Molecular analysis of tobacco carcinogen induced experimental lung tumors and chemoprevention studies

By

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Miss. Rasika R. Hudlikar entitled "Molecular analysis of tobacco carcinogen induced experimental lung tumors and chemoprevention studies" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / at this or any other Institution / University.

Navi Mumbai Miss. Rasika R. Hudlikar

Date:

List of Publications arising from the thesis

Research articles:

- Rasika R Hudlikar, V. Venkadakrishnan, Rajiv Kumar, Rahul A. Thorat, Sadhana Kannan, Arvind D. Ingle, Saral Desai, Girish B. Maru and Manoj B. Mahimkar. Polymeric black tea polyphenols (PBPs) inhibit benzo(a)pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone induced lung carcinogenesis potentially through down-regulation of p38 and Akt phosphorylation in A/J mice. Molecular carcinogenesis, 2017 Feb;56(2):625-640.*doi: 10.1002/mc.22521, PMID: 27377358*

- Rasika R Hudlikar, Venkatesh Pai, Rahul Thorat, Arvind Ingle, Girish B. Maru, Manoj B. Mahimkar. PBPs decreases B(a)P induced DNA adducts in lung by modulation of CYPs and GSTs in dose dependent manner (MS, under review).

- Rasika R Hudlikar, Rahul Thorat, Arvind Ingle, Girish B. Maru, Manoj B. Mahimkar. Effect of pre and post treatment of PBPs on B(a)P induced BPDE-DNA adducts in lung and liver (MS under preparation).

Review:

- Girish B. Maru, Rasika R. Hudlikar, Gaurav Kumar, Khushboo Gandhi, Manoj B. Mahimkar. Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. World J Biol Chem. 2016 Feb 26;7(1):88-99. doi: 10.4331/wjbc.v7.i1.88. Review, *PMID: 26981198*

Others:

- Tanmoy Bhattacharjee, Sneha Tawde, Rasika Hudlikar, Manoj Mahimkar, Girish Maru, Arvind Ingle, C. Murali Krishna. Ex vivo Raman spectroscopic study of breast metastatic lesions in lungs in animal models. J Biomed Opt. 2015 Aug;20(8):85006. doi: 10.1117/1.JBO.20.8.085006. PMID: 26295177.

Conference abstracts

- **Poster presentation**: International Conference on "Global cancer: Occurrence, Causes and Avenues to Prevention" organized by International Agency for Research on Cancer (IARC), Lyon, France, on 7- 10th June 2016. Poster presentation entitled "Modulation of molecular markers by polymeric black tea polyphenols (PBPs) in tobacco carcinogen induced lung carcinogenesis in A/J mice".
- **Oral presentation**: Conference on "Cancer in Asia: bridging the gaps" organized by Indian Association for Cancer Research (IACR), India, on 8- 10 April 2016. Oral presentation on "Modulation of tobacco carcinogen induced early molecular markers by various doses of Polymeric black tea polyphenols (PBPs) in lung and liver of A/J mice". Published in Journal of Carcinogenesis, 2016 and received **an award as Mangala Bamne young scientist Best Oral Presentation**.
- **Poster presentation**: International conference on "New ideas in Cancer-Challenging dogmas", organized by Tata Memorial Centre Platinum Jubilee Celebration, Mumbai on 26-28 February 2016. Poster presentation on "Chemoprevention of lung cancer by Polymeric Black tea Polyphenols (PBPs): preclinical evaluation of potential molecular targets".
- **Oral presentation**: International conference on "Promotion of animal research, Welfare and Harmonization of Laboratory Animal Science" organized by Laboratory Animal Scientist's Association (LASA) India, on 15-16 October 2015. Oral presentation on "Evaluation of chemopreventive efficacy of Polymeric Black tea Polyphenols (PBPs) in carcinogens induced A/J mice model during sequential lung carcinogenesis" and received **an award as Best Oral Presentation**.
- **Poster presentation**: International conference on "Molecular pathway to therapeutics: paradigms and challenges in oncology", organized by Carcinogenesis foundation, USA. Carcinogenesis, 11-13 February 2015, presented a poster "Polymeric Black tea Polyphenols (PBPs) modulates tobacco carcinogen induced cellular responses in A/J mice lung carcinogenesis model." Published in Journal of Carcinogenesis, 2015; 14(Suppl 1): S21–S38, 2015; 14(Suppl 1), Published online 2015 Feb 10.

Rasika R. Hudlikar

Dedicated to Aai, Baba, family and friends who supported unconditionally throughout this journey…!

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Contents

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SYNOPSIOS OF Ph.D. THESIS

1. **Name of the Student:** Rasika R. Hudlikar
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Synopsis

Introduction:

Our understanding about the molecular genetic alterations in sequential development of human cancers is limited (1). Several studies have been conducted using tobacco-specific carcinogens in animal models which focused on specific molecular pathways involved in carcinogenesis including proliferation, apoptosis, inflammation, etc. None of the studies till date have carried out molecular analysis during sequential development of lung tumors induced using cigarette smoke carcinogens (2-4). Our proposed study is comprehensive approach which will enable us to understand the cross talk between these pathways during the early development and progression of cancer. Hence we have proposed the molecular analysis during sequential development of lung tumors in A/J mouse induced by benzo(a)pyrene [B(a)P] and 4-

(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) - potent carcinogens found in tobacco smoke. Extensive studies are carried out on mechanism of actions of these carcinogens. However, modulation of different genetic and molecular signal transduction pathways during the process of sequential progression of lung carcinogenesis remains to be elucidated.

Chemoprevention or dietary phytochemical intervention is emerging as an important modality for cancer prevention. Chemoprevention or dietary and phytochemical intervention is emerging as an important modality for cancer prevention. Cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer [9]. Vegetables and fruits consumption has been hypothesized to influence lung cancer risk. Recent reports convincingly depict that the risk of lung cancers was reduced with increasing variety in fruit and vegetable consumption in current smokers (9). Number of dietary phytochemicals have been shown to protect against free radical(s)/ chemical(s)-induced toxicity and carcinogenicity in experimental model systems. These evaluations have been carried out using in vitro pre-screen tests and in vivo experimental models employing tumor as the end-point. However, these properties are not always reproducible in vivo experimental system(s) and / or clinical trials.

Tea, a commonly consumed beverage derived from the dried leaves of the *Camellia sinensis* plant, has been studied extensively for its health benefits, including cancer prevention [10]. Chemopreventive efficacy of polymeric black tea polyphenols (PBPs) has been established in various experimental models, although much remains to be elucidated *in vivo* to understand their molecular mechanism(s) of observed chemoprevention [11-18]. Therefore the other aim of this study is to examine the

molecular mechanism(s) of PBPs-mediated chemoprevention *in vivo*. This approach may be helpful in identification of several new molecular pathways and potential biomarkers that will be excellent intermediate end-points in monitoring of disease process including early stages of development and progression of tumors, chemoprevention trials and validation of drug effect measurements.

Hypothesis:

Molecular analysis during development of tumors will enable us to understand the cross talk between the pathways during the process of cell transformation. Molecular analysis during the process of chemoprevention by using polymeric black tea polyphenols (PBPs) can be give us an insight about interaction of different biochemical and molecular pathways in sequential lung carcinogenesis / chemoprevention process along with chemoprevention with PBPs.

Objectives:

- To determine the molecular mechanism involved in the sequential development of lung cancer.
- To elucidate the molecular mechanism of the chemopreventive action of PBPs in lung cancer

Extraction and characterization of polymeric black tea polyphenols (PBPs) from black tea

Extraction of PBPs from black tea powder:

Extraction of PBPs from Black tea powder was carried out using well established and previously published method by Krishnan et. al. (8). Briefly, black tea powder (450 g) in a thimble was decaffeinated with chloroform (2.5L) in a Soxhlet continuous extractor, until no colour was present in the solvent (~24 hrs.). Air-dried

black tea powder (447 g) in a thimble was further Soxhlet-extracted with ethyl acetate (2.5L, 24hrs.). The ethyl acetate extract was dried *in vacuo*, and was dissolved in acetone (200 ml) and precipitated with 8 volumes of diethyl ether, thrice, to obtain a precipitate of PBP-1 (~9 g). Whereas the air-dried black tea powder after ethyl acetate extraction constitutes the PBP-2, PBP-3, PBP-4, PBP-5 (~425 g) and other unknown constituents. Mixture of PBP-1 and PBP-2, PBP-3, PBP-4, PBP-5 mixture (air dried tea powder) was used for further study.

Characterization of PBPs:

Characterization of PBPs extracted from black tea powder was carried out using well established and previously published method using thin layer chromatography (TLC) by Krishnan et.al.(8). Isolated PBPs were further characterized using MALDI-TOF analysis.

MALDI-TOF analysis of PBPs :

MALDI -TOF analysis was employed additionally to confirm purity of PBPs. α-cyano-4-hydroxycinnamic acid (HCCA) matrix was used for sample analysis. Vacuum dried PBPs extract(s), with standards (EGCG, TFs,caffeine) were analyzed using reflector mode. Reported spectra of known biological contaminats were found to be absent in PBPs. However, PBPs specific peaks were reported at 876.76 and 854.80 m/z.

Animal Studies:

All animal studies were conducted after approval from the Institutional Animal Ethics Committee as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) guidelines.

Objective 1: To understand the anti-promotion mechanism of PBPs in experimental lung carcinogenesis

Male A/J mice, 6–8 weeks old were received from Laboratory Animal Facility, ACTREC and randomized into four different groups. Polyphenol control (PC) and polyphenol+carcinogen (P+C) groups were primed for 2 weeks with PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water. Animals in vehicle control (VC) and polyphenol control (PC) groups were injected glyceryl trioctanoate (vehicle, i.p., 0.1ml) and continued receiving drinking water and PBPs respectively while carcinogen (C) and polyphenol+carcinogen (P+C) groups were injected B(a)P and NNK (i.p, 3μM of each, 0.1ml) and continued receiving drinking water and PBPs (ad libitum) respectively. Glyceryl trioctanoate or B(a)P and NNK injections were given with the gap of 2 days, once in a week for 8 weeks while PBPs and drinking water were administered ad libitum, prior to (2 weeks), during (8 weeks) and after the carcinogen treatment (4, 6, 10 and 18 weeks) till the end of the experiment. All five lung lobes from three random animals per group were fixed in 10% buffered formalin and paraffin embedded blocks were used for histopathological evaluation of microscopic lung lesions and immunohistochemical analysis (IHC). Tissues from the rest of the animals were snap frozen in liquid nitrogen and stored at -800C until preparation of the extract for immunoblotting.

Tumor incidence, Multiplicity and Latency period:

Macroscopic and microscopic pulmonary lesions:

Macroscopic visible lung tumors 6^{h}, 10^{th}, 18^{th} and 28^{th} weeks post carcinogen treatment was counted using magnifying glass. While paraffin embedded lung tissues from three independent mice in similar time points were processed and four step sections having a thickness of 5 μm each (200 micron apart) were stained with

H&E. Microscopic tumor number and area was evaluated using Image J software. No macro or microscopic lesion was found in the vehicle treated and polyphenol treated group. Reduction in hyperplasia and AAH in both the groups at all the time points was found to be similar but percent reduction in adenomas found to be decreased from 6th week to 18th week post carcinogen. Area of hyperplasia and adenoma at all the time points screened was found to be significantly different in both C and P+C groups.

Analysis of inflammation, cell proliferation and apoptosis markers:

Inflammation, cell proliferation and cellular apoptosis are three known hallmarks of the cancer. Inflammation plays crucial role in the early stages of carcinogenesis. However, balance between cell proliferation and apoptosis plays an important role in disease progression. These parameters were analyzed using IHC and western blotting in different experimental groups at different time points post carcinogen treatment (4^{th},10^{th},18^{th} week).

Cox-2 expression by was found to be decreased by PBPs treatment using immunoblotting which was consistent with the expression levels observed in immunohistochemical staining of tissue sections. Carcinogen treatment resulted in significant increase in levels of PCNA while PBPs treatment resulted in significant decrease in PCNA levels in P+C groups at all time points which was demonstrated by both western blotting as well as calculating the proliferative index. PBPs increases apoptosis which was demonstrated by increased apoptotic index by TUNEL assay and Bax/ Bcl2 expression. To know the role of PBPs in the mechanism involved in affecting the process of lung carcinogenesis, we evaluated the non-phosphorylated and phosphorylated forms of P38 Mitogen activated protein kinase and Akt kinase and its activated phosphorylated forms. Expression

of phosphorylated form of P38 and Akt gets deregulated while native forms remain unaltered.

Objective 2: To understand the anti-initiation mechanism of PBPs in early stages of experimental lung carcinogenesis:

To understand the interplay between various xenobiotic metabolizing enzymes early stages of lung carcinogenesis

Phase I and Phase II metabolizing enzymes are known to play an important role in metabolism of xenobiotics. Balance between the Phase I (CYP1A1 and 1A2) and Phase II (GST mu, pi and alpha) metabolizing enzymes are important to understand the progression of the disease. Hence the interplay of these enzymes was studied using western blot analysis at 1^{st} and 8^{th} week of carcinogen treatment period. Further GST enzyme activity was evaluated by using CDNB as a substrate in both lung as well as liver at both 1^{st} and 8^{th} week of carcinogen treatment. In carcinogen group CYP1A1 as well as CYP1A2 protein expression was found to be increased while it was decreased in polyphenol+carcinogen treated groups at both the time points. GST isoform expressions were found to be high in all polyphenol control groups. However, carcinogen treatment reduced the protein expression significantly at both the time points.

Detection of benzo (a) pyrene diol epoxide-DNA (BPDE-DNA) adducts:

B(a)P forms a covalent binding to DNA of its metabolically active species BPDE resulted by metabolism by various Phase I and II enzymes which may lead to mutations in oncogenes or tumor suppressor genes. The interaction of BPDE with DNA was studied by IHC staining for BPDE–DNA adducts in paraffin-embedded 5 micron thick tissue sections (lung and liver) at the carcinogen (1^{st}, 2^{nd}, 4^{th}, 6^{th}, 8^{th}) treatment weeks in both C and P+C group. Adduct formation was found to be

lowered in the polyphenols treated group in liver and lung as compared to the respective carcinogen group at all the time points (1^{st} , 2^{nd} , 4^{th}, 6^{th} ,8^{th} week) of carcinogen treatment.

Objective 3: To understand the effect of dose response of PBPs on carcinogen metabolism during early stages of lung carcinogenesis

To understand the PBPs dose related changes during early stages of carcinogenesis, dose response study of the PBPs were carried out. Three doses (0.75%, 1.5% and 3%) of black tea derived PBPs were employed to the 6-8 weeks age A/J mice through drinking water. After receiving the animals from animal house they are randomized into 8 different groups as follows:

VC and PC group has received vehicle through i.p. injections and drinking water and various doses of PBPs (0.75, 1.5 and 3%) respectively. While C and P+C had received single B(a)P i.p. injection and drinking water and various doses of PBPs (0.75, 1.5 and 3%) respectively. After 2 weeks of PBP treatment, animals was given i.p injection of vehicle (0.1 ml glycerol trioctanoate) or 3 µmol B(a)P in glycerol trioctanoate (0.1 ml) once in a week and animals were sacrificed 24 hrs. post carcinogen treatment**.** Partial lung and liver tissue was fixed while remaining was dissected out and stored at -80^0 C. Formalin fixed paraffin embedded tissues was used for evaluation of BPDE-DNA adducts using immunohistochemical (IHC) staining. Frozen tissues (both lung and liver) were used for cellular fractionation to obtain the cytosolic and microsomal fractions to evaluate phase II enzymes (GST mu, alpha and pi) and phase I enzymes (CYP 450 1A1, 1A2) expression and activity. BPDE-DNA adducts were found to be decreased in both lung as well as liver treated with various doses of polyphenols as compared to its carcinogen treated group alone. However decrease was found to be significant in 1.5% and 3%

doses but not in 0.75% PBPs dose. Expression and activity of CYPs was induced by carcinogen treatment while it was further decreased by PBPs in dose dependent manner. While PBPs induces GST isoforms irrespective of carcinogen treatment which was further decreased by carcinogen treatment. This modulation was significantly different in group treated with 1.5 and 3% of PBPs however it was marginal in 0.75%. Further we analyzed the effect of increased dose of PBPs i.e. 3% black tea derived PBPs on lung tumor carcinogenicity studies. 3% PBPs decreases B(a)P and NNK induced both macroscopic and microscopic lung tumor multiplicity. However, lung tumor incidence and latency period remains unaltered.

Objective 4: To understand the effect of pre and post treatment of PBPs on early stages of experimental lung carcinogenesis

Effect of PBPs pretreatment on carcinogen metabolism and xenobiotic metabolizing enzymes

Inbred A/J mice (6-8 weeks old) were randomized in to 4 groups: (i) Vehicle control (receiving Glyceryl trioctanoate+ drinking water), (ii) 1.5% PBPs treated (receiving Glyceryl trioctanoate+ 1.5% PBPs) (iii) B(a)P treated (receiving 3µmole B(a)P+ drinking water) (iv) 1.5% PBPs + B(a)P treated (receiving 3µmole B(a)P+ 1.5% PBPs) . Animals were provided standard laboratory diet and normal drinking water or 1.5% black tea-derived PBP extract for 15 days. After 2 weeks of PBP pre-treatment, animals were given single i.p injection of vehicle (0.1 ml glycerol trioctanoate) or 3 µmol B(a)P in glycerol trioctanoate (0.1 ml) and animals were be sacrificed after 24hrs after carcinogen injection. Part of lung and liver tissues was fixed in 10% buffered formalin. While liver, lung and kidney and other tissues were dissected out and stored at -80^0 C. BPDE-DNA adducts were analyzed by IHC in both liver as well as lung tissues. Pretreatment with PBPs

showed decrease in the percent number of positively stained BPDE-DNA nuclei in P+C group as compared to its respective C group.

To elucidate the possible mechanism of decrease in number of BPDE-DNA adducts nuclei, proliferation and apoptosis markers were analyzed in these tissues. PCNA proliferation index was found to be decreased in P+C group as compared to its C group. However apoptosis was found to increase in polyphenols treated group as compared to its respective carcinogen group.

Effect of PBPs post treatment on carcinogen metabolism and xenobiotic metabolizing enzymes

Inbred A/J mice (6-8 weeks old) were given single i.p. injection of vehicle (0.1 ml glycerol trioctanoate) or 3 µmol B(a)P in glycerol trioctanoate (0.1 ml). After 24 hrs of injections, animals were shifted to 1.5% PBPs / plain drinking water treatment. The groups were (i) Vehicle control (receiving Glyceryl trioctanoate+ drinking water), (ii) 1.5% PBPs treated (receiving Glyceryl trioctanoate+ 1.5% PBPs) (iii) B(a)P treated (receiving 3µmole B(a)P+ drinking water) (iv) 1.5% PBPs + B(a)P treated (receiving 3µmole B(a)P+ 1.5% PBPs). After receiving respective drinking water conditions for 24 hrs. animals were sacrificed post-PBPs/drinking water treatment at 24, 48 and 96 hrs. Part of lung and liver tissues was fixed in 10% buffered formalin. BPDE-DNA adducts were analyzed by IHC analysis in both liver as well as lung tissues. Pretreatment with PBPs showed decrease in the percent number of positively stained BPDE-DNA nuclei in poly+carcinogen group as compared to its respective carcinogen group. To elucidate the possible mechanism of decrease in number of BPDE-DNA adducts nuclei, proliferation and apoptosis markers were analyzed in these tissues. PCNA proliferation index was found to be decreased in P+C group as compared to its carcinogen group. However

apoptosis was found to increase in time dependent manner in P+C group as compared to its respective C group.

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Work presented at various conferences:

- **Poster presentation:** International Conference on "Global cancer: Occurrence, Causes and Avenues to Prevention" organized by International Agency for Research on Cancer (IARC), Lyon, France, on 7- 10th June 2016 . Poster presentation entitled "Modulation of molecular markers by polymeric black tea polyphenols (PBPs) in tobacco carcinogen induced lung carcinogenesis in A/J mice".
- **Oral presentation:** Conference on "Cancer in Asia: bridging the gaps" organized by Indian Association for Cancer Research (IACR), India, on 8- 10 April 2016. Oral presentation on "Modulation of tobacco carcinogen induced early molecular markers by various doses of Polymeric black tea polyphenols (PBPs) in lung and liver of A/J mice". Published in Journal of Carcinogenesis, 2016 and received an **award as Mangala Bamne young scientist best oral presentation.**
- **Poster presentation:** International conference on "New ideas in Cancer-Challenging dogmas", organized by Tata Memorial Centre Platinum Jubilee Celebration, Mumbai on 26-28 February 2016. Poster presentation on "Chemoprevention of lung cancer by Polymeric Black tea Polyphenols (PBPs): preclinical evaluation of potential molecular targets".
- **Oral presentation:** International conference on "Promotion of animal research, Welfare and Harmonization of Laboratory Animal Science" organized by Laboratory Animal Scientist's Association (LASA) India, on 15-16 October 2015. Oral presentation on "Evaluation of chemopreventive efficacy of Polymeric Black tea Polyphenols (PBPs) in carcinogens induced A/J mice

model during sequential lung carcinogenesis" and received an **award as best oral presentation.**

- **Poster presentation:** International conference on "Molecular pathway to therapeutics: paradigms and challenges in oncology", organized by Carcinogenesis foundation, USA. Carcinogenesis, 11-13 February 2015, presented a poster "Polymeric Black tea Polyphenols (PBPs) modulates tobacco carcinogen induced cellular responses in A/J mice lung carcinogenesis model." Published in Journal of Carcinogenesis, 2015; 14(Suppl 1): S21–S38, 2015; 14(Suppl 1), Published online 2015 Feb 10.

Publications:

Related to thesis:

- Rasika R Hudlikar, V. Venkadakrishnan, Rajiv Kumar, Rahul A. Thorat, Sadhana Kannan, Arvind D. Ingle, Saral Desai, Girish B. Maru and Manoj B. Mahimkar. Polymeric black tea polyphenols (PBPs) inhibit benzo(a)pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone induced lung carcinogenesis potentially through down-regulation of p38 and Akt phosphorylation in A/J mice. Mol Carcinog. 2017, 56(2):625-640. doi: 10.1002/mc.22521 PMID: 27377358

Review:

- Girish B. Maru, Rasika R. Hudlikar, Gaurav Kumar, Khushboo Gandhi, Manoj B. Mahimkar. Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. World Journal of Biological Chemistry, 2016, February 26; 7(1): 88-99. doi: 10.4331/wjbc.v7.i1.88. PMID: 26981198

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ABC	Avidin-biotin conjugate
ADP	Adenosine diphosphate
AhR	Aryl hydrocarbon receptor
AhRE	Aryl hydrocarbon receptor regulatory element
AHH	Aryl hydrocarbon hydroxylase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activated protein-1
APS	Ammonium persulfate
ARNT	Aryl hydrocarbon nuclear translocator
ARE	Antioxidant response element
ATP	Adenosine tri-phosphate
bHLH	Basic helix-loop-helix
B(a)P	Benzo(a)pyrene
BME	β-Mercaptoethanol
BPB	Bromophenol blue
BPDE	Benzo(a)pyrene diol epoxide
BSA	Bovine serum albumin
Cox-2	Cyclooxygenase-2
CYP 450	Cytochrome P-450
DAB	Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
D/W	Distilled water
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
EC	Epicatechin
ECG	Epicatechin gallate
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol tetra acetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ERK	Extracellular-signal-regulated protein kinase
ER	Ethoxyresorufin
EROD	Ethoxyresorufin O-deethylase
GSH	Glutathione reduced
GST	Glutathione S-transferase

GSSG	Glutathione oxidized
H & E	Haematoxylin and eosin
HRP	Horseradish peroxidase
Hsp	Heat-shock protein
I-3-C	Indole-3-carbinol
ILs	Interleukins
i.p.	Intraperitoneal
IKK	Inhibitory kappa B kinase
IκB-α	Inhibitor of NF-κ B
KCl	Potassium chloride
MALDI	Matrix Assisted Laser Desorption/Ionization
MAPK	Mitogen activated protein kinase
MgCl2	Magnesium chloride
ml	Millilitre
mmol	Millimoles
M	Molar
mM	Millimolar
MR	Methoxyresorufin
MROD	Methoxyresorufin O-demethylase
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NaF	Sodium fluoride
Na3VO4	Sodium orthovanadate
NFκB	Nuclear factor-kappa B
NNK	4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone
nmol	Nanomoles
NQO1	NAD(P)H quinone oxidoreductase1
Nrf2	NF-E2-related factor-2
Nrf1	NF-E2-related factor-1
NSAIDs	Non-steroidal anti-inflammatory drugs
ODC	Ornithine decarboxylase
p38	p38 protein kinase
pmol	Picomoles
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic aromatic hydrocarbons
PBP	Polymeric black tea polyphenol
PBS	Phosphate buffer saline
PCNA	Proliferating cell nuclear antigen
PI3K	Phosphatidylinositol 3-kinase
p-ERK	Phosphorylated extracellular signal-

	regulated kinase
p-JNK	Phosphorylated c-Jun N-terminal kinase
PGE-2	Prostaglandin E-2
PKC	Protein kinase C
PPO	Polyphenol oxidase
PMSF	Phenyl methane sulphonyl fluoride
Prot K	Proteinase K
PVDF	Polyvinylidene difluoride
RT	Room temperature
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulfate
SNPs	Single nucleotide polymorphisms
SOD	Superoxide dismutase
TEMED	N, N, N', N' - Tetramethylene diamine
TA	Total activity
TFs	Theaflavins
TBS	Tris-buffered saline
T-TBS	Tween-20-tris-buffered saline
TPA	12-O-tetradecanoylphorbol-13-acetate
TFu	Theafulvins
TGF-α / -β	Transforming growth factor -α / -β
TLC	Thin layer chromatography
TNF- α	Tumor necrosis factor - α
TPA	12-O-Tetradecanoyl phorbol-13-acetate
TRs	Thearubigins
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
XRE	Xenobiotic response element
XMEs	Xenobiotic metabolizing enzymes

Chapter 1

Introduction

And

Review of literature

Chapter 1: Introduction and review of literature

1.1 Lung Cancer:

Lung cancer has been the most common cancer in the world for several decades. It is one of the most common causes of death in both men and women throughout the world, and can be divided into two broad groups: small cell lung cancer (SCLC), which accounts for about 20-25% of bronchogenic carcinomas and non-small cell lung cancer (NSCLC), which constitutes the rest **(1)**. Lung cancer is the third most prevalent malignancy worldwide amongst both the genders while it is one of the leading causes of death amongst men **(2)**. Globally, lung cancer causes maximum deaths (8201000 cases annually) amongst both the genders, predominant incidence occurring in developed countries. These were estimated to be 1.8 million new cases in 2012 (12.9% of the total), 58% of which occurred in the less developed regions. The disease remains as the most common cancer in men worldwide (1.2 million, 16.7% of the total) with the highest estimated age-standardized incidence rates in Central and Eastern Europe (53.5 per 100,000) and Eastern Asia (50.4 per 100,000) **(Figure1.1)**. In Indian scenario, lung cancer remains to be leading cancer amongst men with 53728 new cases were reported in 2012, accounting for 48697 deaths (an age standardized rate ASR = 11.0), making it top most in terms of incidence **(Figure 1.1)**. As per GLOBOCAN, this number is predicted to increase to around 88831 new cancer cases (for both sexes) by 2020.

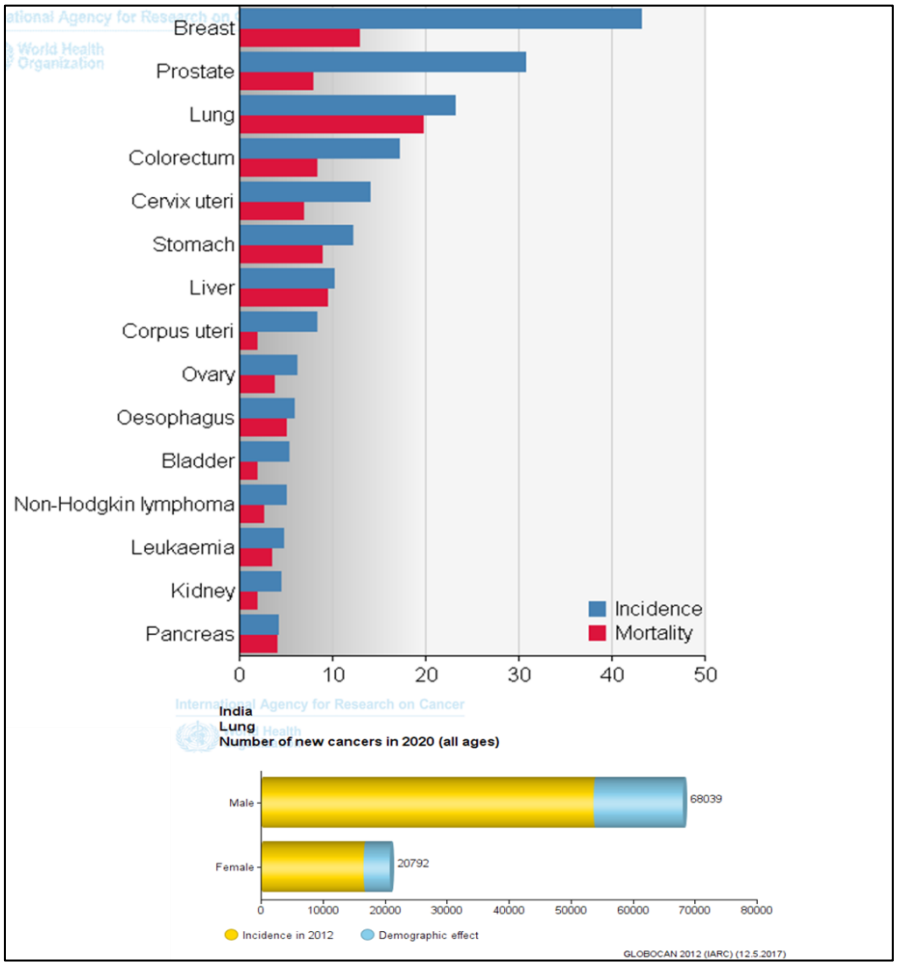


Figure 1.1: Estimated age-standardized incidence & mortality rates in both the sexes and predicted Indian lung cancer incidence in 2020 **(2)**.

1.1.1 Etiology of lung cancer:

There are various etiological factors associated with lung cancer including exposure to Radon gas, asbestos, environmental pollution, second hand smoke and cigarette smoking. However, cigarette smoking remains to be one of the leading causes globally for the lung cancer **(3,4)**. Along with dietary and lifestyle related differences, number of cigarette puffs, and pack size of cigarette/year observed to be the deciding factors for the lung cancer progression. However, second hand smoke and exposure to indoor smoke are one of the leading causes found in non-smokers apart from occupational exposures. The genetic predisposition in oncogenes and tumor

suppressor genes along with genetic and exposure related differences in the individuals viz. genetic polymorphism in carcinogen metabolizing enzymes (phase I and II) and DNA repair genes could be influencing factors for cancer development. Given the large population at increased risk of developing lung cancer, the development of approaches to prevent smoking related lung cancers could have great clinical benefit **(5)**.

1.1.2 Process of carcinogenesis:

Carcinogenesis refers to environmental carcinogen [chemical/physical/biological agent(s)] - induced etiological pathways and processes that lead to cancer. It is a complex, multi-factorial, multi-step, multi-path and in humans a multi-years process comprised of at least three steps *viz*. initiation, promotion and progression **(Figure 1.2) (6)**. Exposure of normal cells in tissues to carcinogenic agent results in genomic DNA damage (in critical genes) and its fixation through a cycle of DNA replication leads to initiation which is an irreversible step. In the promotion process (reversible), clonal expansion of initiated cells occurs due to promotory stimuli resulting in formation of an actively proliferating, pre-malignant tumor cell population. While in progression (irreversible process) additional genetic changes lead to a new clone of tumor cells with increased proliferative, invasive, and metastatic potential. Exposure to environmental carcinogens results in a series of genetic mutations and such alterations in at least two classes of genes, *i.e*., proto-oncogenes and/or tumor suppressor genes have been associated with tumor development. Activation of proto-oncogenes by qualitative or quantitative genetic changes results in enhancement of proliferative signals. Alternatively, environmental carcinogen-mediated loss or inactivation of tumor suppressor genes (normal genes) also leads to tumor development **(7,8)**. Genetic mutations, genomic instability, and series of epigenetic events, such as chronic inflammation are known to play role in transformation of normal to malignant cells. All transformed cells exhibit certain

common characteristics such as sustained proliferation signals, evasion of growth suppressors, resistance to cell death, replicative immortality, and ability to induce angiogenesis and activation of invasion and metastasis **(9)**.

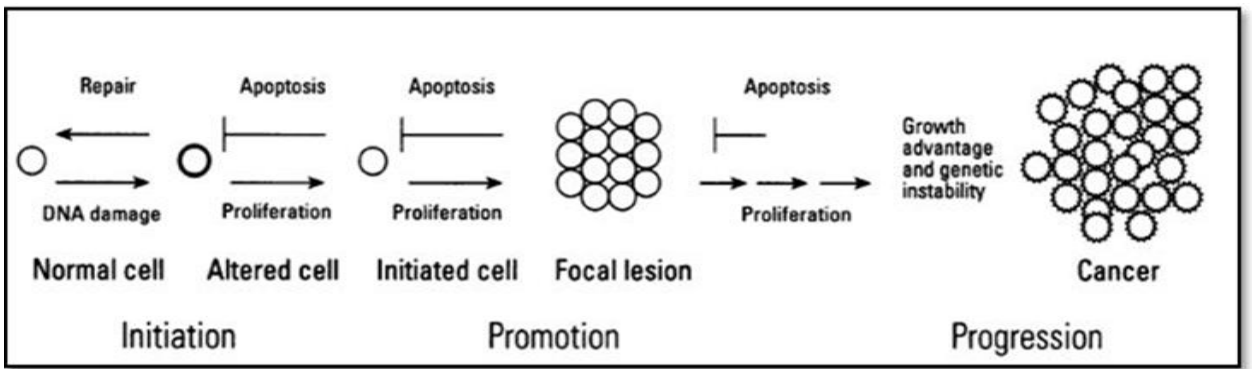


Figure 1.2: Stages in the process of carcinogenesis (Adopted and modified from chemical carcinogenesis-a brief review)

1.1.3 Carcinogens present in tobacco

Tobacco products contain a diverse array of chemical carcinogens that cause cancers of various types. **Table 1.1** summarizes our knowledge of carcinogens in tobacco products.

Table 1 | **Types of carcinogen in tobacco products***

Tobacco smoke			
Chemical class	***Number of compounds‡***	***Representative carcinogens and typical amounts in mainstream smoke (ng per cigarette)§***	
PAH‖	14	BaP	9
		Dibenz[*a*,*h*]anthracene	4
Nitrosamines	8	NNK	123
		NNN	179
Aromatic amines¶	12	4-Aminobiphenyl	1.4
		2-Naphthylamine	10
Aldehydes	2	Formaldehyde	16,000
		Acetaldehyde	819,000
Phenols	2	Catechol	68,000
Volatile hydrocarbons	3	Benzene 1,3-Butadiene	59,000 52,000
Nitro compounds	3	Nitromethane	500
Other organic compounds	8	Ethylene oxide Acrylonitrile	7,000 10,000
Inorganic compounds	9	Cadmium	132
Total	**61**		
Unburned tobacco			
Chemical class	***Number of compounds***	***Representative carcinogens and typical amounts in processed tobacco (ng g^{-1})***	
PAH	1	BaP	0.4–90
Nitrosamines	6	NNK	1,890
		NNN	8,730
Aldehydes	2	Formaldehyde	1,600–7,400
		Acetaldehyde	1,400–7,400
Inorganic compounds	7	Cadmium	1,300–1,6000
Total	**16**		

Table1.1 : Carcinogens present in tobacco (Adopted from Hecht et al, 2003.**(10)**

All of the carcinogens in **Table 1.1** have been formally evaluated by the IARC and, in each case; studies in either laboratory animals or humans have provided sufficient evidence of carcinogenicity. In general, strong carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines and aromatic amines, occur in smaller amounts (1–200 ng per cigarette) than weak carcinogens such as acetaldehyde (nearly 1 mg per cigarette). The total amount of carcinogens in cigarette smoke adds up to 1–3 mg per cigarette (similar to the amount of nicotine, which is 0.5–1.5 mg per cigarette), although most of this consists of weaker carcinogenic agents such as acetaldehyde, catechol and isoprene **(10)**.

B(a)P was the first carcinogen to be detected in cigarette smoke13 and recent studies have implicated its diol epoxide metabolite as the cause of mutations in the TP53 gene. Levels of B(a)P in cigarette smoke are now quite low — typically less than 10 ng per cigarette10 — and the carcinogenic activity of B(a)P in mouse lung is considerably less than that of dibenz[a,h]anthracene and 5-methylchrysene, which are two other PAH carcinogens in cigarette smoke. Unburned tobacco contains fewer carcinogens than cigarette smoke because most are formed during combustion. Levels of PAHs in unburned tobacco are typically quite low **(10)**.

Nitrosamines, particularly the tobacco-specific nitrosamines 4-(methylnitrosamino)- 1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN), are by far the most prevalent strong carcinogens in unburned tobacco products, which include oral snuff, chewing tobacco and other smokeless tobacco products. These agents are formed from tobacco alkaloids during the curing and processing of tobacco, although there have been reports of their presence in green tobacco. Some of the NNK and NNN in cigarette tobacco are transferred to smoke and some is destroyed during cigarette smoking. NNK is a strong, systemic lung carcinogen in rodents, which induces lung tumors irrespective of its route of administration **(11)**. DNA adducts derived from NNK or the related, tobacco-specific nitrosamine NNN, is present at higher levels in lung tissue from lung cancer patients than in that from controls. To note, metabolites of NNK are found in the urine of people who use tobacco in various forms **(12,13).** The changing histology of lung cancer, in which adenocarcinoma has now overtaken squamous-cell carcinoma as the most common lung cancer type. This is consistent with the literature about NNK, which primarily gives rise to adenocarcinoma in rodents **(14)**.

1.2 Mechanism of lung carcinogenesis by tobacco smoke:

Carcinogenesis is a multistep, multifactorial process involving various steps like adducts formation, mutations in important genes and modulation of various signaling kinases (Figure

1.3). Early, young age cigarette smoking habit leads to addiction because of the nicotine present in tobacco smoke. Nicotine, perhaps in concert with other tobacco smoke constituents, is highly addictive. Nicotine is not a carcinogen. However, alkaloids formed by nitrosation of nicotine like NNK, NNN, NAT and related nitrosamines are known as tobacco-specific nitrosamines which are well proven carcinogen in various model systems **(10,11)**. But each puff of a cigarette results in the delivery of a mixture of carcinogens and toxicants along with nicotine. Cigarette smoking increases the risk of all histological types of lung cancer, including squamous cell carcinoma, small-cell carcinoma, adenocarcinoma (including bronchiolar/alveolar carcinoma) and large cell carcinoma **(15)**.

Most cigarette smoke carcinogens are substrates for drug metabolizing enzymes such as the cytochromes P450, glutathione S-transferases, and UDP-glucuronosyl transferases which catalyze their conversion to more water soluble forms that are detoxified and can be readily excreted **(16,17).** But during this process, reactive intermediates such as carbocations or epoxides are produced and these electrophilic compounds can react with nucleophilic sites in DNA such as the nitrogen or oxygen atoms of deoxyguanosine and other DNA bases. The result is the formation of DNA adducts which are critical in the carcinogenic process **(18,19)**. We know that DNA adduction is important because evolution has dictated the development of DNA repair enzymes that can fix damaged DNA. The adducts like 7-methyl-dG, 7-ethyl-dG, O^6-methyl-dG and O^6-ethyl-dG could be formed by direct acting methylating or ethylating agents in cigarette smoke or from the metabolic activation of nitrosamines such as N-nitrosodimethylamine or N-nitrosodiethylamine; while 8-oxo-dG is a well-established product of oxidative damage **(20)**. BPDE-N^2-dG results from reaction of BaP-7,8-diol-9,10-epoxide with DNA. If the DNA adducts persist unrepaired, they can cause miscoding during DNA replication as bypass polymerases catalyze the insertion of the wrong base opposite to the adduct. The result is a permanent mutation. If this mutation occurs in a critical region of an oncogene such as

KRAS or a tumor suppressor gene such as TP53, the result is loss of normal cellular growth control mechanisms and development of cancer. Recent studies have implicated diol epoxide metabolite as the cause of mutations in the TP53 gene while 6-methyl-dG formed due to nitrosamine are known for K-ras mutations in codon 12. Multiple recent studies using next generation sequencing methods demonstrate the presence of thousands of mutations in the lungs of smokers, including in many critical growth regulatory genes like KRAS and TP53 **(21,22)**. Consistently, updates of the TP53 mutation data base (available at: http://www-p53.iarc.fr) demonstrate that lung tumors in smokers, G to T transversions are the most prevalent mutations, followed by G to A transitions. There is significantly more G to T transversions in smokers than in nonsmokers, while G to A transitions is more common in nonsmokers. Hotspots for mutation in the TP53 gene in tobacco smoke associated lung cancers occur at codons 157, 158, 245, 248, 249, and 273 **(23,24)**.

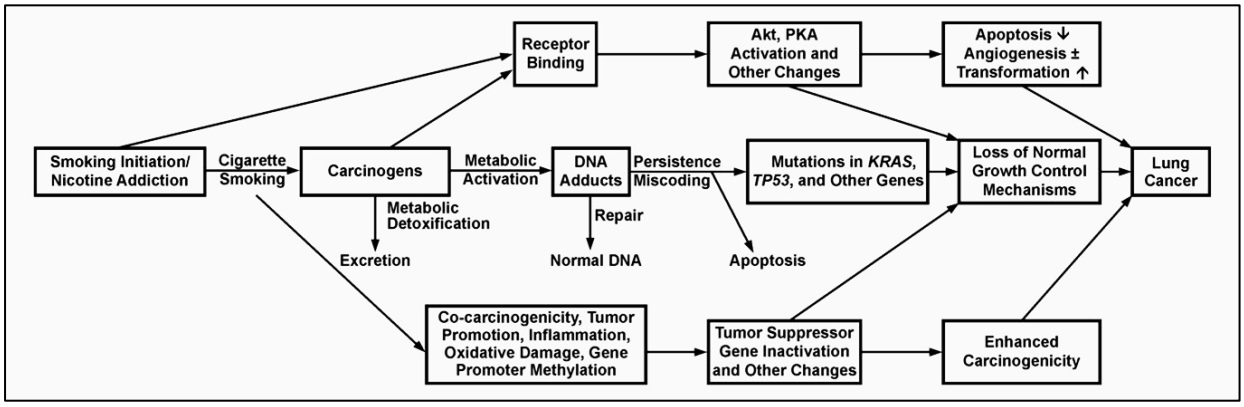


Figure 1.3 : Mechanistic framework for understanding how cigarette smoking causes lung cancer (Adopted from Hecht et al., 2012. **(18).**

Some tobacco smoke constituents such as nicotine and tobacco-specific nitrosamines bind directly to cellular receptors without a metabolic activation process. This can lead to activation of Akt, PKC and other pathways which can contribute to the carcinogenic process **(25)**. Furthermore, cigarette smoke contains compounds that can induce inflammation resulting in enhanced pneumocyte proliferation as well as co-carcinogens, tumor promoters, inducers of oxidative damage and gene promoter methylation, all processes which undoubtedly contribute to lung cancer development.

1.3 Treatment of lung cancer and its limitations:

Late stage diagnosis remains to be one of the challenges associated with the treatment of lung cancer. Combination treatments for non-small cell lung cancer include surgery, chemotherapy and radiotherapy depending upon the stage of the cancer. Computed tomography (CT) scan is used as a diagnostic module for NSCLC. Surgical resection includes removal of the entire lobe (lobectomy) or part of the lobe, removing tumor portion (sengmentectomy or wedge resection) of the lung. In radiation therapy, CT followed by intensity modulated radiation therapy (IMRT) or stereotactic body radiation therapy (SBRT) are commonly postoperative or primary modalities of treatment **(26).** However, side effects of radiation therapy like pulmonary irritation, cough, fever, and radiation pneumonitis is very common **(27)**. Chemotherapy for NSCLC involves common drugs like cisplatin and carboplatin, paclitaxel. Like radiation, the side effects of chemotherapy generally include hair loss, nausea, vomiting, fatigue, skin rash etc. Along with standard chemo and radiotherapy, targeted therapies viz. anti-angiogenesis therapy Bevacizumab (Avastin), tyrosine kinase inhibitors (TKIs) like EGFR inhibitors Erlotinib (Tarceva), Gefitinib (Iressa), Cetuximab are given as drugs for the targeted inhibition **(28).** Evolving concept of immunotherapy which blocks the pathway like PD-1 in lung cancer is given to the patients. Nivolimab (Opdivo) and Pembrolizumab (Keytruda) are commonly used

immuntherupatic drugs for metastatic NSCLC. Following therapy with these drugs, palliative treatments including medication, nutritional support and other therapies are applied **(29,30)**.

Most cancers are treated depending on the specific type, location, and stage. Early detection of tumors is possible for accessible sites like skin, cervix, and oral cancers, but lung being inaccessible site early stage diagnosis is one of the major challenges. After detection, stage of the tumor decides surgery as treatment modality. However, complete surgical removal of the tumor has limitations, as residual tissue is prone to recurrence. Treatment with radiation or surgery followed by radiation causes severe damage to normal tissue function. Chemo and radiotherapy has severe side effects like skin rash, extensive hair fall, nausea, vomiting, bleeding, liver and kidney damage, lung scarring, sterility, numbness, bone marrow damage, diarrhea, mouth ulcers, skin rashes, impotence, bleeding, fall in blood pressure, weakness, fever. Moreover, an increasing trend of chemo/radio-resistance and the recurrence of secondary tumors result in limitations in the fight against cancer.

