PROTECTIVE ROLE OF *ADHATODA VASICA* AND VASICINE IN BIDI SMOKE INDUCED CYTOTOXICITY: AN IMPLICATION FOR RESPIRATORY DISORDERS

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Synopsis 1

ABSTRACT

Tobacco smoking is a major cause of respiratory ailments among both: rural and urban Indians. A large number of toxic chemicals of tobacco smoke are reported to cause various inflammatory diseases by inducing oxidative damage to the exposed biological system. Various natural (majorly from medicinal plants) and artificially obtained medicinal products are in use to combat these inflammatory conditions. *Adhatoda vasica* is one of the most widely used medicinal plants in Indian traditional system which, is known to treat respiratory ailments. Present study was conducted to investigate if, ethanolic extract of *Adhatoda vasica* (AVE) and its active phytocompound Vasicine can combat the toxic effects (cell death, oxidative stress and inflammation) induced by bidi smoke concentrate (BSC) in *in vitro* conditions. As, alveolar epithelial cells are the first ones who get exposed to tobacco smoke during smoking and macrophages are the ones who, neutralize the toxic effect *in vivo*, human lung alveolar epithelial (A549) and human macrophage (THP-1) cell lines were chosen for this *in vitro* studies.

In order to achieve objectives of this study, the lung cells and macrophages were exposed to AVE (0.125 to 8µg/ml, 3h), Vasicine (0.25 to 6µg/ml, 3h), and BSC (0.5 to 15%, 24h), to determine their safe and toxic doses, respectively. The results have shown that BSC could induce toxicity in both the cell lines in a dose dependent manner. LD_{50} dose of BSC was found to be 5% and 3%, for A549 and THP-1 cell lines, respectively. Safe ranges for AVE and Vasicine were found to be 1 to 2 and 0.5 to 3µg/ml, respectively, for A549 cell line and 0.5 to 2 and 2 to 3µg/ml, respectively for THP-1 cell line.

To investigate the protective potential of AVE and Vasicine, both the cell lines were pre-treated with the optimized safe doses of AVE and Vasicine (1h) and then were exposed to toxic doses of BSC in separate sets of experiments and then examined for various parameters, including cell viability. Among the chosen doses for AVE and Vasicine, 2µg/ml of AVE and 3µg/ml of Vasicine, showed significant protective effect as, both could retain the cell viability (90 ± 0.04% and 89 ± 0.03%, respectively in A549 cell) against 5% BSC. For THP-1 cell line also, 2µg/ml AVE and 3µg/ml

Vasicine showed significant protective effect as, they could retain the cell viability (87 ± 0.04% and 88 ± 0.03%, respectively) against 3% BSC.

It was observed that exposure of A549 as well as, THP-1 cells to BSC, resulted in significant increase in production of superoxide [superoxides ($\bullet O_2$-), through % increase in NADPH consumption, from 11 ± 0.4% (Control) to 53 ± 0.9% (5% BSC) in A549 and from 4 ± 1.9% (Control) to 50 ± 0.9% (3% BSC) in THP-1. Nitric oxide radical production was also observed to be increased by 11 ± 0.32% in A549 and 39 ± 5.7% in THP-1. This treatment also increased the leakage of LDH (lactate dehydrogenase) by 19 ± 0.3% in A549 (5% BSC) and 45 ± 3.7% in THP-1 (3% BSC) cells.

Further, studying the status of antioxidants - Superoxide dismutase (SOD) and Catalase (CAT) activity in such a stressed conditions an increase in both the enzyme activities [A549: SOD activity from 9 ± 0.30 U/mg (Control) to 15 ± 0.02 U/mg (5% BSC); THP-1: SOD activity from 29 ± 0.04 U/mg (Control) to 47 ± 0.04 U/mg (3% BSC); A549: CAT activity from 10 ± 0.05 U/mg (Control) to 15 ± 0.04 U/mg (5% BSC); THP-1: 15 ± 0.03 U/mg (Control) to 19 ± 0.04 U/mg (3% BSC)] in the BSC exposed groups were observed. Pre-treatment of cells with optimum safe dose of AVE or Vasicine could maintain these enzymes activities. The integrity of cell membrane and DNA was also maintained by AVE and Vasicine in both the cell lines. Microscopic examination of BSC exposed lung alveolar epithelial and macrophage cells showed cellular apoptotic features such as: blabbed cell membrane, de-shaped nucleus and altered mitochondrial localization and its abundance. Pre-treatment with AVE and Vasicine was observed to prevent these effects.

Along with the above observations it was found that treatment with BSC caused an up regulation of pro-inflammatory markers: Tumour necrosis factor-alpha (TNF-α) and Interleukin -6 (IL-6), also in both the cell lines. In this case also, pre-treatment with AVE and Vasicine seemed to reduce the extent of inflammation by down regulating these pro-inflammatory markers.

Hence, the findings of this study suggest that bidi smoking exerts considerable negative impact on the cell viability, oxidative state, and expression of pro-inflammatory conditions of both, lung as well as, macrophage cell line. These findings further have

implications in analyzing the mechanism of respiratory diseases and disorders in people exposed to tobacco smoke.

The study suggests that AVE and Vasicine both are able to protect cells from the deleterious effects of tobacco smoke in *in vitro* conditions. It is thus, propsoed that, both: the ethanolic plant extract and its active compound Vasicine, can further be explored for their exact molecular mechanism of action, so that we can move towards developing their formulations for the management of respiratory disorders caused lined to tobacco smoking.

Chapter 1

INTRODUCTION

Tobacco smoking (TS) is a major risk factor for respiratory diseases. During tobacco smoking, the lung epithelial cells are exposed to the tobacco smoke as a first line and then the toxic material enters into the system [1]. Further, the immune cells present in the alveolar area (alveolar macrophages etc.) and in blood, also get exposed to these toxic substances due to high vascularity of the lung tissues [2]. Normally, immune cells fight back to cope up with the stress induced by the tobacco smoke and in this process they might succeed or else might add to inflammatory phenomena which can ultimately lead to diseased conditions [3]. The present study was conducted to analyze the extent of the toxic effect of Bidi smoke in *in vitro* conditions, in human lung alveolar epithelial and macrophage (A549 and THP-1) cell lines and to investigate if, the plant *Adhatoda vasica* and its active phytocompound Vasicine could prevent the toxicity caused by Bidi smoke concentrate (BSC) along with investigating their mechanism of action.

