RENGİN REİS

FARMASÖTİK TOKSİKOLOJİ DOKTORA PROGRAMI

2020

T.C.

YEDİTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

# EVALUATION OF PROTECTIVE EFFECTS OF EUCALYPTOL (EUC) AND CURCUMIN (CUR) ON CIGARETTE SMOKE EXTRACT (CSE) INDUCED LUNG CELL TOXICITY *IN VITRO*

DOCTOR OF PHILOSOPHY THESIS

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SUPERVISOR

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## THESIS APPROVAL FORM

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 03.02.2020 and numbered 2020/02-02

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

## BEYAN

Bu tezin kendi çalışmam olduğunu, planlanmasından yazımına kadar hiçbir aşamasında etik dışı davranışımın olmadığını, tezdeki bütün bilgileri akademik ve etik kurallar içinde elde ettiğimi, tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara kaynak gösterdiğimi ve bu kaynakları kaynaklar listesine aldığımı, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını beyan ederim.

20.01.2020

Rengin REİS

## DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

20.01.2020

Rengin REİS

## DEDICATION

This thesis is dedicated to my precious mom and dad with love.

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF SYMBOLS and ABBREVIATIONS

5-LOX: 5-lipoxygenase

AGE: Advanced glycation end products

AP: Argpyrimidine

ARDS: Acute respiratory distress syndrome

ARE: Antioxidant response element

BAL: Bronchoalveolar lavage

BEAS-2B: Human bronchial epithelial cell line

BSC: Biosafety cabinette

CAT: Catalase

COPD: Chronic obstructive pulmonary disease

COX: Cyclooxygenase

cPLA 2: Cytosolic phospholipase A 2

CS: Cigarette smoke

CSE: Cigarette smoke extract

Cu/Zn-SOD: Copper-zinc superoxide dismutase

CUR: Curcumin

CVD: Cardiovascular disease

DCFDA: 2',7' Dichlorofluorescin diacetate

Der p*: Dermatophagoides pteronyssinus*

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DOX: Doxorubicin

DTNB: 6,6′-Dinitro-3,3′-dithiodibenzoic acid

EC-SOD: Extracellular superoxide dismutase

EDTA: Ethylenediaminetetra acetic acid disodium dehydrate

Egr-1: Early growth response factor-1

ETS: Environmental tobacco smoke

EUC: Eucalyptol

FBS: Fetal bovine serum

g: Gram

GI: Gastrointestinal

GSH: Glutathione

GSSH: Oxidized glutathione

$H_2O_2$: Hydrogen peroxide

HBEC: Human bronchial epithelial cells

HO-1: Hemoxygenase-1

$HO^{\bullet}$: Hydroxyl radical

IARC: International Agency for Research on Cancer

IBS: Irritable bowel syndrome

IL-1β: Interleukin 1-Beta

IL-6: Interleukin 6

IU: International unit

i.p.: Intraperitoneal

L: Liter

LC3B: Microtubule-associated protein light chain 3-B

LPS: Lipopolysaccharide

LTB4: Leukotriene B4

MAPK: p38 mitogen-activated protein kinase

MDA: Malondialdeyhde

mg: Milligram

ml: Milliliter

MMP: Mitochondrial membrane potential

Mn-SOD: Manganese superoxide dismutase

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC: N-acetylcysteine

NaOH: Sodium hydroxide

NAPQI: N-acetyl-p-benzoquinone imine

NF-κB: Nuclear factor kappa B

nm: Nanometer

Nrf2: The nuclear factor erythroid 2-like 2

OD: Optical density

OECD: Organization for economic cooperation and development

PBS: Phosphate buffer saline

$PGE_2$: Prostaglandin $E_2$

PI: Propidium iodide

ROS: Reactive oxygen species

SFN: Sulforaphane

SOD: Superoxide dismutase

TBA: Thiobarbituric acid

TNF-α: Tumor necrosis factor-α

UV: Ultraviolet

µg: Microgram

µl: Microliter

µM: Micromolar

## ABSTRACT

**Reis, R. (2020). Evaluation of Protective Effects of Eucalyptol (EUC) and Curcumin (CUR) on Cigarette Smoke Extract (CSE) Induced Lung Cell Toxicity *in vitro.* Yeditepe University, Institute of Health Sciences, Department of Pharmaceutical Toxicology, Ph.D. Thesis, İstanbul.**

Smoking is one of the most important leading cause of worldwide death, over millions of people each year. From toxicological perspective, cigarette smoke (CS) also serving an environmental issue for all living organism, especially human being exposed to passive smoke. Last decades, effects of natural compounds on pulmonary system have been under investigation in order to enlighten their effects on CS mediated respiratory diseases such as chronic obstructive pulmonary disease, asthma and lung cancer. Therefore, protective mechanism of traditionally used eucalyptol (EUC), curcumin (CUR) and their combination on BEAS-2B human bronchial epithelial cell line was investigated *in vitro* in order to understand their impact on adverse outcome pathways involved in cell death, oxidative cell injury and inflammatory response induced by CS extract (CSE). According to exhibited results, EUC, CUR and their combination improved BEAS-2B cell viability, attenuated CSE induced apoptosis and autophagy-dependent cell death. Further, CSE induced oxidative stress remarkably reduced by combination treatment through increase in SOD and CAT antioxidant enzymatic activity and elevated GSH level. Also, intracellular ROS production induced by CSE exposure was prominently decreased with treatments. In parallel, lipid peroxidation was diminished with pre-treatment of combination, significantly. Moreover, Nrf2, master regulator of indirect antioxidant activity, was significantly activated with combination treatment compared to CSE group. Also CSE induced inflammatory response through nitrite and IL-6 release is significantly reduced by combination treatment. In the light of observed results, EUC, CUR and especially their combination might be potential therapeutics through investigated pathways. Furthermore, these results may represent a valuable background for future *in vivo* studies.



**Key words:** Cigarette smoke, pulmonary toxicity, Nrf2, Eucalyptol, Curcumin.

## ÖZET

**Reis, R. (2020). Sigara Dumanı Ekstraktı (SDE) ile İndüklenen Akciğer Hücre Toksisitesine Karşı Ökaliptol (EUC) ve Kurkumin'in (CUR) Koruyucu Etkilerinin *in vitro* Olarak Değerlendirilmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD, Doktora Tezi, İstanbul.**

Sigara tüketimi, dünya çapında her yıl milyonlarca insanın ölümüne neden olan en önemli etkenlerden biridir. Toksikolojik açıdan bakıldığında, sigara dumanı (SD) tüm canlı organizmalar, özellikle de pasif dumana maruz kalan insanlar için çevresel bir sorundur. Son yıllarda, doğal bileşiklerin pulmoner sistem üzerindeki etkileri, kronik obstruktif akciğer hastalığı, astım ve akciğer kanseri gibi SD maruziyeti sonucu görülen hastalıklar üzerindeki etkilerini aydınlatmak amacıyla araştırılmaktadır. Bu nedenle, geleneksel olarak kullanılan okaliptol (EUC), kurkumin (CUR) ve kombinasyonlarının koruyucu mekanizması, BEAS-2B hücre hattı üzerinde SDE ile indüklenen hücre ölümünde yer alan advers yolaklar, oksidatif hücre hasar ve inflamatuvar yanıt *in vitro* olarak araştırılmıştır. Sonuçlara göre, EUC, CUR ve bunların kombinasyonları BEAS-2B hücre canlılığını artırarak, SDE kaynaklı apoptozu ve otofajiye bağlı hücre ölümünü azaltmıştır. Ayrıca, SDE ile indüklenen oksidatif stres, SOD ve CAT antioksidan enzimatik aktivitesindeki artış ve yüksek GSH seviyesiyle kombinasyon tedavisinde önemli ölçüde azalmıştır. Ayrıca, SDE maruziyeti ile indüklenen hücre içi ROS üretimi, EUC, CUR ve kombinasyon muameleleriyle belirgin şekilde azalmıştır. Paralel olarak, kombinasyon muamelesi ile lipid peroksidasyonu önemli ölçüde azalmıştır. Bunların yanında, antioksidan yanıtın dolaylı ana düzenleyicisi nükleer faktör eritroid 2 ilişkili faktör 2 (Nrf2), kombinasyon muamelesiyle SDE grubuna kıyasla önemli ölçüde aktive olmuştur. Nitrit ve IL-6 salınımı aracılı SDE kaynaklı inflamatuar yanıt, kombinasyon muamelesiyle önemli ölçüde azalmıştır. Gözlemlenen sonuçlar ışığında, EUC, CUR ve özellikle bunların kombinasyonları, incelenen yolaklar doğrultusunda potansiyel terapötikler olabilir. Dahası, bu sonuçlar ilerideki *in vivo* çalışmalar için değerli bir altyapı oluşturabilir.



**Anahtar kelimeler**: Sigara dumanı, akciğer toksisitesi, Nrf2, ökaliptol, kurkumin.

# 1. INTRODUCTION AND AIM

Tobacco is one of the most important leading cause of worldwide death, over 8 million of people each year and its consumption as cigarette is considered as biggest health problem (1). Cigarette smoke (CS) is an aerosol containing thousands of chemical substances which are not identified completely and suggested that there might be more than 5600 components in CS (2). From a toxicological perspective, the content of CS which is rich in many lipophilic toxic substances, mutagens, and carcinogens as well as many chemical compounds such as nicotine and heavy metals etc. provide a basis for fatal outcomes (3,4). Furthermore, both passive and direct exposure to tobacco smoke can trigger progression of many diseases not only in target organs, but also in a wide range of vital organs (3). Non-smokers are also at risk because of inhalation of passive or secondhand smoke (5). Exposure to passive smoke may cause detrimental effects on vascular homoeostasis (6), respiratory heart disease, chronic obstructive pulmonary disease (COPD) (2) in general population and particularly hazardous to children (5). Besides, due to gender related differences, women are tend to be affected from CS with further health risks such as still births, cervical cancer and fetal diseases during pregnancy (7). Moreover, recent report of WHO revealed that over 1 million of non-smokers effected by environmental tobacco smoke (ETS), which serves a significant environmental and public health issue (1). Therefore, smoking is still a severe health problem worldwide for both active smokers and ETS exposed individuals.

Recent articles on tobacco consumption in the USA have suggested that tobacco smoke continues to kill about millions of Americans per year with a current 34.3 million smokers in the USA (8). In our country, smoking ratio has been reported to be increased among young generation gradually. Recently, Turkey has been ranked at third in Europe and seventh in world regarding to cigarette consumption according to World Health Survey (9). It is well known that chronic cigarette consumption is one of the most important cause of decrease in life quality and progressive factor for diseases such as COPD, respiratory heart disease and lung cancer (2,10,11). Hence, it is important to enlighten the underlying pathogenesis of cigarette smoke related lung diseases and develop a strategy to protect human health against CS as a part of environmental risk assessment.

Over the last decade, researchers are concerned about the mechanisms of CS induced diseases due to its highly toxic content (2). Consequently, tobacco industry seems

to have decided to work for modified cigarettes or “less harmful cigarettes” nowadays. However, smokers who are economically disadvantaged people in society are not able to reach to modified cigarettes which are marketed as “less harmful, reduced toxicity and carcinogenicity cigarettes with less addictive potential”(12). Therefore, preventive strategies against CS is trend research topic as a part of inhalation toxicology and environmental risk assessment.

Cigarette smoking is still the major reason of lung cancer and its cessation for lung cancer prevention remains as a key strategy. However, a vast majority of interventional strategies such as screening programs, dietary or lifestyle managements and chemoprevention may play important role in people who exposed to CS (13). Recent studies on toxicological basis of cigarette smoking are focused on its deleterious or compensable effects on human and environmental health with studies both *in vivo* and *in vitro* (14–23). According to recent records of U.S National Library of Medicine records (2019) there are 441 studies surveying effects of cigarette smoking and its health effects. 64 of these surveys are recruiting and 5 of them is specifically based on cigarette smoke induced toxicity (24).

In particular, effects of natural chemopreventive agents, vitamins and antioxidants on human system have been investigating in order to enlighten their possible effects on CS induced toxic outcomes such as oxidative and inflammatory stress in respiratory system (10,13,25–30). Natural components derived from medicinal plants such as flavonoids, alkaloids, and terpenoids may have biological effects due to their antioxidant, anti-inflammatory or anti-cancer properties (31). In recent years, distinctive synthetic antioxidants such as ascorbic acid (32,33), vitamin E (26,32,34,35), N-acetylcysteine (NAC) (15,17,27,28) and natural components such as luteolin (10,36), sesaminol (30), silymarin (37), quercetogetin (29), liquiritin (38), and curcumin (CUR) (25,39–41) have been investigated *in vivo* or *in vitro* on experimental pulmonary models, especially CS induced lung toxicity. Since pulmonary diseases such as acute respiratory distress syndrome (ARDS) and emphysema do not have proper treatments yet, further studies about alternative therapy methods are required (31)**.**

Pathological consequences of smoking on lung tissue are mainly based on oxidative cell injury and inflammatory stress due to the mixture components present in cigarette smoke. Reactive oxygen species (ROS) present in CS are thought to be participated for progression of COPD. In spite of the fact that ROS present in CS can not easily pass through airway epithelial cells, ROS level have been found to be augmented

in patients with COPD (33). Therefore, antioxidant therapy and relief of acute pulmonary inflammation might represent important potential for treatment and prevention of cigarette smoking related diseases. An important natural compound, eucalyptol, is a monoterpene mainly extracted from *Eucalyptus* species, has an important mucolytic and spasmolytic action on the respiratory tract. Moreover, eucalyptol (EUC) has clinically proved therapeutic potential on respiratory diseases such as asthma and COPD (42). Effects of EUC against pulmonary destruction are mainly focused on its capacity to repair pulmonary tissue by reducing oxidative stress and lipid peroxidation, preventing/ reducing activation of inflammatory cytokines such as nuclear factor kappa B (NF-κB), tumor necrosis factor (TNF-α), interleukin 1-beta (IL-1β) and interleukin-6 (IL-6) with limited *in vivo* or *in vitro* studies (21,43,44). Another important natural substance, curcumin, is a well-known dietary and polyphenolic compound associated with significantly increased pulmonary function due to its antioxidant and anti-inflammatory capacity (31,39). In addition to these effects on pulmonary diseases, CUR has shown a significant cytoprotective activity on human bronchial epithelial cell line BEAS-2B *in vitro* (35). On the other hand, CUR is a widely studied natural compound against COPD (39,41), lung cancer (45–48) and smoking-related pulmonary injuries (25,40).

Among mentioned natural compounds, promising ones, EUC and CUR, have been previously studied against CS-induced diseases through several pathways. However, their combination on CS-induced bronchial epithelial toxicity, cell death mechanism and their direct/ indirect antioxidant pathways on bronchial epithelia have not been studied yet. Therefore, in the present thesis, protective effects of EUC, CUR and their combination on CS-induced toxicity on human bronchial epithelium cells (BEAS-2B) have been investigated *in vitro*. For this purpose, cell death mechanisms involved in CSE-induced toxicity have been elucidated through possible apoptotic/ necrotic cell death, autophagy and mitochondrial dysfunction within the concept of adverse outcome pathway (49). Since chronic inflammation and redox imbalance are involved in the pathogenesis of smoking induced pulmonary diseases (50,51) pro-inflammatory cytokine release and nitric oxide (NO) metabolite nitrite level have also been evaluated. In addition, CS-induced oxidative stress and lipid peroxidation were evaluated by antioxidant enzyme activity, alteration in intracellular GSH and MDA level and expression of the nuclear factor erythroid 2-like 2 (Nrf2) master protein involved in antioxidant/ antiinflammatory action.

# 2. GENERAL INFORMATION

## 2.1 Cigarette Smoking and Health Risks

Smoking is the leading factor for burden of diseases and a major health problem worldwide (7). According to a report published by World Health Organization (2005), smoking is defined as a critical risk factor for six out of eight diseases. Among these diseases, cancer is still in the first place, which is one of the leading cause of death worldwide (9). Tobacco smoke and secondhand tobacco smoke have been reported as carcinogenic to human according to International Agency for Research on Cancer (IARC) classification (52). Therefore, one of the most important health consequence of smoking is considered as cancer (53).

Recent report by WHO (2019) revealed that 22000 people are dying from exposure to secondhand smoke or active tobacco smoke everyday, which was reported as equal to death of 4 people in each second. In the same report, it was emphasized that any type of tobacco including smokeless tobacco in the form of powdered, cut or leaf have been associated with wide range of diseases. The leading one on the list was indicated as cardiovascular diseases such as stroke and hearth attack. Following, oral and esophageal cancer were found to be related with tobacco product consumption. Moreover, fetal abnormalities such as growth retardation, low birth weight and even fetal loss have been reported (54) A report by Salahuddin et al. (2012) revealed that more than 5 million premature death related to smoking has reported. Also epidemiological studies and clinical follow-ups have suggested that life-span of a smoker decreases 8 years approximately (53). Moreover, it was suggested that 10 millions of smokers are expected to be death by 2025 (55). Overall, tobacco smoke, either passive or active, is directly associated with severe diseases such as fetal abnormalities to cancer.

### 2.1.1 Cancer

Cancer is the most serious consequence of tobacco consumption according to recent WHO report (2019). Lung cancer, is the most frequent type of cancer associated to cigarette smoking. However, tobacco usage (both smoked and smokeless) has been found as related with other cancer types such as oral, esophageal, colorectal, nasal, kidney, pancreatic and liver cancer as well (54).

In cancer initiation and progression, tobacco specific nitrosamines, polycyclic aromatic hydrocarbones (PAH), metals and various carcinogenic chemicals were suggested as contributors of disease. According to animals studies and epidemiological data, each substance have a specific involvement in cancer seen in target tissue. Hecht. (2006) reported that nitrosamines and PAH were mainly involved in the progression of lung, oral and cervix cancer and while benzene is involved in leukemia (56,57). Moreover, each carcinogenic substance identified in cigarette smoke have been reported to have different pathways on cancer initiation and progression. These carcinogens may inactivate tumor supressor gene, decrease rate of apoptosis, lead to an increase in cell transformation or form adducts with DNA (57,58).

Esophageal cancer is the sixth ranked cancer type in terms of mortality among other cancer types. Most important contributors of esophageal cancer were suggested as tobacco smoke and alcohol. A study by Toh et al. (2010) has indicated that current cigarette smoking and alcohol consumption worsens esophageal squamous cell carcinoma progression together, acting as synergistic (59). However, cigarette smoke either passive or active, has been identified as key risk factor for esophageal squamous cell carcinoma progression alone (60). In China, one of the biggest tobacco market in the world, it was reported that indicated esophageal cancer is the fourth frequent cancer type in both male and female smokers (61).

Report in 2011 on tobacco consumption associated health burden in Turkey, cigarette consumption have been found to be responsible for over 70% of not only lung but also upper aerodigestive cancers (62). Oral and nasophageal cancers are the other smoking associated cancer types seen in smokers. Previously, Xu et al. (2018) suggested that these two kind of cancers are following after lung cancer according to relative risk ratio in Chinese smokers (61). Moreover, other forms of tobacco products such as pipe, cigar and bidis have been associated with risk of upper aerodigestive part cancers (63).

Colorectal cancer is the other common cancer seen in worldwide with fourth ranked mortality ratio among others (64). Johnson et al. (2013) suggested that cigarette smoke can be listed as a risk factor for colorectal cancer and inflammatory bowel disease (65).

### 2.1.2 Cardiovascular Diseases

Epidemiological studies on the relation between cigarette smoking and diseases have been emphasized that most of the diseases such as cancer, cardiovascular diseases (CVD) and COPD are strongly associated with tobacco consumption. In the USA, more than two thousand people have been reported to be dead due to CVD and remining risk was associated wtih increased rate of smoking (53). According to a data by Centers for Disease Control and Prevention in 2014, even non-smokers are under the risk of showing early signs of CVD due to passive smoke exposure. Furthermore, smoking cigarettes containing less tar or nicotine level have been indicated in the report as not effective on decreasing CVD risk (66).

Cigarette smoke mainly initiates its toxic effect through disrupting endothelial integrity and leading to vascular dysfunction in CVD (55). Following the initiation of atherosclerosis, serum cholesterol, low (LDL) and high density lipoprotein (HDL) alteration was indicated in smokers. Further, smoking was suggested as an increasing factor for the modification of LDL oxidation and cause of lipid peroxidation (67). Therefore, induced oxidative stress, inflammation and change of lipid profile can be suggested as main mechanistic pathways for tobacco smoking related CVD. Also, Starke et al. (2018) indicated that secondary activation of immune cells and pro-inflammatory cytokine production may be suggested as contributing pathophysiological changes for CVD progression in addition to aforementioned alterations (68). An epidemiological study on non-cancerous Australian population has found that current smoking doubling CVD risk including acute myocardial infarction, heart failure and cerebrovascular diseases (69). In Turkey, population with CVD have different backgrounds. According to Onat (2001), Turkish people having CVD consist of men whose sixty percent is smoking and women in postmenopausal period, which have obesity or diabetes (70). Also, recent report of Turkish Statistical Institute (2015) revealed that CVD is the leading cause of death in Turkish population. Since smoking is the most important preventable factor in the management of CVD, it was stated that smoking cessation, especially before the age of 40, plays an important role in prevention of CVD (71).

Recent statistics have revealed that cigarette smoking is a noteworthy preventable factor for atherosclerosis progression. Therefore, smoking cessation is still considered as an important tool for CVD management (72).

### 2.1.3 Gastrointestinal Diseases

Gastrointestinal (GI) diseases have been previously associated with cigarette consumption. As a risk factor, cigarette smoke has been found to induce peptic ulcers, Crohn's disease and even gastric cancer by inhibiting renewal of gastric cells and interfering gastric mucosal layer. Furthermore, it was suggested that thousands of chemicals in smoke have been related to decrease in gastric blood flow rate and increase mucosal cell death, which delay alleviation of peptic ulcer sypmtoms (73). Recent studies on e-cigarette consumptions have not much different results as well. According to Layden et al. (2019), e-cigarette usage has led to an increase in GI symptoms seen in parallel with respiratory symptoms in patients admitted to emergency department during July 2019 (74). Toxicological mechanism of tobacco smoke on gastrointestinal disease progression has been attributed to presence of nicotine previously. Wu et al. (2004) indicated that nicotine present in cigarette smoke worsens ulceration process and its metabolites act as mutagenic, thus alters cell proliferation, apoptosis and modify tumoriogenesis in gastric cells (75). Similar to Wu's findings, Chu et al. (2012) has suggested that nicotine and nitrosamine derivatives in cigarette smoke have a capability to alter GI integrity and increase inflammation both in stomach and colon (76). In Turkey, aside from stomach cancer, smoking has been found in realtion with GI symptoms. According to a cohort study by Sezgin et al. (2019), upper GI disorder prevalance in Cappadocia area was found to be proportional with smoking rate while lower GI symptoms has shown no relation (77). In another study in Turkey has shown that people diagnosed with irritable bowel syndrome (IBS) have lower rate of smoking compared to normal population. This effect of smoking on IBS has suggested to be due to parasympathic ganglion stimulatory function of nicotine (78).

### 2.1.4 Immune Diseases

Immune system is the other important target of cigarette smoke. In literature, there are several reports indicating immune disorder incidance and smoking (79–81).

Constituents of cigarette smoke, etiher passive or active, are able to interfere with immune system due to its complex mixture (82,83). Thousand of carcinogens and oxidative moieties in fresh smoke also have a capacity to persist in environment even after decades of exposure. However, particulate part of cigarette smoke has an ability to

activate macrophages in lung, acting as pro-inflammatory and pro-coagulant (84). Due to its dual effect, effect of smoke on immunity has contraversial results and interpretations in literature. According to Sopori (2002), epidemiological studies shows that smokers have low incidance in terms of having neurodegenerative and inflammatory dieases (85). In this context, smoking ratio plays an important role on the degree of immune system modulation. Holt (1987) suggested that smokers consuming more then 20 cigarettes per day have shown depressed immunoglobulin levels in contrast to moderate or light smokers. Besides, modification of T-cell ratio in smokers may be reason of low rate incidance of such immunological diseases as farmer's lung and sarcoidosis. However, it was also suggested that hypothesis about immunosupressive effect of cigarette smoke should be taken into consideration and can be considered as questionable (86). Also, indoleamine 2,3-dioxygenase (IDO) pathway has been suggested as in relation with immun modulation in smokers through T-cell activity. According to Pertovaara et al. (2006), IDO activity has been found to be diminished in serum of smokers, thus it was suggested that immunostimulation by smoking might be responsible for decrease in IDO-regulated immunsupression (87).

Studies on smoking induced immune disorders suggested that immune and inflammatory markers such as CRP, IL-15, IL-1β and soluble IL-6 receptor have been found elevated in older and chronic smokers compared to non-smokers (80). Furthermore, due to decreased immune response, smokers have been reported to get viral and bacterial infectious diseases with four fold elevated risk compared to non-smokers (79). According to Martin et al. (2016), e-cigarette usage has also impaired defensive response and suppressed immune system. Therefore, e-cigarette vaping has been related to depression of variety number of immune-associated genes level in nasal mucosa (84). Consequently, smoking cessation has a considerable importance in the clinical management of immune disorders.

### 2.1.5 Type-2 Diabetes Mellitus

Type-2 diabetes mellitus (T2DM) is another associated disease with chronic exposure to cigarette smoke. Clinical findings have indicated that smokers exhibit insulin resistant profile, thus under the increased risk for T2DM (88).

Major bioactive constituent of cigarette smoke, nicotine, has shown a direct effect on glucose homeostasis, thus considered as a contributing factor for T2DM development

(89). Epidemiological data also suggested that heavy metal constituent of cigarette smoke and T2DM is related, limited data is available on their effect on disease prevalance (90,91). In addition, cigarette smoke exposure has been defined as a exacerbating factor for T2DM progression by increasing risk for cardiovascular and kidney diseases (92).

Cessation of smoking has been considered as a modifiable management tool in T2DM treatment. However, it is also emphasized that cessation may lead to weight gain in patients, thus concurrent weight control should be taken into consideration (93).

### 2.2. Cigarette Smoke and Responses of Lung

Respiratory system is the main target of CS which has also been considered as a major player in the pathogenesis of pulmonary diseases such as asthma, COPD and act as a triggering factor for acute symptoms of these diseases (94). More specifically, the primary target of inhaled cigarette smoke is the airway epithelium, which serves a protective barrier for inhaled toxicants (20). Thousands of chemicals present in tobacco smoke are inhaled directly or in the form of their high temperature combustion end products (95). One of the most important respiratory defect is COPD, which is characterized by inflammation in airways, airflow obstruction, destruction in the structure of alveolar wall and imbalance between oxidant and antioxidant defense (14). However, the final effect of the influence of smoke in target respiratory system depends on environmental conditions and individual factors such as pathological status, local homeostasis and genetic predisposition (95). Altogether, cigarette has a severe role in the pathogenesis of majority of pulmonary diseases as far as we know.

#### 2.2.1 Chronic Obstructive Pulmonary Disease

COPD is described as generally preventable disease with charecteristically progressive airflow limitation that is usually linked with an inflammatory response in the pulmonary system to detrimental particles or gases (18). In the USA, COPD has been defined as one of the most important leading cause of death and despite that, COPD remains as an unrecognized public health problem (96). There are suggested good clinical predictors for diagnosis of COPD such as increased sound during breathing, alteration in

peak flow rate and smoking history more than decades (97). COPD in human consists of four major anatomic lesions defined as emphysema, small airway remodeling, vascular remodeling with mucus overproduction and increased blood pressure in pulmonary system and bronchitis according to Churg et al (2008) (98). The pathogenesis of COPD is quite complex, and there are multiple pathways which lead to pulmonary inflammation, cell and tissue destruction, and dysfunction in repair responses of target tissue. Therefore, COPD is described as a multifactoral disease with both systemic and pulmonary outcomes (99).

Among major risk factors for COPD such as organic or synthetic dusts, chemical fumes and fuels, cigarette smoke, is the key factor which has a high capacity to initiate pulmonary inflammatory response through various mechanisms (99). Tamimi et al. (2011) described active cigarette smoking as the most confronted risk factor for progression of COPD with declined first time-forced exhaled volume ($FEV_1$) and high rate of mortality (100). Pathophysiological insight of COPD is mainly attributed to inflammation and oxidative stress in literature. According to Tuder et al. (2012), tobacco smoke has an ability to induce airway inflammation within minutes or hours of exposure in both human and rodents (101). It is reported that destruction of vascular structure of lung and dysfunction in airway barriers were seen as pathophysiological findings at the beginning of COPD (102). Overtime, decrease in the elasticity of lungs is seen and subsequently, hyperinflation and dyspnea occurs due to inability of lung to empty trapped air. Therefore, patients with COPD exhibits an enlarged rib cage and hypoxemia occurs in the latest stage of disease (96).

There are numerous factors responsible for initiation of COPD such as childhood respiratory infections, environmental pollutants or host related factors such as airway hyperresponsiveness, gender and genetic susceptibility (99). However, cigarette smoke plays a crucial role in the initiation and progression of COPD. Clinical trials recorded in U.S National Library of Medicine on modulation of cigarette smoke induced COPD are mainly held on adults (18-64 years old) according to latest report. 9 clinical trials of 94 studies on COPD are still ongoing (58).

It is suggested that cigarette smoke and excessive oxidation induce NF-κB and subsequently, acute inflammatory response appears. Since NF-κB p50 subunit is responsible for maintanence of inflammatory response in lung, NF-κB has been suggested

to have a double-side effect. Furthermore, dysfunction of this subunit reported to be responsible for augmentation of smoking induced inflammatory response (101). Furthermore, smoking has led to an increment of proinflammatory cytokine release from macrophages, epithelial cells, and fibroblast of animal studies and an increase in gene expression of proinflammatory mediators including TNF-α, IL-1β, IL-8 and chemoattractants as macrophage inflammatory protein (MIP) subtypes in lung or in BAL (98). From an another aspect, a well-known tryptophan degrading enzyme and T-cell modulator, indoleamine 2, 3-dioxygenase (IDO) has been reported to be associated with airway inflammation in COPD seen in both smokers and non-smokers. Previously, Maneechotesuwan et al. (2012) indicated that patients diagnosed with COPD has showed lower sputum IDO activity and consequently reduced expression of IL-10 while increased pro-inflammatory cytokine release such as IL-6 and IL-17A. Furthermore, prominent decline in IDO activity of smokers suggested as the cause of neutrophilic inflammation due to imbalance of IL-10 and IL-17A (104).

It is known that cigarette smoke exposure increases inflammatory response in lung, but it also has a negative impact on efficacy of short-term corticosteroid treatment in COPD. Tamimi et al. (2011), indicated that eventhough inhaled corticosteroids are still controversial in the treatment of COPD, it is clear that cigarette smoking was associated with relative resistance corticosteroids and reduced efficacy to given therapy in people with severe COPD and who actively smoke (100). Aside from previously active smoking, a good majority of studies in literature on effects of smoking and progression of COPD has been suggested that even passive/ secondhand smoke has an enormous impact on disease progression. According to Diver et al. (2018), exposure to secondhand smoke during juvenile period was involved with a greater risk of death due to COPD in their future life. Besides, exposure to passive smoke more than 10 hours per week in adults was found in relation to death from COPD, stroke and ischemic heart disease, as well in the same study (105).