1.4 Prevention of lung cancer:

Cancer prevention serves as a promising approach to decrease cancer burden. Cancer prevention approach encompasses a vast area of strategies to prevent causes of cancer. Strategies for lung cancer prevention have been implemented but the success rate is limited. Avoiding occupational exposure to asbestos and radon gas is one of the strategies. Awareness about smoke and smokeless tobacco has remained a challenge for the decades. One of the primary approaches for cancer prevention is to reduce the exposure to well established cancer causative agents such as tobacco, carcinogenic food additives, etc. Lifestyle factors like regular exercise, improved hygiene, decreasing smoking and alcohol consumption, etc., are also important. Some of the approaches towards prevention of lung cancer should be considered as follows:

- Human uptake of tobacco carcinogens as a way of assessing risk and investigating mechanisms
- Individual differences in the metabolic activation and detoxification of carcinogens, which may relate to cancer susceptibility
- Chemoprevention of lung cancer in smokers and ex-smokers.

In this context, the approach of cancer prevention by major changes in lifestyle such as decline in tobacco use and obesity and the use plant foods- i.e. nontoxic chemical/dietary entities termed 'chemoprevention', is considered to be an alternative, probably more realistic, cost effective and fundamental strategy for the control of this dreaded disease. Epidemiological studies in most populations have indicated that consumption of diet rich in vegetables, fruits and fiber may provide protection against number of cancers such as oral, colorectal, breast, stomach, etc. **(31,32).** Since, efforts to eliminate known human carcinogens like tobacco from the environment as preventive strategy has had met with limited success; modulations of diet and lifestyle seem achievable **(33,34).** In fact, alternative agents obtained from various plants to prevent or slow down/delay cancer initiation/promotion/progression have garnered great interest in understanding the efficacy and/or limitations in experimental studies and clinical trials. Hence, the emergence of a new area of research in cancer prevention, i.e. cancer chemoprevention.

1.5 Cancer chemoprevention:

Cancer chemoprevention involves the chronic administration of a synthetic, natural or biological agent to reduce or delay the occurrence of malignancy. The primary goal of chemoprevention research is to identify effective agents and/or develop efficient strategies for clinical trials and ultimately, application to human populations. In order to better practice

chemoprevention, it is imperative to understand the nature and mechanism of carcinogenesis. The scientific rationale for the use of cancer chemoprevention is based on the fundamental concept of multi-step carcinogenesis **(33)**. Based on their mode of action, chemopreventive agents can be classified into two groups: blocking agents, which impede the initiation stage either by inhibiting the formation of carcinogens or preventing the carcinogens from reaching / reacting with macromolecules; and suppressing agents, which arrest or reverse the promotion and progression of cancer, mainly by affecting or perturbing crucial factors that control cell proliferation, differentiation, senescence or apoptosis **(35)**.

Cancer preventive strategies can be classified into primary, secondary, and tertiary prevention. Primary cancer prevention is meant for general population and those at increased risk of cancer development. Secondary prevention is employed in patients with premalignant lesions that may progress to cancer. Tertiary cancer prevention is employed in preventing cancer recurrence (local invasion and/or distant metastasis) or second primary tumor among those who already have developed the disease **(36)**.

1.5.1 Synthetic chemopreventive agents (Non-Steroidal-anti-inflammatory drugs):

Several studies have reported a 40-50% decrease in the relative risk of colorectal cancer in people who are continuous users of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDS) **(37)**, suggesting that these drugs can serve as effective cancer chemopreventive agents. Hixson et al., **(38)** showed that the synthesis of prostaglandins is limited by cyclooxygenase. NSAIDS reversibly interrupted prostaglandin synthesis by inhibiting cyclooxygenase. NSAIDS can prevent tumor formation by their actions on prostaglandins, which can have an immune modulating effect. High levels of prostaglandin E2 can suppress the immune system, which keeps malignant cells in check. Other mechanisms that can explain the antiproliferative antitumor effects of NSAIDS

include: interference with membrane-associated processes, such as G-protein signal transduction and trans membrane calcium influx, and inhibition of other enzymes, such as phospho-diesterase, folate-dependant enzymes, and cyclic adenosine-5`-monophosphatasedependent protein kinase, as well as enhancement of immunologic responses and cellular apoptosis **(36,39)**.

1.5.2 Naturally-occurring chemopreventive agents:

Frequent consumption of fruits and vegetables has been associated with lower incidence of cancers at different organ sites. Several factors can contribute to this association; first, the nutrients in fruits and vegetables, notably vitamin C, vitamin E, folic acid, provitamin A, selenium and zinc which are essential for normal cellular functions, a deficiency in these nutrients can enhance the susceptibility of an individual to cancer. Second, some nutrients, such as vitamin C, vitamin E, selenium and β-carotene, at levels above nutritional needs, can display inhibitory activities against carcinogenesis **(40)**. A third factor is that non-nutritive constituents, such as polyphenols, organosulfur compounds, and indoles have anticarcinogen activities. Finally, fruits and vegetables contribute fibers and bulkiness to the diet. People who consume large amounts of fruits and vegetables can eat smaller amounts of meat and other animal products that can contribute to higher cancer incidence in western countries. Supplementation with these antioxidant nutrients apparently produces a protective effect against cancer **(41)**.

1.5.3 Dietary polyphenols as chemopreventive agents:

Polyphenols constitute one of the largest and ubiquitous groups of phytochemicals. One of the primary functions of these plant-derived polyphenols is to protect plants from photosynthetic stress, reactive oxygen species, and consumption by herbivores. Polyphenols are also an essential part of the human diet, with flavonoids and phenolic acids being the most common ones in food. Not surprisingly, there is a growing realization that lower incidence of cancer in certain

populations can probably be due to consumption of certain nutrients, and especially polyphenol rich diets.

A substantial number of studies in cultured cells, animal models and human clinical trials have illustrated a protective role of dietary polyphenols against different types of cancers **(34,42,43)**. Polyphenols are present in fruits, vegetables, and other dietary botanicals. Some estimates suggest that more than 8000 different dietary polyphenols exist, and these can be divided into ten different general classes based on their chemical structure **(44)**. Phenolic acids, flavonoids, stilbenes and lignans are the most abundantly occurring polyphenols that are also an integral part of everyday nutrition in populations worldwide. Some of the common examples of the most studied and promising cancer chemopreventive polyphenols have shown in the following **Figure 1.4.**

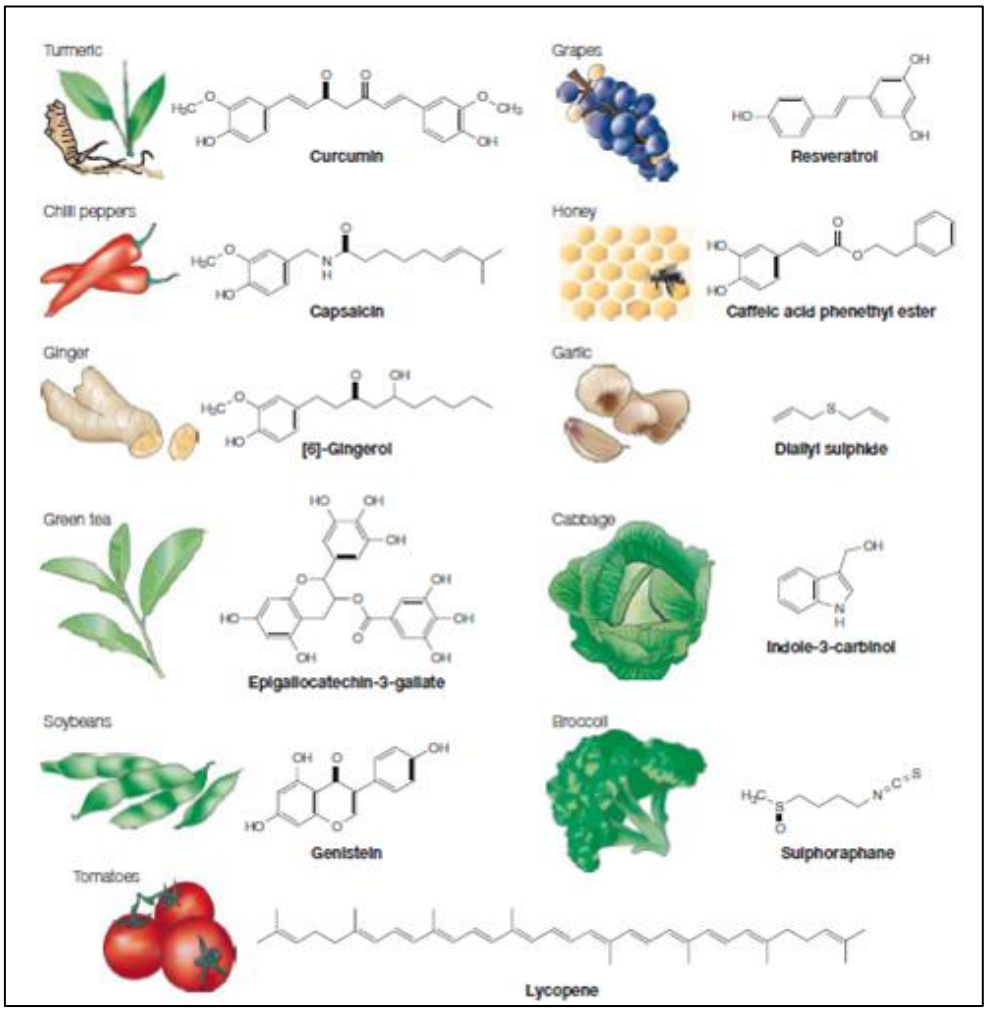


Figure 1.4 : Chemopreventive phytochemicals and their dietary sources (Adopted from Surh et al 2003) **(33)**.

Amongst environmental chemopreventive compounds, diet/beverage-derived agents due to their long history of exposure to humans, high tolerability, low toxicity, and reported biological activities are fast becoming lucrative targets for chemoprevention. Large number of pure compounds and extracts from dietary components has been evaluated in various experimental models for testing the chemopreventive efficacy of dietary phytochemicals **(33,34)**.

1.6 Mechanisms/targets of chemopreventive agents:

Chemopreventive activity of an agent is generally investigated employing carcinogens and/or spontaneously induced tumor and/or appropriate pathways or biomarkers in experimental animals/cell culture assays. In experimental models chemopreventive activity is ascertained based on observed increase in latency period and/or decrease in incidence and/or multiplicity of tumors or by modulation of disease/process associated biomarkers. Alternatively, development of premalignant lesions such as hyperplasia formation has been studied instead of carcinogenesis to study initial development **(45)**. In animal models regression of tumor or tumor xenograft and metastasis has also been reported **(46)**. The mechanism of chemopreventive agents at various stages of carcinogenesis has been shown in the **Figure 1.5**.

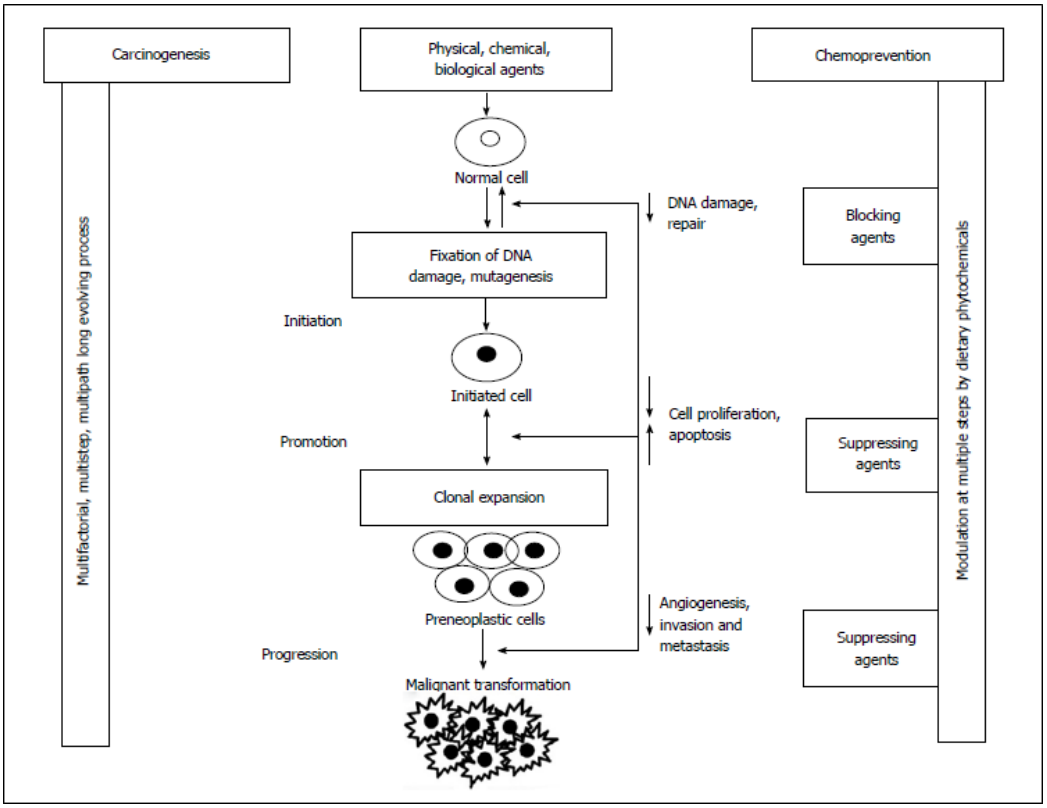


Figure1.5 : Schematic diagram showing multi-factorial, multi-step process of environmental agent(s)-mediated carcinogenesis and step(s) which are modulated by chemopreventive dietary phytochemical(s). (Adopted from Maru et al., 2016) **(36)**

According to the conventional classification originally proposed by Lee Wattenberg, chemopreventive agents are subdivided into two main categories —blocking agents and suppressing agents **(47)**. Blocking agents prevent carcinogens from reaching the target sites, from undergoing metabolic activation or from subsequently interacting with crucial cellular macromolecules (for example, DNA, RNA and proteins). Suppressing agents, on the other hand, inhibit the malignant transformation of initiated cells, in either the promotion or the progression stage. Chemopreventive phytochemicals can block or reverse the premalignant stage (initiation and promotion) of multistep carcinogenesis. They can also halt or at least retard the development and progression of precancerous cells into malignant ones **(Figure 1.5)**.

1.6.1 Anti-initiation targets:

Xenobiotic metabolizing enzymes (phase I and II enzymes) play a crucial role in metabolic activation and detoxification/excretion of xenobiotics entering into the cellular environment in the initiation process of carcinogenesis **(34)**. These metabolizing enzymes thus, could be one of the plausible targets for chemoprevention by cancer chemopreventive agent.

Phase I enzymes:

Phase I enzymes, predominantly cytochromeP450s (CYP450s), are involved in the first step of metabolism where xenobiotics are processed to more electrophilic moieties **(48,49)**. This step can be termed as 'bio activation' which renders pro-carcinogens to reactive intermediates and these in turn can form bio-molecular adducts e.g. DNA-adducts, protein-adducts etc. which mark the process of initiation. Thus, decreased activation of carcinogens due to modulation of the CYP450 enzymes could be one of the plausible targets for chemoprevention to prevent the cancer initiation process.

Cytochrome P 450:

CYP450s constitute a superfamily of haem-thiolate proteins present in prokaryotes and across all eukaryotes. At present, 17 mammalian CYP gene families collectively encode about 60 distinct CYP forms in any given species. CYP450s in gene families 1-4 exhibit broad, but overlapping substrate and product specificities that may vary between corresponding forms from different species **(50)**. Advances in molecular and cell biology during past decade have helped to elucidate the major mechanisms by which drugs and xenobiotics induce the expression of CYP genes. This occurs mainly via receptor proteins that upon inducer binding are transformed into transcriptionally active DNA binding forms.

Phase II enzymes:

In the next step of metabolism, detoxification by phase II enzymes decrease the burden of bio-molecular adducts by eliminating the reactive-intermediates from cellular environment (Patel et al., 2007). Activated pro-carcinogens are conjugated with endogenous bio-molecules like glutathione (GSH) or glucuronic acid by phase II enzymes rendering them less toxic and more water soluble **(51)**. Therefore, enhancement in the activity of detoxifying enzymes by chemopreventive agents would play an important role in blocking the cancer initiation process. The common detoxifying enzymes are glutathione S-transferases (GSTs), UDP-glucuronosyl transferases (UGT), NADPH-quinone oxidoreductase-1(NQO1), epoxide hydrolase, glutamate cystine ligase (GCL), heme-oxygenase -1 (HO-1), γ- glutamyl transpeptidase etc.

Glutathione S-transferases (GSTs):

GSTs catalyze the nucleophilic addition of the thiol of reduced glutathione to a variety of electrophiles **(52)**. In addition, the GSTs bind with varying affinities to a variety of hydrophobic compounds such as heme, bilirubin, polycyclic aromatic hydrocarbons and dexamethasone **(53)**. With the exception of the microsomal enzyme, the GSTs are soluble and dimeric comprising identical or different subunits of 25 to 28 kDa that are grouped into five classes according to structure: alpha, mu, pi, theta and sigma **(52,54)**.

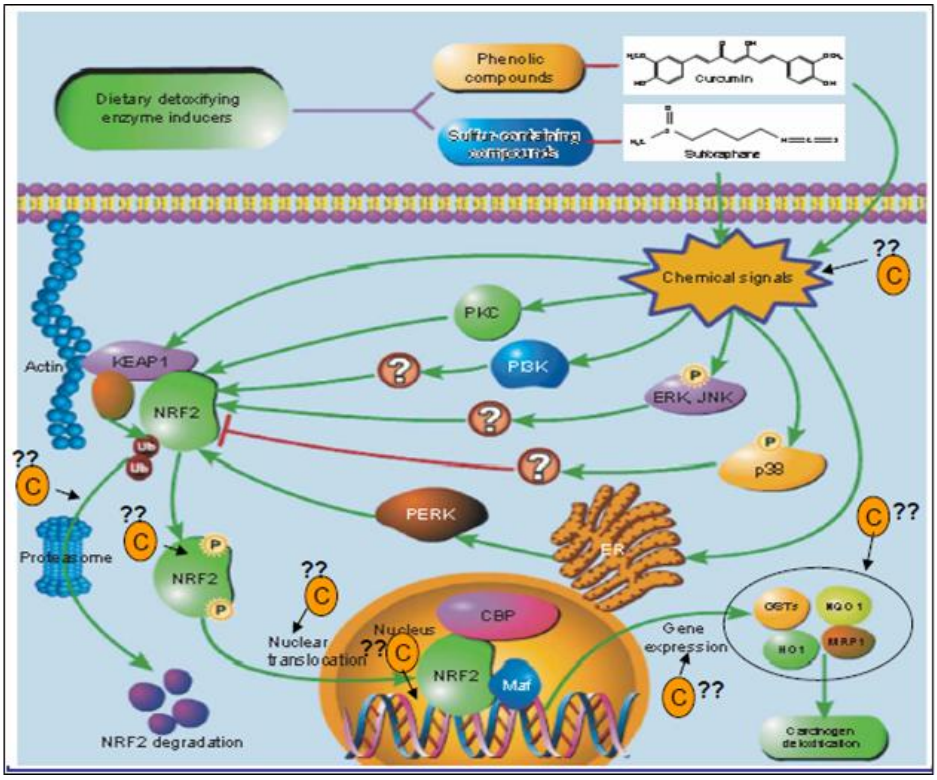


Figure 1.6 : Schematic presentation of steps where chemopreventive agents (C) can potentially modulate phase II enzyme induction (Adopted and modified from **(55)**.

Unlike phase I enzymes, phase II enzymes are regulated by common upstream promoter regulatory element called ARE (anti-oxidant response element). The transcription of ARE-driven genes is regulated, in part by nuclear transcription factor erythroid 2 p45-related factor2 (Nrf2), member of the cap 'n' collar family of basic region leucine b-zip transcription factor. Nrf2 heterodimerises with array of leucine b-zip family members like small maf proteins, jun, fos etc. to either up-regulate or inhibit transcription through ARE **(56)**. Nrf2 under normal un-induced cellular environment is sequestered in cytoplasm by kelch-like ECH-associated protein 1 (Keap1) which, in turn is bound to actin cytoskeleton. Upon activation by ARE inducers, Nrf2 dissociates from Keap1 and translocates to nucleus where it binds to ARE after heterodimerising with other leucine zipper proteins to transcriptionally

activate the downstream genes **(Figure 1.6)**. Activating signals are generally regulated by ROS modulation in cells where a number of signaling molecules might interplay in cell type specific manner **(57)**. Most chemopreventive agents are modulators of cellular ROS and hence activate Nrf2 pathway, which in turn induces phase II detoxifying enzymes **(58).**

DNA adducts:

Reaction of chemical carcinogens with DNA, either directly or following metabolic activation, typically involves covalent binding of an electrophilic carcinogen with a nucleophilic site in DNA. Associations have been observed between DNA adduct formation and mutagenesis and tumorigenesis **(59).**

B(a)P is metabolically activated via a three step process. First, CYP450 catalyze the formation of (7R, 8S)-epoxy-7,8- dihydrobenzo[a]pyrene (B[a]P-7,8-oxide). This is converted to (7R, 8R)-dihydroxy-7,8- dihydrobenzo[a]pyrene (B[a]P-7,8-diol), catalyzed by epoxide hydrolase. B[a]P-7,8-diol then, undergoes another oxidation step catalyzed by CYP450 and other enzymes, producing mainly, (7R,8R)-dihydroxy-(9S,10R)-epoxy-7,8,9,10- tetrahydrobenzo[a]pyrene (BPDE) **(60)**. Among the four possible 7,8-diol-9,10-epoxide isomers of B(a)P, BPDE is formed to the greatest extent in mammalian systems examined to date, and has high tumorigenic activity in murine models. Compounds which in some way decrease the DNA adduct levels or enhance DNA repair would be attractive target for anti-initiation studies. Chemopreventive agents can also act to inhibit initiation process by enhancing DNA repair either by activating cell cycle checkpoints or by inducing or activating DNA repair enzymes **(61)**.

1.6.2 Anti-promotion targets:

During carcinogenesis, initiated cells after accumulating mutations, transform and clonally expand to give rise to tumor and this process is called promotion. This stage is largely characterized by two important cellular events viz., cellular proliferation and apoptosis **(34).**

Cellular proliferation, under normal conditions is a well regulated process where, proliferation signals interplay with cell cycle checkpoint proteins. However, in transformed cells these regulatory processes are over-ridden to cause hyper proliferation under the influence of different mitogenic stimuli. Proliferation can be initiated by different endogenous and exogenous signals like mitogenic stimuli like growth factors, oxidative stress and hormones. Irrespective of the stimuli certain cellular pathways and downstream events remain similar in number of tissues and cell types **(62)**. Thus, agents which can decrease activation of these signaling molecules can suppress the proliferation and hence promotion.

Another important cellular event exploited in chemoprevention is **apoptosis**, which is characterized by cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. General mechanisms for induction of apoptosis are hypothesized to be via stress signals elicited by chemopreventive agents that lead to the loss of mitochondrial membrane potential followed by release of cytochrome c. Consequently, an apoptosome is formed by the cytochrome c, apoptotic protease activating factor-1 (APAF-1) and caspase 9, which later results into activation of downstream effector caspases. Loss of mitochondrial membrane potential is also inhibited by Bcl2 (antiapoptotic protein) or induced by Bax (proapoptotic protein). Furthermore, dietary agent induced activation of p53 (activator of Bax) can also mediate apoptosis in response to DNA damage **(63).** Hence, chemopreventive agents can alter the trigger for apoptosis by altering the expression of anti/pro-apoptotic proteins.

MAP kinases:

Mitogen activated protein kinases (MAPK) are a group of threonine/tyrosine phosphorylated enzymes that are activated by a wide variety of extra-cellular stimuli (mitogens, differentiation factors, stress signals) from the cell surface receptor to the nucleus. They are divided into three different multimember subfamilies: the extracellular signal-regulated

kinases (ERK), the c-Jun N-terminal kinases (JNK) and the p38 kinases. MAPKs mediate their effect through phosphorylation of a wide range of effector proteins, most notably the transcription factors, which in turn lead to changes in the pattern of gene expression **(64)**.

1.7 Tea and tea polyphenols:

Tea is an aqueous infusion prepared from dried leaves, leaf buds and internodes of *Camellia sinensis*. Depending on the manufacturing process, tea is classified into three major types: green tea, black tea and oolong tea **(Figure 1.7)**.

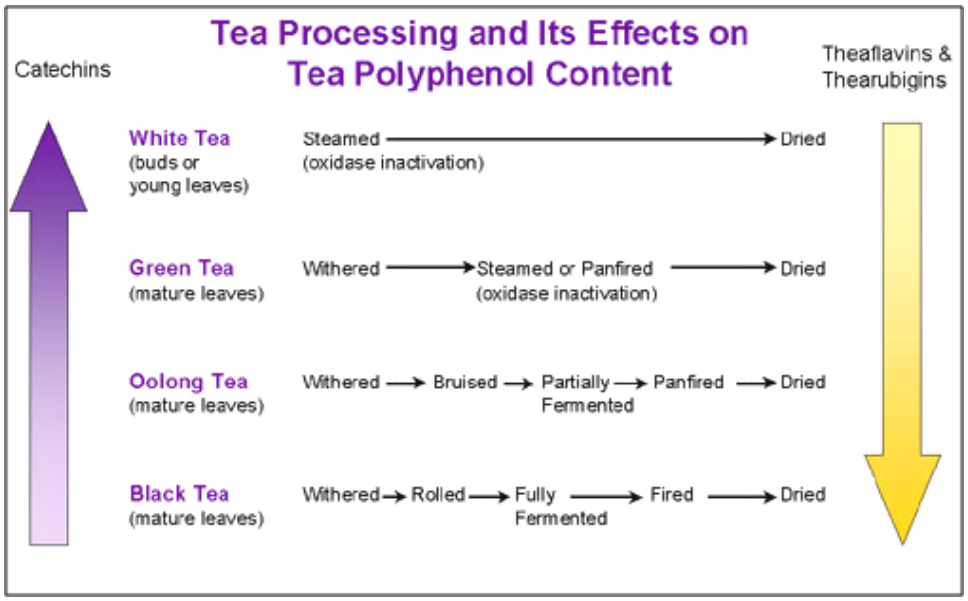


Figure 1.7 : Methods of manufacturing process of different types of tea

(Adapted from www.oregonstate.edu)

Out of total, 76-78% of tea produced and consumed (mainly consumed in Western and some Asian countries) is black tea, 20-22% is green tea (primarily in China, Japan and a few countries in North Africa and the Middle East) and 2% is oolong tea. Oolong tea production and consumption are confined to South-Eastern China and Taiwan **(65,66).** Tea leaves contain more than 700 chemicals, among which the compounds closely related to human

health are flavanoids, amino acids, vitamins (C, E and K), caffeine and polysaccharides. Tea leaves also contain flavonols, such as quercetin and myricitin as well as the nitrogenous compounds, caffeine and theobromine **(67)**.

Leaves of *Camellia sinensis* soon begin to wilt and oxidize if not dried quickly after picking. The leaves turn progressively darker because chlorophyll breaks down and tannins are released. This process of enzymatic oxidation is called fermentation.

1.7.1 Tea polyphenols:

Polyphenols are the most significant group of components in tea, especially the catechin group of the flavanols. The structures of these polyphenols in green and black tea has been depicted in the **Figure 1.8**.

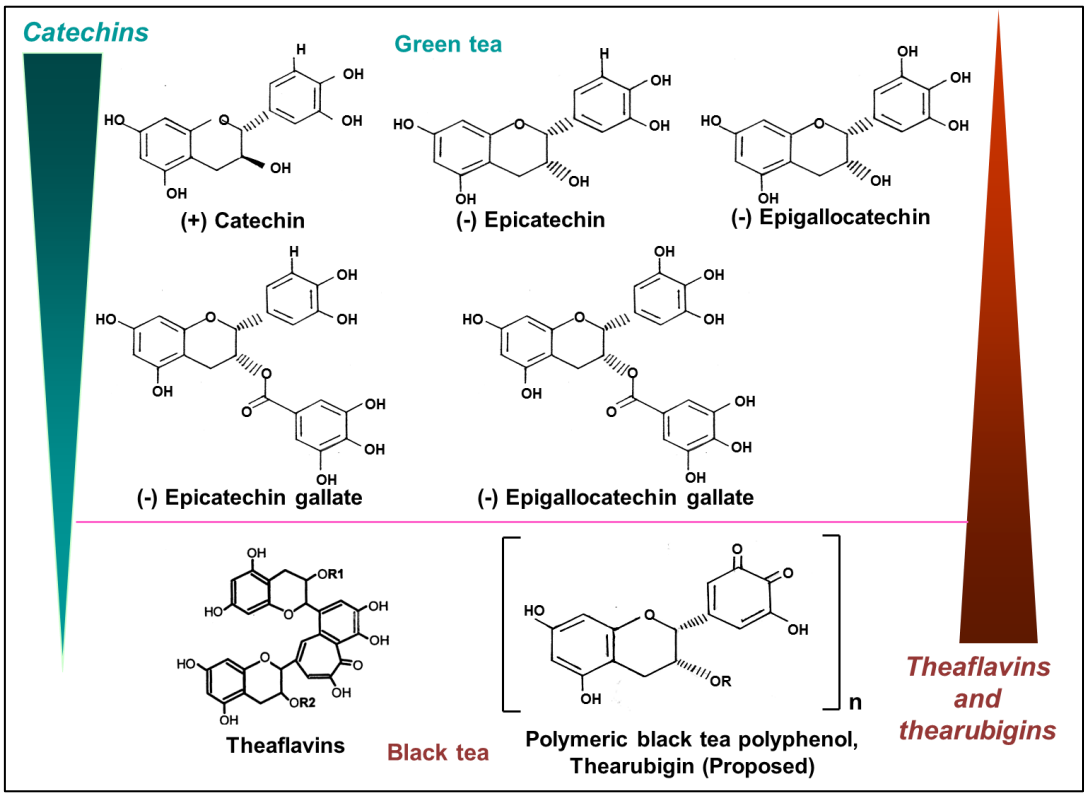


Figure 1.8 : Structure of polyphenols present in tea

The major catechins (a group of polyphenols) in green tea are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−) – epicatechin which together may constitute 30–42% catechins of the dry leaf weight **(35)**. Among these, EGCG is the major polyphenol which accounts for 60% of total catechins and is believed to be the most protective agent in green tea. Among these, EGCG is the major polyphenol which accounts for 60% of total catechins and is believed to be the most protective agent in green tea. For the production of oolong and black tea, the fresh leaves are allowed to wither until their moisture content is reduced to <55% of the original leaf weight, which results in the concentration of polyphenols in the leaves. The withered leaves are then rolled and crushed, initiating fermentation of the polyphenols. Oolong tea is prepared by firing the leaves shortly after rolling to terminate the oxidation and dry the leaves. Normal oolong tea (semi-fermented) is considered to be about half as fermented as black tea. The fermentation process results in oxidation of simple polyphenols to more complex condensed polyphenols to give black and oolong teas their characteristic colors and flavors **(68)**.

During the manufacture of black tea, a major proportion of monomeric free catechins in the fresh green tea leaf undergo PPO-catalyzed oxidative polymerization, to form oligomers-theaflavins (TFs) (500-1000 Da) and polymers– polymeric black tea polyphenols (PBPs)/ thearubigins (TRs) (>1 kD) in a process commonly known as fermentation (Table 1.1) **(69)**. TFs are well characterized group of oligomeric compounds e.g. theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin- 3′-gallate (TF-2b) and theaflavin-3,3′-digallate (TF-3), whereas TRs are a group of polymeric compounds and five different fractions (PBPs 1-5) have been isolated although poorly characterized **(70)**. The polyphenolic components and dry solid weight extracted from green and black tea has been listed below in **Table 1.2.**

	Components	g % of dry solid extracted*	g % of total polyphenols content
Black Tea	Catechins	3-10	30
	Theaflavins	3-6	13
	Thearubigins/PBPs	12-18	47
Green tea	Catechins	30-42	90
	Theaflavins	-	-
	Thearubigins/PBPs	-	-

* % of solid extracted from black tea = 25-35%, PBPs = Polymeric black tea polyphenols

Table1.2 : Polyphenol content in green and black tea (Adopted and modified from Kumar et al., 2010) (35)

Extensive research on green tea/polyphenols has resulted in voluminous literature and evidence that they possess anti-bacterial, anti-viral, anti-oxidative, anti-mutagenic, anti-clastogenic, anti-inflammatory and anti-carcinogenic properties **(71,72)** and possess diverse mechanisms for observed chemopreventive actions **(55,73,74)**. The observed protective activity of green tea has been attributed to the powerful scavenging and antioxidative ability of monomeric catechins and their gallates **(55,69)**.

Studies employing different *in vivo* animal models have demonstrated anti-initiating and anti-promoting activity of BTE and its components in both spontaneous and carcinogen-induced tumor models **(Table 1.3)**. Oral and topical administration of BTPs has been shown to modulate incidence/multiplicity/ latency period or other degenerative changes at various organ sites in experimental animals **(Table 1.3)**.

S. No	Animal Model	Organ	Carcinogen Dose/Route Duration	Black Tea Extract / Polyphenols	Salient Observations	Ref.
Crude black tea extract						
1	SKH-1 mice	Skin	i) UV-A 1.8 x 10^{-3} W/cm^2, 5-15 mins/day, 5x/wk-30 wks ii) UV-B 2.4 x 10^{-4} W/cm^2 , 5-15 mins/day, 5x/wk-30 wks	BTE 1.25-2%, sole drinking fluid, 1 wk prior till end	Decreased multiplicity, increased latency period of skin tumors	**(75)**
2	SKH-1 mice	Skin	i) UV-B 180 mJ/cm^2, 5 mins-1x/day-2 days ii) UV-B 180 mJ/cm^2, single	i) SBTE 0.8%, sole drinking fluid, 2 wks prior till end ii) SBTE, BTF1, BTF2 0.2 mg/cm^2, 30	Decreased incidence, severity of erythema, skin fold thickness	**(76)**
3	Swiss mice	Lung	DEN 20 mg/kg BW, gavage, 1x/wk-8wks	BTE 1, 2, 4%, sole drinking fluid, simultaneously + 20 wks	Decreased incidence, multiplicity of adenoma / adenocarcinoma	**(77)**

4	A/J mice	Lung, Rhabdo-myosarcoma	Spontaneous tumors	i) BT 0.5, 1, 2%, sole drinking fluid, 60 wks ii) Instant BT 0.3-0.6%, sole drinking fluid, 52 wks	Decreased incidence, multiplicity, volume	**(78)**
5	A/J mice	Lung	NNK 103 mg/kg BW, i.p., single	BT 0.6%, sole drinking fluid, star- ting 16 wks after NNK treatment till 52 wks	Inhibition of tumor multiplicity, incidence	**(79)**
Decaffeinated black tea						
6	A/J mice	Lung	NNK 103 mg/kg BW, i.p., single	DBTE 0.3% or 0.6%, sole drinking water i) Starting 2 wk before, till 1 wk after NNK ii) Starting 1 wk after NNK treatment till end	Decreased tumor multiplicity with-out effect on incidence	**(80)**
7	C3H mice	Liver, Lung	DENA 50 µg/kg BW, i.p., 1x/wk- 8 wks	DBT 1.25%, sole drinking fluid, 2 wks prior to & during	Dose dependent decrease in multiplicity in lung and liver	**(81)**

8	SKH-1 mice	Skin	i)DMBA 200 nmole, topical, single ii)UV-B 30 mJ/cm^2, 25 secs, 3 wks after DMBA	DBTE, BT 0.63 % or 1.25%, sole drinking fluid, 2 wks prior to du-ring 31 wks	Decreased incidence, multiplicity, volume of keratoacanthomas, carcinomas	**(82)**
9	SD rats	Esophagus	NMBzA 2.5mg/kg BW,s.c., 2x/wk-5wks	DBT 0.6%, sole drinking fluid, during and after NMBzA treatments till 39 wks	Reduction in tumor incidence, multiplicity	**(83)**
Thearubigins						
10	Swiss bare mice	Skin	DMBA + TPA 20 nmole, topical, single + 1.8 nmole, topical, 2x/wk-40 wks	PBPs 1-5 200µg , topical 20 mins prior to TPA treatment	Decreased multiplicity, incidence of skin papillomas, cell proliferation markers, transcription factors at 10- 40 wks	**(84)**

11	SD rats	Colon	DMH 40 mg/kg BW, s.c., 2x/wk-3wks	PBPE 1.5%, sole drinking fluid, 15 days prior till end	Inhibited colorectal carcinogenesis by decreasing tumor volume, multiplicity, inhibited cell proliferation *via* Wnt / β -catenin pathway	**(85)**
Theaflavins						
12	Mice strain A	Lung	B(a)P 4µg, s.c., single	TFs, EGCG and ECG 0.02 mg, 0.01 mg and 0.004 mg, i.p., till end	Restricted lung carcinogenesis by differential modulation of expression of p53, H-ras, c-myc, cyclin-D1	**(86)**
13	A/J mice	Esophagus	NMBA 3x/wk-5 wks, s.c.	TFs (360ppm), EGCG (1200ppm) sole drinking fluid, 2 wks prior till end	Decreased rate of tumor formation, only TFs decreased the multiplicity	**(87)**
14	A/J mice	Lung	NNK 100 mg/kg BW, i.p., single	TFs 0.1%, sole drinking fluid, 2 days after NNK treatment for 16 wks	Decreased tumor incidence, multiplicity	**(88)**

Table 1.3 : *In vivo* chemopreventive effects of black tea extract/polyphenols/constituents in different rodent models (Adopted and modified from Kumar et al.,2010**) (35)**

Chemopreventive efficacy of BTE / BTPs during different stages of carcinogen-induced tumorigenicity has been demonstrated in several experimental models **(Table 1.3)**. The mechanisms implicated in the inhibition of carcinogenesis by BTPs involve modulation of signaling kinases or xenobiotic-induced activation / translocation of kinases or modulation of tumor-induced responses ultimately leading to effects on multiple signaling pathways and genes. Thus black tea polyphenols also possess protective chemopreventive potential comparable to EGCG, the well-established green tea polyphenol. However, most of the tea extracts are tested in these studies are either whole black tea extracts (BTE) or oligomeric fraction, theaflavin (TF). However, the studies on thearubigins, (TR) or polymeric black tea polyphenols (PBPs), one of the major fractions of black tea are limited. Tea polyphenols such as EGCG and theaflavins have reached clinical trials after their chemopreventive efficacies have been established by various pre-clinical studies **(35,89)**. However, similar studies on TR/PBPs are warranted. Thearubigins or PBPs are structurally and chemically ill-defined heterogeneous polymers of flavan-3-ols and flavano-3-ol gallates with di- and tribenzotropolone skeletons **(70,90)**. We have previously isolated and partially characterized five different PBP fractions **(91)** by modifying PBP isolation method by **(92)**. According to the method and solvents used for isolation, the PBP fractions (PBP 2-5) closely resemble butanol soluble acidic thearubigins (Ozawa et al 1996). These PBP fractions, which are polymeric proanthocyanidine in nature, have shown competence in inhibiting initiation of carcinogenesis by decreasing BPDE-DNA adduct formation *in vitro* and in mouse skin **(93)**. These PBP fractions have shown chemopreventive activity in skin as well as in colon carcinogenesis modulating PKC and Wnt-β catenin signaling pathway respectively **(85,94)**.

Hence, objective of the current study was to investigate the chemopreventive potential of PBPs by evaluation of anti-initiation and anti-promotion properties of PBPs using tobacco carcinogen induced lung carcinogenesis in A/J mice model.

Chapter 2

Aims and objectives

Chapter 2: Aims and objectives

Aims:

- Extraction and characterization of polymeric black tea polyphenols (PBPs) from black tea
- To determine the molecular mechanism involved in the sequential development of lung cancer
- To elucidate the molecular mechanism of the chemopreventive action of PBPs in lung cancer

Objectives:

- To understand the anti-initiation and anti-promotion mechanism of PBPs in experimental lung carcinogenesis
- To understand the effect of dose response of PBPs on carcinogen metabolism during early stages of lung carcinogenesis
- To understand the effect of pre and post treatment of PBPs on early stages of experimental lung carcinogenesis

Chapter 3

Materials and methods

Chapter 3: Materials and methods

Materials

Chemicals and reagents	Source
1-chloro-2,4-dinitrobenzene (CDNB)	Sigma Chemical Co., USA
Acrylamide	Sisco Research Laboratories, India
Ammonium persulphate (APS)	Sisco Research Laboratories, India
Aprotinin	Sigma Chemical Co., USA
Benzo(a)pyrene [B(a)P, purity ~98%]	Sigma Chemical Co., USA
Bis-acrylamide (AR grade)	Sigma Chemical Co., USA
Black tea powder	Brook bond red label, India
β-Mercaptoethanol	Sigma Chemical Co., USA
Bovine serum albumin (BSA)	Sigma Chemical Co., USA
Bromophenol blue (BPB)	Sigma Chemical Co., USA
Coomassie brilliant blue R 250	Sigma Chemical Co., USA
Caffeine	Sigma Chemical Co., USA
Catechin	Sigma Chemical Co., USA
Copper sulphate (AR grade)	Sisco Research Laboratories, India
Dithiothreitol (DTT)	Sigma Chemical Co., USA
Epicatechin	Sigma Chemical Co., USA
Epicatechin gallate	Sigma Chemical Co., USA
Epigallocatechin	Sigma Chemical Co., USA
Ethoxyresorufin (ER)	Sigma Chemical Co., USA
Folin and ciocalteu's phenol reagent	Sisco Research Laboratories, India

Glyceryl trioctanoate	Sigma Chemical Co., USA
Methoxyresorufin (MR)	Sigma Chemical Co.,USA
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 98% purity)	Eagle Picher Pharmaceutical services, USA
Nicotinamide adenine dinucleotide phosphate, Reduced (NADPH)	Sigma Chemical Co.,USA
PageRuler prestained protein ladder	Fermentas Life Sciences, USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Chemical Co.,USA
Poly-L-lysine	Sigma Chemical Co.,USA
Ponceau S	Sigma Chemical Co.,USA
Polyvinylidene difluoride (PVDF) membrane	Amersham Biosciences, UK
Restore western blot restriping buffer	Thermo Scientific, USA
Skimmed milk powder	Sagar milk, India
Sodium fluoride	Sigma Chemical Co.,USA
Sodium orthovanadate	Sigma Chemical Co.,USA
Sodium carbonate	Sisco Research Laboratories, India
Sodium hydroxide	Sisco Research Laboratories, India
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma Chemical Co.,USA
Tris	Sisco Research Laboratories, India
Triton X 100	Sigma Chemical Co.,USA
Tween 20	Sigma Chemical Co.,USA

Solvents	**Source**
Acetone (ultra-pure analytical grade [AR])	Merck Limited, India

Chloroform (AR grade)	S.D. Fine Chemicals Co., India
Diethyl ether (AR grade)	S.D. Fine Chemicals Co., India
Ethyl acetate (AR grade)	S.D. Fine Chemicals Co., India
Ethanol absolute 99.9%	S.D. Fine Chemicals Co., India
Formaldehyde (37-41% w/v)	Sisco Research Laboratories, India
Glacial acetic acid (AR aldehyde free)	S.D. Fine Chemicals Co., India
Glycerol (extra pure AR)	Sigma Chemical Co.,USA
Hydrochloric acid (35-38% AR)	S.D. Fine Chemicals Co., India
Hydrogen peroxide (30% AR)	S.D. Fine Chemicals Co., India
Isopropanol (AR)	S.D. Fine Chemicals Co., India
Methanol (AR)/ (LR)/(HPLC)	S.D. Fine Chemicals Co., India
Sulphuric acid (98% AR)	S.D. Fine Chemicals Co., India
Trifluroacetic acid	S.D. Fine Chemicals Co., India
Acetonitrile	S.D. Fine Chemicals Co., India

Enzymes	**Source**
Proteinase K	Sigma Chemical Co., USA
Ribonuclease A (RNase A)	Sigma Chemical Co., USA

Immunochemical reagents / Kits	**Company**
Rabbit anti-mouse AKT1/2 primary antibody	Cell Signalling Technology Inc., USA
Rabbit anti-mouse p-AKT1/2 primary antibody	Cell Signalling Technology Inc., USA
Rabbit anti-mouse □-actin primary antibody	Abcam, USA
Rabbit anti-mouse Bax primary antibody	Santa Cruz Biotechnology Inc., USA

Rabbit anti-mouse Bcl-2 primary antibody	Santa Cruz Biotechnology Inc., USA
Mouse monoclonal BPDE-DNA adduct (clone 5D11) primary antibody	Hycult Biotechnology (Uden, Netherlands)
Rabbit anti-mouse CYP1A1 primary antibody	Santa Cruz Biotechnology Inc., USA
Rabbit anti-mouse CYP1A2 primary antibody	Abcam, USA
Rabbit anti-mouse Cox-2 primary antibody	Abcam, USA
Rabbit anti-mouse ERK2 primary antibody	Cell Signalling Technology Inc., USA
Rabbit anti-mouse GST alpha primary antibody	Abcam, USA
Rabbit anti-mouse GST mu primary antibody	Abcam, USA
Rabbit anti-mouse GST pi primary antibody	Abcam, USA
Rabbit anti-mouse p38 primary antibody	Abcam, USA
Rabbit anti-mouse p38 primary antibody	Cell Signalling Technology Inc., USA
Rabbit anti-mouse p-p38 primary antibody	Cell Signalling Technology Inc., USA
Rabbit anti-mouse p-p53 primary antibody	Cell Signalling Technology Inc., USA
Horse-radish peroxidase conjugated secondary antibodies (anti-goat/mouse/rabbit)	Abcam, USA
ECL chemiluminescence detection kit	Amersham Biosciences, UK; Advansta solutions, USA Pierce, USA
Vectastain ABC kit	Vector Laboratories, USA
In-situ TUNEL assay kit	Takara Bio.Inc., Japan
GST enzyme activity kit	Sigma Chemical Co., USA

Instruments	**Model**	**Company**

Spectrophotometer	UV – 160 A, UV – 240	*Shimadzu, Japan*
	U – 2001	*Hitachi, Japan*
	Biophotometer 6131	*Eppendorf,Germany*
Spectrofluorophotometer	RF-1501	*Shimadzu, Japan*
Centrifuges		
High – speed	Rota 4-R, Superspin R -V FA	*Plasto Crafts,India*
	Sorvall RC5C, Sorvall RC – 5C plus	*DuPont, USA*
Ultracentrifuge	Sorvall Ultra 80; Centrikon T – 1065	*DuPont, USA* *Kontron, USA*
Table top ultracentrifuge	TL – 100, Optima TLX	*Beckman, USA*
Microfuge	Spin win	*Tarsons, India*
Speed vac concentrator	SVC 1000, AES 1000	*Savant, USA*
Homogenizer	CH – 6010	*Kinematica,Germany*
Upright microscope	Axioimager.Z1	*Zeiss, Germany*
	Eclipse 50i	*Nikon, Japan*
Confocal microscope	LSM 510 Meta	*Zeiss, Germany*
Microtome	RM2145	*Leica Instruments,Germany*
X-ray film developing machine	Optimax	*Protec, Germany*
pH meter	APX 175E	*Ingold, Germany*
Vertical electrophoresis Assembly	Monokin, Minikin, Macrokin	*Techno Source, India*
Electro blot transfer assembly	Technoblot	*Techno Source, India*

	Trans – Blot Cell	*Bio–Rad, USA*
Power packs	Gativaan Power pac 2000	*Techno Source, India;* *Bio–Rad, USA*
ELISA reader	*Spectra Max 190*	*Molecular Devices, USA*
MALDI-TOF	*Ultraflex-II*	*Brucker Daltonic, Germany*

Animal	**Strain (inbred)**	**Source**	**Remark**
Mouse	A/J	Jackson Laboratories, USA.	Tobacco carcinogens induced lung carcinogenesis

Methods

3.1 Extraction and characterization of polymeric black tea polyphenols (PBPs) from black tea powder:

Polymeric black tea polyphenols (PBPs) extract was prepared employing popular brand of black tea powder (Brooke Bond Red Label, India), using a Soxhlet extractor **(Figure 3.1)**.

