Discussion
DISCUSSION

Nobiletin on body weight, lung weight and tumor incidences

In the present investigation the body weight loss observed in lung cancer bearing (Group 2) animals, could be because of cancer cachexia, anorexia which reported to contribute skeletal muscle and adipose tissue wasting are observed in cancer patients (Magesh et al., 2006; Tessitore et al., 1994) malabortion (Pain et al., 1984). The significant increase in body weight in Nobiletin administrated animals (Group 3) could be due to the inhibitory action of the drug on tumor growth, the gradual increase in body weight indicates the antineoplastic property of the drug. Drug control (Group 4) animals do not show any significant variations. These results indicate the positive nature of Nobiletin.

Nobiletin on Tumor Marker Enzymes

Tumor marker indicate the presence or extent of tumor in the body. Tumor marker enzymes were found to be elevated in tissues of lung carcinoma bearing animals, which could be due to the destruction of the neoplastic tissue is of prime importance. The abnormal variations in the marker enzymes reflect the overall change in metabolism that occurs during malignancy (Stefanini, 1985), serves as an indicator of cancer response to therapy (Radkakrishnan et al., 2006). CEA has been extensively studied, oncofetal glycoprotein found to be abnormally elevated in different types of tumors (Sivaramakrishnana et al., 2008; Sakao et al., 2004). High CEA levels was found in 16-23% patients in early stage of lung cancer (Sawabata et al., 2003; 2002). It is usually over expressed on the cell surface of malignant epithelial type tumors and it may offer a survival advantage by allowing adhesion into other cells and further metastasis (Lin et al., 2007). CEA detected high levels in metastasis carcinoma of non digestive organs such as breast, lung, prostate and ovary.
(Devipriya et al., 2006). CEA regarded to its potential role as a marker of early cancer and as a prognostic indicator (Veronesi et al., 2005).

The increased levels of serum CEA level in B(a)P administered animals could be associated with production rates of tumor, its location and stage, size, differentiation and vascularity. In the present investigation Nobiletin treatment lowered the levels of CEA in B(a)P induced lung carcinogenesis, which is a good prognosis for tumor regression, and inhibition of metastasis.

**Nobiletin on Tissue Marker Enzymes**

B(a)P a potent tobacco carcinogen is known to produce enormous amount of free radicals, (Kamaraj et al., 2007) these free radicals and non radical oxidizing species are highly reactive toxic and mutagenesis (Selvendiran et al., 2005; 2003). The analyses of cancer marker enzymes serve as an indicator of cancer response to therapy. Disruption of many biochemical, immunological and molecular properties of the host has been observed in B(a)P mediated cancer conditions (Mikhail et al., 1996). In the present investigation the marker enzymes such as ADA, AHH, GGT, 5'-NT and LDH are specific indicators of lung damage (Vinodhkumar et al., 2006; Durak et al., 1993; Ferringo et al., 1994; Yildrin et al., 1999).

Adenosine deaminase (ADA) is an important enzyme in purine metabolism and has been documented as a tumor marker and increased activities are found in rapidly growing malignancies (Kocic et al., 2003). High level of ADA activity may be a compensatory mechanism against toxic accumulation of its substrate due to accelerated purine and pyrimidine metabolism in the cancerous tissue and cell (Daoud et al., 1978). Increased ADA activity may give selective advantage to cancer cell by causing production of hypoxanthine guanine phosphoribosyl transferase (HGPRT) a key enzyme in salvage pathway which
provide more mononucleotides to cancer cells (Akylo et al., 2001) patients with lung cancer shown to have elevated serum ADA levels (Senthilnathan et al., 2006; Nishihara et al., 1970).

AHH is one of the useful biomarkers in early diagnosis of lung cancer (Chen and Liu, 2000). AHH converts polycyclic hydrocarbons to phenol, dihydrodiols, quinines and epoxides, this enzyme system that is highly inducible in mouse skin as well as in most mammalian tissue is positively correlated with susceptibility to B(a)P cytotoxicity leads to carcinogenesis (Kamaraj et al., 2007). Elevated AHH levels were induced in pulmonary tissue and in serum of animals exposed to B(a)P (Hecht et al., 2005).

γ-GT activity serve as a specific marker for the prognosis of carcinogenic and not useful in diagnosis but also has prognostic value in malignancies such as lung cancer and in malignant melanoma (Obrador et al., 2002). γ-GT is a broad specificity transferase that catalyses the transfer of gamma glutamyl groups from a large variety of peptide donors to a wide range of amino acids and peptide receptors (Valentich and Moris, 1992). γ-GT is cell surface enzyme that cleaves extracellular glutathione thereby providing the increased intracellular glutathione synthesis (Durham et al., 1997). Increased activities of γ-GT were observed in cancer cells (Ngo and Nutler, 1994). Chemical carcinogens that enter liver may initiate some systemic effects that induce γ-GT synthesis. Abnormal high levels of γ-GT are often observed in tumor of lung, liver and variety of tissues, including HCC, malignant squamous carcinoma of skin, mammary tumor and adenocarcinoma of the lung (Jansen and Mason, 1978).

5'-NT are enzymes that hydrolyses nucleotides with a phosphate group on carbon atom 5 of ribose it is found to be widely distributed in tumors tissues and increased activity of the enzymes in leukemia patients has been already reported (Erdemli et al., 2004). Higher activities 5'-NT were reported in lung cancer patients (Durak et al., 1993). 5'-NT a fast
moving 5'-nucleotide phosphodiesterase is found to be elevated in metastases to liver from
tumor of the lung and breast.

LDH a tetrameric enzyme recognized as a potential tumour marker, in assessing the
progression of the proliferating malignant cells, fairly sensitive marker for solid neoplasm
(Engan and Hannisdal 1990; Lippert et al., 1981). Activity of LDH is found to be higher in
malignant tissues (Rogers et al.,1981) the elevated activity of LDH may be due to the over
production by tumour cells or it may be due to the release of isoenzymes from destroyed
tissues. Elevated LDH activity was observed in various type of tumors (Nano et al., 1989).
Sarcoma-180 cells (Saraswathi et al., 1998). LDH is recognized as a potential tumor marker
in assessing the proliferation of malignant cells and increased lung and serum LDH activity
has been reported in experimental lung cancer (Kamaraj et al., 2007) proliferating malignant
cell inhibit very high rate of glycolysis which subsequently leads to elevate LDH activity.

In the present investigation study significant elevation in all the above serum marker
enzymes were observed in B(a)P administrated lung cancer animals, decrease in the
activities of the above mentioned marker enzymes on treatment with Nobiletin suggest the
offers some protective against abnormal cell growth by changing the permeability or
affecting cellular growth. This may be due to the antineoplastic property of Nobiletin. Thus
the Nobiletin pre co-treatment brought down the levels of tissue and serum marker enzymes
close to normal suggesting its membrane stabilizing and protective potential against lung
cancer.