Smoking induced COPD and its mechanism are still investigated topics in literature. It is mentioned that oxidative stress and related consequences in lung and peripheral organs have a great impacy on severity of COPD (51,106–108). Previously, oxidative and carbonyl stress is indicated to be augmented in patients with COPD, especially in the period when acute exacerbations have been experienced. Since cigarette smoke is an oxidative stress promoter, smoking and inhalation of combustion products

have an important role as an additional etiologic factor in COPD progression (109). Also, an important augmentation of malondialdeyhde (MDA) level, product of lipid peroxidation, has been reported in the lung tissue of people diagnosed with COPD (106,109). According to Kluchová et al. (2007), imbalance between oxidants and antioxidants had a greater role in the pathogenesis of COPD and it was reflected through erythrocyte glutathione peroxidase (GSH-Px) activity and MDA level in stable COPD patients. In the same study, it was also suggested no significant differences in the ertyhrocyte SOD and CAT activities were observed among COPD patients on different status (108). Further, it is indicated that oxidative stress and related effects on living system have a wide range of area which is beyond the lung and further tissues. Therefore, oxidative stress mechanisms involved in pulmonary system may contribute to variable the systemic consequences in COPD (51)

Interaction of genes and cigarette smoking have been suggested to have a major role in the predisposition of COPD. According to Hylkema et al. (2007), genes involving in detoxification process like glutathione S-transferase (GST) is a vital support tool against oxidative stress and deletions in GSTM1 and GSTT1 are resulted direct impairment of detoxification, which is similarly seen among cigarette smoke exposed people. In addition, people who consume tobacco more than twenty products per day and with GSTM1 null genotype had higher tendency to suffer from wheezing in compared to an ordinary nonsmoker (110). In addition, it was reported that active smokers who are below the age of 35 and exposed to occupational dust, indoor/ outdoor air pollutants frequently and with $\alpha_1$-antitrypsin deficieny are under the risk of COPD (99). Genetic polymorphism on direct/ indirect antioxidants, metalloproteases, proinflammatory cytokines, and detoxification enzymes have been previously associated to progression of COPD (98). Therefore, genetic predisposition of smokers carries an important role in progression of COPD as well as environmental factors.

Current treatment approaches on COPD such as oral and inhaled corticosteroids are defined as first line therapeutic agents. Eventhough studies based on COPD patients who are smokers or non-smokers or animal models suggested that corticosteroids have an important place in the diminishment of acute exacerbations, induction of antioxidant gene expression is considered as an another target in the therapy (25,109). Therefore, further studies are needed to enlighten mechanism of smoke induced oxidative and inflammatory response to create a target based treatment approach.

### 2.2.2 Asthma

Asthma is a multifactoral disease which consists of complex conditions that result in recurrent, reversible bronchial obstruction and might be seen in any age (111). According to previous NIH Guideline ashtma was defined as long-term inflammatory condition of airways in which play a role such as mast cells, T lymphocytes, eosinophils, macrophages, neutrophils and epithelial cells, particularly (112). It is characterised by erratic respiratory symptoms and airflow limitation with a heterogenous clinical presentation. Asthma is also indicated as a consequence of complex gene and environmental interactions due to its various types and observed degree of airway inflammation (113). In case of asthma, chronical inflammation of lower respiratory tract is seen and its incidence is more frequent among individuals that also have inflammatory disorders of the upper airway. In addition, patients with chronic inflammation experience wheezing, difficulty in breathing, and coughing at late night or at the beginning of morning (112).

Asthma may occur at any age and previously, it was reported that adult type of asthma commonly seen between 40-60 years of age with middle age symptoms while in younger asthmatics, the onset occurs generally since infancy. Moreover, older asthmatics were suggested to need special consideration due to their tendency to be non-allergic and carrying high risk for comorbidities such as COPD and heart failure (114). There are many environmental and socioeconomic factors included in predisposition of asthma disease including disabled housing conditions, allergens, environmental exposures such as traffic and air pollution as well as cigarette smoke (115,116). In recent years, asthma and allergic disease incidence among young people have been increased due to several environmental factors. One of the most important cause of asthma, cigarette smoking, is very common in adolescents and young adults according to a recent report in Korea. According to Lee et al. (2019), tobacco use related asthma and allergic rhinitis have been raised especially among high school students in Korea (117). Similar to Korea, a study in State of Hawaii was suggested that e-cigarette usage is directly linked with increased risk of having asthma among adolescents in high school (118). Another report in the USA suggested that asthma is a common juvenile disease in United States and cigarette smoke either active or passive was described as a major contributing factor for progression of disease (115). According to recent U.S National Library of Medicine records (2019), there are 30 clinical researches based on asthmatic patients who are smokers and 8 of

these trials are on children who are at age between 0-17 (119). Since smoking was described as an important risk candidate for the progression of disease, elimination of cigarette smoke related factors plays a major role in the management of asthma. Previously, Mcleish et al. (2010) suggested that smoking diminishes asthma control, increases mortality risk and asthma exacerbations (120). Moreover, smoking cessation was reported to be highly correlated with lung function improvement and reduces symptoms seen in asthma (120) while chronic smoking has been reported as an important factor which decreases therapeutic efficiency of oral and inhaler corticosteroids in asthma treatment (121,122).

Studies in literature on cigarette smoking and asthma relationship were mainly focused on cigarette smoke induced lung pathology, decreased therapeutic response and increased ashtma exacerbations (121,123,124). According to Hekking et al. (2014), cigarette smoke induced asthma is mainly featured by reduced baseline lung function, extreme cough with sputum and higher IgE level similar to atopic asthma (125). Further, cigarette smoke act as an additive external factor on inflammation and airway remodelling process which lead to accelerated augmentation in lung dysfunction and airflow obstruction. Mucous hypersecretion increases through cigarette smoke induced neutrophil release and it stimulates mucin genes in goblet cells and submucosal glands present in bronchial epithelia (126). Also, prenatal secondhand cigarette smoke exposure has been associated to an increase in airway hyperreactivity and Th2 (T-helper-2-cell) level in murine asthma model (127). Although the mechanism of cigarette smoke induced-asthma is not fully elucidated, it was suggested that cigarette smoke can reduce of HDAC2 (histone deacetylase 2), expression level through phosphoinositide-3-kinaseδ (PI3K-δ)/ Akt signalling activation in ovalbumin induced asthma model in murine. In the same study, Xia et al. (2017) suggested that oxidative stress by cigarette smoking can be defined as key inducer of PI3K-δ/Akt signaling pathway, which is associated to airway inflammation seen in asthma (128).

It is clear that cigarette smoke is an inducer for asthma exacerbations and an important promoting factor for progression of disease. Current treatment strategies for asthma are based on mucosal allergen-specific immunotherapy, identification of molecular pathways that drive T-helper-2-cell polarization (129) and oral/ inhaled corticosteroids (130). Also, mononoclonal Ig E-targeted antibody such as omalizumab biologics such as IL-5 and IL-13 specific drugs are stil under investigation. Because of

the fact that patients serve different symptoms due to variable type of asthma, therapeutic highlights that is necessary to relief different asthma subtypes are inconstant (131). Therefore, phenotype-targeted therapeutics based on clinical signs of asthma subtypes are needed further research.

### 2.2.3 Tuberculosis

Tuberculosis is also one of the most considerable health condition which may be defined as a leading factor for death among different populations in the world. The disease is mainly characterized by differentiated immune response with defective immune cells such as macrophages, CD4 lypmhocytes and monocytes (132). Among risk factors, a suitable host by other means a susceptible human, tubercle bacilus and suitable environmental factors for survival of bacilli for transmission can be described as necessary factors (133). Aside from immunological baseline of disease, internal and external factors such as hormonal alterations and structural and functional anomalies in cilia (132) and cigarette smoking (132,134,135) might be defined as secondary contributor factors for tuberculosis progression.

Relationship between cigarette smoking and tuberculosis have been discussing in literature since 1956, in a publication by Doll and Hill. According to authors, there is a "distinct" relation between tobacco consumption and tuberculosis progression (136). Also, Kiechlet al. (2015) has suggested that cigarette smoking can be described as a possible risk factor for tuberculosis infection and predisposition of disease due to several clinical evidences. In the same study, it was indicated that cigarette consumption was positively correlated with susceptibility of tuberculosis infection (135). However, another report was suggested that active exposure to tobacco smoke might be a risk factor tuberculosis infection with unclear mortality ratio. Thus, Bates et al. (2007) concluded that risk evidence by meta-analysis needed further research to identify whether smoking is an additional risk factor for mortality ratio or not (137). Epidemiological data on cigarette smoking and tuberculosis prevalance have shown different interpretations among populations. A study by Bae et al. (2007) indicated that cigarette smoking is not correlated with tuberculosis ratio significantly in Korean population (138). However, a study held on northern California among patients who newly diagnosed with pulmonary tuberculosis suggested that cigarette consumption increases risk of disease. Further, it was indicated that smoking cessation might be part of prevention programmes on

tuberculosis (139). An observational study in China has also suggested that cigarette smoking is related with increased tuberculosis ratio in Chinese population and eliminating tobacco consumption must be part of tuberculosis management protocol (140). Recently, similar to previous suggestions, Boeckmann et al. (2019) emphasized that smoking cessation plays an active role as a part of tuberculosis theapy (141).

Mechanism of cigarette smoke on disease predisposition and progression has not been fully elucidated. However, recent articles have been suggesting that tobacco smoke may alter T-cell response in lung to *Mycobacterium tuberculosis* by inhibiting IFN-γ production. Moreover, cigarette smoke exposure has led to an increase in bacterial survival in mice infected with *Mycobacterium tuberculosis* and increased susceptibility (142). Cigarette smoking has a negative impact on immune function in lung tissue by altering IFN-γ secretion and latent tuberculosis infection rate through IFN-γ was suggested as more prominent in people at late 60's who chronically smokes (143). However, Altet et al. (2017) interpreted the relationship between smoking and IFN-γ release from a different perspective. According to their study, cigarette smoke has augmented plausibility of false negative result through IFN-γ release assay due to decreased ratio of IFN-γ secretion in people who are diagnosed with active or latent period of tuberculosis. In the same study, it was also emphasized that smoking ratio and lifelong cigarette consumption has augmented IFN-γ secretion and quitting smoking has a major role to provide higher life quality in tuberculosis patients (144).

### 2.2.4 Lung Cancer

Lung cancer is one of the most frequent cancer type seen in our era (145). Moreover, global evaluations appear that lung cancer is comprising the most prominent portion of newly diagnosed cancers (146). It emerges from the cells of respiratory epithelium and can be separated into two main categories. First one, small cell lung cancer (SCLC) can be defined as greater malignant tumor consist of cells showing neuroendocrine background. The other type, non–small cell lung cancer (NSCLC), which accounts for the remaining majority of lung cancer cases, is categorized as three main subtypes such as large cell carcinoma, adenocarcinoma and squamous cell carcinoma, as well (146).

Incidance of lung cancer has been strongly associated with tobacco smoking according to various records and epidemiological studies (56,57,146,147). In the light of epidemiological studies, lung cancer was one of the rarest cancer in the early 20's. However, in the beginning of 50's, cigarette consumption per capita had increased significantly and in parallel, reported lung cancer rate had diminished in a noteworthy degree in the USA (148). Therefore, cigarette smoking rate and consituents have greater role on lung carcinogenesis.

Cigarette smoke is a toxic combination of thousands of chemicals and nearly a hundred of these chemicals were indicated as well-established human carcinogen (56). Polycyclic aromatic hydrocarbons (PAH) are the main carcinogenic compartment of cigarette smoke and also, act as key inducer of drug metabolizing cytochrome P450 family according to both *in vivo* and *in vitro* findings (149). Other identified carcinogens present in cigarette smoke are nitrosamines, aldehydes, phenols and volatile substances. Carcinogenic constituents of cigarette smoke, mainly benzo[α]pyrene and nitrosamine derivatives, are converted to active metabolites and following, form DNA adducts in lung tissue. These adducts are involved in steps of lung carcinegenesis by involving persistent miscoding, hyperplasia, dysplasia and metastasis (150). Besides, they are able to induce further bronchial damage by up-regulating oncogenes and transcriptional factors initiating cancer (16). In addition to these carcinogenes, addictive substance nicotine is known to induce tumors in case of hyperoxia (150). Besides, nicotine is able to alter gene expression responsible for tobacco dependence by up-regulating nicotinic receptors and indirectly conttibutes to tumor progression (147). However, it is not classified as a carcinogenic substance (147,150).

IARC report (2004) on trends in cigarette smoking and lung cancer suggested that smoking prevalance was decreased in developed and transitional countries. However, records of developing countries showed a less significant decreasing profile in terms of cigarette consumption per capita after 2000's compared to the others (151). According to the latest WHO report (2015) on tobacco smoking prevalance, Turkey has shown a decreasing trend on tobacco smoking, especially in smoker population aged between 25-39 years old. In the same report, WHO presumed that tobacco consumer ratio will be decreased from 30% of total population to 19% in 2025, which can be considered as promising for future lung cancer management in Turkey (152). Epidemiological studies have suggested that smoking threshold, number of cigarettes consumed per day, is an

important factor for lung cancer predisposition (153). Therefore, smoking is a well-defined preventable factor involved in initiation, progression and metastasis stages of lung cancer (16).

### 2.3. Toxicity Mechanism of Cigarette Smoke in Lung

#### 2.3.1 Cell Injury and Cell Death

One of the most important toxicological implication of cigarette smoke is cell death in target tissue. Since cigarette smoke contains thousands of chemicals including ROS and reactive nitrogen species (RNS), exposure to cigarette smoke has been reported to lead a decline in cell viability in a dose –depended manner according to various *in vivo* and *in vitro* studies (154–156).

Predictive toxicology studies on a toxicant were defined with adverse outcome pathway (AOP), previously. Vinken and his colleague refers toxicant induced cell death with an initial injury by chemical followed by mitochondrial injury, a key event. Thereafter, disruption of molecular homeostasis occurs and toxicity is seen as an adverse outcome within the concept of AOP. In Figure 1, cell death with AOP concept according to Vinken and his colleague (2017) was summarized (49).

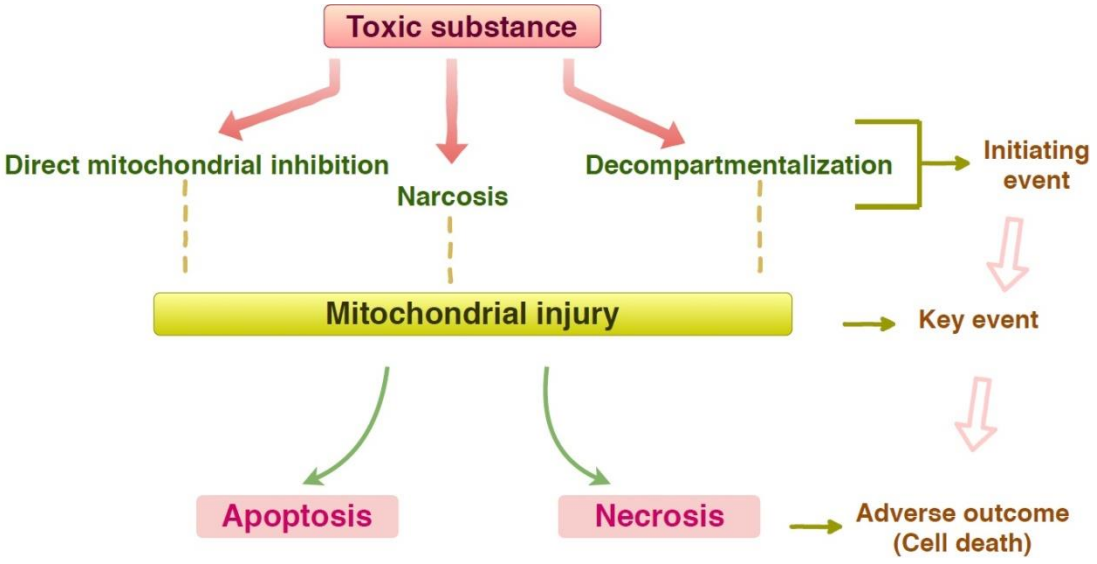



**Figure 1**.Adverse outcome pathway (AOP) of cell death induced by toxic substance

Long term exposure to cigarette smoke is particularly important for cell maintenance due to accumulation of inhaled particles in the airways and alveoli. In acute exposure to inhaled toxicants, clearance capacity of pulmonary system is able to moderate regulation of inhaled substance deposition. However, chronic exposure to toxicants may exceed the clearance capacity of lung, especially of macrophages, and death signal is triggered (156). Similar to macrophages in pulmonary system, airway epithelia is another target for cigarette smoke since its defensive role against inhaled foreign substances. Hence, in case of cigarette smoke exposure, viral infection or exposure to an allergen might trigger apoptosis as a cellular response (157). In airway epithelia, cigarette smoke is a well-known initiating factor that induces death signals through various cascades (156,157). According to previous *in vitro* studies (154–156,158), there are diverse cell death mechanisms involved in cigarette smoke- induced toxicity.

According to recent publication of Nomenclature Committee on Cell Death (2018), interpretation of cell death according to biochemical and morphological aspect has been defined. In the light of recommended nomenclature, cell death has been classified as three distinct types: apoptosis, autophagy-dependent cell death and necrosis (159).

#### 2.3.1.1 Apoptosis

Apoptosis, by other means programmed cell death or individual cellular suicide, is a different concept that refers cell death observed at particular timepoint through homeostasis of organism. Toxicant induced apoptosis depends on exposure duration and dose of toxic substance (160). Cigarette smoke, is an inhaled environmental toxicant and has a capacity to be accumulated in airways depending on exposure frequency and its dose (157,161).

Primary hallmarks of cellular apoptosis incorporate cell shrinkage, DNA and cytoskeleton fragmentation, pyknosis and apoptotic body formation (157). After formation of apoptotic bodies, they are engulfed by macrophages or cells nearby for lysosomal degradation (160,162). Multiple inducers such as oxidative stress, cytokine release or radiation may initiate apoptosis in target tissue. Besides, intrinsic factors may initiate apoptosis through intrinsic caspase (cysteine-aspartate protease) activation (157). There are various types of caspases involved in apoptosis pathways acting as initiator, effector or inflammatory caspase (162). According to White (2011), there are two main

caspase activation pathways remain that are extrinsic and intrinsic pathways. Extrinsic, receptor- mediated or non-mitochondrial pathway is based on the induction of cell death receptors on cellular surface or TNF superfamily by extracellular triggers (157,162). In case of a death signal, receptors such as CD95 or DR4 form a death domain through ligation and thereafter caspase activation occurs (157). The other pathway is defined as intrinsic, stress-mediated or mitochondrial pathway refers that activation of caspases through several stressors such as oxidant exposure, UV or ionized-irradiation, starvation and DNA damage (157,159,162). In this type of apoptosis, caspase activation occurs by stress stimuli instead of receptor activation (162). In addition, functional or structural alterations in organelles such as endoplasmic reticulum (ER) stress may trigger intrinsic pathway (156). This pathway has an important irreversible step through alteration of mitochondrial outer membrane permeability (MOMP) maintained by pro and anti-apoptotic Bcl-2 regulator protein. Following apoptotic stimuli, MOMP is controlled by Bcl-2 associated-X and BAK proteins through pore formation in outer membrane of mitochondria, thus mitochondrial membrane polarity is altered (MMP) (159). This alteration in MMP lead to cytochrome-c release and followingly apoptosome formation. Subsequent to apoptosome formation, caspase 9 cleavage and activation is followed by effector caspase 3 and 7 activation which is resulted to apoptotic cell death. In Figure 2 pathways for apoptosis initiation were depicted below with some revisions (157).

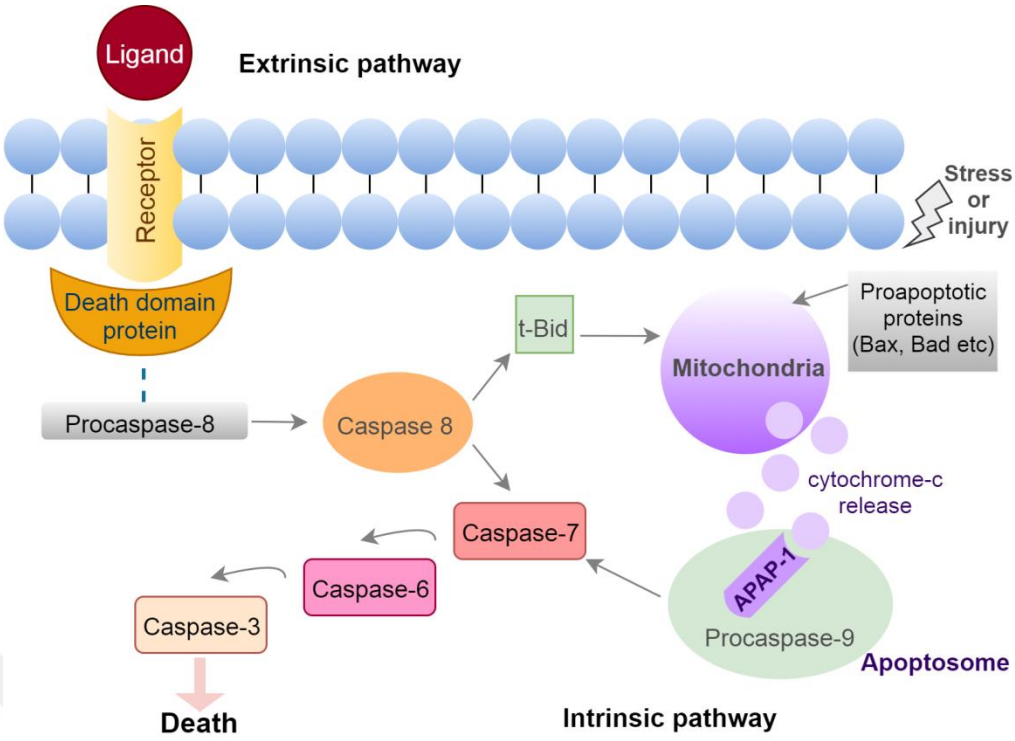



**Figure 2.** Extrinsic and intrinsic pathways involved in initiation of apoptosis.

### 2.3.1.2 Autophagy-dependent cell death

Autophagy can be defined as cell death characterized by excessive cytoplasmic vacuole formation with lysosomal degradation (159). During this lysosome dependent cell death, cytosolic cargo in cytoplasm is formed which is called as autophagosome and followingly fuses with lysosome for further degradation and recycle (163). At basal level, autophagy is seen at very low level due to central inhibition through serine-threonine protein kinase TOR (target of rapamycin) (164). Physiological autophagy can be seen as a protective response to conditions such as starvation, stress or organelle damage. However, excessive autophagy, or its dysregulation might trigger cytolethality (162). Previously, it has been shown that cigarette smoke extract (CSE) has induced autophagy as an adaptive cellular response related with increased oxidative stress and CSE induced inflammation (2,165,166). *In vivo* studies have revealed that COPD patients exposed to cigarette smoke have shown accelerated levels of autophagy in lung tissue of human and mice, previously (167). Furthermore, some biological studies had suggested that autophagy is involved in several responses such as clearance of cilia, cell survival promotion and carcinogenesis suppression (165). According to a recent study, cigarette

smoke exposure had led to an increase in autophagy which is mediated through mitochondria dependent ROS. Also, increased autophagy has been found directly related to increased mucus secretion in cigarette smoke induced COPD in bronchial epithelia. Therefore, it was implicated that inhibition or regulation of autophagy might represent an important therapeutical strategy in COPD (168).

There are several genes and products that have been identified and involved in autophagy process. Since autophagy is a complex process, there are various markers and proteins that are involved suchs as Atg proteins (autophagy-related protein), Beclin-1 and LC3B (microtubule-associated protein lightchain 3B) (164,168). In COPD and cigarette smoking associated diseases it was found that LC3B has a pivotal role in disease progression and underlying mechanism (169). During autophagy-dependent cell death, cytoplasmic free form LC3B-I is converted to autophagosome formation indicator LC3B-II in mammalian system(170). Therefore, ratio of LC3B-II to free form LC3B-I represents autophagosome formation, thus used as an important hallmark for autophagy studies (168). In COPD, it was reported that autophagy through LC3β involved in disease pathogenesis also promotes apoptosis via interacting some apoptosis regulatory proteins such as Fas and caveolin-1 (165). A study by Chen et al. (2010) has suggested that LC3 knockout mice has shown decreased level of apoptosis in lung tissue when they were exposed to cigarette smoke. It was also emphasized that LC3 regulation is an important extrinsic factor for autophagy, thus has a pivotal for COPD management and treatment (171). Also, Beclin-1, another protein involved in autophagy, is regulated by anti-apoptotic protein Bcl-2 and binding of Bcl-2 by Beclin-1 inhibits autophagy. Under stress conditions, Beclin-1 dissociates from Bcl-2, which induces autophagy machinery (164). In addition to LC3B-II/ LC3B-I ratio, expression of several Atg proteins such as Atg4B, Atg12, Atg5 and Atg7 have been reported to be diminished significantly in lung tissue of COPD patients (169). In the light of aforementioned reports (168,169,171) on cigarette smoke induced pathogenesises, this adaptive response in pulmonary system is thought to have an impact on mechanism of cigarette smoke- induced lung disease.

#### 2.3.1.3 Necrosis

Compared to apoptosis, necrotic cell death can be defined as an unorganized cell death induced by factors contributing to cellular stress, loss of homeostasis, cellular lysis and inflammation (49). Necrosis can be triggered by mechanical factors such as toxin

exposure, infection, trauma or heat. As a result of necrotic cell death, disruption of cellular membrane integrity, excessive inflammation and secretion of cell death- related products into extracellular space and mitochondrial swelling are seen characteristically. (172). Therefore, necrosis is classified as a passive form of cell death without nuclear disintegration and cellular programming (173). Biochemical markers indicating necrosis have not been clearly identified yet. However, several techniques are being used to identify primary/ secondary necrotic cells through cell-cell interaction, cytokeratin 18 or caspase release in supernatant or morphological examination (174). Also, several targets such as histone H1/ DNA complex, Hsp90 protein, lupus associated La antigen, HMGB1 and fumarase have been used for imaging of necrosis according to target tissue and desired mechanistic pathway. As an example, histone H1/ DNA complex, Hsp90 and fumarase are specific imaging targets for necrotic cells with disrupted plasma membrane integrity. On the other hand, La antigen and HMGB1are reported to be involved in other biochemical pathways and not specific for necrosis (175).

In lung, direct epithelial necrosis is generally seen due to bacterial exotoxin exposure. Also, immediate diminish of tissue oxygen tension and hypoxia may lead to necrosis (176). However, ROS act as important contributing factor for necrotic cell death. Since cigarette smoke is wealthy of various ROS and free radical species, cigarette smoke induced necrosis also plays an important role in disease pathogenesis. Several studies have suggested that cigarette smoke may induce necrotic cell death in a dose-dependent manner (177–179). Cigarette smoke exposure is generally associated with apoptosis airway epithelia according to *in vitro* studies (29,168,169). However, a recent study by Yoshida et al. (2019) has suggested that ferroptosis, iron- dependent and regulated form of necrotic cell death, is involved in COPD pathogenesis. Since, particulate part of cigarette smoke rich in iron and ROS, exposure to smoke is suggested as a contributor for alteration of iron homeostasis, thus eliciting oxidative stress, inflammation and ferroptosis in both mice model and bronhial epithelial cell line *in vitro* (182). Therefore, depending on dose and target tissue, cell death mechanisms may differ and further studies are needed to clarify toxicity pathways.

### 2.3.2 Inflammation

Cigarette consumption is considered as a risk factor due to its contribution to progression of chronic inflammatory diseases, especially in lung (183). Airway epithelia inflammation basically refers that as a response stimulated by environmental pollutants such as cigarette smoke, dust, allergen etc. which is characterized by mucous and mediator cytokine secretion into the airways (157). Previous findings suggested that, chronic cigarette consumption is associated with activation of inflammatory cytokines in airway epithelia (94), increase in mucus secretion (183) and increased level of neutrophils in BALF and blood (184).

Cigarette smoke is a well-known trigger for inflammatory cascade in pulmonary system. A good majority of studies on health effects of smoking mainly investigated its effects on inflammatory toxic response on airway both *in vitro* (20,37,94,185) and *in vivo* (31,98,128). However, acute effects cigarette smoke on inflammation is not clearly defined. Previously, van der Vaart et al. (2004) suggested that, CO content of cigarette smoke may alter level of some inflammatory cytokines and eosinophils in acute exposure. According to their report, acute exposure to smoke has led to local inflammation and augmented level of neutrophils and macrophages in lung. However, a clear diminish was observed in eosinophil level, which is attributed to anti-inflammatory action of CO and imbalance between Th1 and Th2 cytokines (184). Another clinical study by van der Vaart and colleagues (2005) indicated that non-smokers have lower IL-8 blood level and neutrophils in sputum sample compared to irregular smokers. Despite that finding, they suggested that acute smoking has led to a repression of inflammatory lymphocytes in sputum collected from irregular smokers (186). Also, nitric oxide (NO) another constituent of cigarette smoke, may lead to an augmentation of eosinophil apoptosis, which might be a contributing factor for previous result (100).

Smoking has been also associated with increased level of pro-inflammatory markers such as IL-6, IL-8 and TNF-α in target tissues (187,188). Besides, smoking had been involved in diminished levels of anti-inflammatory IL-10 cytokine level (187). CD8 cell and neutrophil increase by cigarette smoking may also alter therapeutic response in conditions such as asthma and COPD, as well (100). Moreover, increased inflammation triggered by cigarette smoke has been strongly related to dysfunction in goblet cells and mucin exocytosis. According to Cantin (2010), radicals present in cigarette smoke

increase transcription of MUC5AC and increase mucus in airways (189). *In vitro* studies have also evaluated that, cigarette smoke constituents have a capacity to trigger NO release in a dose dependent manner in several cell lines such as murine macrophage (190) and human alveolar epithelia (191). This mechanism is important because, NO acts as highly reactive ROS and involved in inflammation process indirectly. Through stimulation of macrophages by pro-inflammatory cytokines in case of smoke exposure or infection, macrophages became able to release NO and $H_2O_2$ (192).

### 2.3.3 Oxidative Stress

An atom or group of atoms which contains one or more unpaired electrons ($e^-$) are described as free radicals. Free radicals are featuring highly reactive and unstable profile in biological system (193). Oxidative stress can be described as disturbance or imbalance between antioxidants and oxidative agents formed within biological system or due to external exposure, basically. In case of shifting of balance through oxidants, oxidant formation increases in living system and thus, organ capacity diminishes due to reduced amount of antioxidants or decreased reductive capacity of tissue (194). Pyrolysis of tobacco leaf contains many chemicals that induce formation of superoxide from oxygen and starts oxidative stress cascade. Illustration of cigarette smoke induced oxidative stress and antioxidant pathway is shown in Figure 3 pathways (189,194,195).