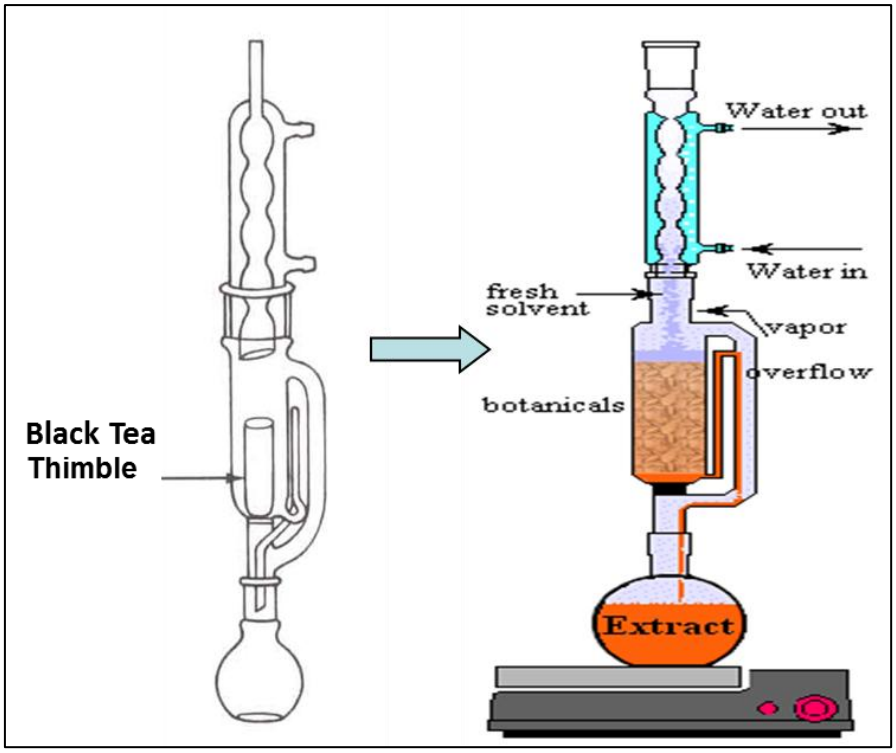


Figure 3.1: Soxhlet extractor for extraction of PBPs.

Extraction was carried out using well established and previously published method by **(95)**. Briefly, 450g of black tea powder was serially extracted in a Soxhlet extractor (Borosil Glass Works Ltd., Mumbai, India) with chloroform (2.5 L) for decaffeination until no color was observed in the solvent (~48 hrs.). Air-dried black tea powder (~447 g) in a thimble was further Soxhlet-extracted with ethyl acetate (2.5L, 48hrs.) which extracts PBP-1, catechins and theaflavins. The ethyl acetate extract was dried *in vacuo*, and was reconstituted with acetone (200ml) and precipitated with 8 volumes of diethyl ether, thrice, to obtain a precipitate of PBP-1 (~9 g). This precipitate was further separated by centrifugation, dried in vacuo, employing rotary flash evaporator and stored at -20^{o}C for further use. Residual tea powder was dried and stored at -20^{o}C **(Figure 3.2)**. The above procedures were carried out in a chemical safety hood with necessary protective gadgets and precautions. To prevent any light mediated effects, containers were covered with brown paper and/or aluminum foil during the course of extraction.

3.1.1 Preparation of PBPs extract:

Preparation of 1.5% black tea derived polymeric black tea polyphenols (PBPs):

Before use, residual tea powder was boiled in autoclaved miliQ water such that it provided 1.5% black tea derived extract, known to contain mixture of PBPs-2, 3, 4, 5. To this aqueous extract, PBP-1was added back to obtain PBP rich extract (free of caffeine, catechins and theaflavins) **(Figure 3.2)**. PBPs was aliquoted and fed to the animals after it was confirmed to be free of other biologically active components such as caffeine, theaflavins and catechins using thin layer chromatography.

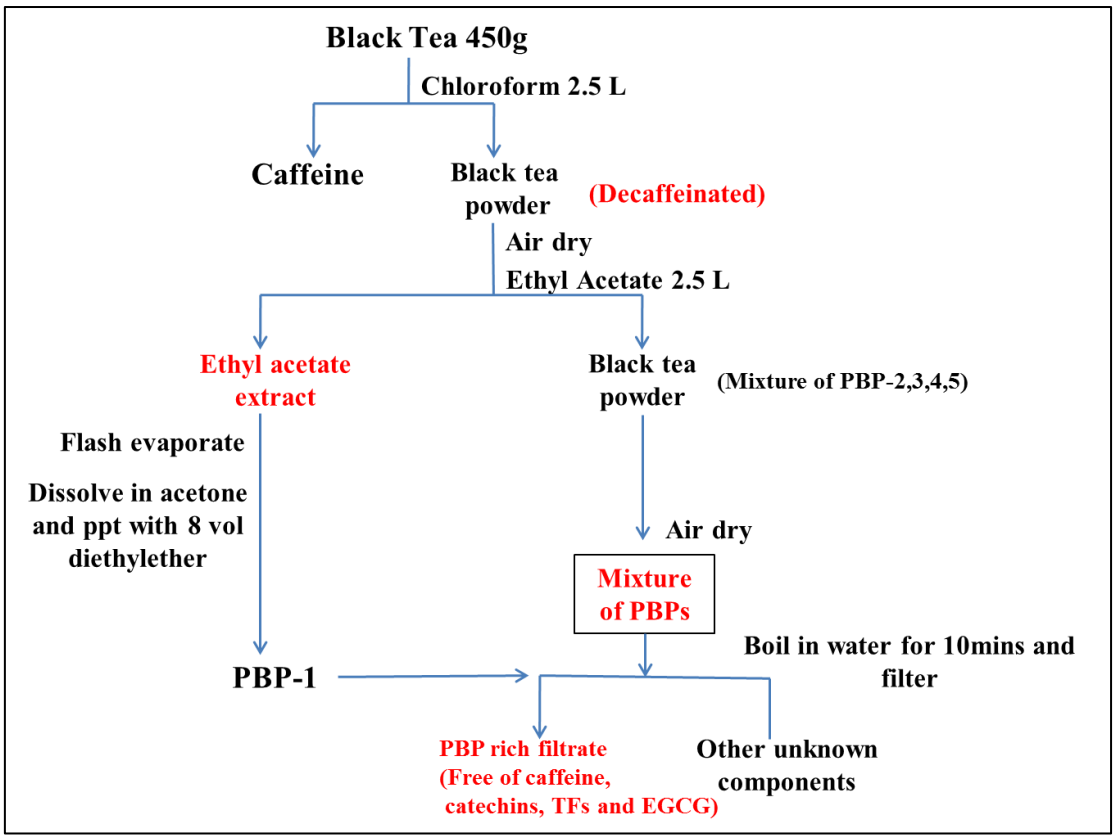


Figure 3.2 : Extraction and preparation protocol for polymeric black tea polyphenols (PBPs) rich extract from black tea powder

3.1.2 Determination of contamination by other black tea components in PBP rich extract / Evaluation of purity of PBP extract:

3.1.2.1 TLC analysis:

To ascertain that PBP fractions were free from other known biologically active, mobile components present in black tea such as caffeine, free catechins [C, EC, ECG, EGC, EGCG, GCG] or TFs, 20-40 µl of each PBP fraction (5 mg dissolved in 1 ml acetone) were spotted on pre-coated silica gel plates containing 254 nm fluorescent indicator and developed in chloroform: ethyl acetate: formic acid (6:4:1) and visualized under UV light. The authentic standards for caffeine and free catechins were also spotted on the same plate for comparison. After confirming the purity, these extracted PBPs were used for all further experiments **(Figure 3.3)**.

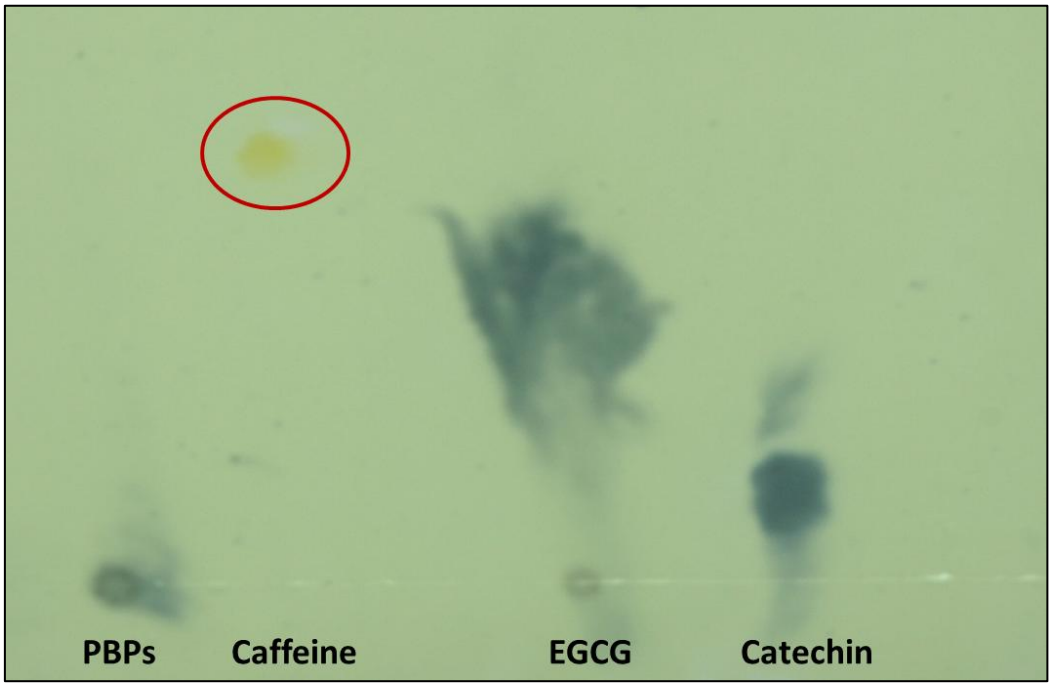


Figure 3.3 : Thin layer chromatogram for PBPs and other biological contaminants like caffeine, EGCG, catechin

3.1.2.2 MALDI-TOF analysis:

Instrumentation and calibration details of MALDI-TOF:

Compounds cocrystallized with matrix are desorbed and ionized by a solid state laser Neodymium/Yettrium aluminium garnet [Nd-YAG] (wavelength 337 nm; 4 ns pulse width) and extracted by 4 kV pulse voltage with time-delayed extraction of 70 ns before entering the time-of-flight mass spectrometer and accelerated under 20 kV. All spectra are recorded with a detector voltage of 2.5 kV and are the average result of 800 laser shots. Laser intensity and sensitivity of detector are variable. MALDI-TOF is calibrated with HCCA matrix as an two point internal calibration [M+H]2+ m/z 190.04987 and [2M+H]+ m/z 37.09246. In interpretation of spectra, peaks at m/z range 100 to 2500 were taken into account.

Protocol for MALDI-TOF:

MALDI TOF analysis was employed additionally to confirm purity of PBPs. α-cyano-4-hydroxycinnamic acid (HCCA) matrix was used for sample analysis. Vacuum dried PBPs extract(s), catechin standard (EGCG), theaflavins and caffeine standard were dissolved (1mg/20µl) in solvent i.e. the mixture of 0.1% trifluroacetic acid (TFA) in 50% acetonitrile (ACN). Purity of PBPs was checked by analysis of 5 independently extracted samples which were mixed to the HCCA matrix solution (1:1 v/v). Aliquot of this mixture (4µl) was put on a spot of 396 array plate and dried at room temperature. MALDI-TOF mass spectra were acquired on a reflectron mode using Bruker Daltonics Ultraflex-II, Germany. The resulting MS data was analyzed using Flex analysis 3.0 (Brucker Daltonic, Germany) software.

Result of analysis of PBPs:

Since black-tea derived contaminants such as caffeine, EGCG and theaflavins are mobile in TLC analysis, while PBPs remain bound to matrix at the origin, even low levels of contamination is possible to detect by TLC. Absence of any mobile analyte in TLC based

analysis of PBPs revealed that the extract was free of biologically active black-tea derived components. To confirm the absence of black tea-derived contaminants, PBP extract(s) were analyzed employing MALDI TOF analysis. We analyzed the spectra of potential contaminants by using commercially available standards for EGCG (Sigma E4143), caffeine (Sigma C0750) and mixture of theaflavins (Sigma T5550) showing its m/z value 459.11, 195.13 and 565.10 respectively which matched with those reported **(Figure 3.4).** None of the PBP extracts showed the presence of any of these contaminants. However all five independently extracted PBP samples showed 2 specific peaks of m/z value 854.8 and 876.7 **(Figure 3.4)** which confirms absence of black tea derived other known biologically active contaminants in PBPs. Hence the observed biological activity can be attributed to exposure to PBPs, the only variable.

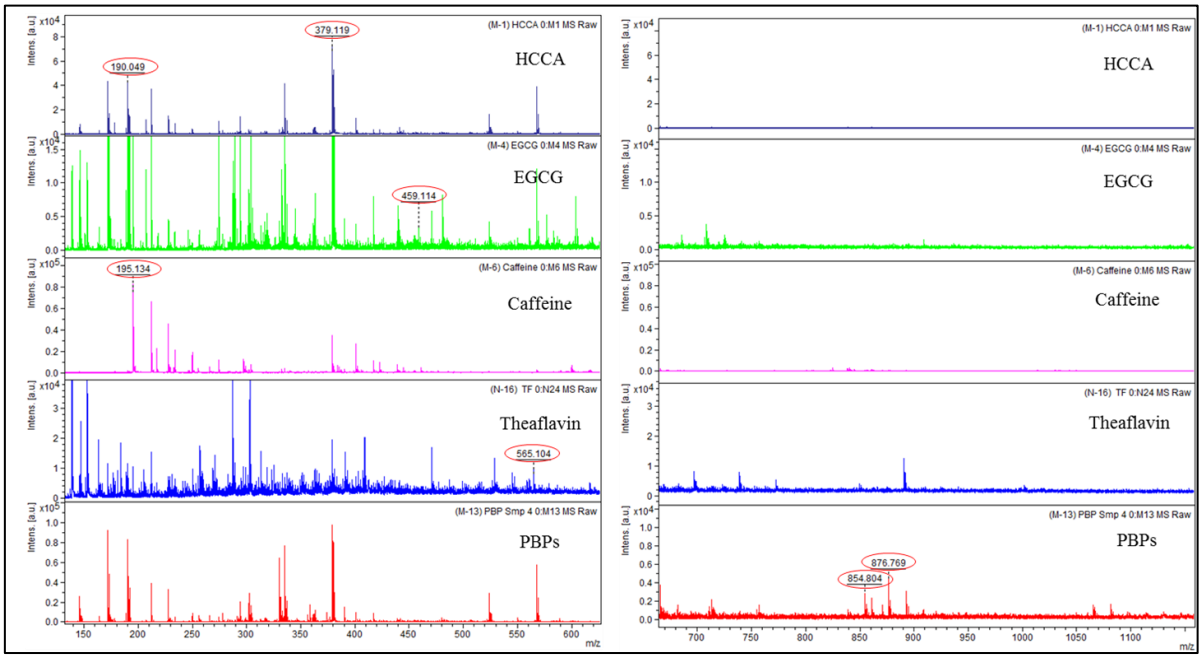


Figure 3.4 : Evaluation of black tea derived contaminants viz. EGCG, caffeine, theaflavins in PBPs extract by MALDI-TOF analysis. Representative MALDI-TOF mass spectra of EGCG (m/z 459.1), caffeine (m/z 195.1), theaflavin (m/z 565.1) using α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix. Absence of signals at those masses in PBPs

extract(s) confirms that PBPs extracts were free from black tea-derived biologically active contaminants. Representative MALDI-TOF mass spectra of one of the five independently extracted PBPs extract showed signals at m/z 854 and 876.

3.2 Animals:

All animal studies were conducted after approval from the Institutional Animal Ethics Committee as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India) guidelines. Inbreed male A/J mice, 6-8 weeks old were obtained from Laboratory Animal Facility (LAF). They were randomly distributed into various groups, housed in polypropylene cages (4-5 per cage) and maintained under standard conditions of 22 ± 2 $^{\circ}$C, 45 ± 10 % relative humidity and 12 h light/dark cycles. Drinking water was passed through Aquaguard® for UV sterilization. Specific-pathogen-free animals were used for all the studies. Details of animal treatments for different objectives have been described separately under each chapter. Animals were sacrificed generally by cervical dislocation. A portion of the tissue was fixed in 10% buffered formalin for histopathological evaluation and immunohistochemical staining while rest of the tissue was snap frozen in liquid nitrogen and stored at -80 $^{\circ}$C until used. Mice belonging to the various treatment groups were observed once a week for net body weight gain/loss, survival, and signs of toxicity such as changes in fur color or texture, behavioral abnormalities and palpable masses during the experimental period.

3.3 PREPARATION OF VARIOUS CELLULAR FRACTIONS

3.3.1 Total cell lysate preparation:

Total cell lysate from mouse (liver and lung) tissues were prepared as described **(96)**. Tissues were weighed (~ 0.2 g) and homogenized in 1-3 ml of ice-cold lysis buffer with freshly added

protease inhibitors* **(Table 3.1)**. All steps were carried out at 0-4°C. The homogenate was incubated on ice for 30 min with intermittent tapping to allow complete lysis to occur. Homogenate was then centrifuged at 14000 x g for 25 min and the supernatant (total cell lysate) was collected, aliquoted, protein content was determined, and stored at -80°C.

Table 3.1: Lysis buffer composition for total cell lysate preparation

Component	**Stock conc.**	**Working conc.**	**For 25 ml buffer**
Tris-HCl, pH 7.4	1 M	50 mM	1.25 ml
NaCl	1 M	150 mM	3.75 ml
EGTA	0.2 M	1 mM	0.125 ml
EDTA	0.5 M	1 mM	0.05 ml
NaF*****	0.5 M	20 mM	1 ml
Na3VO4*****	0.5 M	100 mM	5 ml
NP-40	100%	0.5 %	0.125 ml
Triton X-100	100%	1 %	0.25 ml
PMSF*****	200mM	1 mM	0.125 ml
Aprotinin*****	4mg/ml	10 µg/ml	0.0625 ml
Leupeptin*****	4mg/ml	10 µg/ml	0.0625 ml
β-glycero phosphate	1 M	10mM	0.25 ml
Chilled distilled water (D/W)	-	-	12.95 ml

The stock solutions were autoclaved and stored at 4°C. Aliquots of protease inhibitors were kept at -20°C. Preferably; fresh lysis buffer (according to the requirements) was prepared using individual components at the time of use.

3.3.2 Microsomes and cytosol preparation:

Liver and lung microsomal and cytosolic fractions were prepared by differential centrifugation. All steps were carried out at 0-4°C. The tissues after weighing (liver ~0.6 g; 3 pooled lungs ~0.6 g) were minced with scissors and homogenized in 6 ml (10% homogenate) of chilled 0.15 M KCl in Potter Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged at 9000 x g for 20 min. The supernatant (S9) was then centrifuged for 1 h at 100000 x g in an ultracentrifuge and the supernatant was collected as cytosolic fraction. Microsomal pellet obtained was resuspended in 1-2 ml chilled 0.15 M KCl. Both cytosolic and microsomal fractions were aliquoted, their protein content determined and stored at -80°C.

3.3.3 Determination of protein content:

Determination of protein content of various cellular fractions prepared was done according to the previously detailed procedure **(97).**1 ml miliQ was uniformly added to all the test tube. 5 µl of blank / standard (20 –100 µg BSA solution) / samples (1:5 diluted for liver and lung) were taken in the test tube. To this 1 ml of freshly prepared working CTC solution* was added and vortexed. After an incubation of 10 min at RT, 500 µl of 1 N Folin Ciocalteau (FC) reagent (1 in 6 times diluted, 0.33N) was added and tubes were vortexed and incubated in dark for 30 min at RT. All samples and standards were run in duplicates. Absorbance at 750 nm was measured in a spectrophotometer. A standard curve was prepared using 20–100 µg of BSA. Concentration of protein in samples was determined from the standard curve.

[CTC = 0.1% $CuSO_4$ + 0.2% Sodium potassium tartrate + 10% Na_2CO_3]

[*CTC mixture = CTC: 0.8 N NaOH: 10% Sodium-dodecyl-sulphate (SDS): D/W].

3.4 Protein immunoblotting:

To study the expression of various proteins in cellular fractions that were prepared, SDS-PAGE followed by western blot analysis was employed **(98)**.

Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis:

Polyacrylamide gels are formed by crosslinking monomeric acrylamide with N,N'-methylene bisacrylamide. Cross-links add rigidity and tensile strength and form pores through which the proteins to be separated must pass. The size of these pores decreases as the bisacrylamide:acrylamide ratio increases. The range of protein molecular weight and respective acrylamide concentration to be used is mentioned in **Table 3.2**

Table 3.2 : Effective range of separation of SDS-polyacrylamide gels:

Acrylamide concentration (%) (usually with 0.2-0.5% bisacrylamide)	Linear range of separation (kD)
15	10-43
12	12-60
10	20-80
8	36-94

Resolving gel preparation:

Firstly, the glass plates were assembled with 1.5 mm spacers in between. The two sides and bottom of the glass plate assembly were sealed with 2% molten agar. Simultaneously resolving gel was prepared as below.

Table 3.3: Composition of resolving gels:

Components	**8%**	**10%**	**12%**
30% Acrylamide solution (Acrylamide : Bisacrylamide = 29 : 1)	8 ml	10 ml	12 ml
Tris-HCl pH 8.8 (1.5 M)	7.5 ml	7.5 ml	7.5 ml
10% SDS	0.3 ml	0.3 ml	0.3 ml
Distilled water	13.87 ml	11.87 ml	9.87 ml
10% Ammonium presulfate (APS, freshly prepared)	0.3 ml	0.3 ml	0.3 ml
TEMED (added just before pouring the gel)	0.030 ml	0.030 ml	0.030 ml
Total volume	30 ml	30 ml	30 ml

Resolving gel was poured into the slot between the two glass plates (up to 3/4th their height). A layer of distilled water was poured above the gel; this facilitates the formation of an even upper surface of the gel and prevents the air (oxygen) from affecting the polymerization of the gel. The gel polymerizes in 20-30 min.

Stacking gel preparation:

Table 3.4: Composition of stacking gel

Components	**6%**
30% Acrylamide solution (Acrylamide : Bis acrylamide = 29 : 1)	4.0 ml

Tris-HCl pH 6.8 (0.5 M)	5.0 ml
10 % SDS	0.200 ml
Distilled water	10.58 ml
10% APS (freshly prepared)	0.200 ml
TEMED (added just before pouring the gel)	0.020 ml
Total volume	20 ml

After removing the water layer from above the resolving gel, stacking gel was poured and appropriate comb was immediately inserted. Polymerization takes place in about 20-30 min. Comb was then carefully removed and the wells were washed with distilled water to remove any polymerized/unpolymerized acrylamide therein. The unit was then filled with tank buffer (at a final conc. of 1X, **Table 3.5**).

Sample preparation and gel electrophoresis:

While the gels were polymerizing, protein samples were prepared. Appropriate concentrations of proteins from different cellular fractions were taken. Protein samples were mixed with 3X gel loading dye (Table 3.8) to a final concentration of 1X. The eppendorfs were sealed with parafilm and pulse centrifuged. Samples were denatured by keeping on a float in boiling water bath (100 °C) for 10 min. Samples were then loaded into the wells of polyacrylamide gel and electrophoresed at a constant current of 30 mA for 2-3 h at room temperature (RT). In one of the lanes along-side the samples; molecular weight marker was also run so as to determine the mobility and molecular weights of various proteins in a particular lysate. To check the resolution of proteins and the band pattern of a particular type of lysate, the gel (after the run) was stained with Coomassie blue solution (0.2% Coomassie

blue in destainer) overnight with gentle rocking. The gel was destained the next day by giving several changes of destaining solution (methanol: glacial acetic acid: water = 5:1:4).

Table 3.5 : Buffer compositions for SDS-PAGE

5X tank buffer

Component	Working Conc.	Amount
Tris	125mM	15.1 g
SDS	5%	5 g
Glycine	960mM	72 g
D/W	-	1000 ml

Transfer buffer

Component	Working Conc	Amount
Tris	24.7mM	12 g
Glycine	190mM	57.2 g
Methanol	20%	800 ml
D/W	-	4000 ml

3X loading dye

Component	Working conc. (3X)	For 10ml (3X)	Working conc. (1X)	For 10 ml (1X)
Tris, pH 6.8 (1M)	0.19 M	1.9 ml	0.063M	0.63 ml
Glycerol	30%	3 ml	10%	1 ml
20% SDS	6%	3 ml	2%	1 ml
1% Bromophenol blue (BPB)	0.06%	0.006 g	0.02%	0.002 g
D/W	-	8.5 ml	-	9.5 ml
B- mercaptoethanol (BME)*	15%	0.15 ml	5%	0.05L

*****Make volume to 8.5 ml (for 3X) or 9.5 ml (for 1X) with D/W. Before use, to 850 µl (for 3X) or 950 µl (for 1X) of buffer add 150 µl (for 3X) or 50 µl (for 1X) of BME.

Transfer of proteins (electro blotting, western blot analysis):

Alternatively, proteins separated by SDS-PAGE were identified by blotting onto an adsorbent porous Polyvinylidene difluoride (PVDF) membrane, which gives a mirror image of the gel. Proteins were transferred at 4 °C, employing a constant voltage of 100V for 45-50 min. A cassette clamping the gel and membrane tightly together in between the two scotch brite pads was put in the transfer apparatus containing transfer buffer (Table 3.8). A current is applied from electrodes situated at either side of the cassette. The buffer is often chilled to avoid heating effects.

Following electro-blotting, to check for the transferred proteins the nitrocellulose/ PVDF membrane was stained with Ponceau S (2% Ponceau S dye in 30% trichloroacetic acid and 30% sulphosalicylic acid. This stock was diluted 1:10 with 1% glacial acetic acid prior to use. The efficiency of transfer was also checked by staining the transferred-gel with Coomassie blue to detect any residual protein in the gel. Subsequently the membrane was washed with T–TBS (20mM Tris-HCl, 500mM NaCl, 0.1% (v/v) tween 20, pH 7.4) for ~ 5 min to remove the colour of Ponceau S or Fast green. After blocking with 5% non-fat skimmed milk or 5% BSA in TTBS (depending upon the protein), membranes were probed with 1:500-1:2000 dilutions of appropriate primary antibody at 4°C, overnight (*antibody dilutions were made in 2.5% milk in TTBS or 1-3% BSA). Next day, blots were washed with TTBS (15 min x 3) and incubated with 1:1000-1:5000 dilutions of anti-rabbit or anti-goat horse radish peroxidase conjugated-secondary antibodies* at RT for 1h. Membranes were then washed with TTBS (15 min x 2) followed by a wash with TBS for 15 min. Immunoreactive bands were then

visualized with enhanced chemiluminescence reagent as per the manufacturer's instructions followed by autoradiography. Further, for loading controls the blots were washed with TTBS (10 min x 2) followed by incubating with 2-3 ml of restore western blot restriping buffer (Thermo Scientific) at 37 °C for 10-15 min. The blots were again rigorously washed with TTBS (10 min x 5), until smell of BME is eliminated from the washing buffer. Blots were blocked with 5% non- fat milk protein or 3% BSA. Blots were then re-probed with appropriate primary antibody for loading control proteins (β-actin for total cell or cytosolic proteins) and processed as described above. Densitometry of various analyte proteins was performed using Image J 1.43 (NIH) software by inverting the blot image, subtracting the background and then calculating the mean intensity of each band in a single blot by drawing/selecting the largest band area. Similarly, the intensity of the respective loading controls from the same blot was determined. The intensity of the analyte protein was divided by that of the respective loading control to get normalized relative optical density. Data i.e. mean ± SD of three independent samples in each group were compared and presented.

3.5 Measurement of enzyme activity

3.5.1 Measurement of phase I enzyme activity

Measurements of CYP1A1 and 1A2 enzyme activity

The activity of CYP1A1 and1A2 isozymes was measured using specific probe drugs as described **(99,100)**. These isozymes dealkylate the ethoxy and methoxy groups of ethoxyresorofin (ER) and methoxyresorofin (MR) respectively to yield the product resorufin that was measured in a spectrofluorophotometer. Briefly, the various assay components were added in the test tube: 10 µl of 6.25 mM MgSO4, 10 µl of 60 µM EDTA, 10 µl of 5 µM

ER/MR, 1 mg microsomal protein and 0.1 M sodium phosphate buffer, pH 7.4 to make up the volume to 990 µl. The tubes were incubated for 5 min in a 37°C water bath. Reaction was initiated by addition of 10 µl NADPH (100 µM) and was continued at 37°C for 5 min. The reaction was terminated by the addition of 2 ml chilled methanol (HPLC grade). The tubes were centrifuged at 4000 x g for 10 min to pellet the precipitated microsomal protein. The supernatant was used to determine the amount of resorufin formed, in a spectrofluorophotometer, at Ex = 550 nm and Em= 585 nm. Reaction mixture without NADPH served as blank. Resorufin formed in the test samples was calculated from the standard curve of 0-5 µM of resorufin under identical experimental conditions. Results were expressed as nmoles resorufin formed per min per mg protein.

3.5.2 Measurement of phase II enzyme activity

Measurement of total GST enzyme activity:

GST activity was assayed in cytosol prepared from mouse liver and lungs using specific substrate, 1-chloro-2,4-dinitrobenzene (CDNB) by Glutathione S-Transferase (GST) Assay Kit Sigma-Aldrich as per manufacturer's instructions (St. Louis, MO, USA). A complete assay mixture without enzyme served as the control. GS-DNB conjugate formed was measured spectrophotometrically in 96-well plate at 340 nm. Absorbance was read in the plate reader at 340 nm immediately after preparing the reaction tests, and every minute thereafter to obtain at least 6 time points. GST specific activity was calculated in each group and expressed as µmol /ml/min

The activity was calculated using following formulae:

$(\Delta A340)/min = A340(final\ read) - A340(initial\ read) /reaction\ time\ (min.)$

GST activity = (ΔA340)/min* V (ml)* dilution factor/ε_{mM} * V_{enz} (ml)

ε_{mM} (mM^{-1} cm^{-1}) - the extinction coefficient for CDNB conjugate at 340 nm , 5.3 mM-1 (path length - 0.552 cm)

3.6 Immunohistochemical staining:

The expression of Bax, Bcl-2, PCNA, Cox-2, Phospho-HSP27 (Ser82) in mice lungs belonging to various treatment groups were determined by immunohistochemical staining in formalin-fixed, paraffin-embedded, 5 µm thick tissue sections mounted on poly-L-lysine coated glass slides. Sections were deparaffinized with xylene and rehydrated through graded series of alcohol and finally washed with distilled water and 1X PBS. To quench the endogenous peroxidase activity of the tissues, sections were incubated with 3% H_2O_2 in methanol for 30 min in dark. Excess of methanol was removed by three successive washes with 1X PBS for 5 min each. Non-specific binding was blocked by 3% horse or goat serum (from Vectastain Elite kit) for 1 h at 37°C in humidified chamber. Sections were then, incubated with primary antibodies for PCNA (dilution 1:50)/cox-2 (dilution 1:25)/Bax (dilution 1:25) and Bcl-2 (dilution 1:25) overnight at 4 °C. The following day, after two washes with 1X PBS (5 min each), sections were incubated with biotinylated anti-horse or anti-goat secondary antibody (from Vectastain Elite kit) for 30 min at 37°C for 1 h in a humidified chamber. Before and after incubating with Vectastain ABC reagent system for 30 min at 37°C for 1 h, slides were washed with 1X PBS. This was followed by detection using diaminobenzidine (DAB) as the chromogen and Mayer's haematoxylin as the counterstain. Images were captured with Zeiss Microscope (at magnification X100 and X400), with Axiocam MRc5 digital camera attached to it. For negative or isotype controls, the primary antibody was replaced with PBS/ TBS or respective antibody serum (used at respective antibody concentration). In each batch, slides with positive control(s) and PCNA were

processed simultaneously. For nuclear staining of protein, semi quantitative analysis was done by Image J 1.43 (NIH) software. PCNA labeling (Mitotic) index was calculated by counting the number of positively stained cells × 100/total number of cells in photomicrographs of at least 10 different randomly selected fields/section/animals, with at least five animals per group. More than 1000 cell were counted/section /animals.

3.7 Terminal Deoxynucleotidyl Transferase biotin-dUTP Nick End Labelling (TUNEL) Assay for measuring apoptotic index:

Formalin-fixed, paraffin-embedded 5 μm tissue sections of mouse (liver and lungs) tissues were assayed for apoptosis as per the guidelines of *in-situ* TUNEL assay kit. The tissue sections were deparaffinized with xylene and rehydrated through graded series of alcohol and finally washed with distilled water and 1X PBS. Slides were washed by immersing in 0.85% NaCl for 5 min and then with 1X PBS. Tissue sections were fixed by immersing the slides in 10% buffered formalin in PBS for 15 min at RT. Slides were then washed twice with 1X PBS, 5 min each. Tissue sections were permeabilised by incubating with 20 μg/ml proteinase K solution in PBS for 30 min at RT. After washing with 1X PBS, sections were refixed by immersing the slides in 10% buffered formalin in PBS for 5 min at RT and then washed twice with 1X PBS. Sections were then equilibrated for 10 min with equilibration buffer. The fragmented DNA of apoptotic cells was then labeled with the help of terminal deoxynucleotidyl transferase, recombinant, (rTdT) enzyme and biotinylated nucleotides (supplied with the TUNEL assay kit). The sections were incubated with rTdT mix (i.e. a mixture of equilibration buffer, biotinylated nucleotide mix and rTdT enzyme) for 1 h at 37 °C in a humidified chamber. For negative controls, a control incubation buffer without rTdT enzyme (i.e. a mixture of equilibration buffer, biotinylated nucleotide mix and autoclaved water) was prepared. For positive controls, tissue sections treated with DnaseI was used. Reaction was terminated by immersing the slides in 2X SSC buffer for 15 min at RT. Slides

were then washed three times with 1X PBS, 5 min each, to remove unincorporated biotinylated nucleotides. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 in methanol for 30 min in dark. Slides were then washed with 1X PBS and incubated with Streptavidin HRP solution (1:500 in PBS) for 30 min at 37^0C in a humidified chamber and then washed twice with 1X PBS before proceeding for DAB staining. Diaminobenzidine was employed as the chromogenic substrate and slides were counterstained with Mayor's haematoxylin. Slides were further dehydrated, cleared in serial ethanol gradient and xylene and finally mounted with D.P.X. The nuclei of the apoptized cells were stained brown in color. Apoptotic index was calculated by counting the number of positively stained cells × 100/total number of cells in photomicrographs of at least 10 different randomly selected fields/section/animals, with at least five animals per group. More than 1000 cell were counted/section /animal.

BPDE-DNA adducts measurement:

The interaction of BPDE with DNA was studied by immunohistochemical staining using monoclonal antibody recognizing BPDE-DNA adducts as described **(101)**. The formalin-fixed, paraffin embedded 5 μm tissue sections (on poly L-lysine coated slides) were deparaffinized using two successive changes of xylene for 15 min each and then immersing the slides in xylene + alcohol (in equal ratio) for 10 min. Rehydration of tissue sections was performed by sequential incubations for 5 min each in absolute ethanol, 95% and 70% ethanol. Sections were washed successively with tap water, distilled water (D/W) and 1X phosphate buffer saline (PBS) (9.1 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.6 mM KCl, and 135 mM NaCl, pH 7.4) for 5 min each. Sections were incubated for 1 h at 37 °C in a humidified chamber with RNase (100 μg/ml) in Tris buffer (8.1 mM Tris base, 1 mM EDTA, 0.4 M NaCl, pH 7.5). Sections were then washed with 1X PBS, treated with proteinase K in Tris

buffer (10 µg/ml) at room temperature (RT) for 7 min and washed with 1X PBS. To denature the DNA, slides were incubated with 4 N HCl for 7 min at RT and then neutralized with 50 mM Tris base for 5 min at RT. After washing the slides with 1X PBS, non-specific binding was blocked by incubating sections with 3% normal horse serum (prepared in PBS) at 37 °C for 1 h in a humidified chamber. Sections were then incubated with anti BPDE-DNA monoclonal antibody clone 5D11 (1:20 dilution) overnight at 4 °C. For each reaction, a negative control was included by replacing the addition of primary antibody with PBS buffer on one of the sections on the same slide. Next day, after washing twice with PBS for 5 min each, slides were incubated with biotinylated anti-mouse secondary antibody for 30 min at 37 °C for 1 h in a humidified chamber. Following washing of the sections twice with 1X PBS (5 min each), endogenous peroxidase was blocked by treating the slides with 3% H_2O_2 in methanol for 30 min at RT in dark. To remove excess of methanol, sections were washed thrice with 1X PBS for 5 min each and then, incubated with Avidin-Biotin Complex (ABC) reagent (from Vectastain Elite kit) for 30 min at 37 °C in a humidified chamber. The slides were rinsed with 1% Triton-X 100 (prepared in PBS), thrice for 30 sec. After washing with PBS, sections were stained with diaminobenzidine (DAB) solution (8 mg DAB in 10 ml D/W + 10 µl H_2O_2), till visible brown coloration appeared on the sections. The slides were washed immediately with water, counterstained with haematoxylin and washed with tap water. Slides were dehydrated, cleared in serial ethanol and xylene and cover slip was mounted with D.P.X. Images were captured with Zeiss Microscope (Imager Z1) to which an Axiocam MRc5 digital camera was attached. For calculation of percent positive nuclei of BPDE-DNA adduct staining, semi-quantitative analysis was conducted using Image J 1.43 (NIH) software which was done by counting the number of positively stained cells × 100/ total number of cells in photomicrographs of lung/liver sections. At least 10 different randomly selected

fields and/or minimum 1000 cells were counted at 400x magnification with at least three mice per group.

3.8 Statistical analysis:

Densitometry and cell counting were carried out using Image J 1.43 (NIH) software. Densitometry of western blots or various analyte proteins was carried out using Image J 1.43 (NIH) software as described in section 3.6.5. Statistical analysis was performed using SPSS 15.0 software. Data was presented as mean ± SE. Means of all data was compared by ANOVA with post-hoc Bonneferoni's test. $p \leq 0.05$ was considered statistically significant.

Chapter 4

To understand the anti-promotion and anti-initiation mechanism of PBPs in experimental lung carcinogenesis

Chapter 4: To understand the anti-promotion and anti-initiation mechanism of PBPs in experimental lung carcinogenesis

4.1 Introduction:

Lung cancer associated with cigarette smoking is a leading cause of death in both men and women worldwide **(102)**. Cigarette smoke or environmental exposure to various carcinogens including major classes like polycyclic aromatic hydrocarbons (PAHs) and nitrosamines are major risk factors associated with lung carcinogenesis. Benzo(a)pyrene [B(a)P] from PAH and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) from tobacco-specific nitrosamines are potent carcinogens present in tobacco smoke which have been proven to be associated with lung carcinogenesis. These carcinogens are proven to be carcinogenic in *in vitro* and *in vivo* model systems independently or in combination **(10).** Minimizing the exposure to environmental smoke and cessation of cigarette smoking has been demonstrated as one of the successful approaches to reduce lung cancer risk and prolong the quality of life**.**

Plant–derived natural compounds are receiving increasing attention as chemopreventive agents because of low toxicity and high tolerability. Tea is rapidly emerging as a potential chemopreventive beverage and tea polyphenols such as (-)-epigallocatechin-3-gallate (EGCG, the most effective monomeric catechin in green tea) and TFs (oligomeric black tea polyphenols) have reached clinical trials. Their chemopreventive efficacies were established by various preclinical studies without any remarkable toxicity **(103,104)**. Thearubigins (TR) or polymeric black tea polyphenols (PBPs), which contributes as the most abundant constituent of the total polyphenolic fraction of black tea, are polymeric fraction of polyphenols formed in the processing of the black tea due to oxidation process. Despite being major polyphenolic component (>40%), studies on PBPs are limited. PBPs are pro-anthocyanidin, heterogeneous polymers of flavano-3-ols and flavan-3-ol gallates with di- and

tri-benzotropolone skeletons **(70,90)**. Earlier studies have shown PBPs to possess anti-initiating activities as judged by their ability to inhibit carcinogen-induced DNA adduct formation *in vitro* and in mouse skin. The study has further showed that PBPs inhibit Benzo(a)pyrene [B(a)P]-induced activation of phase I enzymes such as cytochrome P450 1A1 and 1A2, and/or enhance the levels/activities of phase II enzymes **(93,105)**. Anti-promotion action of topically applied PBPs has already shown to inhibit the carcinogen induced cell proliferation in mouse skin through MAPK pathway and 1, 2-dimethylhydrazine induced colorectal carcinogenesis via Wnt/β-catenin pathway **(85)**.

Considering the chemoprevention potential of black tea derived PBPs in various carcinogenesis model systems, the current study was undertaken to understand the chemopreventive potential (anti-promotion and anti-initiation) of 1.5% black tea derived PBPs on the process of sequential lung carcinogenesis. In the present study the anti-promotion potential of PBPs on the process of lung carcinogenesis measured as tumor incidence, multiplicity and latency period using a well-established B(a)P and NNK induced lung carcinogenesis model in A/J mice. We observed that PBPs treatment decreased B(a)P and NNK (i.p., 3μM of each) induced microscopic lung lesions like hyperplasia, atypical adenomatous hyperplasia and adenomas at 6th, 10th and 18th week post-carcinogen treatment. PBPs significantly decreased the multiplicity of carcinogen induced lung adenomas by modulating the processes associated with inflammation, cell proliferation and apoptosis with phosphorylation of signaling kinases like p38 and Akt during sequentially studied lung carcinogenesis process. The study also highlights the anti-initiation potential of PBPs by inhibition of CYPs while induction of GSTs isoform decreasing the B(a)P induced BPDE-DNA adducts in both liver and lung tissue.