1. Tobacco smoke

1.1 Prevalence and habit of tobacco smoking: Tobacco smoking is popular all over the world and India is a home for approximately 275 million tobacco users [4]. Several means of using tobacco are available in the market and these include cigarettes, cigars, blunts, cigarillos, bidis, chuttas and kereteks. “Bidi” or “beedi” is a slim, hand-rolled, unfiltered cigarette. The bidis are known as the “poor man’s cigarettes”, as these are smaller and cheaper than cigarettes and, are perhaps the cheapest tobacco smoking product in the world. Number of bidis smoked per day, duration of smoking and the age of initiation, are some of the key factors that determine the mortality rate in a tobacco smoking population [5].

1.2 Chemistry of tobacco smoke: Tobacco smoke (TS) contains around 10^{15} to 10^{17} oxidants/free radicals and 4700 other components, including carcinogens, oxidants, reactive aldehydes, quinones, and semiquinones per puff. All of these have the potential to cause inflammation and damage to the cells. Tobacco smoke can be divided into two phases: tar and gas-phase. Both phases contain a large number of reactive oxygen and nitrogen species (ROS & RNS) like superoxide ($\cdot O_2^-$), hydroxyl ($\cdot OH$) and peroxyl

(·RO_2), and RNS like nitric oxide (·NO), nitrogen dioxide (·NO_2^-) and peroxynitrite ($ONOO^-$), including phenols and quinine etc. [6].

The toxic compounds and free radicals of tobacco smoke (as discussed above), get absorbed into the blood stream from the respiratory tract from where they reach to various organs of the body like: heart, pancreas, liver and kidney etc. thus, causing toxicity in those organs/tissues [7]. On the other hand, the particles from the particulate fraction of the smoke get adhered to lung tissue and causes injury due to the adhered toxins and oxidant released over hours to days, resulting in progressive cellular injury and mucus membrane destruction.

1.3 Statistical scenario: According to the World Health Organization, tobacco-attributable mortality is projected to increase from 1.5 million deaths in 1990 to 3·0 million annually by 2020 in India [8]. Tobacco-related deaths are projected to increase to more than 8 million deaths a year by 2030 [9].

2. Respiratory disorders: Lung diseases are some of the most common medical conditions in the world. Tens of millions of people suffer from lung disease in the Unites States every year [10]. Air pollution, smoking, infections, and genetic predisposition are majorly responsible for most of these pathological conditions [11]. Asthma and chronic obstructive pulmonary disease (COPD) are the most common inflammatory lung diseases which are known to be caused by exposure to environmental stressors such as pollution, smoking, UV radiation and dust etc. [12]. Asthma is a chronic inflammatory disorder of the airways characterized by episodes of reversible breathing problems due to airway narrowing and obstruction. These episodes can range in severity from mild to life threatening [13]. COPD is a preventable and treatable disease characterized by airflow limitation that is not fully reversible [14]. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases (typically from exposure to cigarette smoke) [15].

2.1 Tobacco smoking and respiratory diseases: As, mentioned before, tobacco smoking has been a major cause for respiratory diseases. Epidemiological and clinical studies have shown that smokers are more likely to develop diseases like emphysema, asthma and smoker's cough etc. [16]. Smoking cigarettes causes numerous changes in

the lungs and airways such as, mucus producing cells in the lungs and airways, grow in size and number thereby, increases the amount of mucus produced and loss of function of cilia, as a result, the lungs and airways get irritated and inflamed [17]. The air ways become narrow and the airflow in the lungs reduces. When lung tissues are destroyed, the number of air spaces and blood vessels in the lungs also decrease and the smoker's lungs become more susceptible to allergies, and infections [18]. Prolonged exposure to tobacco smoke can even lead to lung cancer [19].

2.2 Respiratory disorders and oxidative state of a biological system: Respiratory diseases like, Asthma and Chronic obstructive pulmonary disease (COPD) are inflammatory lung diseases. Oxidative stress is one of the most common factors causing inflammation [20]. The term *"oxidative stress"* is defined as the adverse condition resulting from an imbalance in cellular oxidants and antioxidants. Oxidative stress results when reactive species like free radicals, reactive oxygen or nitrogen species (ROS & RNS) etc. are not adequately removed or neutralized in a biological system [21]. The balance between oxidants and antioxidants "redox homeostasis", is a crucial event in living organisms and subjecting cells to oxidative stress can result in oxidative damages to biological molecules of the cells like, proteins, carbohydrates, DNA, RNA, mtDNA, membrane lipids etc. and so can lead to various types of metabolic dysfunction and cell death [22].

Experimental studies showed that materials like: the airborne particulate matter (PM) and tobacco smoke induce production of ROS/RNS in the exposed biological system [23]. This type of increase in oxidative stress has been implicated in the activation of mitogen-activated protein kinase (MAPK) family members and activation of transcription factors such as NF-κB (nuclear factor) and AP-1 (activator protein-1) [24]. These signaling pathways have been implicated in many important processes like, inflammation, apoptosis, proliferation, transformation and differentiation [25]. ROS are generated endogenously along with the routine metabolic reactions such as, electron transport during respiration, and remain in balance. Oxidative reactions can also be triggered exogenously by external agents such as, air pollutants or cigarette smoke etc. [26]. Increased levels of ROS have been shown to affect the extracellular environment impacting a variety of physiological processes and inflammation etc. [27]. It is proposed that ROS produced by phagocytes at the site of inflammation, is a major cause of the

cell and tissue damage associated with many chronic inflammatory lung diseases including asthma and chronic obstructive pulmonary disease (COPD) [29].

2.3 Redox state of cells in a smoker: As, discussed before, tobacco smoke disturbs the redox state of the exposed biological system. Tobacco itself contains huge number of free radicals/ROS and RNS which are delivered to the exposed system directly. Besides this, various components of tobacco smoke induce formation of reactive species in the exposed biological system. Normally, endogenous defence mechanisms play a key role in combating the harmful effects of ROS but, in a smoker, oxidants level may exceed over the antioxidants, and can impair the physiological functions [30]. Subsequent induction of oxidative stress initiates toxic effects in cells and tissues, which has been implicated in several human lung diseases like asthma and COPD etc. [31].

2.3.1 Role of oxidants: Reactive species induction has been shown to interfere with the cell signaling pathways, apoptosis, gene expression as well as, in activation of several other signaling cascades (Figure 1) thus, prompting a vicious cycle of OS in several pathological conditions. Increased levels of ROS & RNS have been reported to mediate altered gene expression [32]. $ONOO^-$ radical has been reported to mediate (formed due to reaction between ·NO and $\cdot O_2$-) activation of nuclear transcription factor (NF-κB) which further increases ·NO formation and the cycle continues [33]. Thus, an overload of ROS and RNS along with an absence/lack of endogenous antioxidant compensatory mechanism to abolish them, leads to activation of several other stress-sensitive intracellular signaling pathways [34]. On the other hand damage to cells occurs as a result of ROS-induced alterations of macromolecules, as well [35]. These include lipoperoxidation of polyunsaturated fatty acids in membrane lipids, protein oxidation, DNA strand breakage, RNA oxidation, mitochondrial depolarization and apoptosis [36]. Tobacco smoke has also shown to mutate nuclear protein p53 leading to apoptosis [37].