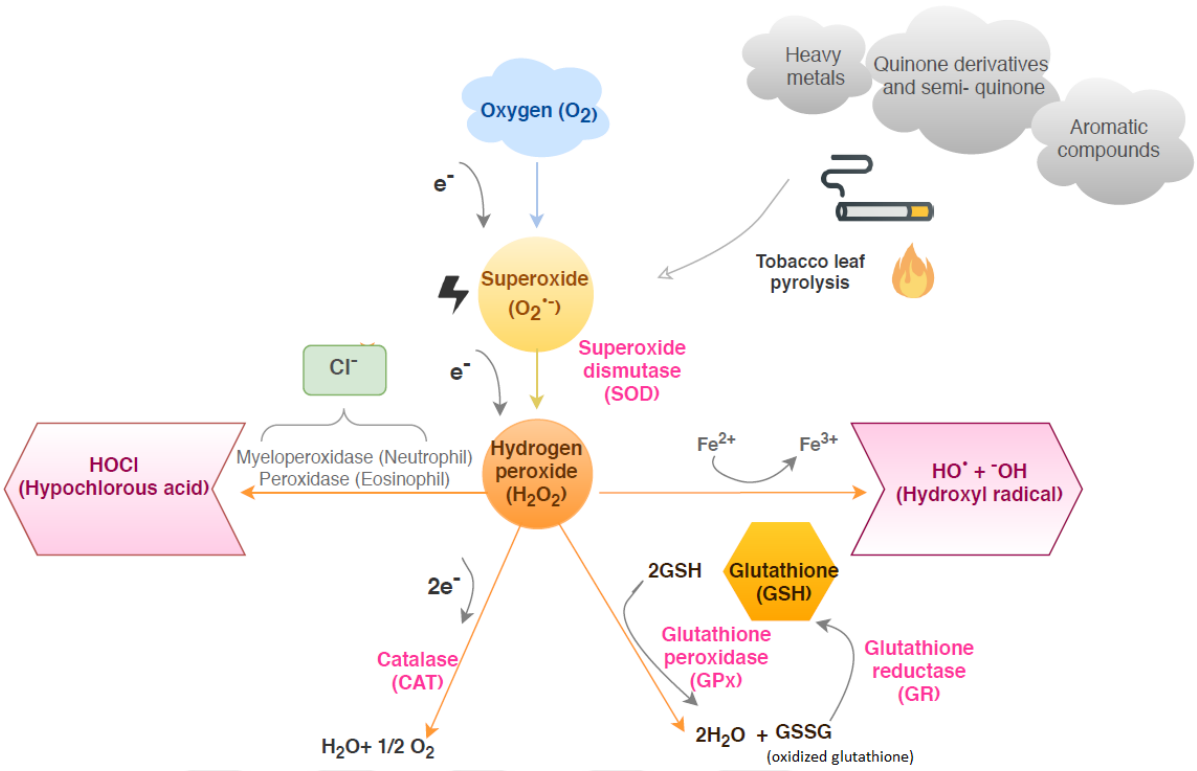



**Figure 3.** Illustration of CS induced oxidation and antioxidant pathways.

Beside many toxic chemicals, cigarette smoke contains thousand of ingredients which are not totally identified. Reactive oxygen species, present in cigarette smoke has been strongly associated to initiation and progression of lung diseases (33). Two main parts present in cigarette smoke, tar and gas phase, both contains free radicals and non-radical oxidants. Especially presence of high amount of superoxide ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) are thought to be important biological radicals for disease progression by initiating oxidative stress and lipid peroxidation cascade (196). As primary target of cigarette smoke, lung epithelia, acts as a barrier against many toxins, allergens, irritants as well as cigarette smoke and inhaled pollutants (197). Therefore, many carbonyl compunds and ROS is directly inhaled through lung and creating basis for harmful effects. According to previous studies (198–200), ROS generation via cigarette smoke exposure and free radicals within smoke itself create an imbalance between oxidants and antioxidant system. Hence, this imbalance leads to an alteration in cellular defence mechanism in lung and causes lung diseases such as COPD and fibrosis (197). Moreover, these reactive species interfere with enzymes, macromolecules, lipids and DNA or RNA structure, disrupt cellular function and structure (201). In cell based level, augmented levels of oxygen tension gives rise to increased ROS formation. Therefore, as

a target organ, impaired respiratory function and diminished energy production might be seen in lung (202).

Free radicals and ROS that are responsible for destructive pulmonary toxicity and their toxicity mechanisms are summarized below.

### 2.3.3.1 Superoxide Radical ($O_2^{\bullet-}$)

$O_2^{\bullet-}$ radical or anionic form of $O_2$ is produced by binding an extra electron to molecular oxygen and act as a powerful reductant and poor oxidant in biological system (201,203). It has a high capacity to react with thiol groups in cellular structure and enzymes and leads to GSH (glutathione) depletion. This process triggers progression of further stress, and thus inactivates cellular or enzymatic function (201). Furthermore, $O_2^{\bullet-}$ has an ability to start Haber-Weiss reaction, which leads to the production of highly reactive hydroxyl ($HO^{\bullet}$) radical in the presence of ferrous iron (Reaction 1) (201,204).

$O_2^{\bullet-} + H_2O_2 \rightarrow HO^{\bullet} + {}^{-}OH + O_2$ (Reaction 1)

Due to its restricted short-life, $O_2^{\bullet-}$ is converted into oxygen and $H_2O_2$ spontenously or by SOD (superoxide dismutase) enzyme (Reaction 2) (201) .

$2O_2^{\bullet-} + 2H^{+} \rightarrow O_2 + H_2O_2$ (Reaction 2)

Superoxide is also able to react with NO to form peroxynitrite (ONOOH) and peroxynitrous acid ($HNO_3$), that later decomposes to $NO_2$ and hydroxyl-like radicals (195) .

### 2.3.3.2 Hydroxyl Radical ($HO^{\bullet}$)

$HO^{\bullet}$ radical is one of the most abundant and strong radical for biological systems. In the presence of ferrous iron, $O_2^{\bullet-}$ and $H_2O_2$ are able to form $HO^{\bullet}$ radical as summarized above in Reaction 2 (201). $HO^{\bullet}$ radical has relatively short half-life because of its high energy gain during abstracting a hydrogen atom and producing water. Therefore, it can be said that $HO^{\bullet}$ radical have a reaction capacity spontaneously in its microenvironment in biological system, which makes trapping $HO^{\bullet}$ harder by an antioxidant (195). Furthermore, biological system, consisting of high proportion of water is vulnerable in case of exposure to X and gamma ray to form hydrogen radikal ($H^{\bullet}$) and $HO^{\bullet}$ radical. This reaction is described below in Reaction 3 (201).

$H_2O \rightarrow$ (X or ɣ ray) $\rightarrow H^{\bullet} + HO^{\bullet}$ (Reaction 3)

### 2.3.3.3 Hydrogen Peroxide ($H_2O_2$)

$H_2O_2$ is itself not a free radical, but considered within ROS category. It is a potent oxidizing agent with slower reaction rate. Major source of $H_2O_2$ is spontenous or SOD mediated dismutation of $O_2^{\bullet-}$ into oxygen and $H_2O_2$ described in Reaction 2, previously. Another source of $H_2O_2$ generation of biological system is peroxidase enzymes (201). Presence of myeloperoxidases in neutrophils also contribute to $H_2O_2$ toxicity by forming a powerful oxidant, hypochlorous acid (HOCl) (195).

### 2.3.3.4 Hypochlorous Acid (HOCl)

Similar to $H_2O_2$, HOCl itself is not free radical and classified as ROS. HOCl has an important biological function on destruction of bacteria by phagocytic cells (201). As mentioned before, myeloperoxidase derived $O_2^{\bullet-}$ dismutation produces $H_2O_2$ and then HOCl (195). Reaction of $H_2O_2$ derived formation of HOCl is given in Reaction 4 (201).

$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$

HOCl has a high oxidation capacity and interacts with thiol groups, aminoacids, thioethers, hemoproteins and nucleotides (201).

### 2.3.3.5 Singlet Oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$), a highly reactive ROS, can be spontenously formed by activation of triplet oxygen and SOD mediated dismutation of $O_2^{\bullet-}$ . Also, reaction of $H_2O_2$ with HOCl gives singlet oxygen (195). It can mainly be detected by photoemission through dimol reaction (Recation 5) in biological system. However, impact of $^1O_2$ in toxic response still remains unclear (194).

$^1O_2 + {}^1O_2 \rightarrow 2^3O_2 + h\nu$ ( $\lambda$=634 nm, 703 nm) (Reaction 5)

### 2.3.3.6 Nitrogen Derived Radicals

In this subgroup, nitric oxide and peroxynitrite can be given as example. Nitric oxide is a nitrogen derived free radical, an important neurotransmitter and host-defense substance produced by phagocytes. It is produced by L-arginine and NADPH in living organisms via four different forms of nitric oxide synthase (NOS) (194). $NO^{\bullet}$ is classified

as free radical due to its formation reaction, combination of a nitrogen atom and an oxygen atom with an unpaired electron (201). One of the most important toxicity mechanism of $NO^{\bullet}$ is based on its high reactivity with other free radicals such as with $O_2^{\bullet-}$, which leads to formation of highly reactive peroxynitrite ($NO_3^-$) (Reaction 6). Further, protonated peroxynitrite rapidly form hydroxyl radical and $NO_2^-$ (Reaction 7). Also, carbondioxide ($CO_2$) reacts with peroxynitrite to produce other contributers of its toxicity, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ given in Reaction 8 (194).

$O_2^{\bullet-} + NO^{\bullet} \rightarrow NO_3^-$ (Reaction 6)

$HNO_3 \rightarrow NOO^{\bullet} + HO^{\bullet}$ (Reaction 7)

$NO_3^- + CO_2 \rightarrow ONOOCOO^-$

$ONOOCOO^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$ (Reaction 8)

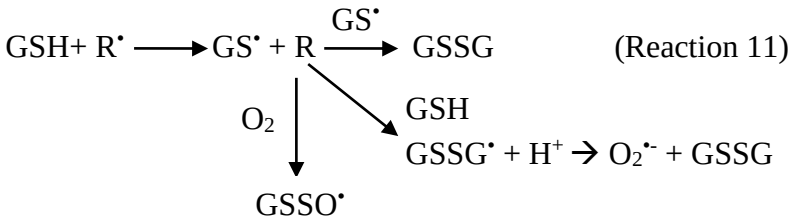
In the presence of molecular oxygen, NO is able to give $NO_2$ (Reaction 9). Rapid reaction of NO with produced $NO_2$ leads to nitrous acid ($N_2O_3$) formation and then $HNO_2$ (Reaction 10) (201).

$2\ NO + O_2 \rightarrow 2\ NO_2$ (Reaction 9)

$NO_2 + NO \rightarrow N_2O_3 + HNO_2$ (Reaction 10)

**2.3.3.7 Sulfur Derived Radicals**

They can be produced from endogenous structures present in organism carrying thiol compounds. The most important example for this group is non-enzymatic antioxidant reduced glutathione (GSH) (194). GSH is an atypic tripeptide exists in most of living organisms. It occurs in reduced and disulfide (GSSG) forms, and as mixed disulfides with numerous leukotrienes, proteins and other metabolites (189). Several drugs and chemical exposures may trigger formation of glutathionyl radicals by interacting with GSH. Mechanism of reactions for sulfur derived radical formation is defined in Reaction 11 (194).



### 2.3.3.8 Carbon Derived Radicals

In living organisms, carbon-derived (centered) radicals are considered as short half-lifed due to their rapid reaction with molecular oxygen. Their main formation pathway is abstraction of hydrogen atom from PUFA (polyunsaturated fatty acids) (194). This process is also called as initiation of lipid peroxidation, thus it will be explained in section 2.3.4.

### 2.3.4 Lipid Peroxidation

Lipid peroxidation is basically defined as attack of oxidants on lipid structure carrying carbon-carbon double bond, most importantly PUFA structure (205). In biological system, biomembranes and organelles such as mitochondria and endoplasmic reticulum, are vulnerable to lipid peroxidation due to unsaturated fatty acid structure in their membrane phospholipids (201). This process is also an important tool in research due to its products to enlighten and identify pathogenesis of many diseases. Lipid peroxidation progress is mainly based on three main mechanisms. These are defined as oxidation reactions mediated by free radicals, non-enzymatic oxidation reactions and enzymatic oxidation reactions (206). Non-enzymatic lipid peroxidation is based on initiation of peroxidation reactions by the presence of ferrous iron, copper, azo compunds, lipid hydroperoxides, tertiary butyl hydroperoxides and cumene hydroperoxides. Enzymatic lipid peroxidation can be defined as peroxidation reactions started by either natural enzymes in biological system or their substrates. Microsomal fractions in the presence of NADPH and ferrous iron are able to start lipid peroxidation reactions. In addition, xanthine- xanthine oxidase system produces superoxide and produced radical may reduce ferric iron into ferrous iron and initiate lipid peroxidation as well (201).

Lipid peroxidation is a complex chain reaction initiated by free radicals and results in damage of these oxidants on target lipid structure (207). Oxidants such as free radicals or nonradical structures assault lipids, especially PUFAs that contain hydrogen abstraction from a carbon, with oxygen addition ended with lipid peroxyl radicals and hydroperoxides (205). Therefore it can be described as a complex of propagation reactions initiated by structures containing one or more unpaired electrons (201). The

most common ROS that can initiate lipid peroxidation by destroying lipid structure are hydroxyl ($HO^{\bullet}$) and hydroperoxyl ($HO^{\bullet}_2$) radicals (205). $HO^{\bullet}$, a water-soluble and highly reactive ROS that can attack cellular macromolecules unspesifically, is responsible for lipid peroxidation and thus, initiation of many chronic diseases. It can be produced by Fenton reaction in the presence of ferric iron and $H_2O_2$. Further, Haber-Weiss reaction of $O_2^{\bullet-}$ with ferrous iron produces ferric iron and catalyzes redox cycling (194,201,205). On the other hand, hydroperoxyl radical, protonated version of $O_2^{\bullet-}$, is capable of producing $H_2O_2$ and in the presence of transiton metals, $H_2O_2$ may further generate $HO^{\bullet}$. Therefore, $HO^{\bullet}_2$ can be defined as a strong radical capable of disruption of membrane structure through chain oxidation of phospholipids (205). Chain reactions seen in lipid peroxidation process, initiation, propagation and progression steps were given below (194).

Initiation step:

$A\text{-}B \rightleftharpoons A^{\bullet} + B^{\bullet}$

Propagation (H abstraction):

$A^{\bullet} + LH$ (lipid molecule) $\rightleftharpoons AH + L^{\bullet}$

Propagation (lipid peroxidation):

$L^{\bullet} + O^{\bullet}{=}O^{\bullet} \rightleftharpoons LOO^{\bullet}$

$LOO^{\bullet} + LH \rightleftharpoons LOOH + L^{\bullet}$

Termination:

$LOO^{\bullet} + B^{\bullet} \rightleftharpoons LOOB$

#### 2.3.4.1 Peroxyl Radical ($ROO^{\bullet}$)

Peroxyl radical is one of the most important carbon derived radical due to its ability to abstract a hydrogen atom from neighbouring lipid structure and produces lipid peroxides (201). This process is defined as propagation step, described previously in section 2.3.4.

### 2.3.4.2 Alkoxyl Radical (RO•)

Alkoxyl radical is another type of carbon derived radical and has a short life in biological systems. Hence, they are intermediate products of reaction between peroxyl and hydroxyl radicals (194).

### 2.3.4.3 Lipid Peroxidation Products

Toxicological aspect of lipid peroxidation is mainly attributed to generation of highly reactive aldehydes from oxidized lipids. Formation of these aldehydes has a crucial role in the pathogenesis of many diseases due to their ability to form adducts with proteins and macromolecules (208). Major products of lipid peroxidation are defined as lipid hydroperoxides (LOOH). Among them, secondary products of peroxidation process are aldehydes, such as malondialdeyhde (MDA), 4- hydroxynonenal (4-HNE) and hexanal (195,201,205). As a convenient marker, MDA is highly screened in laboratories as a lipid peroxidation product with highest mutagenic activity while 4-HNE is the most toxic one among these end products (205). In Figure 4 and Figure 5, molecular structure of MDA (209) and 4-HNE (210) are given, respectively.

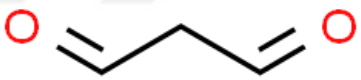



**Figure 4**. Chemical structure of MDA.

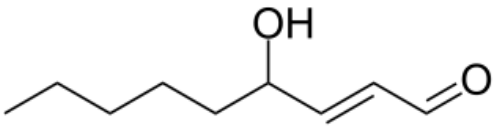



**Figure 5.** Chemical structure of 4-HNE.

4-HNE is mainly produced by transformation of *n*-6 PUFAs by 15- LOX (lipoxygenase) enzyme whereas MDA can be formed by both enzymatic and non-enzymatic pathways. In Figure 6, MDA formation and metabolism pathway is depicted (205).

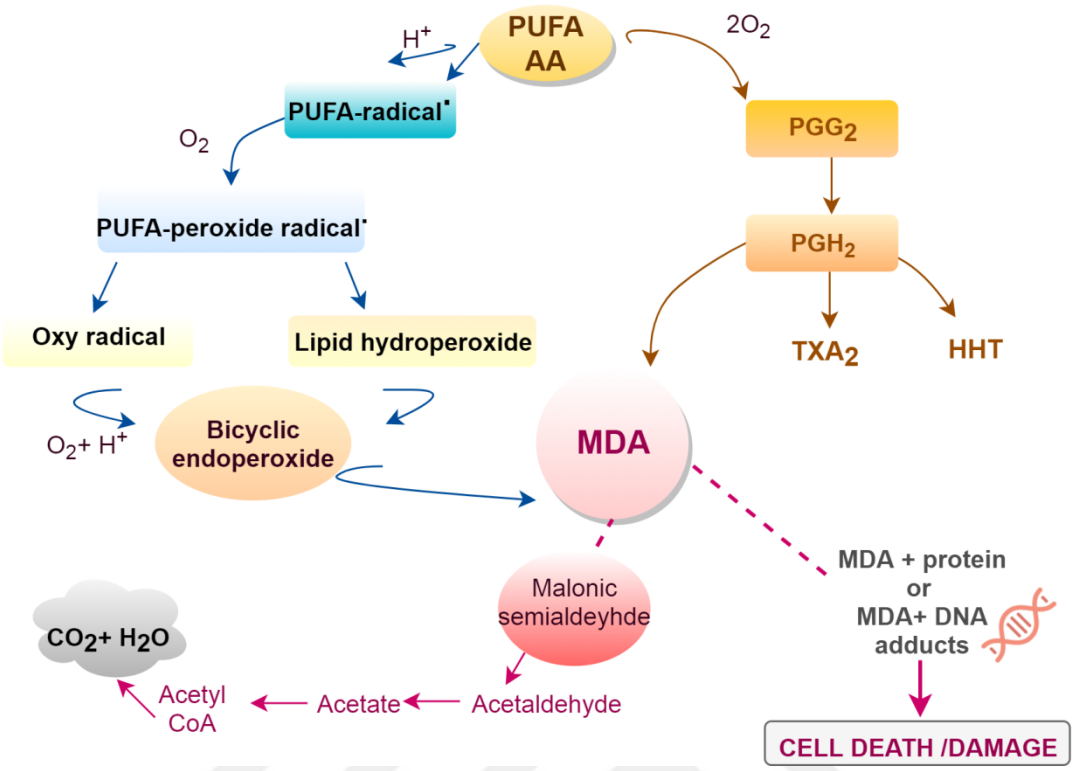



**Figure 6.** MDA formation and metabolism pathway.

MDA can be generated *in vivo* by AA decomposition or as an enzymatic side product of PUFA. AA: Arachidonic acid; PUFA: Polyunsaturated fatty acid; $TXA_2$: Thromboxane $A_2$; HHT:12-l-hydroxy-5,8,10-heptadecatrienoic acid; MDA: Malondialdeyhde; $PGG_2$: Prostaglandin 2, PGH2: Prostaglandin H2.

### 2.3.5 Antioxidant Defense Systems

Lung, is one of the most important human organ that directly exposed to high oxygen pressure and thus, vulnerable to ROS formation and related oxidation reactions (211). Since oxidative stress has been proved as an important contributor for pathogenesis of many pulmonary diseases, defense mechanism and antioxidant response in lung has a major role in prevention and treatment of lung diseases (212).

#### 2.3.5.1 Enzymatic Antioxidants

**Superoxide Dismutase (SOD)**

Superoxide dismutases (SOD) are classified as metaloenzymes that are responsible for dismutation of $O_2^{\bullet-}$ radical into $H_2O_2$ and triplet oxygen (194). $O_2^{\bullet-}$ is one of the most abundant ROS in biological system, thus the first defense mechanism against $O_2^{\bullet-}$ is dismutation of this highly reactive radical by SOD enzyme (195). Beside

being one of the most important endogenous enzymatic antioxidant in living system, it is also called as first line defense against ROS (213).

Aerobical organisms have several types of this enzyme while mammaians have two types as cytosolic and mitochondrial. Cu and Zn containing form (Cu/Zn-SOD or SOD1) is responsible for extracellular dismutation of $O_2^{\bullet-}$ whereas mitochondrial SOD (Mn-SOD or SOD2) has Mn as cofactor (194). Further, there is an extracellular SOD called as EC-SOD mainly located in extacellular fluid. Despite the fact that function and impact of EC-SOD in lung still remains unclear, it is thought that EC-SOD is responsible for regulation of NO level by controlling $O_2^{\bullet-}$ anion available (214). In previously given Figure 1, function of SOD is shown.

**Catalase (CAT)**

Catalase (CAT) enzyme is a 240 kDa protein and acts as an important endogenous antioxidant defence in all tissues that utilize oxygen (213). As a defence mechanism against ROS and free radicals, CAT detoxifies ROS produced in peroxisomes (194). Main substrate of CAT, $H_2O_2$, can be produced by dismutation of $O_2^{\bullet-}$ and break downs into $H_2O$ and $O_2$ within seconds by CAT enzyme (194,213). Mechanism of CAT enzyme on $H_2O_2$ degradation is shown in Reaction 12 (194).

$2\ H_2O_2 \rightarrow 2H_2O + O_2$ (Reaction 12)

**Glutathione Peroxidase (GPx)**

Glutathione peroxidase (GPx), is a selenium dependent endogenous antioxidant enzyme, and responsible for reduction of $H_2O_2$ and produce GSSG (oxidized glutathione) and $H_2O$ (194). In such cases such as increased metabolism of fatty acids and induction of fatty acyl CoA oxidase, capacity of CAT may be exceeded. Therefore, GPx acts as an important antioxidant enzyme to degrade excessive ROS in cytosol (195). In mammalian biological system, there are five known types of GPx have been reported. Seventyfive percent of cellular GPx is mainly found in cytosol (GPx1) and remaining is present in mitochondria. In liver, there is also plasma GPx (GPx3) (195). In addition to degradation of $H_2O_2$, GPx also catalyzes reduction of peroxide radicals into oxygen and alcohols. Mechanism of action of GPx is given in Reaction 13 (214).

**Glutathione Reductase (GR)**

Glutathione reductase (GR) catalyzes NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) dependent conversion of oxidized glutathione into its reduced form, GSH. GR is found in cyoplasm, mitochondria and nuclear fraction of cellular system. However, in terms of toxicological aspect, impact of subcellular GR is still not clearly identified (194,214). However, *in vivo* studies suggested that GR deficient mice have shown a great tendency to be vulnerable against oxidant related stress. In Reaction 13, function of GR is shown below (194).

$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$

$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH$

$2\ NADPH + GSSG \rightarrow 2\ GSH + NADP^+$ (Reaction 13)

**Indirect Enzymatic Antioxidants**

Enzymatic antioxidants generally generally have a capability to remove ROS directly. However, indirect enzymatic systems involved in this removing process have an important role in ROS scavenging (194,201).

One of the most important indirect enzymatic antioxidant system is conjugation enzymes involved in metabolism. These enzymes are responsible for diversion of reactive substances into biologically removable form. Glutathione S-transferase is the most important one involved in the process of conjugation of electrophilic intermediates and GSH to form glutathione S-conjugates (194). Also, UDP- glucuronyl transferase system is able to remove electrophilic intermediates as conjugated form and prevent their interactions with cellular units and further radical formation (194,201).

Another important enzyme system is NAD(P)H quinone oxidoreductase system involved in the process of quinone reduction. In Figure 7, process of quinone reduction by flavoenzyme NADPH quinone oxidoreductase to form hydroquinone is depicted (194).

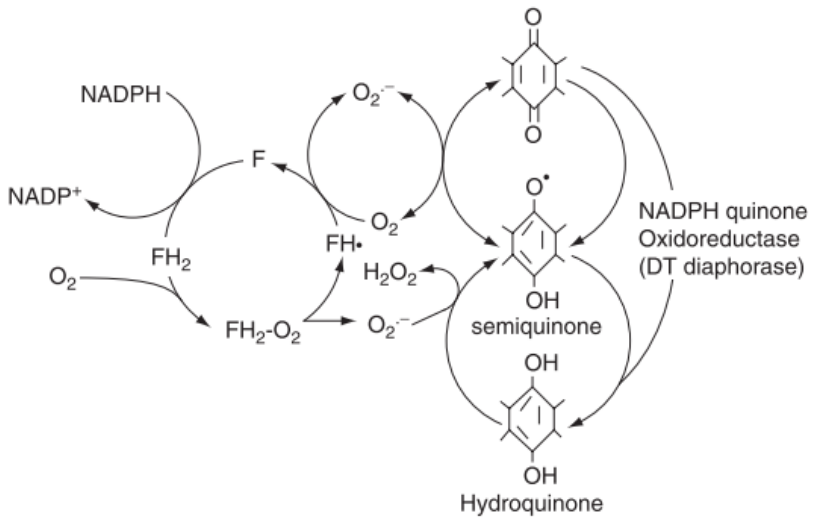



**Figure 7.** NADPH quinone oxidoreductase function and redox cycling.

NADPH supply is the other crucial part of antioxidant defence system in biological system (194). NADPH level and mitochondrial oxidative stress have been reported as inversely correlated and NADPH reserve has been identified as essential for mitochonrial protection against oxidative stress by maintaining GSH/ GSSG ratio (215). Also, NADPH acts as an essential cofactor in thioredoxin reductase mediated thiol reduction (216).

### 2.3.5.2 Non-enzymatic Antioxidants

Non-enzymatic antioxidants can be found as either endogenous or exogenous. The most common non-enzymatic antioxidants can be defined as glutathione (ɣ-glutamylcysteinylglycine), α-tocopherol (vitamin E), ascorbic acid (vitamin C) and carotenoids (194).

#### Glutathione

Glutathione and cysteine mainly act as direct radical scavenger and cofactor. In biological system their nucleophilic part, thiol group, is able to react with electrophilic species without enzymatic intervention (194).

**Low Molecular Weight Antioxidants**

In this subgroup, α-tocopherol, ascorbic acid and carotenoids can be given. Their major mechanism of action based on preventing chain radical formation reactions and trasnferring produced radical away from target areas as lipophilic membranes (195).

Among these antioxidants, α-tocopherol has an ability to break chain reactions in membrane by reducing lipid peroxyls into the form of hydroperoxides. Further, it reduces radical chain reaction at propogation stage, forming relatively stable tocopherol radical (194,195,201). The formed tocopherol radical then can be reduced by endogenous antioxidants such as glutathione ar vitamin C into the form of vitamin E (194). However, it was concluded that pharmacological doses of vitamin E in *in vivo* studies with limited benefit and did not reflect *in vitro* results (195).

On the other hand, carotenoids, mostly β-carotene, are reported to be efficient against singlet oxygen, specifically. β-carotene, acts as a transferer of excitation energy of singlet oxygen and forming a carotenoid triplet(195). Due to its low toxicity and radical scavenging capacity, it is used in cancer prevention clinical trials previously. However, results are failed to be found as beneficial, which was attributed to its pro-oxidant action (194).

**Metal Binding Proteins**

Metal binding proteins act as a defence mechanism in the initiation and propagation stages of chain reactions. In these reactions, these metal binding proteins such as ferritin, transferin, lactoferrin and ceruloplasmin are transferred and bound to proteins and stored (194,195). Therefore, they are called as indirect preventive antioxidants by involving in chelation process of metals in ROS producing reactions (e.g., Fenton reaction) instead of acting as a protective antioxidant (194).

### 2.3.4 Nrf2-ARE Pathway

Nrf2 (nuclear factor erythroid-2 related factor 2) is a key transcription factor in regulation of antioxidant system (217). Its pivotal role on regulation of antioxidant system is based on managing expression of antioxidant response element (ARE)-controlled cytoprotective and antioxidant genes (218). Therefore, Nrf2 is considered as a master regulator of antioxidant response in biological system (212).

Nrf2 is encoded by *NFE2L2* gene and under basal condiditons, Nrf2 expression is regulated at the protein level (219). In healthy condition, Nrf2 is maintained as sequestered form in cytoplasm (217) and has a short half-life around 20-30 minutes owing to its degradation by ubiquitin proteasome system (220). However, in case of oxidative stimulation, Nrf2 translocates to nucleus as an antioxidant response and triggers ARE (217). Nrf2 can be activated by agents that have oxidative, pro-oxidative and antioxidative properties as well (197). Main control of this translocation into nucleus held by homodimer protein called as Keap-1 (Kelch-like ECH-associated protein 1) (220). Keap-1, is a Cullin-3 (Cul3) dependent substrate protein (217), acts as cytoplasmic inhibitor of Nrf2. Under oxidative stress or in the presence of an antixidant, Nrf2 dissociates from Keap-1 and translocates into nucleus to bind ARE which activates cytoprotective genes (197). Concisely, Nrf2 translocation is regulated by several mechanisms including its suppression by Keap-1 or activation by ARE inducers such as polyphenolic antioxidants (221). In Figure 8, representative action of Nrf2/Keap1/ARE signaling was given (222,223).

