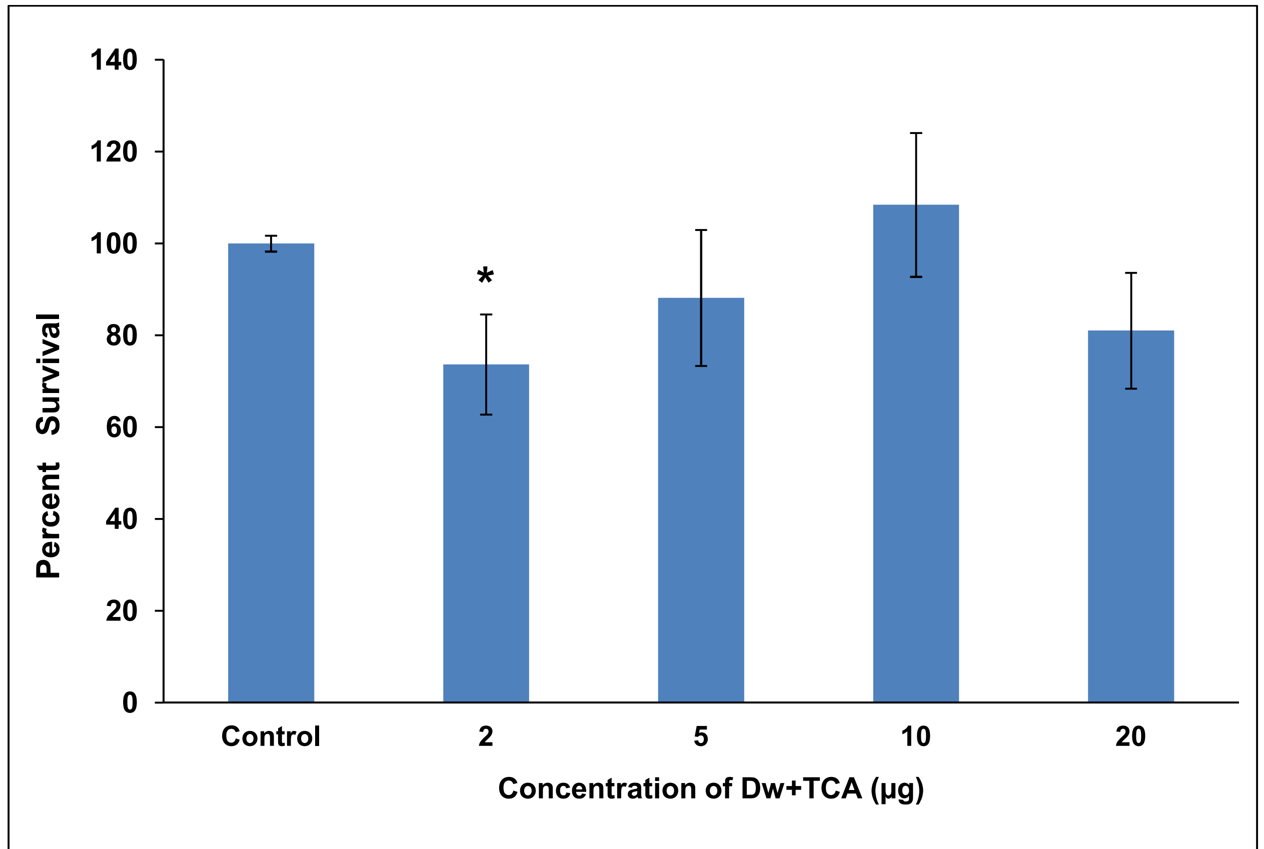


Figure 4.3d: The percent viability of A549 cells treated with Dw+TCA pollen extract. The results are expressed as mean ± standard deviation. Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls.

A549 cells were treated with different concentrations of pollen extract dark white without TCA (Dw-TCA) to see their toxicity on cells. The cells treated with 2µg of Dw-TCA showed significant 26% decrease in cell viability as compared to untreated cells that served as control (0.54±0.01** vs. 0.73±0.01). The cells treated with 5 µg showed significant 17% increase in cell viability as compared to control (0.85±0.02** vs. 0.73±0.01). The cells treated with 10 µg showed non-significant 17%

decrease as compared to control (0.60±0.07 vs. 0.73±0.01). The cells treated with 20 µg showed significant 45% decrease as compared to control (0.41±0.02** vs. 0.73±0.01) suggesting these concentrations are toxic to the cells as shown in Figure 4.3e and Table 4.1.

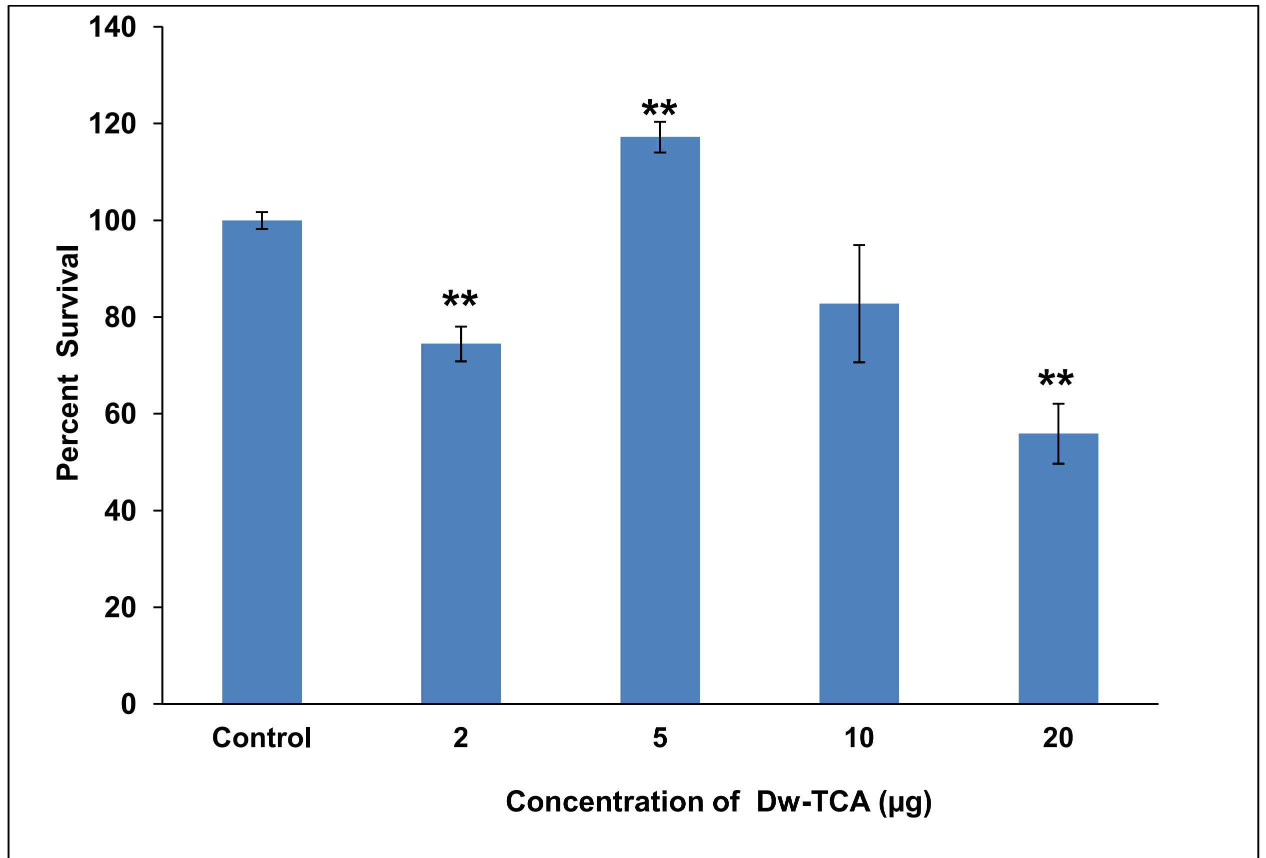


Figure 4.3e: The **percent viability of A549 cells treated with Dw-TCA pollen extract.** The results are expressed as mean ± standard deviation. Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls.

A549 cells were treated with different concentrations of pollen extracts of light white without TCA (Lw-TCA) in order to check cytotoxicity. The cells treated with 2 µg of Lw-TCA showed significant 44% decrease in cell viability as compared to untreated cells that served as control (0.41±0.02*** vs. 0.73±0.01). The cells treated with 5 µg

showed significant 29% increase as compared to control (0.52±0.01*** vs. 0.73±0.01). The cells treated with 10 µg showed significant 45% decrease that results in cytotoxicity as compared to control (0.40±0.04** vs. 0.73±0.01). The cells treated with 20 µg showed significant 59% decrease as compared to control (0.30±0.02*** vs. 0.73±0.01), hence toxic to the cells as shown in Figure 4.3f and Table 4.1.