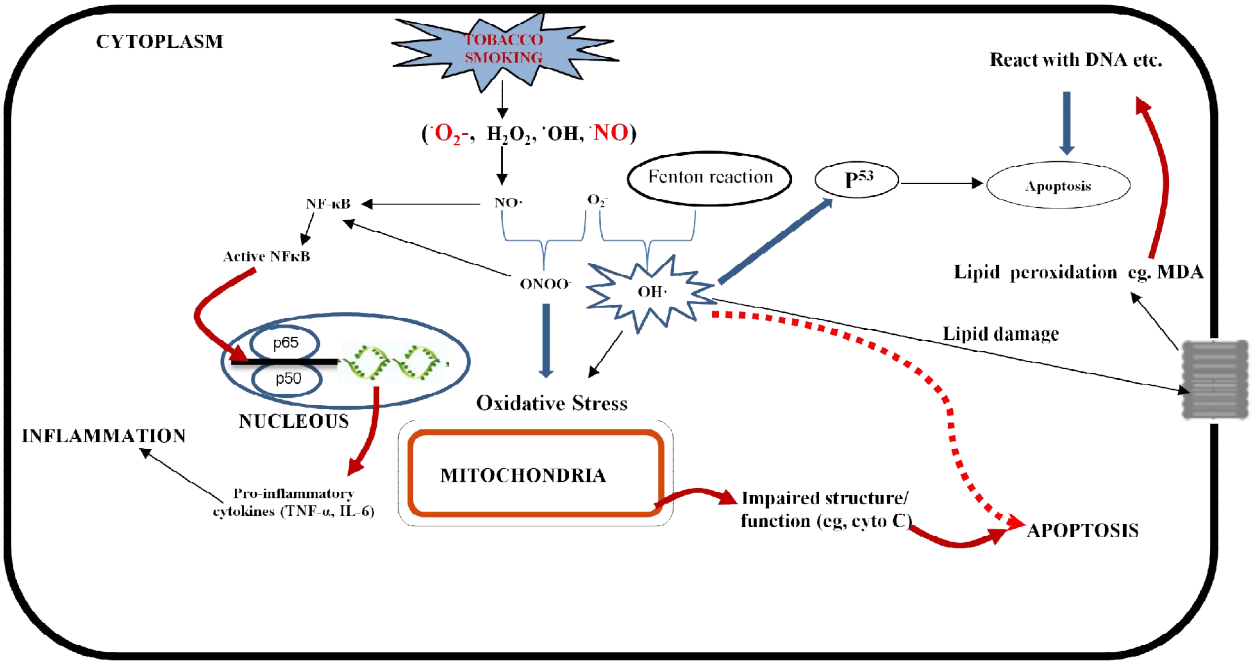

Figure 1. ROS-induced cellular oxidative damage and inflammatory response. Schematic representation of the multiple pathways by which the exposure to reactive oxygen species originated by tobacco smoke can induce cellular damage and inflammation.

2.3.2 Role of Antioxidants: As discussed before, normally, there is balance between oxidants and antioxidants in the cells. The reactive species like ·O_2- radicals thus generated, get scavenged by the antioxidant enzyme like Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase etc. Superoxide dismutase is a prime antioxidant that scavenges the excess superoxide radicals in the cells. The activity of the enzyme (SOD) has been found to have variations in the results obtained by various scientists (decreased or increased or showed no change) in several respiratory study models [38].

Superoxide ions further can be dismutated to H_2O_2 by superoxide dismutase. H_2O_2 is a more stable and lipid soluble molecule which, can go through cell membranes and can reach other parts of the cell. It also has a longer half life than $O_2^{\cdot -}$ but gets further scavenged by catalase and glutathione peroxidase to water and the damage is prevented [86].

2.3.3 Oxidative stress and tobacco smoking: As discussed above exposure to tobacco smoke lead to excessive production of free radicals like ·O_2- and ·NO, etc. which may lead to several losses including loss of membrane integrity of the cells as well as, of its

various other cell organelles including mitochondria. In mitochondria it mainly affects inner membrane phosphoprotein Cardiolipin [39]. This leads to opening of mitochondrial permeability transition pore releasing of Bax-α, and cytochrome c. Kuo et al. proposed two main mechanisms for cigarette smoke-induced apoptosis in rat models [38]. The first one relies on the activation of p38/JNK-Jun-FasL signalling. The second is mediated by p53 stabilization, increased Bax/Bcl-2 ratio, and release of cytochrome c. It also alters the function of mitochondria and nucleus in smoker's lung cells [40]. All these events trigger apoptosis leading finally to cell death [41].

2.3.4 Oxidative stress and inflammation: ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors, such as: NF-κB and AP-1, and other signal transduction pathways, such as: mitogen-activated protein (MAP) kinases and phosphoinositide-3-kinase (PI-3K), leading to enhanced gene expression of pro-inflammatory mediators (TNF-α & IL-6) etc. which further initiate inflammation causing several inflammatory diseases [42].

3. Therapeutic options for inflammatory respiratory diseases

3.1 Modern day's therapy: Currently, many therapeutic options are available for the treatment of inflammatory respiratory diseases. For example, three lines of anti-inflammatory treatment are available for asthma: 1) inhaled glucocorticoids, which have multiple mechanisms of action; 2) cysteinyl-LT inhibitors and 3) β_2-agonists which are very effective bronchodilators, act predominantly on airway smooth muscle, and also exert a mild anti-inflammatory action. All these synthetic drugs effectively alleviate oxidative and inflammatory injury but several adverse side effects like: increased rate of pneumonia, shakiness, heart palpitations, dry mouth and urinary tract symptoms etc., are also found to be associated with most of them and so limit their widespread clinical use and acceptance [43]. Instead, herbal products from traditional medicines could be considered to be the better options owing to the fact that they are comparatively safer, economic and commonly available. Furthermore, due to the wide acceptance of traditional medicines among the population, phytopharmaceuticals with proven antioxidant and anti inflammatory properties could become a suitable therapeutic alternative to current medication.

3.2 Respiratory disorders and Ayurveda: Plant kingdom has been an important source of therapeutic agents since thousands of years. World Health Organization (WHO) estimates that, up to 80% of people still rely mainly on traditional remedies such as: herb(s) and their formulation(s), for the treatment of various diseases [44]. India has about 45,000 plant species and several thousands of them have been claimed to possess medicinal properties to treat different diseases including respiratory ailments [45], few of them are included in the table below (table1).