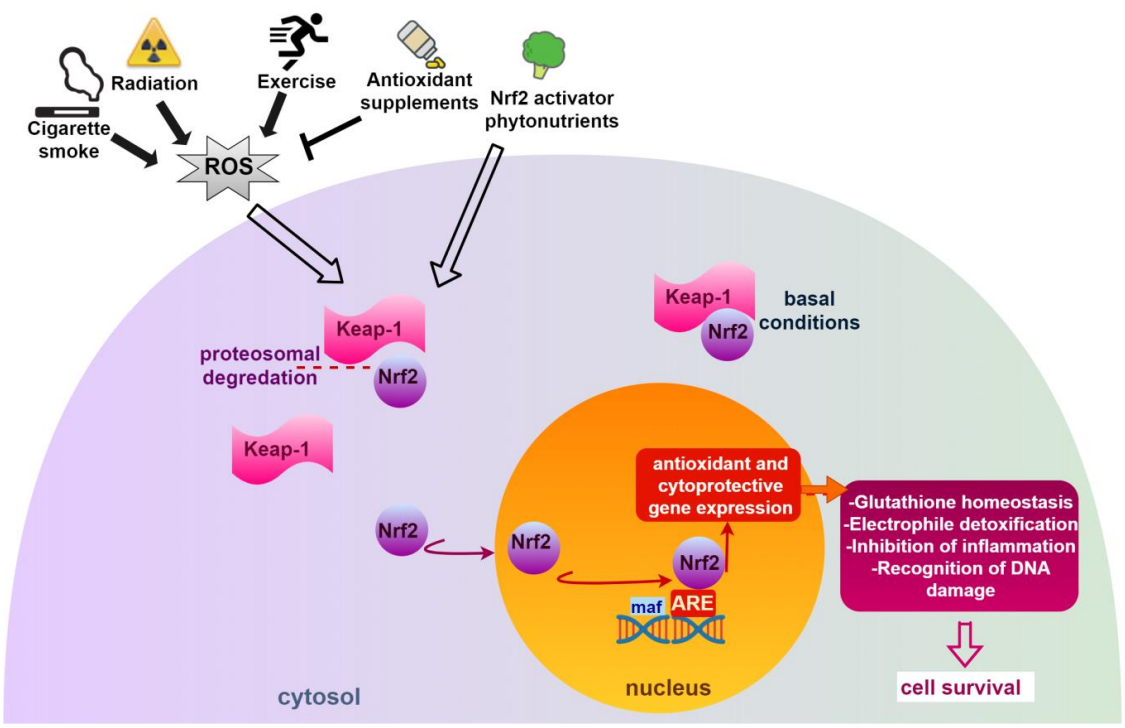



**Figure 8.** Nrf2/Keap1/ARE signaling.

Nrf2 can be activated by ROS or Nrf2 activator nutrients, which is resulted as Keap1/ Nrf2 proteasomal degradation and Nrf2 nuclear translocation. ARE: Antioxidant response element, Keap-1:

Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid-2 related factor 2; maf: musculoaponeurotic fibrosarcoma bZIP transcription factor

Nrf2 is majorly expressed in alveolar macrophage and epithelium, thus has a crucial protective role in lung by activating antioxidant and cytoprotective gene expression cascade (218). Previously, it was suggesed that Nrf2 is involved in lung response induced by cigarette smoke exposure. Therefore, Nrf2 expression is considered as a molecular target for both disease progression and therapeutical management of cigarette smoke induced lung injury (101). Since oxidative damage is a crucial contributing factor for cigarette induced lung disorders, regulation of oxidative stress through Nrf2 and further antioxidant gene expression have pivotal role on identification of underyling molecular mechanisms of disease progression (84).

Recent *in vivo* studies have revealed that interference with Nrf2 mediated antioxidant defence led to an increased susceptibility against cigarette smoke induced toxicity and damage in experimental animals (84,218,224). Stämpfli et al. (2009) indicated that deficiency in Nrf2 transcription factor and related detoxifiying enzymes increases risk for COPD (84). Another study on mice also suggested that deletion of Nrf2 has increased pulmonary inflammation caused by cigarette smoke exposure due to altered regulation of antioxidantive response (98). Similarly, Iizuka et al. (2005) and Rangasamy et al. (2004) have found that cigarette smoke exposure exacerbated emphysema and inflammation degree seen in Nrf2 deficient mice (225,226). Kensler et al. (2007) revealed that two major Nrf2 regulated genes involved in redox regulation, thioredoxin and GSH system, were induced in lung tissue of smoke exposed wild-type mice as a response to cigarette smoke exposure (227). In addition to its dominant role on antioxidative response, Nrf2 also acts as a regulator on inflammatory and pro-inflammatory mediator release such as COX-2, cytokines and iNOS during early phase of inflammatory damage seen in lung (228). According to several studies on Nrf2 deficient mice, it has been reported that environmental stimulants such as diesel particles or toxins known to induce pulmonary inflammation such as ovalbumin and bacterial lipopolysaccharide have diminished inflammation and extensified emphysema symptoms (227).

In the light of literature, Nrf2 targeting is considered as a potential therapeutic tool for the management of oxidative and inflammatory lung diseases. Owing to its multiple regulatory effects on oxidative stress, inflammation and cytoprotection, Nrf2 activation

might be beneficial for smoking induced pulmonary diseases. Therefore, there is a need for further studies.

## 2.4. Impact of EUC, CUR and NAC on Smoking-Dependent Lung Damage

### 2.4.1. Eucalyptol

Eucalyptol (1,8-cineole or 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) is isolated from essential oils of mainly eucalyptus, cardamom, daphne and sage (229). Chemical structure of EUC has been given in Figure 9 below (230).

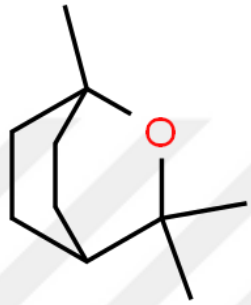


**Figure 9**.Chemical structure of EUC

EUC has been frequently used in cosmetic products as well as a penetration increasing agent for percutaneous drugs (231). According to English based-literature, there are various studies on EUC and its biological activity. These studies are based on a wide range of study area focused on its antioxidant (232–235), anti-inflammatory (236–238), antimicrobial (232,239–242), antimutagenic (243–245) and anticancer (246,247) effects. In various preclinical studies on animal models have also shown that EUC has a protective efficacy against hepatic necrosis, ethanol-induced gastric injury and colon damage in induced colitis (42). Above all, EUC has important functions in aromatherapy as nasal decongestant and as anticough agent. It has been used to treat bronchitis, sinusitis, rhinitis and asthma due to its possible anti-inflammatory action (231). Therefore, there has been variable number of research on EUC to date with unknown antioxidant and anti-inflammatory activity. According to literature, it was suggested that anti-inflammatory action of EUC is based on its inhibiting properties on TNF-α (tumor necrosis factor alpha), inflammatory cytokines and production of prostaglandins (231,248). Furthermore, previous studies indicated that EUC severely inhibited TNF-α and IL-1β level, which have major role on airway mucus hypersecretion in asthma (249). Also, EUC has been

reported as monocytes stimulative *in vitro*, which seems to be responsible for its bronchodilator effect in asthma (248). EUC has also diminished formation of leukotriene B4 (LTB4) and prostaglandin $E_2$ ($PGE_2$) in monocytes of people diagnosed with asthma (249). Since airway inflammation has a crucial part in the pathogenesis of a great majority of pulmonary diseases such as COPD, asthma and chronic bronchitis, substances have anti-inflammation properties are widely used in the treatment of airway diseases seen in both lower and upper region of pulmonary system (250). EUC has been used traditionally to treat respiratory disorders due to its secretolytic and anti-inflammatory action in the airways. Eventhough EUC application has been reported as beneficial for COPD patients, the mechanism of the action of this natural substance still remains unclear (43).

Limited studies on EUC and its action against inhaled toxicants have been suggesting that it is beneficial against airway inflammation (21,43,44,249). Previously, Lee et al. (2016) found that EUC treatment inhibited the *Dermatophagoides pteronyssinus* (Der p), an airway allergen, -induced cytokine protein expression, phosphorylation of p38 mitogen-activated protein kinase (MAPK) and Akt and intracellular toll-like receptor 4 expression in human bronchial epithelial cells (HBEC) (249). Another *in vitro* study by Zhou et al. (2007) also suggested that pre-treatment with EUC significanlty decreased the expression of Egr-1 (early growth response factor-1), a transcription factor that act as regulatory in the expression of many important genes for inflammation, in LPS-stimulated THP-1 cells. According to Zhou et al. (2007), EUC has shown a dose dependent effect on expression of Egr-1. In the same study, its expression was significantly increased in both whole cell and nuclear fraction of LPS treated group. However, pretreatment with EUC has showed no affect on the expression of NF-κB in the nuclear fraction of THP-1 cells stimulated with LPS (250). Another study on J774A.1 murine macrophage was also suggested that Eucalyptus oil blocked mRNA expression of inducible nitric oxide synthase and NO level induced by LPS and interferon-γ (251). Supplementary to other *in vitro* findings, Juergens et al. (1998) suggested that EUC has inhibited metabolism of of arachidonic acid and IL-1β secretion through a steroid similar pathway in cultured human monocytes. Therefore, in the same study EUC was indicated as a suitable therapy for airway inflammation seen in asthma (252).

In addition to limited *in vitro* studies on EUC, there are several *in vivo* studies investigating effect of EUC on pulmonary diseases and underlying mechanism. In an *in vivo* COPD and emphysema modelling induced by LPS+ *Klebsiella pneumoniae* in rats

investigated effects of Eucalyptus oil (30- 300 mg/ kg) for 4 months. According to this study, Eucalyptus oil significantly diminished inflammatory cell level in bronchoalveolar lavage fluid (BALF), TNF-α and IL-β level in lung tissue. Also, it significantly led to a decrease in MDA level and up-regulated superoxide dismutase (SOD) activity. These findings showed that Eucalyptus oil could exert a protective effect against lung injury through not only inflammation pathway but also improvement of antioxidant system (44). A double-blind clinical research in Germany has also showed that EUC repressed metabolism of arachidonic acid and cytokine production in human monocytes compared to placebo treatment. More interestingly, it was suggested long-term therapy with EUC provided an equivalent activity to 3 mg of prednisolone in patients with severe asthma (253). According to two different studies of Kennedy-Feitosa et al. (2015; 2019), EUC promoted lung repair in mice emphysema model (21) and diminished inflammation and oxidative stress on mice lung exposed to smoke both acute and chronic time interval (254). Similar to this study, Gondim et al. (2019) suggested that EUC showed a promising effect on short-term cigarette smoke exposure induced acute lung injury in mice at an oral dose of 300 mg/kg (255).

EUC and its biological activity on pulmonary diseases are still under investigation and its possible protective activity on cigarete smoke induced lung toxicity on *in vitro* modelling is limited. Therefore, further studies on EUC and its effect on cigarette smoke induced target organ toxicity still remains unclear. In the present thesis, EUC is investigated against cigarette smoke extract (CSE) induced bronchial epithelial cell toxicity *in vitro* through molecular pathways based on cell death, oxidative stress and inflammation on target tissue.

### 2.4.2. Curcumin

CUR (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a lipophilic polyphenol yielded by *Curcuma longa*, and has been extensively investigated for its chemopreventive potential (13). It is also extensively used as spice in Asian cuisine and is involved in ayurvedic medicine (256). Several assumed mechanisms of action for CUR are based on its radical scavenging activity, anti-inflammatory, anticancer, antiangiogenic, proapoptotic, anti-mutagenic and immune-modulatory actions (13,257,258). According to preclinical studies, CUR is rapidly metabolized and

conjugated in the liver, therefore having limited systemic bioavailability (257). Chemical structure of CUR was given in Figure 10 below (259).

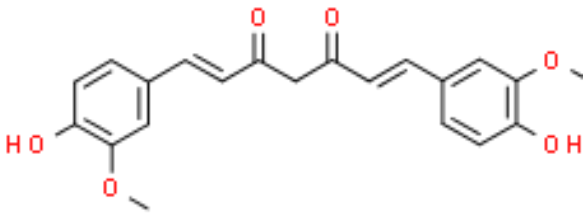



**Figure 10.** Chemical structure of curcumin (CUR)

Excessive research over years has suggested that CUR has therapeutic capacity against broad scale of diseases such as cancer, pulmonary and renal diseases, neurological diseases, metabolic diseases as well as various other inflammatory diseases (260). Majority of bioactivity studies on CUR have mainly focused on its antioxidant capacity (257,261–263) and its anti-inflammatory activity (262,264–266) in literature both *in vivo* and *in vitro*. Anti-inflammatory activity of CUR was attributed to its ability to block phosphorylation of 2-cytosolic phospholipase A 2 (cPLA) and affect arachidonic acid metabolism as well as decreasing cyclooxygenase 2 (COX-2) and 5- lipoxygenase (5-LOX) inhibition according to Hong et al. (2004) in LPS-stimulated RAW 264.7 cells (267). Also, CUR has attenuated acute pancreatitis through an antioxidant, anti-inflammatory and anti-apoptotic pathway in mice *in vivo*. In mentioned study, mice were exposed to low (i.p. CUR 50 mg/ kg) and high (i.p. CUR 200 mg/ kg) doses of CUR for 3 days and it was seen that serum amylase, myeloperoxidase and 4-Hydroxynonenale level and NF-kB protein expression were decreased in pancreatic tissue of both groups treated with CUR compared to mice with acute pancreatitis (268).

Regarding to its biological activity on inflammatory diseases, CUR, is also a highlighted natural substance for treatment of inflammatory pulmonary diseases. Over recent years, the therapeutic/ preventive properties of CUR on several pulmonary diseases that are specified with abnormal inflammatory reactions seen in asthma, COPD, ARDS, acute lung injury and pulmonary fibrosis have slowly been elucidated. According to recent *in vitro* and *in vivo* data in literature, it has been shown that CUR showed pleiotropic behaviour, regulating NF-kB transcription factor, cytokines such as IL-6 and TNF-α and adhesion molecules like intercellular adhesion molecule-1 that are regulatory

on inflammation and cancerogenesis (269). Previously, Moghaddam et al. (2009) suggested that CUR inhibited intrinsic and extrinsic inflammation response and supressed development of K-ras-induced lung cancer in mice (270).

Aside from its anti-inflammatory action, CUR also exerts both direct and indirect antioxidant effects by scavenging ROS and induces expression of cytoprotective proteins through Nrf2 (nuclear-factor-erythroid-2-related factor 2) pathway and triggers nuclear translocation of Nrf2. Therefore, it is considered as "bifunctional antioxidant" (260). Furthermore CUR is described as a strong scavenger of the superoxide anion, the hydroxyl radical, and nitrogen dioxide (35). Recent studies on CUR and its biological effect on smoking induced lung toxicity and lung inflammation have mainly focused on its anti-inflammatory and antioxidant action on target tissue. According to a study by Yuan et al. (2018), CUR has exerted anti-inflammatory action on LPS-stimulated BEAS-2B cells through supression of NF-κB activation and inhibition of COX-2 expression. In the same study, murines also exposed to both LPS and cigarette smoke and CUR decreased alleviated airway inflammation by inhibiting degradation of IκBα (inhibitory subunit of NF-κB alfa) and COX-2 expression (41). In an *in vitro* study on BEAS-2B cell line, it is suggested that CUR pre-treatment protected cells from BaP-induced cytotoxicity, reduced cell cycle arrest, and reversed BaP-induced radical production in a significant level (35). Previously, Suzuki et al. (2009) found that oral CUR administration 100 mg/ kg for three weeks attenuated cigarette smoke induced pulmonary inflammation and emphysema in mice. As a part of same study Suzuki et al. (2009) suggested that CUR significantly augmented expression of Nrf2 dependent antioxidant genes like hemoxygenase-1 (HO-1) dose dependently in mouse primary alveolar macrophages *in vitro* (25). There are several studies on CUR in literature evaluated its proliferative property against a pulmonary toxic substance. However, Vanella et al. (2017) suggested that CUR did not have a significant effect on cell proliferation against cigarette smoke induced cytotoxicity in BEAS-2B cell *in vitro.* In their study, CUR combined with Vitamin B2, Carnitine and NAC have showed a promising effect on cigarette smoke induced intracellular ROS level and induce antioxidant genes such as HO-1, Nrf2 and peroxisome proliferator-activated receptor ɣ co-activator-1 α (PGC-1α) as well (34). Since Nrf2 targetting has become an important therapeutical strategy for COPD within the last decade, Nrf2 activators such as CUR have an extensive part in new researches (228). Therefore, in the present thesis, CUR and its combination with EUC were

investigated within the aspect of their antioxidant and anti-inflammatory effects as well as their presumed cytoprotective bioactivity.

### 2.4.3. *N*-Acetylcysteine

*N*-acetylcysteine, also known as *N*-acetyl cysteine, *N*-acetyl-L-cysteine or NAC is a precursor of cysteine aminoacid, that is crucial for the formation of glutathione, and has a broad-range of clinical use in practice (219). It is a well-known antidote for paracetamol toxicity due to its capacity to act as a synthetic glutathione source (219,271). In Figure 11, chemical structure of NAC has been given below (272).

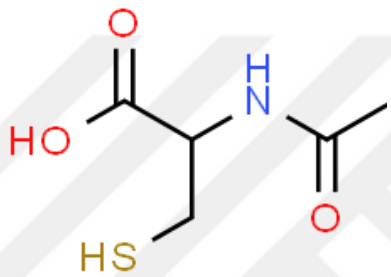



**Figure 11.**Chemical structure of N-Acetylcysteine (NAC)

Aside from being spesific antidote for paracetamol toxicity, NAC also has an important mucolytic effect in COPD and other inflammatory conditions of the airways (271). Responsible pathways of underlying above mentioned therapeutic effects are clearly defined. In particular, the mucolytic action is based on ability of NAC to alter structure of disulphide bridges present in glycoproteins of mucus, thus reducing viscosity. As a paracetamol poisoning antidote, NAC restores the deposits of glutathione in liver, which is the main endogenous nucleophilic structure responsible for neutralisation of exogenous electrophilic structures such as N-acetyl-p-benzoquinone imine (NAPQI), toxic endproduct of acetaminophen (273). Furthermore, NAC is also used in various clinical conditions such as doxorubicin-induced cardiotoxicity, several types of cardiac injury, acute respiratory distress syndrom and psychiatric disorders (274). Beyond its use in clinical and experimental research, NAC also has been promoted as a food supplement over the last decade in market (275).

Main action of NAC as a potential pharmacological antioxidant is attributed to its ability to scavenge free radicals and ability to augment intracellular glutathione in *in vivo* and *in vitro* experiments in literature (276). GSH and NAC are widespread antioxidants due to their ability to minimize oxidative stress and capacity to down regulate negative effects associated to oxidative stress (276,277). Glutathione mainly has preventive action on lipid peroxidation of cellular membranes as well as other lipid structured targets. As a by-product of GSH, it is sugggested that cysteine residues of NAC has a major role on glutathione maintenance and metabolism (277). Therefore, main idea of taking NAC as a supplement is based on its role on GSH maintanence as well as its ability to prevent the accumulation of intracellular free radicals.

As an important antioxidant and mucolytic agent, NAC has been widely used in studies investigating toxicity of cigarette smoke related pulmonary diseases as a therapeutic more recently. Since cigarette smoke involves thousands of chemicals including ROS, NAC has been used as a ROS scavenger in these studies (15,17,27,34,278,279). Previously, Antognelli et al. (2014), used NAC as a pretreatment in BEAS-2B cells to evaluate whether NAC is effective on some molecular pathways regulating ROS-induced apoptosis or not. In that study, they have found that NAC prevented deposition of the pro-apoptotic AGE (advanced glycation end products), argpyrimidine (AP) within the cells and reduced programmed cell death through mitochondrial pathway (28). Also, NAC is previously mentioned as an effective treatment in the regulation of interleukin (IL) synthesis, which is an important mechanism involved in acute lung injury. Matsumoto et al. (1998) found that NAC is effective on human bronchial cells through an antioxidant-susceptible pathway which is also involved in diminishment of IL-8 secretion through IL-lα stimulated bronchial epithelial cells. Therefore, NAC on acute lung injury have been suggested as a protective treatment due to scavenging ROS, their intermediate products and stimulating glutathione synthesis in a dose dependent manner (280). Aside from its modulatory effect on ROS scavenging and IL secretion, NAC has an important role on Nrf2-ARE pathway. In a previous study on BEAS-2B cells, Lee et al. (2011) has found that NAC blocked sulforaphane (SFN) induced decrease in BEAS-2B cell growth and supressed increase of Nrf2 protein levels in both nuclear and whole cell lysates related to SFN depended ROS production (281). Furthermore, van Schooten et al. (2002) suggested that NAC has been exerted a protective action on cancer due to its potential to inhibit mutagens and/or carcinogens induced

pathological, cytological, clastogenic, metabolic and molecular alterations in experimental rodent models. According to van Schooten et al. (2002), NAC has an impact on carcinogenicity by tobacco smoke in humans by modulating cancer associated biomarkers in specific organs such as mouth floor and soft palate by decreasing frequency of micronuclei formation in these target organs. Besides, in the mentioned study it is observed that NAC showed an inhibitory effect of toward the formation of lipophilic-DNA adduct in bronchoalveolar lavage (BAL) cells as well as of 7,8-dihydro-8-oxo-2'-deoxyguanosine adducts (279). However, the chemopreventive capacity of NAC in humans have not been fully enlightened yet and further studies are needed. According to recent clinical trial database of NIH, there are 27 studies performed involving NAC as a treatment for smoking reduction, prevention of smoking induced inflammation and oxidative stress in smokers or cigarette smoking conditions (282). Protective effect of NAC against cigarette smoke induced toxicity on target organs has been well-documented according to literature, especially its effect on cigarettte smoke induced oxidative stress condition. Therefore, in the present study, NAC is used as positive control for protective activity against cigarette smoke induced bronchial epithelial toxicity through different pathways.

# 3. MATERIAL AND METHODS

## 3.1 Materials

### 3.1.1 Chemicals

| | |
|---|---|
| 1,1,3,3-Tetramethoxypropane (TMP) | Sigma Aldrich; USA |
| 2-Mercaptoethanol | Merck KGaA; USA |
| 2-Thiobarbituric acid (TBA) | Sigma Aldrich; USA |
| Acetic acid | Riedel de Haen; Germany |
| Acrylamide | Intron Biotechnology; Germany |
| Anti LC3B antibody | Abcam;USA |
| AntiNrf2 antibody | Abcam;USA |
| APS | BioRad; Japan |
| Bio-Rad Clarity Western ECL substrate | Bio-Rad Laboratory; USA |
| Bovine serum albumine (BSA) | Sigma Aldrich; USA |
| CAT from bovine liver | Sigma Aldrich; USA |
| Cellular ROS Assay Kit | Abcam; USA |
| Cobalt (II) nitrate hexahydrate | Alfa-aesar; Germany |
| Copper sulfate ($CuSO_4$) | Riedel de Haen; Germany |
| Curcumin | Sigma Aldrich; Germany |
| DIC | SantaCruz Biotechnology; USA |
| Dimethyl sulfoxide | Sigma Aldrich; Germany |
| Doxorubicin HCl | Sigma-Aldrich; Germany |
| DTNB | Sigma Aldrich; USA |
| DTT | Thermo Scientific; Lithuania |
| Dulbecco's Modified Eagle Medium | Gibco; USA |
| EDTA | Alfa Aesar; Germany |
| Ethanol | UmayLab; Turkey |
| Eucalyptol | Fluka; Germany |
| Fetal bovine serum (FBS) | Gibco; USA |
| Folin-Ciocalteu's phenol reagent | Sigma Aldrich; USA |
| Glutathione reduced (GSH) | Sigma Aldrich; USA |
| Glycerol | Sigma Aldrich; Germany |
| Glycine | BioShop; Canada |
| HEPES Buffer | BioFroxx; Germany |

| | |
|---|---|
| Human IL-6 ELISA Kit | eBioscience; |
| Hydrogen peroxide (30% v/v) | Sigma Aldrich; USA |
| IL-6 Human ELISA Kit | eBioscience; USA |
| Lamin B1 antibody | Abcam; USA |
| Magnesium chloride | Sigma Aldrich; USA |
| Methanol | UmayLab; Turkey |
| N-(1-naphtyl) ethylenediamine | Fluka; Germany |
| N-Acetylcysteine (NAC) | Sigma Aldrich; Germany |
| NP-40 Protein Grade Detergent | EMD Millipore; USA |
| o-phosphoric acid | Merck; Germany |
| Penicillin-Streptomycin antibiotic | Gibco; USA |
| Phenylmethylsulfonyl fluoride (PMSF) | SantaCruz Biotechnology; USA |
| Pierce BCA Assay Kit | Thermo Scientific; USA |
| Potassium chloride | Sigma Aldrich; Germany |
| Protease inhibitor (PI) | SantaCruz Biotechnology; USA |
| Pyrogallol | Sigma Aldrich; USA |
| Rhodamine | Sigma Aldrich; USA |
| RIPA lysis buffer | SantaCruz Biotechnology; USA |
| Sodium carbonate anhydrous ($Na_2CO_3$) | Sigma Aldrich; USA |
| Sodium chloride (NaCl) | Sigma Aldrich; Germany |
| Sodium dodecyl sulfate (SDS) | Sigma Aldrich; Germany |
| Sodium hydroxide (NaOH) | Riedel de Haen; Germany |
| Sodium nitrite ($NaNO_2$) | Riedel de Haen; Germany |
| Sodium phophate dibasic dihydrate | Merck; Germany |
| Sodium potassium tartarate | Sigma Aldrich; USA |
| SQ | SantaCruz Biotechnology; USA |
| Sulfanilamide | Fluka; Germany |
| Tetramethylethylenediamine | BioFroxx; Germany |
| Trichloroacetic acid (TCA) | Riedel de Haen; Germany |
| Trizma HCl | Sigma Life Science; USA |
| Trypsin | Gibco; USA |
| Tween 20 | Fischer Scientific; USA |
| β-Actin (mouse) antibody | SantaCruz Biotechnology; USA |

### 3.1.2 Equipment

| | |
|---|---|
| 12 well plate | TPP; Germany |
| 6 well plate | SPL LifeScience; Korea |
| 96 well plate | Isolab; Tukey |
| Bead bath | Lab Armour; Cornelius, OR |
| Biotek plate reader | BioTek; USA |
| CellQuest Flow Cytometer | Becton Dickinson; USA |
| Centrifuge | Thermo Scientific SL 8R; Germany |
| Chemidoc™ XRS system | Bio-Rad Laboratory; USA |
| Class-II Biosafety cabinette | ESCO Class-II Airstream; Singapore |
| $CO_2$ tube | Habaş; Turkey |
| Deep-freezer | Arçelik; Istanbul, Turkey |
| Eppendorf tubes (1.5- 5 ml) | Eppendorf; Germany |
| Falcon tubes (15- 50 ml) | Falcon; Germany |
| Guava® easycyte flow cytometer | Merck KGaA; Darmstadt, Germany |
| Incubator | Binder CB-150; Germany |
| Micropipette (100-1000 µl) | Pipetman |
| Micropipette (10-100 µl) | Pipetman |
| Micropipette (1-10 µl) | Pipetman |
| Microplate washer | BioTek; USA |
| pH meter | Mettler Toledo MP220; US |
| Plate reader | Thermo Multiscan; Finland |
| Powerpac universal power supply | Bio-Rad Laboratory; USA |
| Primovert microscope | Carl Zeiss; Germany |
| PVDF Membrane | GE HealthCare LifeScience; Germany |
| Refrigerator | Arçelik; Istanbul, Turkey |
| Sterile filter (0.22 µm) | GVS; Germany |
| Sterile serological pipette (5-10 ml) | SPL LifeScience; Korea |
| Sterile syringe | Hayat Lab; Turkey |
| T-25/ 75/ 175 Flask | TPP; Germany |
| Tissue homogenizer | Heidolph Silent Crusher; Germany |

### 3.2. Methods

#### 3.2.1 Preparation of Cigarette Smoke Extract

Cigarette smoke is subdivided into two main phases consist of tar phase (total particulate matter) and gas phase (16). In a perspective on human consumption, the gas phase is noteworthy, due to its ability to pass through the alveolar epithelium directly and inducing toxic response in tissues both in lung and peripheral organs through systemic circulation. Also, the gas phase extract is wealthy of toxic and stable structures which exert various cytotoxic effects in a wide range of cells (283). Therefore, gas phase of cigarette smoke extract (CSE) was preferred for the present study.

Research grade cigarettes (3R4F containing 9.4 mg tar and 0.73 mg nicotine/ cigarette) from Kentucky Tobacco Research and Development Center at University of Kentucky (Lexington, KY) were used for extraction. CSE was prepared with slight modifications (181,284). Extract was prepared by pumping smoke, using a 50 ml syringe from 10 of 3R4F cigarettes into 40 ml of culture medium with 2 s puff duration, 35 ml puff volume and 60 s puff cycle as represented in Figure 12. The procedures were performed in the fume hood to prevent excessive inhalation of produced smoke. Following this, CSE was filtered by 0.22-µm filter, supplemented with 5% fetal bovine serum and 100 IU/ml penicillin- 100 µg/ml streptomycin and vortexed. This preparation was considered as 100% CSE and aliquots were kept at -80 $^{o}$C for further studies. For each experiment, CSE was diluted with complete cell culture medium to provide required experimental concentration of CSE.

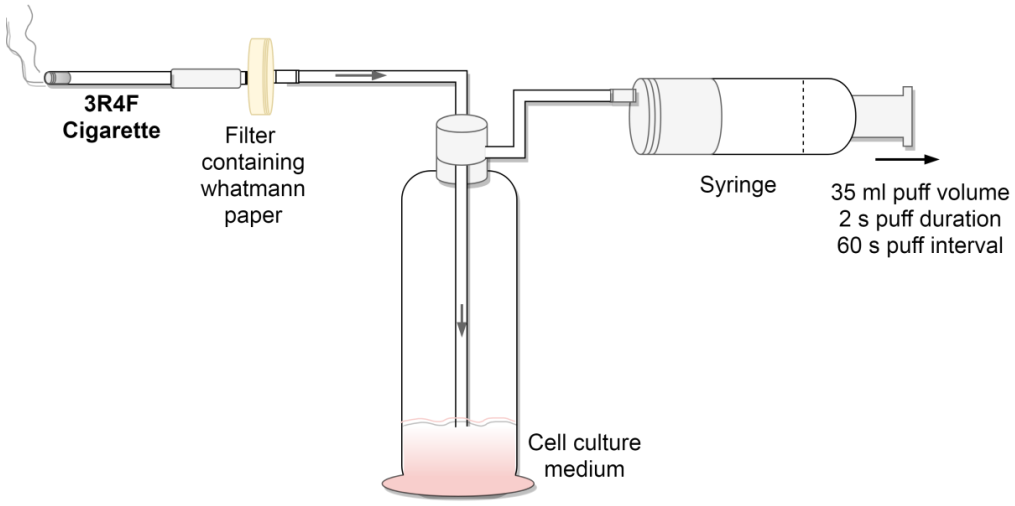



**Figure 12.** Schematic diaghram of CSE preparation

Used equipment during this extraction process was given in Figure 13.