4.2 Methods:

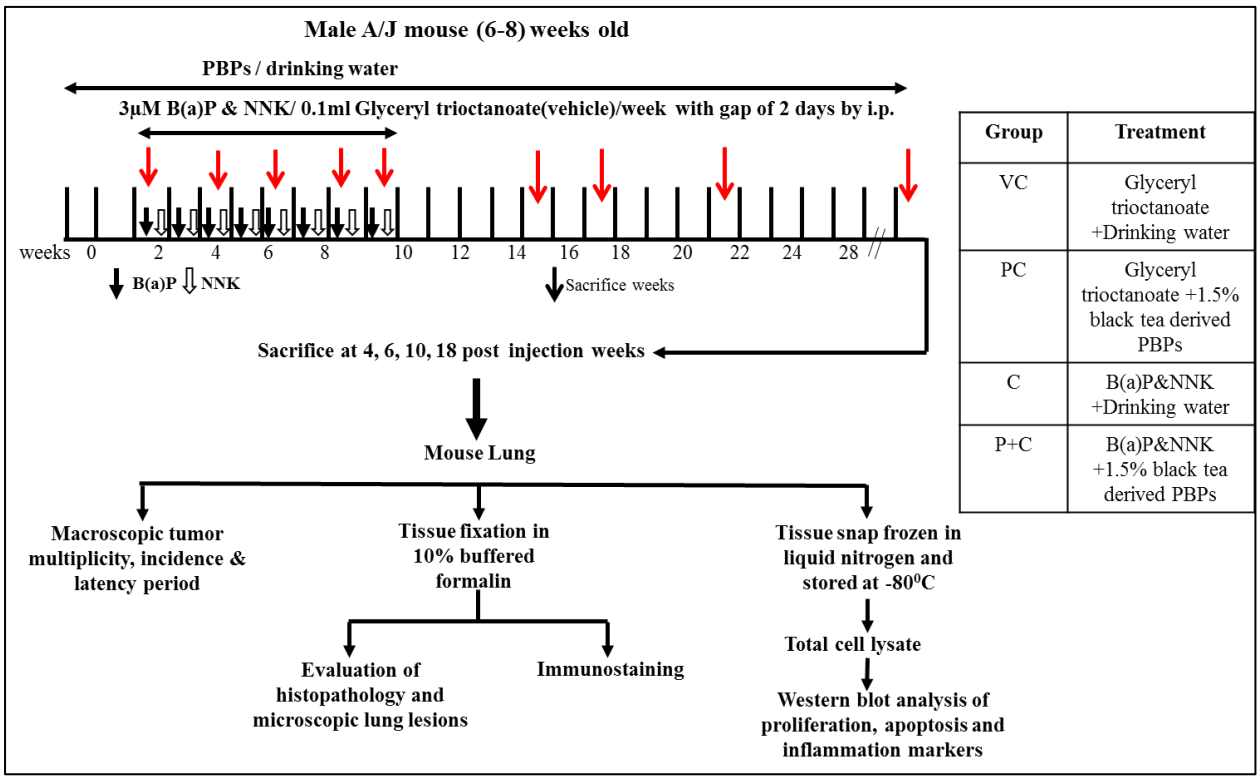
4.2.1 Isolation and preparation of PBP rich extract:

To study the mechanism(s) of anti-promoting activity of PBPs on B(a)P and NNK induced lung carcinogenesis in A/J mice, PBPs were isolated and 1.5% PBP rich extract was prepared with well-established method **(95)** as described earlier in materials and methods (Chapter 3, section no.3.1, page no. 41-47)**.**

4.2.2 Animal treatment:

All animal studies were conducted after approval from the institutional animal ethics committee (sanction no: 09/2011) endorsed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. A/J mice obtained from Jackson Laboratory, USA were inbred at Laboratory Animal Facility, ACTREC, India and were housed under standard conditions: 22 ± 2°C, 55 ± 5% relative humidity, and 12-h light/dark cycle. Animals received a standard pellet diet *ad libitum*. Male A/J mice, 6–8 weeks old were received from Laboratory Animal Facility, ACTREC and randomized into four different groups. Polyphenol control (PC) and polyphenol+carcinogen (P+C) groups were primed for 2 weeks with PBPs as the sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water. Animals in vehicle control (VC) and polyphenol control (PC) groups were injected glyceryl trioctanoate (vehicle, i.p., 0.1ml) and continued receiving drinking water and PBPs respectively while carcinogen (C) and polyphenol+carcinogen (P+C) groups were injected B(a)P and NNK (i.p, 3μM of each, 0.1ml) and continued receiving drinking water and PBPs (*ad libitum*) respectively. Glyceryl trioctanoate or B(a)P and NNK injections were given with the gap of 2 days, once in a week for 8 weeks. While PBPs and drinking water were administered *ad libitum*, prior to (2

weeks), during (8 weeks) and after carcinogen treatment (4,6,10 and 18 weeks) till the end of the experiment (**Figure 4.1**). Fresh bottle of PBPs were employed every alternate day. Body weights were monitored after receiving the animals and before the sacrifice time points. Animals were sacrificed by cervical dislocation at 1st and 8th week of carcinogen treatment & 4, 6, 10, 18 weeks after administration of the last dose of carcinogen. Macroscopic/visible lung tumors were counted at each time point using magnifying glass. Lung tissues were perfused with sterile 0.15 M KCl and excised. All five lung lobes from three random animals per group were fixed in 10% buffered formalin and paraffin embedded blocks were prepared. These blocks were used for histopathological evaluation of microscopic lung lesions and immunohistochemical analysis (IHC). Tissues from rest of the animals were snap frozen in liquid nitrogen and stored at -80°C until preparation of the extract for immunoblotting.



Group	Treatment
VC	Glyceryl trioctanoate +Drinking water
PC	Glyceryl trioctanoate +1.5% black tea derived PBPs
C	B(a)P&NNK +Drinking water
P+C	B(a)P&NNK +1.5% black tea derived PBPs

Figure 4.1: Experimental design for studying the effect of PBPs on B(a)P and NNK induced lung carcinogenesis in A/J mice model 6-8 weeks old male A/J mice were randomized into four different groups such as vehicle control(VC), polyphenol control (PC), carcinogen (C), and polyphenol+carcinogen (P+C) as shown in the tabular format. Polyphenol control (PC) and polyphenol+carcinogen (P+C) groups were primed for 2 weeks with PBPs as the sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water. After 2 weeks of priming, B(a)P and NNK(3µM each/0.1ml) in glyceryl trioctanoate or only glyceryl trioctanoate were intraperitoneally injected with the gap of 2 days, once in a week for 8 weeks (shown by solid and hollow arrow respectively) while PBPs or drinking water were administered orally prior to (2 weeks), during (8 weeks) and after the carcinogen treatment throughout the experimental period in male A/J mice. Treatment groups are as shown in the tabular format. Sacrifice was done at 1, 2, 4, 6, 8 weeks of carcinogen treatment and 4, 6, 10, 18 weeks after the last carcinogen treatment (shown by red arrow).

4.2.3 Detection of PBPs exposure related signals in sera of exposed animals:

Five sera (50µl) samples each from vehicle and PBPs exposed animals were extracted with methanol (500µl, HPLC grade) and mixtures were incubated at -20º C overnight. Deproteinized supernatants collected after centrifugation were vacuum dried, further reconstituted and processed for MALDI-TOF analysis for detection of PBPs / metabolites related signals as earlier in materials and methods (Chapter 3, section no.3.1.2.2, page no. 45-47).

4.2.4 Evaluation of macroscopic lung lesions:

At 4, 6, 10, 18 weeks post carcinogen treatment, mice were sacrificed by cervical dislocation and macroscopic lung tumors were evaluated. Macroscopic/visible lung tumors were counted at each time point using magnifying glass in three independent animals in each group at all the time points. The evaluation was carried out by two independent persons to avoid the bias. Tumors were evaluated and expressed as mean ± S.D. in each group. Tumor incidence was evaluated as number of animals developing tumors out of total animals treated in each group. Tumor multiplicity was evaluated as number of tumors developed per animal in total animals developing tumors. Latency period was evaluated as the time point at which appearance of first tumor after the carcinogen treatment has terminated.

4.2.5 Evaluation of microscopic lesions (number and area) and their histopathology:

Evaluation of number and area of microscopic pulmonary lesions was carried out as described **(106,107)**. Briefly, formalin-fixed lung tissues from three independent mice were processed through series of graded alcohol, embedded in paraffin blocks. Four step sections (each 200 μm apart) with thickness of 5 μm each were cut and stained with haematoxylin and eosin. Each stained section was tile scanned using LSM 510 Meta Carl Zeiss confocal microscope (Zen software) at final 100X magnification **(Figure 4.2)**. Proliferative lesions in lungs were classified as hyperplasia, atypical adenomatous hyperplasia (AAH) and adenoma independently by two pathologists based on recommendations published by The Mouse Models of Human Cancers Consortium **(108)**. Proliferative lesions were counted and recorded in each step section and the total number of each type of lesion per mouse (n=3) was expressed as average number of each lesion per mouse (sum of each lesion in three mice divided by three). Area of all proliferative foci in each tile scanned step section was marked independently by two pathologists and lesion size was evaluated using Image J software (Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA,

http://imagej.nih.gov/ij/, 1997-2015). Image J was precalibrated for conversion of pixels into square micrometer according to magnification used for image acquisition (100X). Individual lung lesion was outlined using free hand drawing tool and its size was measured as square micrometer. Area measurement was carried out by analyzing step sections from 3 independent mice and expressed as an average area of each type of lesion per mouse.

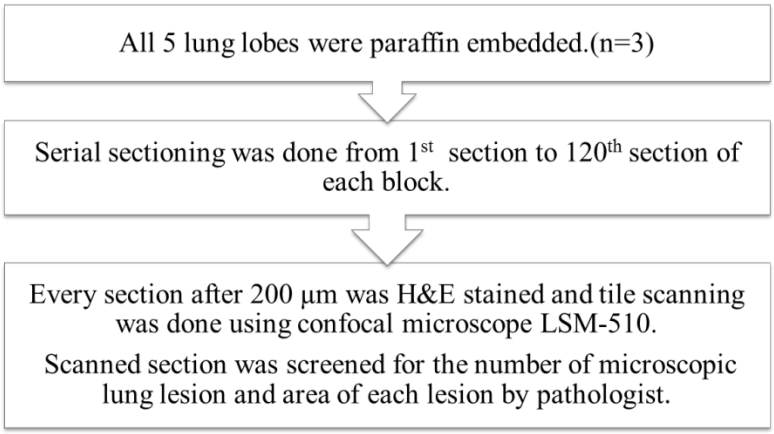


Figure 4.2: Flowchart showing tile scanning protocol employed for evaluation of number and area of microscopic lung lesions at week 6, 10 and 18 post carcinogen treatment.

4.2.6 Protein immunoblotting:

To understand the anti-promotion mechanism mediated by PBPs, various hallmarks of cancer like cell proliferation, apoptosis and inflammation were studied using total cell lysates. Total cell lysates (TCL) of lung tissues from week 4, 10 and 18 post carcinogen treatment were prepared by a previously described cell fractionation procedure presented in materials and methods (Chapter 3, section no.3.3.1, page no. 47-48). For preparations of total cell extract, whole lung tissues from all the treatment groups were used. The expressions of various biomarkers along with various signaling kinases (non-phosphorylated/native and phosphorylated/active forms) were studied by SDS-PAGE followed by immunoblotting as

described in materials and methods (Chapter 3, section no.3.4, page no. 52-55). β-actin was used as loading control. The antibodies used for the immunoblotting and its dilution are as mentioned in the **Table 4.1.**

To understand the anti-initiation mechanism by PBPs, various isoforms of CYPs (CYP1A1 and CYP1A2) and GSTs (GST mu, pi and alpha) were studied using microsomal and cytosolic cellular fraction respectively. Both liver and lung cellular fractions were prepared and used to study expression of CYPs and GSTs. In the absence of a well-accepted standard internal control, quantitative analysis was done by normalizing the CYP1A1/1A2 immunoblot with a prominent band visualized on membrane after Fast green staining in microsomal fraction while β-actin was used as the loading control for cytosolic fraction. Densitometry of various analyte proteins and their respective loading controls from the same blot was performed using Image J 1.43 (NIH) software as described in materials and methods (Chapter 3, section no.3.4, page no. 52-55).

Protein	% gel	Amount of protein	Primary antibody dilution	Secondary antibody dilution
PCNA	12	TCL, 50µg	1:2000	1:4000
Bax	12	TCL, 50µg	1:1000	1:4000
Bcl_2	12	TCL, 50µg	1:1000	1:4000
Cox-2	12	TCL, 50µg	1:2000	1:5000
P38	12	TCL, 50µg	1:2000	1:4000
Phospho-p38	12	TCL, 50µg	1:1000	1:3000
Akt	12	TCL, 50µg	1:2000	1:4000
Phospho-Akt	12	TCL, 50µg	1:1000	1:3000
CYP1A1	12	Microsome,50µg	1:1000	1:4000
CYP1A2	12	Microsome,50µg	1:1000	1:4000
GST mu	12	Cytosol,50µg	1:1000	1:4000
GST pi	12	Cytosol,50µg	1:1000	1:4000
GST alpha	12	Cytosol,50µg	1:1000	1:4000

Table 4.1 : List of antibodies used for immunoblotting and its dilution

4.2.7 Immunohistochemical staining and analysis:

For immunohistochemical (IHC) staining, all the lung lobes of treated animals were fixed in 10% buffered formalin. 5 µm thick section of paraffin embedded tissue was used for all the IHC staining. The expression of PCNA, Bax, Bcl2, Cox-2 was studied in week 4,10 and 18 post carcinogen treatment while BPDE-DNA adducts were assayed in week 1,2,4,6 and 8 of carcinogen treatment as described in materials and methods (Chapter 3, section no.3.6, page no. 57-61). Detection was conducted using Vectastain ABC system kit (Vector Laboratories, Burlingame, CA, USA). DAB was employed as the chromogenic substrate, and slides were counterstained with Mayer's haematoxylin. Images were captured with a Zeiss microscope (Imager Z1) to which an Axiocam MRc5 digital camera was attached. For negative or isotype

controls, the primary antibody was replaced with PBS/ TBS or respective antibody serum (used at respective antibody concentration) **(Table 4.2)**.

For PCNA nuclear protein staining and BPDE-DNA adducts staining, semi-quantitative analysis was conducted using Image J 1.43 (NIH) software. PCNA labeling index and %nuclei with BPDE-DNA adducts was calculated by counting the number of positively stained cells × 100/ total number of cells in photomicrographs of lung sections. At least 10 different randomly selected fields and/or minimum 1000 cells were counted at 400x magnification in at least three mice per group.

In PCNA labeling index, comparisons of non-lesion area in all the groups at 4th, 10th and 18th weeks were done with its respective controls. Proliferation labeling index in the lesion area of P+C group was calculated and compared with its respective C group in all the weeks. Due to absence of any microscopic lesions, week 4 was not considered in the lesion area proliferation index analysis.

Target	Location of antigen	Primary antibody dilution	Secondary antibody dilution
PCNA	Nuclear	1:250	1:50
Bax	Cytoplasmic	1:300	1:50
Bcl2	Cytoplasmic	1:300	1:50
Cox-2	Nuclear, ER membrane	1:200	1:50
BPDE-DNA adducts	Nuclear	1:20	1:50

Table 4.2: Antibodies used for immunohistochemical staining

4.2.8 Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay:

Apoptosis was assayed in formalin-fixed, paraffin-embedded 5 μm tissue sections, according to the manufacturer's instructions (TUNEL assay kit, Promega, Madison, WI, USA), as described in materials and methods (Chapter 3, section no.3.7, page no. 58,59). The nuclei of the apoptotic cells were stained fluorescent green and counterstained with 4', 6-Diamidino-2-phenylindole (DAPI). Slides were mounted by Vectashield (Vector Lab Cat. # H-1000). Images were captured using Zeiss confocal LSM 780 at 40x (oil immersion) magnification. The apoptotic index was calculated by counting the cells with positively stained nuclei × 100/total number of cells in photomicrographs of lung sections of at least 10 different randomly selected fields (~ 1000 nuclei/ mice), with at least three mice per group.

4.2.9 Statistical analysis:

Statistical analysis was performed using SPSS 21.0 software. Initial and final body weights among the different groups were compared using non-parametric Kruskal-Wallis test followed by Mann-Whitney test with Bonneferoni's correction. Surface lung tumors within the groups were compared using non-parametric Mann-Whitney Wilcoxon test while microscopic lung lesions were compared using Poisson regression, which represents the counts or number of events where few or no events occur. For microscopic tumor area analysis, data was log transformed and comparison of area of each lesion between C and P+C groups was done using regression analysis. Mean of each analyte proteins was normalized with its respective loading controls in western blotting and data was compared with analysis of variance (ANOVA) with post-hoc testing. Cell counting was conducted for proliferation index (lesion and non-lesion areas) and apoptosis index using Image J (NIH) software. Data was represented as mean ± SD and compared using ANOVA with Bonneferoni's correction. $P<0.05$ was considered to be statistically significant.

4.3 Results:

4.3.1 General observations and body weight:

There were no signs of toxicity and no mortality was observed in any of the groups. At the time of sacrifice, no gross changes in kidney, spleen, liver, stomach or intestine were seen at varying treatment periods. Final body weights were found to be higher at the termination time points in all the groups as compared to its initial body weights probably suggesting lack of toxicity in the treated groups, although statistical significance was not attained **(Table 4.3)**.

Groups	Weeks	n	Body weight	
			Initial	Final
VC	4	10	21.78±2.29	25.04±1.98
PC		9	22.01±2.55	25.19±2.15
C		10	22.62±2.54	24.98±2.19
P+C		10	22.16±2.81	24.23±2.12
VC	6	14	20.73±3.49	23.06±3.65
PC		13	21.21±2.29	24.11±2.39
C		10	20.60±2.38	22.81±2.36
P+C		10	19.33±2.46	21.49±2.55
VC	10	9	23.86±3.12	26.8±4.01
PC		19	22.96±2.44	25.51±2.33
C		9	23.33±1.87	25.76±1.98
P+C		8	21.59±1.83	23.91±1.79
VC	18	8	21.61±2.46	24.25±1.38
PC		9	22.06±1.23	24.72±1.01
C		8	21.6±2.27	24.47±2.03
P+C		8	22.29±1.42	24.9±2.54

Table 4.3 : Initial and final body weights of animals treated in various groups

4.3.2 Detection of PBPs exposure related signals in sera of exposed animals:

MALDI-TOF analysis of five sera extracts each from vehicle and PBPs exposed (28 weeks) animals did not show any PBPs/metabolites exposure related signals probably due to very low levels of analytes.

Analysis of macroscopic lung tumor multiplicity

4.3.3 PBPs reduced the pulmonary macroscopic tumor multiplicity:

Mice were sacrificed at sequential time points i.e. 4^{th}, 6^{th}, 10^{th} and 18^{th} week after administration of last dose of carcinogen and macroscopic/visible lung tumors were counted. Both VC and PC showed no tumors at any of the time points studied. Macroscopic tumors were not observed at 4^{th} and 5^{th} week post-carcinogen treatment. However 100% tumor incidence was seen at 6^{th}, 10^{th} and 18^{th} week post-carcinogen treatment in both C and P+C groups **(Table 4.4)**. Latency period was found to be 6 weeks post-carcinogen in both C as well as P+C group. Average tumor multiplicity was found to be increasing in C group from 6^{th} to 18^{th} week post-carcinogen treatment **(Figure 4.3)**. PBPs treatment resulted in significant decrease in average tumor multiplicity in P+C group as compared to its respective carcinogen treated animals at 6^{th}, 10^{th} and 18^{th} week post-carcinogen treatment **(Table 4.4)**. However, only decrease at 18^{th} week post-carcinogen treatment attained statistical significance, probably because of lower tumor number at 6^{th} week and high deviation at 10^{th} week.

Group	Weeks	n	Lung tumors		p value
			Incidence (%)	Multiplicity #	
VC	4	10	0	0	
PC		9	0	0	
C		10	0	0	
P+C		10	0	0	
VC	6	14	0	0	
PC		13	0	0	
C		10	100	1 ± 1.18	0.606
P+C		10	100	0.64 ± 0.67	
VC	10	9	0	0	
PC		19	0	0	
C		9	100	8.2 ± 2.97	0.095
P+C		8	100	6.0 ± 1.76	
VC	18	8	0	0	
PC		9	0	0	
C		8	100	25.6 ± 1.51	**0.001**
P+C		8	100	20 ± 3.02*	

Table 4.4 : Macroscopic lung tumor incidence, multiplicity at various sacrifice weeks (4, 6, 10, 18) post carcinogen treatment.

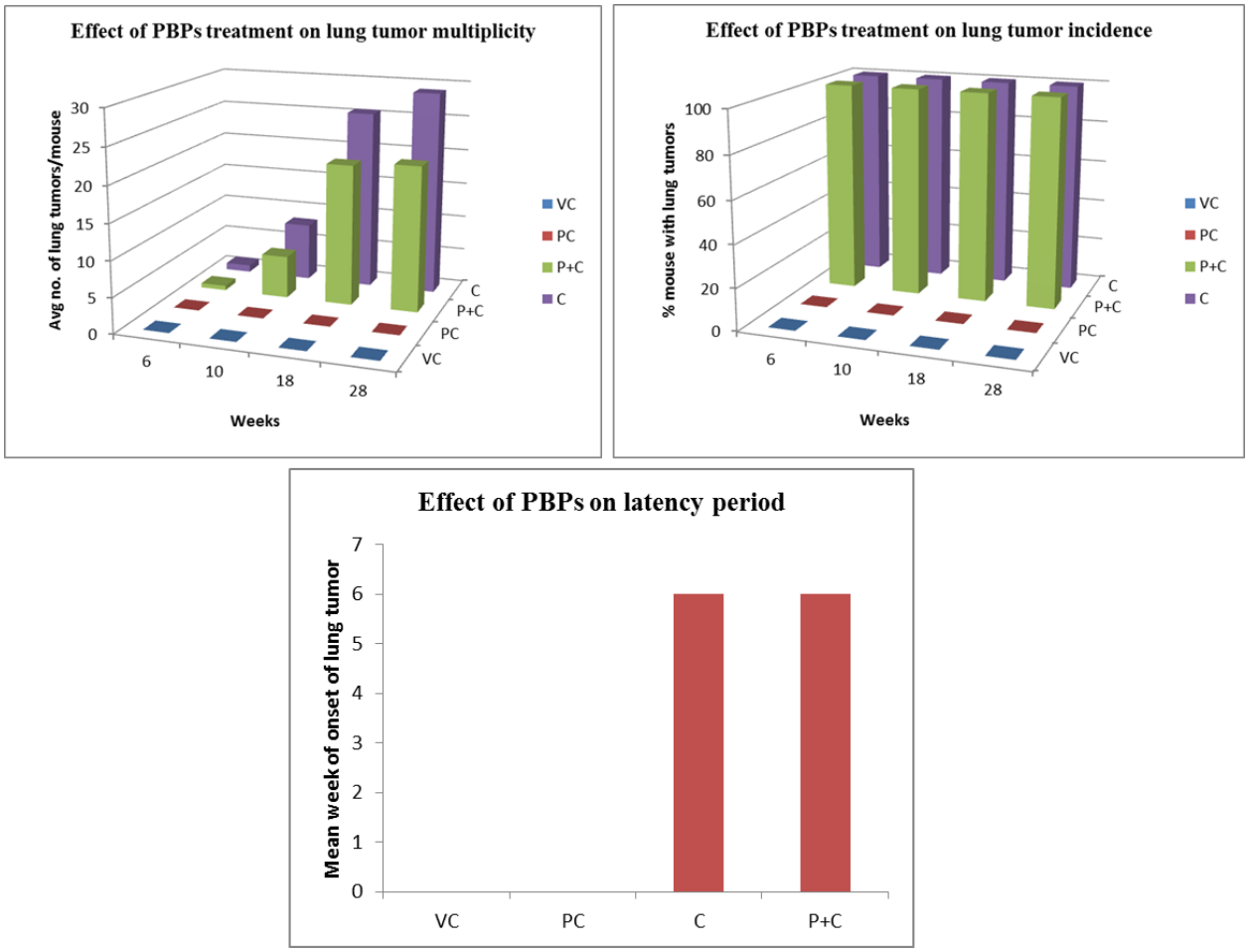


Figure 4.3: Effect of PBPs on macroscopic lung tumor incidence, multiplicity and incidence

Macroscopic lung tumors in each mouse was evaluated and expressed as % mice with lung tumors **(Figure 4.3)**. Mice developing tumors were evaluated using magnifying glass and represented average no. of lung tumors per mice.

Analysis of microscopic lung lesions:

4.3.4 PBPs reduced the number of microscopic pulmonary lesions:

The microscopic lesions observed in lung tissues of mice were classified as hyperplastic foci, AAH and adenoma based on established criteria **(108).** Briefly, hyperplasia was assigned based on increased number of cuboidal, columnar, ciliated or mucous cells without atypia with normal architecture of bronchioles and alveoli. AAH as focal, diffuse lesions involving alveoli and

terminal bronchioles which consist of relatively uniform atypical cuboidal to columnar cells with dense chromatin of cellular and nuclear atypia. Adenomas as small size, well circumscribed and well demarcated lesion with typical features indicated benign character and absence of vascular invasion **(108)**. Tile scan image of representative lung tissue cross sections showing microscopic lung lesions in treated group are depicted in (**Figure 4.4).** VC and PC groups showed no microscopic lung lesion while C and P+C groups showed all three types of lesions. At weeks 4, 5 post-carcinogen treatment no lesions in C as well as P+C group were observed. Microscopic foci were observed at 6^{th} week post-carcinogen treatment in both C and P+C groups in all the animals. Mice treated with polyphenol+carcinogen showed significant reduction in number of hyperplastic foci at both 6^{th} and 10^{th} week post-carcinogen treatment as compared to its carcinogen treated counterpart but not at 18^{th} week probably due to lower number of hyperplastic foci. However, mice treated with polyphenol+carcinogen showed significant reduction in adenomatous foci at 10^{th} and 18^{th} week but not at 6^{th} week post-carcinogen treatment probably because of large intragroup variation **(Figure: 4.5).** On the contrary, although PBPs lowered the number of AAH lesions, it didn't attain statistical significance because of lower number of AAH foci.

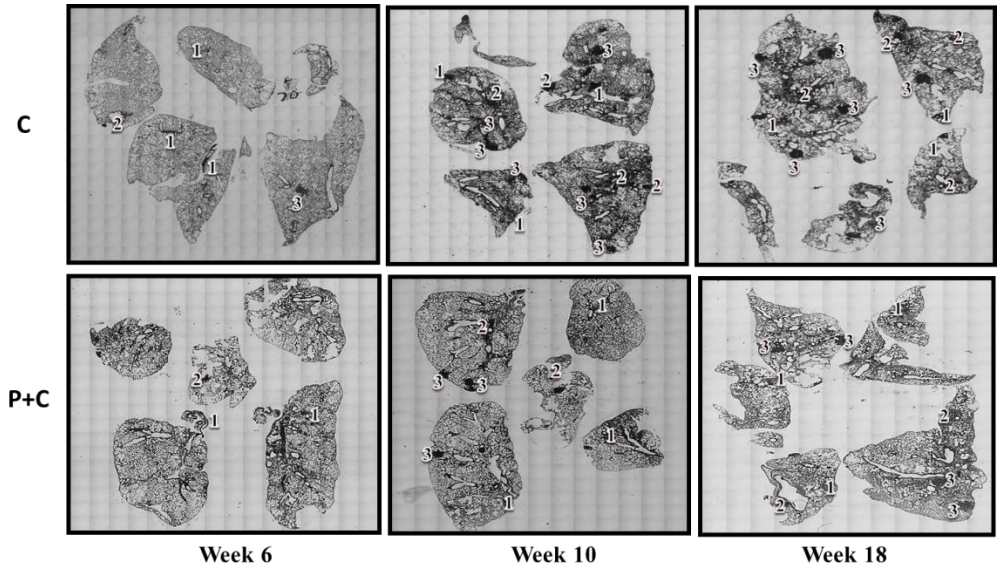


Figure 4.4 : Effect of PBPs on multiplicity of microscopic lung lesions

Representative tile scan images showing microscopic lung lesions in C and P+C groups at 6th, 10th and 18th weeks post-carcinogen treatment (magnification 100X). The numbers 1, 2, 3 represent pulmonary hyperplastic lesion, atypical adenomatous hyperplasia and adenoma respectively.

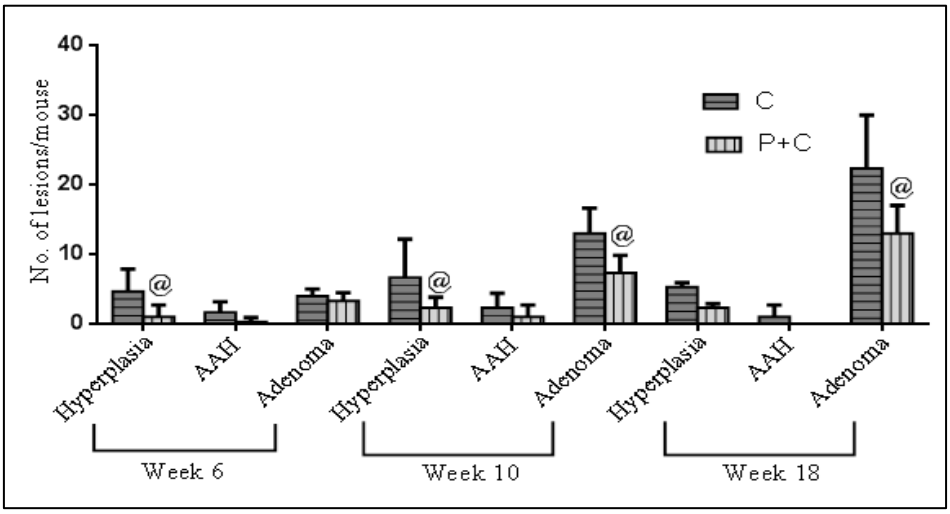


Figure 4.5: Effect of PBPs on multiplicity of microscopic lung lesions

Number of each proliferative lesion assigned by pathologist were counted in each step section (200 micron apart) and the total number of each type of lesion per mouse was expressed as an

average number of each lesion per section (sum of each lesion in three step sections divided by three) in 3 independent animals in C and P+C group and plotted in a graph. @ significantly different from C, $p<0.05$, Poison regression. Data represented as mean±SD of three observations.

4.3.5 PBPs reduced the area of microscopic pulmonary lesions:

Average area of hyperplasia in polyphenol+carcinogen treated mice was reduced at 6th, 10th and 18th week post-carcinogen treatment as compared to its respective carcinogen treated mice; however, statistical significance was observed only at 10th and 18th week post-carcinogen but not in 6th because of small lesion area to detect the changes, although differences in the numbers were significant **(Figure: 4.6).** Similarly, average area of adenoma in P+C group mice was significantly reduced at 10th and 18th week as compared to carcinogen alone group but no statistical difference was observed in 6th week due to low number and small area of lesions **(Figure: 4.6)**. PBPs treatment decreased average area of AAH however these differences were not statistically significant.

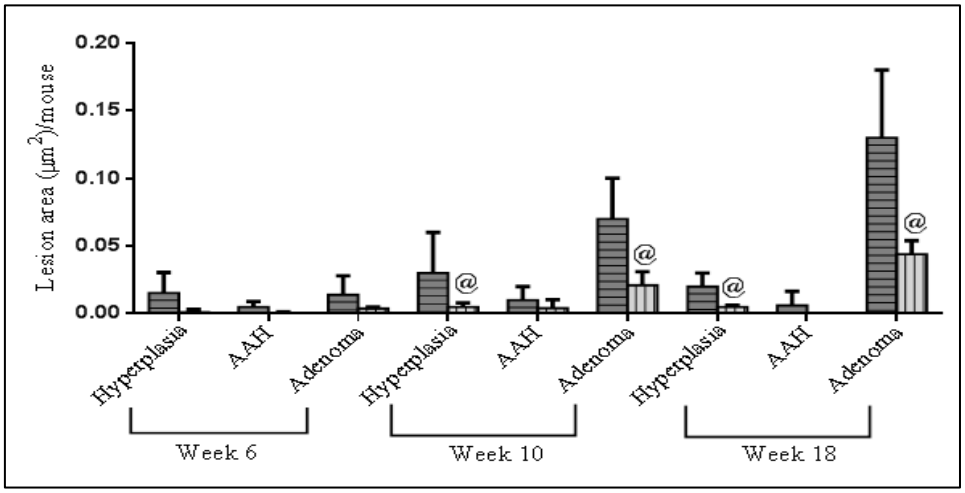


Figure 4.6 : Effect of PBPs on area of microscopic lung lesions

The area of lesion were counted using *Image J* software in each step section and total area of each type of lesion per mouse was expressed as average area of each lesion per section, @ significantly different from C, $p<0.05$, log transformation and regression analysis. Data represented as mean±SD of three observations.

Analysis of molecular markers:

To ascertain the contribution of cell proliferation and apoptosis along with inflammation in observed decrease in number and size of pulmonary lesions with PBPs administration, these markers were studied at 4^{th}, 10^{th} and 18^{th} week post-carcinogen treatment. 4^{th} week post-carcinogen treatment was selected to understand the modulation of processes, if any, before the formation of the tumors.

4.3.6 PBPs decreased the B(a)P and NNK induced inflammation during lung carcinogenesis:

Levels of Cox-2, a well-known marker of inflammation, were evaluated during the sequential carcinogenesis i.e. 4^{th}, 10^{th} and 18^{th} week post-carcinogen treatment period. Cox-2 protein levels in vehicle and polyphenol controls were found to be similar while in carcinogen treated groups, they were significantly higher compared to respective vehicle controls at all the time points studied. In carcinogen treated groups there was time related decrease in the levels of Cox-2 which was observed after cessation of carcinogen treatment i.e. 4^{th} to 18^{th} week. Time related decrease in Cox-2 levels was further enhanced by PBPs at 4^{th} and 10^{th} week but not in 18^{th} week in P+C group as compared to its time matched carcinogen group **(Figure 4.7**). In spite of time related and polyphenol mediated decrease in levels of Cox-2, it never attained the levels observed in respective vehicle control groups **(Figure 4.7**). Observed relative differences in Cox-2 expression by immunoblotting were in good concordance with the expression levels observed in immunohistochemical staining of tissue sections. PBPs

mediated decrease in carcinogen-induced Cox-2 levels suggests anti-inflammatory activity of PBPs **(Figure 4.7**).

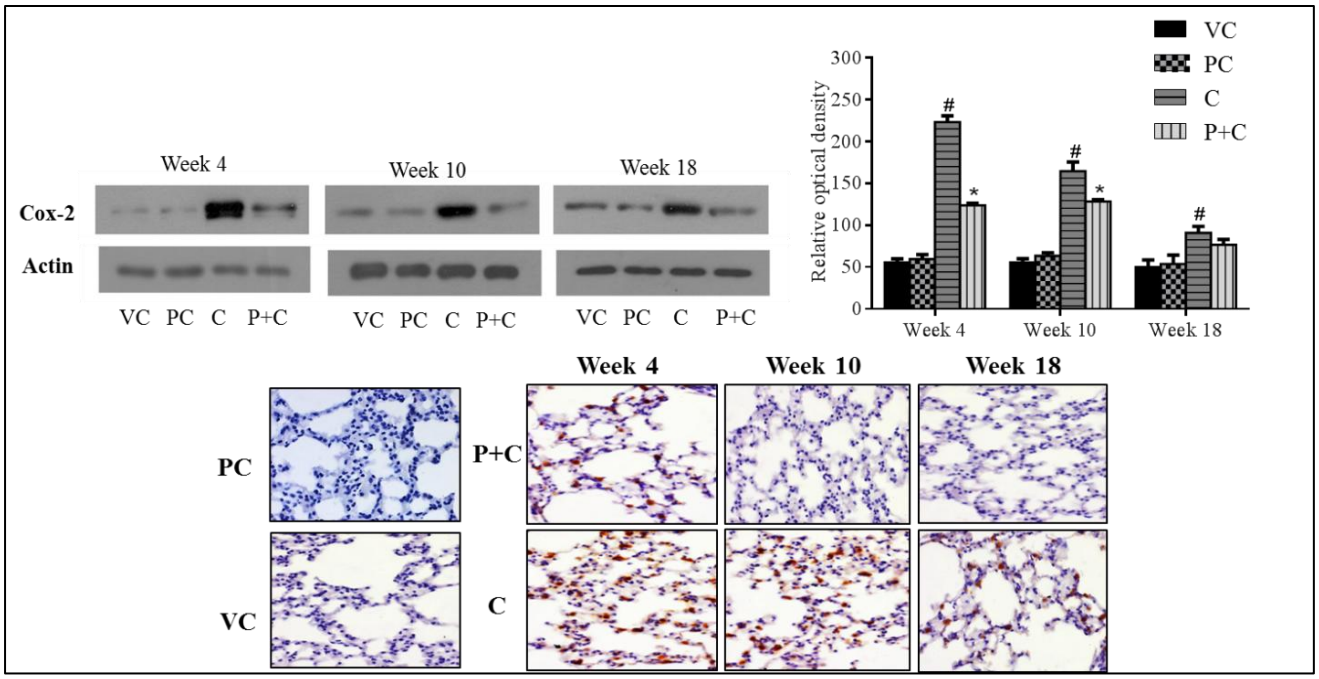


Figure 4.7: Effect of PBPs on B(a)P and NNK mediated alteration of inflammation marker in A/J mice

Representative blots and relative densitometry levels of Cox-2 protein at various sacrifice time points (4^{th}, 10^{th} and 18^{th} weeks) in lung total cell extract analyzed by immunoblotting. Beta actin was used as a loading control. Representative photomicrograph showing immunohistochemical detection of Cox-2 positive cells (magnification at 200X) at various sacrifice weeks in formalin fixed, paraffin embedded lung tissue sections. Data represented as mean±SD of three observations. #, significantly different from VC;*, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

4.3.6 PBPs inhibited B(a)P and NNK mediated cell proliferation:

To assess the effect of PBPs on B(a)P and NNK mediated cell proliferation in sequential lung carcinogenesis, lung tissues were analyzed for the cell proliferation marker, proliferating cell

nuclear antigen (PCNA) at 4^{th}, 10^{th} and 18^{th} week post-carcinogen treatment. Vehicle and polyphenol controls showed no significant difference in PCNA levels at any time point studied **(Figure 4.8**). Carcinogen treatment resulted in significant increase in levels of PCNA when compared to respective vehicle control and it was significantly higher with passage of time (18^{th} >10^{th} > 4^{th} weeks). PBPs treatment resulted in significant decrease in PCNA levels in P+C groups at all-time points as compared to its respective carcinogen group, showing higher decrease at later time points (18^{th} >10^{th} > 4^{th} week).

In spite of PBPs mediated decrease in PCNA; the levels were relatively higher in P+C group as compared to vehicle control group at respective time points. Relative differences in protein expression were further substantiated by immunohistochemical staining in lung tissue sections depicting significant decrease in PBPs mediated enhancement in PCNA during B(a)P and NNK induced lung carcinogenesis **(Figure 4.8**). PCNA index was unaltered in vehicle and polyphenol control groups at all the three study weeks. In C and P+C groups, PCNA labeling index was calculated in both non-lesion and lesion(s) (hyperplasia, AAH and adenoma) areas. In non-lesion areas, carcinogen induced proliferation was observed by increase in PCNA index at 4^{th}, 10^{th} and 18^{th} weeks. PBPs reduced the carcinogen induced cell proliferation which was evident by decrease in proliferation index in non-lesion area at all the time-points **(Figure 4.8**). In lesion areas carcinogen induced increase in PCNA index was observed in hyperplasia, AAH and adenoma at 10^{th} and 18^{th} week. PBPs significantly decreased carcinogen induced proliferation by observed decrease in PCNA index in all the lesions, however decrease in PCNA index in AAH was not found to be statistically significant at 10^{th} week **(Figure 4.8**). Time related increase in proliferation index was observed in the lesion area at 10^{th} and 18^{th} week in carcinogen treated group (adenoma > AAH > hyperplasia) as compared to vehicle control, which is notably higher than that of the non-lesion areas. It is important to note that observed increase in proliferation index in non-

lesion area in carcinogen group was twofold higher than that in vehicle control. However in lesion areas the increase in proliferation index in carcinogen group was 4 to 5 fold higher than that of its respective vehicle control group. Interestingly treatment of PBP significantly reduced proliferation in both lesions as well as non-lesion areas compared to its time matched carcinogen group, however observed reduction was pronounced in lesion areas. Hence, PBPs mediated decrease in PCNA levels in P+C group demonstrate anti proliferative activity of PBPs.

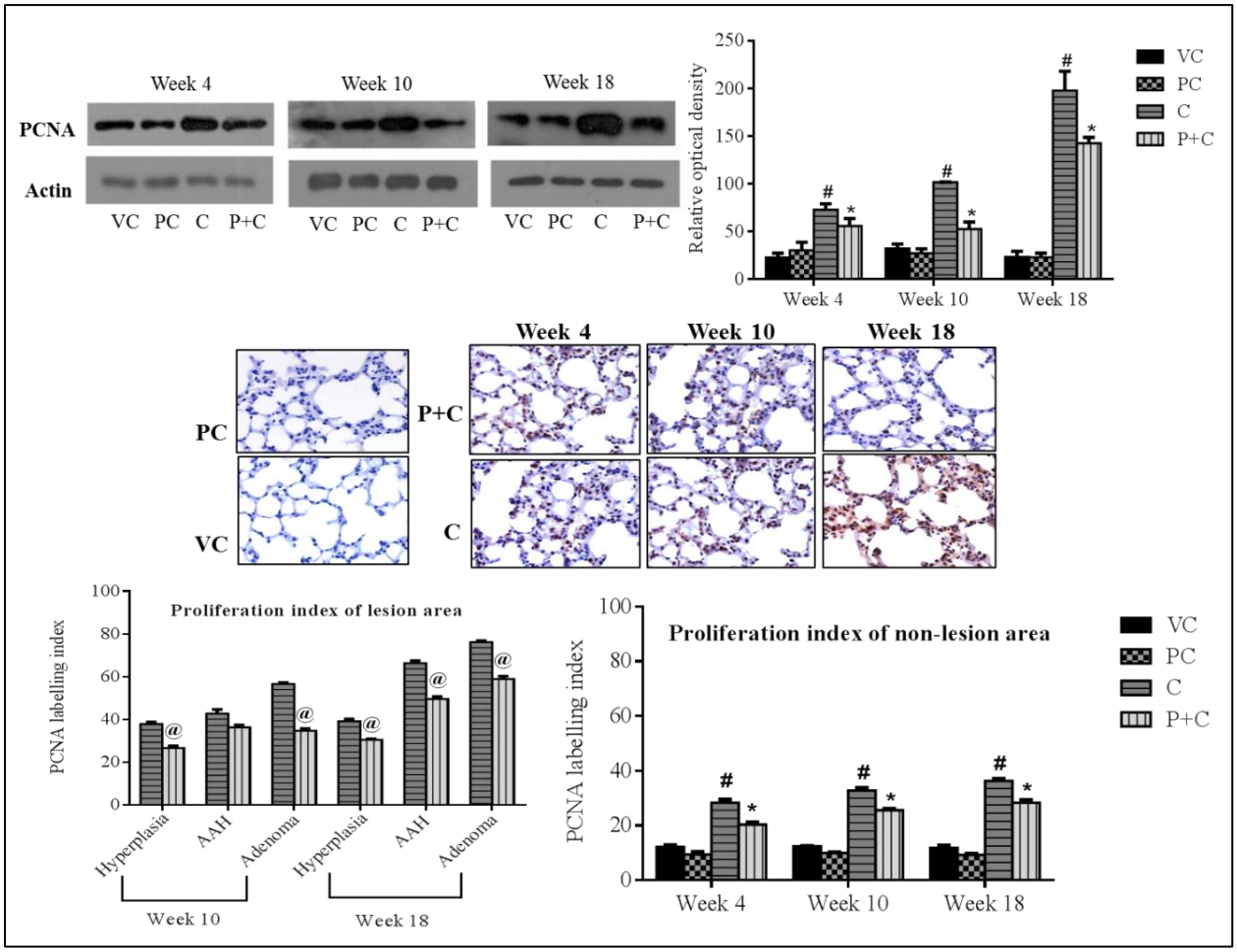


Figure 4.8: Effect of PBPs on B(a)P and NNK mediated alteration of cell proliferation marker in A/J mice

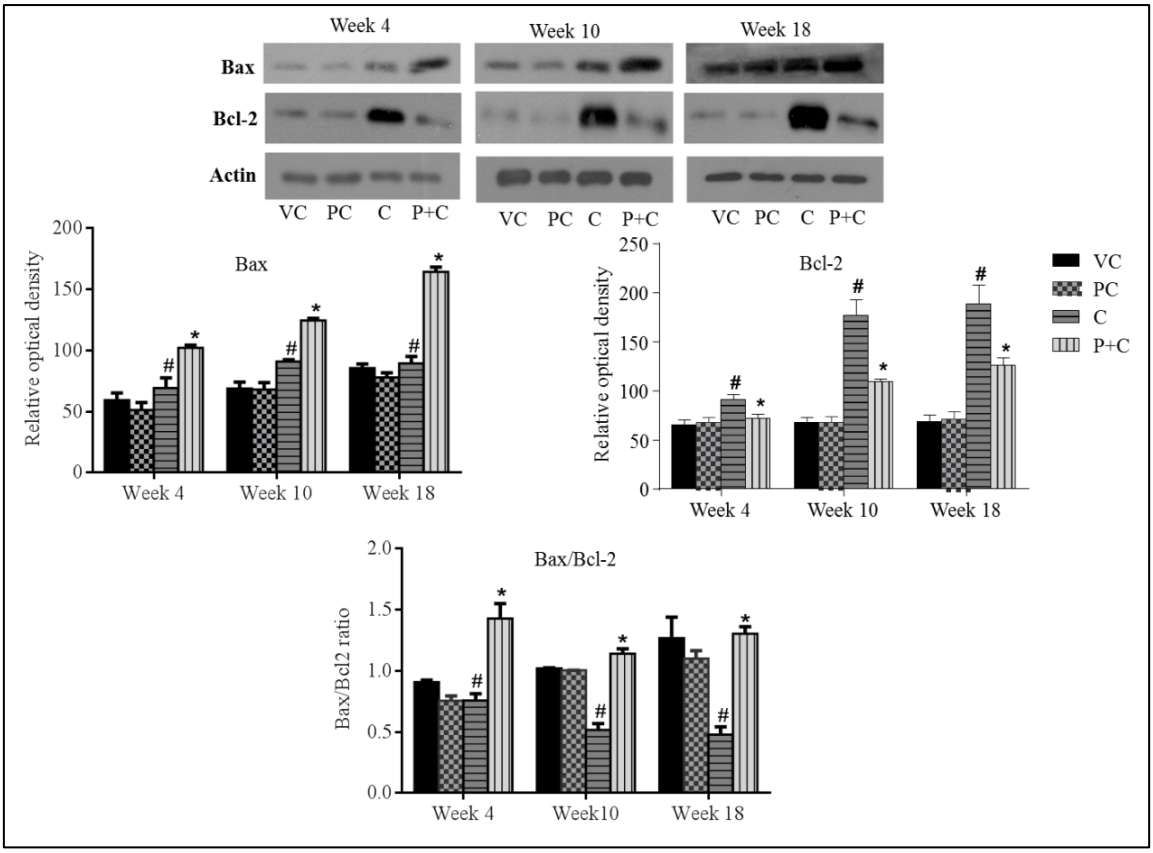
Representative blots and relative densitometry levels of PCNA protein at various sacrifice weeks (4^{th}, 10^{th} and 18^{th} weeks) in lung total cell extract analyzed by immunoblotting. Beta

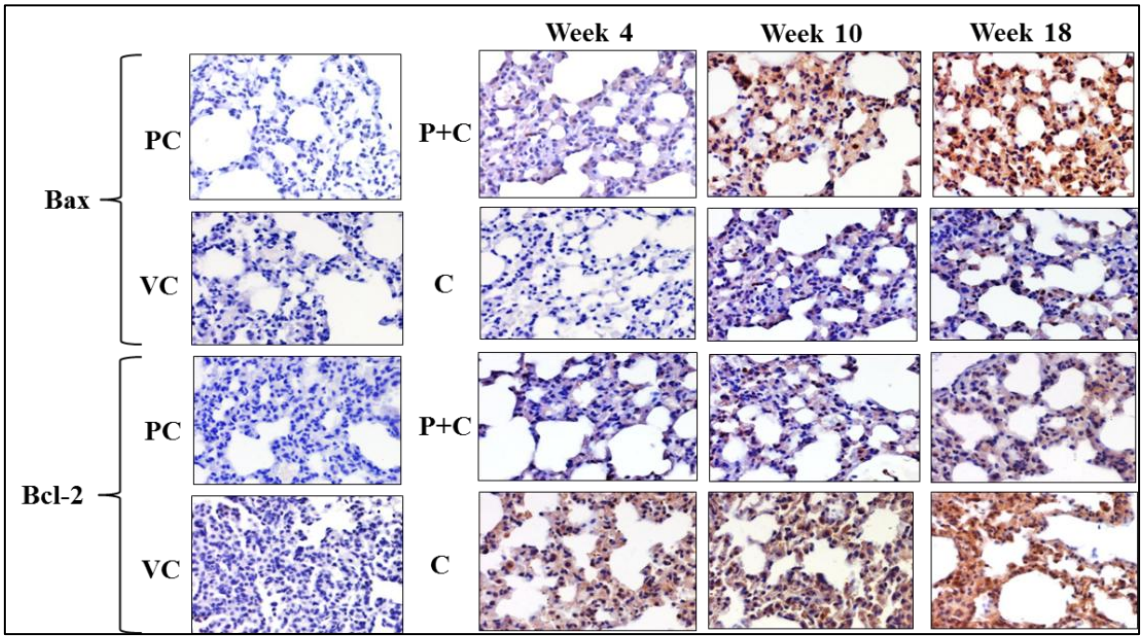
actin was used as a loading control. Representative photomicrograph showing immunohistochemical detection of PCNA positive cells (magnification at 200x) at various sacrifice weeks in lung tissue sections. PCNA index was calculated as number of positively stained cells × 100/ total number of cells. Semi quantitative analysis was conducted by Image J 1.43 (NIH) software by counting the brown-stained nuclei in photomicrographs of at least 10 different randomly selected fields from lesion and non-lesion areas. Data represented as mean±SD of three observations. #, significantly different from VC;*, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

4.3.7 PBPs enhanced B(a)P and NNK mediated apoptosis:

The levels of proapoptotic protein, Bax and antiapoptotic protein Bcl-2 were evaluated at 4^{th}, 10^{th} and 18^{th} week post-carcinogen treatment. Levels of Bax in vehicle and polyphenol control groups were not significantly different at any of the time points. Bax expression in carcinogen treated animals showed increase from 4^{th} to 18^{th} week as compared to respective vehicle control group. Like PCNA, Bax expression in carcinogen treated animals showed time dependent increase from 4^{th} to 18^{th} week. PBPs treatment further enhanced carcinogen- induced Bax expression at all the time points studied as compared to only carcinogen treated mice **(Figure 4.9).** Levels of Bcl-2 were similar in vehicle or polyphenol control groups at various time points studied. Bcl-2 expression was higher in carcinogen treated group as compared to its respective vehicle control at all the time points. Similar to Bax, time dependent overexpression of Bcl-2 was observed in carcinogen treated animals as compared to respective vehicle control. Bcl-2 expression showed significant polyphenol mediated decrease in P+C group as compared to only carcinogen treated group at all the time points studied **(Figure 4.9)**. As relative difference can't be accurately judged by measurement of individual proapoptotic Bax or antiapoptotic Bcl-2, Bax/Bcl-2 ratio was calculated. Ratio of Bax/Bcl-2 was similar in vehicle or polyphenol control groups. Carcinogen treated group showed significant decrease in the ratio compared to respective vehicle

control while PBPs treatment showed significant increase compared to only carcinogen treated group at various time points studied. PBPs mediated increase in the Bax/Bcl-2 ratio was the effect of relatively more pronounced increase in proapoptotic Bax expression consistently at all the time points **(Figure 4.9**). Concomitant to immunoblotting, relative differences in levels of Bax and Bcl-2 were further substantiated by immunohistochemical staining of lung tissue section confirming the proapoptotic activity of PBPs **(Figure 4.9**). Apoptotic index was unaltered in vehicle and polyphenol control groups at all the three study weeks. Time dependent increase in apoptotic index was significantly high in carcinogen treated group (week 4 < week 10 < week 18) as compared to its respective vehicle control. The observed increase in apoptotic index in carcinogen group was 5 to 7 fold higher than its respective vehicle control. PBPs treatment further enhanced the apoptotic index in P+C group which was evident from statistically significant increase in apoptotic nuclei at all the time-points. The increase in the apoptotic index in P+C group was about 1.5 fold higher than that of its respective carcinogen group **(Figure 4.9**). Hence, PBPs mediated modulation in Bax and Bcl2 expression along with increase in apoptotic index in P+C group demonstrate apoptotic activity of PBPs.