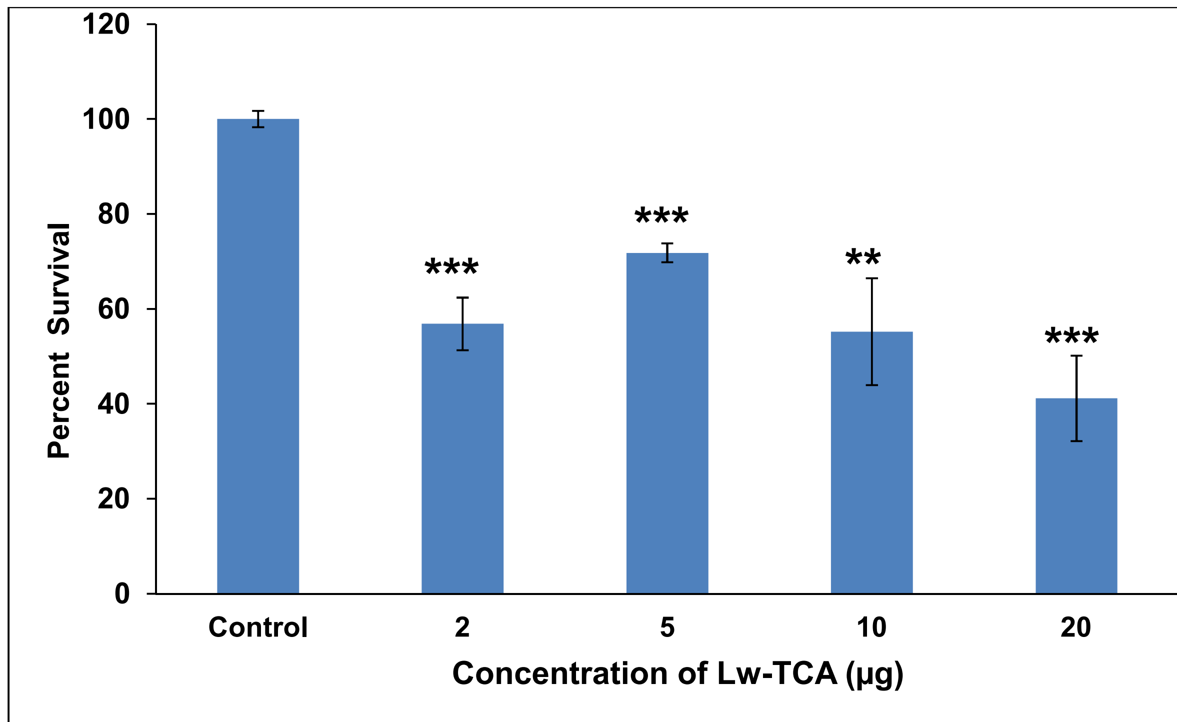


Figure 4.3f: The percent viability of A549 cells treated with Lw-TCA pollen extract. The results are expressed as mean ± standard deviation. Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p\leq 0.05$, $^{**}p\leq0.01$ and $^{***}p\leq0.001$ when pollen extract treated samples were compared with untreated controls.

Table 4.1: Table showing the OD and percent viability estimated by MTT assay of the A549 cells with different concentrations of various pollen extracts.

Control	**Samples conc. ⟶**	**2µg**	**5µg**	**10µg**	**20µg**
0.73±0.01 (100)	**Dy+TCA**	0.61±0.06 (83)	0.70±0.05 (95)	0.76±0.11 (104)	0.52±0.14 (71)
0.73±0.01 (100)	**Dy-TCA**	0.62±0.11 (85)	0.66±0.01* (90)	0.60±0.09 (82)	0.41±0.02 (56)
0.73±0.01 (100)	**Ly+TCA**	0.61±0.10 (84)	0.73±0.12 (100)	0.98±0.13 (134)	0.82±0.20 (113)
0.73±0.01 (100)	**Dw+TCA**	0.54±0.01* (73)	0.85±0.02 (88)	0.60±0.07 (108)	0.41±0.02 (81)
0.73±0.01 (100)	**Dw-TCA**	0.54±0.01** (74)	0.85±0.02** (117)	0.60±0.07 (82)	0.41±0.02** (55)
0.73±0.01 (100)	**Lw-TCA**	0.41±0.02*** (56)	0.52±0.01*** (71)	0.41±0.04** (55)	0.30±0.02*** (41)

A549 cells treated with various extracts of yellow and white were used to access their toxicity on the cells. Where Dy+TCA, Dy-TCA is Dark yellow prickly poppy pollen extract with and without TCA treatment respectively; Ly+TCA is Light yellow prickly poppy pollen extract with TCA treatment; Dw+TCA, Dw-TCA is Dark white prickly poppy extract with and without TCA respectively, Lw-TCA is Light white prickly poppy pollen extract without TCA. Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls.The results are expressed as mean ± standard deviation, the value in parenthesis are percent change.

Table 4.2: Different concentrations of pollen extract samples which were used in future experiments.

Sr. No.	Samples	Concentration
1.	**Dy+TCA**	10 µg
2.	**Dy-TCA**	5 µg
3.	**Ly+TCA**	20 µg
4.	**Dw+TCA**	10 µg
5.	**Dw-TCA**	5 µg

4.4 Effect of Pollen Extract on Lipid Peroxidation

Oxidative stress leads to the oxidation of lipids present in cell membranes resulting in formation of malondialdehyde (MDA) with thiobarbituricacid (TBA) which forms thiobarbituric acid reactive species (TBARS). The mean MDA formation estimated in Con A was increased significantly by 1-fold as compared to untreated control cells (1.49±0.07** vs. 0.70±0.07). H_2O_2, Dy+TCA, Dy-TCA, Ly+TCA and Dw-TCA showed 1-fold, 2-fold, 88%, 67%,33% significant increase respectively in MDA content as compared to control untreated cells (1.54±0.13** vs. 0.70±0.07, 2.16±0.28* vs. 0.70±0.07, 1.32±0.10* vs. 0.70±0.07, 1.17±0.08* vs. 0.70±0.07, 0.93±0.10* vs. 0.70±0.07) respectively as shown in Figure 4.4a and Table 4.3a.

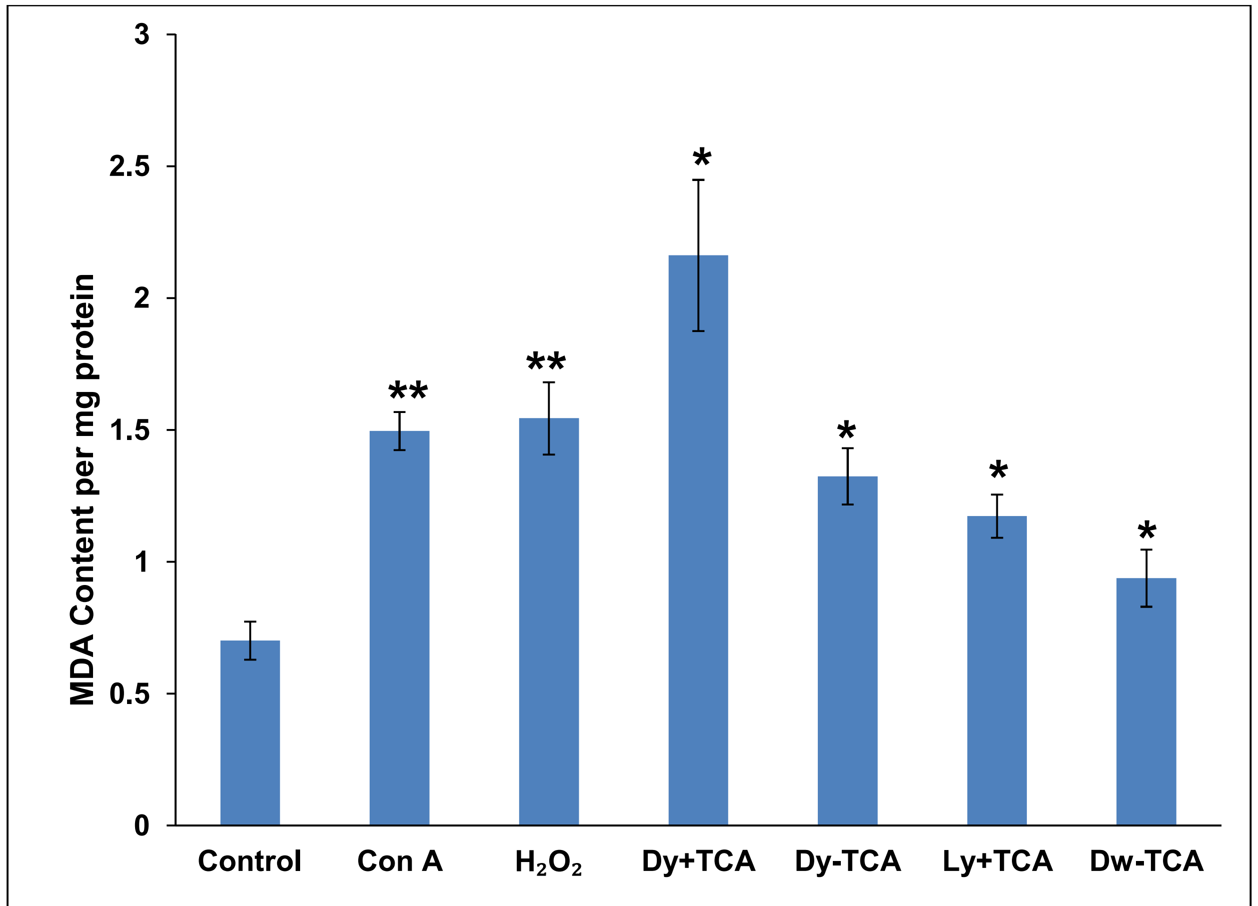


Figure 4.4a: The TBARS activity in total cell lysates of A549 cells treated with Con A, H_2O_2, various yellow and white extract with and without TCA treatment. The results were expressed as mean ± standard deviation (n=3). Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls.

The mean MDA formation estimated in Dw-TCA treated samples was increased significantly by 41% as compared to untreated control cells (0.99±0.10*vs. 0.70±0.07). Allopurinol showed 19% decrease in MDA content as compared to Dw-TCA treated cells (0.80±0.02 vs. 0.70±0.07). NAC showed significant 49% increase as compared to Dw-TCA treated cells ($1.48 \pm 0.12^{\#\#}$ vs.0.70±0.07) respectively. DPI and NF-kB blocking peptide showed significant 69% and 78% decrease respectively as compared with Dw-TCA treated cells ($0.30 \pm 0.11^{\#\#}$ vs. 0.70±0.07), ($0.22 \pm 0.02^{\#\#}$ vs. 0.70±0.07) as shown in Figure 4.4b and Table 4.3b.