**Figure 13.** CSE preparation system used in present thesis.

### 3.2.2 Cell Culture and Viability

*In vitro* models investigating effects of cigarette smoke on target organ generally have been performed by mimicking human system with specific cell lines. In respiratory system, epithelial and endothelial cells function as a borderline and forming an essential barrier for the maintanence of respiratory function of lung (181). In this context, toxicological pathways directly related to CS induced lung pathophysiology were studied on BEAS-2B cell line (20,169,185,198,200,285). Human bronchial epithelial cell line BEAS-2B (ATCC, USA) were cultured in DMEM high-glucose medium supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin and 5% fetal bovine serum and incubated at 37°C with 5% $CO_2$. Preparation of complete cell culture medium was described below:

| **Complete medium** | |
|---|---|
| DMEM high-glucose medium | 47.5 ml |
| FBS | 2.5 ml |
| Antibiotics (Penicillin+ Streptomycin) | 0.5 ml |
| Final volume: | 50 ml |

For detection of cell viability, water soluble MTT (3- (4,5-Dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide) was used. Preparation of MTT (5 mg/ml stock) was described below:

**For MTT (5 mg/ml stock):**

| | |
|---|---|
| MTT powder | 250 mg |
| PBS | 50 ml |

Prepared 5 mg/ml MTT stock solution was aliquoted as 1 ml portions and kept at -20 $^{o}$C within aluminium foil covered eppendorf tubes. Freshly diluted (1:10, v/v) with complete cell culture medium for each experiment.

The assay protocol is based on the calculation of cell viability through metabolic activity in BEAS-2B human healthy bronchial epithelial cell. The yellow, water soluble MTT is metabolically reduced to blue-violet, water-insoluble formazan in living cells. The number of living cells was calculated by determining the color intensity after formazan was dissolved in isopropanol. Based on this assumption, BEAS-2B cells ($3x10^4$) were seeded to a 96 well culture plate and left to incubation for 24 hours to form a semi-confluent layer. To evaluate cytotoxicity profile of CSE, CSE was applied 1-50% (v/v) for 4 hours. Following, dose exhibiting approximately 70% cell viability, 20% CSE (v/v), was used for all experiments. For dose selection of EUC, CUR and NAC, cells were exposed to various concentrations of EUC (6.25-200 μM), CUR (5-20 μM) and NAC (250-2000 μM) to observe their non-cytotoxic profile. After 2 hours of pre-treatment, cells were exposed to 20% (v/v) CSE and incubated for additional 4 hours. After incubation, formazan formation for each dilution was determined and compared to the values obtained from the control cultures. Cell viability is directly related to the amount of blue-violet-colored formazan read at 570 nm as optical density by plate reader (Thermo Scientific, Finland) and the decrease in viability as compared to the negative control (non-treated cells) was calculated using the following equation:

$$\textbf{Viability of cell (\%)} = \frac{100 \times OD_{570e}}{OD_{570b}}$$

$OD_{570e}$: Mean of measured optical density of tested substance
$OD_{570b}$: Mean of measured optical density of the negative control group

Concentrations where the calculated cell viability is 70% or greater relative to the control were used for further experiments. The test procedure was performed as triplicates.

### 3.2.3 Annexin V-PI Staining

To determine the amount of apoptotic/ necrotic cell death induced by CSE, annexin staining was carried out using an Annexin-V FLUOS staining kit (Roche, Germany) by method of Sur et al. (2012) with slight modifications (286). Briefly, the cells ($3x10^5$) were seeded in six-well plates and incubated for 24 h. Doxorubicin (2 µM), an anticancer drug and 10% DMSO (v/v) were used as positive controls (287) and non-treated cells were used as negative control. After treating the cells with EUC (50 µM), CUR (5 µM), EUC+CUR and NAC (1 mM) for 2 h, cells were exposed to CSE for 4 h. Following CSE exposure, cells were collected and washed once with PBS. Subsequently, the cells were re-suspended in solution containing 100 µl of incubation buffer and 3 µl of Annexin-V-Fluos labeling reagent and then incubated for 10 min at 37ºC in the dark. Following incubation, 1 µl of propidium iodide was added and within 2 min the Guava® easyCyte Single Sample Flow Cytometer system (Merck KGaA, Darmstadt, Germany) was used to quantify the number of viable, apoptotic and necrotic cells. The data were analyzed by Guava InCyte (Merck KGaA, Darmstadt, Germany) software.

### 3.2.4 Preparation of Cell Lysate

BEAS-2B cell lines were seeded in T-25 flasks at 37°C with 5% $CO_2$ for 24 hours to form an 80-90% confluent layer. Then, cells were be exposed to EUC (50 µM), CUR (5 µM), EUC+CUR and NAC (1 mM). Following exposure, cells were trypsinized and centrifuged. After this, remaining cell pellet was washed with PBS and centrifuged at 1000 g, 4ºC for 10 minutes. After centrifugation of the cell suspension, cell pellet was transferred to 1.5 mL eppendorf tubes containing 1 mL of PBS. The cells were homogenized at a gradually increasing speed from 30,000 rpm to 60,000 rpm for 30 seconds with tissue homogenizer. Completely lysed cells were centrifuged at 14,000 rpm, 4ºC for 15 min and the supernatants were stored at -80ºC for the determination of protein content, MDA level, and oxidative stress parameters.

### 3.2.5 Determination of Protein Content

The protein content in samples was determined according to the method published by Lowry et al. (1951) with slight modifications (288). Briefly, 50 µl cell lysate was mixed with 150 µl cupper reactive containing sodium carbonate, sodium potassium tartarate and copper sulfate in 96 well plate and incubated for 10 min at room temperature.

After incubation, 50 µl folin reagent was added each well and incubated at 50ºC for 10 min and absorbance was measured at 690 nm spectrophotometrically. For determination of protein content, different concentrations of bovine serum albumine (1 mg/ ml) were used as standard. Results were expressed as µg/ ml. Reactives of Lowry method were described below:

**Copper reactive components**:

| | |
|---|---|
| Sodium carbonate | 500 mg |
| Sodium hydroxide | 100 mg |
| | up to 5 ml $dH_2O$ |
| Sodium potassium tartarate | 10 mg |
| | up to 500 µl $dH_2O$ |
| Copper sulfate solution (1%) | 5 mg |
| | up to 500 µl $dH_2O$ |

**Copper reactive (freshly prepared):**

| | |
|---|---|
| Sodium carbonate solution | 1820 µl |
| Copper sulfate solution (1%) | 90 µl |
| Sodium potassium tartarate solution | 90 µl |
| Final reactive solution volume: | 2000 µl |

**Folin & Ciocalteu's phenol solution (10%, v/v)**

| | |
|---|---|
| Folin & Ciocalteu's phenol reagent | 500 µl |
| $dH_2O$ | 4500 µl |

### 3.2.6 Determination of CSE Induced Oxidative Stress

#### 3.2.6.1 Determination of GSH Level

GSH level was measured from cell lysates prepared previously by modification of a method performed by Sedlak et al. (1968) (289). Briefly, 50 µl of cell lysate was mixed with 10 µl DTNB solution and 150 µl of buffer solution in 96-well plate and incubated at 37ºC in dark for 30 min. After incubation period, absorbance of yellow-colored chromophore was measured at 412 nm wavelength by UV spectrophotometer. PBS was used as blank. Each measurement was performed triplicate. Results were expressed as µmol/ g protein. Solutions used for measurement of GSH level was described below:

**Buffer solution (pH 8.2):**

| | |
|---|---|
| Trizma Base | 1.51 g |
| EDTA | 3.65 mg |
| dH2O | 50 ml |

**DTNB Solution, 10µM (Ellman's Reagent):**

| | |
|---|---|
| DTNB | 1.35 mg |
| MeOH | 340 µl |

**GSH Stock Solution, 10 mM:**

| | |
|---|---|
| GSH | 3.07 mg |
| PBS | 1 ml |

**GSH Standards:**

| GSH stock vol. | Diluent (PBS) vol. | Final GSH Concentration (mM) |
|---|---|---|
| 200 µl | 800 µl | 2 mM |
| 100 µl | 900 µl | 1 mM |
| 50 µl | 950 µl | 0.5 mM |
| 25 µl | 975 µl | 0.25 mM |
| 12.5 µl | 987.5 µl | 0.125 mM |

### 3.2.6.2 Determination of CAT Activity

CAT activity was measured from previously prepared cell lysates by modification of a method performed by Hadwan et al. (2018) (290). This method is based on the correlation between carbonato-cobaltate (III) complex and catalase enzyme. Turn of hydrogen peroxide ($H_2O_2$) into molecular oxygen and water ($H_2O$) in a chemical reaction controlled by CAT was evaluated through absorbance of the carbonato-cobaltate (III) complex in 2 minutes. According to present method, decrease in color intensity associates with increased CAT activity. Briefly, 50 µl of cell lysate is mixed with twice amount of 10 mM $H_2O_2$ in tubes, mixed with vortex and incubated in 37 °C for 2 min in eppendorf tubes. Following, 600 µl of working solution containing phosphate buffer (pH 7.4), sodium bicarbonate and cobalt (II) nitrate hexahydrate was added and mixed with vortex immediately. Reaction tubes were kept in dark for 10 min and 250 µl of reaction for each group were transferred to 96-well plates. Distilled water was used as blank. Absorbance were recorded at 440 nm spectrophotometrically and results were expressed as U/ mg protein. For calculation of CAT activity, given volumetric amounts and formula were followed:

**Calculation of CAT Activity:**

| Reagents | Test | Standard | Blank |
|---|---|---|---|
| Cell lysate | 50 µl | - | - |
| Blank ($dH_2O$) | - | 50 µl | 150 µl |
| $H_2O_2$ | 100 µl | 100 µl | - |
| Working solution | 600 µl | 600 µl | 600 µl |

According to the rate constant of a first-order reaction (k) equation by method of Hadwan et al. (2018) was used to determine catalase activity, given below (290):

$$\textbf{Catalase Activity of test kU} = (2.303/ t) \times \log (S^0/ S)$$

t: time
$S^0$: absorbance of standard tube
S: absorbance of test tube

Reagents used for CAT activity measurement were given below:

**Components for working solution:**

| | |
|---|---|
| Potassium phosphate monobasic (a) | 0.68 g<br>up to 100 ml $dH_2O$ |
| Sodium dihydrogen phosphate (b) | 0.89 g<br>up to 100 ml $dH_2O$ |
| Phosphate buffer (pH 7.4) | 40 ml (a) + 60 ml (b) |
| Sodium bicarbonate | 9 g<br>up to 100 ml $dH_2O$ |
| Cobalt (II) nitrate hexahydrate | 2 g<br>up to 100 ml $dH_2O$ |

**Working solution (freshly prepared):**

| | |
|---|---|
| Cobalt (II) nitrate hexahydrate | 5 ml |
| Phosphate buffer (pH 7.4) | 5 ml |
| Sodium bicarbonate | 90 ml |
| Final working solution volume: | 100 ml |

**Hydrogen peroxide solution (freshly prepared):**

| | |
|---|---|
| Hydreogen peroxide stock (9.8 M, 30% v/v) | 11 µl<br>up to 10 ml $dH_2O$ |
| Final $H_2O_2$ concentration: | 10 mM |

### 3.2.6.3 Determination of SOD Activity

SOD activity was determined according to method of Marklund et al. (1974) with modifications (291). This method is based on ability of intracellular SOD to inhibit autooxidation of pyrogallol to pyrogallol-ortho-quinone in reaction medium. Briefly, 10 µl of cell lysate prepared from confluent T-25 cell culture flasks were incubated with 150 µl of reaction mixture and 50 µl of EDTA in 96 well plate. Following, 2 mM pyrogallol was added to each reaction mixture and changes in absorbance were recorded immediately for 450 seconds at 420 nm. Each measurement was recorded for 30 seconds intervals for total of 15 recordings. First measurement after addition of pyrogallol to reaction medium was accepted as time 0. Distilled water was used as blank for all

measurements. The amount needed for inhibition of 50% of pyrogallol autooxidation was accepted as 1 U of SOD activity. SOD activity for each group were performed duplicate. Results were expressed as U/ ml. Calculation of % inhibition of pyrogallol autooxidation was performed according to following formula given below:

$$\textbf{\% Inhibiton of pyrogallol autooxidation} = \frac{(\Delta OD_{blank} - \Delta OD_{test})}{\Delta OD_{control}} \times 100$$

$\Delta OD_{blank}$: Difference in the absorbance of blank for 300 sec
$\Delta OD_{sample}$: Difference in the absorbance of sample for 300 sec

Calculation SOD activity was performed according to following formula given below:

$$\textbf{SOD Activity (U/ ml)} = \frac{\text{\% Inhibition of pyrogallol autooxidation}}{50}$$

Reaction mixture and preparation of pyrogallol were explained below:

**SOD reaction mixture (freshly prepared, conditioned at 37 ºC for 15 min):**

| | |
|---|---|
| Tris-HCl Buffer, 50 mM (pH 8.2) | 605 mg |
| | up to 50 ml $dH_2O$ |
| EDTA, 1 mM | 17.55 mg |
| | up to 10 ml $dH_2O$ |

**Pyrogallol stock solution (freshly prepared, kept in dark):**

| | |
|---|---|
| Pyrogallol, 2 mM | 2.52 mg |
| HCl (37% v/v, 12 M) | 8.83 µL |
| | up to 10 ml distilled water |

### 3.2.6.4. Determination of Lipid Peroxidation

Thiobarbituric acid (TBA), a chromogenic compound, was used for MDA analysis with a slight modification of the methods of Buege et al. (1978) and Daraei et al.

(2012) which is based on the pink coloration of TBA by reacting with MDA in acidic medium and at the appropriate temperature (292,293). Reaction mixture for MDA measurement was described below:

**For TBA Mix (4x):**

| | |
|---|---|
| TBA | 37 mg |
| TCA | 1500 mg |
| HCl | 208 µl |
| $dH_2O$ | up to 10 ml and mixed at 70 $^oC$. |

**TBA Mix (1x) freshly prepared for 20 samples**:

| | |
|---|---|
| TBA Mix (4x) | 1 ml |
| BHT (0.03%, w/v) | 120 µl dissolved in EtOH |
| $dH_2O$ | 3 ml |
| Final volume of TBA mix: | 4 ml |

**Dilution of standards:**

| | |
|---|---|
| Tetramethoxypropane (TMP) | 6.08 µmol/ ml |
| Stock TMP (3.04 nmol/ml) | 5 µl TMP |
| | up to 10 ml $dH_2O$ |

**TMP Standards**

| Standard concentration | Stock TMP vol. | Diluent ($dH_2O$) vol. |
|---|---|---|
| 0.0019 nmol/ml | 6.25 µl | |
| 0.0038 nmol/ml | 12.5 µl | |
| 0.0076 nmol/ml | 25 µl | up to 10 ml $H_2O$ |
| 0.0152 nmol/ml | 50 µl | |
| 0.0304 nmol/ml | 100 µl | |

Briefly, 200 µL TBA mix (1x) containing TBA, trichloroacetic acid, HCl and BHT (butylhydroxytoluene) was vortexed with 100 µL cell lysate or standard solution (tetramethoxypropane) in 1.5 ml eppendorf tubes and incubated in hot water bath for 30 min at 95°C. After cooling for 5 min, absorbance of 100 µl of pink colored chromophore for each group were transferred to 96-well plate and read at 540 nm wavelength on UV

spectrophotometer. Each measurement was performed as triplicate. Results were expressed as nmol/ g protein.

### 3.2.7 Intracellular ROS

Protective effect of groups against CSE induced intracellular ROS level was determined by Cellular ROS Assay Kit (Abcam, Germany) according to flow cytometry protocol. Basically, 2',7'–dichlorofluorescin diacetate (DCFDA, also known as $H_2$DCFDA and as DCFH-DA), a fluorosence sensitive dye that evaluates quantitative amount of hydroxyl, peroxyl and other ROS in stimulated cells. DCFDA is deacetylated by cellular esterases and form a non-fluorescent compound. In stress conditions, it is oxidized by produced ROS within cells into 2',7'–dichlorofluorescein (DCF). DCF is a fluorescent compound that gives signal by fluorescence spectroscopy with excitation / emission at 495 nm/ 529 nm. In this study, BEAS-2B cells were seeded in 12-well plate ($3x10^5$ cell/ well) and harvested for 24 hours. Following day, pre-treatment with NAC, EUC, CUR and EUC+ CUR were applied for 2 h. Later, 4h of CSE (20%, v/v) exposure was performed. At the end of exposure, cells were trypsinized and collected in 400 µl PBS and 100 µl of 25 µM DCFDA was added to each flow cytometer tube and incubated for 30 min at 37°C with 5% $CO_2$ in dark. Cells pre-treated with cell culture medium were used as negative control while 100 µM of tert-butyl hydroperoxide (THBP) for 4 h were used as positive control in experiment. Data were analyzed by CellQuest Pro Software. Preparation of 1x buffer, DCFDA and THBP were described below:

**Solutions used for ROS assay:**

| | |
|---|---|
| DCFDA (20mM) stock | 1.25 µl |
| 1x Buffer | 998.75 µl |
| Final DCFDA concentration: | 25 µM |
| | |
| THBP (50 mM) | 1 µl |
| Cell culture medium | 500 µl |
| Final THBP concentration: | 100 µM |

| | |
|---|---|
| 10x Buffer | 100 µl |
| Autoclaved $dH_2O$ | 900 µl |
| Final volume of 1x buffer: | 1000 µl |

### 3.2.8. Mitochondrial Memrane Potential (MMP)

Mitochondrial membrane potential determination was performed with two different assays. For live cell imaging, glass slides were sterilized an then located on center of 6-well plates. Following, $3x10^5$ cells/ well was seeded on glass slide. After 24 hours of incubation at 37ºC and 5% $CO_2$, same treatments were followed as previous experiments. At the end of treatment period, each well was washed with PBS and incubated with 5 µg/ ml cationic dye Rhodamine 123 prepared with incomplete media at 37ºC and 5% $CO_2$ for half an hour. Following the incubation, cells were gently washed with PBS for 3 times for 5 min. At the end of last washing step, plate was covered with aluminium foil until confocal imaging. As positive control, 10 and 20 µM of FCCP (Carbonyl cyanide p-(trifluoromethoxy) phenyihydrazone) exposure for 15 min before rhodamine 123 incubation were performed. Images were captured by Zeiss Zen Software (Germany) at 63x focus at excitation/ emission at 507/ 529 nm.

For quantitave analysis of MMP, $3x\ 10^5$ cells/ well were seeded in 12-well plates and stained with Rhodamine 123 as described previously, but 20 min of incubation with dye was followed. At the end of staining, cells were trypsinized, centrifuged at 300 x*g* for 5 min and washed with PBS for 3 times each for 5 minutes. At the end of last washing step, 500 µl of cell suspensions were tranferred to flow tubes and change shift compared to FCCP was measured at excitation/ emission at 550/ 580 nm.

### 3.2.9 Western Blot Analysis

#### 3.2.9.1 LC3B Expression

To determine autophagy-dependent cell death, autophagosome marker LC3B expression was assessed by Western blotting. After 2 h pre-treatment with NAC, EUC, CUR and EUC+CUR, CSE 20% (v/v) was added in T-25 flasks for 4 h, cells were harvested and washed twice with PBS. Collected cell pellets were lysed in commercial RIPA lysis buffer (10 mM Tris-HCl, pH 8, 0.32 mM sucrose, 5 mM EDTA, 2 mM dithiothreitol, 1mM phenylmethyl sulfonylfluoride, and 1% Triton X-100). After

incubation on ice for 15 min, extracts were clarified by centrifugation at 14,000 rpm for 10 min at 4°C; the supernatants were collected for western blot analysis of LC3B detection and total cell proteins were isolated. Proteins were loaded to electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (14%). The gel separated proteins were transferred to polyvinylidine fluoride membranes and the membranes were blocked with 5% non-fat dry milk powder in TBST (10 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h and then probed with primary antibodies [LC3B 1:3000 (Abcam, USA) in TBST containing milk powder 5%] overnight at 4ºC. The following day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 in TBST; SantaCruz Biotechnology, CA, USA).To require an equal amount of protein was loaded in each case, Pierce bicinchoninic acid (BCA) protein assay kit was used according to kit protocol. 20 µg protein were loaded for each group to determine expression of LC3B.

### 3.2.9.2 Nrf2 Expression

To evaluate antioxidant response, cytosolic and nuclear Nrf2 expression was assessed by Western blotting. After 2 h pre-treatment with NAC, EUC, CUR and EUC+CUR, cells were exposed to CSE 20% (v/v) in 60 mm cell culture petri dishes for 4 h. At the end of incubation, cells were harvested and washed twice with PBS. Collected cell pellets were lysed in nuclear and cytosolic lysis buffer separately to isolate proteins in both nuclear and cytosolic fraction. Briefly, trypsinized cell pellets were centrifuged 1,000xg for 5 min at 4 ºC. After centrifugation, collected pellets were lysed in cytosolic lysis buffer described below.

**Cytosolic lysis buffer (100 ml):**

| | |
|---|---|
| HEPES (pH 7.9) | 238 mg |
| $MgCl_2$ | 14.3 mg |
| DTT | 7.71 mg |
| KCl | 74.5 mg |
| PMSF (0.2 mM) | 1% (v/v) (freshly added) |
| PI | 1% (v/v) (freshly added) |
| Nonidet P-40 (0.6%, v/v) | 600 µl |
| | up to 100 ml $dH_2O$ |

100 µl cytosolic buffer added to each pellet and incubated for 15 min in ice. Following, each pellet is vortexed and centrifuged at 3,000xg for 15 min at 4°C. Supernatants were collected as cytoplasmic protein fraction. Remaining pellet was washed with wash buffer described below.

**Wash buffer (100 µl per sample):**

Cytosolic lysis buffer without Nonidet P-40 was used for washing of cell pellets used for nuclear protein isolation.

For each washing step, pellet was resuspended in wash buffer and centrifuged at 3000xg for 15 min. After washing, remaining pellets were lysed in nuclear lysis buffer described below. Pellets were resuspended and incubated in ice for 30 min with intermitent mixing and vortexing. Following, suspension was sonicated for 10 s and centrifuged at 16,000xg for 20 min at 4°C. Supernatants were collected as nuclear protein fraction. To require an equal amount of protein was loaded in each case, Bradford reagent was used according to kit protocol. 20 µg protein were loaded for each group to determine expression of Nrf2.

**Nuclear lysis buffer (100 ml):**

| | |
|---|---|
| HEPES (pH 7.9) | 476.6 mg |
| $MgCl_2$ | 14.3 mg |
| DTT | 7.71 mg |
| KCl | 14.9 mg |
| NaCl (400 mM) | 2.33 g |
| Glycerol (25%, v/v) | 25 ml |
| EDTA | 5.84 mg |
| PMSF (0.2 mM) | 1% (v/v) (freshly added) |
| PI | 1% (v/v) (freshly added) |
| | up to 100 ml $dH_2O$ |

To require an equal amount of protein was loaded in each case, Bradford reagent was used according to kit protocol. 20 µg protein were loaded for each group to determine expression of Nrf2.

Proteins were loaded to electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (14%). The gel separated proteins were transferred to polyvinylidine fluoride membranes and the membranes were blocked with 5% non-fat dry milk powder in TBST (10 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 0.05% Tween-20) at room temperature for 1 h and then probed with primary antibodies [Nrf2 1:3000 (Abcam USA) in TBST] overnight at 4$^{o}$C. The following day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 in TBST; SantaCruz Biotechnology, CA, USA). For whole cell protein extracts and cytoplasmic protein extracts, β-actin (SantaCruz Biotechnology, CA, USA) was used as loading control (1:5000 in TBST) while rabbit polyclonal anti-Lamin B1 Ab (Abcam; USA, 1:5000 in TBST) was used as loading control for nuclear protein extracts. The blots were visualized using Bio-Rad Clarity Western ECL substrate (Bio-Rad Laboratory, USA) within Bio-Rad Universal Hood II- ChemiDoc™ XRS system (Bio-Rad Laboratory, USA) and all data were analyzed with ImageLab Software 6.0.1 (Bio-Rad Laboratory, USA). Preparation of SDS-PAGE gel, running and transfer buffer were desrcibed below:

**SDS-PAGE Gel (For 2 gels):**

| | **Stock** | **Stacking (4%)** | **Separating (14%)** |
|---|---|---|---|
| Acrylamide | 30% | 667 µl | 4.67 ml |
| $dH_2O$ | - | 3.05 ml | 2.71 ml |
| Buffer | 4x | 1.25 ml | 2.5 ml |
| APS | 10% | 25 µl | 100 µl |
| TEMED | - | 5 µl | 20 µl |
| Total | - | 4997 µl | 10 ml |

**10x Running buffer (pH 8.5)**

| | |
|---|---|
| 25 mM Tris base | 30.28 g |
| 190 mM glycine | 144.1 g |
| 0.1% SDS (10% stock) | 100 ml (10 g powder) |
| $dH_2O$ | up to 1L |

**1x Running buffer (for 1 L)**

| | |
|---|---|
| 10x Running buffer | 100 ml |
| $dH_2O$ | up to 1L |

**10x Wet transfer buffer (pH 8.3)**

| | |
|---|---|
| 25 mM Tris base | 30.28 g |
| 192 mM glycine | 144.1 g |
| $dH_2O$ | up to 1L |

**1x Wet transfer buffer (for 1 L)**

| | |
|---|---|
| 10x wet transfer buffer | 100 ml |
| 20% MeOH | 200 ml (freshly added) |
| 0.05% SDS (stock 10%) | 500 mg |
| $dH_2O$ | up to 1L |

**Blocking buffer**

| | |
|---|---|
| Milk powder (non-fat) | 500 mg |
| TBST (1x) | 10 ml |

**10x TBS (1 Liter, pH 7.4)**

| | |
|---|---|
| Tris base | 12 g |
| NaCl | 44 g |
| $dH_2O$ | up to 1L |

**1x TBST (1 Liter, pH 7.4)**

| | |
|---|---|
| 10x TBS | 100 ml |
| $dH_2O$ | 900 ml |
| 0.1% Tween 20 (100% stock) | 1 ml |

**Destaining Solution**

| | |
|---|---|
| 40% MeOH | 200 ml |
| 10% Acetic acid | 50 ml |
| $dH_2O$ | up to 500 ml |

**Commasie Blue**

| | |
|---|---|
| 50% MeOH | 250 ml |
| 5% Acetic acid | 25 ml |
| Brilliant Blue | 2 g |
| $dH_2O$ | 500 ml |

### 3.2.10 Determination of Nitrite Level

Nitric oxide accumulation was assessed through its oxidation product, nitrite level. Briefly, cell supernatants of cytotoxicity assay were used for its quantitative detection. Nitrite, metabolite of nitric oxide and indirect marker of inflammation, was examined by using Griess method in culture medium (294). Briefly, 50 µl of the cell supernatants were mixed with equal amount of Griess reagent at 25 $^oC$ and incubated for 5 min. Absorbances correlated with nitrite concentration were detected at 540 nm using a microplate spectrophotometer. 100 µM of sodium nitrite was used as standard as serially diluted to 12.5 µM. Preparation of Griess reagent was described below.

**Griess reagent:**

**Solution 1 (kept at 4$^o$C in dark)**

| | |
|---|---|
| Sulfanilamide (1%, w/v) | 300 mg |
| *o*-phosphoric acid | 1.5 ml |
| | up to 30 ml $dH_2O$ |

**Solution 2 (kept at 4$^o$C in dark)**

| | |
|---|---|
| N-(1-naphtyl)ethylenediamine (0.1%, w/v) | 30 mg |
| | up to 30 ml $dH_2O$ |

Solution 1 and solution 2 were mixed 1:1 (v/v) freshly for each experiment.

### 3.2.11 Determination of IL-6 Level

Cell supernatants of MDA assay were collected for IL-6 analysis. IL-6 level induced by CSE was masured by Human IL-6 ELISA kit according to manufacturer's instruction. 50 µl of cell supernatant was used and each group was applied as duplicate.

### 3.2.12 Statistical Analysis

All data were presented as mean± standard deviation (SD) in studies. Data were analyzed using GraphPad Prism 6.0 statistical Software (San Diego, California, USA) with one-way ANOVA followed multiple comparison by Dunnett's test. The value of $p<0.05$ was regarded as a statistically significance.

# 4. RESULTS

## 4.1. CSE Induced Cell Death

### 4.1.1. Cytotoxicity by MTT Assay

Cytotoxic effects of different concentrations of CSE on BEAS-2B cell line has been shown in Figure 14. According to results 15-50% of CSE has showed significant reduction on cell viability and 20% CSE has been selected for further experiment with a cell viability of 70.45± 2.28% compared to non-treated control group.

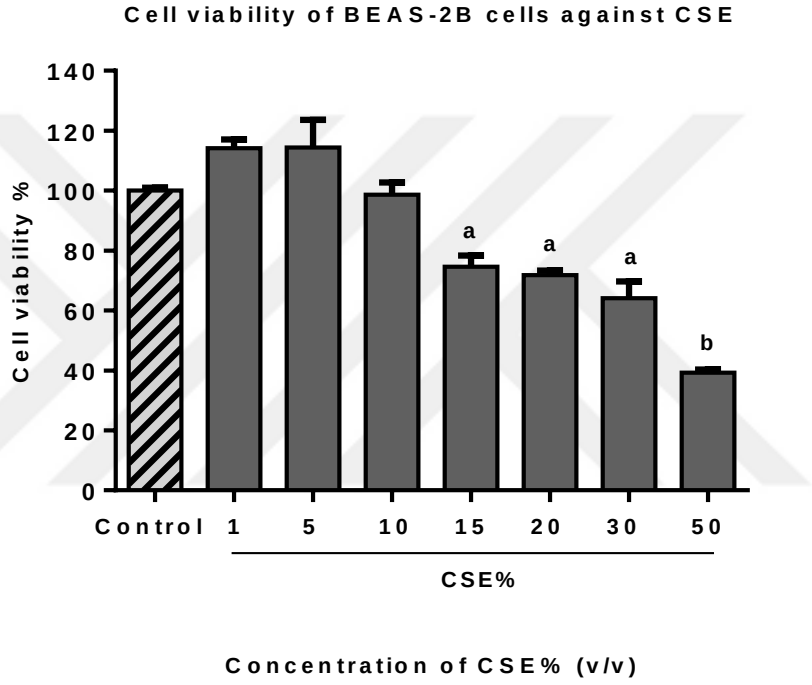



**Figure 14.** Dose-dependent cytotoxicity of CSE in BEAS-2B

CSE: Cigarette smoke extract. Statistical differences were indicated for control vs groups as $^{a}p<0.001$,$^{b}p<0.0001$. The data are shown as mean ± SD.

Experimental doses used for identificatiob of cytotoxicity profile of EUC, CUR and NAC have shown in Figure 15. According to MTT assay, EUC (50 μM), CUR (5 μM) and NAC (1 mM) have showed the highest cell viability thus, were selected for further studies.