**Nobiletin on the Status of Lipid Peroxidation in Lung and Serum**

The B(a)P is a very effective carcinogen to induce enormous amounts of free
radicals, which in turn reacts with lipids causing lipid peroxidation (LPO) (Kim et al.,
2006;Selvendiran and Sathisekaran,2004; Sikkim and Muke, 2000;). Lung is exposed to
higher levels of oxygen than most of other tissues, the level of reactive oxygen species (ROS) in the lung is further increased by cigarette smoke, inflammation, pollutants, chemicals and carcinogen (Cugell and Camp, 2004; Chruch and Pryor, 1985, Harris and Sun, 1984). Free radicals and non radicals oxidizing species are regularly produced in animals treated with carcinogens and in human tissue, accumulating evidences suggest that these free radicals and electrophilies mediated lipid peroxidation, occupies a significant position in carcinogenesis and is the most studied biologically relevant free radical chain reaction (Banakar et al., 2004). The effect of many antioxidants is to scavenge radicals and consequently to inhibit lipid peroxidation, chemical carcinogen compound strongly involved in the generation of reactive oxygen species, such as peroxides, hydroxyl and superoxide anion radicals leading to cellular oxidative damage through DNA strand break and lipid peroxidation (Singletary et al., 1996).

Lipid peroxidation widely recognized as a primary toxicological event is caused by the generation of free radicals from a variety of sources including organic hydroperoxides and redox processes (Halliwel, 1994; Fridovich, 1986). The secondary events includes changes in membrane structure, permeability and fluidity lysozomal destabilization and stimulation of apoptosis (Dorman et al., 1995). The various primary and secondary product of lipid peroxidation in biological system are decomposed aldehydes are formed, one intensely studied aldehyde is malonidialdehyde (MDA) is commonly used as a marker for the lipid peroxidation (Machlin and Bendich, 1987). Polycyclic aromatic hydrocarbon (PAH) has been extensively as a prototype carcinogen and cause the production of malonidialdehyde (Wu et al., 2001).

LPO is regarded as one of the basic mechanism of cellular damage caused by free radicals (Esterbauer et al., 1991). An increase in LPO indicates serious damage to cell
membranes, inhibition of several enzymes, cellular function and cell death (Pompella et al., 1991). Increased levels of LPO products play a role in the early phases of tumor growth (Rice-Evans and Burdon, 1993). Involvement of free radicals in B(a)P induced lung carcinogenesis was confirmed by the over production of 8-hydroxy guanine in lung and liver of B(a)P administrated mice (Mikhail et al., 1996). LPO and modulation of various cellular molecular pathway reactive free radical generated during cytochrome P450 dependent metabolism of B(a)P has been implicated during the pathogenesis of lung carcinogenesis (Das Rajat et al., 2007). Our result are consistent with the above findings, phytochemicals are reported to exhibit a wide range of biological activities including antioxidants and free radical scavenging activities (Beutner et al., 2001). Nobiletin major chemical constitute of the essential oil from Citrus karma Raf (Rutaceae) showed significant inhibition for the oxidation of linoleic acid in the beta-carotene-linoleic acid system indicating the possible role of Nobiletin in antioxidant activity (Malhotra et al., 2009).

LPO is initiated by the free radicals through peroxidative degradation of membrane lipids rich in poly-unsaturated fatty acid (PUFA) and, ultimately, terminates in the formation of stable products such as malondialdehyde (MDA) and hydro-peroxides. Hence, MDA and hydro-peroxides function as markers of LPO. Moreover, antioxidant such as GST and GSH plays an important role in the elimination of ROS and protects against oxidative stress. In accordance with the present findings, the significant decrease in the activities/levels of GST and GSH was due to exhaustion of the activities of enzymes as a result of oxidative stress. Depletion of GSH impairs the ability of the cells to protect against the free radicals and results in enhanced LPO during pulmonary carcinogenesis in B(a)P-administered mice (Kosower, N.S et al., 1976). Nevertheless, Nobiletin treatment bolster the antioxidant defense system as depicted by the increased tissue levels of GST and GSH. The supposition
was that Nobiletin functioned as an antioxidant/anti-lipid propagating agent against oxidative damage through termination of peroxyl radical-mediated reaction.

The Nobiletin could protect the cell to the oxidative stress induced by exogenous addition of H₂O₂ could protect normal lymphocytes from diseases related to oxidative stress, including cancer (Roberto et al., 2009). The nobiletin biotransformation extract have free radical scavenging activities and inhibit lipid peroxidation (Mario et al., 2009). Monoterpenes in daily diet would be relevant for offering some protection against lipid peroxidation and inherent mutagenic risk. Nobiletin supplementation negatively controlled spontaneous MDA formation, plasma MDA concentration in supplemented animals were significantly lower than in not supplemented rats and also greatly inhibited the oxidation stress induced by DMBA (Pattanaik, U et al., 1996). Nobiletin generated a greater amount of ROS followed by more cell death, makes H₂O₂ scavenging system break down more rapidly with decrease of GSH (Tanaka, S et al., 2004).

In the present investigation study, an increase in the levels of lipid peroxides were observed in B(a)P administrated lung cancer animals when compared to control animals and in nobiletin treated animals the levels were significantly reduced after treatment when compared to control. There is extensive evidence that supplementation of Nobiletin can enhance antioxidant enzymes. The antioxidant enzymes may reduce the carcinogen-DNA interaction by providing a large nucleophilic pool for electrophilic carcinogens thus quenching effect signifying its potent anti-peroxidative effect.

**Nobiletin on Pulmonary Antioxidant (Enzymic And Non Enzymic) Defense System**

Cells have an elaborate defense system for protection against free radical induced damage that involve generation of GSH, cysteine and antioxidant enzymes including GSH, GPx, SOD and CAT (Murakami, 2000). ROS are shown to be generally destructive to
biological material and consequently play a role in host defense (Suzuki, R. et al., 2004). Antioxidants are present in appropriate amount and localization thus the intracellular levels of ROS are regulated (Reid, 2006). They include, small molecules thioredoxin, ascorbic acid, vitamin E, uric acid and GSH and antioxidant enzymes such as CAT, GPx, GR, GST and SOD. Effective antioxidants defense are organized at multiple levels and include prevention, interception and repair. Natural antioxidant exhibit dynamic balance between the amount of free radicals generated in the body and antioxidant defense system that quench or scavenge them and protect the body against their deleterious effects (Kolanjiappan et al., 2002). Antioxidant are capable of inhibiting the ROS production and there by reducing the associated intracellular oxidative stress (Feng et al., 2001). The antioxidant enzyme plays an important role as a protective enzyme against ROS in tissue and comprise the cellular defense system (Guillaume et al., 2001). Antioxidant enzymes are the main scavengers of free radicals function as the inhibitors at both initiation and promotion or transformation stages of carcinogenesis (Jansen et al., 1993). Nobiletin is effective at both the initiation and promotion/progression stage of induced cancer (Maltzman et al., 1998). It is widely accepted that diet rich in terpenoids, alkaloids and flavonoids have a good antioxidant capacity. Nutraceutical contained in daily diet like nobiletin (terpene found in citrus fruit like lemon and orange peel) and other compounds are reported to be an excellent antioxidants. Nobiletin monoterpenes whose potential antioxidant and chemopreventive role has been reported (Sun, 2007; Chow et al., 2002; Morse and Toburen, 1996).