Table 1: Herbs and their active constituents, used to treat respiratory disorders.

Medicinal Plant	**Active compound**
Mentha piperita (Peppermint)	Menthol
Eucalyptus obliqua (Eucalyptus)	Cineole
Zingiber officinale (Ginger)	Gingerol, gingerdione and shogaol etc.
Glycyrrhiza glabra (Mulethi)	Glycyrrhizin
Lobelia laxiflora (Lobelias)	Lobeline
Adhatoda vasica (Vasaka)	Vasicine

These herbs are reported to combat the respiratory disorders due to their strong antioxidant potential and them also posses different types of phytoconstituents (such as, phenolic and flavonoids) which may have their specific targets. These herbs are easily available at a cheaper price and people clutch trust on them due to their traditional uses [46]. Thus, WHO also supports, encourages and proposes remedies through medicinal plants in different healthcare programmers.

Although, most of the medicinal plants carry antioxidant properties and many types of phytoconsituents. Compound like: polyphenols and flavonoids etc., capture the free radicals by donating hydrogen atoms or electrons, thus neutralizing them and decreasing the load of OS in cells but, overcoming OS is not the only way the phytoconstituents may work, there may be several other specific targets for each of the phytoconstituent of the plant, responsible for its therapeutic potential [47]. Even many of the phytocompounds within one plant, may also have their own unique ability to act in a

"multi-targeted manner" thereby, may be helpful in several ways to control the pathological conditions. In the present study we are mainly focusing on the antioxidant behavior of the herb with a further step towards its anti-inflammatory properties.

It has been seen many a times that a purified active compound from a plant does not meet the efficacy of the crude extract of the plant [48]. So, it is required to understand the mechanism of action of most of herbs/their formulations/active constituents. We have investigated one of the major active phytoconstituent Vasicine of the AV to move towards the above said direction.

3.2.1 The plant – *Adhatoda vasica*

- **Introduction:** *Adhatoda vasica* is a valuable plant and it has been proven for its medicinal properties against a broad array of diseases specially, for the respiratory ailments like: dry cough, asthma, bronchitis, common cold, smoker's cough and many others like: menstrual disorders, eye infections, skin diseases, sore throat, bleeding diarrhea, etc. [49]. It has also been reported to be abortifacient, hepatoprotective, sedative, antiulcer, antispasmodic, anti-allergic, anti-inflammatory, anti-tubercular, and anthelmintic etc. [50].
- **Taxonomical and geographical distribution:** *Adhatoda vasica* belongs to the family Acanthaceae. It is an evergreen shrub growing throughout Punjab, Bengal, Manipur and Kerala etc., at an altitude of 135 m [51]. The plant is also seen distributed in Sri Lanka, Upper and Lower Myanmar, Southern China, Laos, and the Malay-Peninsular and Indonesian Archipelago [52]. The plant is commonly known as "Vasaka" in Sanskrit, "Arusha" in Hindi [53].
- **Chemical constituents:** Few of the main chemical constituents present in AV are Vasicine, 2'-hydroxy-4-glucosyloxychalcone, Vasicol, Vasicinone, Vasicinol and Deoxyvasicinone [54] etc.
- ***In vitro*/*in vivo* and clinical studies with plant/plant extract:** Antioxidant nature of the herb AV and its components are suggested to be its main characteristic, responsible for their physiological effects [55]. Several studies have been carried out to investigate the antioxidant potential, anti inflammatory activity and other therapeutic potentials of different extracts of AV. Few important studies are summarized here as follows:

a. The methanolic extract of *Adhatoda vasica* was evaluated for anti-inflammatory activity [57]. The alkaloid fraction showed potent anti-inflammatory activity at a dose of 50µg/pellet (in hen's egg chorioallantoic membrane model).

b. Kumar et al. (2005) investigated the hematological changes in the blood of Swiss albino mice after the treating them with ethanolic extract of AV (800mg/kg body weight, 6-30 d post irradiation intervals). AV leaves extract could significantly increased GSH content and decreased LPO level [58].

c. Wahid et al. (2010) have worked upon the antioxidant and anti-inflammatory activity of ethanolic extract of *A.vasica* against carrageenan and formalin-induced inflammation in albino rats. They showed that ethanolic extract of *A.vasica* possess antioxidant and anti-inflammatory activities and suggested that it may be due to the presence of flavonoids and other polyphenolic moieties in it, which supports the use of this plant in traditional medicine [58]. It was suggested from this report that the ethanolic and aqueous extracts of leaves of plant *Adhatoda vasica* has anti-inflammatory activity and are comparable to the standard drug (Indomethacin) [59].

3.2.2 *In vitro/ in vivo* and clinical studies with Vasicine

Vasicine is a quinazoline alkaloid ($C_{11}H_{12}N_2O$) (Molecular Weight: 188.2) (28) (Figure 2).

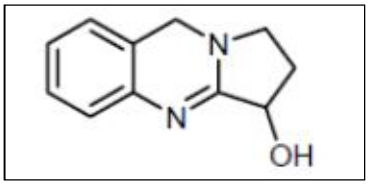

Figure 2: Structure of Vasicine

Srinivasrao et al. (2006) have worked upon the antioxidant and anti-inflammatory activity of Vasicine against ovalbumin and aluminum hydroxide induced lung damage in rats. They had shown that, Vasicine treatment had increased in the activity of various antioxidases like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase and reduced glutathione [60].

Gupta et al. (1977) had suggested that, the bronchodilatory activity of Vasicine works through respiratory sensors and peripheral receptors [55]. Again in 1999, Dhuley et al. reported that Vasicine: 2,4-diethoxy-6,7,8,9,10,12-hexahydroazepino [2,1-b]

quinazolin-12-on exhibited marked bronchodilator activity on contracted trachea or constricted tracheo-bronchial tree [56].

Various other experimental evidences have also reported the antioxidant and anti-inflammatory properties of Vasicine [61]. Pure Vasicine and its derivatives are worked upon to investigate their bronchodilatory and antitussive effects. One of those derivatives is Bisolvon/bromhexine (N-cyclo-N-methyl-(2-amino-3, 5-dibromo-benzyl) amine hydrocloride) has been reported to possess mucus liquefying/expectorant activity by Amin et al. and Sharafkhaneh et al. [62, 63].