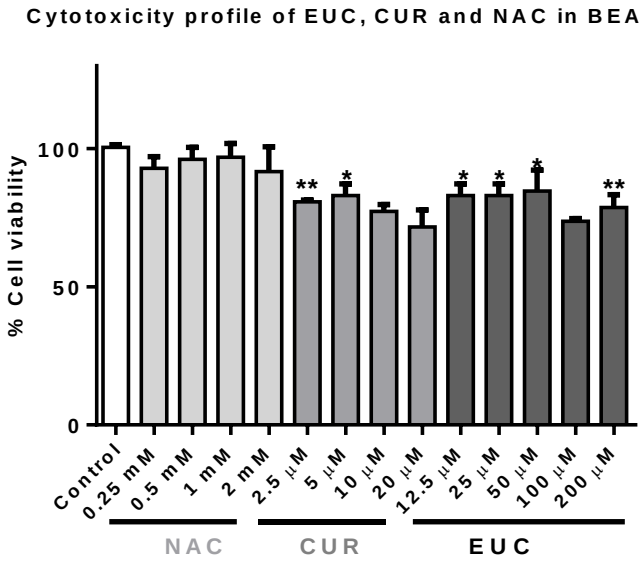



**Figure 15.** Cytotoxicity profile of EUC, CUR and NAC on BEAS-2B.

Statistical differences were indicated for control vs groups as $^{*}p<0.05$, $^{**}p<0.01$. CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

As seen in Figure 15, highest protective activity was observed with 1 mM NAC pre-treatment, which was used as positive control. Following the protective effect of NAC, 50 μM EUC and 5 μM CUR lead to an increase in cell viability among other experimental doses, respectively. CUR, has led to a dose-dependent decrease in cell viability (5-20 μM) while EUC has not exhibit a dose-dependent cytotoxic profile up to 200 μM. Highest cell viability was seen with 50 μM for EUC. Therefore, 5 μM of CUR and 50 μM of EUC were selected for further experiments.

Protective effect of NAC, EUC and CUR against CSE (20%, v/v) induced toxicity on BEAS-2B cell line has been shown in Figure 16.

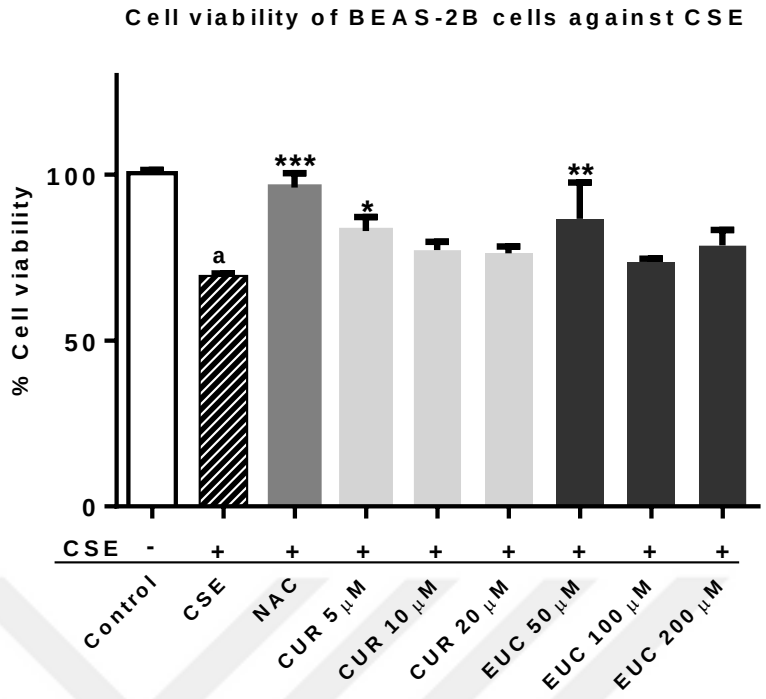



**Figure 16.** Cell viability of pre-treated and CSE exposed BEAS-2B cell

Statistical differences were indicated for CSE vs groups as $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and control vs CSE as $^{a}$ $p<0.0001$. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

According to MTT results given above, doses exhibited highest cell viability against CSE, 50 μM for EUC and 5 μM for CUR, and their combination were selected for further experiments as well. 1 mM NAC, with the highest protective activity, was selected as positive control for further experiments.

Cytotoxicity results of combination treatment has been shown in Figure 17. According to MTT test, there is no observable summative effect of combination treatment on cell viability compared to EUC and CUR are both used alone against CSE induced toxicity. Effect of these doses of EUC, CUR and their combination on cell viability against CSE exposure on BEAS-2B cell line were given in Figure 17 below.

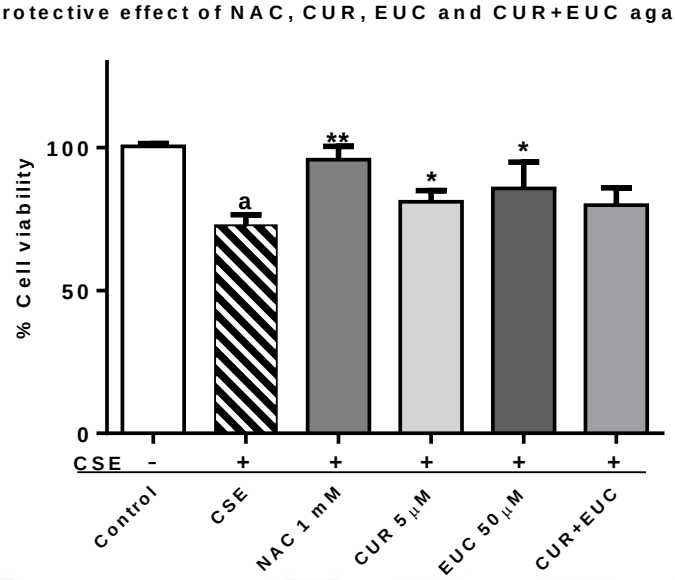



**Figure 17.** Protective effect of NAC, EUC, CUR and EUC+CUR against CSE

Statistical differences were indicated for CSE vs groups as $^{*}p<0.05$, $^{**}p<0.01$ and control vs CSE as $^{a}p<0.001$. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

### 4.1.2. Apoptotic Cell Death

CSE exposure led to a remarkable early apoptosis as seen in Figure 18, 19 and 20. CSE treatment led to an early apoptosis is 26.64% while late apoptosis was 11.09%. As similar to MTT assay, live cell ratio of BEAS-2B cell line was decreased prominently (61.73%). In Figure 18, cells dyed with Annexin V, PI and Annexin V+PI were shown. Shift to R2 region showed late apoptotic cell ratio stained with both Annexin V+PI, while early apototic cells were stained with Annexin V and necrotic cells were stained with PI only. According to Annexin V- PI staining, CSE increased early and late apoptotic cell rate and pre-treatments were markedly attenuated the CSE-induced early apoptosis. The most effective impact on CSE-induced early apoptosis was seen with 1 mM NAC pre-treatment with a non-significant difference from other pre-treatments.

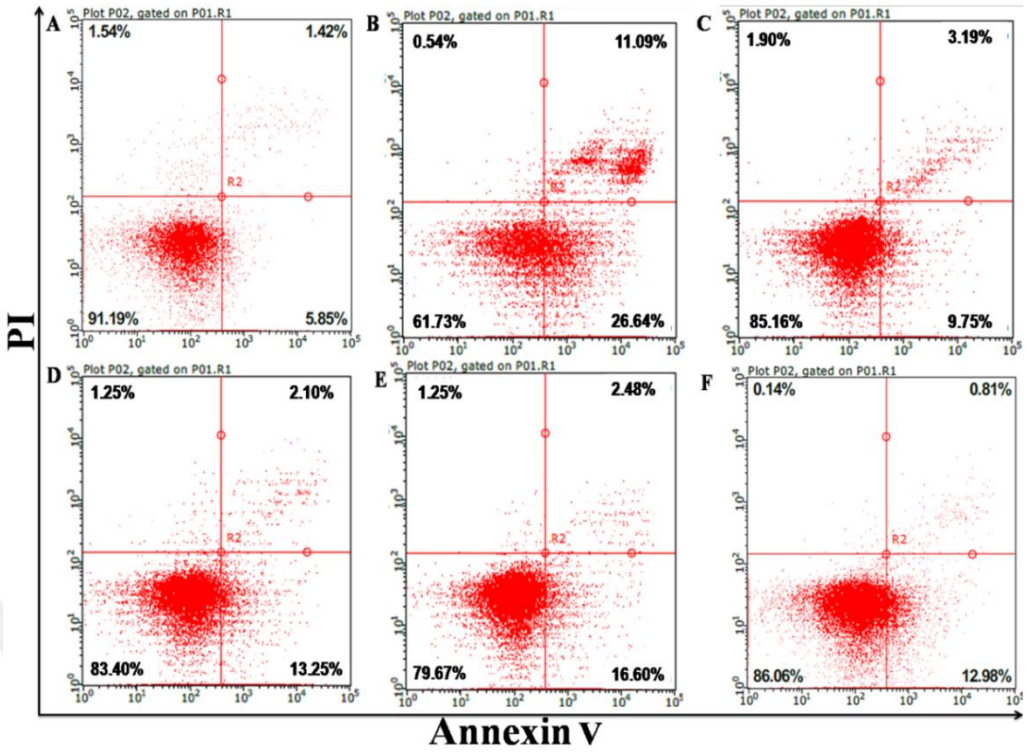



**Figure 18**.CSE-induced apoptosis and effects of treatments on BEAS-2B cells

BEAS-2B cells were pretreated with groups + 20% CSE for 4 h and stained with Annexin V-PI kit. (A) Control, (B) 20% CSE, (C) 20% CSE + 1 mM NAC, (D) 20% CSE + 50 µM EUC, (E) 20% CSE + 5 µM CUR, (F) 20% CSE + EUC+CUR. CSE: Cigarette smoke extract; CUR: Curcumin; EUC: Eucalyptol; NAC:*N*-acetylcysteine.

However, it was seen that pre-treatment with CUR has induced apoptosis in a dose-dependent manner. Besides, pre-treatment with other groups did not lead to a dose dependent effect in the increase of apoptotic rate. In Figure 19, induction of early apotosis in cells pre-treated with 5-20 µM of CUR for 2h and exposed to CSE for 4h has been shown.

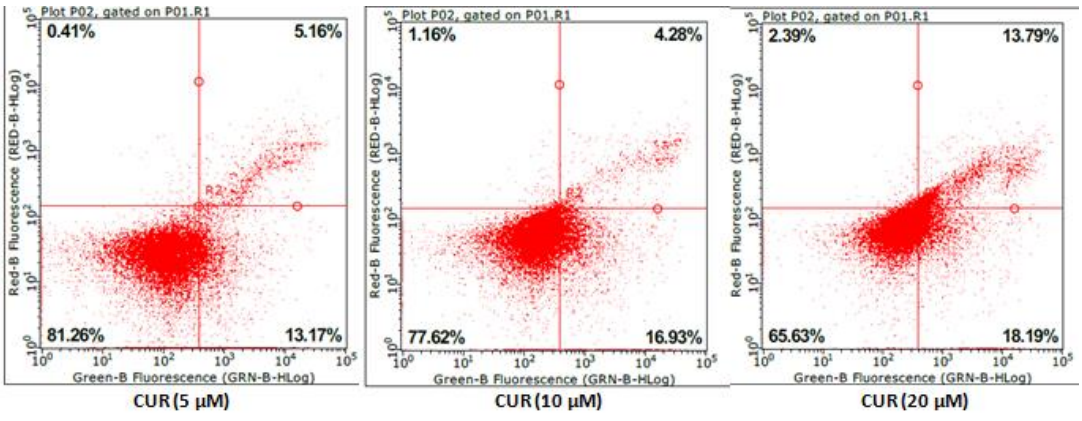



**Figure 19.** Dose-dependent apoptosis induced by CUR pre-treatment.

In Figure 20, bar graph of apoptotic anc necrotic cells treated with CSE has been shown. CSE exposure significanly induced both apoptotic and necrotic cell deatj. Pre-treatment with NAC significanlty reduced early apoptosis induced by CSE while combination pre-treatment was the most effective on reducing CSE-induced late apoptosis ratio.

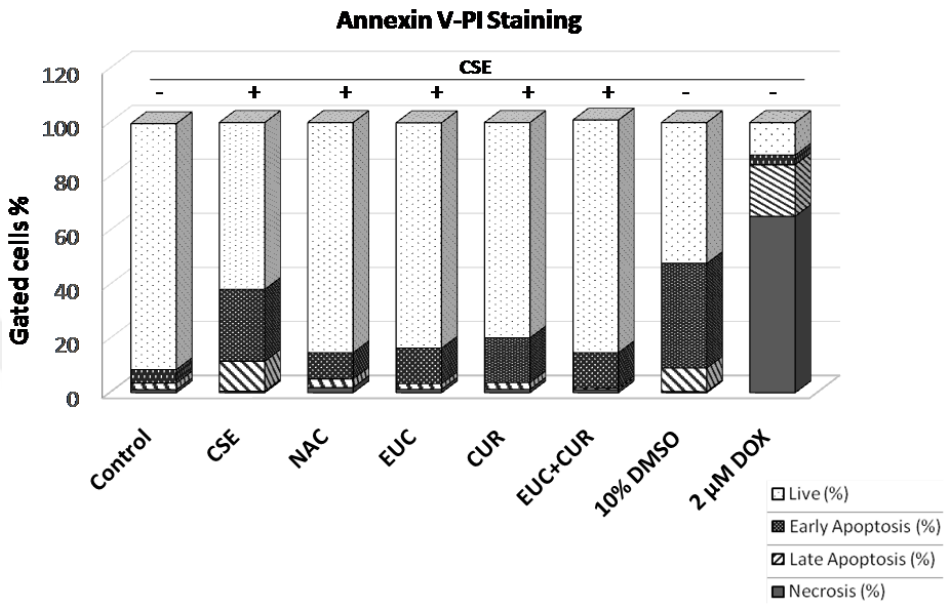



**Figure 20**.Bar graph of groups after Annexin V- PI labelling

BEAS-2B cell line was quantified by FACS (Fluorescence-activated cell sorting) after CSE exposure. 10% DMSO was used as positive control for apoptosis and 2 µM doxorubicin was used as positive control for necrosis. BEAS-2B: human bronchial epithelial cell line; CSE: cigarette smoke extract; CUR: curcumin (5 µM); DMSO: dimethylsulfoxide (10% v/v); DOX: doxorubicin (2 µM); EUC: eucalyptol (50 µM); NAC: N-acetylcysteine (1 mM), PI: propidium iodide.

According to Annexin V-PI staining, highest apoptotic rate was observed with positive control 10% DMSO, which was followed by CSE exposure alone. Lowest apoptotic cell rate was seen with NAC and combination pre-treatment, respectively. In Figure 21, total apoptotic rate of groups were given.

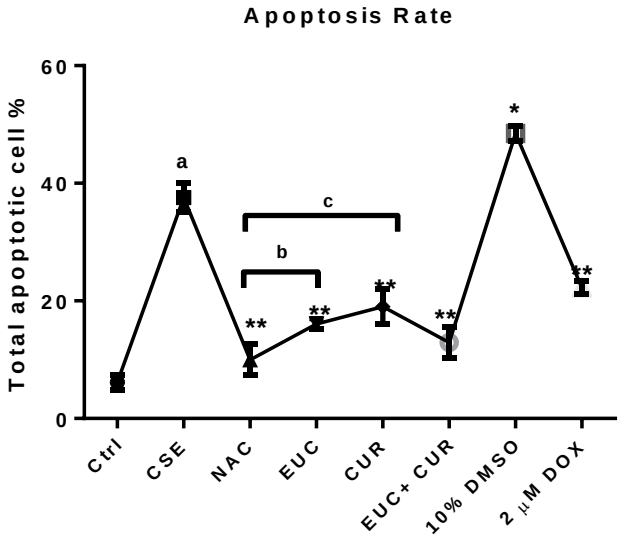



**Figure 21.** CSE induced total apoptosis rate in BEAS-2B cells.

a: Statistical difference between control vs CSE $p<0.0001$; $^{*}$: CSE vs groups, $p<0.0001$; $^{**}$: CSE vs groups, $p<0.001$; b: NAC vs EUC, $p<0.05$; c: NAC vs CUR, $p<0.01$. 10% DMSO was used as positive control for apoptosis and 2 μM DOX was used as positive control for necrosis. CSE: cigarette smoke extract; CUR: curcumin (5 μM); DMSO: dimethylsulfoxide (10% v/v); DOX: doxorubicin (2 μM); EUC: eucalyptol (50 μM); NAC: N-acetylcysteine (1 mM).

### 4.1.3. Autophagy-dependent cell death

Autophagy dependent cell death was evaluated with LC3B expression and rate of LC3B-II/ LC3B-I. Pre-treatment with all substances down-regulated expression of LC3B and diminished ratio of LC3B-II/ LC3B. Cells exposed to CSE has showed important autophagosome formation through increased LC3B expression compared to non-treated control group. The most attenuating effect on CSE induced autophagy rate was seen with NAC pre-treatment, which was followed by EUC+CUR and EUC pre-treatment, respectively. CUR pre-treatment has also declined CSE induced autophagy rate, but it was statistically insignificant. Figure 22 shows LC3B expression induced by CSE.

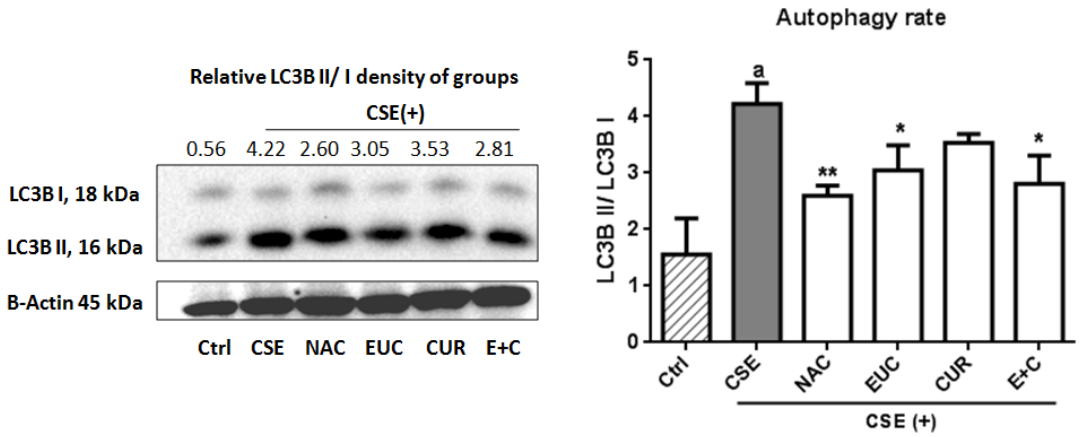



**Figure 22.** Antioxidants down-regulated CSE-induced autophagy rate

Statistical significance between Ctrl vs. CSE has shown with [a] $p<0.0001$; differences between CSE vs. groups *p<0.05; **p<0.01. Ctrl: Control; CSE: Cigarette smoke extract; CUR: Curcumin; EUC: Eucalyptol; NAC: *N*-acetylcysteine, E+C: Eucalyptol and curcumin. The data are shown as mean ± SD.

## 4.2. CSE Induced Oxidative Stress

### 4.2.1. GSH Level

According to the result of GSH assay, CSE lead to a significant increase in oxidative stress by reducing GSH level significantly. Pre-treatment with NAC, which is also synthetic precursor of glutathione, increased GSH level significantly more than basal level. Similar increase was also seen with pre-treatment with 50 μM EUC and combination treatment. Also, pretreatment with 5 μM of CUR, has showed a significant effect on GSH restoration by increasing its level nearly to the basal conditions. Results were given in Figure 23 below.

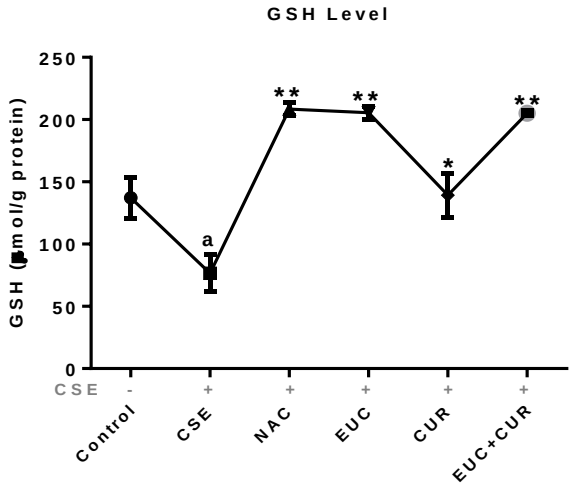



**Figure 23.** GSH level of CSE exposed BEAS-2B cell line

a: Statistical difference between control vs CSE $p<0.001$; *: CSE vs groups, $p<0.05$; **: CSE vs groups, $p<0.01$. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

### 4.2.2. CAT Activity

According to the results of CAT activity, CSE led to a significant increase in oxidative stress by diminishing CAT enzyme activity significantly. Pre-treatment with NAC, EUC and combination tretament EUC+CUR have increased CAT activity compared to CSE group. Also, pretreatment with 5 μM of CUR, has showed a significant effect on CAT activity. However, it showed lesser effect on CAT enzyme activity among other treatments. Results were given in Figure 24 below.

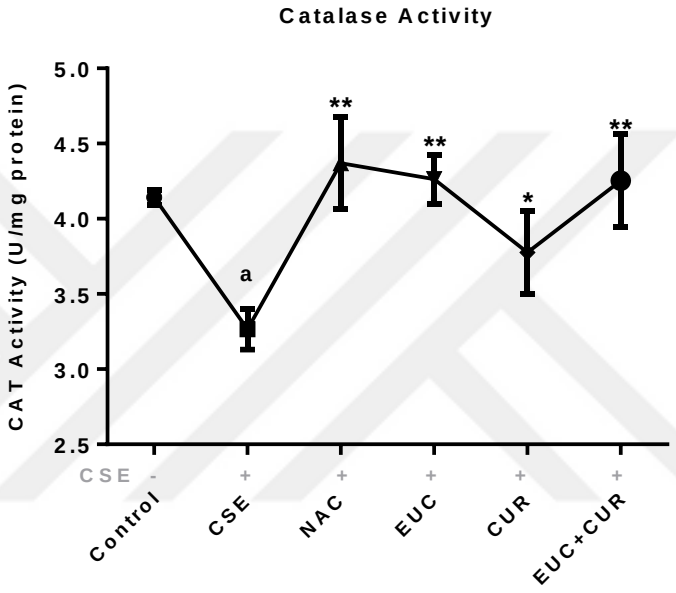



**Figure 24.** CAT activity of CSE exposed BEAS-2B cell line

a: Statistical difference between control vs CSE $p<0.001$; *: CSE vs groups, $p<0.05$; **: CSE vs groups, $p<0.01$. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

### 4.2.3. SOD Activity

CSE exposure has lead to a slight decline in SOD acitivity in CSE group, which was statistically insignificant. Pre-treatment with NAC, EUC and E+C combination enhanced SOD activity, and NAC was the most promising effective treatment. However, SOD activity of CUR pre-treated group was similar to CSE group, which was not statistically different than that of control group. Results of SOD enzymatic acitivity were given in Figure 25 below.

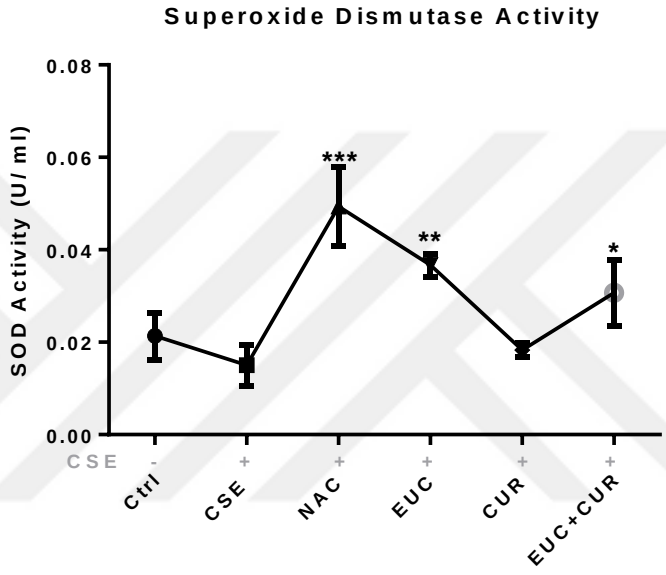



**Figure 25.** SOD activity of CSE exposed BEAS-2B cell line

Statistical difference between CSE vs groups *p<0.05; **p<0.01 and *** p<0.0001. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

### 4.2.4. Lipid Peroxidation

As shown in Figure 26, cytoprotective doses of NAC, CUR, EUC and EUC+CUR are effective against CSE induced lipid peroxidation on BEAS-2B cell line. According to statistical results, 1 mM NAC pre-treatment against increased MDA levels induced by CSE is the most effective treatment. This efficiency was followed by 50 μM EUC pre-treatment. Combination with CUR has showed a similar decline on MDA level, as well. Pre-treatment with 5 μM CUR caused a slight decrease on MDA level, albeit it was statistically insignificant.

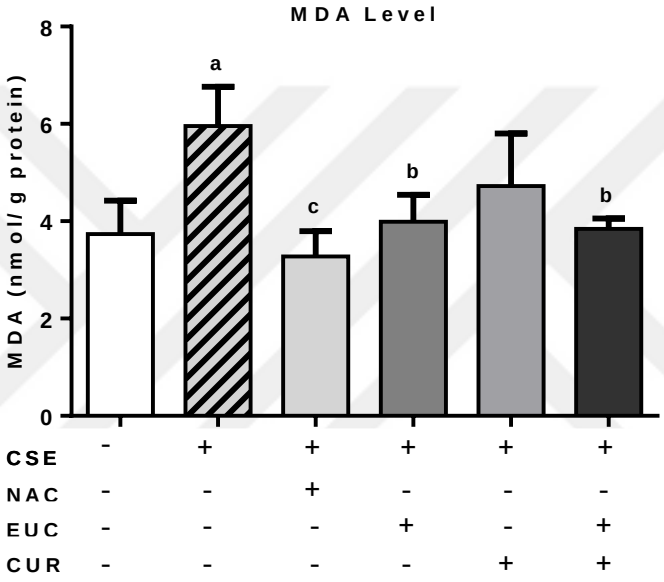



**Figure 26.** CSE induced lipid peroxidation on pre-treated BEAS-2B cell line

a: Statistical difference between control vs CSE; b: CSE vs groups, $p<0.05$; c: CSE vs groups, $p<0.01$. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

### 4.3. Nrf2 Expression

Protein expression of Nrf2 in two different cellular fraction, in cytosol and nuclei, was investigated on BEAS-2B cell line. Isolated protein fractions of whole cell, cytosol and nucleus have shown that CSE exposure led to Nrf2 nuclear translocation, as shown in Figure 27.

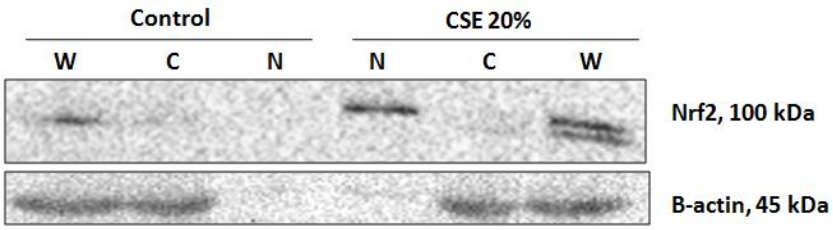



**Figure 27.** Nuclear translocation of Nrf2 by CSE exposure

Control group was treated without CSE. CSE groups were treated with 20% (v/v) CSE for 4 hours. CSE: Cigarette smoke extract; W: Whole cell lysate; C: Cytosolic fraction; N: Nuclear fraction.

Pre-treatment with NAC with or without CSE exposure, has led to a significant cytosolic accumulation of Nrf2 in cytosolic fraction of BEAS-2B cell line. However, pre-treatment with EUC, CUR and their combination have led to a decline in cytosolic Nrf2 expression with or without CSE exposure. In Figure 28, cytosolic Nrf2 expression was shown.

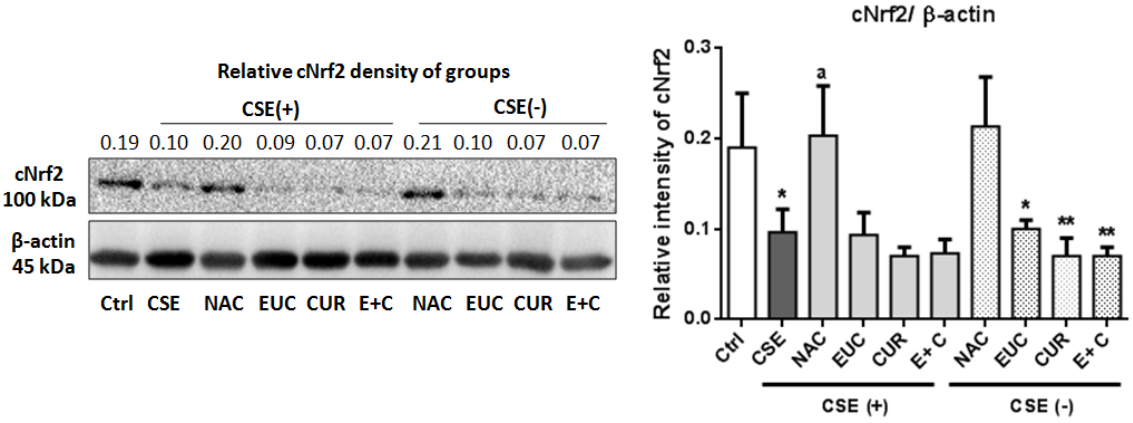



**Figure 28.** Cytosolic expression of Nrf2 (cNrf2)

a: Statistical difference between CSE vs NAC $p<0.01$; Ctrl vs groups *$p<0.05$ and **$p<0.01$. Ctrl: Control; CSE: Cigarette smoke extract; CUR: Curcumin; EUC: Eucalyptol; NAC: *N*-acetylcysteine, E+C: Eucalyptol and curcumin. The data are shown as mean ± SD.

Nuclear Nrf2 expression was significantly increased with CSE exposure. As shown in Figure 29, pre-treatment with EUC and EUC+CUR have significantly increased nuclear translocation of Nrf2. Moreover, all treatments increased nuclear expression of Nrf2 without CSE, but the most promising effect was observed with combination treatment, acting as Nrf2 activator itself. Especially EUC and combination pre-treatment followed by CSE exposure has led to a significant nuclear Nrf2 expression in BEAS-2B cell line.