SOD convert superoxide radicals to hydrogen peroxide is widely distributed in cells having oxidative metabolism and believed to protect such cells against the toxic effect of superoxide anion (Fridovich, 1975). Superoxide dismutase protect against oxygen free radicals by catalyzing the removal of superoxide radicals hydrogen peroxide mediated LPO.
(Ekaambaram et al., 2007). Which in metabolized by CAT and GPx that damage the membrane and biological structure.

CAT is a heme protein widely distributed in all tissues and catalyses the breakdown of hydrogen peroxide to water it protect the cellular constituent against oxidative damage (Shimeda et al., 2005). In the lung, catalase is localized mainly in alveolar macrophages and alveolar epithelium (Kinnula, 1995). This enzyme is relatively constitutive, no major induction has been reported by cytokines or oxidants in the lung. Catalase has not usually been connected to malignancies, but its expression in mesothelioma is high (Kahllos, 2001). Suggesting that this enzyme may also be connected to highly resistant invasive tumors. This phenomenon may also have clinical significance since inhibition of catalase in vitamin by aminotriaxole has shown to potential oxidant toxicity in mesothelioma cells (Kinnula, 1998). Nobiletin exerted a biphasic effect on cell proliferation; the increase in cell proliferation was related to decrease in H$_2$O$_2$ levels by the increase in catalases and peroxidases activities (Roberto et al., 2009).

GPx plays a predominant role in detoxification of peroxides from the cells and tissues (Thirunavukarusu et al., 2001) to non toxic product and scavenges the highly reactive lipid peroxides in the aqueous phase of cell membrane (Vijayalakshmi et al., 1997). GPx coupled GSH reductase catalysis the conversion of oxidized glutathione to reduced glutathione and simultaneously NADPH is oxidized to NADP$^+$ (Kinnula, 1995). B(a)P induces the oxidation of mitochondrial NADPH (Zhuohan et al., 1994).This causes an increase in the NADP$^+/\text{NADPH}$ ratio. The low availability of substrate NADPH may be responsible for the decrease in the activity of GR. The lowered activity of GR decreases the conversion of oxidized GSH level, which in turn decreases the conversion of oxidized GSH in to reduced GSH level, which in turn decreases the activity of GPx.
GST is a multifunctional protein that perform functions ranging from catalyzing the detoxification of electrophilic compound to protective against peroxidative damage (Balaji Raghavendra et al., 2005). GR play a major role in regenerating GSH from GSSG thus maintaining the balance between redox couple (Buzby et al., 1980). This enzyme requires reducing equivalent (NADPH) for its activity, which provided by the action of G6PD.

The induction of antioxidant enzymes are a major strategy for protecting cell against a variety of endogenous and exogenous toxic compound such as ROS and chemical carcinogens. Nobiletin protect the cells to the oxidative stress induced by exogenous compounds. In the present investigation study shows a reduction in the activities of the antioxidant enzymes SOD, CAT, GPx, GST, GR and G6PD in lung cancer bearing animals. It can absorb free electrons also known as free radicals and hold them. This stops further free radical damage to cells. Nobiletin supplementation in the diet influenced GST activity, GST isoenzymes levels, GSH levels, Glutathione peroxidase activity (Esther et al., 1998). Thus, Nobiletin known for its antioxidant property. On Nobiletin treatment, the activities of these enzymes inclined to near normal. This may be due to the effect of Nobiletin to quench oxygen derived free radicals by donating hydrogen atom or an electron to chelate redox-active metals and inhibit lipoxygenases (Prakash et al., 2007). Hence, antioxidant status has been suggested as a useful tool in estimating the risk of oxidative damage induced carcinogenesis. Enzymatic antioxidants like SOD, CAT, GPx, GST and GR synergistically scavenge reactive oxygen species (ROS) and prevent LPO.

**Non Enzymic Antioxidants**

Reactive Oxygen Species (ROS) have been implicated in the pathology of many diseases. ROS are involved in cell growth, differentiation, progression and death (Mates et al., 1999). They play a major role in cancer initiation and promotion. Exposures of cells to
free radicals gives rise to DNA damage, causing mutagenesis and ultimately cell death, if the antioxidant system is faulty.

Maintenance of normal cell function in the presence of O₂ largely depends on the efficacy of the defense mechanism against free radical mediated oxidative stress (Forman and Fisher, 1982). Reduced glutathione, vitamin C and α-tocopherol comprise the non-enzymatic antioxidant components which protects the cells against the deleterious effects of the free radicals.

Normal human lung is efficiently protected and buffered against exogenous free radicals. Besides classical antioxidant enzymes (AOEs), epithelial lining fluid contains lower molecular weight antioxidants and proteins including a tripeptide glutathione (reduced glutathione, GSH), mucin GSH dependent enzymes and catalase that are located in the cytosolic compartment and peroxisomes. Among these antioxidants GSH is abundantly localized to the epithelial lining fluid of human lung and its reaction pathways are tightly linked to the reactions of other thiol containing proteins, that participate not only in scavenging of H₂O₂ but also in the regulation of the redox balance of the cells.

Reduced glutathione, chemically γ-glutamyl cysteinyl glycine is a predominant non-protein thiol present in virtually all cell types. Glutathione is ubiquitous in animals. GSH play an important role in detoxification of xenobiotic compound, in the oxidation of ROS and free radicals and decreased GSH levels signify increased oxidative stress. GSH depletion in the respiratory tract has been associated with increased risk of lung damage and pulmonary disease. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. It fulfills a wide variety of important functions such as detoxification of electrophiles, serves as a transfer vehicle for cystene and renders protection against ROS conjugation. The reduced glutathione in
tissues keeps up the cellular level of vitamin C and vitamin E in active forms. These vitamins also exist in inter convertible form and participate in neutralizing free radicals. When there is reduction, the level of GSH, the cellular levels of vitamin C and vitamin E are also lowered. In our investigation study, we observed a decline in GSH levels in lung cancer bearing animals, which may be due to excess utilization of this antioxidant for tumor cell proliferation. Nobiletin supplementation elevated GSH levels in Group 3 animals.

The ascorbate molecules is involved in the feedback inhibition of the lysosomal glycosidase responsible for the malignant invasiveness. Vitamin C, an important antioxidant acts in tissue, involving ROS in aqueous phase and it has been reported that the tissue concentration of vitamin C is a good indicator of oxidative stress. In cell and lining fluid of the respiratory tract lactoferin and vitamin E associated with lung surfactants have an important role in ROS pulmonary oxidant–antioxidant balance. Vitamin E is a principal lipid soluble antioxidant in cell membrane that protect critical cellular structure against oxidative damage. Vitamin E is the major lipid soluble peroxy radical scavenger, which can limit LPO, terminating chain reactions initiated in the membrane lipids. It is the most significant antioxidant of its kind in animal cell and it can protect against carcinogenesis and tumor growth. Vitamin C protects cell membrane and lipoprotein particles from oxidative damage by regenerating the antioxidant from vitamin E. Thus vitamin C and vitamin E act synergistically in scavenging wide variety of ROS. The concentration of vitamin E has been inversely correlated to LPO (Selvendiran et al., 2003).