Week 4
Week 10
Week 18
Bax
Bcl-2
Actin
VC PC C P+C
Bax
Relative optical density
Week 4 Week 10 Week 18
Bcl-2
Relative optical density
Week 4 Week 10 Week 18
VC
PC
C
P+C
Bax/Bcl-2
Bax/Bcl2 ratio
Week 4 Week10 Week 18


Week 4
Week 10
Week 18
Bax
PC
VC
P+C
C
Bcl-2
PC
VC
P+C
C

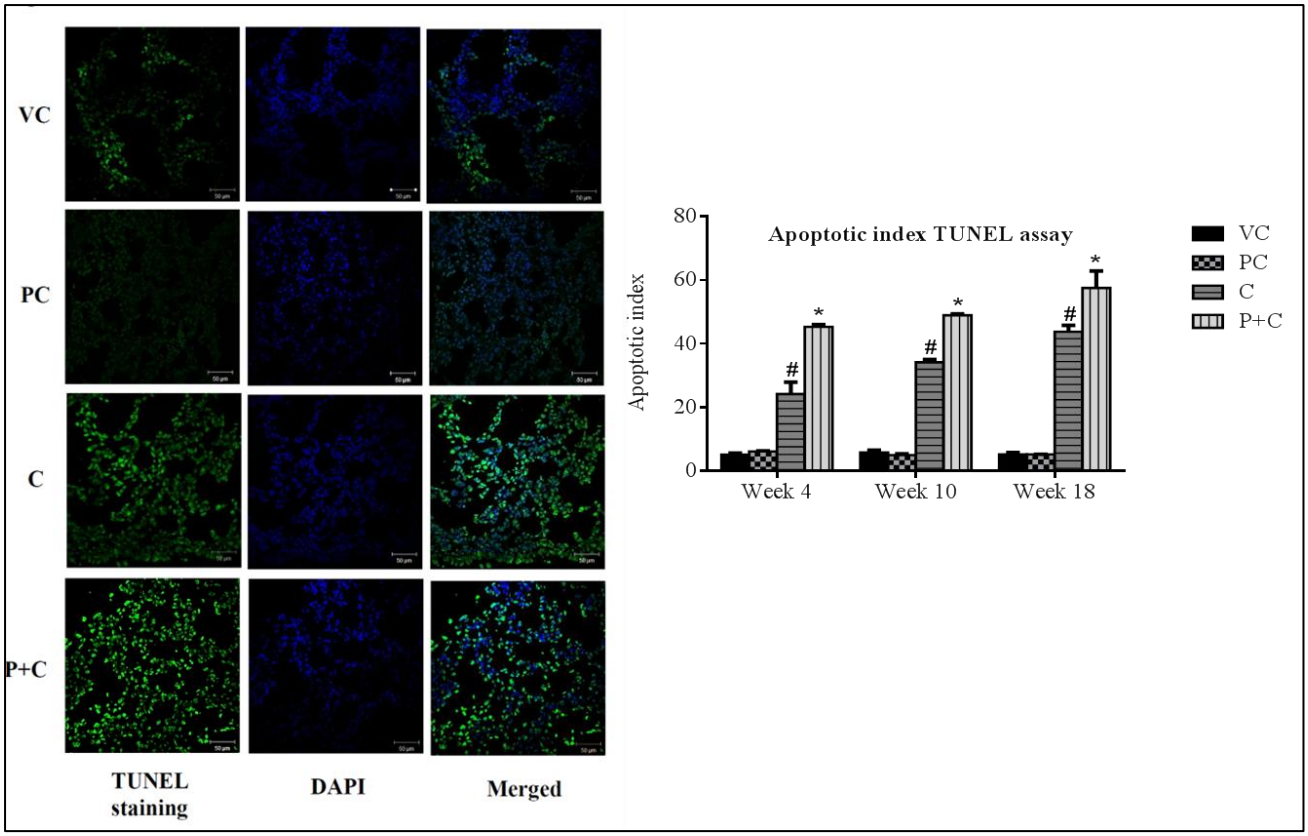


Figure 4.9: Effect of PBPs on B(a)P and NNK mediated alteration of apoptosis markers in A/J mice

Representative blots and relative densitometry levels of Bax, Bcl2 protein at various sacrifice weeks (4^{th}, 10^{th} and 18^{th} weeks) in lung total cell extract analyzed by immunoblotting. Beta actin was used as a loading control. Extent of apoptosis in lung tissues was determined by calculating the ratio of normalized band density of Bax and Bcl-2 in each treatment groups. Representative photomicrograph showing immunohistochemical detection of Bax, Bcl2 positive cells (magnification at 200x) at various sacrifice weeks in lung tissue sections. Representative photomicrographs showing fluorescence detection of apoptotic cells using TUNEL assay in formalin fixed, paraffin embedded tissues of lung (magnification 400X). The apoptotic index was calculated by counting the cells with positively stained nuclei × 100/total number of cells in photomicrographs of lung sections of at least 10 different randomly selected fields (~ 1000 nuclei/ mice), with at least three mice per group. Data

represented as mean±SD of three observations. #, significantly different from VC;*, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

4.3.8 PBPs decreases activation of p38 and Akt in A/J mice:

PBPs have been observed to modulate cell proliferation, apoptosis and inflammation. In order to delineate the potential mechanism of PBPs-mediated effect on cell proliferation and apoptosis of PBPs via modulation of cellular kinases in B(a)P and NNK induced lung carcinogenesis, we assessed phosphorylated and non-phosphorylated forms of major downstream kinases, p38 and Akt. Expression of non-phosphorylated forms of p38 and Akt remained unchanged at all the time points in all the animal groups **(Figure 4.10)**. However, significant increase was observed in the phosphorylated forms of p38 and Akt in carcinogen treated group from 4^{th} to 18^{th} week post-carcinogen treatment as compared to its respective vehicle control **(Figure 4.10).** It is noteworthy, that time dependent increase in phosphorylation of p38 and Akt was observed in post- carcinogen treatment groups from 4^{th} to 18^{th} week similar to that observed in case of PCNA and Bax. PBPs treatment resulted in decrease in the phosphorylated p38 and Akt when compared to only carcinogen treated groups from 4^{th} to 18^{th} week. In spite of PBPs-mediated decrease in phosphorylated p38 and Akt, their levels were higher than those in vehicle controls. The observed differences in the levels of proteins involved in cellular proliferation and apoptosis along with signaling kinases suggest anti tumorigenic potential of PBPs via modulation of kinase function.

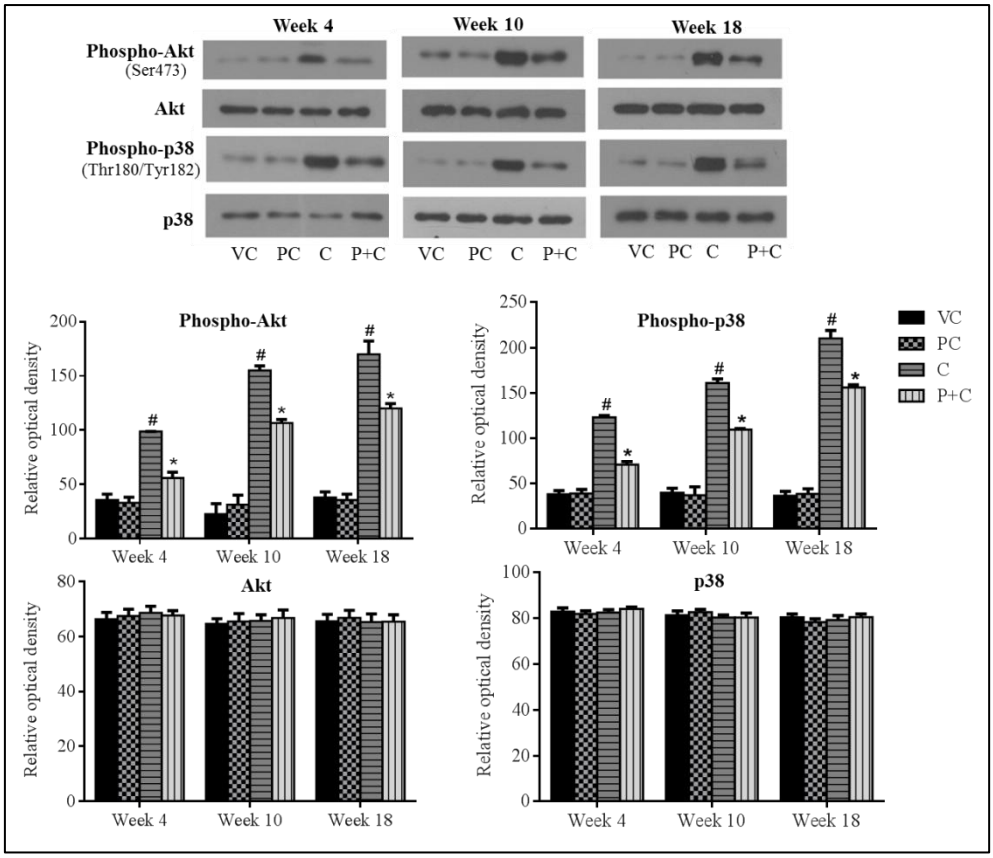


Figure 4.10 : Effect of PBPs on B(a)P and NNK mediated activation of p38 and Akt kinases in A/J mice.

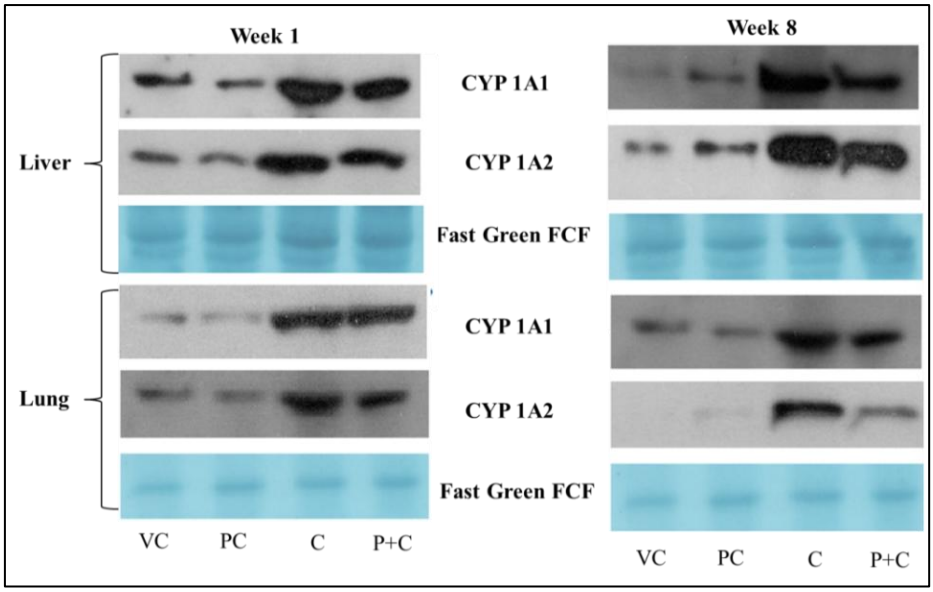
Representative blots showing the protein levels of p38, Akt, phospho-p38 and phospho-Akt in lung total cell lysates. Protein levels of phospho-p38 and phospho-Akt were normalized to that of respective total kinase. Data represented as mean±SD of three observations. #, significantly different from VC;*, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction).

4.3.9 PBPs inhibited B(a)P and NNK induced expression of CYP isozymes in liver and lung:

VC and PC showed basal level expression of both CYP1A1 and 1A2 in liver and lung. B(a)P and NNK induced the expression of CYP 1A1 in lung and liver at 1A2 at week 1 and 8.

However, pretreatment with PBPs decreased the carcinogen induced expression of both the isoforms of CYPs at week 1 and 8 **(Figure 4.11)**.

It is noteworthy that tissue related differences were observed at week 1 and 8 of carcinogen treatment. Decrease in expression of CYP1A1 was observed to be significant in liver while that of CYP1A2 isoform in lung showing modulation of CYP isoform in tissue specific manner **(Figure 4.11**).



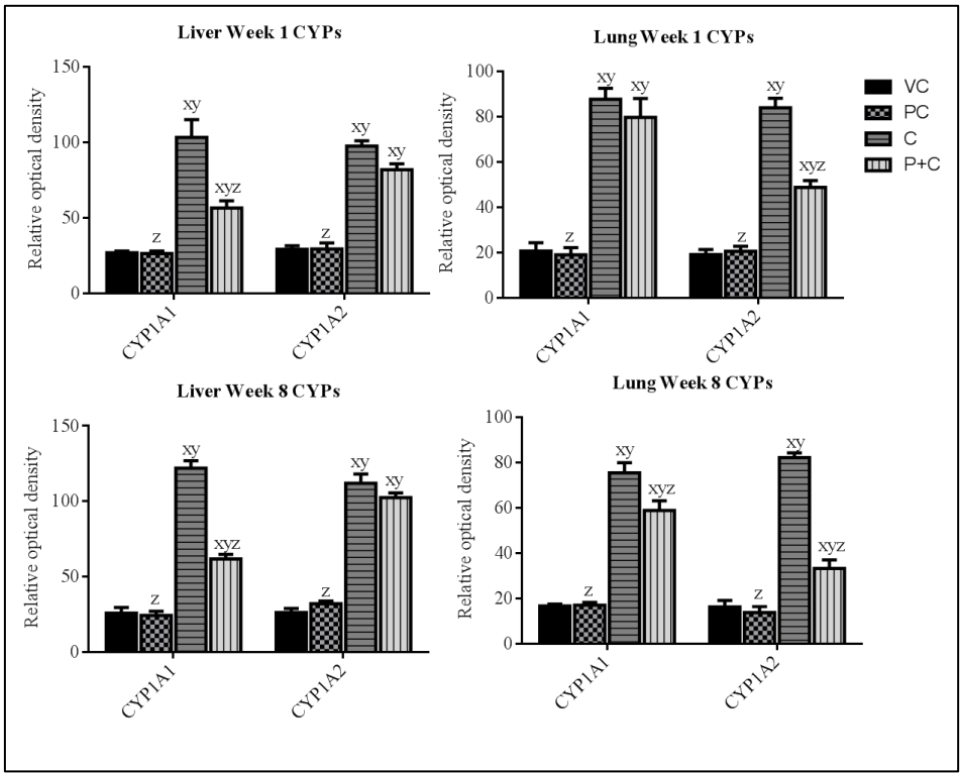


Figure 4.11: Effect of PBPs on B(a)P and NNK mediated decrease in expression of CYP1A1 and CYP1A2 in A/J mice.

Representative blots and densitometric analysis showing the protein levels of CYP1A1 and CYP1A2 in lung and liver microsomal fraction. In absence of a well-accepted standard internal control, quantitative analysis was done by normalizing the CYP1A1/1A2

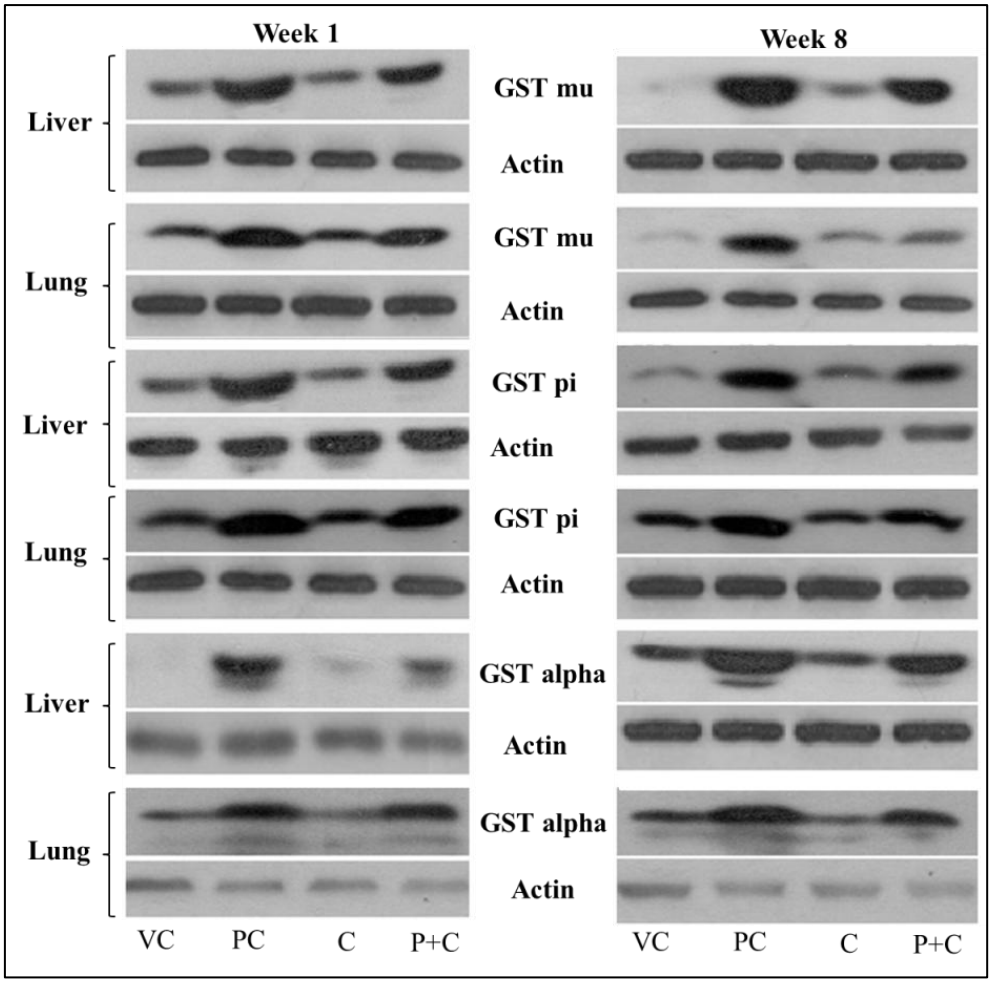
immunoblot with a prominent band visualized on membrane after fast green staining. Data represented as mean±SD of three observations of liver or lungs (three pooled lungs = 1 sample). x= significantly different from VC, y=significantly different from its respective PC, z=significantly different from C. $p<0.05$, ANOVA followed by Bonneferoni's correction.

4.3.10 PBPs induced GST isozymes (GST mu, pi and alpha) in liver and lung:

To understand the interplay of GSTs with PBPs pretreatment, expression of various isoforms of GSTs (mu, pi and alpha) were studied in liver and lung using immunoblotting. In both liver and lung, vehicle control group showed basal expression of various GST isozymes. GST expression in carcinogen group was comparable to vehicle control groups in all the isoforms. Significant over expression of all GST isoforms were observed in polyphenol control group at week1 and 8 in both liver and lung **(Figure 4.12)**.

To note, the expression of GST mu isoform in polyphenol control group was observed to be highest at both the study time points in both liver and lung. GST pi was observed to be second most up regulated isoform after GST mu in both liver and lung. In polyphenol+carcinogen treated group, expression of PBPs induced GST isoforms was down regulated by carcinogen treatment (**Figure 4.12**).

At week 1, significant down regulation of GST mu, pi and alpha was observed in P+C group as compared to its polyphenol treated group in both liver and lung. In week 8, significant down regulation was observed in GST mu and pi in liver while GST mu and alpha in lung. Observed differences in the GST isoforms can be attributed to the physiological differences at the tissue level.



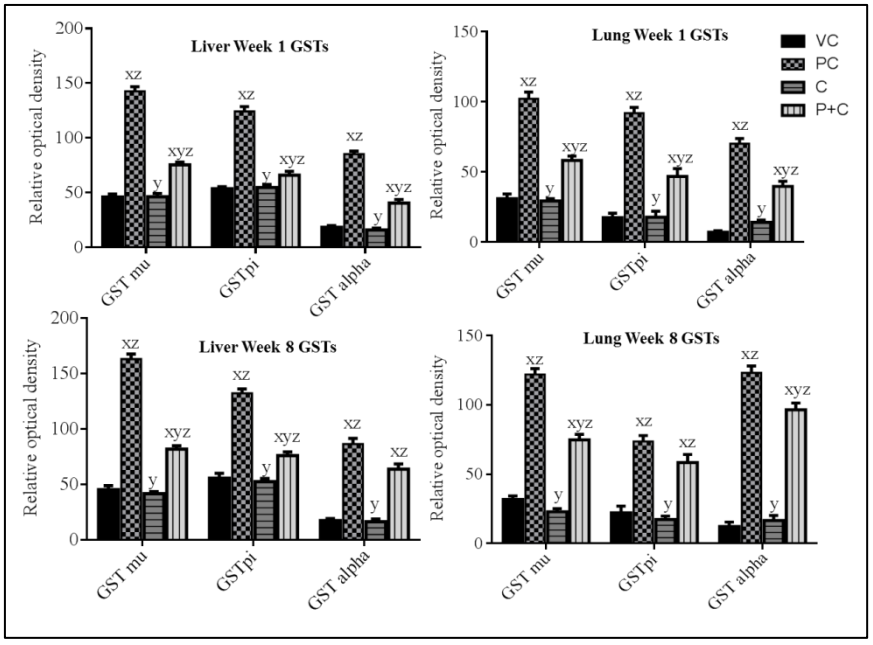
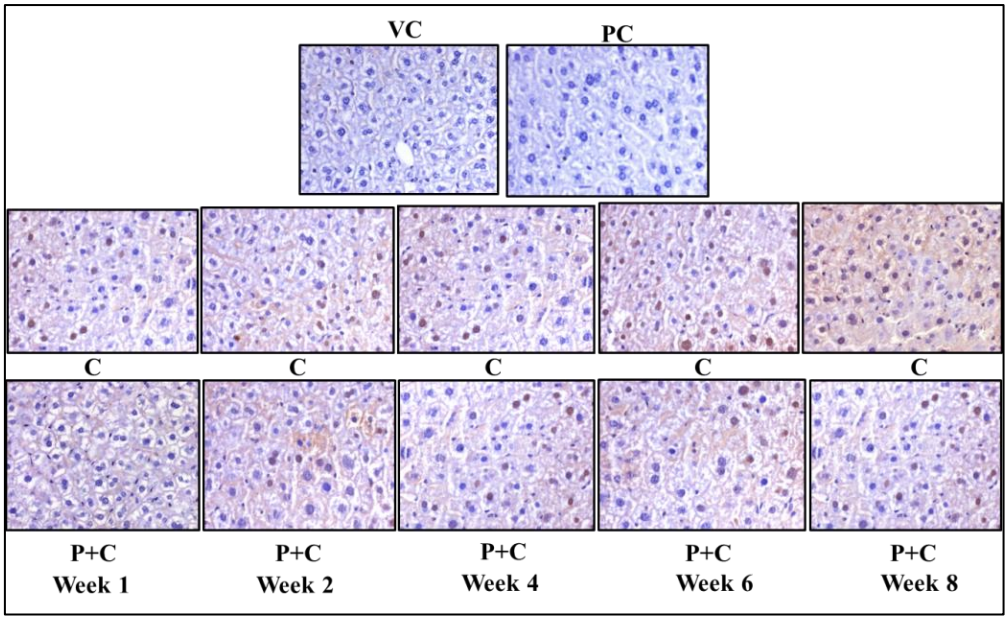


Figure 4.12: Effect of PBPs on B(a)P and NNK mediated decrease in expression of GST isozymes in A/J mice.

Representative blots showing the protein levels of GST isozymes (mu, pi and alpha) in lung and liver microsomal fraction. Beta actin was used as a loading control. Data represented as mean±SD of three observations of liver or lungs. x= significantly different from VC, y=significantly different from its respective PC, z=significantly different from C. $p<0.05$, ANOVA followed by Bonneferoni's correction.

4.3.11 PBPs decreased number of BPDE-DNA adducts containing cells in liver and lung:

BPDE-DNA adducts were evaluated in the liver and lung at carcinogen treatment weeks 1, 2, 4, 6, 8. BPDE-DNA adducts were not detected in liver and lung of animals receiving glyceryl trioctanoate in vehicle and polyphenol control while detectable levels of adducts were observed in carcinogen and polyphenol+carcinogen group by IHC staining **(Figure 4.13)**. B(a)P induced average number of BPDE-DNA adducts in liver was two times higher than that of lung. Time related increase in BPDE-DNA adducts were observed in carcinogen treated group in both liver and lung. However, PBPs treatment decreased the carcinogen induced formation of BPDE-DNA adducts in both the tissues (**Figure 4.13**). Importantly, PBPs treatment mediated decrease in the BPDE-DNA adducts in liver was comparatively higher than in lung, depicting the biochemical changes in the metabolic hub i.e. liver and lung.


VC
PC
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Week 1
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Week 6
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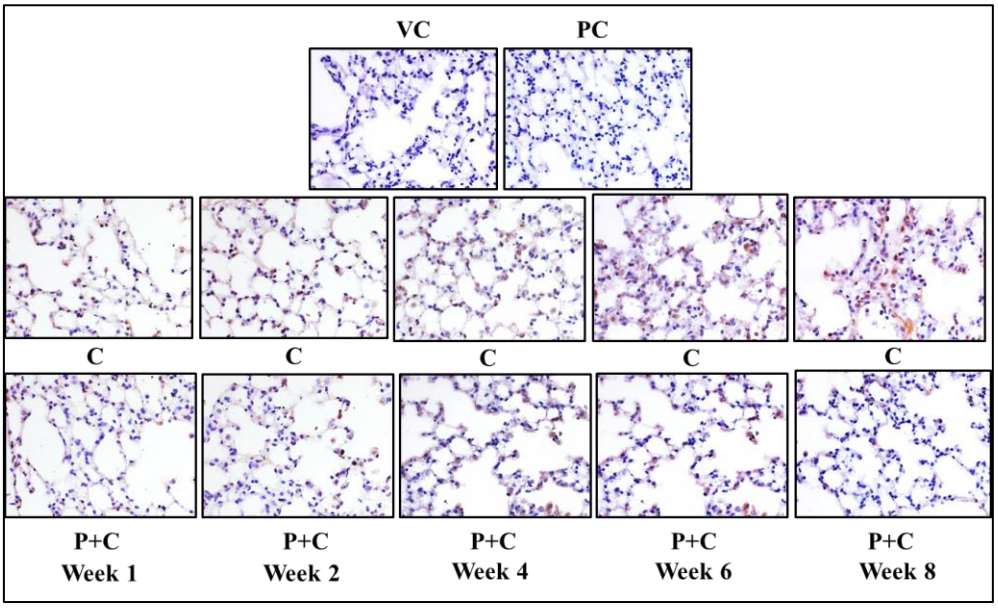
Week 8


VC
PC
C
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Week 1
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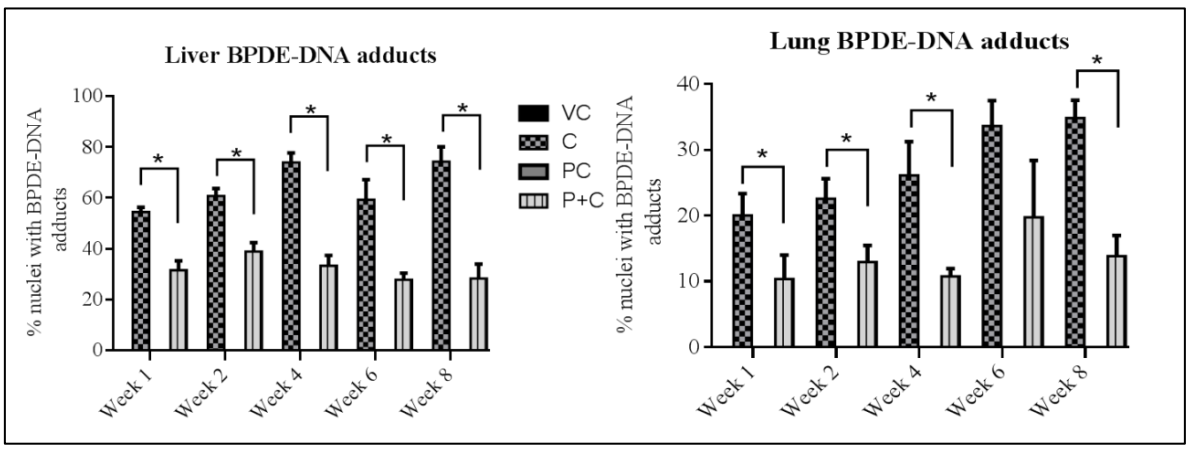

Liver BPDE-DNA adducts
% nuclei with BPDE-DNA adducts
100
80
60
40
20
0
VC
C
PC
P+C
Week 1
Week 2
Week 4
Week 6
Week 8
Lung BPDE-DNA adducts
% nuclei with BPDE-DNA adducts
40
30
20
10
0
Week 1
Week 2
Week 4
Week 6
Week 8

Figure 4.13 : Effect of PBPs on BPDE-DNA adducts in A/J mice.

Representative photomicrographs showing immunohistochemical detection of BPDE-DNA adducts positive cells (magnification at 400x) at various sacrifice weeks (1^{st}, 2^{nd}, 4^{th}, 6^{th} and 8^{th} weeks) in formalin fixed, paraffin embedded lung tissue sections. Data represented as mean±SD of three observations.*significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

4.4 Discussion:

In the present study, chemopreventive efficacy of orally administered PBPs was evaluated by measuring its effect on incidence / multiplicity and/or delay in the latency period of both macroscopic and microscopic lung tumors in B(a)P and NNK induced lung carcinogenesis in A/J mice model. The number as well as area of the microscopic lung lesions like hyperplasia, AAH and adenoma was analyzed as per the recommendations and guidelines by Nikitin *et al.*, 2004 **(108).**

In the present study, we demonstrated orally administered PBPs decreases macroscopic lung tumor multiplicity as well as multiplicity and area of microscopic lung lesions induced by B(a)P and NNK at 10 and 18 weeks post-carcinogen treatment. The observed decrease in tumor multiplicity and / or lesion areas have not attained the statistical significance at early time points possibly because of the a) low tumor numbers b) very small tumor size and, c) possible variation in the doses of PBPs received by animals due to intra group variation in uptake of drinking water resulting in variable doses of PBPs. However, PBPs administration did not affect the incidence of B(a)P and NNK induced lung tumors and latency period. Similar observations were reported previously in other studies using different chemopreventive agents like phenethyl isothiocyanate,

butylated hydroxyanisole, myo-inositol, resveratrol and lycopene which affect tumor multiplicity without affecting incidence **(35,109)**. Oral administration of 2% crude black tea extract and 0.6% decaffeinated black tea extract (mixture of free catechins, theaflavins and PBPs) have resulted in the decrease in the tumor multiplicity by 31% and 65% respectively, without affecting the tumor incidence in NNK induced lung carcinogenesis model **(77,80).**

Earlier reports on crude black tea extract has been shown to be affect various pulmonary microscopic lesions like hyperplasia while, PPE and caffeine has been shown to reduce progression from lung adenoma to adenocarcinoma in experimental model **(79,110).** In our study, PBPs significantly decreased number and size of both hyperplasia and adenomas at all the time points studied indicating PBPs act on both early as well as late proliferative lesions in lung carcinogenesis process. However, observed decrease in AAH did not attain the statistical significance due to low occurrence and small size of the foci.

In our study, administration of 1.5% black tea derived PBPs has decreased the lung tumor multiplicity by ~ 21%. This observation when compared to reported chemopreventive activity of various crude and decaffeinated extracts of black tea **(77,80)** suggest PBPs to be major contributory components in black tea mediated chemopreventive activity. PBPs have been reported to be heterogeneous polymers of flavano-3-ols and flavan-3-ol gallates with di- and tri-benzotropolone skeletons **(70,90).** Ideally demonstration of presence and evidence correlating the circulating or tissue levels of PBPs and / or their metabolic products with biological activity is needed to attribute observed chemopreventive effects to PBPs. However currently the information on structural / molecular characterization and methods for detection of PBPs and / or their products are not available due to complexity of oxidation product (PBPs) comprised of unknown numbers of isomeric structures **(70,111,112)**. Additionally our attempts to detect exposure related signals in MALDI-TOF analyses of sera from exposed animals were not successful probably because of very low levels of circulating PBPs / metabolites. However, observed PBPs-mediated increase in expression of protein and activity of GST in lung suggest its

exposure to parent PBPs and / or their metabolic products after oral administration of PBPs. Under such circumstances the exposure to PBPs was the only variable in our study and MALDI-TOF analysis of PBPs extract was used in our study which has demonstrated them to be free of other biologically active black tea-derived contaminants (e. g. caffeine, catechins, theaflavins), observed chemopreventive activity could be attributed due to PBPs.

In our experiment oral administration of PBPs was done two weeks prior, during and subsequent to the carcinogen treatment. Hence observed chemopreventive effects can be interpreted to be due to both anti-initiation as well as anti-promotion activity of PBPs. However, chances for predominant effects of anti- promotion activity of PBPs are more likely due to prolonged treatment of PBPs after cessation of carcinogen treatment. Similarly observed chemopreventive effects can be attributed to parent and/or metabolic products of PBPs, hence observed bioactivity is likely to be due to PBPs and / or their products. To the best of our knowledge, this is the first report demonstrating chemopreventive effects of major black tea polyphenols, PBPs/ thearubigins exerting the chemopreventive effects on both macroscopic as well as microscopic lung lesions by decreasing the tumor multiplicity.

As reported in the literature, A/J mice are known to develop spontaneous lung tumors **(113)**. One notable difference in our observation is none of the control groups developed visible or microscopic spontaneous lung tumors till the end of experiment i.e. 36 weeks of age. Ruling out all the possibilities, the diet employed for the animals is the only potential factor which can influence absence of spontaneous lung tumors. In previous studies AIN-76/AIN-93 synthetic diet was used wherein spontaneous lung tumors were observed even at 16 weeks of age **(113)**. However diet employed in our study is majorly plant derived which are known to induce the Phase II metabolizing enzymes which are responsible for conjugation reaction and delay the spontaneous or carcinogen induced tumors **(114).** Despite using plant derived diet; demonstration of significant chemopreventive activity attributable to PBPs exposure suggest that PBPs mediated

chemopreventive effect would have been pronounced if synthetic diet devoid of any chemopreventive agents was used.

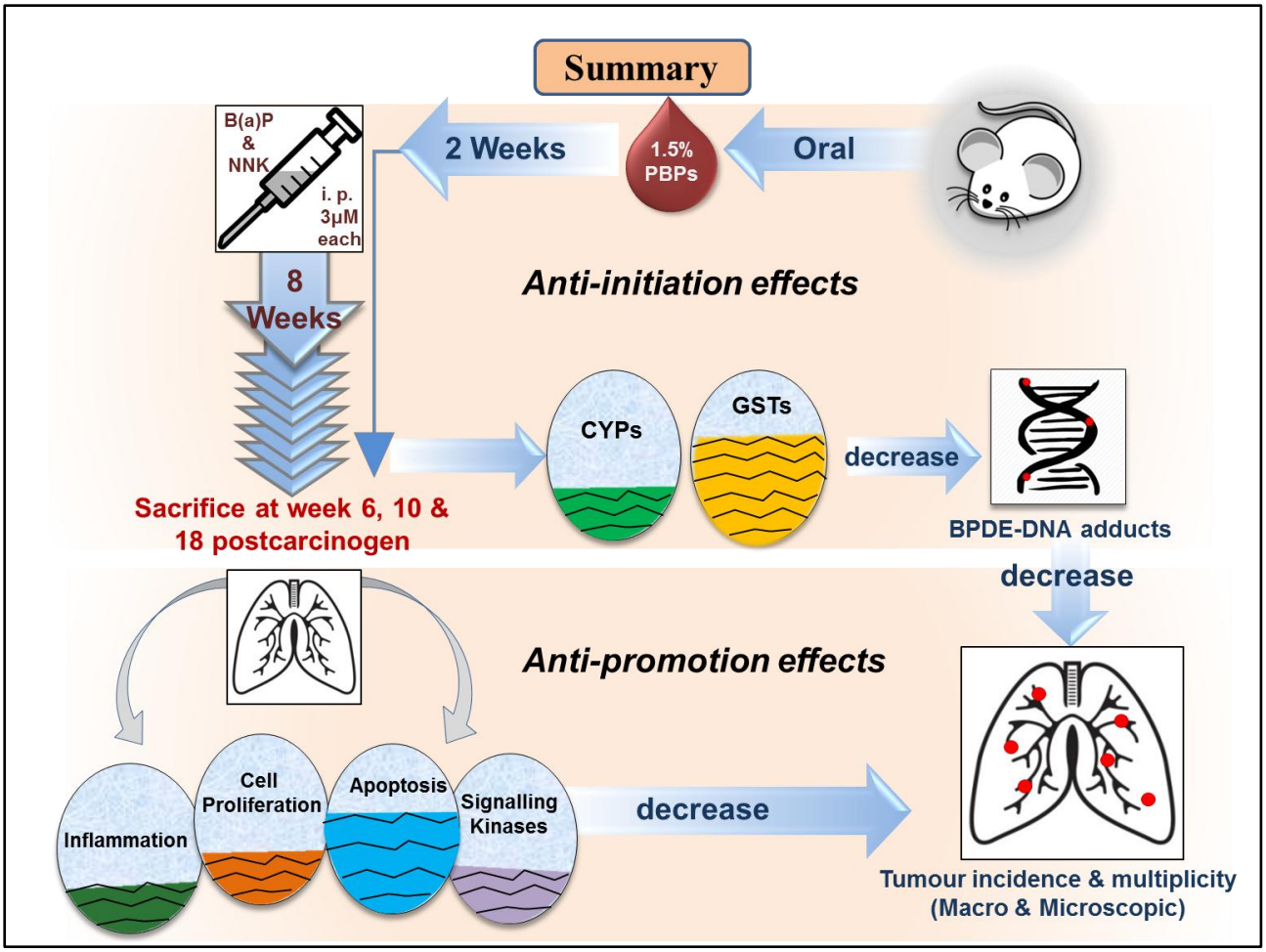
To explain the observed decrease in lung tumor multiplicity and to understand the mechanism of chemoprevention by PBPs we have also analyzed molecular markers of inflammation, cell proliferation and apoptosis. In our study, Cox-2 expression was found to be modulated by PBPs at all the time points studied. Interestingly anti-inflammatory effect of PBPs was more pronounced at early weeks as compared to late weeks. Previous studies have shown that dietary administration of crude black tea polyphenols modulates the AOM-induced Cox-2 and iNOS gene expression in tumors **(115).** Similarly, black tea extract rich in theaflavins (TFs) and EGCG has been shown to inhibit B(a)P-induced Cox-2 expression in mouse lungs **(116)**. We have also observed modulation of process of proliferation and apoptosis by PBPs in sequential lung carcinogenesis. Decrease in PCNA along with Bcl-2 down regulation and Bax overexpression occurred progressively during lung tumorigenesis with PBPs treatment. It is noteworthy that we observed decrease in PCNA expression / proliferation index as well as modulation of Bax/Bcl-2 ratio and apoptotic index even before the appearance of first microscopic tumor, which further reassured the anti-promotion efficacy of PBPs in B(a)P and NNK induced lung carcinogenesis. However it is important to note that despite significant decrease in carcinogen induced proliferation index by PBPs, it never matched with vehicle control. Thus PBPs decreased carcinogen induced cell proliferation and increased apoptosis which in turn is responsible for observed decrease in lung tumor multiplicity and lesion areas. Our results showing decrease in proliferative index and increase in apoptosis are consistent with previous reports on theaflavins and caffeine in NNK induced lung carcinogenesis model **(79).** Furthermore, dietary administration of black tea polyphenols decreased the DMBA-induced Bax/Bcl-2 ratio in hamster buccal pouch carcinogenesis model **(117).** Thus PBPs could be one of the potential contributing components in observed anti promotion mechanism of whole black tea extract.

Studies on EGCG and theaflavins in various model systems have demonstrated modulation of MAP kinases and Akt/PKB signaling pathway **(118).** Previously pre-treatment of mouse skin with PBPs has shown to decrease the TPA-induced phosphorylation of ERK and p38 during tumor development, while expressions of total ERK and p38 were unaltered indicating that signaling kinase functions are one of the targets for chemoprevention by PBPs **(84).** Interestingly our results showed significant decrease in phosphorylated forms of p38 and Akt at post-carcinogen treatment weeks which are in agreement with observed decrease in various biomarkers of cell proliferation and enhancement of apoptosis described earlier, suggesting modulation by the signaling kinases. It gives suggestive evidence that PBPs either as a parent compound or its metabolic products with differences in structure and molecular weights (when compared to monomeric catechins) also retain ability to modulate carcinogen-induced signaling kinase functions.