As, in smokers the respiratory diseases are found to be linked with free radicals and reactive oxygen and nitrogen species, it was postulated by us that, the plant *Adhatoda* and the molecule Vasicine may be useful in the conditions where tobacco smoke is the major cause for initiating or deteriorating the conditions, as well. No scientific evidence exists till date analyzing this plant for its protective potential in tobacco smoke induced toxicity state in human lung model system investigating the mechanism of action.

So, the present study was undertaken to investigate the efficiency of AVE and Vasicine in protecting the cells (lung alveolar A549 and macrophage THP-1cell line) against the toxicity caused by BSC analyzing their probable mechanism of action.

AIMS AND OBJECTIVES

2. Hypothesis: We hypothesize that as, *Adhatoda vasica* and Vasicine have been shown to have very good antioxidant and anti-inflammatory potential in various pathological conditions, and they might be able to combat the toxicity, oxidative stress and inflammatory reactions, caused by bidi smoke.

2.1 Aim of the study: To investigate the protective role of *Adhatoda vasica* and Vasicine in bidi smoke induced toxic conditions in A549 and THP-1 cell lines.

2.2 Objectives of the study:

- Preparation and characterization of *Adhatoda vasica* extracts.
- Determination of the toxic doses of bidi smoke and safe doses for the *Adhatoda vasica* extract and Vasicine for A549 and THP-1 cell lines.
- Investigating if, optimal does of *Adhatoda vasica* extract and Vasicine can protect the alveolar epithelial cells and macrophages against the stress/toxicity caused by bidi smoke.
- Investigating the mechanism of action for *Adhatoda vasica* extract and Vasicine in protection at cellular, organelle and molecular level.

Chapter 2

Deals with literature survey on: respiratory disorders, tobacco smoking, oxidative stress and medicinal plant used for respiratory disorders with core focus on *Adhatoda vasica* and its active compound Vasicine.

Chapter 3

METHODOLOGY

Phytochemical analysis of AV extracts was performed in order to characterize it before investigating the biological activity. This was followed by various biological assays (as mentioned below) in order to achieve the rest of the objectives of this study.

3.1 Preparation and characterization of plant material (leaves) and plant extracts

3.1.1 Preparation of ethanolic, methanolic, ethyl acetate, chloroform, aqueous extracts

A. Preparation of Ethanolic extract: 100 g leaves powdered of *A. vasica* were exhaustively extracted with 250 ml ethanol (99%) in a Soxhlet extractor for 72h at 60ºC. The supernatant was then collected and filtered. This liquid extract was then dried and concentrated in a rotary evaporator, under reduced pressure at less than 40°C, to get respective type of extract. The extract was collected and stored at -80°C until further analysis.

B. Preparation of other extracts: 100 g leaves powdered of *A. vasica* were soaked in respective solvent (methanol/ethyl-acetate/chloroform/water) for 24h at room temperature. The supernatant was then collected and filtered. This liquid extract was then dried and concentrated in a rotary evaporator, under reduced pressure at less than 40°C, to get respective type of extract. The extract was collected and stored at -80°C until further analysis.

3.1.2 Characterization of *Adhatoda vasica* extracts

This was achieved at two levels of analysis: qualitative analysis and quantitative analysis

A. Qualitative phytochemical analysis

- Biochemical tests for carbohydrates, amino acids, sterols, terpenoids, alkaloids, phenolic compounds, flavonoids, tannins and anthraquinone by standard methods [64]
- TLC analysis using Vasicine as marker compound – by standard method [65].

B. Quantitative phytochemical analysis

- Determination of total phenolics content – by standard method (Folin-Ciocalteau assay) [66,67]
- Determination of total flavonoids content – by standard method [68,69]
- Determination of total tannins content – by standard method [70]
- HPTLC analysis using the marker compound Vasicine.
- High Performance Liquid Chromatographic (HPLC) analysis

C. The antioxidant property of AVE was analyzed through

- DPPH scavenging activity – by standard method [71]
- ABTS scavenging activity – by standard method [71]
- Reductive ability of AVE – by standard method [73]
- Hydrogen peroxide scavenging activity – by standard method [72]
- Superoxide radical scavenging activity – by standard method [73]

- Nitric oxide radical scavenging activity – by standard method [73]

3.2 Preparation and Standardization of Bidi Smoke Concentrate (BSC)

- Preparation of BSC – by the method of Lannan et al. 1994 with slight modifications [74]
- Standardization of BSC by spectrophotometric, UPLC, and ^{1}H NMR analysis using Nicotine as a reference content.

3.3 Assessment of toxicity of BSC and investigation of protective potential of AVE and Vasicine

3.3.1 Treatments with BSC, AVE and Vasicine, individually and in combinations: effect on cell viability:

a. MTT assay– Briefly, 3 x 10^5 cells of human lung epithelial and macrophage cell lines were treated with different dose ranges (0.5 - 5µg/ml of AVE, 0.25 - 5µg/ml of Vasicine for 3h, 0.5 - 15% BSC, for 24h). Cells in each well were further incubated with 10µl MTT for 3 - 4h in a 5% CO_2 incubator, maintained at 37˚C. 100µl of DMSO was then added and the plate was incubated for another 10min in dark at room temperature. Finally, the treatment plate was read in an ELISA plate reader using 570nm filter [75].

b. Morphological analysis: The effect of treatment with BSC, AVE and Vasicine and their combinations on the morphology of A549 and THP-1 cells were analyzed under an inverted microscope.

3.3.2 Effects of various treatments on cell membrane integrity of A549 and THP-1 cells through:

a. Trypan blue exclusion assay – by standard method [76].

b. Lactate dehydrogenase (LDH) leakage assay – In this method, 500µl sodium pyruvate (30 mM), 20µl NADH (6.6 mM) and 250µl Tris-HCl buffer (0.2 M, pH7.3), were mixed and incubated at 25˚C for 5min. Then, 20µl of each of the supernatants from the treated/untreated sets were added in this reaction mixture, and the decrease in absorbance (340nm) over time was recorded for 30min [77].

c. Estimation of lipid peroxidation by TBARS assay – In this method, the cell lysate (50µg protein) obtained after respective treatments was incubated in 500µl of buffered which (175 mM KCl and 10 mM Tris, pH7.4) medium at 25˚C for 5min. After incubation, 50µl of sample was taken and mixed with 450µl TBARS reagent and heated at 80 - 90˚C for 15min. The mixture was then cooled in ice and after centrifugation, the

O.D. of the supernatant was measured at 535nm and the percentage MDA formed was calculated [78].

3.3.3 Effect on mitochondrial localization

a. 10N-nonyl acridine orange (NAO) staining - NAO staining was performed for analyzing the distribution of mitochondrial Cardiolipin – by standard method through fluorescence microscopy [79].