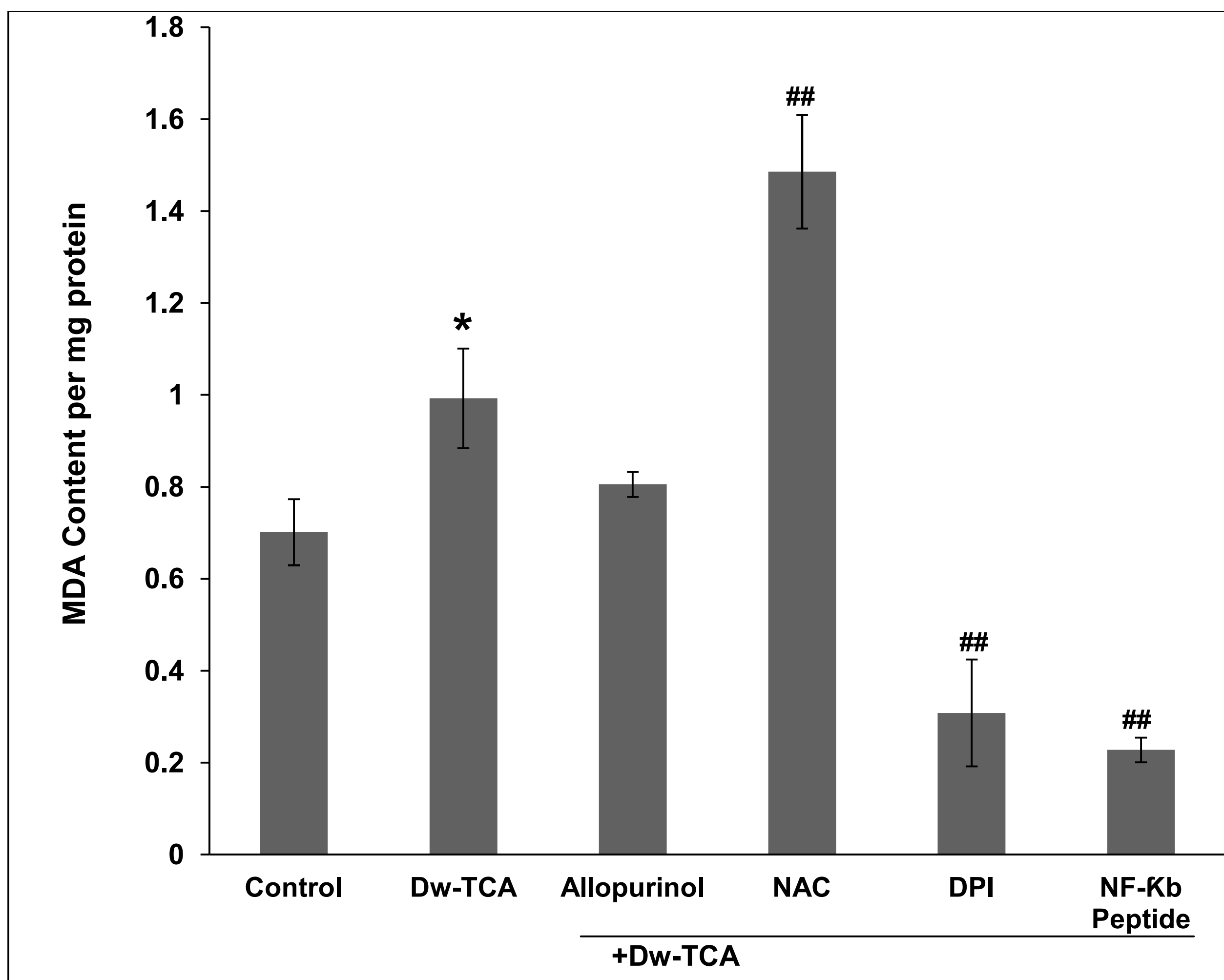


Figure 4.4b: The TBARS activity in total cell lysates of A549 cells treated with Dw-TCA pollen extract in presence of various inhibitors. The results were expressed as mean± standard deviation (n=3). Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls and $^{\#\#}p \leq 0.01$ when Dw-TCA treated with various inhibitors were compared with only Dw-TCA treated samples.

4.5. <u>Pollen Extracts Induced MPO Activity</u>

The mean MPO activity estimated in A549 cell lysates were high when Con A treated cells compared with untreated control cells (60.7±4.08 vs. 9.20±1.48). There was 6.6 fold increase in MPO activity in Con A treated cells as compared to control

cells. The MPO activity in cells treated with H_2O_2 was increased when treated with untreated control cells (129.7±8.17 vs. 9.20±1.48). Dark yellow extracts with TCA showed no change when treated with untreated control cells (9.43±1.44 vs. 9.20±1.48). Dy-TCA, Ly+TCA, Dw+TCA showed significant 68%, 63% and 41% increase in MPO activity as compared to untreated control cells (15.4±1.69*** vs. 9.20±1.48), (15.0±3.49** vs. 9.20±1.48), (12.9±3.82* vs. 9.20±1.48), (9.43±2.04 vs. 9.20±1.48) respectively as shown in Figure 4.5a and Table 4.3a.

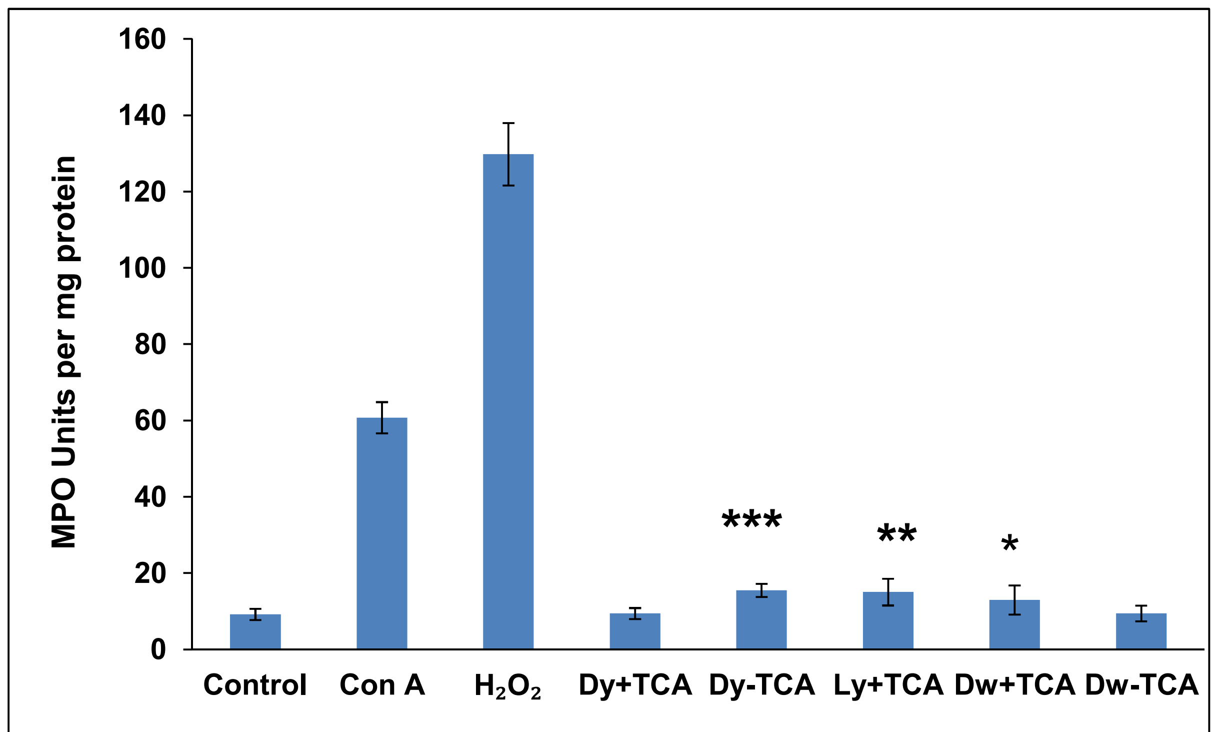


Figure 4.5a: The MPO activity in total cell lysates of A549 cells treated with Con A, H_2O_2, yellow and white extract with and without TCA treatment. The results were expressed as mean ± standard deviation (n=3). The significance of results was evaluated by student *t*-test and $^{*}p \leq 0.01$ and $^{**}p \leq 0.001$ and $^{***}p \leq 0.001$when pollen extracts treated samples were compared with untreated controls.

The MPO activity showed no change when Dw-TCA treated cells were compared with untreated control cells (9.43±2.04 vs. 9.2±1.69). NAC showed 8-fold increase when compared with only Dw-TCA treated control cells (91.7±3.68 vs. 9.4±2.04). DPI showed 46% increase when treated with Dw-TCA treated control cells

(13.8±3.8 vs. 9.4±2.04). FCCP showed 1.7-fold increase and NF-ƙB blocking peptide showed significant 3.9-fold increase when compared with treated Dw-TCA control cells (26.1±2.6 vs. 9.4±2.04), (46.96±4.08$^{###}$ vs. 9.4.1±1.69) respectively as shown in Figure 4.5b and Table 4.3b.