In our present investigation study the decreased vitamin E content in lung cancer bearing animals might be due to excessive utilization of this antioxidant for quenching enormous free radicals produced in these conditions. Vitamin E acts a chain breaking antioxidant by donating its labile hydrogen atom from phenolic OH group to propagating
lipid peroxyl and alkoxyl radicals intermediates of LPO, thus terminating the chain reactions. The supplementation of Nobiletin to the experimental animals would have improved various cellular antioxidants and thiol content in tissues, which in turn reduces free radical formation during lung carcinogenesis. Nobiletin generated a greater amount of ROS followed by more cell death, makes H$_2$O$_2$ scavenging systems breakdown more rapidly with decrease of GSH. Nobiletin was effective against oxidative stress by countering the depleted antioxidant molecule and antioxidant enzymes.

**Histopathological Studies in the Lung of Control and Experimental Group of Animals**

Histopathological examination of lung section (H and E staining) of control and experimental group of animals were done because of the similarities in the histopathology and tumor progression stages between mouse and human lung adenocarcinomas, the mouse lung tumor model has been used extensively to evaluate the efficacy of putative lung cancer chemopreventive agents (Herzog et al., 1997). Group I control animals revealed normal architecture with small uniform nuclei. Group II lung cancer bearing animals, the lung showed alveolar damages and more number of pyknotic nuclei. Further the alveolar damage accompanied by increased number of hyperchromatic, irregular nuclei in the cells of an alveolar walls. Group III lung cancer bearing animals treated with Nobiletin animals markedly reduced the alveolar damage with near normal architecture. This shows the protective nature of Nobiletin. Group IV Nobiletin alone treated animals showed no appreciable change of histopathological abnormalities as that of control animals, indicating the non toxic nature of Nobiletin.

**Nobiletin on microsomal phase I and phase II xenobiotic metabolizing enzymes**
**Phase I and Phase II enzymes**

The primary site for xenobiotic metabolism is lung and many xenobiotics are substrate for cytochrome P₄₅₀ catalysed oxidations. Cytochrome P₄₅₀ enzymes are responsible for the metabolic conversion of many drugs to the polar metabolites via Phase I and Phase II reactions to earlier excretion. Cytochrome P₄₅₀ is a key enzyme responsible for metabolic activation and the carcinogenic potential of B(a)P. Furthermore, the lung epithelium take part in the detoxification of tobacco smoke component through metabolic activation by phase I and Phase II enzymes indeed alveolar macrophages clear the airway of tobacco smoke particles and therefore constitute an important defense mechanism. It is widely accepted that metabolic activation of xenobiotics by phase I enzymes is required for their cytotoxic, mutagenic and carcinogenic activities. Carcinogens such as polycyclic aromatic hydrocarbons require metabolic activation to cause DNA damage and cancer. Xenobiotic metabolizing enzymes play a major role in regulating toxic, oxidative damaging, mutagenic and neoplastic effect of chemical carcinogen. Phase I metabolism involves oxidative, reductive and/or hydrolytic reactions that cleave substrate molecules to produce a more polar moiety. Phase II reactions involve conjugation of certain endogenous molecules to the products of phase I reaction.

Most chemical carcinogens require transformation by phase I metabolizing enzymes into a more reactive form able to bind to DNA. If the resulting mutation is not repaired, it may initiate or promote the carcinogenesis process. The reactive chemical group introduced by phase I enzymes (or the original carcinogen) can be detoxified through conjugation by phase II metabolizing enzymes into a water-soluble compound which can then be eliminated from the body. In the current report, the Nobiletin has been shown to reduce the risk of cancer by inducing phase I and phase II detoxification enzymes in the liver that metabolize
carcinogens into harmless products in mammary carcinogen. The blocking chemopreventive effect of Nobiletin and other monoterpenes during the initiation phase of mammary carcinogenesis are likely to the induction of phase I and phase II carcinogen metabolizing enzymes, resulting in carcinogen detoxification. Chemopreventive dose of dietary Nobiletin induce total cytochrome P<sub>450</sub>. Cytochrome P<sub>450</sub> enzymes in liver microsome has shown to oxidize Nobiletin to several oxidation products 1,2 and 8,9-epoxides. Nobiletin inhibit DNA-adduct formation in liver, lung, spleen and kidney and to increase the levels of member of the cytochrome P<sub>450</sub> 2B and 2C families. Nobiletin induce carcinogen metabolizing enzymes especially the cytochrome P<sub>450</sub> II B sub family. Nobiletin inhibit the action of carcinogen in the target cells by enhancing the detoxification systems (kimura et al., 1996). In the present investigation the increased levels of Cyt P<sub>450</sub>, Cyt b5 and increased activities of phase I xenobiotic metabolizing enzymes namely NADPH Cyt p450 reductase and epoxide hydrolase in B(a)P administered lung cancer bearing animals.

The chemoprevention of Nobiletin may be related inactivate the ultimate carcinogen by induction of phase II enzymes results in protection against toxicity and chemical carcinogenesis, especially during the initiation phase. Several lines of evidence indicate that phase II xenobiotic metabolizing enzymes, such as glutathione-S–transferase (GST) and NAD(P)H (quinine-acceptor) oxidoreductase (NQO), play a major role in the cellular detoxification of oxidative damaging, genotoxic and carcinogenic chemicals. Phase II detoxification enzymes such as GST, UDP- GT and DTD are consider to be a major mechanism of protection against chemical stress and initiation of carcinogenesis. They are widely distributed enzymes that detoxify carcinogen either by destroying their reactive centers or by conjugating them to endogenous ligands facilitating their excretion. Mounting
evidences have suggested a relevant mechanism between the induction of phase II detoxifying enzymes and cancer chemoprevention.

Among the phase II enzymes GSTs are a family of enzymes that catalyses conjugation of reactive chemicals with GSH (reduced glutathione) and play a major role in protecting cells. After generating conjugated GSH these are subsequently eliminated via a GSH conjugate-recognizing transport. GSTs are a family of soluble proteins, which conjugate xenobiotics with glutathione. Metabolites after glutathionylation are more hydrophilic and thus biologically inactive. Therefore, they are readily excreted in bile or urine as conjugates. This action is thus believed to be the major mechanism for the detoxification of reactive ultimate carcinogens. It is now generally accepted that the GSTs are encoded by at least eight different gene families (including classes alpha, mu, pi, theta, zeta, omega, sigma and kappa) of cytosolic GSTs. Class Pi GST (GSTP1-1), one of the GST isozymes, can profoundly alter susceptibility to chemical carcinogenesis possibly through glutathione (GSH) conjugation to carcinogens.