3.3.4 Effect on nucleus and DNA integrity

a. PI staining- for analyzing the effect on nucleus integrity – by standard method through fluorescence microscopy [80].

b. DNA fragmentation assay – by standard method

Briefly, DNA was isolated from treated and untreated cells (5×10^5). Equal amount DNA from sample was loaded for gel electrophoresis on agarose gel (1%) and analyzed [81].

3.3.5 Study into the level of oxidative stress – whole cell analysis

a. Effect on oxidants:

- **NADPH oxidase assay** – by standard method [82]
- **Nitric oxide radical scavenging assay** – by standard method [83]

b. Effect on antioxidants:

- **NBT assay** - for determination of enzymatic antioxidant status (Superoxide dismutase by standard method [73].
- **Catalase assay** - Determination of enzymatic antioxidant status by standard method [73].

3.3.6 Investigation the expression of pro-inflammatory markers (TNF-α and IL-6):

- **RNA isolation –** by standard method [84]
- **Reverse transcription of RNA –** by standard method [84]
- **Semi-quantitative RT-PCR –** by standard method [84]

Chapter 4

RESULTS

4.2 Preparation and characterization of plant extracts

4.2.1 Preparation of extracts and percentage yield of extracts

Five different extracts (ethanolic, methanolic, ethyl acetate, chloroform and water) were prepared and the percentage yield of five different crude extracts were in the order of water > ethanol > methanol > chloroform > ethyl acetate extracts, respectively.

4.2.2 Characterization of *Adhatoda vasica* extracts

A. **Qualitative analysis**

- **Biochemical tests** were performed with all five extracts (ethanolic, methanolic, ethyl acetate, chloroform and water) of AV and they showed presence of: alkaloids, phenolics, flavonoids, saponins, reducing sugars, tannins, amino acids, and anthraquinone etc.

Table 2: Phytochemical present in various extracts of *Adhatoda vasica*

Type of extracts of *Adhatoda vasica*	Phenolics compounds	Alkaloids	Flavonoids	Saponins	Reducing sugars	Tannins	Amino Acids	Anthraquinone derrivatives
Ethanolic	++	++	+	+	+	++	+	+
Methanolic	+	+	+	+	+	++	+	-
Ethyl acetate	-	+	-	-	-	-	-	-
Chloroform	-	+	-	-	-	+	-	-
Water	-	-	-	-	-	+	-	-

(+) Presence of phytochemical compounds, (-) absence of phytochemical compounds.

B. **Thin Layer Chromatography** showed the presence of various bands in all the extracts indicating many phytoconstituents present in all the extracts. Vasicine was present only in methanolic and ethanolic extract.

C. **Quantitative analysis**

a. Quantitative analysis on the plant extracts have shown the amount of Phenolic (88.77 ± 1.21mg/g GAE/g; 67.20 ± 0.31mg/g; 21.07 ± 0.21mg/g; 18.40 ± 2.44mg/g and

35.12 ± 0.43mg/g), Flavonoid (55.28 ± 1.01mg/g; 55.82 ± 0.23mg/g; 51.79 + 0.62mg/g; 46.84 ± 0.42mg/g and 45.16 ± 0.12mg/g) and Tannin (25.00 ± 0.41mg/g; 23.50 + 0.21mg/g; 06.12 ± 0.52mg/g; 05.60 ± 1.31mg/g and 05.82 ± 0.10mg/g) dry weight of sample content present in ethanolic, methanolic, ethyl acetate, chloroform and water extracts, respectively.

b. **High Performance Liquid Chromatographic (HPTLC) analysis of** these five extracts showed:

- Numerous colored, well defined bands indicating the presence of numerous phytocompounds in the *Adhatoda vasica* extracts
- The ethanolic and methanolic extracts showed similarity in their band pattern, indicating the extraction of similar types of phytocompounds.

c. The marker compound (Vasicine) gave a peak with R_f value 0.45 in the two extracts (ethanolic and methanolic). HPTLC chromatogram showed the presence of Vasicine in only two extracts of AV in the concentration sequence: ethanolic > methanolic, respectively

d. **HPLC analysis** also confirmed the presence of Vasicine (4.15 ± 0.24%).

As, ethanolic extract of AV has shown the highest amount of marker compound Vasicine and most of the of the past studies also have shown the significance of ethanolic extract for the biological activity of the plant, ethanolic extract of *Adhatoda vasica* was chosen for the study

D. **Antioxidant potential of AVE**

AVE possess strong reductive ability as well as, DPPH, ABTS, H_2O_2, $\cdot O_2$-, ·NO scavenging activity. And the IC_{50} values of AVE in DPPH, ABTS, H_2O_2, $\cdot O_2$- and ·NO scavenging assays obtained were 64µg/ml, 200µg/ml, 62µg/ml, 40µg/ml and 58µg/ml, respectively.

4.2 **Preparation and characterization of Bidi Smoke Concentrate (BSC)**

- BSC (100%) was prepared as mentioned before and its absorbance at 260 nm (O.D. range: ~0.4) was noted, to normalize its preparation every time.
- BSC was characterized through spectrophotometric, ^{1}H NMR and UPLC analysis with respect to its Nicotine (as marker) content.

4.3 **Assessment of toxicity of BSC and investigation of protective potential of AVE and Vasicine**

a. **Effect on cell viability:** MTT assay performed after exposing the cells to different doses to AVE and Vasicine, showed that 1 and 2µg/ml AVE and, 2 and 3µg/ml (for 3h) Vasicine maintained the cell viability near to control for both the cell lines. BSC treatment was found to be toxic to both the cell lines in a dose dependent manner and almost 50% cell death was obtained on treatment with 5 and 3% BSC for A549 and THP-1 cells, respectively.

This toxic effect of BSC was found to be prevented by pre-treating the cells with the above optimized safe doses of AVE or Vasicine, before exposing them to tobacco smoke as pre exposure to AVE or Vasicine could retain the cell viability (~90%) even after exposing the cells to BSC for both the (A549 and THP-1) cell lines.

b. **Morphological analysis:** Microscopic observations confirmed the cytotoxicity as, various structural abnormalities were observed in BSC- treated cells and, suggested the occurrence of apoptosis in the both the cell lines. These deleterious effects were found to be prevented by pre-treating the cell lines with AVE or Vasicine.