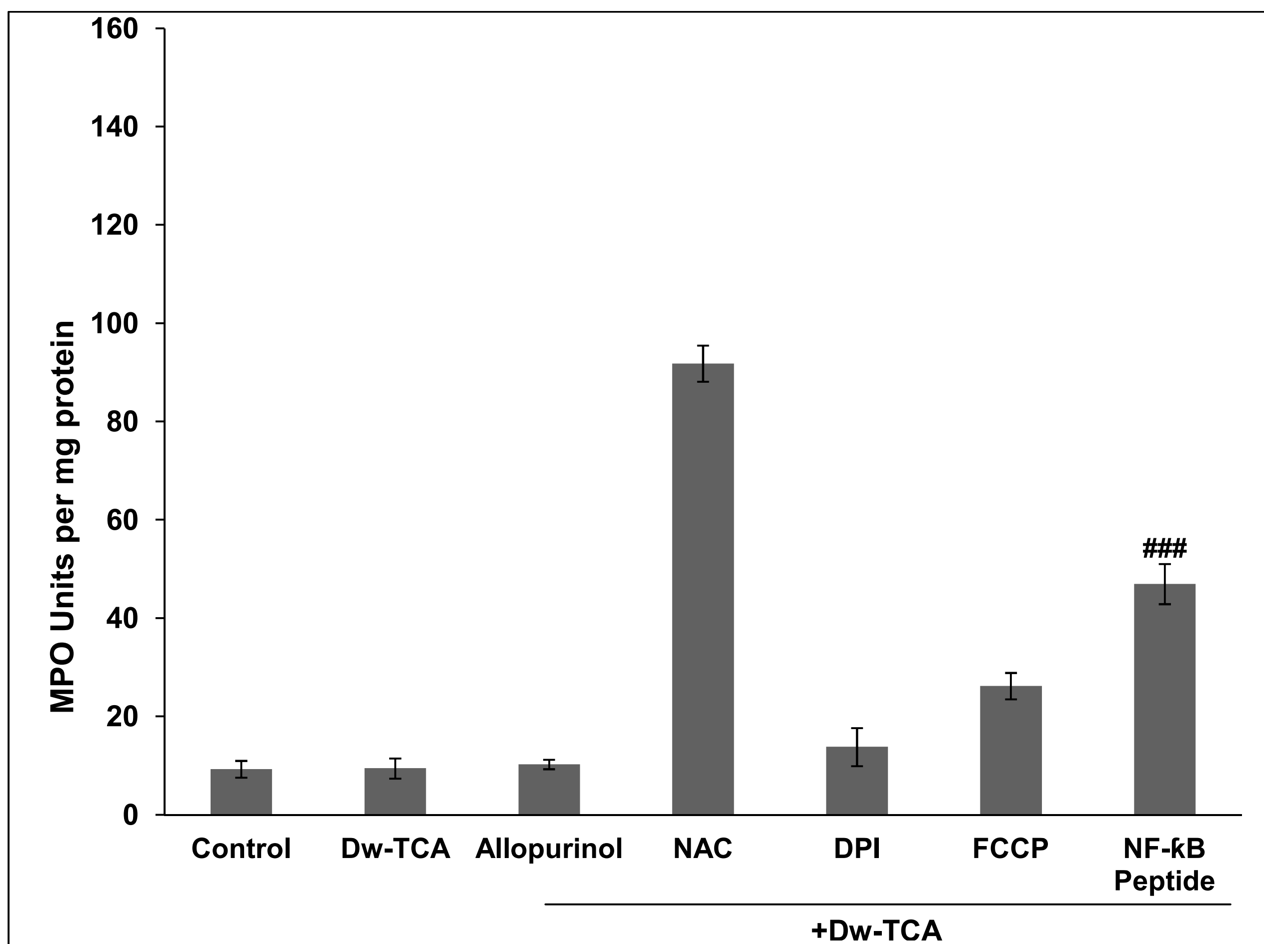


Figure 4.5b: MPO estimation in total cell lysates of A549 cells treated with Dw-TCA and various inhibitors. The results were expressed as mean ± standard deviation (n=3). Student *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, when pollen extract treated samples were compared with untreated controls and $^{##}p \leq 0.01$ when Dw-TCA treated with various inhibitors were compared with only Dw-TCA treated samples.

4.6 Effect of Pollen Extracts on Nitric Oxide Levels

The Griess test or NO assay is a chemical analysis test which detects the presence of organic nitrite compounds, uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NEDD) in acidic conditions. The amount of NO is determined by standard curve plotted by using Sodium Nitrite ($NaNO_2$) as a standard as shown in Figure 4.6a.

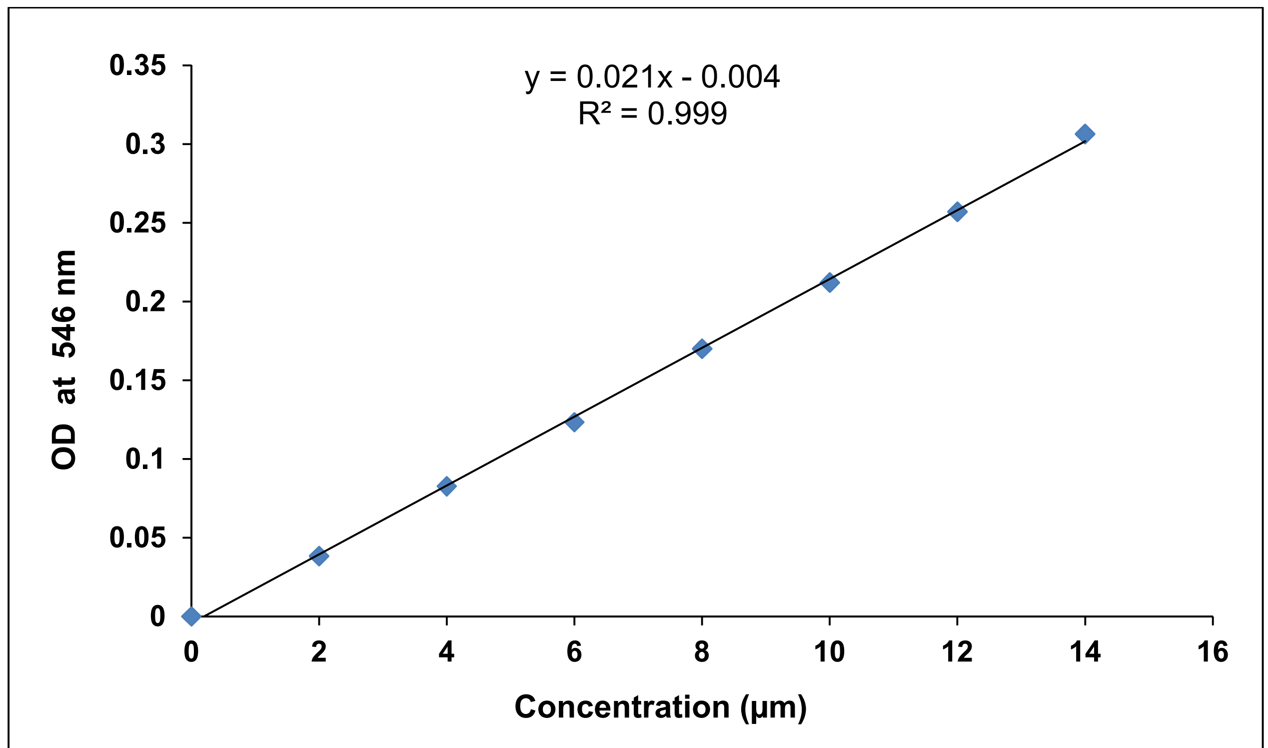


Figure 4.6a: Standard curve for NO with $NaNO_2$.

The mean of concentration of nitrite showed no significant change when ConA treated cells compared with untreated control cells (24.3±0.40 vs. 22.8±0.18). H_2O_2 treated cells showed increase in nitrite concentration by 15% when compared with untreated control cells (26.4±0.47 vs. 22.8±0.18). Dy+TCA showed an increase of about 7% when compared with untreated control cells (24.7±0.97** vs. 22.8±0.18). Dy-TCA, Ly+TCA, Dw+TCA and Dw-TCA showed no significant change in nitrite content as shown in Figure 4.6a (shown as percent change) and Table 4.3a. No

significant change was observed in nitrite concentration when the cells were treated with Dw-TCA pollen extract in presence of various inhibitors (data not shown).

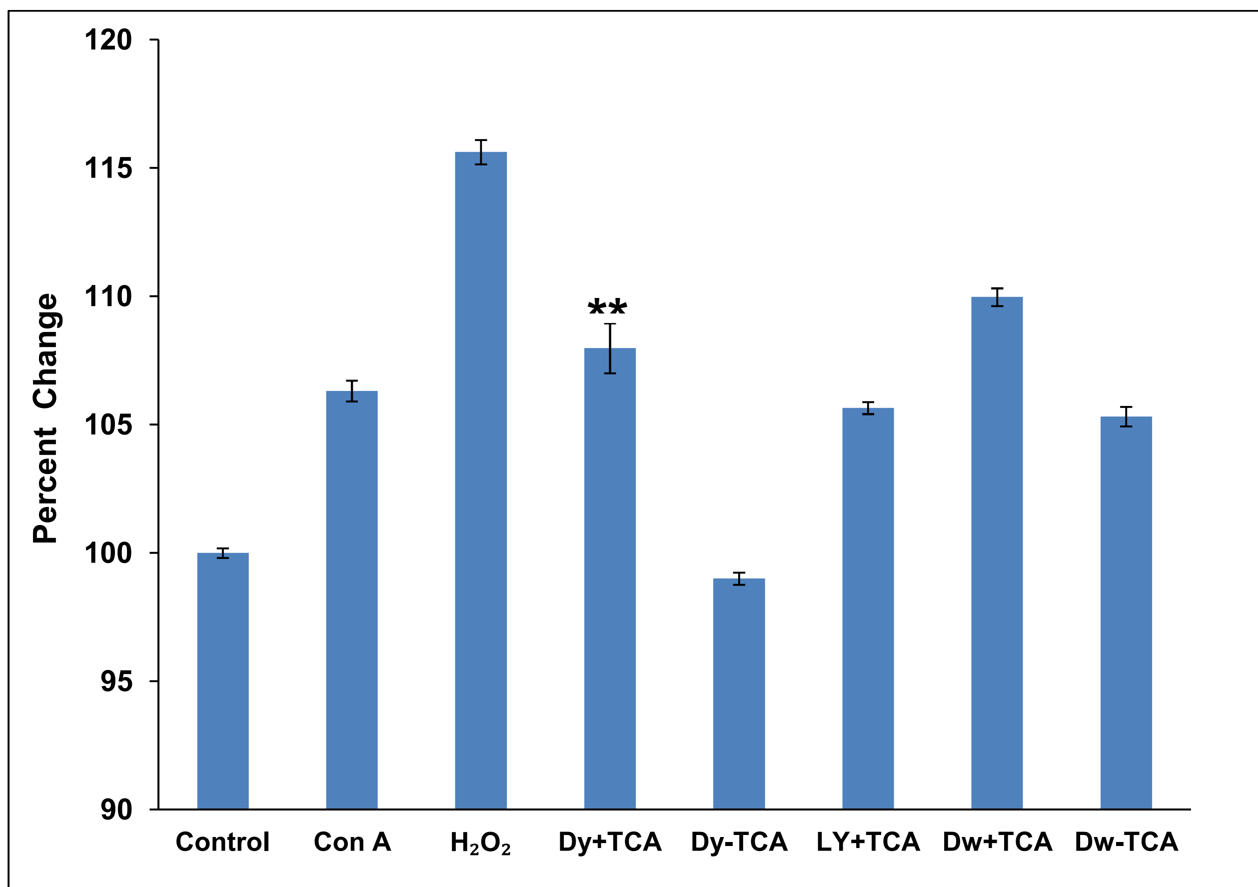


Figure 4.6b: NO estimation in total cell lysates of A549 cells treated with Con A, H_2O_2, yellow and white extract with and without TCA. The results were expressed as mean ± standard deviation (n=3). The significance of results was evaluated by student *t*-test and $^{*}p \leq 0.01$ and $^{**}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls.