B(a)P and NNK induced lung carcinogenesis model in A/J mice has been extensively used to understand the complex interactions of carcinogens and to test the effect of various chemopreventive agents with varying dose, duration and route **(119-121).** Administering B(a)P and NNK by i.p. injections offered several advantages such as a) effectively delivers the complete dose of carcinogen, b) avoids direct interaction with PBPs which are administered orally c) prevents development of the fore stomach tumors as in case of gavage **(122)**. Despite the fact that defined doses of B(a)P & NNK were administered by i.p. route; while PBPs was given through drinking water *ad libitum* resulting in absorption and uptake of probably part or low levels of PBPs continuously over a period of time (resulting in very low levels of circulating PBPs and/or its products), detectable effects were observed at different time points studied. Future studies on measurement of circulating or tissue levels of parent (PBPs) or product (metabolites of PBPs) compounds will enhance our understanding about modulation of various biomarkers in the chemopreventive trials. Since, we have used single dose of PBPs, minimal effective dose cannot

be ascertained from the given study. However, dose response study employing various doses of PBPs will further address the issues about absorption and bioavailability.

Thus by using B(a)P and NNK induced lung carcinogenesis model, we deciphered for the first time the chemopreventive efficacy of PBPs which was evident by decrease in the tumor multiplicity as well as low number and size of the microscopic lesions (hyperplastic and adenomatous foci) at various time points studied. Our study further attempted to understand the molecular mechanisms underlying the given chemopreventive efficacy exhibited by PBPs which is apparent by marked reduction in inflammation, cell proliferation and increase in the apoptosis may be possibly governed by lowered phosphorylation of p38 and Akt. Our study further suggests that black tea interventions offered throughout the life are likely to present an effective approach in decreasing carcinogen-induced cell proliferation while increasing the probability of initiated cell being apoptosised. PBPs administration through drinking water suggests that polymeric and relatively high molecular weight compounds or their products are getting absorbed/ metabolized and exert their chemopreventive effect through various pathways including signaling kinase function.


Summary
B(a)P & NNK
i. p. 3µM each
2 Weeks
1.5% PBPs
Oral
8 Weeks
Anti-initiation effects
CYPs
GSTs
decrease
BPDE-DNA adducts
Sacrifice at week 6, 10 & 18 postcarcinogen
decrease
Anti-promotion effects
Inflammation
Cell Proliferation
Apoptosis
Signalling Kinases
decrease
Tumour incidence & multiplicity (Macro & Microscopic)

Chapter 5

To understand the effect of dose response of PBPs on carcinogen metabolism during early stages of lung carcinogenesis

Chapter 5: To understand the effect of dose response of PBPs on carcinogen metabolism during early stages of lung carcinogenesis

5.1 Introduction:

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental agents, many of which have been identified as potent human carcinogens **(123,124)**. Exposure to complex mixtures of PAHs has been associated with development of skin, lungs and breast cancer. Benzo(a)pyrene, a well- established ubiquitous carcinogen belonging to the PAHs group of compounds, is metabolically activated by phase I enzymes (or CYP1A class of cytochrome P450 (CYP450) enzymes) to form a highly mutagenic reactive electrophile, benzo(a)pyrene diol-epoxide (BPDE). Though phase II enzymes catalyze the conjugation of BPDE, some of the reactive electrophiles interact covalently with DNA to form adducts that mark the early initiation event. Unrepaired/misrepaired adducts may lead to mutation in genes involved in proliferation, growth and apoptosis and finally to a diseased condition such as cancer **(60)**. Thus dose and/or route of administration of carcinogen and chemopreventive agent which in turn balances the interplay between phase I and II enzymes play an import2ant role in carcinogen metabolism and hence modulation of expression of these enzymes plays crucial role during chemoprevention.

Tea polyphenols such as epigallocatechin gallate (EGCG) and theaflavins have been shown to influence carcinogen metabolism by altering cellular metabolism (phase I and II enzymes) in a number of preclinical studies **(58,125)**. PBPs, the most abundant polyphenols in black tea are structurally and chemically ill-defined because of its complexity due to number of isomeric compounds and matrix reactivity. Previously isolated and partially characterized PBPs fractions have shown to have anti initiation activity by inhibiting formation of BPDE-DNA adducts in mouse skin and demonstrating decrease in liver microsomal CYP activity *in*

vitro **(93)**. Furthermore, previous studies have also indicated that PBPs may decrease B[a]P-induced DNA adducts by inhibiting B[a]P-induced activation of phase I enzymes such as CYP450 1A1 and 1A2 **(93,126)**. However, the knowledge about dose related responses of black tea polyphenols on carcinogen metabolism via modulation of xenobiotic metabolizing enzymes is limited. Along with dose, efficacy of chemopreventive agent is mainly attributed to its pharmacological bio distribution including bioavailability, tissue competent metabolism and its circulating levels in the body fluid.

Considering the previous studies which demonstrate the chemopreventive potential of either each fractions of PBP or 1.5% black tea derived PBP rich extract in different invivo and invitro systems, it was pertinent to evaluate the dose related effects of PBP rich extract. In the present study we evaluated effect of 0.75, 1.5 and 3% black tea derived PBP rich extract on expression and activity of phase I & II enzymes and BPDE-DNA adducts in B(a)P induced A/J mice model in liver and lung. Dose related chemopreventive efficacy, if any, was further evaluated using a well-established B(a)P and NNK induced lung carcinogenesis model in A/J mice. The chemopreventive efficacy of 1.5% and 3% black tea derived PBP rich extract was measured by analyzing macroscopic and microscopic lung tumor incidence, multiplicity.

5.2 Methods:

5.2.1 Isolation and preparation of various doses of PBP rich extract:

To understand the dose response effect of PBP rich extract in A/J mice model, PBPs were isolated using previously discussed protocol (Chapter 3, section no.3.1, page no. 41-47) and various doses of black tea derived PBP rich extract was prepared as shown in the **Figure 5.1.**

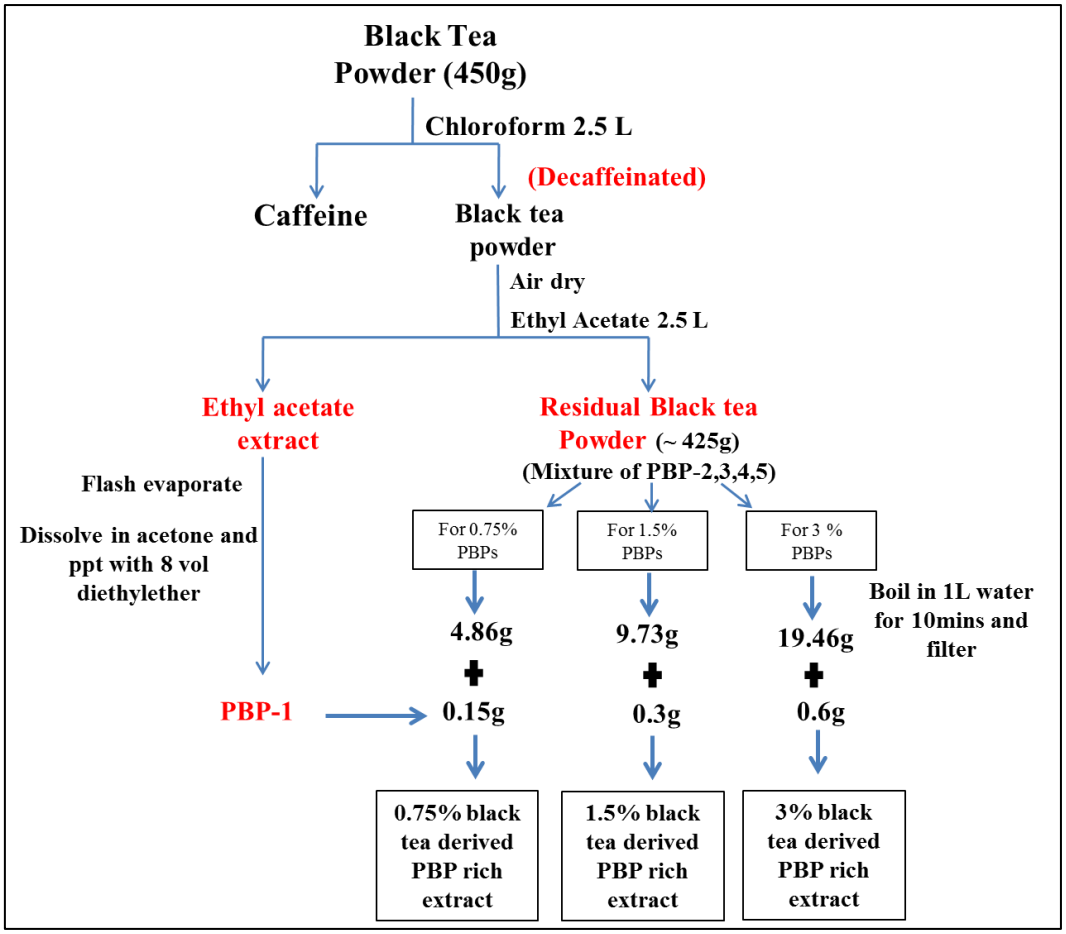


Figure 5.1 : Flowchart showing preparation of various doses of PBPs (0.75%, 1.5% and 3% black tea derived PBPs) from black tea powder.

Briefly, residual tea powder was weighed and boiled in autoclaved miliQ water in such a proportion which will provide 0.75%, 1.5% and 3% black tea derived extract which is known to contain mixture of PBPs-2, 3, 4, 5. To this aqueous extract, PBP-1 was added back accordingly to obtain PBP rich extract **(Table 5.1)**. Various doses of PBP rich extract were aliquoted and administered to the animals after it was confirmed to be free of other biologically active components such as caffeine, theaflavins and catechins using thin layer chromatography as well as MALDI-TOF analysis as discussed in materials and methods (Chapter 3, section no.3.1.2.2, page no. 46-48). Total solids (mg)/ml were measured and UV

absorption spectra was recorded (scan at 190-340nm) for all three doses of PBP rich extract as described **(91)** to confirm the administration of various doses of PBPs.

% of PBPs	Components (g/liter)	
	PBP-1	Residual tea powder (contains PBP-2,3,4,5)
0.75%	0.15	4.86
1.5%	0.3	9.73
3%	0.6	19.46

Table 5.1 : The amount of PBP-1 and residual tea powder to obtain various doses of PBP rich extract. The amount of residual tea powder which was weighed and boiled in autoclaved miliQ water to which PBP-1 was added back to obtain PBP rich extract in such a proportion which contained 0.75%, 1.5% and 3% original black tea derived PBPs.

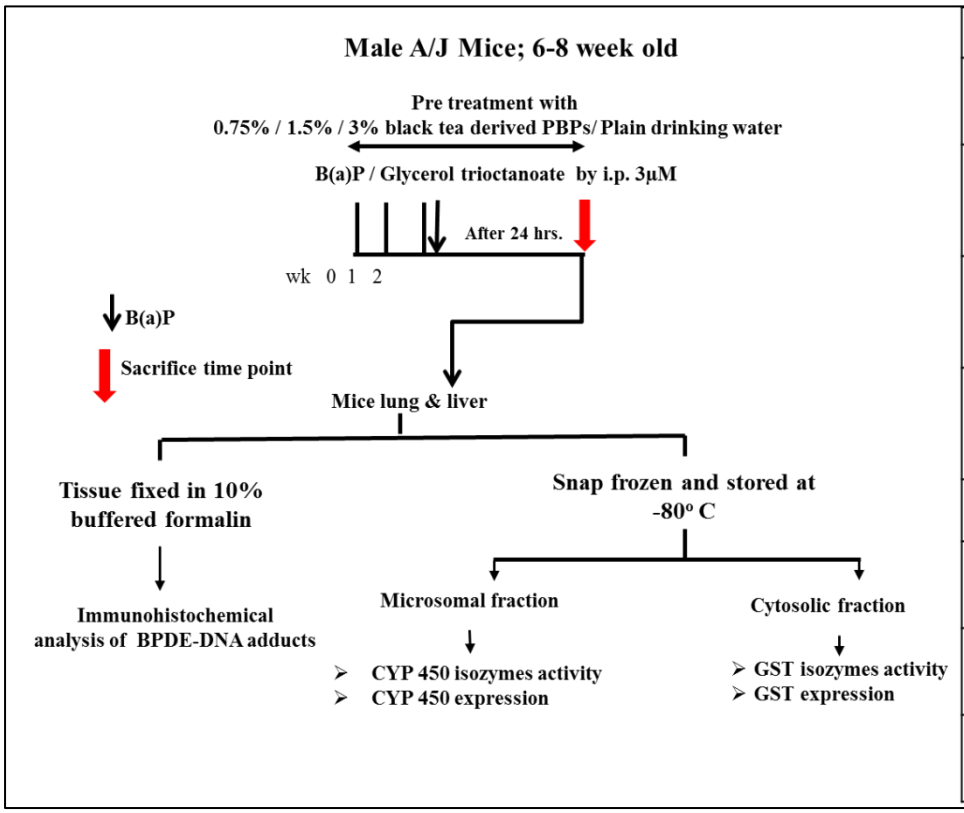
5.2.2 Animal treatment:

All animal studies were conducted after approval from the institutional animal ethics committee (sanction no: 09/2011) endorsed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. A/J mice obtained from Jackson Laboratory, USA were inbred at Laboratory Animal Facility, ACTREC, India and they were housed under standard conditions: 22 ± 2°C, 55 ± 5% relative humidity, and 12-h light/dark cycle. Male A/J mice (6-8 weeks old) were used for the study which received a standard pellet diet *ad libitum*. Fresh bottle of PBPs rich extract/ drinking water were employed every alternate day. Body weights were monitored after receiving the animals and before the sacrifice time points. At the end of the experiment, animals were sacrificed using

cervical dislocation. Lung and liver tissues were perfused with 0.15M KCl, excised. All five lung lobes and part of liver from three random animals per group were fixed in 10% buffered formalin and paraffin embedded blocks which were used for histopathological evaluation as well as immunohistochemical analysis (IHC). Tissues from the rest of the animals were snap frozen in liquid nitrogen and stored at -80°C until preparation of the extract for immunoblotting.

5.2.2.1Evaluation of biochemical effects:

Male A/J mice, 6–8 weeks old were obtained from Laboratory Animal Facility, ACTREC and randomized into eight different groups **(Figure 5.2)**. Polyphenol control (0.75%PC, 1.5%PC, 3%PC) and polyphenol+carcinogen (0.75%P+C, 1.5% P+C, 3%P+C) groups were primed for 2 weeks with 0.75, 1.5 and 3% black tea derived PBPs rich extract as a sole source of drinking water respectively. While vehicle control (VC) and carcinogen(C) groups received normal drinking water. Animals in vehicle control (VC) and polyphenol control groups (0.75%PC, 1.5%PC, 3%PC) were injected glyceryl trioctanoate (vehicle, i.p., 0.1ml) and continued receiving drinking water and 0.75, 1.5 and 3% black tea derived PBPs rich extracts respectively. While carcinogen (C) and polyphenol+carcinogen groups (0.75%P+C, 1.5% P+C, 3%P+C) were injected B(a)P (i.p, 3µM, 0.1ml) and continued receiving drinking water and 0.75, 1.5 and 3% black tea derived PBPs rich extract (*ad libitum*) respectively. Single i.p. injection of glyceryl trioctanoate or B(a)P was given, while PBPs rich extracts and drinking water were administered *ad libitum*, prior to (2 weeks), during and after injection (24 hr) till the end of the experiment **(Figure 5.2)**. Animals were sacrificed 24 hr after single injection of glyceryl trioctanoate or B(a)P.

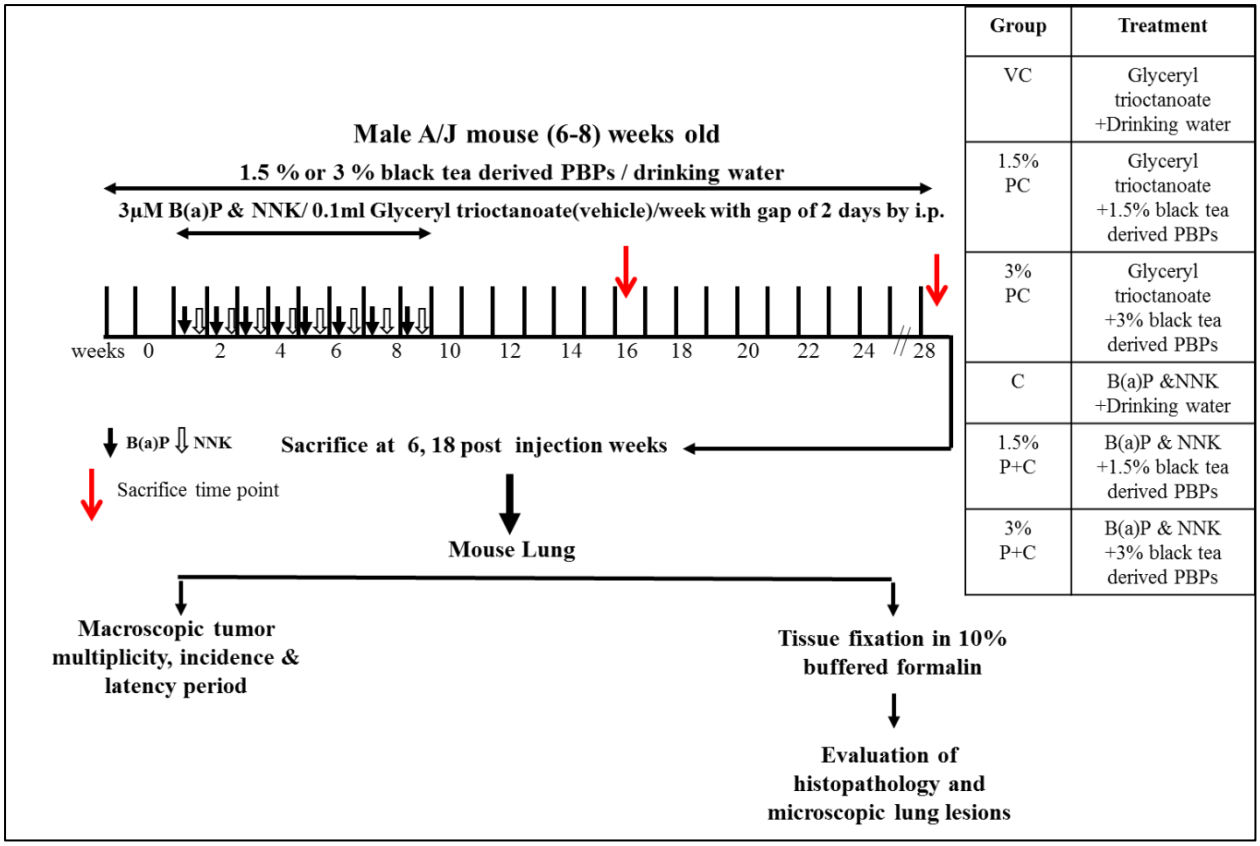


Group	Treatment
VC	Glyceryl trioctanoate +Drinking water
0.75% PC	Glyceryl trioctanoate +0.75% black tea derived PBPs
1.5% PC	Glyceryl trioctanoate +1.5% black tea derived PBPs
3% PC	Glyceryl trioctanoate +3% black tea derived PBPs
C	B(a)P +Drinking water
0.75% P+C	B(a)P +0.75% black tea derived PBPs
1.5% P+C	B(a)P +1.5% black tea derived PBPs
3% P+C	B(a)P +3% black tea derived PBPs

Figure 5.2: Experimental design for studying the dose response effect of PBPs on B(a)P induced biochemical changes in lung and liver of A/J mice model 6-8 weeks old male A/J mice were randomized into eight different groups such as vehicle control(VC), Polyphenol control (0.75%PC, 1.5%PC, 3%PC), carcinogen (C) and polyphenol+carcinogen (0.75%P+C, 1.5% P+C, 3%P+C) as shown in the tabular format. Polyphenol control (0.75%PC, 1.5%PC, 3%PC) and polyphenol+carcinogen (0.75%P+C, 1.5% P+C, 3%P+C) groups were primed for 2 weeks with respective doses of PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water. After 2 weeks of priming, single intraperitoneal injection of B(a)P (3µM/0.1ml) in glyceryl trioctanoate or only glyceryl trioctanoate were given (shown by thin arrow) while PBPs or drinking water were administered orally throughout the experimental period. Treatment groups are as shown in the tabular format. Sacrifice was done at 24 hr post carcinogen treatment (shown by red arrow).

5.2.2.2 Evaluation of effects on tumor incidence and multiplicity of various doses of PBPs:

To study dose related effect, if any, of (1.5 and 3%) black tea derived PBPs rich extracts on B(a)P and NNK induced lung carcinogenesis model was employed. Animals were randomized into six different groups. Polyphenol control (1.5% PC and 3% PC) and polyphenol+carcinogen (1.5% P+C and 3% P+C) groups were primed for 2 weeks with respective doses of black tea derived PBPs as the sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water **(Figure 5.3)**. Animals in vehicle control (VC) and polyphenol control (1.5% PC and 3% PC) groups were injected glyceryl trioctanoate (vehicle, i.p., 0.1ml) and continued receiving drinking water and black tea derived PBPs rich extracts respectively while carcinogen (C) and polyphenol+carcinogen (1.5% P+C and 3% P+C) groups were injected B(a)P and NNK (i.p, 3μM of each, 0.1ml) and continued receiving drinking water and black tea derived PBPs rich extracts *(ad libitum)* respectively. Glyceryl trioctanoate or B(a)P and NNK injections were given with the gap of 2 days, once in a week for 8 weeks while PBPs rich extracts and drinking water were administered *ad libitum*, prior to (2 weeks), during (8 weeks) and after the carcinogen treatment (6 and 18 weeks) till the end of the experiment **(Figure 5.3)**. Animals were sacrificed at 6 and 18 weeks after administration of the last dose of carcinogen. Sacrifice weeks were selected as per earlier study time points i.e. week 6 and 18 to understand the chemopreventive efficacy in terms of delay in the latency period and/or decrease in tumor incidence and/or multiplicity respectively **(127)** . Macroscopic/visible lung tumors were counted at both time points using magnifying glass. The number and area of microscopic pulmonary lesions were evaluated as described (Hudlikar et al. 2017).



Group	Treatment
VC	Glyceryl trioctanoate +Drinking water
1.5% PC	Glyceryl trioctanoate +1.5% black tea derived PBPs
3% PC	Glyceryl trioctanoate +3% black tea derived PBPs
C	B(a)P &NNK +Drinking water
1.5% P+C	B(a)P & NNK +1.5% black tea derived PBPs
3% P+C	B(a)P & NNK +3% black tea derived PBPs

Figure 5.3 : Experimental design for studying the effect of various doses of PBPs (1.5% and 3%) on B(a)P and NNK induced lung carcinogenesis in A/J mice model 6-8 weeks old male A/J mice were randomized into four different groups such as vehicle control(VC), polyphenol control (1.5%PC, 3%PC), carcinogen (C), and polyphenol+carcinogen (1.5%P+C, 3%P+C) as shown in the tabular format. Polyphenol control (1.5%PC, 3%PC) and polyphenol+carcinogen (1.5%P+C, 3%P+C) groups were primed for 2 weeks with PBPs as the sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water. After 2 weeks of priming, B(a)P and NNK(3µM each/0.1ml) in glyceryl trioctanoate or only glyceryl trioctanoate were intraperitoneally injected with the gap to 2 days, once in a week for 8 weeks (shown by solid and hollow arrow respectively) while PBPs or drinking water were administered orally prior to (2 weeks), during (8 weeks) and after the carcinogen treatment till the end of the experiment throughout the experimental

period in male A/J mice. Treatment groups are as shown in the tabular format. Sacrifice was done at 6th and 18th weeks after the last carcinogen treatment (shown by red arrow).

5.2.3 Protein immunoblotting:

Liver and lung microsomal and cytosolic fractions were prepared by previously described cell fractionation procedure in materials and methods (Chapter 3, section no.3.3.1, page no. 47-49). Fractions were aliquoted, their protein content was determined using Lowry method and stored at -80°C. Microsomal fraction (50 µg) for CYP1A1, CYP1A2 and cytosolic fraction (50 µg) for GST mu, GST pi, GST alpha expression respectively were used to study expression of proteins using standard immunoblotting SDS-PAGE protocol as described in materials and methods (Chapter 3, section no.3.4, page no. 50-55) and conditions mentioned in the **Table 5.2**. Immunoreactive bands were visualized with enhanced chemiluminescence reagent followed by autoradiography.

Protein	% gel	Amount of protein	Primary antibody dilution	Secondary antibody dilution
CYP1A1	12	Microsome,50µg	1:1000	1:4000
CYP1A2	12	Microsome,50µg	1:1000	1:4000
GST mu	12	Cytosol,50µg	1:1000	1:4000
GST pi	12	Cytosol,50µg	1:1000	1:4000
GST alpha	12	Cytosol,50µg	1:1000	1:4000

Table 5.2 : Antibodies used for immunoblotting and its dilution

5.2.4 Phase I and phase II enzyme activity:

Microsomes for CYPs while cytosols for GSTs were employed with equal protein concentration (1mg) for all the activity assays. CYP1A1 and 1A2 employing isozyme-

specific probe drugs-ethoxyresorufin and methoxyresorufin, were used respectively, as described earlier in materials and methods (Chapter 3, section no.3.5, page no. 55,56). The product resorufin formed from O-dealkylation of ethoxyresorufin and methoxyresorufin was measured fluorimetrically at Ex = 550 nm and Em = 585 nm. The activity was expressed in terms of nmoles of product formed/min/mg of protein.

The total GST activity was assayed using common substrate, 1-chloro-2,4-dinitrobenzene (CDNB), by Glutathione S-Transferase (GST) assay kit as per manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA) as described earlier in materials and methods (Chapter 3, section no.3.5, page no. 56,57). A complete assay mixture without enzyme served as the control. GS-DNB conjugate formed was measured spectrophotometrically in 96-wells plate at 340 nm. The activity was expressed in terms of µmole of product formed/ml/min.

5.2.5 Immunohistochemical analysis and Terminal Deoxynucleotidyl Transferase fluorescein-dUTP Nick End Labelling (TUNEL) assay:

The immunohistochemical (IHC) staining of the lung tissues for proliferation cell nuclear antigen (PCNA), BPDE-DNA adducts and TUNEL assay was carried out on 5 µm thick sections of paraffin embedded whole lung tissue using protocol described earlier in materials and methods (Chapter 3, section no.3.6 & 3.7 page no. 57-59). In each batch, slides with positive control(s) were also processed simultaneously. For negative or isotype controls, the primary antibody was replaced with PBS. Images were captured with a Zeiss microscope (Imager Z1) to which was attached an Axiocam MRc5 digital camera. Semi-quantitative analysis was conducted using Image J 1.43 (NIH) software. Number of nuclei with BPDE-DNA adducts staining/ PCNA labeling index/apoptotic index was calculated by counting the number of positively stained cells × 100/ total number of cells in photomicrographs of lung/liver sections. At least 10 different randomly selected fields and/or minimum 1000 cells

were counted at 400x magnification with at least three mice per group. Quantitative analysis of staining intensity of BPDE-DNA adducts positive nuclei was performed by IHC profiler **(128)**, which is an open source plug-in for the quantitative evaluation and automated scoring of immunohistochemistry images of tissue samples.

5.2.6 Digital image analysis for BPDE-DNA adduct containing cells:

IHC photomicrographs were used for developing semi-automated analysis protocol, namely IHC profiler **(128)**. Images were analyzed for the staining intensity count as described earlier by **(129)**. Briefly, a color de-convolution plug-in was used to un-mix the pure DAB and haematoxylin stained areas that left a complimentary image. The pixel intensities of separated DAB images range from 0 to 255. Value 0 represents the darkest shade whereas 255 represent the lightest shade of the DAB brown color in the image. To select the DAB-stained (brown) nuclei, the threshold feature of the Image J 1.43 (NIH) software was used. Further to assign an automated percentage of pure DAB staining patterns in the nucleus, a macro was developed and plugged in the Image J 1.43 (NIH) software to obtain an automated counting of the pixel wise percentage contribution of high, medium and low positive pixels/intensity in an image i.e. the number of pixels of a specific intensity value vs. their respective intensity zone. For measurement of BPDE-DNA adducts [similar areas of tissue sections (mm2) and number of cells (~1000 cells/section/animal)], total intensity in percentage [of nuclei containing percentage of high, medium and low intensity] was analyzed within different treatment groups. After determining these numbers, the program applied them to a simple algebraic formula as shown below to determine the actual number of high /medium/low positive intensity.

Percentage of high positive/medium intensity/low positive intensity =

Percentage of high positive/medium positive/low positive DAB colour intensity pixels X score of the zone

---

Total number of pixels in the image

In order to determine the total percentage intensity (of adducts containing nuclei), the following formula was used.

Total percentage of intensity (Adducts containing cells) =

Percentage of (high positive intensity + medium positive intensity + low positive intensity)

5.2.7 Statistical analysis:

Statistical analysis was performed using SPSS 21.0 software. Data are presented as mean ± SD. Means of (western blot analysis) data were compared using ANOVA with post-hoc testing. Statistical comparisons of levels of number of BPDE-DNA adducts among the groups were made using Student's T test. While intensity of BPDE-DNA adducts was compared amongst the group using ANOVA with Bonneferoni's correction. $P<0.05$ was considered statistically significant. Surface lung tumors within the groups were compared using non-parametric Mann-Whitney Wilcoxon test while microscopic lung lesions were compared using Poisson regression, which represents the counts or number of events where few or no events occur.

5.3 Results:

5.3.1 General observations:

The 0.75%, 1.5% and 3% black tea brew-derived PBP rich extract contained average 2.35, 5.33 and 10.25 mg of total solids per milliliter of drinking water respectively **(Figure 5.4)**.

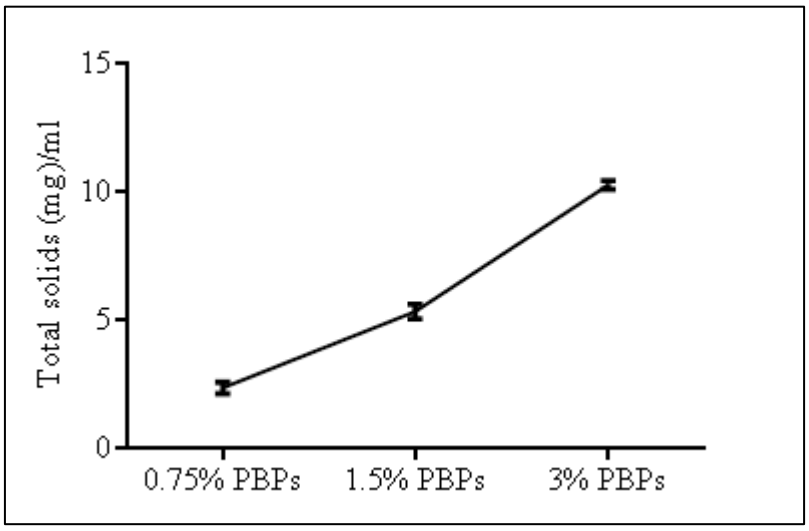


Figure 5.4 : Total solids (mg)/ ml in various doses of black tea derived PBP rich extract (0.75%, 1.5% and 3%) administered to A/J mice

The percentage yield of PBP rich extract and total solids obtained from PBP extract were reproducible. The UV spectra of PBP rich extract, showed two absorption maxima, namely λmax1 and λmax2 in the range of 210–219nm and 263–273 nm respectively as described **(91) (Figure 5.5)**.

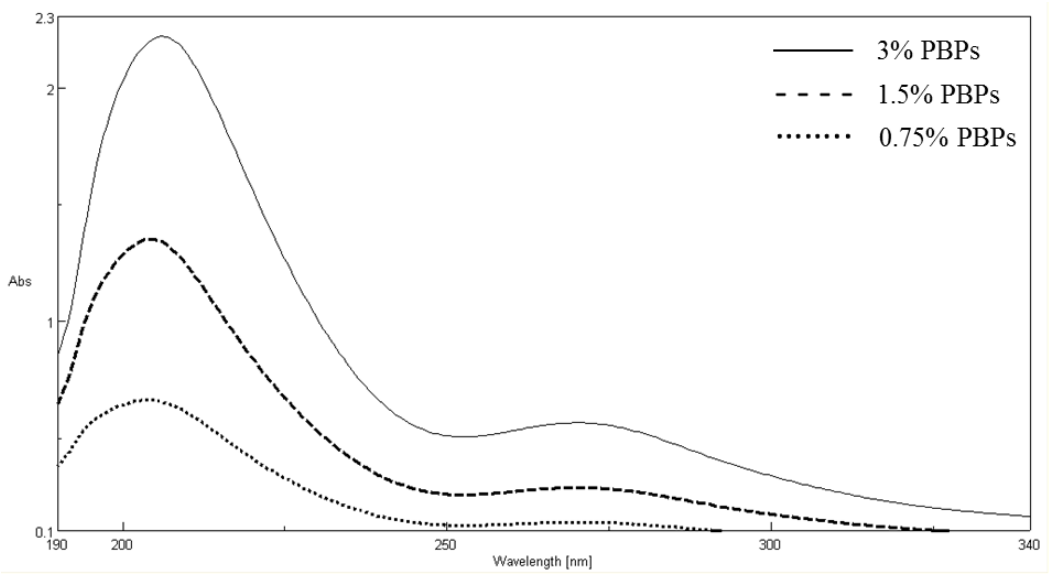


Figure 5.5 : UV spectra of various doses of PBP rich extract (0.75%, 1.5% and 3%) administered to A/J mice

Dose related increasing absorbance of PBP rich extract and weight of solids per unit volume confirmed the dose-related (0.75, 1.5, 3% tea-derived) yield / extraction in PBP rich extracts.

There were no signs of toxicity and no mortality was observed in any of the treated groups. No significant difference was found in initial and final body weights in animals belonging to different treatment groups **(Table 5.3)**.

Group	Week	n	Body weight	
			Initial	Final
VC	6	14	20.73±3.49	23.06±3.65
1.5% PC		13	21.21±2.29	24.11±2.39
C		10	20.60±2.38	22.81±2.36
1.5% P+C		10	19.33±2.46	21.49±2.55
VC		12	21.33±1.49	22.16±2.25
3% PC		11	20.21±2.49	21.10±1.15
C		8	20.60±1.26	22.31±2.46
3% P+C		8	21.53±2.36	23.69±1.65
VC	18	8	21.61±2.46	24.25±1.38
1.5% PC		9	22.06±1.23	24.72±1.01
C		8	21.60±2.27	24.47±2.03
1.5% P+C		8	22.29±1.42	24.90±2.54
VC		8	21.41±1.56	22.35±2.18
3% PC		9	21.16±1.43	23.77±1.11
C		7	22.41±2.23	23.84±2.04
3% P+C		7	24.15±2.02	24.90±1.54

Table 5.3 : Initial and final body weights of animals treated in various groups

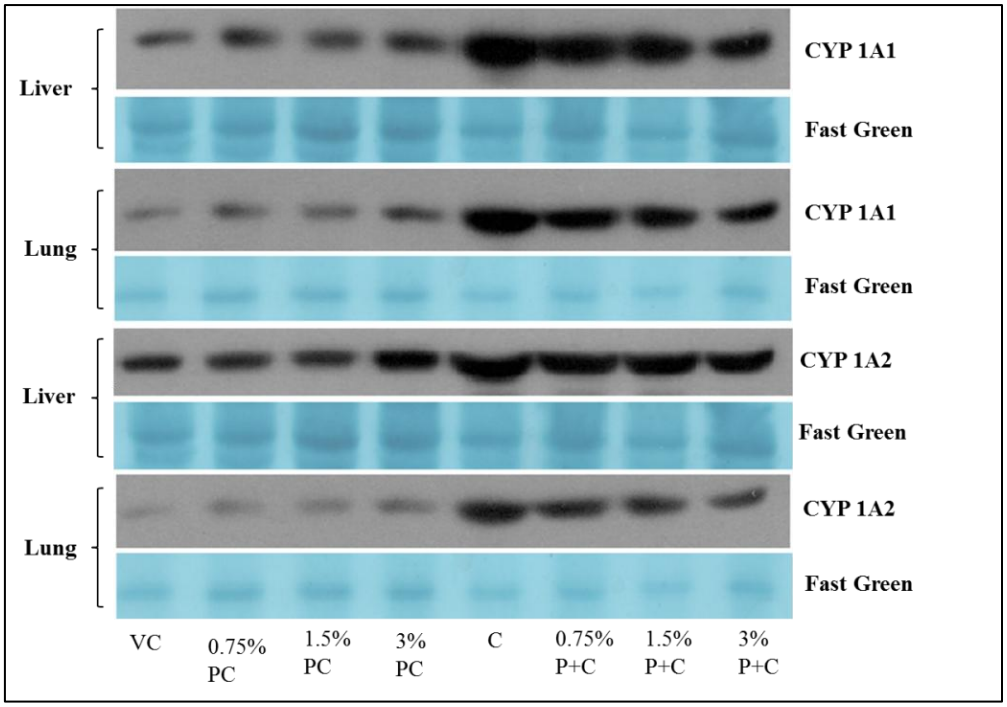
5.3.2 Pretreatment with various doses of PBP rich extract inhibited B(a)P induced expression of CYP isozymes in liver and lung:

VC and PC (0.75% PC, 1.5% PC & 3% PC) showed basal level expression of both CYP1A1 and 1A2 in liver and lung. In both tissues, B(a)P induced the expression of both CYP1A1 and 1A2 in C group as compared to its vehicle treated controls. Further, all the doses of PBPs decreased the B(a)P induced CYPs expression in P+C treated group however basal level expression of CYPs observed to be unaltered **(Figure 5.6)**. Despite various dose related PBPs

induced decrease in levels of CYP1A1 and CYP1A2, it never attained the levels observed in respective vehicle control groups.

In liver, CYP1A1 expression was found to be significantly decreased in P+C group as compared to its carcinogen group. Across the various doses of PBPs, 3% P+C group showed dose dependent significant decrease as compared to 1.5% P+C group. As contrast to CYP1A1, PBPs induced decrease in CYP1A2 expression in P+C group which could not attain the statistical significance at all three doses when compared to respective carcinogen treated group (**Figure 5.6**).

In lung, across the various doses of PBPs, 3% P+C group showed significant decrease in expression of carcinogen induced CYP1A2 as compared to 1.5% P+C and 0.75% P+C group and observed to be dose dependent (significant decrease in 3% > 1.5% > 0.75%). However, dose dependent decrease was not observed in carcinogen induced CYP1A1 expression in P+C treated with various doses of PBPs (**Figure 5.6**).



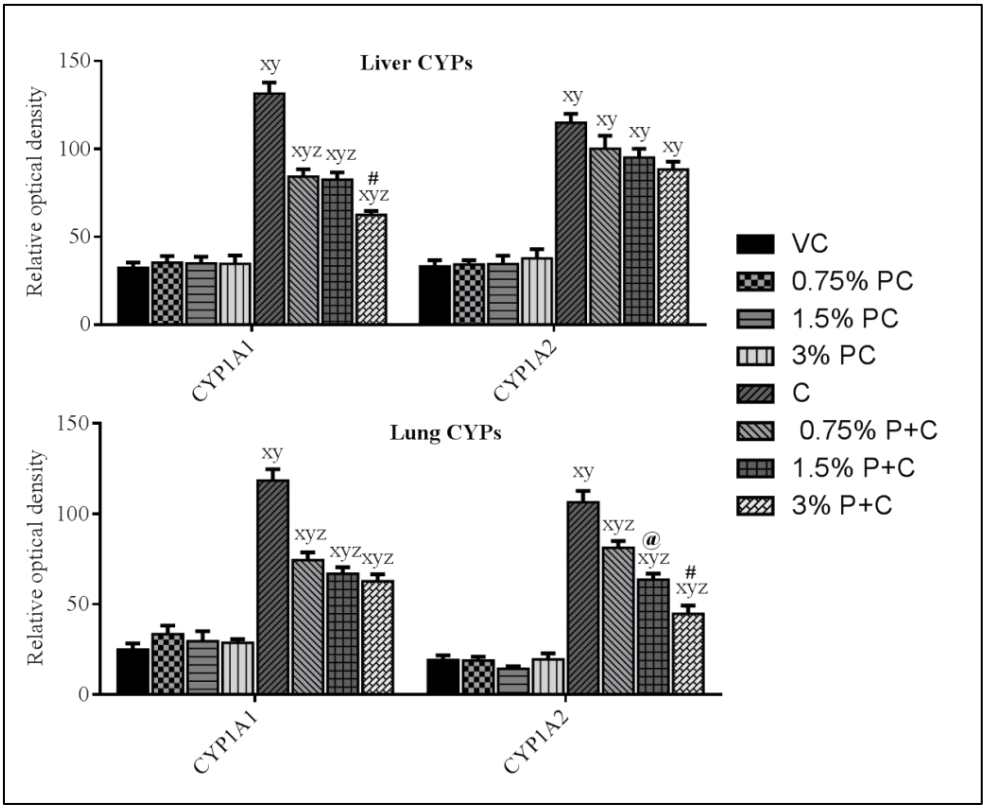


Figure 5.6 : Pretreatment with various doses of PBP rich extract inhibited B(a)P induced expression of CYP isozymes in liver and lung

Representative blots and densitometric analysis showing the protein levels of CYP1A1 and CYP1A2 in lung and liver microsomal fraction. In absence of a well-accepted standard internal control, quantitative analysis was done by normalizing the CYP1A1/1A2 immunoblot with a prominent band visualized on membrane after fast green staining. Data represented as mean±SD of three observations (three pooled lungs = one sample). X= significantly different from VC, y= significantly different from its respective PC, z= significantly different from C, @= significantly different from 0.75% P+C, # = significantly different from 1.5% P+C. ($p<0.05$, ANOVA followed by Bonneferoni's correction).

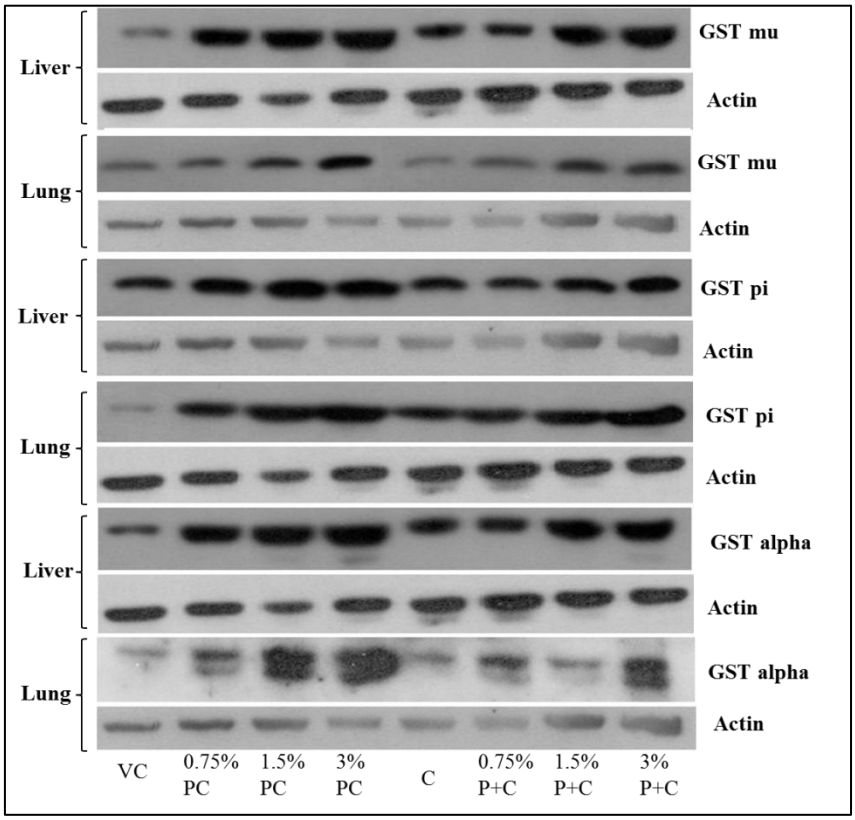
5.3.3 Pretreatment with various doses of PBP rich extract induced the expression of GST isozymes in liver and lung:

To understand the interplay of GSTs with PBPs pretreatment, expression of various isoforms of GSTs (mu, pi and alpha) were studied in liver and lung using immunoblotting. In both liver and lung, vehicle control group showed basal expression of various GST isozymes. GST expression in carcinogen group was comparable to vehicle control groups in all the isoforms. Significant up regulation of all GST isoforms were observed in PC groups treated with various doses of PBP rich extract (0.75, 1.5 and 3%) irrespective of carcinogen treatment as compared to vehicle treatment group **(Figure 5.7)**. However, decrease in PBPs induced expression of GSTs was observed in P+C group in both liver and lung.

In liver, amongst various doses of PBP rich extract administered GST mu and GST pi showed dose dependent increase in PC group (3%PC > 1.5%PC > 0.75%PC). Carcinogen treatment in 1.5% P+C and 3% P+C group decreased the polyphenol induced expression of GST mu and pi isozymes significantly as compared to its respective polyphenol controls. However, in

0.75% P+C group, the decrease was marginal and hence the GST expression levels in this group for all GST isozymes were comparable to carcinogen treatment group (**Figure 5.7**).

Concomitant to liver, lung GST mu expression was observed to be higher as compared to other isoforms in PC group. Amongst three GST isoforms, GST mu and GST alpha showed dose dependent significant increase in expression in PC group (3% > 1.5% > 0.75%). However, observed carcinogen mediated decrease in expression of GST mu and pi was significant in dose dependent manner in P+C group as compared to its respective polyphenol controls **(Figure 5.7)**.