Glutathione S-Transferase (GSTs) belong to a group of detoxification enzymes that also require intracellular thiol tripeptide. GSH for their catalytic activity with the multidrug resistance proteins (MRPs) in the transport of various drugs from the cells (Zhang, 1998). A negative correlation was observed between GST enzyme activity and tumor incidences in the mucosa along the human gastrointestinal tract, suggesting the importance of GSTs in cancer prevention. It has been reported that antioxidant properties of Nobiletin would related to induction of total cytochrome P450 and of phase II enzymes glutathione-s-transferase and UDP-glucuronyl transferase (Elegbede et al., 1993).

Glucuronidation, catalysed by UDP-GT family of enzymes is a major metabolic pathway of endogenous steroids, bile acids, drug and carcinogen. Several phytochemicals
are known to cause elevation in the activities in the GST through the induction of the microsomal detoxification enzymes UDP-GT gene complexes.

DTD another phase II enzymes is a flavoprotein that catalyze two-electron reduction of quinines, quinione imines and nitrogen oxide. The reaction prevent the formation of semiquinone. Reduction of quinines and nitrogen oxide might also make them available for conjugation with UDP-glucuronic acid and facilitating their excretion. Hence DTD act as an early cellular defense against tumorigenesis. There is substantial evidence that phase II drug metabolizing enzymes, e.g., GST, NQO1, epoxidehydrolase, hemoxygenase and UDP-glucuronosyl-transferase, plays an important role in the detoxification of electrophilic toxicants and their induction protects against carcinogenesis and mutagenesis.

Nobiletin treatment lead to an enhanced B(a)P detoxification and elimination as well as reduction of BPDE-DNA adduct formation. Nobiletin effectively inhibited the formation of rat tracheal epithelial cell by B(a)P the mechanism of inhibition of nobiletin its ability to stimulate xenobiotic metabolizing enzymes. Thus our present data suggests that Nobiletin may exert its beneficial effect through modification of the metabolic activation and/or detoxification of the carcinogen by modulation of phase I and II drug metabolizing enzymes during lung carcinogenesis. Hence in the present investigation study suggest that the enzymes modifying capability of Nobiletin might plays an important role in its anticarcinogenesis potency against B(a)P induced experimental lung cancer.

**Nobiletin on TCA Enzymes**

Alterations in mitochondrial functions i.e., maintenance of ion homeostasis or ATP supply, have repeatedly been suggested to contribute to cellular transformation (Pedersen, 1978). The NAD\(^+\)/NADP\(^+\) linked citric acid cycle enzymes regulate the NADH levels through the allosteric stimulation by ADP, the content of which increase with breakdown of
ATP. These NAD+/NADP+ dependent major enzymes of citric acid cycle ICDH, α-KDH, MDH are involved in the maintenances of the reduced redox state in mitochondria in order to provide the reducing power to generate ATP via oxidative phosphorylation, unlike the other citric acid cycle enzymes, which are soluble in the mitochondrial matrix, the FAD dependent SDH is an integral protein of the inner mitochondrial membrane that is a strategic location for the regeneration of its prosthetic group (Anandakumar et al., 2008).

SDH is a marker enzyme in TCA cycle and succinate, phosphate and ATP promote its activity. Being a regulatory enzyme its property is altered when it is solubilised. ICDH refers to the NADP+ dependent enzyme, which in several tissues has dual localization being in part of cytoplasmic and in part of mitochondria. Availability of oxalate is controlled by another chief enzyme in TCA cycle namely MDH, which converts malate to oxaloacetate. Decreased activities of these enzymes might be due to the alteration in cancer cell morphology, ultrastructure and ability of mitochondria to undergo metabolic changes and also the number of mitochondria is drastically reduced in cancer cells (Anandakumar et al., 2008b). Nobiletin has chemoprotective effect against rodent and human cancer. This activity is observed both at initiation and promotion.

In the present investigation study, we have observed decreased activities of major TCA cycle key enzymes such as ICDH, SDH, MDH and α-KDH in lung cancer bearing mice and on Nobiletin pretreatment increased the activities of these mitochondrial enzymes close to normalcy suggesting its chemoprotective nature.

Nobiletin on electron transport complexes

Electron transport and oxidative phosphorylation alone require the coordinated action of five enzyme complexes, which together are composed of different structural proteins. A reduction in enzyme content could arise in a failure of assembly of electron
transfer chain complexes or enhanced rates of degradation of complexes. NADH-ubiquinone oxidoreductase, also known as Complex I is a multi subunit integral membrane complex of the mitochondrial ETC which catalyzes electron transfer from NADH to ubiquinone. Coupled to the transfer of electrons, protons are vectorially translocated across the mitochondrial inner membrane to establish an electrochemical gradient used for the synthesis of ATP. Cytochrome Bc1 complex (Complex III) is considered to be crucial for the activity of the entire respiratory chain and appears to be well coupled with succinate dehydrogenase (Complex II). Cytochrome c oxidase is the terminal enzyme of the mitochondrial respiratory chain, catalyzing the reduction of molecular oxygen with electrons from reduced cytochrome c and concomitantly conserving the reaction energy by pumping protons across the inner mitochondrial membrane. A possibility is that the phospholipid membrane environment, surrounding the protein complexes may become relatively less optimal during cancer progression. An increased LPO has been reported to alter the lipid environment of the membrane thus affecting the activity of these respiratory chain enzymes in B(a)P treated animals (Senthilnathan et al., 2006).

Decrease in the activities of ETC complexes may in turn promote the leakage of electron from the mitochondrial inner membrane associated with electron transport complexes contributing to increased mitochondrial ROS generation. Decreased activities of ETC complexes were observed in B(a)P induced lung cancer animal. Nobiletin inhibits the synthesis of ubiquinone (co enzyme Q10) in tumor cell mitochondria there by reducing the amount of chemical energy produced to meet metabolic needs.

The results of our present investigation demonstrate the chemoprotective efficacy of Nobiletin in modulating LPO and restored the membrane structure leading to marked increase in the activities of these complexes enzymes, mitochondrial antioxidants and TCA
cycle enzymes. These observations are important because Nobiletin could conceivably protect against chemically induced carcinogenesis. Biochemical parameters carried out in our study that proved the mitochondrion stabilizing effect of Nobiletin during B(a)P induced lung cancer.

**Nobiletin on Lyosomal Enzymes**

The lysosomal compartment is responsible for the controlled recycling of cellular organelles and macromolecules. Lyosomes are essential for controlled intracellular digestion of cellular components by different pathways such as autophagy, heterophagy and endocytosis. The location of acid hydrolases from the lysosomes to the cytosol leads to cellular injury and death. Lysosomal hydrolases participate in the digestion of endocytosed and autophagocytosed material inside the lysosomal/autolysosomal compartment in acute cell death, when released into the cytosol and in cancer progression following their release into extracellular space. Lysosomal alteration are common in cancer cells the increased expression and altered trafficking of lysosomal enzymes participates in tissue invasion, angiogenesis and sensitization to the lysosomal death pathway. Lysosomal acid comprise a variety of proteases, nucleases, glycosidase, sulfatases and lipases.