4.7 Effect of Pollen Extracts on $p47^{phox}$ and NF-kB Levels

4.7a. ELISA for $p47^{phox}$ Levels

ELISA was done to estimate the $p47^{phox}$ content in all groups. Figure 4.7a shows the $p47^{phox}$ levels in total cell lysates of samples made from A549 cell. Increase of about 8-fold was obtained when H_2O_2 treated cells are compared with untreated

control cells (0.72±0.024 vs. 0.072±0.006). Con A and Ly+TCA showed significant decrease in $p47^{phox}$ content when compared with untreated control cells (0.01±0.001*** vs. 0.072±0.006, 0.011±0.013** vs. 0.072±0.006) respectively. Dw-TCA showed significant 33% decrease when compared with untreated control cells (0.04±0.004** vs.0.072±0.006). Allopurinol showed no change when compared with only Dw-TCA treated cells (0.041±0.009 vs. 0.049±0.004). NAC showed 1-fold increase when compared with Dw-TCA treated cells ($0.13±0.01^{##}$ vs. 0.049±0.006). FCCP, DPI, NF-kB showed no significant change when compared with only Dw-TCA treated cells, (0.05±0.008 vs. 0.049±0.004), (0.05±0.008 vs. 0.7049±0.006) respectively as shown in Figure4.7a and Table 4.4.

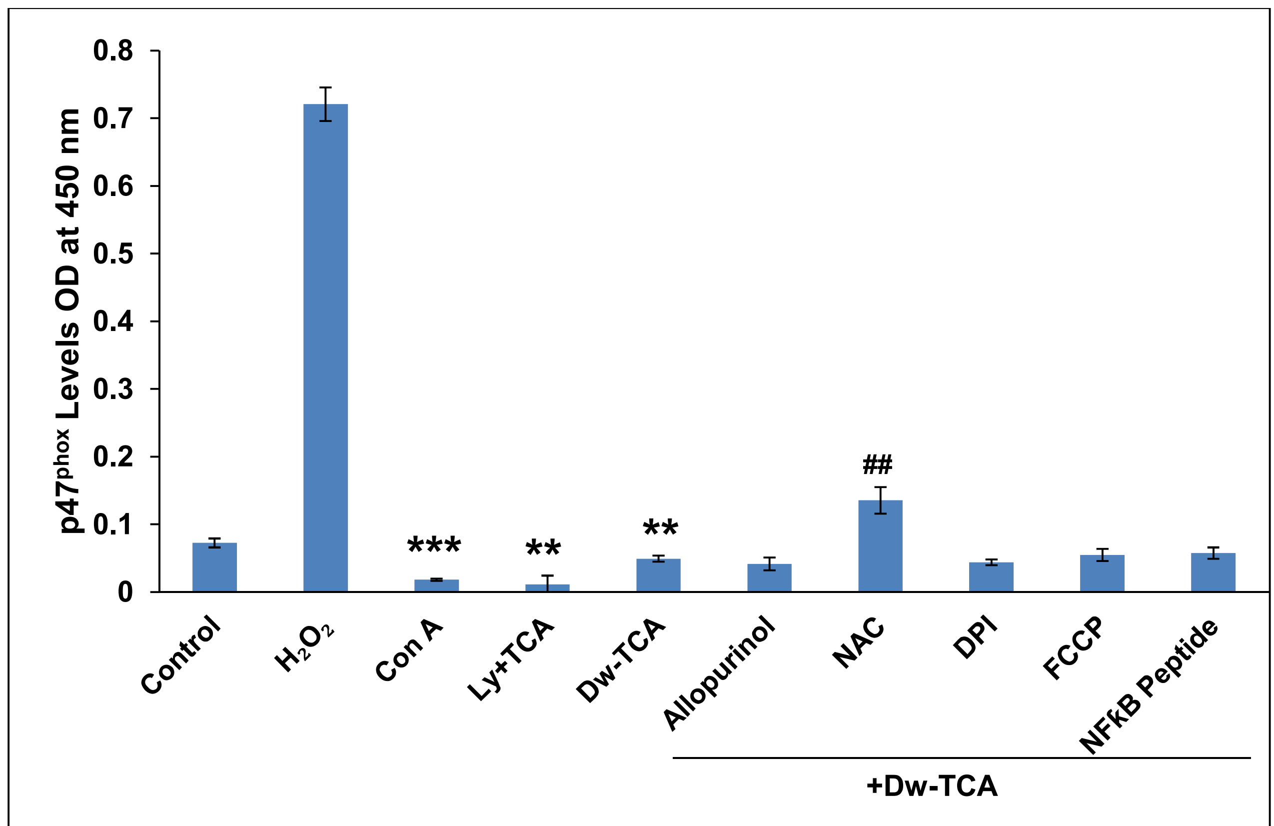


Figure 4.7a: ELISA for $p47^{phox}$ levels in total cell lysates of A549 cells. Student *t*-test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p\leq 0.05$, $^{**}p\leq0.01$ and $^{***}p\leq0.001$ when pollen extract treated samples were compared with untreated controls and $^{##}p\leq0.01$ when Dw-TCA pollen treated with various inhibitors were compared with only Dw-TCA treated samples.

4.7b. Levels of NF-kB Estimated in Total Cell Lysates by ELISA

Figure 4.7b, shows the NF-kB levels in total cell lysate of A549 cells. An increase of about 5-folds was observed when H_2O_2 treated cells were compared with untreated control cells (0.88±0.02 vs. 0.14±0.01). Con A showed significant 52% decrease when compared with untreated control cells (0.07±0.01** vs. 0.14±0.01). Ly+TCA showed significant 42% decrease and Dw-TCA decreases significantly by 70% when compared with untreated control cells (0.08±0.003** vs.0.14±0.01) and (0.04±0.01***vs. 0.14±0.01). Allopurinol showed a significant 50% decrease when compared with only Dw-TCA pollen extract treated cells (0.02±0.009$^{\#}$ vs. 0.04±0.01). NAC, DPI, FCCP and NF-kB showed significant increase of 5-fold, 2-fold, 2-fold and 4-fold increase when compared with the cells treated only with Dw-TCA pollen extract (0.25±0.02$^{\#\#\#}$ vs. 0.04±0.01),(0.11±0.05 vs. 0.04±0.01), (0.94±0.04 vs. 0.04±0.01),(0.21±0.07 vs. 0.04±0.01) respectively as shown in Figure 4.7b and Table 4.4.