4.3.1 **Effects on cell membrane integrity**

a. **Trypan blue exclusion assay:** In trypan blue exclusion assay we observed that percentage of dead cells was increased under the toxic effects of BSC. These effects were prevented by pre-treating the cells with the optimized doses of AVE or Vasicine.

b. **LDH assay:** Exposure of cells to toxic doses of BSC had shown an increase in LDH (19 ± 0.4% in A549 (5% BSC) and 45 ± 0.3% in THP-1 (3% BSC) cells) enzyme activity in culture medium of the cells thus, confirming alteration in plasma membrane integrity. These effects were prevented by pre-treating the cells with the optimized doses of AVE or Vasicine.

c. **TBARS assay:** Exposure of cells to toxic doses of BSC had shown an increase in percentage MDA (17 ± 0.3% in A549 (5% BSC) and 33 ± 0.3% in THP-1 (3% BSC) cells) formation in their cell membrane thus, confirming lipid peroxidation in the cells. These effects were prevented by pre-treating the cells with the optimized doses of AVE or Vasicine.

4.3.2 **Effect on mitochondrial localization:** Exposure to BSC had induced alteration in mitochondrial localization and abundance which was maintained by pre-treatment of both the cell lines with AVE or Vasicine.

4.3.3 **Effect on nucleus**

a. **PI staining of nucleus:** To further observe the effect of BSC on nuclear and chromatin integrity for both the cell lines, PI staining was performed. BSC-induced cells showed changes in nuclear morphology, decreased cell density and condensed nuclear content as compared to control. When the BSC-induced cells were pre-treated with AVE and Vasicine, it showed the numbers of nuclei seen in the field is similar to control and morphology also reaching similar to control (round and less fluorescent) for both the cell lines.

b. **DNA fragmentation assay:** Total genomic DNA was isolated from the cells (with or without treatment(s)) and DNA fragmentation patterns were analyzed on 1% agarose gel. DNA pattern in the samples treated with toxic doses of the stressors, indicated induction of apoptosis, for both the cell lines which was observed to be prevented by the pretreatment of the cells with AVE or Vasicine.

4.3.4 **Analysis of redox state of cells under various treatment conditions**

a. **Effect on oxidants:** Under such BSC-induced stressed conditions, we found a significantly high level of increased NADPH oxidase enzyme activity (53 ± 0.9% in A549 (5% BSC) and 50 ± 0.9% in THP-1 (3% BSC) cells) and nitric oxide production activity (11 ± 0.32% in A549 (5% BSC) and 39 ± 5.7%) in THP-1 (3% BSC) cells) in the treated cells. The protective effect of AVE and Vasicine pre-treatment was confirmed in such stressed conditions as, a decreased $\cdot O_2$- and ·NO radical production was observed in both A549 and THP-1 cells.

b. **Effect on antioxidant levels:** Treatment with toxic doses of BSC was found to increase SOD (15 ± 0.02 U/mg protein in A549 (5% BSC) and 47 ± 4.0 U/mg protein in THP-1 (3% BSC) cells) and Catalase activity (15 ± 0.04 U/mg in A549 (5% BSC) and 19 ± 0.04 U/mg in THP-1 (3% BSC) cells). However pretreatments with AVE or Vasicine were found to maintain their level near to normal (control) after exposing them to BSC, in both the cell lines.

c.

4.3.5 **Effect on pro-inflammatory markers (TNF-α and IL-6)**

It was observed that the expression of pro-inflammatory markers TNF-α and IL-6, was up regulated in both the cell lines, by BSC treatment (RT-PCR analysis). Whereas AVE or Vasicine pre-treatment could decrease their levels in comparison to BSC treated groups.

Chapter 5

DISCUSSION

The present study was conducted to evaluate protective potential of *Adhatoda vasica* and Vasicine, over tobacco smoke induced toxicity to human alveolar epithelial and macrophage cell line. This research has implications towards finding a better treatment in tobacco smoke induced pathological conditions of respiratory system.

Characterization is a necessity step for any herbal product; the study begins with the phytochemical analysis of the five different extracts (ethanolic, methanolic, ethyl acetate, chloroform and water) of *Adhatoda vasica* (AV). It revealed the presence of many saturated and unsaturated compounds in *Adhatoda vasica* extract which, might be responsible for the medicinal importance of AV. Biochemical analysis of all five extracts of *Adhatoda vasica* had shown the presence of phenolics, flavonoids, alkaloids, anthraquinone, reducing sugars, amino acids, saponins and tannins in different proportions and combinations.

Phenolic compounds and flavonoids are the major constituents in most of the medicinal plants that are reported to possess antioxidant and free radical scavenging activity. They act by interfering with free radicals and other reactive species and so prevent oxidation of lipids and other biomolecules [85, 86]. These compounds are known for their hydrogen or electron donating and metal ion chelating properties and, many findings have established an inverse relationship between the consumption of antioxidant rich plants and the incidences of human diseases [87]. Polyphenols have been reported to modulate the activity of a wide range of enzymes and cell receptors thus, affecting basic cellular functions like cell cycle, apoptosis etc. [88].

In our study, we have found that ethanolic extract of *Adhatoda vasica* possess higher amount of these phytochemicals (phenolic compound, flavonoids and tannins, 88.77mg

GAE/g, 55.28mg Rutin/g; 25.00mg GAE/g, respectively) as compared to other extracts (methanol/ethyl acetate/chloroform/water) and so was chosen for this study.

Further the extract was characterised by the advanced techniques like HPTLC and HPLC. Chattopadhyay et al. (2004) have reported 2% Vasicine in ethanolic extract of *Adhatoda vasica* in their study, through HPLC analysis [89] however, in the present study more than double (4.15 ± 0.24%) of the amount of Vasicine was found to be present in AVE. Percentage of any phytoconstituent depends upon location, weather farming practices and harvesting practices etc and might be the cause for the difference in the content of Vasicine in AVE.

Most of the medicinal plants are reported to be strong antioxidants and higher antioxidant potential has been shown to be correlated with their medicinal values. Hence, to evaluate the intrinsic antioxidant potential of AVE, antioxidant assays like: DPPH, ABTS, H_2O_2, $\cdot O_2$-, ·NO scavenging and reducing power assays were performed and we found that AVE has strong reductive ability as well as, DPPH, ABTS, H_2O_2, $\cdot O_2$- and ·NO scavenging ability.

Tobacco has been known to have toxic effects on many biological systems [90]. It had been shown that, tobacco smoke (mostly cigarette have been used) can induce considerable oxidative damage in the biological systems including respiratory system exposed to it [90]. As respiratory tract is the first system exposed to smoke during tobacco smoking they become the first system to get affected by tobacco smoke.