An increase in the expression, secretion and/or activity of various lysosomal proteases has been demonstrated in numerous human tumors including breast, lung and brain cancer (Berchem et al., 2002). A complete correlation between expression of lysosomal hydrolases and aggressiveness of breast, prostate or gastric cancer has been reported. The diagnostic and prognostic potential of lysosomal proteases as cancer markers has previously been evaluated. It has been shown that in some types of tumors, the high content of lysosomal proteases is correlated with a higher risk of recurrence and with a poor
prognosis. Beside their usefulness as marker, lysosomal enzymes and act as genuine player in cancer development.

Tumor invasion and metastasis are associated with altered lysosomal trafficking and increased expression of the lysosomal proteases termed cathepsins (Nicole Fehrenbacher and Maria Jaattela, 2005). The main class of lysosomal proteases is represented by the cathepsins that are possibly involved in autophagic digestion of discrete areas of cytoplasm and mitochondrial proteins. Proteases of the cathepsin family are among the best studied lysosomal hydrolases. Cathepsins can be divided into three subgroups according to their active-site amino acid, i.e., cysteine (B, C, H, F, K, L, O, S, V, W, and X/Z), aspartate (D and E) and serine (G) cathepsins (Rawlings and Barrett, 1999). Apart from their function in general protein turnover, certain cathepsins perform more specific functions in the control of cell-cycle progression, antigen presentation, epidermal homeostasis and hair follicle morphogenesis. Cathepsins have interesting functions outside the lysosomal compartment degradation of extracellular matrix when secreted to the extracellular space and execution of programmed cell death when released to the cytosol. Cathepsins have been implicated in cancer progression, in particular the cysteine cathepsins B and L and the aspartate cathepsin D. High expression levels of these cathepsins offer a reliable diagnostic marker for poor prognosis (Kos and Lah, 1998).

An over expression of cathepsin D in cancer cell is associated with increased risk of metastasis. The aspartyl protease cathepsin D can influence multiple tumor progression steps such as cell proliferation, angiogenesis and apoptosis of interest, its catalytically inactive form is still able to promote tumor progression and angiogenesis suggesting that this protease could stimulate tumor growth by acting, directly or indirectly, as a mitogenic factor on both cancer and endothelial cells independently of its catalytic activity (Devipriya
et al., 2006). Moreover, cytosolic cathepsin D levels in endometrial adenocarcinoma correlate with the extent of tumor differentiation and myometrial invasiveness.

The lysosomal protease is over expressed in primary breast cancer where its concentration has been correlated with rapid development of metastasis. Metastasis human breast cancer cells transfected with an antisense construct against the protease presented tumor growth in nude mice compared with control. These result point to the crucial role of cathepsin D in cancer development. In breast carcinoma high level of cathepsin D are correlated with poor prognosis.

Cathepsin B is an other important lysosomal protease and its activity appear to be positively correlated with the metastatic potential of cancer. Tumors, which overexpress cathepsin-B and exhibit a powerful capacity for invasiveness in the early stage of carcinoma. In a mouse model of pancreatic cancer, pharmacologic inhibition of cysteine cathepsin B activity impaired angiogenic switching in progenitor lesions and inhibited tumor growth, vascularity and invasiveness. Cathepsin-B contributes to biochemical processes underlying tumor metastasis, high level of expression in breast, colon, colorectal, lung carcinomas and associated with significantly shorter survival in non-small lung cancer (Sukoh et al., 1994).

B(a)P is known to produce enormous of free radicals and these free radicals can induce very fast lysosomal disruption creating intra lysosomal iron-mediated redox reaction. The free radical mediated oxidative stress has been reported to induce lysosomal disruption in alveolar cells leading to the release of lysosomal enzymes in to the serum (Yin et al., 2005).

Our result are agreement with these findings as we have observed increased activities of lysosomal proteases cathepsin D, cathepsin B, together with β-D-glucosidase, β-
D-N-galactosidase, β-D-glucuronidase, β-D-N acetylglucosaminidase, acid phosphatase in the lung and serum of cancer bearing animals

Thus in the present investigation study shows that B(a)P administration resulted in pronounced damage to oxidative damage to lysosomes in the lung tissue, Nobiletin supplementation was effective in preventing these change and preserving lysosomal stability which showed the chemopreventive function of Nobiletin in ameliorating lysosomal abnormalities during B(a)P induced lung carcinogenesis.

**Impact of Nobiletin on Inflammatory, Metastatic and Proliferative Studies**

**Effect of Nobiletin on Mast Cell Density**

Mast cells (MCs) are a group of long living cells of bone marrow origin that are commonly found in the skin, gastrointestinal and respiratory system. These cells produce, store and release a high number of bioactive mediators especially cytokines. MC numbers increased in association with various malignant tumors (Lampiasi et al., 2007). Mast cell possess pleiotropic properties for cell growth arrest (apoptosis) as well as tumor promotion and angiogenesis. There is also evidence that tumor associated mast cell accumulation promotes tumor growth and metastasis in some condition. Recently, MC accumulation has also been shown in lung cancer.

The decrease in ratio of mast cells to tumor cells are was correlated with reduced survival rates in patients with pulmonary adenocarcinoma. A significant relationship exist between the presences of mast cell and tumor angiogenesis in lung cancer. The protective effect of essential oil of Protium hepatophyllum resin the major constituents were nobiletin in mice and rats significantly inhibited mast cell degranulation that has anti-inflammatory activity. These above findings corroborate with our present observation there was increased level of toluidine blue stained mast cells in the lung section of lung cancer induced animals,
thus suggesting that recruitment of mast cells increased in lung cancer. Whereas Nobiletin treated animals showed significantly decreased the level of mast cells in the lung. Thus one of the mechanisms by which Nobiletin inhibited angiogenesis and invasion is through the stabilization of mast cells.

**Effect of Nobiletin on Cell Proliferation**

Cell proliferation plays an important role in multistage carcinogenesis and involve multiple genetic alteration, hence assessment of cell proliferation is an crucial means to designate the complications of hyperplastic, premalignant and malignant lesion associated with carcinogenic process. Therefore in our present investigation we have focused on the analysis of glycoproteins and proliferating cell nuclear antigen (PCNA) to reveal the anti-proliferative effect of Nobiletin against B(a)P induced lung carcinogenesis.

**Nobiletin Regulates the Expression of Proliferating Cell Nuclear Antigen (PCNA)**

Proliferating cell nuclear antigen (PCNA) is a highly conserved 36 KDa nuclear protein, associated with DNA replication and cell proliferation, also known as cyclin or auxiliary protein essential co-factor for DNA polymerase gamma and DNA polymerase. Expression of PCNA increases at the end of G1 period, reaches its maximum in S-phase, decline during G2 phase, and is absent during the mitotic phase and in quiescent cells. PCNA as a cell proliferation marker has its application in toxicology and as a prognostic marker in human tumors (Miyamoto S et al.,2008).

PCNA was first identified with the use of autoantibodies present in some patients with lupus erythematosus. Nuclear immunoreactivity of PCNA was also found in the proliferative compartment of normal adult tissue and was used as an index of cell proliferation in various tumors including lung cancer. Since PCNA is considered as a
marker of proliferation it may likely be related to tumor aggressiveness, vascular invasion and clinical behavior.