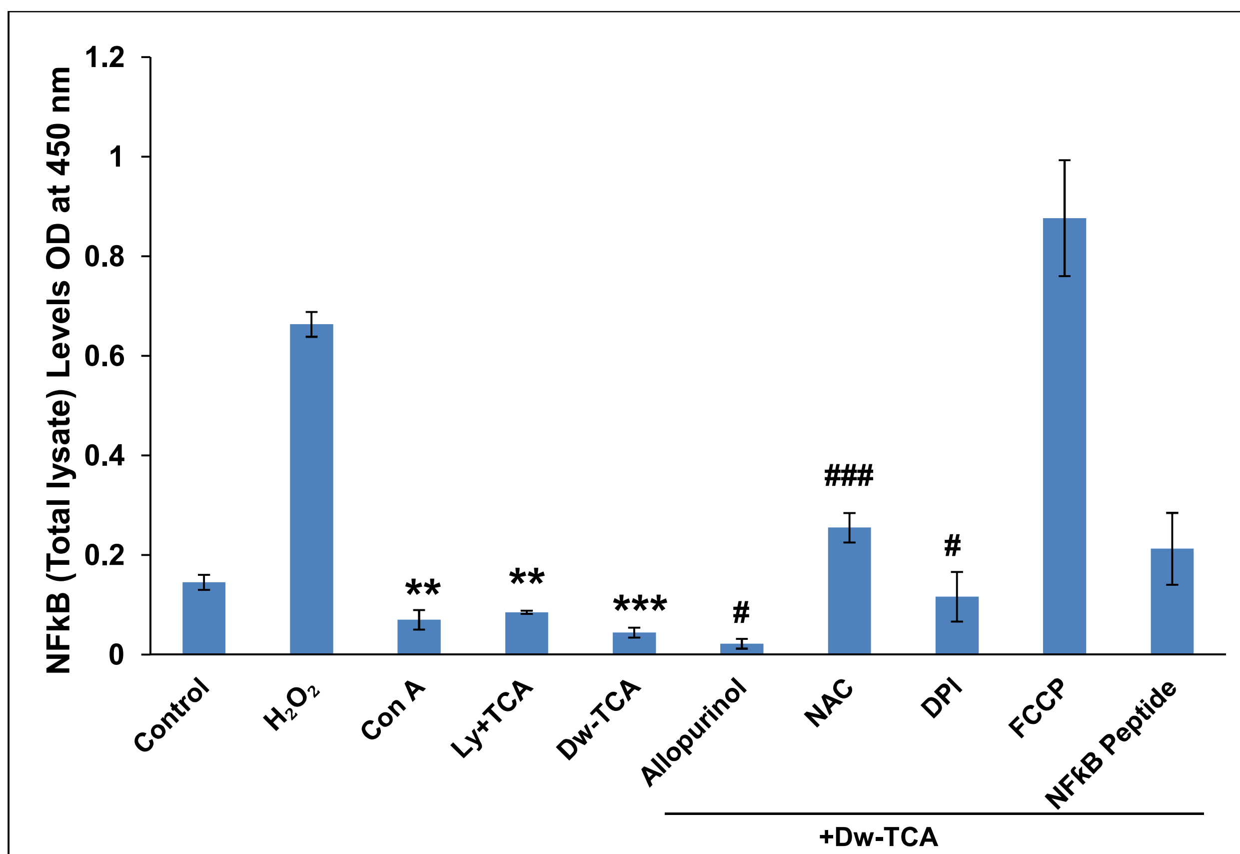


Figure 4.7b: ELISA for NF-kB levels in total cell lysates of A549 cells. Student *t*-test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls and $^{\#\#}p \leq 0.01$ when Dw-TCA pollen extract with various inhibitors were compared with only Dw-TCA treated samples.

4.7c. Levels of NF-kB Estimation in Nuclear Extracts by ELISA

Figure 4.7c shows level of NF-kB in nuclear extract samples of A549 cells. A significant decrease of about 18% was observed when H_2O_2 treated cells were compared with untreated control cells (0.006±0.002** vs. 0.03±0.013). Con A showed 64% increase when compared with untreated control cells (0.05±0.02 vs. 0.03±0.013). Dw-TCA showed 48% decrease when compared with untreated control cells (0.017±0.00* vs. 0.03±0.013). Allopurinol, NAC, DPI and FCCP showed significant increase when compared with Dw-TCA treated cells (0.02±0.009

vs.0.017±0.008; 0.046±0.003 vs. 0.017±0.008), (0.043±0.009[##] vs. 0.017±0.008), (0.06±0.02[##] vs.0.017±0.008) respectively. NF-kB showed decrease of 10% when compared with Dw-TCA treated cells (0.016±0.011 vs. 0.017±0.008) as shown in Figure 4.7c and Table 4.4.

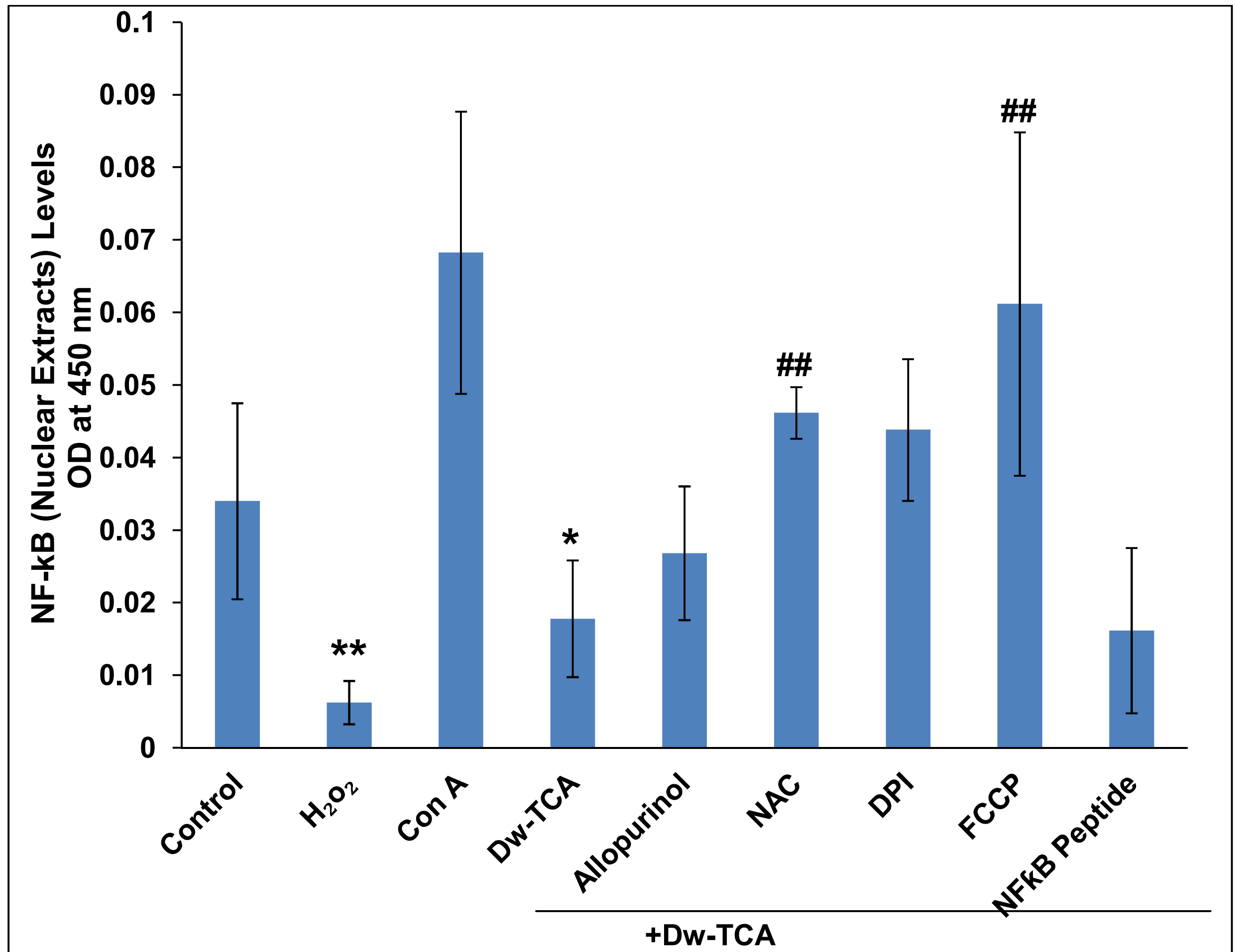


Figure 4.7c ELISA for NF-kB levels in nuclear extracts of A549 cells. Student *t*-test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls and $^{\#\#}p \leq 0.01$ when Dw-TCA with various inhibitors were compared with only Dw-TCA treated samples.

Table 4.3 a: Table showing the data from the various parameters such as TBARS, MPO and NO assay.

Samples	TBARS Assay	MPO Activity	Nitric Oxide Level
Control	0.70±0.07 (100)	9.20±1.48 (100)	22.8±0.18 (100)
Con A	1.49±0.07** (213)	60.7±4.08 (660)	24.3±0.40 (106)
H_2O_2	1.54±0.13** (220)	129.7±8.17 (1410)	26.4±0.47 (115)
Dy+TCA	2.16±0.28* (308)	9.43±1.44 (102)	24.7±0.97** (107)
Dy-TCA	1.32±0.10* (188)	15.48±1.69*** (168)	22.6±0.23 (99)
Ly+TCA	1.17±0.08* (167)	15.04±3.49** (163)	24.1±0.23 (105)
Dw+TCA	-	12.9±3.82* (141)	25.1±0.34 (109)
Dw-TCA	0.93±0.10* (133)	9.43±2.04 (102)	24.1±0.38 (105)

A549 cells were treated with Con A, H_2O_2, and various poppy pollen extracts. The total cell lysates and were submitted to spectrophotometric analysis for TBARS, MPO activity and NO levels as described in materials and methods. The Student's *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extracts treated samples were compared with untreated controls. The results are expressed as mean± standard deviation, the value in parenthesis are percent change when compared with untreated controls. TBARS expressed in nanomoles per mg of protein, MPO activity expressed in units of MPO per mg protein and NO is expressed as nitrite in µM/mg protein.

Table 4.3 b: Table showing the data from the various parameters such as TBARS, MPO and NO assay when treated with Dw-TCA pollen extracts in the presence of various inhibitors.

Samples	TBARS Assay	MPO Activity
Control	0.70±0.07 (100)	9.29±1.69 (100)
Dw-TCA	0.99±0.108* (141)(100)	9.43±2.04 (101) (100)
Dw- TCA+Allopurinol	0.80±0.02 (81)	10.2±0.96 (108)
Dw-TCA+NAC	1.48±0.12## (149)	91.7±3.68 (972)
Dw-TCA+DPI	0.30±0.11## (31)	13.8±3.87 (146)
Dw-TCA+FCCP	-	26.1±2.68 (277)
Dw-TCA+NF-ĸB Peptide	0.22±0.02## (22)	46.9±4.08### (497)

A549 cells were treated with Con A, H_2O_2, and various poppy pollen extracts. The total cell lysates were submitted to spectrophotometric analysis of TBARS, MPO activity and NO levels as described in materials and methods. The Student's *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p\leq 0.05$, $^{**}p\leq 0.01$ and $^{***}p\leq 0.001$ when pollen extract treated samples were compared with untreated controls and $^{\#\#}p\leq 0.01$ when Dw-TCA with various inhibitors were compared with only Dw-TCA treated samples. The results are expressed as mean± standard deviation, the value in parenthesis are percent change when compared with untreated controls. TBARS expressed in NO oxide is expressed as nitrite in μM/mg protein.

Table 4.4. Table showing ELISA data for $p47^{phox}$ and NF-kB levels when the cells were treated with different pollen extracts and Dw-TCA pollen extracts in presence of various inhibitors.