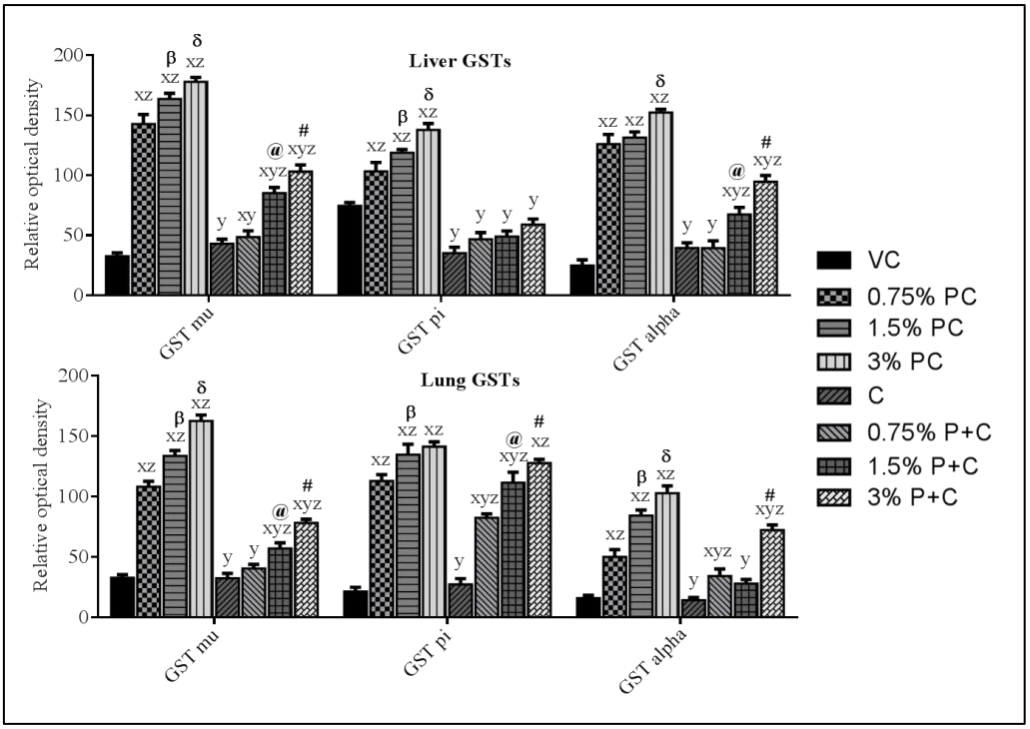


Figure 5.7 : Pretreatment with various doses of PBP rich extract induced the expression of GST isozymes in liver and lung

Representative blots showing the protein levels of GST isozymes (mu, pi and alpha) in lung and liver microsomal fraction. Beta actin was used as a loading control. Data represented as mean±SD of three observations. x= significantly different from VC, y= significantly different from its respective PC, z= significantly different from C, @= significantly different from 0.75% P+C, # = significantly different from 1.5% P+C, β= significantly different from 0.75% PC, δ= significantly different from 1.5% PC. ($p<0.05$, ANOVA followed by Bonneferoni's correction).

5.3.4 Pretreatment of various doses of PBP rich extract modulates activity of CYP and GST isozymes in liver and lung:

As CYPs and GSTs being xenobiotic enzymes, the relative differences can't be accurately judged only by studying expression of these proteins, hence enzyme activity of these proteins were calculated in the fractionated microsomal and cytosolic lysate respectively using

specific substrates in both liver and lung. Vehicle and polyphenol control showed basal activity CYP isozymes i.e. CYP1A1 (EROD) and CYP1A2 (MROD) in both the tissues. Concomitant with the GST expression; pretreatment with all the doses of PBPs induced the GST activity significantly as compared to its vehicle control group in liver as well as lung. Importantly, the increase in carcinogen induced CYPs activity and PBPs induced GST activity in liver being a metabolic hub was observed to be 6 to 8 times higher than the lung.

Amongst different isoforms of CYPs, CYP1A1 activity (EROD) was observed to be consistently higher in both C and all P+C groups as compared to CYP1A2 (MROD) in both liver and lung.

Across the various doses of PBPs treatment, although PBPs alone did not alter the basal activity of CYPs in either of the tissues, pretreatment with orally administered PBPs showed significant dose related decrease in B[a]P-induced CYP1A1/1A2 activities in both liver as well as lung (**Figure 5.8)**. Although PBPs alone did not alter the basal activity of CYPs in either of the tissues, pretreatment with orally administered PBPs showed significant dose related decrease in B[a]P-induced CYP1A1/1A2 activities in both liver as well as lung confirming the anti-initiation potential of PBP rich extract **(Figure 5.8)**.

Amongst the various does of PBP rich extract in PC group, increasing trend of GST activity (3% PC > 1.5%PC > 0.75%PC) was found to be significant as compared to its respective lower dose in both liver and lung. Dose related increasing trend in PBPs induced GST activity trend was observed in both liver and lung in P+C group (3% PC > 1.5%PC > 0.75%PC), however statistical significance was attained only in liver and not lung due to high intragroup variation (**Figure 5.8**).

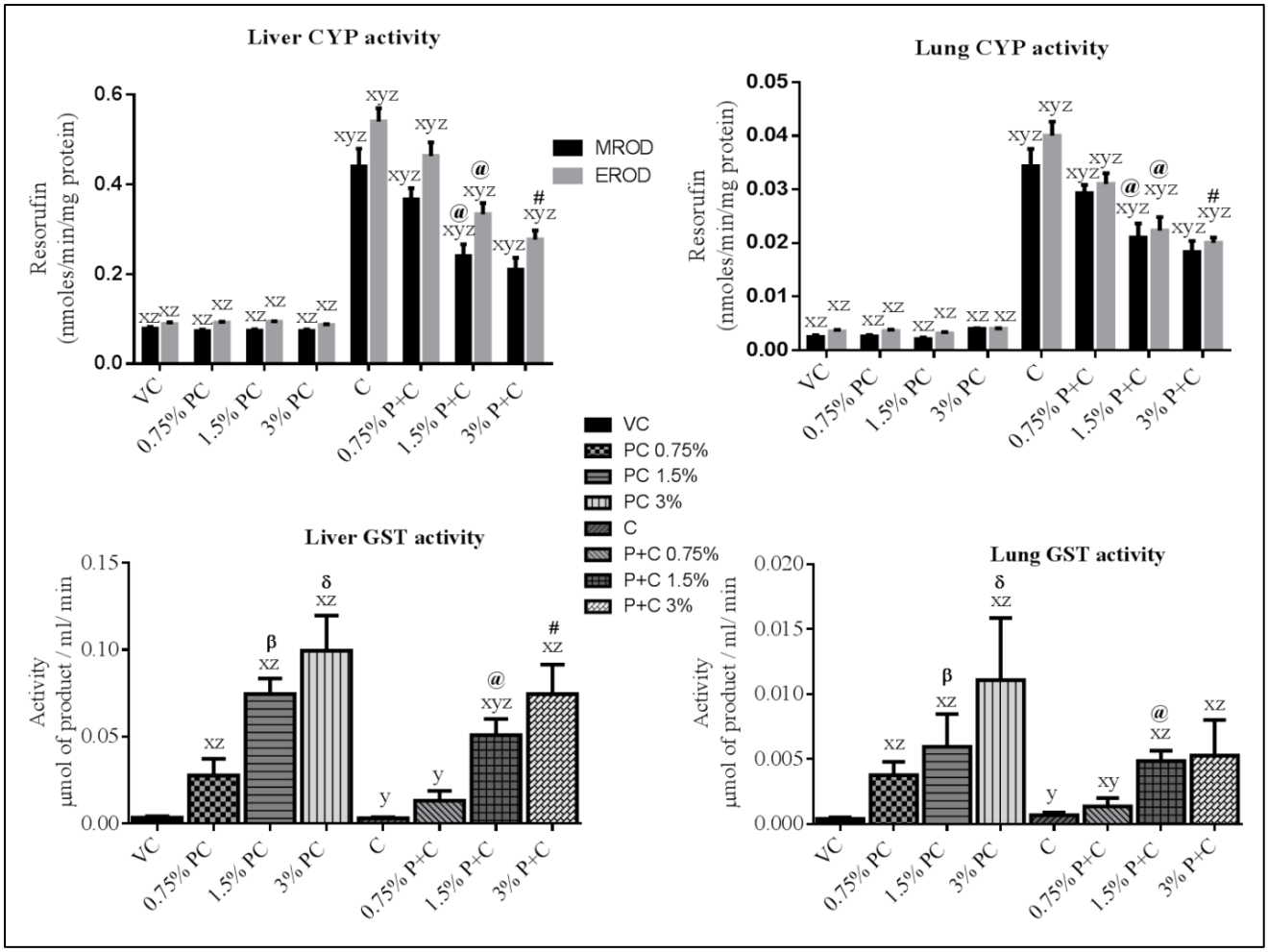


Figure 5.8 : Pretreatment of various doses of PBP rich extract modulates activity of CYP and GST isozymes in liver and lung

Isozyme-specific probe drugs were employed to measure enzyme activity of CYP1A1 and CYP1A2 in microsomes prepared from mouse in liver and lungs. Enzyme activity was expressed as nanomoles of resorufin formed per minute per milligram protein. Pan GST activity was measured in cytosolic fraction using 1-Chloro-2, 4-dinitrobenzene (CDNB) as a substrate from mice liver and lung. Enzyme activity was expressed as µmole of product formed per minute. Data represented as mean±SD of three observations. x= significantly different from VC, y= significantly different from its respective PC, z= significantly different from C, @= significantly different from 0.75% P+C, # = significantly different from 1.5%

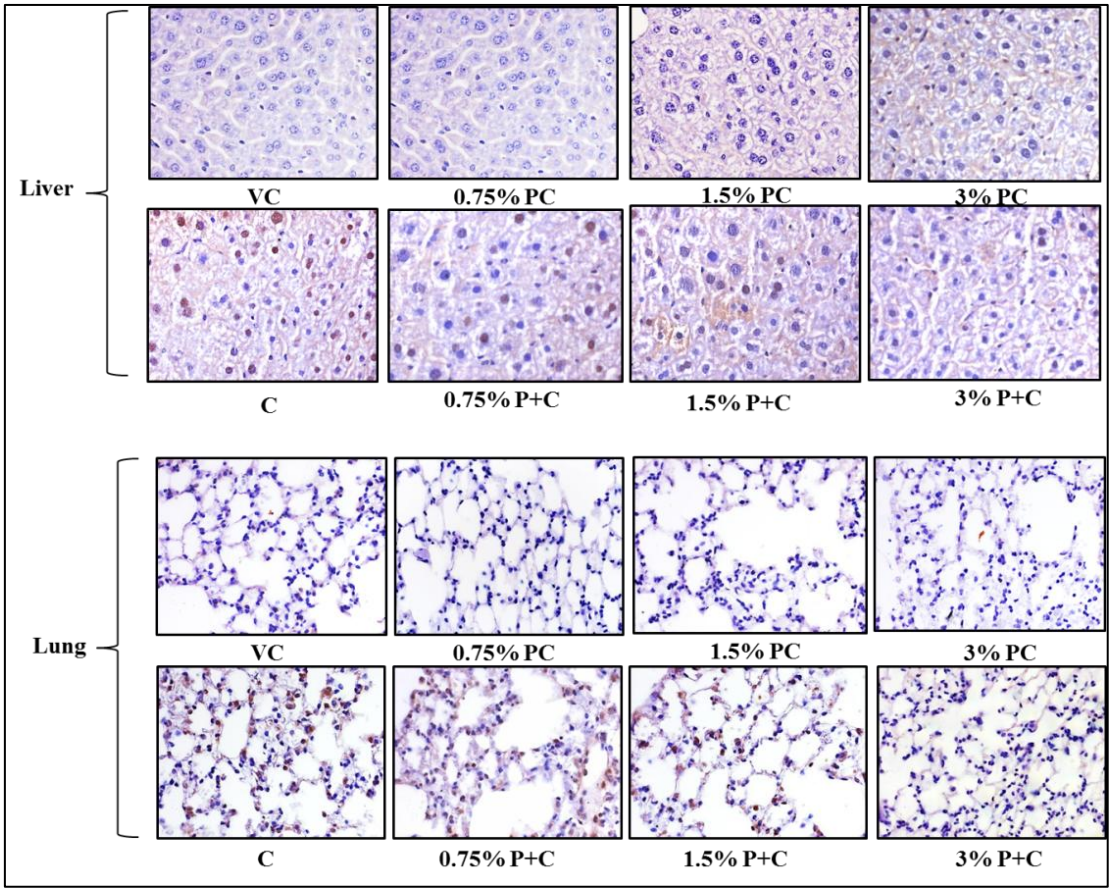
P+C, β= significantly different from 0.75% PC, δ= significantly different from 1.5% PC. ($p<0.05$, ANOVA followed by Bonneferoni's correction).

5.3.5 Pretreatment of various doses of PBP rich extract decreased number and intensity of BPDE-DNA adducts containing cells in both liver and lung:

In animals receiving glyceryl trioctanoate in vehicle and polyphenol control, BPDE-DNA adducts were not detected in liver and lung while detectable levels of adducts were observed by IHC staining following 24 hr. of B(a)P injection in carcinogen group **(Figure 5.9)**. It is important to note that, the % nuclei with BPDE-DNA adducts formed in liver was two times higher as compared to lung in carcinogen alone treated group. In liver and lung, number of % nuclei with BPDE-DNA adducts in 1.5%P+C and 3%P+C groups were found to be significantly lower as compared to respective carcinogen treated group **(Figure 5.9)**. Dose dependent decrease in number of BPDE-DNA adducts containing cells in P+C group was found to be significant in 1.5% P+C group as compared to previous dose in both lung and liver.

To analyze the B(a)P induced DNA adducts containing nuclei along with number of nuclei positive with BPDE-DNA adducts, the amount/number of DNA adducts formed per nucleus were analyzed using imaging based software. Carcinogen group showed highest total intensity including high and medium density cells denoting highest number of DNA adduct formed per nucleus in both liver as well as lung **(Figure 5.9)**. The observed decrease in total adduct intensity in both liver and lung tissues treated in the P+C group (0.75% P+C, 1.5% P+C, 3% P+C) with carcinogen treated group was predominantly attributed to reduction in both high and medium intensity stained nuclei. However it is interesting to note that percent low intensity stained nuclei containing cell remains unaltered across various doses of PBP

rich extract administration in P+C group in (0.75% P+C, 1.5% P+C, 3% P+C) both liver and lung. Together, results suggest that various doses of PBPs pretreatment led to decrease in number of cells containing BPDE-DNA adducts in both liver and lung.



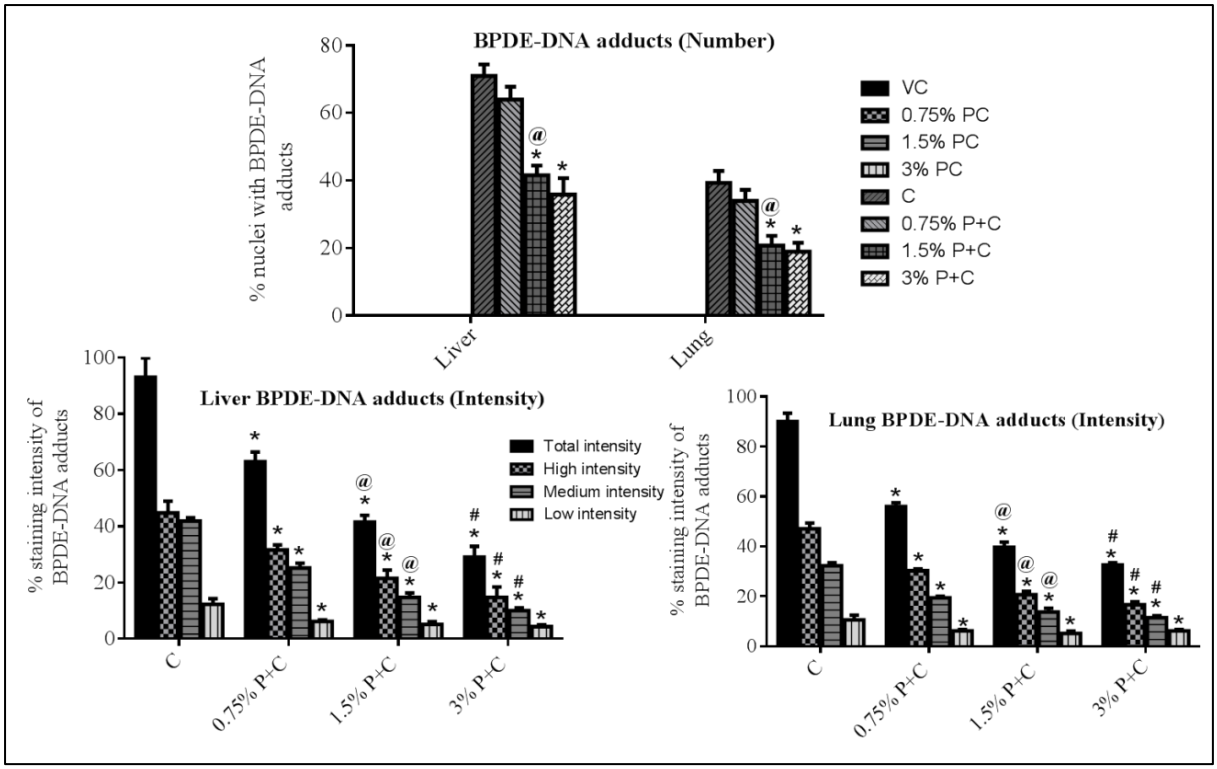


Figure 5.9 : Effect of pretreatment with various doses of PBP rich extract on levels of BPDE-DNA adducts in lung and liver in A/J mice.

Representative photomicrographs showing immunohistochemical detection of levels of BPDE-DNA adducts in paraffin embedded tissue sections of A/J mice liver and lung pretreated with various doses of PBP rich extract (magnification 400x). Percent positive nuclei with BPDE-DNA adducts were calculated by counting the cells with positively stained nuclei × 100/total number of cells in photomicrographs of lung sections of at least 10 different randomly selected fields (~ 1000 nuclei/ mice), with at least three mice per group. Percent staining intensity of BPDE-DNA adducts containing nuclei were calculated by digital image analysis as described under materials and methods. Data represented as mean±SD of three observations. *= significantly different from C, @= significantly different from 0.75% P+C, # = significantly different from 1.5% P+C. ($p<0.05$, ANOVA followed by Bonneferoni's correction).

Analysis of macroscopic lung tumor multiplicity:

5.3.6 Pretreatment of various doses of PBP rich extract decreased the pulmonary macroscopic tumor multiplicity in B(a)P and NNK lung carcinogenesis model:

Mice were sacrificed at 6th and 18th week after administration of last dose of carcinogen and macroscopic/visible lung tumors were counted. Both VC and any of the PC (1.5% PC and 3% PC) showed no tumors at both time points studied. Macroscopic tumors were observed in carcinogen as well as polyphenol+carcinogen treated mice at week 6. Hence latency period was found to be 6 weeks post carcinogen in both C and P+C group. At week 6, all the animals developed visible tumors in carcinogen treated group. However, only 2 out of 8 treated animals in 3% P+C group developed macroscopic tumors as that of all the animals developing tumors in 1.5% P+C group. Hence 3%P+C group showed decrease in average tumor incidence by 75% which was observed to be significant as compared to its carcinogen treated group **(Table 5.4)**.

However, all the animals developed visible pulmonary tumors at week 18 in 1.5%P+C and 3%P+C group as that of carcinogen treated group showing no decrease in tumor incidence. Average tumor multiplicity was found to be increasing in C group from 6th to 18th week post-carcinogen treatment. PBPs treatment resulted in significant decrease in average tumor multiplicity in both 1.5% and 3% P+C group as compared to its respective carcinogen treated animals at both 6th and 18th week post-carcinogen treatment **(Table 5.4)**. Dose dependent significant decrease was observed in tumor multiplicity in 3% P+C group as compared to 1.5%P+C group demonstrating the dose dependent chemopreventive potential of black tea derived PBP rich extract.

Group	Week	n	Lung tumors			
			Incidence (%)	p Value	Multiplicity	p Value
VC	6	14	0		0	
1.5% PC		13	0		0	
C		10	100	NS	1.00 ± 1.18	NS
1.5% P+C		10	100		0.64 ± 0.67	
VC		12	0		0	
3% PC		11	0		0	
C		8	100	0.007	2.00 ± 1.31	0.001
3% P+C		8	25@		0.25 ± 0.46*	
VC	18	8	0		0	
1.5% PC		9	0		0	
C		8	100	NS	25.60 ± 1.51	0.001
1.5% P+C		8	100		20.00 ± 3.02*	
VC		8	0		0	
3% PC		9	0		0	
C		7	100	NS	25.40 ± 2.23	0.001
3% P+C		7	100		12.29 ± 1.50*ε	

Table 5.4 : Effect on lung tumor multiplicity and incidence treated with various doses of PBP rich extract in B(a)P and NNK induced A/J mice model

The effect of various doses of PBPs at week 6 and week 18 post carcinogen treatment is indicated in table. Out of total carcinogen treated animals in each group (C and P+C), number of animals developing tumors was calculated and compared with carcinogen treated group ($^{@}$ $p<0.05$, Fisher's exact test). Surface tumors were counted in each sacrifice weeks and compared with carcinogen treated group (*$p<0.05$, Mann-Whitney Wilcoxson test). Surface tumors in 1.5% P+C group was compared with 3% P+C group ($^{\varepsilon}$ $p<0.05$, Mann-Whitney Wilcoxson test).

Analysis of microscopic lung lesions:

5.3.7 Pretreatment of various doses of PBP rich extract decreased the number of microscopic pulmonary lesions in B(a)P and NNK lung carcinogenesis model:

The microscopic lesions observed in lung tissues of mice were classified as hyperplastic foci, AAH and adenoma based on established criteria **(108)** and counting of each lesion was done with already published protocol **(127)**.

VC and both the PC groups (1.5 and 3% PC) showed no microscopic lung lesion while C and P+C groups (1.5 and 3% P+C) showed all three types of lesions. As contrast to macroscopic lung tumors, microscopic foci were observed at 6^{th} week post-carcinogen treatment in both C and 3% P+C groups in all the animals. At week 6, significant decrease in average number of hyperplasia was observed in 1.5 and 3% P+C group as compared to its C group. 3% P+C group showed statistical decrease in adenomatous lesions at week 6 than 1.5% P+C group as compared to its respective carcinogen group. Interestingly, decrease in the adenoma in 3%P+C group was significant as compared to 1.5%P+C group signifying dose dependent effect of PBPs **(Figure 5.10)**.

At week 18, significant reduction in average number of adenomatous lesions were observed in both 1.5%P+C and 3%P+C group as compared to its respective carcinogen group. To note, decrease in 3%P+C was observed to be significant as compared to decrease in 1.5% P+C group **(Figure 5.10)**. However, decrease in average number of hyperplasia and AAH couldn't attain statistical significance due to high intragroup variation and lower number foci respectively.

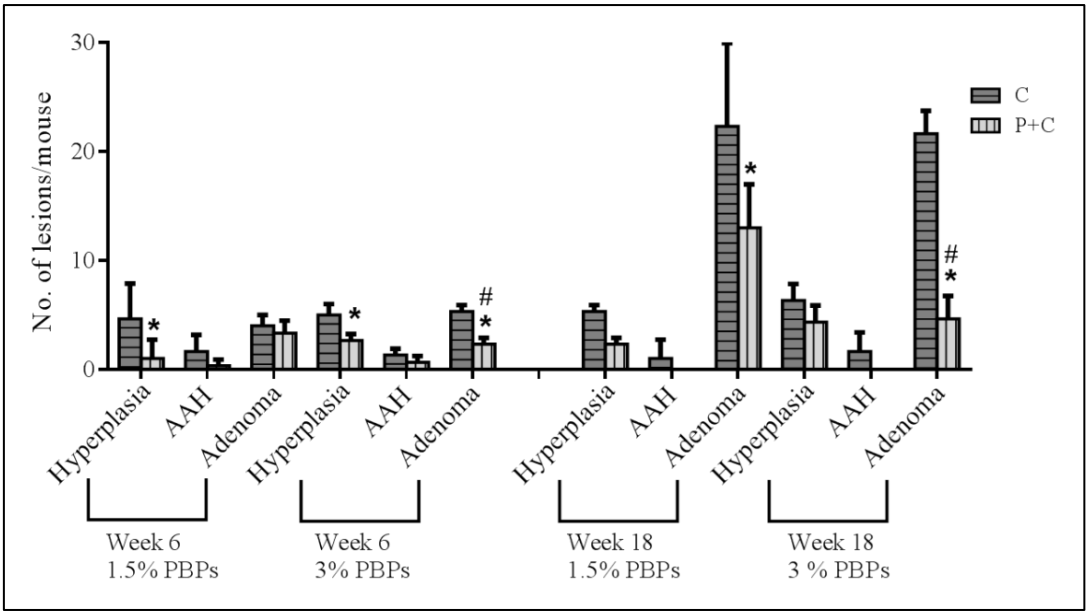


Figure 5.10 : Effect of various doses of PBP rich extract (1.5% and 3%) on multiplicity of microscopic lung lesions

Number of each proliferative lesion assigned by pathologist were counted in each step section (200 micron apart) and the total number of each type of lesion per mouse was expressed as an average number of each lesion per section (sum of each lesion in three step sections divided by three) in 3 independent animals in C and respective P+C group and plotted in a graph. *****significantly different from C, # = significantly different from 1.5% P+C. $p<0.05$, Poison regression.

5.3.8 Pretreatment of various doses of PBP rich extract reduced the area of microscopic pulmonary lesions in B(a)P and NNK lung carcinogenesis model:

At week 6, average area of hyperplasia and adenoma in 3% P+C group was significantly reduced as compared to its respective carcinogen treated mice **(Figure 5.11)**. However, in 1.5%P+C group decrease in these lesions couldn't attain the statistical significance due to high intragroup variation. Importantly, increased dose of PBP rich extract (3% P+C group)

decreased the area covered by adenomatous lesions significantly as compared to the area covered by adenomatous lesions in 1.5% P+C group. As contrast to week 6, at week 18 significant decrease in area of hyperplasia and adenoma was observed in both 1.5%P+C and 3% P+C group as compared to its respective carcinogen treated group **(Figure 5.11)**. Increased dose of PBPs significantly decreased the average area occupied by adenomatous lesions in 3% P+C group as compared to 1.5% P+C group. However, decrease in average area of hyperplasia and AAH was not in dose dependent manner.

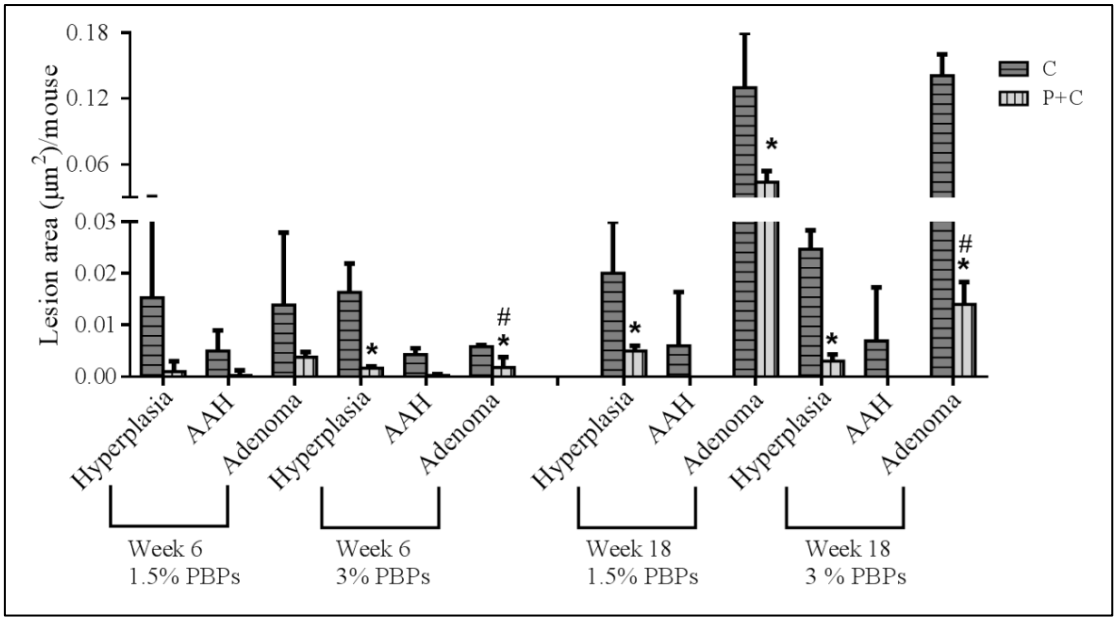


Figure 5.11 : Effect of various doses of PBP rich extract (1.5% and 3%) on area of microscopic lung lesions

The area of lesion was counted using *Image J* software in each step section and total area of each type of lesion per mouse was expressed as average area of each lesion per section in 3 independent animals in C and respective P+C group and plotted in a graph. *****significantly different from C, # = significantly different from 1.5% P+C. $p<0.05$, Data was subjected to log transformation and regression analysis.

5.3.9 Pretreatment of various doses of PBP rich extract decreased B(a)P and NNK induced cell proliferation while induced apoptosis in A/J mice model:

To assess the effect of various doses of PBP rich extract on B(a)P and NNK mediated cell proliferation and apoptosis in lung carcinogenesis, lung tissues were analyzed for the cell proliferation marker, proliferating cell nuclear antigen (PCNA) and TUNEL assay for apoptosis at 6th and 18th week post-carcinogen treatment. In proliferation and apoptotic index, vehicle and polyphenol controls showed no significant difference at any time point studied. Polyphenol treatment has reduced the carcinogen induced cell proliferation in P+C group at both the weeks in dose dependent manner. Dose dependent significant decrease in proliferation index in 3%P+C group showed significant decrease as compared to 1.5% P+C group **(Figure 5.12)**. As contrast to proliferation, the observed increase in apoptotic index was further increased by polyphenol treatment in P+C group at both the time points studied. Apoptotic index in P+C group was found to be significantly higher in dose dependent manner (3%P+C > 1.5%P+C) **(Figure 5.12)**. Hence PBPs mediated increase in apoptotic index and decrease in cell proliferation demonstrating the dose response effect of PBP rich extract.

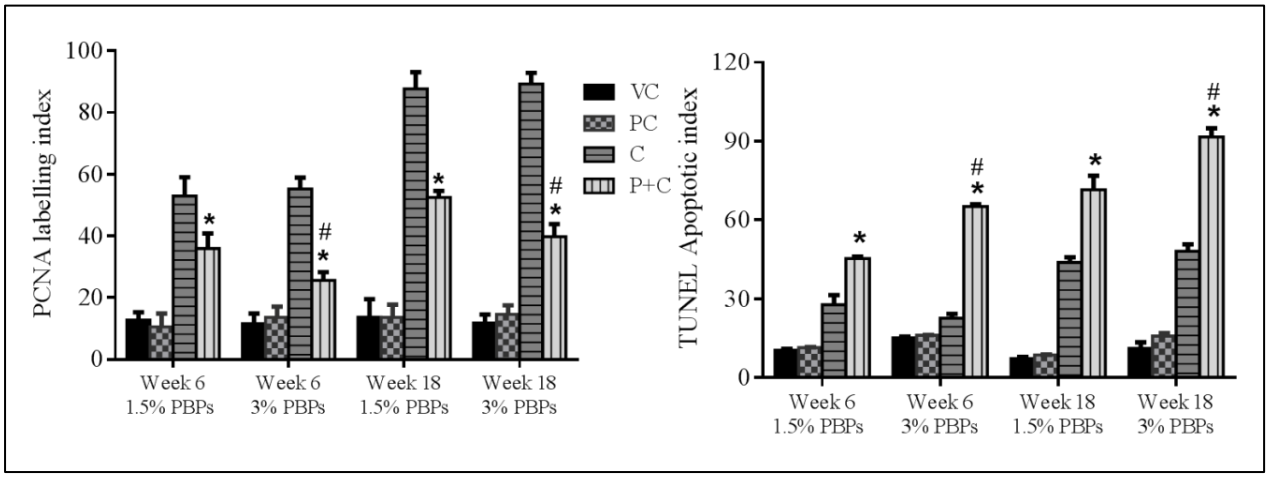


Figure 5.12 : Effect of various doses of PBP rich extract (1.5% and 3%) on cell proliferation and apoptosis in B(a)P and NNK induced A/J mice model

TUNEL apoptotic index and PCNA proliferation index was calculated as number of positively stained cells × 100/ total number of cells. Semi quantitative analysis was conducted by Image J 1.43 (NIH) software by counting the brown-stained nuclei in photomicrographs of at least 10 different randomly selected fields from lesion and non-lesion areas. Data represent Mean ± SD of three observations. *****significantly different from C, # = significantly different from 1.5% P+C. $p<0.05$, ANOVA followed by Bonneferoni's correction.

5.4 Discussion:

Chemopreventive efficacy of black tea polyphenols has been established in various experimental models **(109)**. Though few mechanistic studies have been carried out with PBPs *in vitro* **(35)** much remains to be elucidated *in vivo* to understand the dose response effect of PBPs for observed mechanism of chemoprevention. The major aim of our study was to delineate the dose related effects of 0.75%, 1.5% and 3% of black tea derived PBP rich extract, if any, using experimental lung carcinogenesis model. We studied the effect of administration of various doses of black tea derived PBP rich extract (0.75, 1.5 and 3%) on biochemical parameters and lung carcinogenicity in A/J mice model. Pretreatment with various doses of PBPs showed dose related decrease in B(a)P induced expression and activity of CYP1A1 in liver while CYP1A2 in lung. Dose dependent significant increase in PBPs mediated over expression and activity of GST isoforms (alpha in liver while pi in lung) were observed in polyphenol treated groups. Significant dose related decrease in number and intensity of BPDE-DNA adducts were observed in liver and lung. 1.5% and 3% black tea derived PBPs showed dose mediated decrease in lung tumor incidence and multiplicity which was further correlated with different molecular markers like cell proliferation (diminished

PCNA) and enhanced apoptosis (increased TUNEL apoptotic index) in B(a)P and NNK model.

Carcinogen metabolism plays a very crucial role in the process of initiation during cell transformation. Carcinogens entering into the cellular environment are metabolized by xenobiotic metabolizing [phase I (functionalization) and phase II (conjugation)] enzymes (XMEs), rendering them into more water soluble compounds **(35)**. Hence, these XMEs could be one of the probable targets for cancer chemoprevention. We demonstrated for the first time that PBPs (thearubigins), one of the major black tea polyphenols, when administered orally, modulate phase I and phase II enzyme expression and activity ultimately decreasing the carcinogen-adducts formation in liver and lung in dose dependent manner. Phase I enzymes predominantly cytochrome P450s (CYPs) are a diverse superfamily of haem-containing enzymes which process xenobiotic to more electrophilic moieties by addition of functional groups **(35,130)**. These electrophilic moieties will further react with cellular macromolecules like DNA and proteins to form adduct. Thus, decreased activation of carcinogens due to modulation of CYPs could be one of the main targets for prevention of the cancer initiation process. Our study has shown that administration of PBPs decreased the expression of CYP1A1 in liver while CYP1A2 in lung in dose dependent manner depicting differences in the metabolic competence of the tissues.

Aryl hydrocarbon receptor (AhR) is a ligand dependent transcription factor which binds to xenobiotic response element (XRE) to activate the transcription of a battery of xenobiotic metabolizing enzymes **(130,131)**.The present study showed that pre-treatment with various doses of PBPs attenuated the B(a)P-induced CYP1A *in vivo*. One of the possible reasons for the observed decrease in CYP1A may be B(a)P-induced new synthesis of AhR protein as well as nuclear translocation of AhR which in turn would be responsible for the observed PBP mediated decrease in AhR-DNA binding and subsequent CYP1A transactivation. However, in our study

0.75% fraction did not influence CYP1A expression, as against that observed with 1,5% and 3% PBP rich fraction. This could possibly be due to the lack of sensitivity of the assay system employed or due to the low circulating levels of parent or product achieved after administration of 0.75% PBP rich fraction. It may be important to mention that oral administration of PBPs mediated decrease in B(a)P-induced CYP1A1 and CYP1A2 could be either due to direct effect of PBPs on AhR protein itself, or due to the probable i*n vivo* interaction of PBPs with other AhR-associated proteins like hsp90, XAP2 and p23, or ARNT, the heterodimeric partner of AhR. However, the interaction of PBPs with other heterodimeric partners remains to be elucidated.

Dietary polyphenon-B (0.05%, mixture of different catechins, caffeine, oligomers and polymers of tea polyphenols) has been shown to decrease the dimethylbenz (a) anthracene (DMBA)-induced increase in the levels of total CYPs in buccal pouch and liver of hamsters **(132,133)**. Polyphenone-B has also showed to inhibit p-dimethylaminoazo-benzene (DAB)-induced levels of total CYPs, CYP 1A1 and activity of CYP1A1, 1A2 and 2B **(134)**. The findings in present study also illustrate significant inhibition of carcinogen-induced CYP1A1/1A2 protein expression and activity upon pretreatment with various doses of PBP rich extract though, basal levels of protein remained unaltered. These results suggest that PBPs mediate inhibition of carcinogen-induced CYP450s. It is noteworthy that although, PBPs have been observed to modulate expression of several B(a)P induced molecular and biochemical markers, their levels continued to be significantly different from animals administered with water or PBPs alone. Moreover throughout the evaluation, various doses of PBPs has been generally affecting the carcinogen-induced markers, without influencing their basal levels, suggesting that its effects on normal functioning or physiology are likely to be minimal. Therefore, toxicity and/or related problems may not be encountered. This is in agreement with the documented effects of oral administration of PBPs alone or PBPs followed by tumor promoter in mouse skin **(84)**.

During the course of xenobiotic metabolism, phase I enzymes predominantly CYP450 metabolize the xenobiotics to more reactive electrophilic moieties **(49)** which in turn are conjugation by phase II enzymes **(51)**. Therefore, enhancement in the activity of detoxifying enzymes by chemopreventive agents would play an important role in blocking the initiation process. Interestingly, data in the present study indicate PBPs mediated enhancement in the activity of phase II enzyme, GST, in mice as reported with BTE and DBTE **(135)**. The observed orally administered PBP mediated increase in GST isoform expression and activities in hepatic as well as extra hepatic tissue like lung were in agreement with the previous study carried out using black tea **(125,136)**. Further, mice pre-treated with various doses of PBPs and subsequently challenged with B(a)P showed enhanced activity of GST, its isoforms suggesting increased detoxification of B(a)P by PBPs induced conjugating enzymes *in vivo*. The bZip transcription factor Nrf2 plays a key role in regulating the gene expression of cytoprotective enzymes through ARE **(53)**. Additionally, enhanced nuclear translocation of Nrf2 and increased Nrf2-AREbinding by oral administration of PBPs were reported in lung and liver tissues **(105)**. The cause-effect relationship between Nrf2 and GST isoform induction however, cannot be established in the present *in vivo* experimental conditions and warrants further investigations with Nrf2 knockout mice.

The electrophilic intermediate formed as a result of metabolic activation of carcinogen interacts with cellular biomolecules. Since DNA-adduct formation marks the process of initiation; the observed PBPs mediated decrease in DNA-adducts in target/non-target tissues appears to play an important role in blocking the initiation process. However, the role of PBPs in dose dependent enhanced repair of DNA-adduct *in vivo* still remain unexplored and needs further investigations to add to the anti-initiating mechanism(s) of this polyphenol. Furthermore, pretreatment with various doses of PBPs decreased the B(a)P-induced DNA damage which could either attribute to the observed PBP mediated decrease in B(a)P-induced CYP450s or free radical scavenging activity of PBPs.

It is known that the types, number and genomic location of carcinogen DNA-adducts and cell turn-over in a tissue may be significant factors in tumor development and discrimination as target Vs. non-target organ. This differences were observed in the present study, in which the number of nuclei with DNA adducts in liver were found to be almost two times higher than that of lung. Liver being metabolic hub number of adducts formed are more but which is not target organ as tumors are observed only in lung as a result of tissue specific metabolism. However, consistent with our previous study reported **(127)**, no histopathological changes in the tissues were observed with prolonged exposure to B(a)P showing lack of hepatotoxicity. B(a)P being a contact carcinogen does not lead to hepatocarcinogenicity, however interestingly we have observed almost similar anti-initiating effects of PBP rich extract against B(a)P in target (lungs) as well as non-target tissue (liver, the hub of metabolizing enzymes).

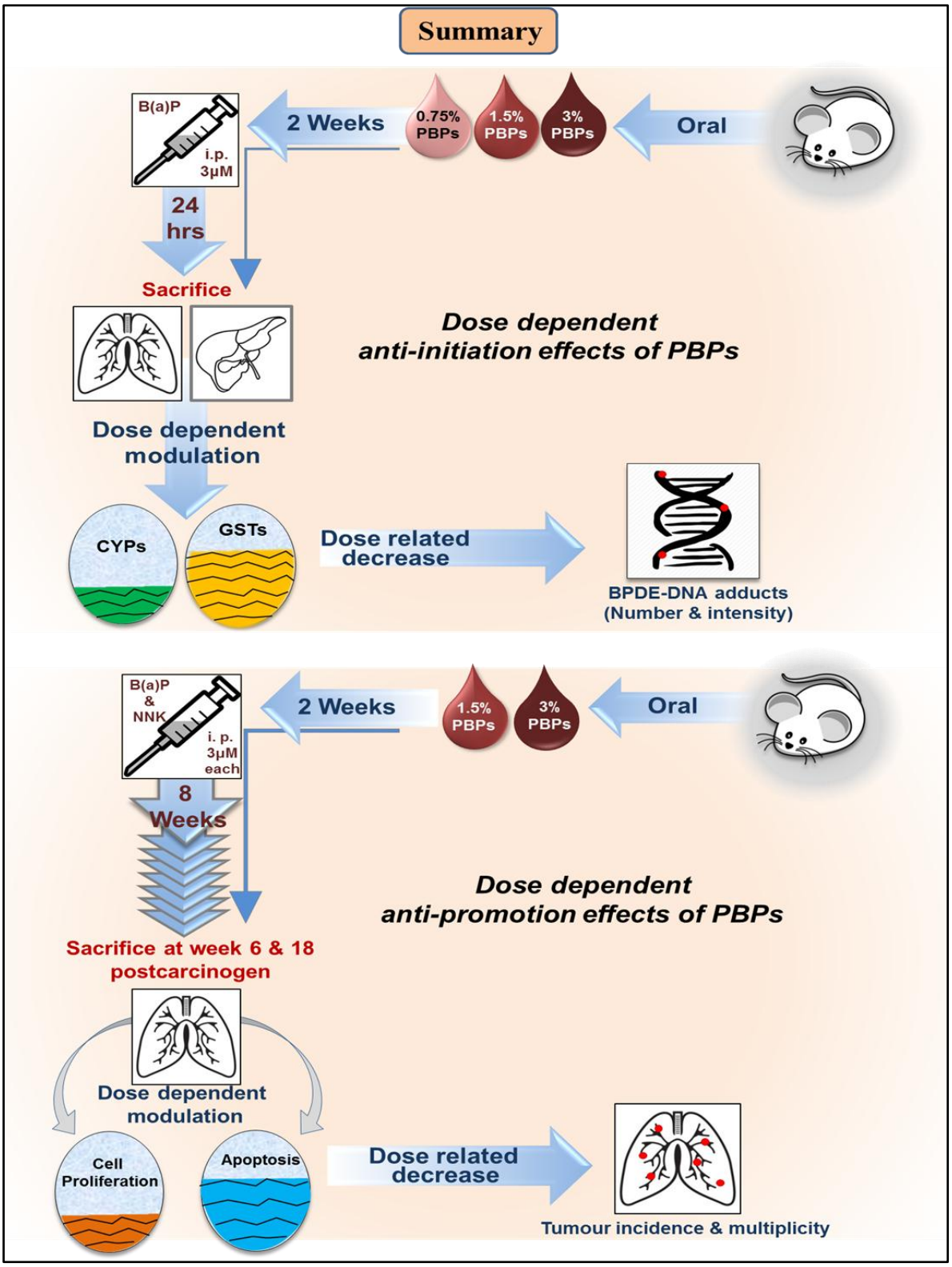
In the 28 week long term carcinogenicity experiment, no significant difference in initial and final body weight was suggestive evidence that increased dose (1.5% and 3%) of PBP rich extract exert chemopreventive effect without any toxicity. We further demonstrated administration of 1.5% and 3% PBP rich extract decreased macroscopic lung tumor incidence, multiplicity as well as number and area of microscopic lung lesions induced by B(a)P and NNK at 6 and 18 weeks post-carcinogen treatment in dose dependent manner. Chemopreventive efficacy of PBPs in our study was consistent with previously reported studies using different chemopreventive agents like phenethyl isothiocyanate, butylated hydroxyanisole, myo-inositol, resveratrol and lycopene which affect tumor multiplicity, incidence without affecting latency period **(36,137)**. Earlier reports on crude black tea extract has been shown to be affecting various pulmonary microscopic lesions like hyperplasia while, PPE and caffeine has been shown to reduce progression from lung adenoma to adenocarcinoma in experimental model **(79,110).** In our study, PBPs significantly decreased number and size of both hyperplasia and adenomas at all the time points studied in dose dependent manner indicating PBPs act on both early as well as late proliferative lesions in lung carcinogenesis process. However, observed decrease in AAH did not attain the statistical

significance due to low occurrence and small size of the foci. In lung carcinogenicity study, we administered 1.5% and 3% PBP rich extract two weeks prior, during and subsequent to carcinogen treatment. Hence the observed chemopreventive effects can be interpreted to be due to both anti-initiation as well as anti-promotion activity of PBP rich extract. Similarly observed chemopreventive effects can be attributed to parent and/or metabolic products of PBPs, hence observed bioactivity is likely to be due to PBPs and / or their products.

To explain the observed decrease in lung tumor multiplicity and understand the mechanism of chemoprevention by PBPs, we have also analyzed molecular markers of cell proliferation and apoptosis. It is noteworthy that decrease in proliferation index as well as increase in apoptotic index was observed to be dose dependent in both the time points studied. However it is important to note that despite of significant dose related decrease in carcinogen induced proliferation index by PBPs, it never matched with vehicle control. Thus PBPs decreased carcinogen induced cell proliferation and increased apoptosis which in turn is responsible for observed decrease in lung tumor multiplicity and lesion areas.