PCNA immunohistochemistry may be a useful prognostic indicator of malignancy. The detection of PCNA is a common way to study the proliferating activity of transformed cells. Increased number PCNA positive cells in B(a)P Induced lung cancer animal signifies the hyperproliferative activity of the tumor cells. Reducing cell proliferation is one of the hallmark of cancer chemoprevention and thus Nobiletin treatment markedly reduced the expression of PCNA that suggest its anti-proliferative effect during lung carcinogenesis.

Proliferating cell nuclear antigen (PCNA), a highly conserved nuclear protein of DNA polymerase-delta, has been found to be a useful marker to assess tumor cell proliferation and progression (Keshgegian A et al., 1995). Elevated expression of PCNA in the lung of B(a)P-induced animals indicates the hyper proliferative activity of tumor cells. Reducing cellular proliferation was one of the hallmarks of controlling the carcinogenic process (Jagan S et al., 2008); hence, the effect of Nobiletin on cell proliferation in the B(a)P-administered mice was assayed by PCNA as a proliferative marker. Decreased expression of PCNA upon Nobiletin treatment depicts antiproliferative efficacy of nobiletin. The result of this study states that nobiletin diminished PCNA expression in rat mammary carcinogenesis.

Proliferating tumor cells, their surrounding host stromal cells and tumor-infiltrating inflammatory/immune cells create a tumor microenvironment that reflects a persistent inflammatory state (Ariztia EV et al., 2006). Within the tumor microenvironment, various proinflammatory mediators participate in a complex inflammatory signaling that facilitates extravasation of tumor cells through the stroma, thereby fostering tumor progression (Philip M et al., 2004).
Effect of Nobiletin on Inflammation

Role of Nobiletin on TNF-alpha, IL-6, COX-2 and NF-kB

Among the major molecular players involved in the inflammation-to-cancer axis are proinflammatory cytokines, namely TNF-a, COX-2 and NF-kB. As a representative inflammatory cytokine with pleiotropic functions, TNF-a plays a dual role in carcinogenesis. While a high concentration of TNF-a is destructive to tumor vasculature and causes necrosis, it may stimulate the growth of fibroblasts and certain tumor cells (Noguchi M et al., 1998). Several preclinical studies have suggested TNF-a as an endogenous tumor promoter (Ben-Baruch A et al., 2003, Tselepis C et al., 2002).

IL-6 is another major proinflammatory cytokine that participates in carcinogenesis (Rose-John S et al., 2007). IL-6 modulates the expression of genes involved in cell cycle progression and inhibition of apoptosis, primarily via the JAK-STAT signaling pathway (Kai H et al., 2005). An elevated level of IL-6 has been implicated in the pathogenesis of different cancers (Schneider MR et al., 2000, Chung YC et al., 2003).

Cyclooxygenase (COX) is the rate-limiting enzyme in the production of prostaglandin from arachidonic acid. COX-2, an inducible form of cyclooxygenase, serves as an interface between inflammation and cancer (Aggarwal BB et al., 2006). Aberrant induction of COX-2 has been implicated in the pathogenesis of various types of malignancies, including those arising in the lung (Hida T et al., 1998, Ristimaki A et al., 1997, Yip-Schneider MT et al., 2000). Different transcription factors are abnormally turned on or switched off in various human malignancies. Among these, NF-kB has been most extensively investigated because of its ubiquitous presence and multiple functions.

The induction of proinflammatory cytokines such as IL-6 and TNF-a and COX-2 several adhesion molecules are mediated via transcriptional activation of NF-kB (Naylor
MS et al., 1993, Lawrence T et al., 2007). Henceforth, NF-kB has been identified as a potential molecular bridge between inflammation and cancer (Pikarsky E et al., 2004). While the cytokine expression is regulated primarily by NF-kB, the tumor cell-derived cytokines further stimulate NF-kB-mediated transcription of proinflammatory genes in tumor cells, tumor associated stromal cells and host tissues, thereby creating a sustained chronic inflammatory state within the tumor microenvironment (Lu H et al., 2006). In our present study, the increased expressions/levels of TNF-a, IL-6 and COX-2 in lung cancer bearing animals may perhaps be due to enhanced activity of NF-kB during lung carcinogenesis.

NFkB one of the ubiquitous redox sensitive transcription factors, regulates many biological processes including cellular proliferation, differentiation and inflammation. The main inducible form of NFkB is heterodimeric consisting of the p50/p65 subunits. In resting cells NFkB resides along with its cytosolic repressor inhibitory protein IkB. Exposure with different stimuli including oxidative stress and phorbol esters causes the phosphorylation and degradation of IkB by cytoplasmic IkB kinase (IKK) which results in the nuclear translocation of NFkB. In the nucleus, it regulates the transcription of array of target genes including proinflammatory mediators, such as iNOS, COX-2, various cytokines, chemokines, and adhesion molecules (Pahl, 1999; Baeuerle and Baltimore, 1996; Chao et al., 2010).

Previous findings implicate that activation of NFkB triggers transcriptional up-regulation of COX-2 and proinflammatory cytokines, such as IL-6 and TNF-a (Wu et al., 2008). The present study findings show that cutaneous application of TPA leads to the activation of NFkB in mouse which was strongly suppressed by nobiletin pretreatment suggesting the strong anti-inflammatory and therefore anti-tumour promoting potential of nobiletin. Immunohistological staining and anti-proliferative potential of nobiletin against
TPA induced early tumour promotional changes in mouse skin. Inhibition of COX-2 expression, production of proinflammatory cytokines and activation of NFkB provide the molecular basis for the anti-inflammatory potential of nobiletin.

**Effect of Nobiletin on Metastasis**

**Role of Nobiletin on Matrix Metalloproteinase’s (MMP-2 & MMP-9)**

Mast cells activate matrix metalloproteinases (MMPs) expression. MMPs has been found to be increased in virtually every type of human cancer and correlates with advanced stage, invasive and metastatic properties and resulting in poor prognosis. Early expression of matrix metalloproteinases, either by cancer cells themselves or by surrounding stromal cells, helps to remodel the extracellular matrix (ECM) and release ECM and/or membrane bound growth factors (GFs), which provides a favorable micro environment for the establishment of primary cancer. Both MMP-2 and MMP-9 have been implicated in the induction of the angiogenic switch in different model systems (Egebald et al., 2002). Angiogenic switch can occur very early in some cancers, even before cancer cell progression with increased vessel density seen in pre-cancerous lesions.

MMPs are required for migration, metastatic growth and angiogenesis for better growth. Spreading of cancer cell to the surrounding environment is one of the primary reasons for the mortality in lung cancer. From the results of the present research it is evident that Nobiletin treatment suppresses the MMP-2 and MMP-9 protein and mRNA expression in cancer induced animals. In the present study, it can be speculated that increase in the levels of mast cells responsible for the increased levels of MMP-2 & MMP-9 in the B(a)P induced cancer bearing animals. Nobiletin treatment resulted in decreased mast cells number thereby might have decreased the expression of MMP-2 and MMP-9.