Sample	Anti-$p47^{phox}$	Anti- NF-kB	
	Total Lysate	Total Lysate	Nuclear Extract
Control	0.07±0.006 (100)	0.14±0.01 (100)	0.034±0.013 (100)
H_2O_2	0.72±0.02 (991)	0.88±0.02 (609)	0.006±0.002** (18)
Con A	0.01±0.001*** (25)	0.07±0.01** (48)	0.056±0.024 (164)
Ly+TCA	0.011±0.01** (15)	0.08±0.003** (58)	0.03±0.01 (95)
Dw-TCA	0.04±0.004** (68) (100)	0.04±0.01*** (30) (100)	0.017±0.08* (143) (100)
Dw-TCA+Allopurinol	0.04±0.009 (84)	0.02±0.009# (50)	0.02±0.009 (150)
Dw-TCA+NAC	0.135±0.01## (275)	0.25±0.02### (571)	0.04±0.003 (259)
Dw-TCA+DPI	0.04±0.004 (89)	0.11±0.05 (261)	0.04±0.009## (246)
Dw-TCA+FCCP	0.05±0.008 (111)	0.94±0.04 (2108)	0.06±0.02## (343)
Dw-TCA+NF-kB peptide	0.05±0.008 (116)	0.21±0.07 (476)	0.016±0.01 (90)

The total cell lysates and nuclear extracts of A549 cells treated with various poppy pollen extracts were used for measuring the levels of $p47^{phox}$ and NF- kB using ELISA. The Student's *t*- test was performed to evaluate the significance of the results, the data was considered as statistically significant at $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ when pollen extract treated samples were compared with untreated controls and $^{\#\#}p \leq 0.01$ when Dw-TCA with various inhibitors were compared with only Dw-TCA treated samples.

Chapter V

Discussion

One of the most common reasons for increased allergic reactions that has occurred during the last few decades is chronic inflammation (Romagnani, 2004). Chronic inflammation plays a very important role in the development, progression and in increasing the risk of lung cancer and various other inflammatory diseases (Yazdanbakhsh *et al.*, 2002). The cytokines, chemokines, iNOS, NF-kB activation and ROS/RNS produced during inflammation are the important factors that link inflammation and cancer.

High levels of oxidative stress in the lungs is observed in several pulmonary diseases such as asthma, COPD, interstitial lung disease, cystic fibrosis, pulmonary hypertension, acute chest syndrome, sickle cell disease, acute lung injury [including acute respiratory distress syndrome (ARDS)], and severe respiratory failure in infants as well as in healthy chronic smokers (Dalle-Donne *et al.*, 2006).

In present study, the inflammatory responses induced by pollen extracts of yellow (*Argemone mexicana*) and white prickly poppy (*Argemone ochroleuca)* extracts were studied. Yellow and white prickly poppy pollens were considered as one of the most common aeroallergens those are present in nearby region of Bathinda and Barnala in the Malwa region of Punjab. In this study, A549 cells were used which are Adenocarcinomic human alveolar basal epithelial cells.

MTT assay was performed to determine the cytotoxicity of various concentrations of pollen extracts from yellow and white prickly poppy. The results obtained indicated that yellow and white prickly poppy with or without TCA enhanced proliferation in A549 cells at lower concentrations, and higher concentrations were being toxic to the A549 cells.

In a previous study from our laboratory, it was shown that yellow prickly poppy pollen extract exposure within limited period of time lead to increased inflammation (NOX, NO and AOPP levels) and oxidative stress (protein carbonyls and lipid peroxidation) in A549 cells (unpublished data). In the present study, studies were extended to delineate the mechanism responsible for inducing inflammation and oxidative stress.

Oxidative stress-induced peroxidation of membrane lipids can be very damaging because it can lead to the alterations in the biological properties of the membrane, such as the degree of fluidity, and can lead to inactivation of many membrane-bound receptors and enzymes, which in turn may impair normal cellular function and increase tissue permeability. Lipid peroxidation contributes to and amplifies cellular damage resulting from generation of oxidized products, some of which are chemically reactive and covalently modify critical macromolecules. Products of lipid peroxidation have therefore commonly been used as biomarkers of oxidative stress/damage. Lipid peroxidation generates a variety of unsaturated aldehydes, such as malondialdehyde (MDA), 4- hydroxy-2-nonenal (Juhasz *et al.*, 2009), and various 2-alkenals (Devasagayam *et al.*, 2003).

In this study, TBARS assay was used to measure whether yellow and white prickly poppy extracts induce lipid peroxidation which is a conventional marker of oxidative stress. A significant increase in lipid peroxidation was observed when A549 cells were treated with yellow and white prickly poppy extracts. In previous studies, inflammations due to ragweed pollen extract (Hussain *et al.,* 2007) induced cytotoxic and inflammatory signals which subsequently activate oxidative stress-induced expression of inflammatory cytokines via NF-κB-dependent mechanism in mice (Yadav *et al.*, 2009).

To rule out the possible source of ROS responsible for inducing oxidative stress, the present study used various inhibitors such as Allopurinol, DPI and FCCP which inhibit the classical sources of ROS such as Xanthine oxidase, NOX and mitochondria respectively. In addition, also used the NF-kB blocking peptide and NAC to inhibit the NF-kB pathway and general ROS production respectively along with pollen extracts.

The lipid peroxidation was significantly decreased in DPI and NF-kB blocking peptide treated samples suggesting the role of NOX and NF-kB induced ROS in enhancing the lipid peroxidation. Previous study by Kruzel*et al.* (2006) demonstrated

that protein lectoferrin decreased ragweed pollen extract (Hussain *et al.,* 2007) induced in ROS levels (Kruzel *et al.*, 2006).

MPO is mainly expressed in macrophages and neutrophils. It is a classical heme peroxidase that uses H_2O_2 produced by neutrophils in order to oxidize a variety of aromatic compounds. It is a unique enzyme because it readily oxidizes chloride ions to the strong non-radical oxidant i.e. HOCl which is a bactericidal oxidant. Many species of bacteria are killed readily by a myeloperoxidase/hydrogen peroxide/chloride system (Hampton *et al.*, 1998). The release of ROS and HOCl by neutrophils causes damage to important biological structures, such as proteins, carbohydrates, lipids, and nucleic acids which may enhance inflammatory responses. It is a classical marker of inflammation and the present study showed that the MPO activity was not altered when A549 cells were treated with various yellow and white prickly poppy extracts with and without TCA whereas the positive controls such as Con A and H_2O_2 showed significant increase in MPO activity. Previous study showed the secretion of granule proteins from neutrophils of 11 pollen-atopic asthmatic patients and the release of MPO from neutrophils was significantly increased without altering the functioning of the lungs (Carlson *et al.*, 1992).

The MPO level was increased significantly in NAC, DPI, FCCP and NF-kB blocking peptide treated samples suggesting the role of ROS scavenger, NOX, mitochondrial uncoupler and NF-kB in inflammation. Others have reported the link between MPO and oxidative damage of proteins and lipids suggesting that the enzyme has the ability to promote lipoprotein oxidation by pathways involving HOCl and tyrosyl radical which is a pivotal agent in the development of atherosclerotic lesions (Daugherty *et al.*, 1994).

NO is a conventional marker of RNS. Present study showed that there is an increase in NO level in A549 cells when treated with yellow and white pollen extracts. Our data is consistent with a previously reported study which showed accumulation of nitrite, a potent signaling molecule and a precursor of NO formation in mammalian system, when pollen was present in the media (Bright *et al.*, 2009).

In the present work the A549 cells when treated with various inhibitors showed no change in NO level. However, a previous study showed increased iNOS production *in vivo* model which might be due to the sensitivity of the model selected (Hiramoto *et al.*, 2013).

NOX family NADPH oxidases are enzymes dedicated to the generation of ROS, which serve distinct roles in a variety of cell types. ELISA is an assay that uses an enzyme linked to an antibody as a marker for the detection of a specific protein. To measure the levels of $p47^{phox}$ when A549 cells were treated with inhibitors and pollen extracts ELISA assay was used. The NOX levels were found to be significantly decreased in pollen extracts treated samples where as it was found to be increased in cells treated with mitochondria uncoupler and NAC suggesting role of mitochondrial ROS during pollen allergies.

The NF-kB levels were found to be decreased in pollen extracts treated samples but the A549 cells when treated with mitochondria uncoupler (FCCP) elevated the NF-kB levels, suggesting the role of mitochondrial ROS in activating NF-kB. Similarly, in the nuclear extracts the NF-kB levels were found to be significantly decreased in pollen extract treated samples but the A549 cells when treated with FCCP and the NOX inhibitor elevated the NF-kB levels suggesting the role of mitochondria and phagocytic ROS in activating NF-kB during pollen allergies.

From the present work, it is clear that during pollen allergies there is an accumulation of oxidative stress as confirmed by the increased TBARS level hence resulting in lipid peroxidation. Interestingly, NO and MPO which are the conventional markers of inflammation did not show any change in their levels when treated with various pollen extracts. Involvement of NADPH oxidase and NF-kB pathway has been elucidated as a marker of ROS production in the present study. The levels NOX activity and NF-kB measured using ELISA showed significant decrease in their levels indicating some other pathway/source responsible for generating ROS and hence increasing lipid peroxidation. The increased levels of NOX and NF-kB in presence of inhibitors suggested that mitochondrial ROS and phagocytic ROS are very important

in activating NF-kB pathway. It has also been reported to be involved in various pollen allergies studied in various *in vitro* and *in vivo* models (Hampton *et al.*, 1998; Li *et al.*, 2011). Gilles *et al.* (2009) reported that the pollen-derivedphytoprostanes (PPE_1) inhibit IκBα degradation in the cytoplasm, p65 translocation to the nucleus and NF-κB binding to κB binding sites on the DNA and that in turn inhibit LPS induced IL-12 production that ultimately blocks NF-kB signaling thereby, reducing the transcription of IL-12 p40 subunit gene (Gilles *et al.*, 2009).