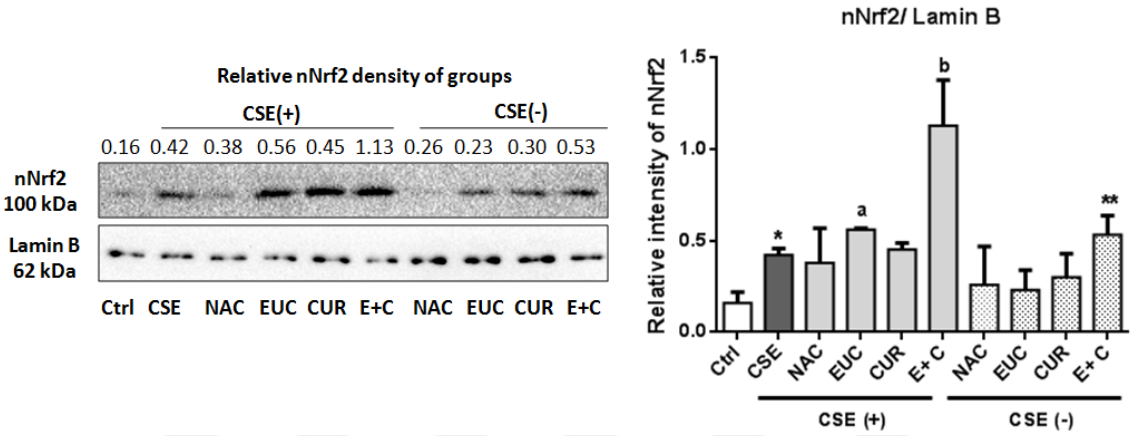



**Figure 29.** Nuclear translocation of Nrf2 (nNrf2)

a: Statistical difference between CSE vs EUC $p<0.05$; b: CSE vs E+C $p<0.0001$; differences between Ctrl vs groups, *$p<0.01$, **$p<0.0001$. Ctrl: Control; CSE: Cigarette smoke extract; CUR: Curcumin; EUC: Eucalyptol; NAC: *N*-acetylcysteine, E+C: Eucalyptol and curcumin. The data are shown as mean ± SD.

## 4.4. Intracellular Reactive Oxygen Species

Intracellular ROS levels were significantly up-regulated with CSE exposure in each group. With CSE exposure for 4 hours, relative produced ROS level was observed around 40%. EUC and CUR combination has showed more potent ROS scavenging potential compared to each treatment alone. According to the results, pre-treatment with NAC has showed a greater decline in intracellular ROS production compared to other pre-treatments. In Figure 30 and Figure 31, representative histogram of increased ROS accumulation and relative ROS % induced by CSE has been given, respectively.

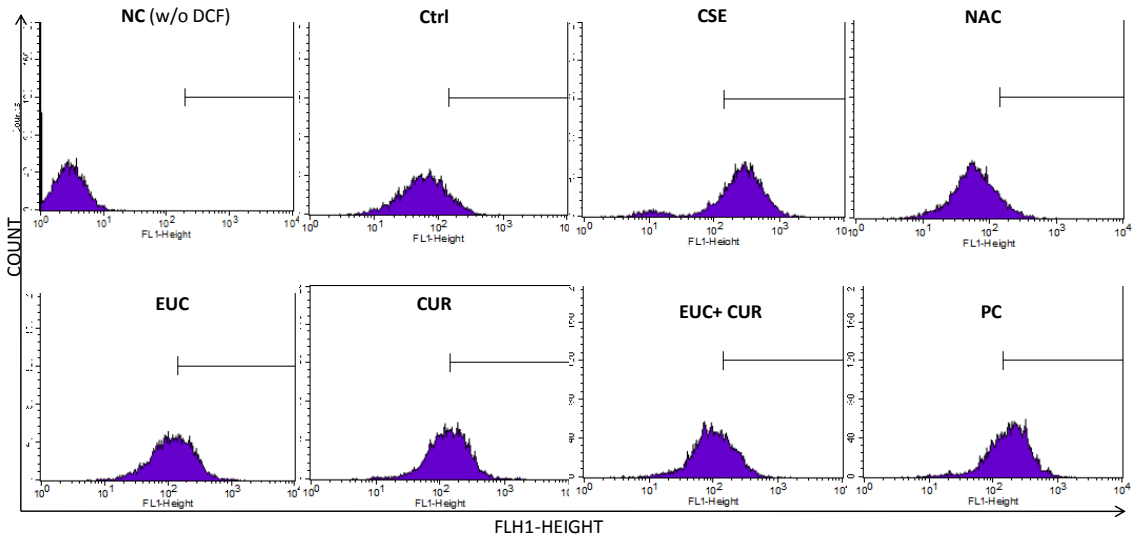



**Figure 30.** Representative histograms for percentage of increase of ROS accumulation in CSE exposed groups

Enhancement of intracellular ROS level, observed via the shift of the signal curve obtained for the treated cells to the right compared with that of the control cells. CSE: cigarette smoke extract; CUR: curcumin (5 μM); EUC: eucalyptol (50 μM); NAC: *N*-acetylcysteine (1 mM); PC: positive control THBP (100 μM).

In Figure 30, representative histograms for percentage of increase of total ROS accumulation in CSE exposed groups have been shown above. The shift of the signal curve obtained for the treated cells to the right compared with that of the control cells showed increase of total ROS induced by CSE. According to shift of signals, CSE led to a significant increase in total ROS production. According to results, CSE significanlty increased total ROS accumulation in BEAS-2B cells, while NAC was the most effective pre-treatment on reducing ROS accumulation induced by CSE. Also, combination pre-treatment reduced ROS production efficiently compared to EUC and CUR pre-treatments alone. Differences between groups have been depicted with overlay of signals and with bar graph of independent experiments in Figure 31.

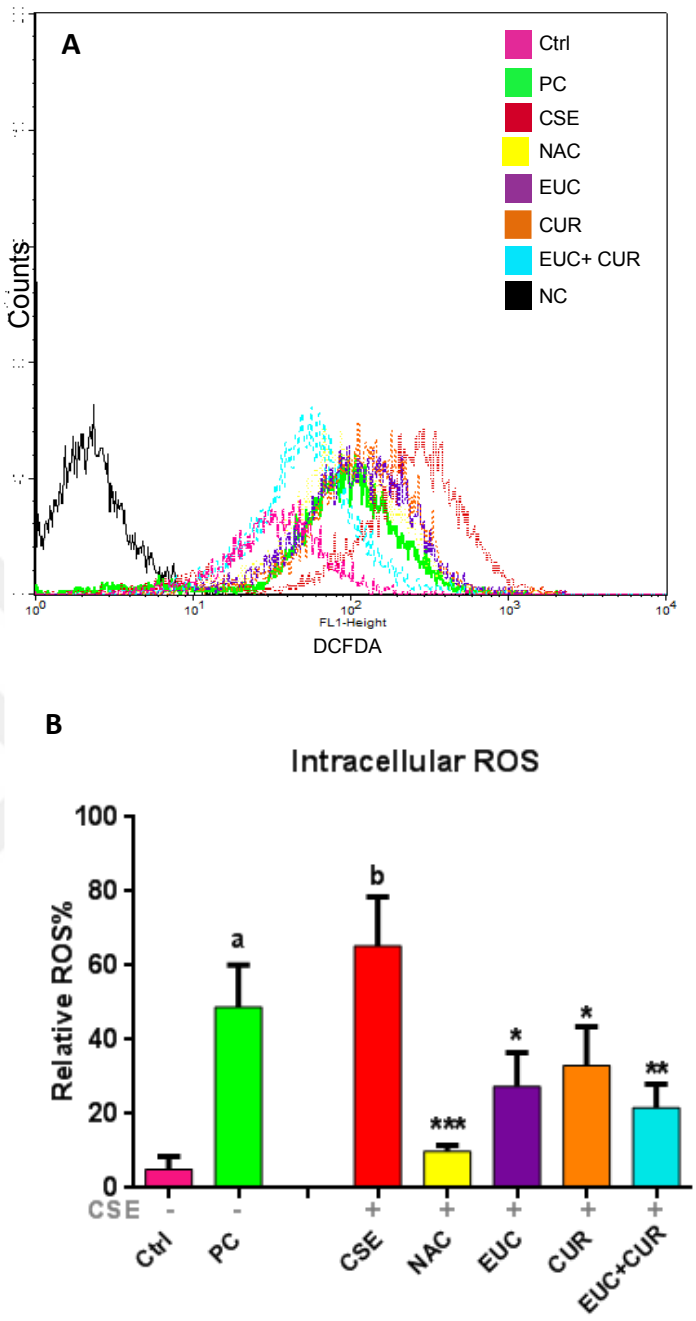



**Figure 31.** Intracellular ROS accumulation in cells exposed to CSE

(A) Enhancement of intracellular ROS level, observed via the shift of the signal curve obtained for the treated cells (presented in different colours) to the right compared with that of the control cells (presented as pink curve). (B) Bar graph presenting the mean relative ROS percentage of three independent experiments. Statistical significance between CSE vs groups $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.0001$; Ctrl vs groups $^{a}p<0.001$; $^{b}p<0.0001$. CSE: cigarette smoke extract; CUR: curcumin (5 µM); EUC: eucalyptol (50 µM); NAC: *N*-acetylcysteine (1 mM); PC: positive control THBP (100 µM). The data are shown as mean ± SD.

### 4.5. Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP, $\Delta\Psi_M$) measured by confocal imaging with Rhodamine 123 dye showed that CSE exposure 20% (v/v) did not lead to a significant change in MMP. FCCP, a mitochondrial oxidative phosphorylation uncoupling agent, led to a decline in MMP according to qualitative images. Figure 32 shows the mitochondrial membrane potential of groups dyed with Rhodamine 123.

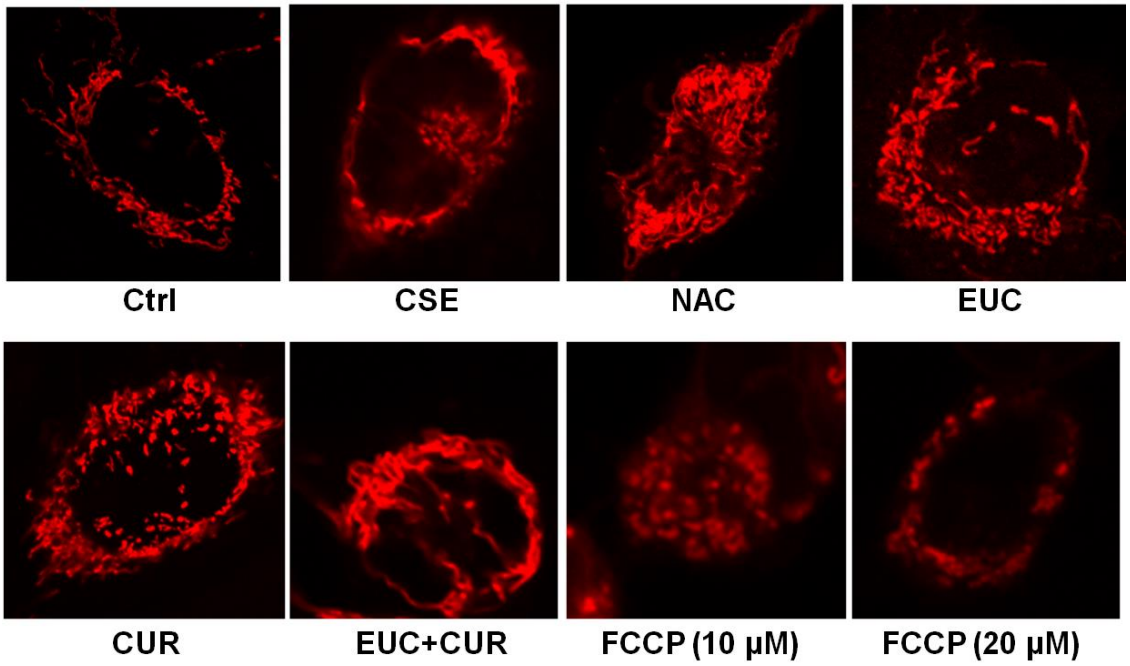



**Figure 32.** Representative images of mitochondrial membrane dyed with Rhodamine 123 (63x)

Ctrl: Control; CSE: Cigarette smoke extract; CUR: Curcumin; EUC: Eucalyptol; FCCP: Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (PC); NAC: N-acetylcysteine, E+C: Eucalyptol and curcumin

### 4.6. Evaluation of Inflammatory Response

#### 4.6.1. Nitrite Level

CSE was significantly induced nitrite production on BEAS-2B cell line compared to non-treated medium control group. According to results, 2 h pre-treatment with NAC has showed a lack of reducing effect on nitrite level. However, EUC and CUR have showed a slight reducing effect on nitrite level at experimental doses, which were statistically insignificant. However, their combination attenuated CSE induced nitrite

production significantly. CSE induced nitrite level and effect of pre-treatments were shown in Figure 33 below.

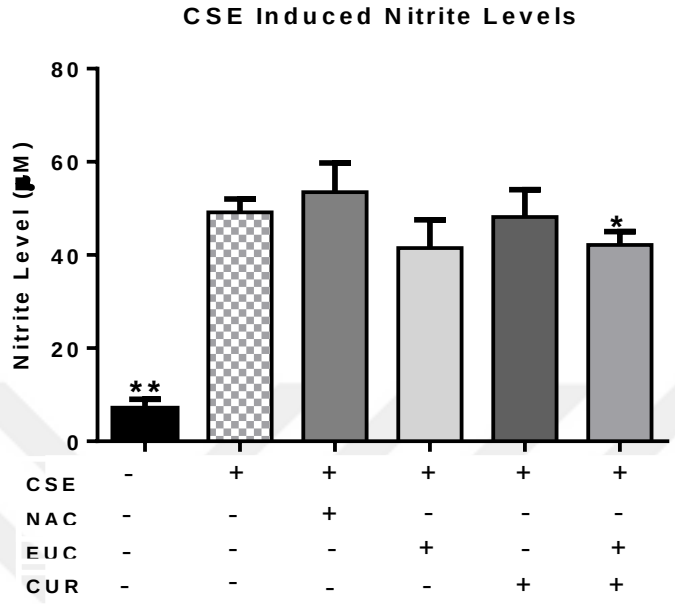



**Figure 33.** CSE induced nitrite levels and effect of pre-treatments

Statistical significance between CSE vs groups *p<0.05; **p<0.0001. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: N-acetylcysteine. The data are shown as mean ± SD.

### 4.6.2. IL-6 Level

According to results, 20% CSE exposure to BEAS-2B cells has increased IL-6 level compared to non-treated control group, significantly. Similar to the result of other inflammatory marker nitrite level, IL-6 level was not changed significantly by NAC pre-treatment compared to CSE group. However, combination treatment has led to an important decline in CSE induced IL-6 level. EUC and CUR pre-treatment have also alleviated increased IL-6 level. In Figure 34, CSE induced IL-6 level was shown.

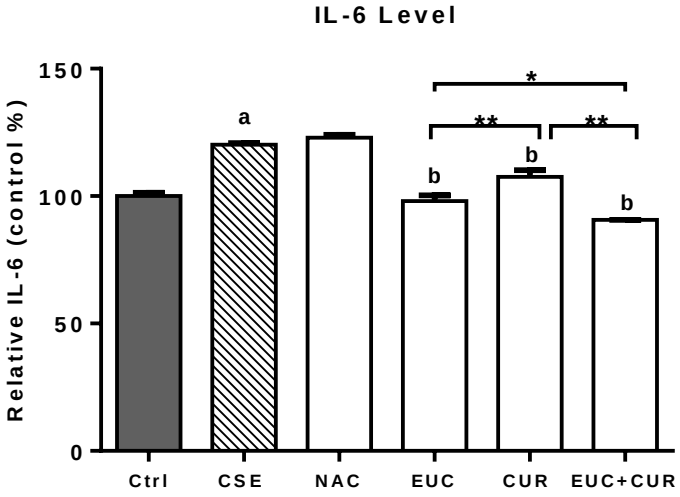



**Figure 34**. Relative IL-6 level of CSE exposed BEAS-2B cell line

a: Statistical difference between Ctrl vs CSE $p<0.0001$; b: CSE vs groups $p<0.0001$. Differences between two groups: *$p<0.001$ and **$p<0.0001$. CSE: cigarette smoke extract; CUR: curcumin; EUC: eucalyptol; NAC: *N*-acetylcysteine. The data are shown as mean ± SD.

# 5 DISCUSSION AND CONCLUSION

Cigarette smoking is considered as one of the most important contributing factor for chronic disease progression and a public health problem (295). According to latest report of Organization for Economic Cooperation and Development (OECD), daily smoking rate of Turkey has been ranked fourth among all countries while it has the third highest smoking ratio among OECD countries (296). Also, public area where smoking has taken place may have toxicological impact on both human health and environment (297). Therefore, cigarette smoking poses an important environmental hazard as well as public health concern, especially in our country.

It is widely known that, cigarette smoke contains many toxic substances which are carcinogenic, mutagenic and main cause for diseases related with oxidative injury (14,68,298). Moreover, chronic inflammation and cigarette smoke induced oxidative stress act as major drivers for lung pathogenesis (109,298). Therefore, bronchial epithelia is reported to be susceptible to cigarette smoke exposure as a primary target. In this study, it was aimed to identify whether EUC, CUR and their combination act as ameliorative on oxidative and inflammatory response induced by CSE or not in BEAS-2B toxicity *in vitro*. For this purpose, mitochondrial dysfunction, apoptosis/necrosis and autophagy dependent-cell death, oxidative stress parameters and Nrf2 expression and as an indicator of inflammatory response, IL-6 release as well as CSE-induced nitrite level has been evaluated *in vitro* in BEAS-2B cell line.

In present study, for the demonstration of CSE induced bronchial epithelial damage, several doses of CSE has been applied. In cell viability results, it was seen that CSE (15-50%, v/v) has induced bronchial epithelial damage in BEAS-2B cell line in a dose dependent manner and following 20% CSE (v/v) has been selected for further studies. On the other hand, lower doses of CSE (1-5%, v/v) led to a slight increase in cell viability surprisingly, which was statistically insignificant. Some studies also found similar results which revealed lower doses of CSE may increase cell viability with MTT (192,295) or neutral red uptake assay (299).

For the assessment of protective activity of EUC, CUR and their combination, highest cell viability observed doses against 20% CSE have been used for all experiments. EUC, has been used as 50 μM for 2 h in this study, enhanced cell viability of BEAS-2B

cells exposed to CSE for 4 h, significantly ($p$<0.05). According to limited *in vitro* data in literature, Lee et al. (2016) suggested that EUC up to 10 µg/ ml (64.82 µM) has showed cytoprotective effect against airway irritant *Der p* in BEAS-2B cell line through MTT assay (249). CUR is a well-known traditional medicine and has been widely used as protective against pulmonary diseases (269,300), thus it is widely studied in literature against pulmonary toxicity. In our study, 5 µM CUR has showed the slightest effect on cell viability ($p$<0.05) among treatments. Previous report by Vanella et al. (2017), it was indicated that 5 µM CUR pre-treatment had no proliferative action on BEAS-2B cells exposed to CSE (34). However, *in vitro* study by Zhu et al. (2014) on BEAS-2B cell line suggested that CUR pre-treatment reversed BaP-induced cytotoxicity up to 5 µM (35). Similar to the results of Zhu and colleagues, increased dose of CUR has led to a prominent decline in cell viability in the present study. Also, recent finding by Li et al. (2019) indicated that 2.5- 7.5 µM CUR have been found to ameliorate CSE-induced cytotoxicity in BEAS-2B cell line (301). Similarly, Zhu et al. (2019) indicated that CUR treatment up to 5 µM for 24 h is non-cytotoxic to BEAS-2B cells (302), which was supportive to our observations as well. Combination of selected doses of EUC and CUR has also showed protective effect on cytotoxicity induced by CSE in BEAS-2B cells. However, that increase was not statistically significant compared to CSE alone and no summative effect was seen with EUC+ CUR in the present study. Also, positive control NAC (1 mM), a potent radical scavenger and mucolytic agent, showed the most promising effect on cell viability against CSE ($p$<0.01). Since it is used as a positive control in the present study, a moderate dose according to literature and our MTT results, 1 mM NAC was used in the present study with the highest cell viability. Previous *in vitro* studies indicated that NAC is protective against CSE or inhaled toxicant induced cytotoxicity in BEAS-2B cell line up to 10 mM (28,34,276). Therefore, it can be suggested that NAC has a broad therapeutic range in BEAS-2B cells, and suitable as a positive control in pulmonary toxicity studies.

Aside from mitochondrial dysfunction, other cell death pathways such as apoptosis, necrosis and autophagy involved in CSE induced toxicity were also investigated in the present study. Dose, content and time of exposure of CSE might differentiate type of cell death in target tissue (181,303). In this study, CSE has led to an increase in apoptotic cell rate significantly compared to control according to Annexin V-PI staining ($p$<0.0001). According to previous findings, CSE induced apoptosis is related with activation of cleaved caspase-9 and caspase 8 (169). Also, Kwak et al. suggested

that CSE exposure increases BEAS-2B apoptotic cell death in a time and dose- dependent manner through anti-apoptotoic Bcl-xL decline and apoptotic caspase-3 activation (295). However, pre-treatment with EUC, CUR, and combination as well as NAC have attenuated CSE induced apoptotic cell death ratio according to quantification with Annexin V-PI staining. Effect of EUC on apoptotic cell death has been discussed in previous studies on antitumor effects. Previously, EUC (5-10 μM) has been suggested to induce apoptosis in two human leukemia cell lines, Molt 4B and HL-60 through increased nuclear DNA fragmentation and morphological examination. However, these apoptotic changes were not observed with KATO-III human stomach cancer cell line (304). According to Bhowal et al. (2017), EUC has led to a specific induction of apoptotic cell death in HCT116 and RKO human colon cancer cell lines through cleaved PARP and caspase-3 (305). Only available information about apoptotic effect on EUC on healthy cell was reported by Hibasami et al. (2002), which indicated that up to 15 μM EUC did not induce apoptotic cell death in human lypmphocyte cells isolated from healthy subjects (304). In the present study, 50 μM EUC pre-treatment significantly reduced CSE induced apoptotic cell ratio in BEAS-2B cell line. However, caspase- dependent pathway and other hallmarks of apoptosis should be elucidated to enlighten its mechanism. On the other hand, CUR, has also reduced apoptotic cell ratio induced by CSE exposure in BEAS-2B cells. However, it was observed that CUR enhanced apoptosis in a dose-dependent manner in the present study. Similarly, CUR has showed a dose-dependent increase in apoptotic cell rate in several healthy cell lines *in vitro* according to previous findings, which were attributed to its ability to boost ROS production in a dose-dependent manner (306,307). Protective role of CUR on apoptosis was held in a study by Zhu et al. (2014), which suggested that CUR attenuated BaP induced- apoptosis in BEAS-2B cell line through p53 and PARP-1 dependent pathways. In the study of Zhu and colleagues, it was also indicated that low dose (5 μM) CUR acted as cytoprotective while higher doses acted as pleiotropic and led to apoptosis induction in human bronchial epithelial cell (35), which correlates the findings of present study. Therefore, it might be suggested as CUR has dual effects on cell survival, depending on dose. In this study, combination of EUC and CUR has also attenuated apoptotic cell ratio compared to CSE group ($p<0.0001$). However, this alleviation with combination pre-treatment was statistically not different with the apoptotic rate observed with EUC treatment alone, thus no summative effect has been identified. On the other hand, NAC led to the most prominent decline in CSE

induced apoptosis in BEAS-2B, which was also correlated with cell viability results. Previous finding on NAC has been suggested that NAC attenuates ROS mediated-apoptotic cell death in BEAS-2B cells by declining nuclear DNA fragmentation. Moreover, it was indicated that higher radical scavenging capacity of NAC, mainly $H_2O_2$ and $O_2^{\bullet-}$ anion, was involved in anti-apoptotic action of NAC in BEAS-2B cells against wood dust (28). Recent data on CSE –induced BEAS-2B toxicity indicated that 5 mM NAC pre-treatment for 30 min is effective against CSE-induced apoptosis through ASK-1/p38 MAPK signaling pathway, which is induced in case of oxidative stress (181). Therefore, it might be suggested that preventive action of NAC against ROS production and consequent oxidative injury are involved in attenuating action of NAC on apoptotic cell death. To summarize, parameters such as caspase- 3 and procaspase- 8 involved in extrinsic apoptosis or effect on p53 tumor supressor function should be identified to elucidate the mechanism of CSE induced apoptosis and protective effects of subjected treatments for further.

Another investigated cell death mechanism is autophagy involved CSE induced toxicity in the present study. This lysosome dependent self-degradation of cell is initiated by autophagosome formation (164). According to reports on COPD and cigarette smoking associated diseases, it was found that LC3B has a pivotal role in disease progression and underlying mechanism (169). During autophagy-dependent cell death, cytoplasmic free form LC3B-I is converted to autophagosome formation indicator LC3B-II in mammalian system (170). In the present study, autophagy rate (LC3B-II/ LC3B-I) through autophagosome formation was significantly increased with CSE exposure in parallel with increased apoptosis rate. Similar to our finding, Chen et al. (2016) reported that CSE may lead to apoptosis in parallel with autophagy dependent cell death through LC3B expression (168). Findings on the association of autophagy and apoptosis suggested that autophagy protein LC3B is involved in up-regulation of apoptosis by interacting with Fas protein. Also, autophagy inhibition by LC3B knockdown protected BEAS-2B against CSE induced apoptosis, significantly (169). Therefore, observed apoptosis induction seen with CSE exposure might be in relation with LC3B- related pathway, Fas protein is needed to be evaluated, though. In present study, pre-treatment with EUC reduced CSE induced autophagy rate significantly by diminishing CSE induced LC3B-II/ LC3B-I ratio ($p<0.05$). In literature, Pereira (2018) reported that EUC (600 µg/ ml) induces LC3B-II accumulation in MCEC-I mouse endothelial cell line, thus

increased autophagy rate (308). Since there is no other available data on EUC- autophagy relationship in literature, it might be suggested that this is the first report indicating diminishing effect of EUC on CSE induced autophagy rate through LC3B-II/ LC3B-I rate in BEAS-2B cells. On the other hand, CUR pre-treatment has led to a slight decrease in this autophagy hallmark, which was insignificant compared to CSE group. Previous findings mainly focused on anticancer effect of CUR through autophagy induction, thus it was indicated that high doses of CUR (50-100 μM) act as autophagy inducer on A549 human lung adenocarcinoma (47,309) through various pathways. However, literature data on CUR-mediated autophagy response in pulmonary system as a protective mechanism is not sufficient to interpret remaining result. In parallel to results of apoptotic cell death, combination treatment significantly reduced CSE- mediated autophagy through LC3B expression. However, combination treatment did not differ significanlty as observed with EUC alone, interpreted as no summative effect CSE induced autophagy dependent-cell death. Positive control NAC has showed the most prominent effect by reducing autophagosome formation compared to CSE group ($p<0.01$). Overall, it might be suggested that not mitochondrial dysfunction, but also apoptosis and autophagy-dependent cell death are involved in CSE-induced toxicity in BEAS-2B. Moreover, CSE-mediated apoptotis and autophagy might be target adverse outcome and these two pathway are in relation. Previous data has also indicated that autophagy is involved in smoking related COPD pathogenesis and altering cell senescence. Furthermore, accumulation of p62, another important regulatory protein involved in autophagy process has reported to be increased in healthy bronchial epithelial cells (HBEC). However, CSE exposure to isolated HBEC from smoker COPD patients showed impaired autophagy functions through LC3B, p62 and Atg5 expression compared to autophagy response seen in healthy HBEC cells, and promoting cell senescence (310). Therefore, cell death mechanims, especially autophagy, plays an important role in COPD progression and other smoking related pulmonary pathogenesis (29,169). Targeting of autophagy pathways and their relation with extrinsic apoptotic factors such as Fas might represent a potential therapeutic and preventive strategy for the management of smoking induced pulmonary diseases.

Since great majority of cigarette smoke toxicity is attributed to oxidative injury and lipid preoxidation (181,198,311), modulatory effect of EUC and CUR was investigated through enzymatic antioxidant activity, non-enzymatic antioxidant level,

MDA level, Nrf2 pathway as well as intracellular ROS production in the present study. Cigarette smoke induced damage of human lung epithelia cells is associated with increased oxidative stress and Nrf2 related pathways. Previous studies suggested that, CSE is a well-defined ROS and free radical source, and exposure to CSE reduces antioxidant enzyme activity and increases MDA level in human bronchial epithelia as a sign of lipid peroxidation *in vitro* (30,312).

In this study, 4 h CSE exposure to BEAS-2B significantly reduced CAT activity ($p<0.001$), intracellular GSH level ($p<0.001$) and up-regulated MDA level ($p<0.05$) compared to control. However, in contrast to *in vitro* findings in literature (30,312), SOD enzymatic activity was slightly decreased in the present study, which might be due to low relative $O_2^{\bullet-}$ content of CSE or unstability of $O_2^{\bullet-}$. Previously, $O_2^{\bullet-}$ anion was indicated as an unstable ROS present in gas phase of CSE (313). Therefore, observed result is suggested to be due to unstable nature of $O_2^{\bullet-}$ in used CSE.

In our study, pre-treatment with EUC has attenuated CSE-induced oxidative stress through enhancing activity of both SOD and CAT compared to CSE group in BEAS-2B cells *in vitro* ($p<0.01$). Also, intracellular GSH has replenished significantly compared to both CSE and control groups ($p<0.01$). Especially in GSH replenishment, EUC has showed similar results with NAC, therefore it might represent an important source for GSH deposition in biological system. EUC's effect on oxidative stress parameters was insufficient *in vitro*. However, numerous *in vivo* studies have suggested that EUC has antioxidative properties against cigarette smoke-induced lung emphysema in mice (21) as well as paracetamol-induced hepatotoxicity (314) and exercise-induced oxidative stress (315) by up-regulating enzymatic antioxidant activity of SOD, CAT and GPx. In addition to mentioned studies, it has showed a reducing effect on elevated MDA levels induced by cigarette smoke in mice (254) and crowding stress in *Oncorhynchus mykiss* fish *in vivo* (316). Recently, Kennedy-Feitosa et al. (2019) have suggested that 1-10 mg/ml EUC treatment via inhalation acted as antioxidant against cigarette smoke induced pulmonary emphysema model in mice. According to their study, EUC in higher doses acted as redox regulator, up-regulated antioxidant mechanism by increasing SOD activity and led to a decline in MDA level (21). Similar to their findings, Lin et al. (2017) have suggested that Eucalyptus oil showed protective effect against LPS induced COPD model in mice. In their study, pre-treatment with Eucalyptus oil significantly reduced MDA level and up-regulated SOD activity in lung tissue of mice (44). In parallel to *in vivo* findings

on smoke-induced oxidative stress and lipid peoxidation (21,254), EUC also attenuated CSE-induced MDA elevation in BEAS-2B cells *in vitro*. Therefore, it may be suggested that EUC, might be an important therapeutic against cigarette induced bronchial damage owing to anti-oxidative properties.