**Effect of Nobiletin on Apoptosis**
Apoptosis is an evolutionarily conserved suicide programme residing in all cells. It leads to cell death through a tightly regulated process resulting in the removal of damaged or unwanted cells. It also plays an important role in the development of various diseases including cancer (Evan and Littlewood, 1998). Apoptosis has been recognized to maintenance of tissue homeostasis by the specific elimination of excessive cells (Nayfield et al., 1991). Apoptosis process play a crucial role in both proliferation and turnover of cells in various tumors. Additionally, induction of apoptosis of cancer cell is recognized as a valuable tool for cancer treatment. Recently, considerable attention has been focused on dietary and/or pharmacological manipulation of apoptosis as a novel and promising strategy for cancer chemoprevention as well as therapy.

Nobiletin provoke apoptosis in many experimental studies. Nobiletin has been reported to induce apoptosis on tumor cells (Hata et al.,2003). Hence interest has been focused on the manipulation of apoptotic process for the treatment and prevention of cancer. Many efforts have been directed towards the search for compounds that influence apoptosis.

Hence in present investigation study attempts were made to explore the possible mechanism of induction of apoptosis by Nobiletin in B(a)P induced lung carcinogenesis. In our present investigation, agarose gel electrophoresis, mast cell staining and the expression of some apoptosis related proteins & gene expression therefore analyzed to reveal the apoptosis inducing effect of Nobiletin in different dimension.

**Nobiletin on Expression of p53, Bel 2,Bax and Caspases-3**

**Nobiletin Induced Apoptosis Through P53 Expression**

The p53 gene which is strongly implicated in animal and human carcinogenesis is a significant regulator of the process of apoptosis. p53 recruitment in response to various genotoxic stresses is an important cellular response to maintain the integrity of the genome.
A loss of p53 function result in an enhanced frequency of genomic rearrangement or genetic instability (Livingstone et al., 1992). The p53 mutation are recognized to be the most common genetic change in human cancer and p53 act as tumor suppressor gene while apoptosis pathway is regulated to induction of p53. This pathway is held in check by the antiapoptotic gene Bcl-2. Although the induction of the p53 protein has been studied extensively, a vast majority of the studies have been done using in vitro models. Induction of p53 after B(a)P exposure has been seen in mouse. The PAH and its metabolite result in rapid accumulation of the p53 gene products in human and mouse cell (Vaziri and Faller, 1997).

The p53 is a tumour suppressor protein, whose function is opposite to Bcl 2 was observed not only in the nuclei associated with heterochromatin but also in the cytosol. p53 stops a cell cycle in the G1 phase when its DNA is damaged. If this damage could not be repaired by itself, the cell would undergo apoptosis. p53 a tumor suppressor protein is believed to play an integral role in cellular response pathway to DNA damage. B(a)P induced protein accumulation also involved p53 promoter activation. The p53 is a transcription factor that plays a key role in the cellular response to DNA damage and has been called the guardian of genome (Kelman, 1997) or watchman of the cell cycle.

The p53 gene alterations play an important role in lung cancer. In general, p53 protein expression is very low in normal cells and this low expression level is caused by its short half-life. Mutations in the p53 gene often greatly prolong the half-life of protein. The cellular p53 protein levels increase upon exposure to B(a)P and its metabolites and suggested that this increase is due mainly to an increase in p53 protein stability (Khan et al.,1997). In lung cancer p53 mutational patterns are different between smokers and non-smokers with an excess of G-T transversion in smokers associated cancer, recent studies have indicated that there is a strong coincidence of G-T transversion hot spots in lung cancer
and sites of preferential formation of PAH adduct along with p53 gene. The p53 mutation in
lung cancer can be attributed to direct DNA damage from cigarette smoke carcinogen. p53
mutation are common in lung cancer, the chemical carcinogen such as B(a)P in cigarette
smoke cause G-C to T-A transversion at p53 codon 157,248 and 249 and that non tumors
lung tissue from smokers with lung cancer carry a high p53 mutational load at the codons.

In the studies, p53 expression was found to be high in the Nobiletin treated animals. The p53 levels were found to be very much lowered in lung cancer bearing animals than the normal animals and the over expression were seen in Nobiletin treated animals. This may prove the apoptotic property of the Nobiletin.

Nobiletin on the expression of caspases 3, Bax , Bcl-2

Caspases are proteases that cleaves other proteins and therefore essential for
apoptotic cell death .In response to apoptotic stimuli, such as radiation, APAF-1 binds to
Cytochrome c and procaspase 9 in the presence of adenosine triphosphate to form
multiprotein complex called the apoptosome (Watson, 2004). Formation of apoptosome
results in the activation of procaspase 9 by autocatalytic cleavage initiating a cascade of
downstream effectors caspase especially caspase 3 which cleaved several cellular proteins
ultimately leading to apoptosis. Activation of procaspase 3 requires proteolytic processing
of its inactive zymogen into activated p17 and p19 subunits. Treatment with Nobiletin has
been shown to not only up regulate the expression of procaspase 3 but also its activation
during the preneoplastic condition of B(a)P induced lung carcinogenesis. This implies the
pro-apoptotic efficacy of Nobiletin.

The Bax is an apoptosis promoting member of the Bcl-2 protein family are anti-
apoptotic protein that are found to be over expressed in wide variety of cancers. The Bcl-2
protein is known to form hetero dimers with the Bax protein \textit{in vivo} and the molar ratio of Bcl-2 to Bax determines whether apoptosis is induced or inhibited in Several tissues. The Bax protein controls cell death through its participation in disruption of mitochondria and subsequent Cytochrome c release and is also considered to be one of the primary p53 targets (Marzo et al., 1998).

In our experiment, there was decreased expression of pro apoptotic Bax with subsequent increased expression of anti apoptotic Bcl-2 in both B(a)P induced lung cancer animals. Bcl-2 can block mitochondrial permeability transition pore opening thereby prevents the release of caspases activators from mitochondria. Over expression of Bax has been shown to accelerate apoptosis where as Bcl-2 represses the death function of Bax. The proto oncogene Bax form a heterodimer with Bcl-2 and accelerate the process of apoptosis. Thus ratio between Bcl-2/Bax might be one of the critical factors of cells threshold for undergoing apoptosis. Treatment with Nobiletin decreased the expression level of Bcl-2 and increased Bax concentration thereby decreasing the Bcl-2: Bax ratio.

Nobiletin resulted in higher ROS generation, depletion of GSH, accompanied by increased caspases activity and also triggered a series of effect involving Cytochrome c, cleavage of caspases-3 and poly (ADP ribose) polymerases, and a shift in Bad: Bcl –xl ratio in favour of apoptosis. Nobiletin induces apoptosis by down regulation expression of Bcl-2 and mutant p53 in human leukemia cells \textit{in vitro} (Guo et al., 2006). Nobiletin up-regulates Bax coupled with release of Cytochrome c from mitochondria leading to increased caspases-3 and caspases-9 (Guo et al., 2006; Gao et al., 2006). These observation indicate that the relationship between p53, Bcl-2/Bax ratio, caspases-3 activation and enhanced apoptosis induced by Nobiletin treatment resulted in the restriction of B(a)P induced lung carcinogenesis.