In our *in vitro* system we have exposed human alveolar epithelial and macrophage cell lines to tobacco smoke (from bidi) followed by MTT assay, evaluating cytoxocity potential of BSC on both the cell lines (A549 and THP-1). Bidi is an Indian form of hand rolled cigarette which have been known to have more drastic toxic effects to the exposed personals. In India, low income people mostly use bidi as; these are cheaper and are more addictive. We observed a significant reduction in number of metabolically active functional cells of these cell lines after exposing them to bidi smoke extract. Occurrences of symptoms of “apoptosis” were observed in microscopic analysis and DNA fragmentation assay. This indicates towards an arrested proliferation pathway or triggering of a death pathway due to this exposure.

We further analyzed the causes and extent of damage due to this cytotoxicity. Analyzing the results at plasma membrane level it was found that its integrity was compromised as indicated by an increase in the leakage of LDH enzyme after exposing the cells with the LD_{50} doses of BSC. Several studies have shown that, free radicals generated from molecular oxygen during the treatment with toxic agents, attack the membrane lipid bilayer, and create a superoxide radical mediated chain reaction [91]. In our study also we observed that there is an increase in lipid peroxidation in both the cell lines (A549 and THP-1) when exposed to toxic doses of BSC. Lipid peroxidation is a process where increased level of oxidants cause loss in membrane integrity which might have caused LDH release from the treated cell lines. It was observed that pre-treatment the cells with the optimized doses of AVE or Vasicine could reduce the level as compared to BSC exposed cells.

While investigating the effect of BSC on mitochondria, it was found that BSC could alter mitochondrial localization and abundance, whereas, pre-treatment of the cells with the optimized doses of AVE or Vasicine could maintain it.

In smokers, the $^{\cdot}O_2$- has been reported to increase the peroxynitrite and nitric oxide production, thus pushing the cell towards apoptosis [92]. Also, both nitric oxide synthase and NADPH oxidase are key generators of free radicals which modify cellular proteins and initiate redox signaling [93] the latter being considered as an important contributor to OS in lung, as well [94]. We have examined the oxidative state of the cells exposed to tobacco smoke and found that, high amount of OS is generated by this stressor. When we estimated nitric oxide production it was found that BSC could increase NO production up to 11% in A549 and 39% THP-1 cells in comparison to control. THP-1 cell lines were found to be more sensitive to BSC treatment. It was found that treatment with 2µg/ml AVE and 3µg/ml Vasicine could maintain the toxic effect induced by BSC. Vasicine treatment could control NO production also which is reported to be the major initiator for oxidative stress and inflammatory cascade [95].

NADPH oxidase enzyme activity was also found to be significantly increased (53% in A549 50% in THP-1 cells) under the effect of BSC and in their case also AVE and Vasicine were able to decrease the enzyme activity as compare to BSC treated group in both the cell lines.

In order to restore this oxidant: antioxidant imbalance, SOD and CAT has been reported to play a strong role and, the observations from our study coincide with it [95, 97]. Exposure of cells to the stressors showed an increase in SOD as well as, CAT activity thus, indicating that lung cells has inbuilt capacity to fight against these toxic effects. Pre-treatment of A549 and THP-1 cells with AVE and Vasicine caused decrease or maintained SOD and CAT activity at higher toxic doses of 5% and 3% BSC, respectively, thereby indicating more utilization of this enzyme under oxidatively stressed condition.

ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors, such as nuclear factor (NF-κB) and activator protein (AP-1), and other signal transduction pathways, such as mitogen-activated protein (MAP) kinases and phosphoinositide-3-kinase (PI-3K), leading to enhanced gene expression of pro-inflammatory mediators (TNF-α and IL-6) which stimulates inflammatory markers and produce inflammation [98]. In our study, also BSC was found to indicate all these features including increase in OS, cell death, and increase in expression of pro-inflammatory markers (TNF-α and IL-6) in both cell lines (A549 and THP-1). AVE and Vasicine treatment before BSC-exposed groups decreased the expression of these pro-inflammatory markers (TNF-α and IL-6) which is less than BSC treated groups of A549 as well as, THP-1 cell lines.

Chapter 5

CONCLUSION

On the basis of results obtained following conclusion can be made from the present study:

1. Ethanolic extract of *Adhatoda vasica* possess considerable amount of the known antioxidants: Phenolics, Flavonoids and Tannins, which might be responsible for their intrinsic antioxidant and free radical scavenging activity of the extract.
2. The active phytoconstituent (Vasicine) was also present in the highest amount in Ethanolic extract of *Adhatoda vasica*.

3. Bidi smoke causes deleterious effects on cell viability of A549 and THP-1 cell lines, in a concentration dependent manner and LD_{50} doses for the two cell lines were 5% and 3% BSC, respectively.
4. Increasing concentrations of bidi smoke:
    a. Disturbed the oxidative state of both the cell lines in terms of increase in MDA (17 & 32%), NADPH (53 & 50%), NO (12 & 39%), SOD (15 & 47%), Catalase (15 & 19%) for both A549 and THP-1 cell lines, respectively.
    b. Altered the chemistry and integrity of biomolecules such as, MDA from membrane lipids and DNA integrity, which is possibly caused by the altered oxidative state of the cells. Toxic doses of BSC could induce increase in expression of pro-inflammatory markers: TNF-α and IL-6, in both the cell lines.
5. Pre-treatment of the cells with the optimized doses has shown a decrease in cell death caused by bidi smoke up to 92% and 96% for both A549 and THP-1 cell lines, respectively.
6. Both AVE and Vasicine could protect both (A549 and THP-1) cell lines against BSC induced toxicity.
7. The reasons suggested to be responsible for the protection are:
    a. Both (AVE and Vasicine) have shown increase in the level of antioxidants and decrease in the level of oxidants and so might have prevented the damage caused by the oxidants, in both the cell lines.
    b. Maintenance of the oxidative state of the cells might have further-
        - Protected cell membrane and DNA integrity.
        - Preserved the localization of mitochondria and intactness of mitochondrial Membrane
        - Maintained the cellular anti-inflammatory fighting capability by regulation of the pro-inflammatory markers: TNF-α and IL-6.

FUTURE PROSPECTS

1. Confirmation of cell death by apoptosis

    - TUNEL assay: DNA Fragmentation.
    - Gene expression of apoptotic markers like, Bax and Caspase 9 (through RT-PCR and Western blotting)
    - NF-κB directed activation/inhibition of target gene in nucleus can be analyzed through expression analysis of the NF-κB.

2. Since, *in vitro* study cannot completely mimic the pathological situation *in vivo*, the investigation should be extended to *in vivo* and clinical study level in all the three study groups.

3. Isolation and investigation of the biological activity of the other phytoconstituents of *Adhatoda vasica* in the same model might give us better alternatives over the currently used drugs.

4. As, antioxidant activity might not be the only mechanism of action for this herb and Vasicine for its activity a deeper analysis might also needed to pin point the specific target(s) responsible for this protective activity.

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