CUR, has been widely studied substance due to its anti-oxidative properties (261–263). An earlier *in vitro* finding suggested that CUR acts as preventive against oxidative stress by up-regulating SOD, GPx and CAT enzymatic activity between 5- 20 μM while reducing lipid peroxidation by lowering MDA level at moderate doses (5-10 μM) in RAW264.7 murine macrophage cells. However, it was also indicated that higher dose of CUR might increase oxidative injury due to its abilitty to boost ROS production in a dose-dependent manner (306). *In vitro* studies on antioxidant property of CUR has been mainly focused on different pathways instead of direct antioxidant enzyme activity in pulmonary system (34) and limited data is available on CUR's effect on oxidative stress seen in BEAS-2B cell line. In the present study, CUR pre-treatment significantly increased intracellular GSH level and CAT activity compared to CSE group ($p<0.01$). However, no significant effect was seen on SOD activity. Also, lipid peroxidation was diminished with CUR pre-treatment. However, this affect was statistically insignificant. Therefore, it may be suggested that CUR at low dose (5 μM) not acting as potent as EUC on enhancing SOD activity and attenuating lipid peroxidation induced by CSE. *In vivo* findings of CUR on oxidative stress suggested that CUR is able to downegulates various antioxidant systems such as Nrf2, heat shock protein 70, HO-1, and thioredoxin (317). Hence, investigation of these pathways to elucidate antioxidant property of CUR might be important in CSE-induced oxidative stress in BEAS-2B cells.

Combination treatment has showed similar results yielded with EUC treatment alone, by enhancing SOD and CAT activity as well as up-regulating intracellular GSH and reducing MDA level. Combination treatment did not exhibit synergistic effect on quenching oxidative stress mediated by CSE exposure in BEAS-2B cells. However, intracellular GSH level is significantly restored with combination pre-treatment similar to 1 mM NAC. Therefore, EUC and CUR combination might be a potential therapeutic agent on pulmonary diseases drived by oxidative injury.

Among treatments, GSH precursor NAC exhibited the most prominent effect on relieving CSE-induced oxidative stress in BEAS-2B, owing to its confirmed antioxidant

activity in pulmonary system both *in vitro* (34,180,192) and *in vivo* (15,279) by enhancing antioxidant enzymatic activity, replenishing intracellular GSH store and reducing lipid peroxidation.

ROS production is another important contributing factor for COPD pathogenesis. Also, ROS present itself in cigarette smoke is able to induce mitochondrial dysfunction and altering MMP and ATP levels (33). Therefore, intracellular ROS production and consequent alteration in MMP is important for disease pathogenesis. CSE is a well-defined ROS source and it is confirmed that CSE exposure induces intracellular ROS level in pulmonary system (33,181). In this study, intracellular ROS production was significantly increased with 4 h CSE exposure in BEAS-2B cells compared to control. EUC pre-treatment previously has been found as protective against $H_2O_2$ induced ROS production *in vitro*. Porres-Martinez et al. (2015) suggested that essential oils yielded from *Salvia lavandulifolia* showed a potent inhibiton on cellular ROS production in U373-MG human astrocytoma cell line. In their study, EUC and α-pinene were identified as main components of *Salvia lavandulifolia* and it has been indicated that EUC (25 μM) was more potent on inhibiting ROS production up to compared to α-pinene (318). In our study, EUC decreased CSE-induced intracellular ROS formation in BEAS-2B cells by reducing relative ROS from 60% to 38%, approximately. These result is also correlated with anti-oxidative enzyme activity described previously. The other compound CUR has previously been indicated as a potent ROS inducer in KBM-5 chronic myeloid leukemia cell line *in vitro* by Sandur et al. (2007) (319). Similarly, Lee et al. (2011) revealed that CUR act as a potent anti-cancer agent by elevating intracellular ROS level and ROS-dependent autophagy as well as conseguent cell death in HCT-116 human colon cancer line (320). However, due to target and dose-dependent differences, CUR may act as alleviating on intracellular ROS production. In a study on BV2 microglial cells, it was reported that 100 μg/ ml CUR alleviated intracellular ROS production induced by LPS, consequently prevented ROS- mediated neuroinflammation (321). Bargezar et al. (2011) suggested that CUR is an efficient ROS scavenger up to 4 μM in L-6 rat muscle cell line *in vitro*. In the same study, it was emphesized that having two phenolic sites on its structure serves a good capacity for H donation. Also, penetration increase of lipophilic CUR into polar cytoplasm enhances its ROS scavenging capacity (322). Similar to Bargezar and his colleagues findings, Zhang et al. (2018) recently suggested that liposomal formulation of CUR is more potent in reducing ROS and oxidative stress as

well as lipid peroxidation in BEAS-2B cells by altering low water soluble nature of CUR and increasing cellular uptake. Besides, anti-cancer acitivity has been reported to be more potent due to increased cellular uptake, dose-dependent increase in ROS-mediated oxidative injury and consequent cell death up to 100 μM (48). Our results may relate to previous *in vitro* findings with healthy cell lines, and 5 μM CUR pre-treatment for 2 h in BEAS-2B cells decreased intracellular ROS production compared to CSE group ($p$<0.05). However, lowest activity seen with CUR pre-treatment compared to other groups might be due to high lipophilic and low stable nature of CUR, by lowering its cellular uptake and intracellular penetration into polar medium. Therefore, CUR with different formulations increasing its cellular uptake and bioavailibility might exhibit higher bioavailibility compared to free CUR and might reduce ROS more efficiently. On the other hand, a well-known antioxidant, NAC has showed the most promising effect on CSE induced ROS production by decrasing relative ROS level below 20% in BEAS-2B cells in the present study, which is similar to numareous findings on different cell lines (34,323,324) as well as seen with BEAS-2B (276). This effect of NAC has been attributed to its thiol group interaction with ROS as well as being GSH precursor. Furthermore, it was suggested that hydropersulfide formation from NAC-derived thiols may act as direct radical scavenger (275).

In addition to intracellular ROS formation, consequent mitochondrial dysfunction and alteration in MMP is an important pathway for identification of toxicity mechanism of CSE on BEAS-2B cells. Earlier studies have suggested that mitochondrial dysfunction is involved in toxicity mechanism of smoking (325). Therefore, investigation of mitochondrial ROS level, alteration in mitochondrial membrane function, swelling and change in oxidative phosphorylation are needed for further identification of underlying toxicity mechanism. Banzet et al. (1999) revealed that tobacco smoke is involved in mitochondrial depolarization and consequent apoptotic cell death in time and dose-dependent manner. In the same study, NAC showed a significant effect on maintanence of mitochondrial membrane potential, thus alleviating ROS mediated oxidative injury and apoptosis (154). In our study, MMP was not determined quantitatively, thus qualitative images did not meet the criteria for a proper interpretation of CSE induced MMP alteration and consequent mitochondrial dysfunction. Therefore, further quantification is needed for MMP alteration in all groups.

Other important pathway is Nrf2-related antioxidant response in pulmonary system. Nrf2 is relatively abundant in lung tissue due to high antioxidation/ detoxification capacity. Nrf2 pathway is involved in recovery of lung tissue in case of lung inflammation, cigarette smoke exposure or epithelial injury, by boosting cytoprotective and antioxidant genes (326). Cigarette smoke, a well defined airborne oxidant, has been reported to enhance electrophilic attack and activates nuclear translocation of Nrf2 which up-regulates antioxidant defense as a cellular response (10,181). Previous data on Nrf2 pathway and pulmonary system have been indicated that, cigarette smoke is an important activator of Nrf2 as an environmental stress source and targeting of Nrf2 might represent a therapeutical strategy in treatment of chronic airway diseases (218,327). *In vitro* studies with bronchial epithelial cells suggested that pharmacological Nrf2 activators (14) or natural compounds activating Nrf2 pathway (197,311) may be useful for further management of COPD and other oxidant induced airway diseases.

In our study, 4 h CSE exposure significantly induced Nrf2 nuclear translocation in human bronchial epithelial cells as a cellular response to increased oxidative stimuli. On the other hand, pre-treatment with EUC, CUR and their combination followed by CSE exposure increased nuclear expression of Nrf2. However, these pre-treatments were acted as Nrf2 activators without CSE exposure. Therefore, it is suggested that EUC, CUR and their combination attenuated CSE induced oxidative injury through Nrf2 activation and possibly augmented ARE related genes mediated by Nrf2 pathway. Previous findings on EUC have different conclusions on Nrf2 activation. Porres-Martinez et al. (2018) suggested that EUC isolated from *Salvia lavandulifolia* essential oil acted as Nrf2 activator and regulated cellular redox balance in $H_2O_2$-induced oxidative injury in U373-MG astrocytes *in vitro* (318). Fewer studies have been focused on therapeutic mechanism of EUC through Nrf2 pathway both *in vitro* and *in vivo*. A study by Kennedy-Feitosa et al. (2019) suggested that EUC promoted lung repair in cigarette smoke-induced emphysema in mice. However, Nrf2 activation by EUC was interpreted as conclusive due to lack of protein transcription. Therefore, protective effect of EUC was attributed to its anti-inflammatory activity via NF-κB reduction and potential ROS scavenger activity (21). On the other hand, recent study by Jiang et al. (2019) has revealed that EUC isolated from essential oil of *Artemisia vulgaris* L. showed protective effect on paracetamol-induced liver damage by activating Nrf2 nuclear translocation. In addition, EUC has been found to act as Keap-1 inhibitor, thus led to a decline in cytoplasmic accumulation of

Nrf2 (314). Similar to *in vitro* findings, EUC exhibited a significant Nrf2 activation against CSE exposure in the present study. Our results showed that Nrf2 activation was significantly increased with EUC, which might promote cell survival in CSE exposed BEAS-2B. However, there was no opportunity to measure Keap-1 expression in this study. Therefore, EUC induced nuclear translocation of Nrf2 might be attributed to its Keap-1 inhibitory action within the light of previous studies. EUC might be useful in pulmonary diseases by Nrf2 mediated antioxidant pathway. However, further *in vivo* and clinical findings are needed. Also CUR, powerful Nrf2 activator according to literature, defined as a promising therapeutic agent for management of cigarette smoke induced COPD and other pulmonary diseases (220,328). Previous study by Suzuki et al. (2009) suggested that CUR showed ameliorative effect on mouse alveolar macrophage cells exposed to CSE by up-regulating antioxidant gene expression mediated by Nrf2 pathway in a dose-dependent manner *in vitro* (25). Similar to Suzuki and his colleagues study, it was reported that moderate dose of CUR (10 μM) was found reduce oxidative stress induced by $H_2O_2$ in murine macrophages by enhancing antioxidant enzyme activity *via* Nrf2 activation (306). However, in a study on lung carcinogenesis, CUR was described as weak Nrf2 inducer with low bioavailibility and bis[2-hydroxybenzylidene]acetone derivative of CUR has been reported to have a potent activity on Nrf2 nuclear translocation. Also, it was reported that this derivative of CUR had shown protective effect on $As^{3+}$ induced carcinogenesis on human lung epithelia through Nrf2 activation while CUR was failed to exhibit any effect both *in vivo* in mice and *in vitro* (329). In the present study, CUR led to slight Nrf2 activation in BEAS-2B cells compared to CSE exposed group, which was statistically insignificant. On the other hand, combination of EUC and CUR showed the most promising effect on Nrf2 activation compared to CSE group in present study. Therefore, EUC and CUR combination might be a potential therapeutic agent on pulmonary diseases drived by oxidative injury. However, Nrf2-dependent antioxidant gene expression such as HO-1, glutamate-cysteine ligase and NAD(P)H-quinone oxidoreductase-1 should better be investigated by further studies to elucidate the adaptive response driven by EUC and CUR. On the other hand, pre-treatment with NAC, significantly reduced CSE mediated Nrf2 activation. Therefore, it is suggested that CSE induced Nrf2 activation is up-regulated due to excessive ROS generation in the present study. This effect of NAC has been previously attributed to direct radical scavenger capacity as well as improving activity of antioxidant enzymes such as SOD and CAT (330). Similarly, NAC treatment has been found to interfere with

nuclear translocation of Nrf2 by Li et al. (2004). According to their study, cysteine residues on Keap-1, main inhibitor of Nrf2, might interefere with ubiquination of Nrf2 and thus, proteosomal degradation (331). Since NAC has cysteine in its structure, cysteine and Keap-1 interaction might be another reason for inactivation of Nrf2 by NAC pre-treatment in the present study. In addition, according to previous findings, Nrf2 is involved in up-regulation of antiapoptotic proteins Bcl-2 and Bcl-xL, thus preventing apoptotic cell death (219). Therefore, Nrf2 activation by EUC and CUR might be involved in attenuation of CSE induced apoptosis. For further evidence, expression of Bcl-2 and Bcl-xL are needed to be investigated, though.

Chronic inflammation is another well defined and contributing factor for progression of cigarette smoke induced pulmonary diseases according to numerous studies (38,55,102). Therefore, in the present study, several inflammatory markers induced by CSE has been investigated. NO is one of the most important oxidant present in cigarette smoke and also involved in the inflammatory response against smoke exposure (332). Also, pro-inflammatory cytokine IL-6, is defined as an important marker in COPD pathophysiology (50), was evaluated to determine CSE induced inflammation in BEAS-2B cells.

Presence of NO in the present study was measured indirectly through its breakdown product, nitrite (333). According to the results, CSE increased nitrite level significantly in bronchial epithelial cells as an inflammatory response. EUC has been identified as an important anti-inflmmatory agent, especially on pulmonary system (21,249,255). Previous study by Yadav et al. (2017) suggested that Eucalyptus oil and its major constituent EUC ameliorated LPS induced inflammation on murine alveolar macrophage cell line by decreasing NO an pro-inflammatory cytokine release *in vitro* (334). Also, previous reports have indicated that EUC reduced NO in human monocytes stimulated by LPS (335). In the present study, EUC decreased nitrite level in BEAS-2B cells compared to CSE group. However, observed decline was statistically insignificant. On the other hand CUR, has been defined as an important anti-inflammatory agent and used traditionally in respiratory diseases as well as chronic inflammatory diseases (265,269). A finding by Zhao et al. (2015) suggested that CUR and its metabolites are able to reduce LPS-stimulated nitrite release in RAW264.7 murine macrophage cells (336). Similarly, CUR has alleviated lipoteichoic acid induced-neuroinflammation in BV-2 microglial cells *in vitro* (337). In our study, CUR slightly reduced CSE induced nitrite

level in BEAS-2B cells. However, that was statistically insignificant. *In vivo* findings indicated that CUR reduced LPS-induced inflammation and NO level in mice (338). In addition, CUR is reported to be a potent anti-inflammatory agent in acute lung injury and decrease NO level in mice, significantly (339). However, our results do not reflect *in vivo* results on respiratory system. On the other hand, combination treatment of EUC and CUR diminished nitrite level induced by CSE exposure in BEAS-2B cells, which might be suggested that combination acting as more potent on NO mediated inflammatory response induced by CSE. However, other pathways involved in anti-inflammatory mechanism should be taken into consideration.

IL-6, an important pro-inflammatory cytokine involved in inflammatory lung disease pathogenesis was also induced by 4h CSE exposure in our study. Similar to our findings, studies with BEAS-2B cell line indicated that CSE exposure sigificantly up-regulates IL-6 level (30,340). *In vivo* and clinical studies also indicated that cigarette smoke exposure, asthma and lung infections increases IL-6 release as an inflammatory response (50,341). Also, Nrf2 pathway is involved inflammatory response, these results may be associated with Nrf2 mediated inflammatory response induced by CSE. According to Vomund et al.(2017), Nrf2 is an important mediator for inflammation by inhibiting iNOS (219) as well as release of pro-inflammatory cytokines such as TNF-α and IL-6 (219). Therefore, it is suggested that Nrf2 activation seen with combination pre-treatment might be involved in the attenuation of CSE induced inflammation in the present study. Previously, EUC has been reported to diminish IL-6 level in murine alveolar macrophage cells stimulated by LPS *in vitro* (334). Also, it has showed dose-dependent induction in LPS-induced IL-6 release in human monocytes (42). In our study, EUC exhibited a reduction in IL-6 level in BEAS-2B cells compared to CSE group ($p$<0.0001). *In vivo* findings also suggested that EUC acted as a potent anti-inflammatory agent through inhibition of NF-κB activation and reversing IL-6 release in pulmonary tissue (21). Since Nrf2 activation is involved in IL-6 release by up-regulating anti-inflammatory gene expression and mediating pro-inflammatory cytokine release (219), EUC induced Nrf2 activation might be also involved in observed reduction of IL-6 level in this study. Also, it was suggested that CUR alleviated inflammatory response in airways by reversing TNF-α, IL-1 and IL-6 release (39,257,270,342) or modulating NF-κB pathway (301) in several studies. In our study, CUR decreased IL-6 level but exhibited less potent profile on reduction of CSE induced inflammatory response through IL-6

release among EUC and combination treatments. Similar to nitrite release, combination treatment exhibited the most promising effect on reduction of CSE-induced IL-6 release. Therefore, combination treatment showed summative effect on inflammation parameters and acted as an efficient anti-inflammatory agent compared to EUC and CUR alone on CSE-induced BEAS-2B inflammatory response. Also, CSE induced autophagy has been associated with increased pro-inflammatory cytokine release according to recent data of Xu et al. (2019). In the study of Xu and colleaugues, inhibition of autophagy through MAPK and PI3K/Akt/mTOR pathway and LC3B-II significantly reduced IL-1β, IL-6 and IL-8 release. Consequently, it was suggested that CSE- induced inflammation is mediated through autophagy (2). In the light of mentioned study, in addition to antioxidative effect of EUC and CUR, regulation of pro-inflammatory cytokine release induced by CSE might be involved in their mechanism of action in previously discussed down-regulation of CSE induced-autophagy. Therefore, future studies focused on MAPK pathway-mediated inflammation, inflammatory cytokine release and related autophagy protein expressions altered by EUC and CUR might be beneficial for further elucidation. Lastly, NAC pre-treatment exhibited no effect on reduction of inflammatory markersin the present study. Previously, it was suggested that high dose NAC (150 mg/ kg) administration alleviated symptoms seen in acute lung injury model in mice by reducing IL-6 level (343). Also, NAC has been suggested as potent reducer of NF-κB activation in airways (278) and effective on IL-8 inhibition in bronchial epithelia *in vitro* (280). However, similar to our findings, James et al. (2003) suggested that NAC treatment (300 mg/kg) was not found effective on APAP induced liver nitrite, nitrate, IL-1β and IL-6 level (344). These results seen with NAC are suggested as pre-treatment with 1mM NAC for 2 hours may not be sufficient to exhibit any effect on inflammatory markers or might be related with its weak Nrf2 activation observed in BEAS-2B cells in this study.

In Figure 35, suggested mechanism of action of EUC, CUR, EUC+CUR, and NAC against CSE induced toxicity in BEAS-2B cells have been depicted within the light of observed results.

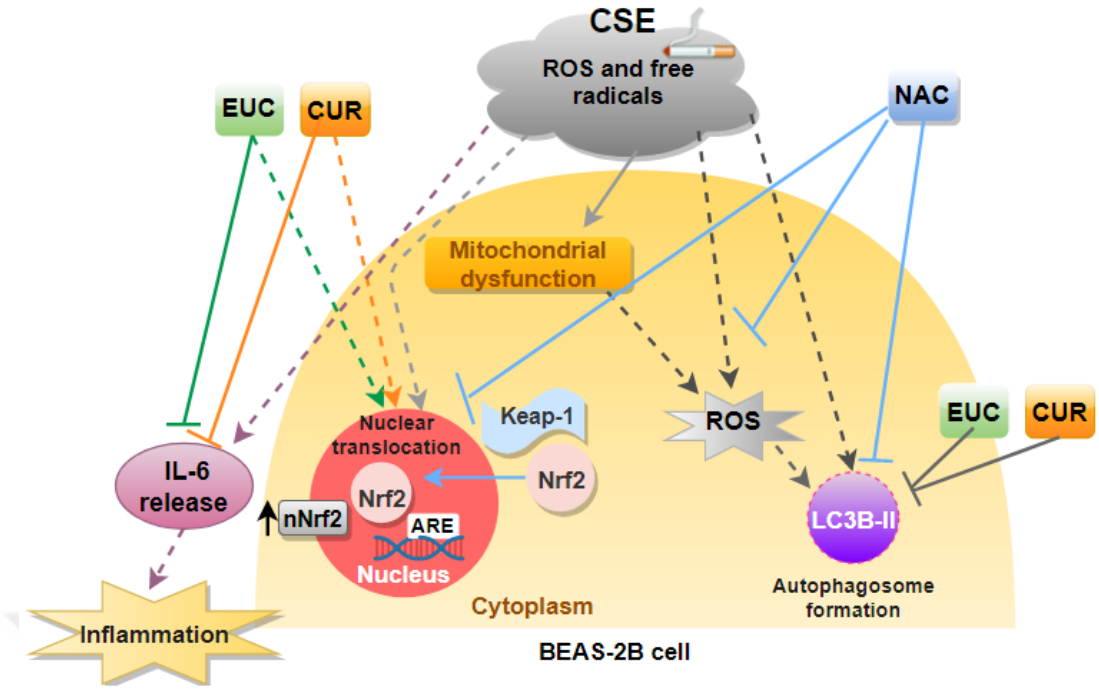



**Figure 35.** Effect of substances on CSE induced toxicity in BEAS-2B cells.

ARE: Antioxidant response element, CUR: Curcumin, EUC: Eucalyptol; IL-6: Interleukin 6; Keap-1: Kelch-like ECH-associated protein 1; LC3B-II: Microtubule-associated protein light chain 3-B Nrf2: Nuclear factor erythroid-2 related factor 2; ROS: Reactive oxygen species; NAC: *N*-acetylcysteine.

In conclusion, this study demonstrates that EUC, CUR and their combination resisted CSE induced oxidative stress and inflammation by enhancing antioxidant enzyme activity and activating Nrf2 pathway, which might promote bronchial epithelial cell survival. Therefore, owing to its regulatory effect on cytoprotection, inflammation and oxdative injury, Nrf2-ARE pathway might represent an important tool for interpretation of pulmonary toxicity, as well as therapeutic target for cigarette smoke induced lung pathogenesis. However, Nrf2-driven cyoprotective gene expression should be further elucidated. Also, further studies on cigarette smoke induced inflammatory response in relation with cell death are needed for mechanistic targetting of therapeutics. Besides, effect of these therapeutics should be enlighten in case of chronic CSE exposure. Results obtained with combination of EUC and CUR might represent a valuable background for further *in vivo* studies. However, there are still need for *in vitro* chronic inhalation exposure methods demonstrating lifetime exposure. Also, aside from tobacco cigarette, there are still restricted information about long-term health risks of "less harmful cigarettes" and new generation cigarettes as well as their third-hand smoke embedded on

environment. Therefore, detailed studies are needed for both public health and within the concept of environmental risk assessment.

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factor-κB as potential target. *Mol Med Rep*. 2015;**11**(4):3087-3093.

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# 7 CURRICULUM VITAE

**Kişisel Bilgiler**

| | | | |
|---|---|---|---|
| **Name** | Rengin | **Surname** | REİS |
| **Doğum Yeri** | Trabzon | **Doğum Tarihi** | 08.10.1991 |
| **Nationality** | TC | **ID No** | 44821901740 |
| **E-mail** | renginreis@gmail.com | **Tel** | +905354010922 |

**Education**

| **Level** | **Proficiency** | **Institution** | **Graduation Year** |
|---|---|---|---|
| **Ph.D.** | Toxicology | Yeditepe University | 2020 |
| **B.A.** | Faculty of Pharmacy | Yeditepe University | 2015 |
| **High School** | - | Kanuni Anatolian High School | 2009 |

| **Language Skill** | **Exam Degree** |
|---|---|
| English | YÖKDİL: 82.5 (03.11.2019) |
| Korean | TOPIK-I: 94 (19.10.2019) |

**Job experience**

| **Görevi** | **Kurum** | **Süre (Yıl - Yıl)** |
|---|---|---|
| Research and Teaching Assistant | KTU Faculty of Pharmacy | 2016-2020 |

**Computer skills**

| Program | Skill |
|---|---|
| Microsoft Word | Very good |
| Microsoft Excel | Very good |
| GraphPad Prism 6.0 | Good |
| Mendeley | Good |

**Scientific Works**

**Publications in SCI, SSCI, AHCI Indexed Journals**

| |
|---|
| Sipahi H., **Reis R**., Dinç Ö., Kavaz T. , Dimoglo A., Aydin A., "In vitro biocompatibility study approaches to evaluate the safety profile of electrolyzed water for skin and eye", Human & Experimental Toxicology,38:1314-1326,2019. |
| Kırmızıbekmez H., İnan Y. , **Reis R**., Sipahi H., Gönen A.C., Yeşilada E., "Phenolic compounds from the aerial parts of Clematis viticella L. and their in vitro anti-inflammatory activities", Natural Product Research, 33(17):2541-2544,2019. |
| Bayram F., **Reis R**., Tuncer B., Sipahi H., "The İmportance of the Structure Similarity of Drugs Used for Depression And Inflammation, Two Comorbid Diseases", Current Topics in Medicinal Chemistry, 2018 |
| **Reis R**., Charehsaz M. , Sipahi H., Doğan Ekici A.I., Macit Ç., Akkaya H., et al., "Energy Drink Induced Lipid Peroxidation and Oxidative Damage in Rat Liver and Brain When Used Alone or Combined with Alcohol", Journal of Food Science, 82(4);1037-1043, 2017 |
| Charehsaz M., Coşkun Ş., Ünalan H.E., **Reis R**., Helvacioglu S., Kumar Giri A. , et al., "Genotoxicity Study of High Aspect Ratio Silver Nanowires", Toxicological & Environmental Chemistry, 99(5-6): 837-847,2017. |

**Diğer dergilerde yayınlanan makaleler**

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| Şenyüz C.Ş., **Reis R.,** Sipahi H., "Drugs Used in the Treatment of Obesity Since Decades and Their Adverse Effects", J Lit Pharm Sci, 8:.61-75, 2019 |
| Doğan İ.S., Sellitepe H.E., Kayıkçı N., Sipahi H., **Reis R**., Yayli N., "Synthesis and anticancer (MCF-7, PC-3) activities of new 2-hydroxy-2,2-bis(4-substitutedphenyl)-N '-[(1E)-(3/4- substitutedphenyl)methylene]-acetohydrazides", Organıc Communıcatıons, 11:142-148, 2018 |
| **Reis R**., Sipahi H., "İnflamasyon ve Depresyon", Hacettepe University Journal of the Faculty of Pharmacy, 1:52-62, 2017 |
| **Reis R.**, Sipahi H., Aydın A., "Possible Haematological Abnormalities Induced by Herbal Tea Consumption: A Review ", J Blood Disord Transfus, 7:1-5, 2016 |

**International Conference Proceedings**

| |
|---|
| **Reis R.**, Orak D., Çimen H., Sipahi H., "Eucalptol and Curcumin Protects Against Cigarette Smoke Extract Induced Bronchial Epithelial Damage Through Nrf2 Pathway", 6th Pulmonary Drug Delivery Workshop, İstanbul, TÜRKIYE, 11-13 Eylül 2019 (Oral presentation) |
| **Reis R**., Sipahi H., Kavaz T. , Türkay Ö., Özçelik H., Aydın A., "In Vitro Approaches To Evaluate The Irritation Potential Of Electrolyzed Water For Skin And Eye: A Biocompability Study", 12th International Symposium on Pharmaceutical Sciences (ISOPS), Ankara, TÜRKIYE, 26-29 Haziran 2018, no.121, pp.103-103 (Oral presentation) |
| **Reis R**., Sipahi H., Kavaz T. , Türkay Ö., Aydın A., "Electro-activated Water as a Wound Healing Agent In vitro", Society of Toxicology 57th Annual Meeting, San Antonio, ABD, 11-15 Mart 2018, vol.162, no.1, pp.505-505 |
| **Reis R**., Sipahi H., Zeybekoğlu G., Çelik N., Kırmızıbekmez H., Aydın A., "Hydroxytyrosol: the main responsible for the anti-inflammatory activity of olive pits is revealed", 37th International Winter-Workshop on Clinical, Chemical and Biochemical Aspects of Pteridines and Related Topics, Innsbruck, AVUSTURYA, 20-23 Şubat 2018, vol.29, no.1(Oral presentation) |

**Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar**

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|---|
| Doğan İ.S., Gümüş M., Orak D., Reis R., Sipahi H., "Microwave-assisted Modified Biginelli Synthesis of 5-methyl-11,12-dihydro-5,11-methano-[1,2,4]triazolo[1,5-c][1,3,5]benzoxadiazocine Derivatives as Potential Anti-inflammatory Activities.", International Multidisciplinary Symposium on Drug Research and Development (DRD2019), Malatya, TÜRKIYE, 1-3 Temmuz 2019, vol.1, no.1, pp.10-14 |
| Hamitoğlu M., Coşkun Ş., Reis R., Helvacıoğlu S., Ünalan H.E., Aydın A., "Toxicological Evaluation of High Aspect Ratio Silver Nanowires ", 12th International Nanoscience and Nanotechnology Conference, Kocaeli, TÜRKIYE, 3-5 Haziran 2016, pp.313-313 |
| Hamitoğlu M. , Reis R., Helvacıoğlu S., Sipahi H., Çiçek G., Güzelmeriç E., et al., "Styrax Liquidus Üzerinde Toksikolojik Araştırmalar: Güncel Bulgular", XXII. Bitkisel İlaç Hammadeleri Toplantısı, Trabzon, TÜRKIYE, 31 Ağustos - 5 Eylül 2016, ss.74-74 |
| Akyüz G.S., Celep M.E., Hamitoğlu M. , Helvacıoğlu S., Reis R., Yeşilada E., "Cornus mas L. Meyve Çekirdeklerinin Antioksidan, Antimutajenik Aktiviteleri ve Oksidatif Hasara Karşı Koruyucu Etkisi ", XXII. Bitkisel İlaç Hammadeleri Toplantısı, Trabzon, TÜRKIYE, 31 Ağustos - 5 Eylül 2016, ss.115-115 |
| Reis R., Sipahi H., Aydın A. , "Bitki Çaylarının Tüketiminin Hematolojik Parametreler Üzerine Muhtemel Etkileri", Gıda, Metabolizma &Sağlık: Biyoaktif Bileşenler ve Doğal Katkılar Kongresi, İstanbul, TÜRKIYE, 28 Kasım 1916 - 28 Kasım 2016, ss.224-224 |
| Reis R., Charehsaz M. , Sipahi H., Aydın A., Doğan Ekici A.I., "Energy Drink Intake Alone or Combined with Alcohol-Induced Lipid Peroxidation and Hepatotoxicity in Rat", IVEK 2nd International Convention of Pharmaceuticals and Pharmacies, İstanbul, TÜRKIYE, 27-29 Kasım 15, pp.170-170 |

**Diğer (Görev Aldığı Projeler/Sertifikaları/Ödülleri)**

| |
|---|
| Doktora Bursu, Yeditepe Üniversitesi, Ağustos 2015 |
| Üniversite Öğrencileri Yurt İçi Araştırma Projeleri Destek Programı, TÜBİTAK, Mart 2015 |
| Reis R,Charehsaz M., Sipahi H., Aydın A., Ekici, A.I., "Poster Bildirisi Ikincilik Ödülü, İlaç ve Eczacılık Kurumu Derneği, Kasım 2015 |
| Reis R, "Yeditepe Üniversitesi Eczacılık Fakültesi, 2015 Mezunları Dönem Birinciliği, Yeditepe Üniversitesi, Haziran 2015 |