The present study has provided preliminary, but very important data showing relation between oxidative stress and inflammation during pollen allergies. The detailed mechanism involved needs further investigation. The precise mechanism of the pollen extracts remains to be elucidated and whether pro/anti-inflammatory cytokines are related to these inflammation markers will be done in near future.

Chapter VI

Summary and Conclusions

Long term exposure to any type of oxidative stress leads to chronic inflammation. The increase in risk of chronic inflammation may lead to lung cancer. The present study was conducted to evaluate stress responses which gets activated when exposure to aeroallergens such as pollen grains. The pollen grains are important aeroallergens causing allergic disorders like rhinitis and asthma. The various mediators of inflammation like ROS/RNS, cytokines and chemokines form a link between oxidative stress, inflammation and cancer.

This study involves with the induction of oxidative stress by pollen extracts derived from various varieties of yellow prickly poppy in small lung carcinoma A549 cells. To rule out the pathway responsible for pro-inflammatory and oxidative stress inducer, the inhibitors of the classical ROS sources such as NADPH oxidase, Xanthine oxidase, mitochondria and general ROS scavenger (NAC) were used. In addition, NF-kB blocking peptide was also used to see whether blocking the NF-kB pathway can attenuate the pollen induced stress. It was observed that there was no significant difference in inducing inflammation and/or oxidative stress in the purified pollen antigen versus the crude pollen extracts.

Lipid peroxidation was significantly increased in pollen extract treated cells whereas no change in MPO activity and NO level were observed. The NF-kB and NOX levels were significantly reduced in pollen extract treated cells whereas the levels were normalized when treated with NOX inhibitors (DPI) and mitochondria uncoupler (FCCP) suggesting the role of mitochondrial and phagocytic ROS in activating NF-kB. This study signifies that the poppy pollen which is very common in Malwa region of Punjab may play a key role in enhancing the allergen related complications and chronic lung diseases.

In future studies, the detailed mechanism and role of other mediators of inflammation like cytokines and chemokines will be studied which will elucidate correlation between pollen extracts and the chronic disease state.

Chapter VII

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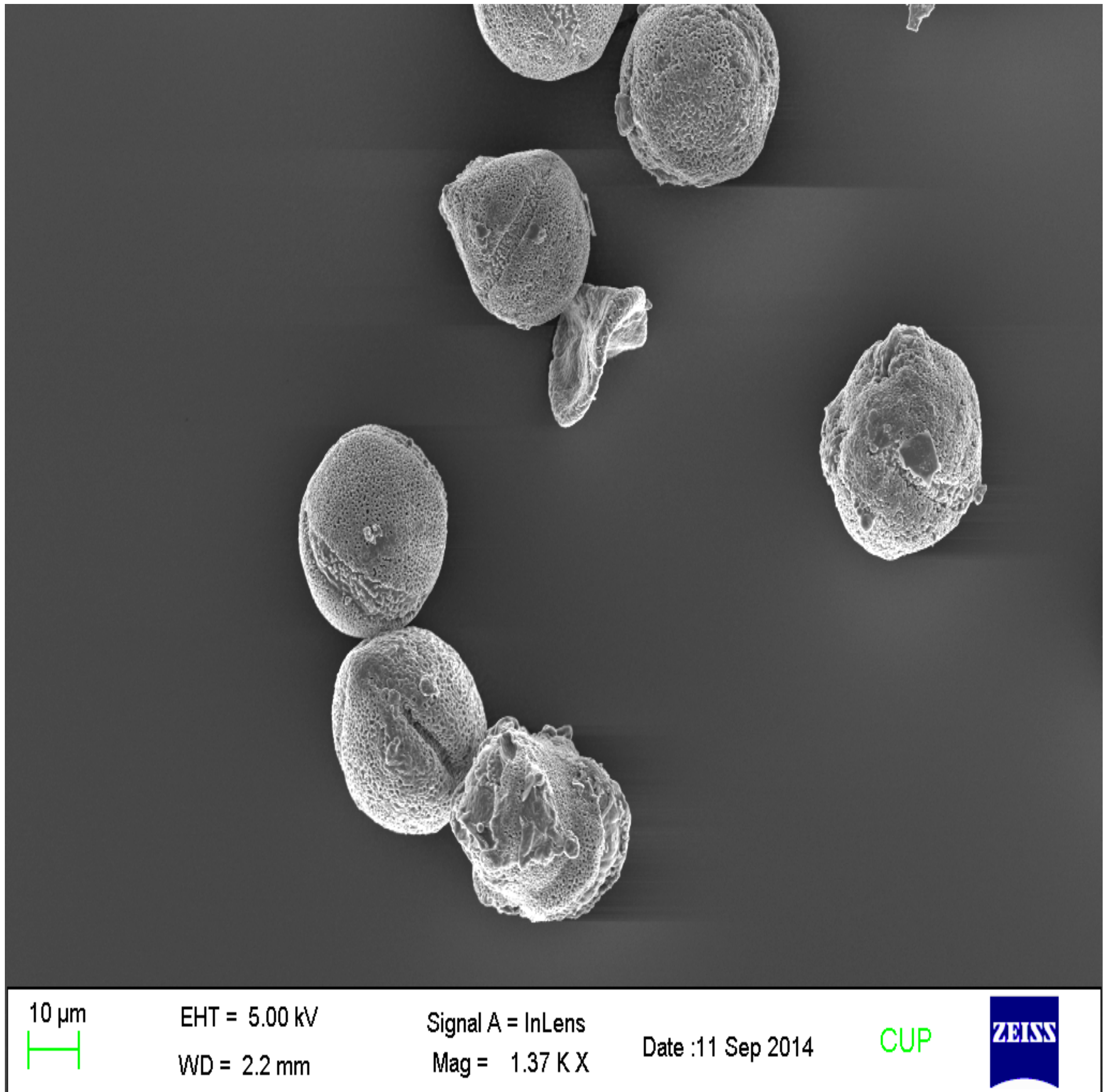
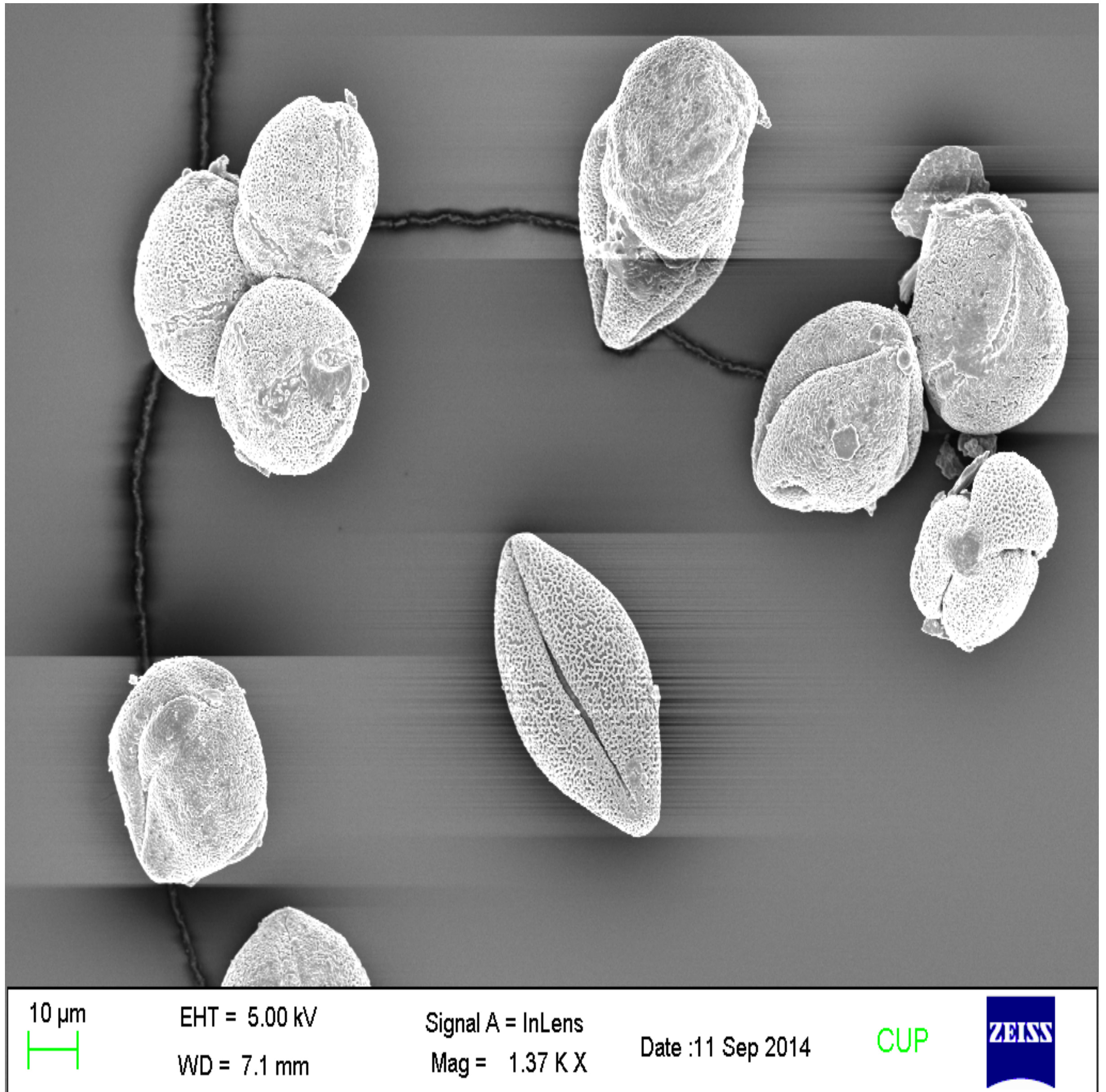
Annexure I



Electron Micrograph of Yellow Prickly Poppy Pollen



Electron Micrograph of White Prickly Poppy Pollen