To the best of our knowledge, this is the first report demonstrating dose dependent anti-initiation and anti-promotion chemopreventive efficacy of PBPs in *in vivo* system. Using B(a)P induced A/J mice model, we deciphered the dose dependent anti-initiation mechanism of black tea derived PBP rich extract evident by inhibition in carcinogen induced expression and activity of CYP (CYP1A1 and 1A2) and PBPs induced GST isoforms (mu, pi and alpha) in both liver and lung. This modulation of XMEs further led to dose related decrease in number and intensity of B(a)P induced BPDE-DNA adducts containing nuclei. We further demonstrated chemopreventive efficacy of various doses of PBPs in well-established B(a)P and NNK induced lung carcinogenesis model . We observed the dose dependent decrease in macroscopic and microscopic lung tumor incidence and multiplicity which was apparently marked by reduction in

the cell proliferation and increase in the apoptosis. It is suggestive evidence that oral administration of various doses of black tea derived PBP rich extract exert its chemopreventive potential (anti-initiation and anti-promotion) without showing any remarkable effects like toxicity, bioabsorption and bioavailability.


Summary
B(a)P
i.p.
3µM
2 Weeks
0.75% PBPs
1.5% PBPs
3% PBPs
Oral
24 hrs
Sacrifice
Dose dependent anti-initiation effects of PBPs
Dose dependent modulation
CYPs
GSTs
Dose related decrease
BPDE-DNA adducts (Number & intensity)
B(a)P & NNK
i. p.
3µM each
2 Weeks
1.5% PBPs
3% PBPs
Oral
8 Weeks
Dose dependent anti-promotion effects of PBPs
Sacrifice at week 6 & 18 postcarcinogen
Dose dependent modulation
Cell Proliferation
Apoptosis
Dose related decrease
Tumour incidence & multiplicity

Chapter 6

To understand the effect of pre and post treatment of PBPs on early stages of experimental lung carcinogenesis

Chapter 6

To understand the effect of pre and post treatment of PBPs on early stages of experimental lung carcinogenesis

6.1 Introduction:

Cancer chemoprevention can be defined as the use of natural or synthetic compounds to prevent, suppress, delay, or reverse the development of invasive cancer **(34)**. Based on their mode of action, chemopreventive agents which impede the initiation stage either by inhibiting the formation of carcinogens or preventing carcinogen from reacting with other macromolecules are classified as blocking agents **(35)**. One effective strategy for chemoprevention is the blockade of DNA damage caused by carcinogenic insult. This can be achieved by reducing the formation of reactive carcinogenic species and stimulating their detoxification via modulating phase I and II enzymes **(55)**.

Anti-initiating and/ or anti-promoting activities of tea polyphenols have been well documented in experimental model systems at different organ sites when administered prior to, during or following exposure to a carcinogen **(35)**. Topical application of crude black tea extract has already showed to decrease UV-B induced skin tumor multiplicity when given prior and during the exposure. Decaffeinated black tea extract post carcinogen treatment has shown to decrease the lung tumor multiplicity in A/J mice model **(80)**. Studies on skin carcinogenesis have shown that pretreatment with PBP rich extract has decreased the expression of CYP1A1 and 1A2 in liver **(93)**. However, comprehensive analysis about anti-initiation mechanism of PBPs in carcinogen induced lung carcinogenesis model remains to be elucidated.

We conducted two independent experiments to dissect out the anti-initiation mechanism of black tea derived PBPs using B(a)P induced A/J mice model. In first experiment we pretreated the mice with 1.5% black tea derived PBPs/ water for 2 weeks and then administered the B(a)P/vehicle while in the second set of experiment we initially administered B(a)P and then shifted mice to 1.5% black tea derived PBPs/ water followed by sacrifice at following time points viz.24, 48 and 96 hr. Thus, administration of pre and post treatment of PBPs helped to dissect out its anti-initiation mechanism. The liver (nontarget) and lung (target) tissues were analyzed for various biomarkers: DNA adducts formation, inflammation, proliferation, apoptosis and analysis of DNA damage.

6.2 Methods:

6.2.1 Isolation and preparation of PBP rich extract:

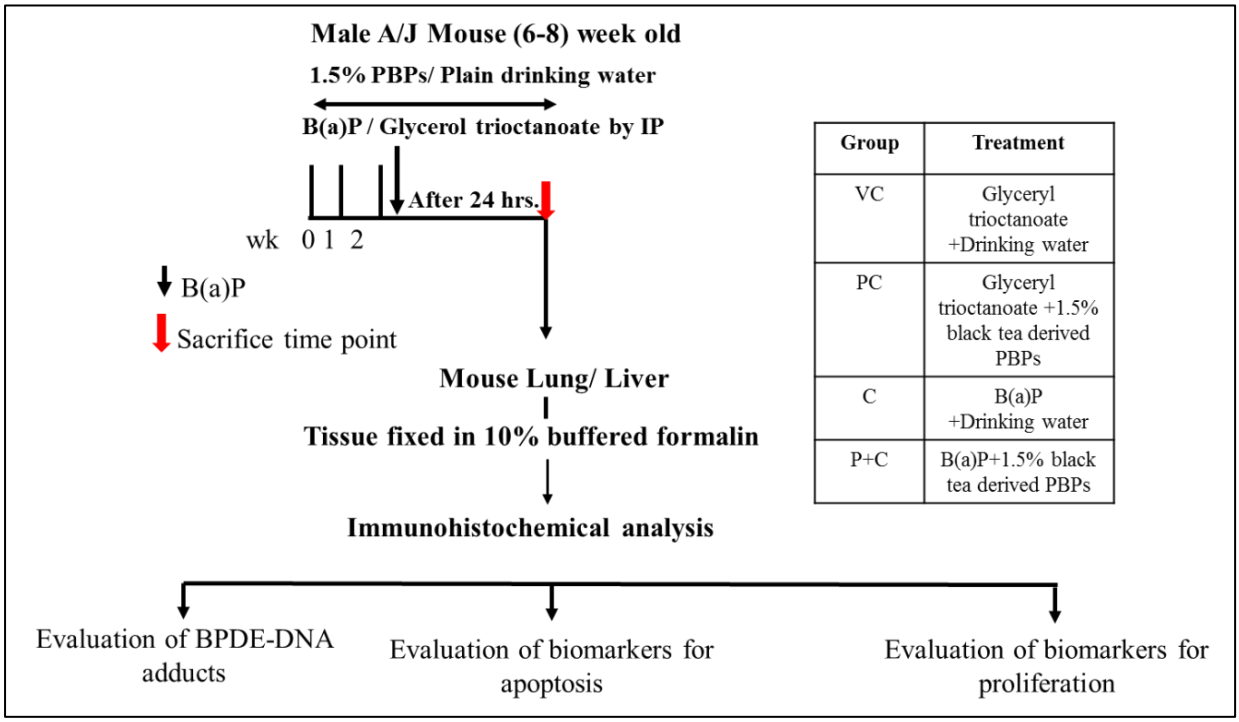
To study the mechanism(s) of anti-promoting activity of PBPs on B(a)P and NNK induced lung carcinogenesis in A/J mice, PBPs were isolated and 1.5% black tea derived PBP rich extract was prepared with well-established method **(91)** as described earlier in materials and methods (Chapter 3, section no.3.1, page no. 41-47).

6.2.2 Animal treatment

6.2.2.1 Animal protocol to evaluate the effect of pre-treatment with 1.5%black tea derived PBP rich extract on B(a)P induced A/J mice model:

Inbred male A/J (6–8 weeks old) obtained from Lab Animal Facility; ACTREC was randomized in to 4 groups. Polyphenol control (PC) and polyphenol+carcinogen (P+C) groups were primed for 2 weeks with PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water **(Figure 6.1)**. Animals in vehicle control (VC) and polyphenol control (PC) groups were injected glyceryl

trioctanoate (vehicle, i.p., 0.1ml) and continued receiving drinking water and PBPs respectively while carcinogen (C) and polyphenol+carcinogen (P+C) groups were injected B(a)P (i.p, 3µM, 0.1ml) and continued receiving drinking water and PBPs (*ad libitum*). Fresh bottle of PBPs were employed every alternate day. Post 24 hr vehicle/ B(a)P injection, animals were sacrificed by cervical dislocation. Lung and liver tissues were perfused with 0.15M KCl, excised and part of the tissue was fixed in 10% buffered formalin **(Figure 6.1)**.



Group	Treatment
VC	Glyceryl trioctanoate +Drinking water
PC	Glyceryl trioctanoate +1.5% black tea derived PBPs
C	B(a)P +Drinking water
P+C	B(a)P+1.5% black tea derived PBPs

Figure 6.1 : Experimental design for studying the effect of pretreatment with PBPs on B(a)P induced in A/J mice model

6-8 weeks old male A/J mice were randomized into four different groups such as vehicle control (VC), polyphenol control (PC), carcinogen (C), and polyphenol+carcinogen (P+C) as shown in the tabular format. Polyphenol control (PC) and polyphenol+carcinogen (P+C) groups were primed for 2 weeks with PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen(C) groups received normal drinking water. After 2 weeks of priming, B(a)P (3µM/0.1ml) in glyceryl trioctanoate or only glyceryl trioctanoate were

intraperitoneally injected once (shown by black arrow), while PBPs or drinking water were administered till the end of the experiment throughout the experimental period in male A/J mice. Treatment groups are as shown in the tabular format. Sacrifice was done after 24 hr. post carcinogen injection (shown by red arrow).

6.2.2.2 Animal protocol to evaluate the effect of post-treatment with 1.5% black tea derived PBP rich extract on B(a)P induced A/J mice model:

Inbred male A/J (6–8 weeks old) mice were obtained from Lab Animal Facility, ACTREC and were given single i.p. injection of glyceryl trioctanoate (vehicle, 0.1 ml) or B(a)P in glycerol trioctanoate (3 µmol in 0.1 ml). After 24 hr. of injections, mice were shifted to 1.5% PBPs / plain drinking water according to the group **(Figure 6.2)**. The groups were (i) Vehicle control (receiving Glyceryl trioctanoate+ shifted to drinking water), (ii) Polyphenol control (receiving Glyceryl trioctanoate+ shifted to 1.5% PBP rich extract) (iii) Carcinogen (receiving B(a)P+ shifted to drinking water) (iv) Polyphenol+carcinogen (receiving B(a)P+ shifted to 1.5% PBP rich extract). After receiving respective drinking conditions for 24 hr., animals were sacrificed at 24, 48 and 96 hr. post treatment **(Figure 6.2)**. Lung and liver tissues were perfused with 0.15M KCl, excised and part of the tissue was fixed in 10% buffered formalin. Rest of the tissues was snap frozen in liquid nitrogen and stored at -80^{0}C until preparation of the tissue extract.

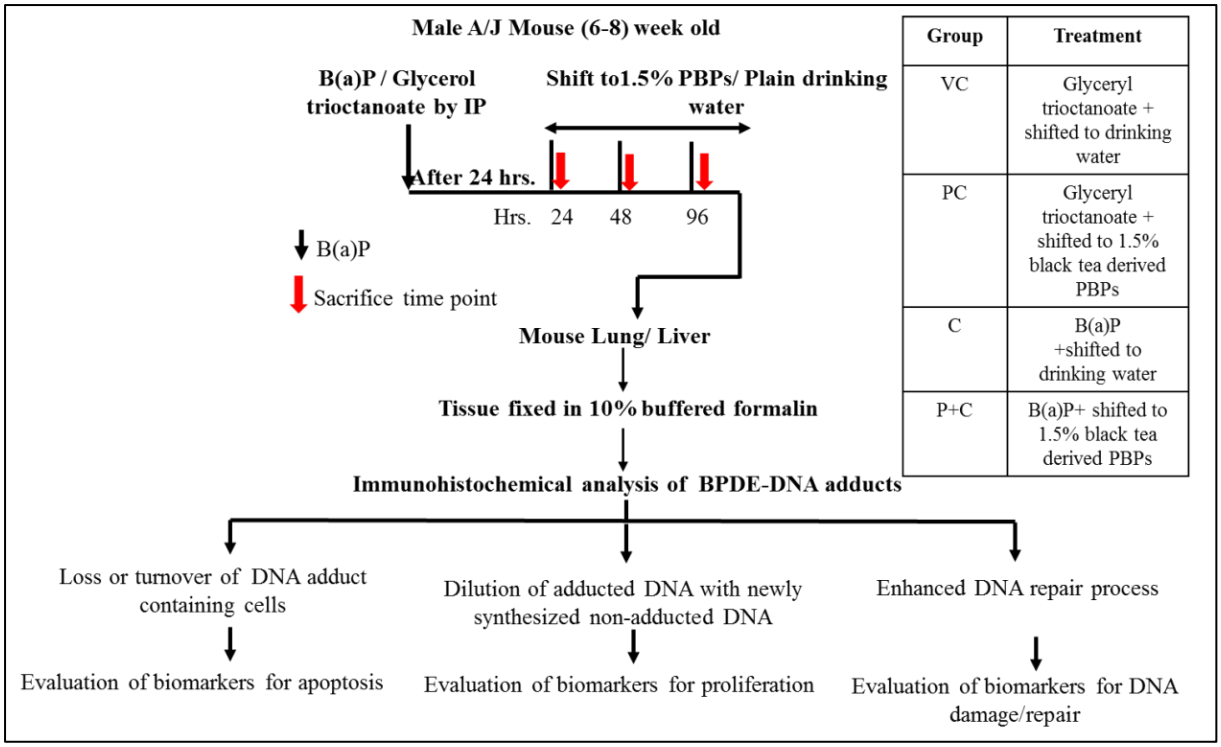


Figure 6.2 : Experimental design for studying the effect of post treatment with PBPs on B(a)P induced in A/J mice model

6-8 weeks old male A/J mice were obtained and were given single injection of B(a)P (3µM/0.1ml) in glyceryl trioctanoate or only glyceryl trioctanoate via intraperitoneal route (shown by black arrow). After 24 hr. post injection, animals were shifted to 1.5% PBPs or drinking water. Treatment groups are as shown in the tabular format. Animals were randomized into four different groups such as vehicle control (VC), polyphenol control (PC), carcinogen (C), and polyphenol+carcinogen (P+C) as shown in the tabular format. After receiving respective drinking water conditions for 24 hr., animals were sacrificed at 24, 48 and 96 hr. post treatment (shown by red arrow).

6.2.3 Analysis of bronchioalveolar lavage (BAL) fluid:

Mice were sacrificed and their lungs were lavaged three times with 0.15M KCl. The recovered BAL (BAL) fluids from animals of all the treatment groups were centrifuged and cell counts were obtained using grid hemocytometer. The Cell suspension was cytospinned and subjected to H7E staining. Number of different cells like macrophage, neutrophils, lymphocytes and lung epithelial cells was analyzed by counting at least 500 cells on slides.

6.2.4 Immunohistochemical staining and analysis:

For immunohistochemical (IHC) staining, all the lung lobes of treated animals were fixed in 10% buffered formalin. 5 µm thick section of paraffin embedded tissue was used for all the IHC staining. The expression of BPDE-DNA adducts and PCNA were assayed in pre as well as post treatment liver and lung tissues in all the treatment groups as described in materials and methods (Chapter 3, section no.3.6, page no. 57-61). For the evaluation of BPDE-DNA adducts and PCNA proliferation index, semi quantitative immunohistochemical detection system was employed in a paraffin embedded subsequent sections from the same portion of the tissue to achieve relevant and meaningful comparison. Detection was conducted using Vectastain ABC system kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was employed as the chromogenic substrate, and slides were counterstained with Mayer's haematoxylin. Images were captured with a Zeiss microscope (Imager Z1) to which an Axiocam MRc5 digital camera was attached. For negative or isotype controls, the primary antibody was replaced with PBS/ TBS or respective antibody concentration **(Table 6.1)**.

Evaluation of PCNA and BPDE-DNA adducts was carried out by semi-quantitative analysis using Image J 1.43 (NIH) software. PCNA labeling index and % nuclei with BPDE-DNA adducts was calculated by counting the number of positively stained cells × 100/ total number

of cells in photomicrographs of lung and liver sections. At least 10 different fields were randomly selected and/or minimum 1000 cells were counted at 400x magnification. Per group at least three mice were screened for the given assay.

Protein	Location of antigen	Primary antibody dilution	Secondary antibody dilution
PCNA	Nuclear	1:250	1:50
BPDE-DNA adducts	Nuclear	1:20	1:50

Table 6.1: Antibodies used for immunohistochemical staining

6.2.5 Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay:

Apoptosis was assayed in formalin-fixed, paraffin-embedded 5 μm tissue sections, according to the manufacturer's instructions (TUNEL assay kit, Promega, Madison, WI, USA), in pre as well as post PBP treated animal liver and lung tissues in all the treatment groups as described in Materials and methods (Chapter 3, section no.3.7, page no. 58,59). The nuclei of the apoptized cells were stained using DAB as a chromogenic substrate and slides were counterstained with Mayer's haematoxylin. Images were captured with Zeiss microscope (Imager Z1) to which an Axiocam MRc5 digital camera was attached. The apoptotic index was calculated by counting the cells with positively stained nuclei × 100/total number of cells in photomicrographs of lung sections of at least 10 different randomly selected fields (~ 1000 nuclei/ mice), with at least three mice per group.

6.2.6 Protein immunoblotting:

Total cell lysates of lung tissues from 24, 48 and 96 hr. post PBP treatment was prepared by a previously described cell fractionation procedure presented in materials and methods (Chapter 3, section no.3.3.1, page no. 47-49). For preparations of total cell extract, whole lung tissues from all the treatment groups were used. The expressions of biomarkers for DNA damage were also studied by immunoblotting to understand the interplay of biomarkers **(Table 6.2)**. β-actin was used as loading control. Densitometry of various analyte proteins and their respective loading controls from the same blot was performed using Image J 1.43 (NIH) software as described in Materials and methods (Chapter 3, section no.3.4, page no. 50-55).

Protein	% gel	Amount of protein	Primary antibody dilution	Secondary antibody dilution
p-ATR	10	TCL, 50µg	1:1000	1:4000
p-Chk1	15	TCL, 50µg	1:1000	1:4000
Actin	As per the protein	As per the protein	1:2000	1:4000

Table 6.2 : List of antibodies used for immunoblotting and its dilution

6.2.7 Statistical analysis:

Statistical analysis was performed using SPSS 21.0 software. Proliferation, apoptotic index was calculated in mice lung and liver tissues and represented as mean ± SD from three observations. Means were compared using ANOVA with Bonneferoni's correction. Mean of each analyte proteins were normalized with its respective loading controls in western blotting and data were compared with analysis of variance (ANOVA) with post-hoc testing. Data were represented as mean ± SD and compared using ANOVA with Bonneferoni's correction. $p<0.05$ was considered to be statistically significant.

6.3 Results

6.3.1 General observations and body weight:

There were no signs of toxicity and no mortality was observed in any of the groups. At the time of sacrifice, no gross changes in kidney, spleen, liver, stomach or intestine were seen at varying treatment periods.

6.3.2 Effect of pre-treatment with black tea derived PBP rich extract on B(a)P induced A/J mice model:

6.3.2.1 Pretreatment with 1.5% black tea derived PBP rich extract inhibited B(a)P induced inflammation:

The presence of inflammatory cells in the BAL was observed in all the groups. The number of lymphocytes, macrophages and lung epithelial cells was found to be predominantly present in the BAL fluid. Importantly, lymphocytic infiltration was found to be predominant in the carcinogen treated group **(Figure 6.3)**. Hence the decrease in the specific cell type i.e. lymphocytes, was observed in P+C group as compared to carcinogen control group. However, the inflammatory effect on neutrophils and macrophages was found to be marginal. To note, the decrease in the lymphocytic count in P+C group was comparable to the vehicle treated group **(Figure 6.3)**.

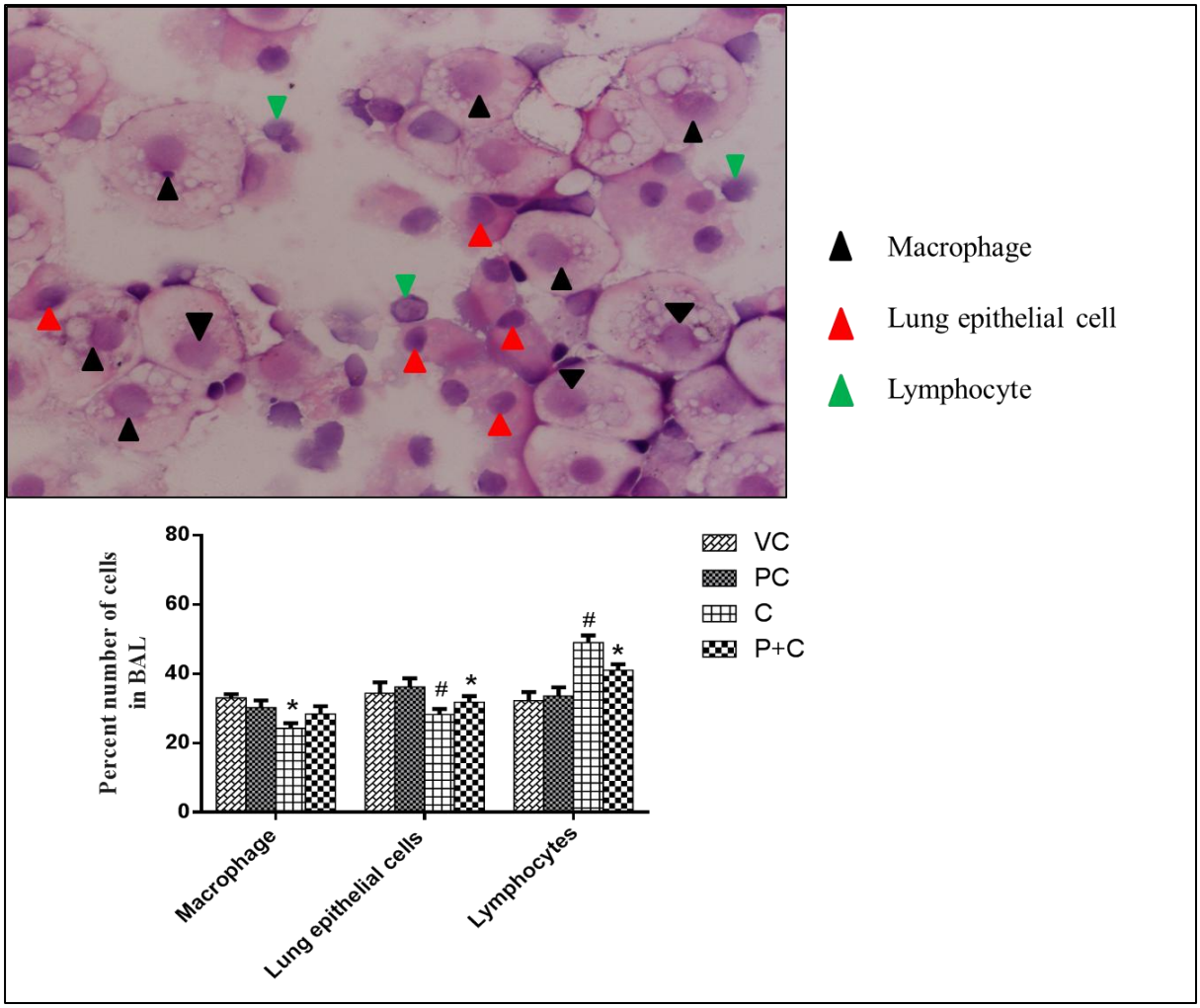


Figure 6.3 : Effect of pretreatment with 1.5% black tea derived PBP rich extract on B(a)P induced inflammation

Representative photomicrographs showing H&E staining of BAL fluid in A/J mice (magnification 400x). A/J mice were sacrificed and their lungs were lavaged three times with 0.15M KCl. The recovered BAL fluid from animals of all the treatment groups was centrifuged and cell counts were obtained using grid hemocytometer. The cell suspension was cytospinned and subjected to H&E staining (magnification at 400x). Number of different cells like macrophage, neutrophils, lymphocytes and lung epithelial cells was analyzed by counting at least 500 cells on slides with at least five mice per group. Data represented as

Mean±SD of five observations. # = significantly different from VC, ***** = significantly different from C. $p<0.05$, ANNOVA followed by Bonneferoni's correction.

6.3.2.2 Pretreatment with 1.5% black tea derived PBP rich extract inhibited formation of B(a)P induced BPDE-DNA adducts:

Animals in vehicle and polyphenol control, BPDE-DNA adducts were not detected in liver and lung while detectable levels of adducts were observed by IHC staining following 24 hr. of B(a)P injection in carcinogen group. It is important to note that, the % nuclei with BPDE-DNA adducts formed in liver was two times higher in liver as compared to lung in carcinogen control group **(Figure 6.4)**. Pretreatment with 1.5% black tea derived PBP rich extract significantly reduced the % nuclei with BPDE-DNA adducts in the P+C as compared to C group **(Figure 6.4)**. However, the reduction in the % positive nuclei in liver was found to be higher as compared to that in the lung.

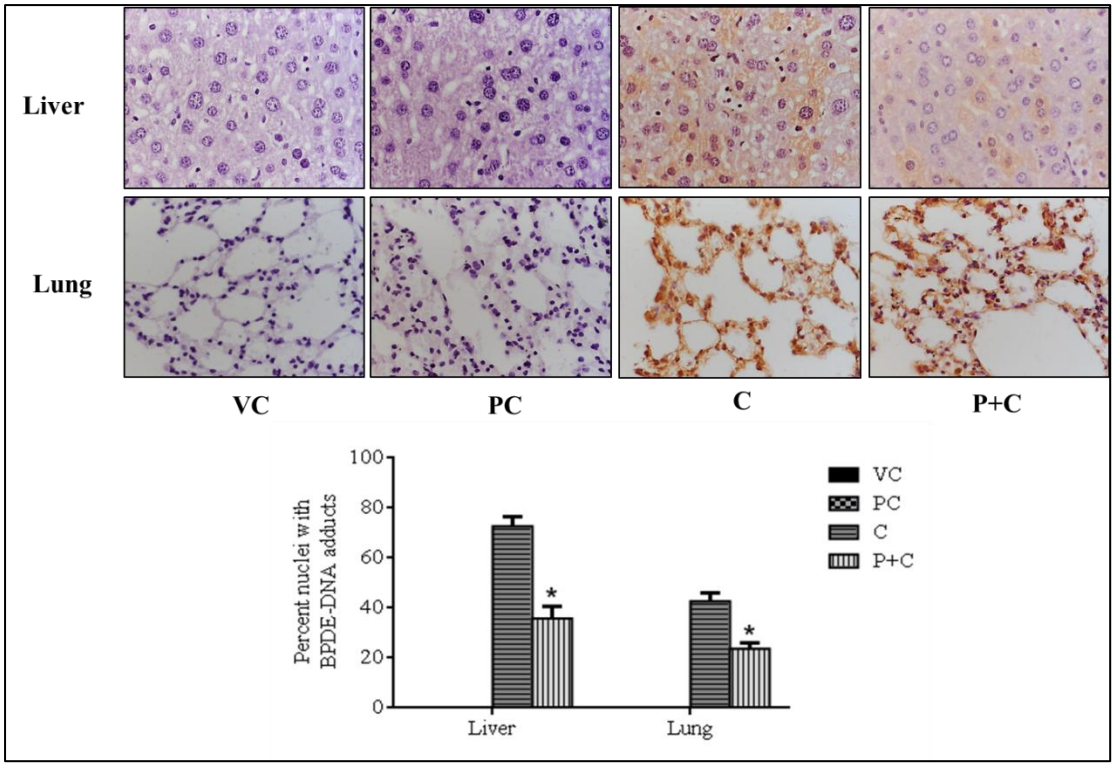


Figure 6.4 : Effect of pretreatment with 1.5% black tea derived PBP rich extract on B(a)P induced BPDE-DNA adducts in liver and lung

Representative photomicrographs showing immunohistochemical detection of BPDE-DNA adducts positive cells (magnification at 400x) using formalin fixed, paraffin embedded tissue sections. Percent nuclei with BPDE-DNA adducts was calculated by counting the number of positively stained cells from at least 10 different randomly selected fields (~ 1000 nuclei/ mice). Data represented as mean±SD of three observations.*significantly different from C ($p<0.05$, Student's T test)

6.3.2.3 Pretreatment with 1.5% black tea derived PBP rich extract inhibited B(a)P induced cell proliferation:

To assess the effect of pretreatment of PBP rich extract on B(a)P induced cell proliferation in liver and lung, tissues were analyzed for the cell proliferation marker, proliferating cell

nuclear antigen (PCNA). The expression in vehicle and polyphenol control group showed basal level of the proliferation marker and was comparable. Carcinogen treatment resulted in significant increase in levels of PCNA when compared to respective vehicle control. To note, the increase in proliferation was found to be higher in liver as compared to lung tissue. However, PBPs treatment resulted in significant decrease in PCNA levels in P+C group as compared to its respective carcinogen group **(Figure 6.5)**. Decrease in the proliferation index in liver was comparable to the decrease in the proliferation index in lung tissue. Hence PBP rich extract lower B(a)P induced cell proliferation showing anti-initiation effect.

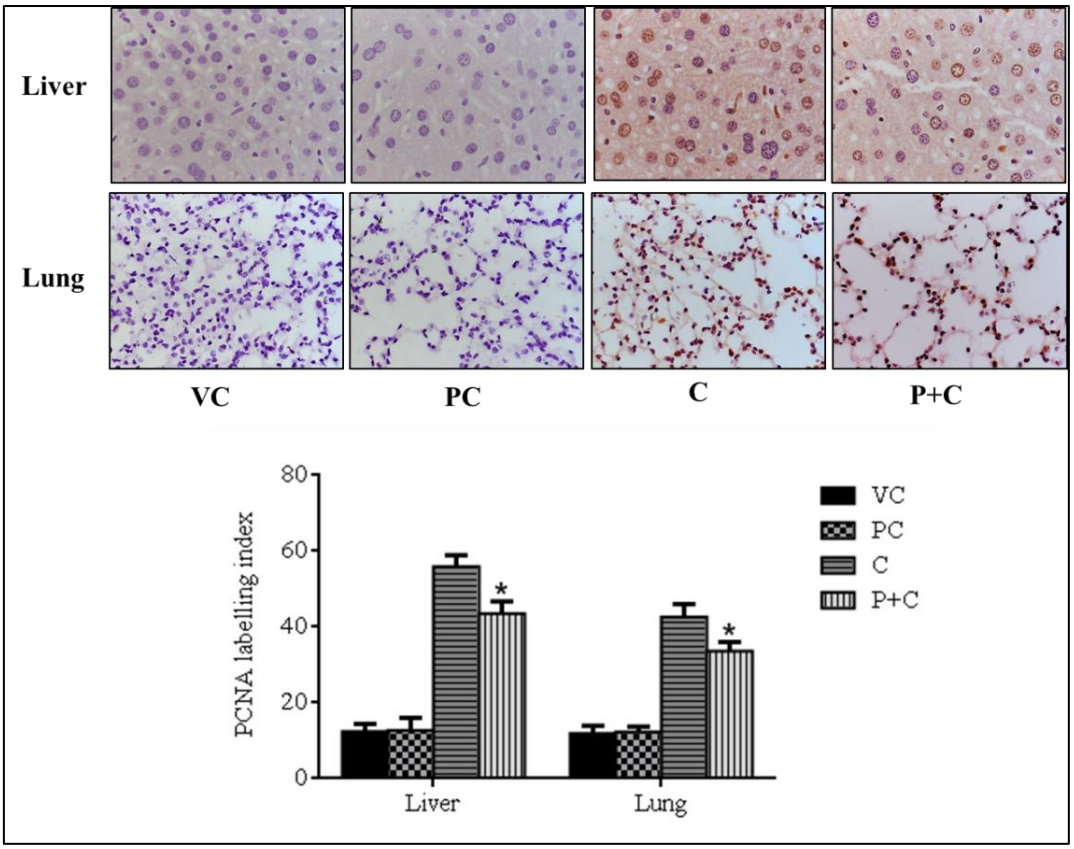


Figure 6.5 : Effect of pretreatment with 1.5% black tea derived PBP rich extract on B(a)P induced cell proliferation in liver and lung

Representative photomicrograph showing immunohistochemical detection of PCNA positive cells (magnification at 400x) at various sacrifice weeks in liver and lung tissue sections. PCNA index was calculated as number of positively stained cells × 100/ total number of cells. Analysis was conducted using Image J 1.43 (NIH) software by counting the positively stained nuclei in photomicrographs from at least 10 different randomly selected fields. Data represented as mean±SD of three observations. *, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

6.3.2.4 Pretreatment with 1.5% black tea derived PBP rich extract enhanced apoptosis:

To evaluate the effect of pretreatment of PBPs on apoptosis, lung and liver tissues were analyzed for TUNEL mediated apoptotic index. Apoptotic index was found to be unaltered in vehicle and polyphenol control groups. The level of apoptosis was found to be increased in carcinogen treated group as compared to its vehicle control group. The number of apoptotic nuclei in P+C group was observed to be significantly higher as compared to carcinogen control group depicting PBP rich extract increases carcinogen induced apoptosis **(Figure 6.6)**. Apoptotic index in P+C group in liver was found to be higher as compared to lung tissue.

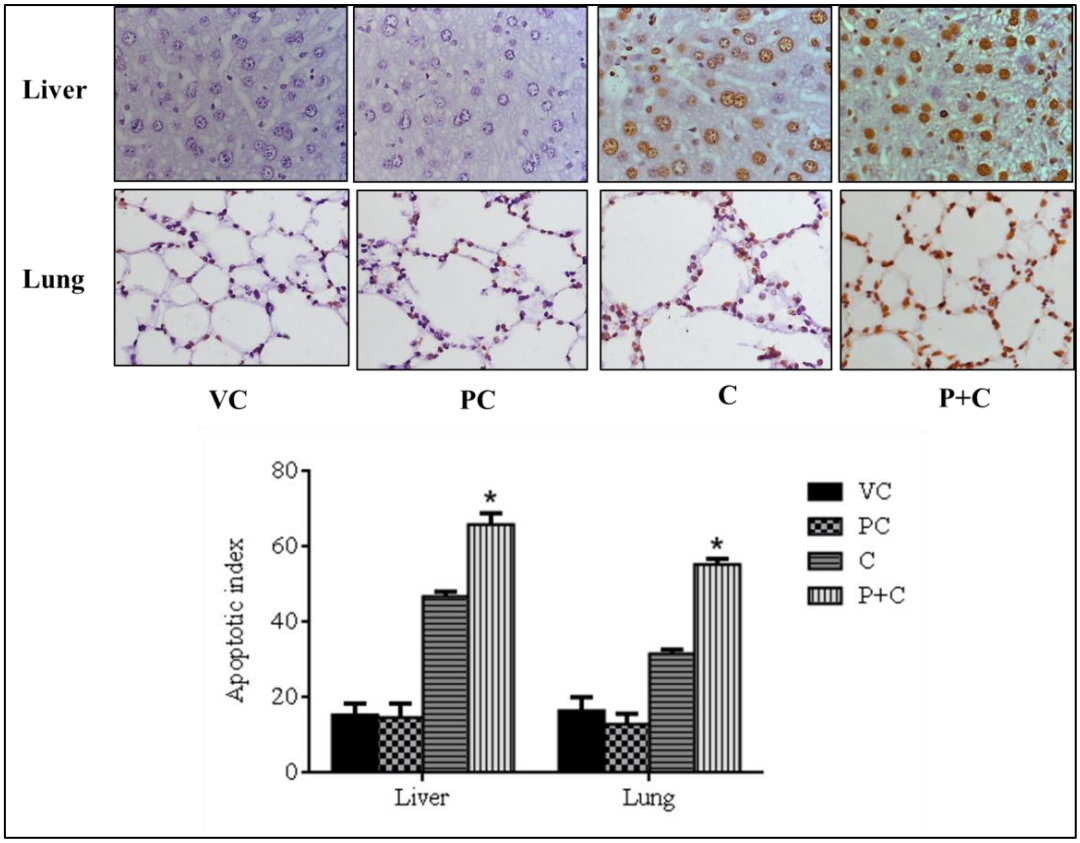


Figure 6.6 : Effect of pretreatment with 1.5% black tea derived PBP rich extract on apoptosis in liver and lung

Representative photomicrograph showing immunohistochemical detection of apoptotic cells using TUNEL assay (magnification at 400x) in liver and lung tissue sections in all the groups. Apoptotic index was calculated as number of positively stained cells × 100/ total number of cells. Analysis was conducted using Image J 1.43 (NIH) software by counting the positively stained nuclei in photomicrographs from at least 10 different randomly selected fields. Data represented as mean±SD of three observations. *, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

6.3.3 Effect of post-treatment with 1.5% black tea derived PBP rich extract on B(a)P induced A/J mice model:

6.3.3.1 Post treatment with 1.5% black tea derived PBP rich extract enhanced the disappearance of B(a)P induced BPDE-DNA adducts:

To understand the effect of PBP post treatment on nuclei with BPDE-DNA adducts in liver and lung, tissues were evaluated at 24, 48 and 96 hr. post PBP treatment. BPDE-DNA adducts were not detected in liver and lung of animals treated with vehicle and polyphenol control, while detectable levels of adducts were observed in carcinogen and polyphenol+carcinogen group. To note, number of BPDE-DNA adducts positive nuclei in liver was two times higher than that of lung in carcinogen group **(Figure 6.7)**. Post carcinogen treatment with polyphenol enhanced the disappearance of nuclei positive for BPDE-DNA adducts in P+C group in both liver and lung tissue ($P+C_{24\ hr.} < P+C_{48\ hr.} < P+C_{96\ hr.}$). Interestingly, time related disappearance of DNA adduct nuclei in liver being metabolically active tissue was observed to be higher than in the lung tissue. Hence, post treatment with PBP rich extract enhanced the disappearance of B(a)P induced BPDE-DNA adducts.

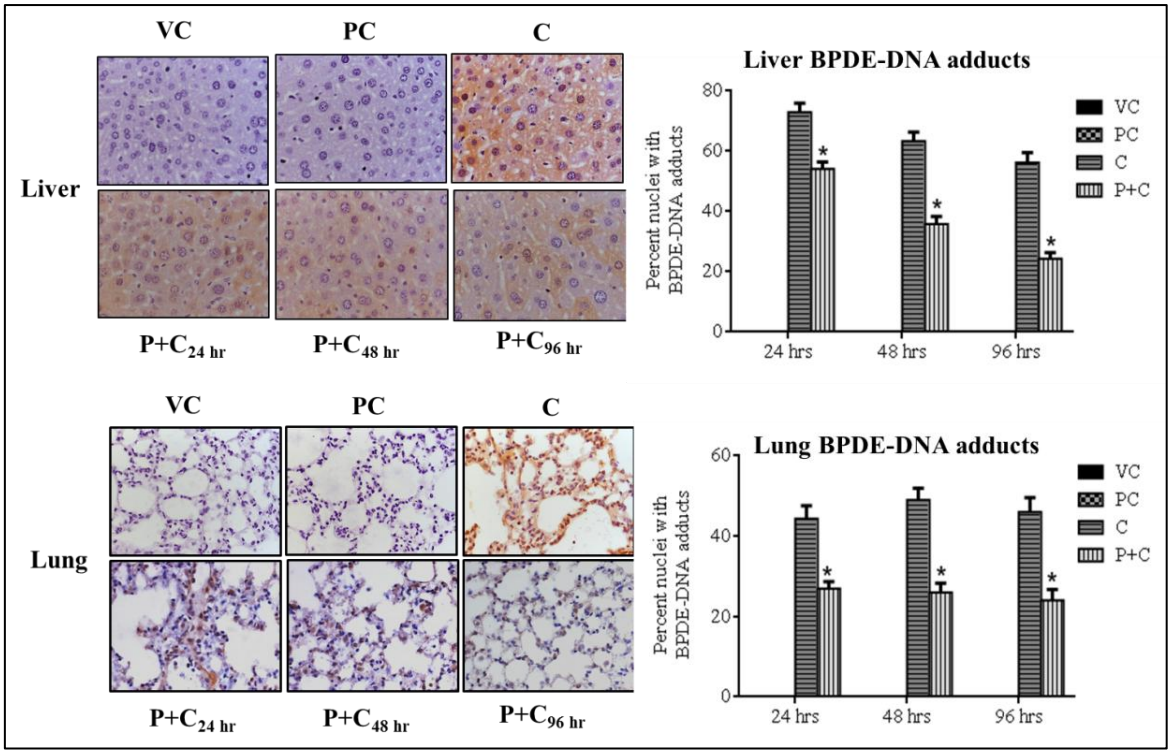


Figure 6.7 : Effect of post treatment with 1.5% black tea derived PBP rich extract on B(a)P induced BPDE-DNA adducts in liver and lung

Representative photomicrographs showing immunohistochemical detection of BPDE-DNA adducts positive cells (magnification at 400x) using formalin fixed, paraffin embedded tissue sections at 24,48 and 96 hr. post PBP treatment. Percent nuclei with BPDE-DNA adducts was calculated by counting the number of positively stained cells from at least 10 different randomly selected fields (~ 1000 nuclei/ mice). Data represented as mean±SD of three observations.*significantly different from C ($p<0.05$, Student's T test).

6.3.3.2 Post treatment with 1.5% black tea derived PBP rich extract decreased B(a)P induced inflammation:

To assess the effect of PBP post treatment on inflammation, lung lavage was analyzed for inflammatory cells in BAL. The appearance of lymphocytes, macrophages and lung epithelial

cells was found to be predominant in all the treated groups. The number of neutrophils in all the groups was found to be negligible. The number of inflammatory cells in vehicle and polyphenol controls was found to be comparable in all the time points. In carcinogen treated group, the number of lymphocytes was found to be significantly higher than its vehicle control group **(Figure 6.8)**. Interestingly, time related decrease in the lymphocytic infiltration was observed post 24, 48 and 96 hr. PBP treatment in carcinogen group. However, number of lymphocytes was found to be decreased in P+C group over the period of time (24hr>48hr>96hr). Time dependent differences were found to be lacking in number of macrophage and neutrophils.

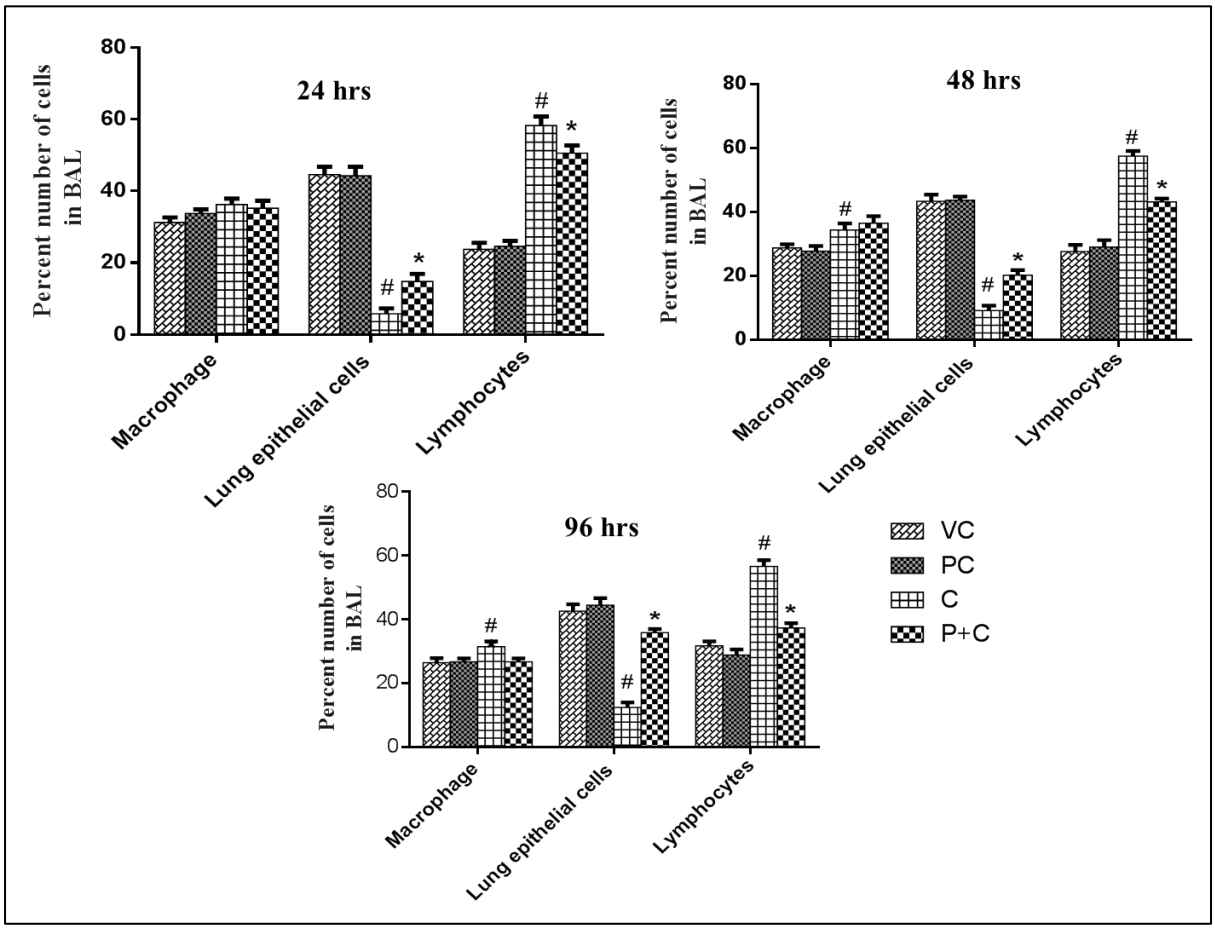


Figure 6.8 : Effect of post treatment with 1.5% black tea derived PBP rich extract on B(a)P induced inflammation

Representative photomicrographs showing H&E staining of BAL fluid in A/J mice (magnification 400x). A/J mice were sacrificed and their lungs were lavaged three times with 0.15M KCl. The recovered BAL fluid from animals of all the treatment groups was centrifuged and cell counts were obtained using grid hemocytometer. The cell suspension was cytospinned and subjected to H&E staining. Number of different cells like macrophage, neutrophils, lymphocytes and lung epithelial cells was analyzed by counting at least 500 cells on slides with at least five mice per group. Data represented as Mean±SD of five observations. # = significantly different from VC, ***** = significantly different from C. $p<0.05$, ANNOVA followed by Bonneferoni's correction.

The decrease in the number of nuclei positive for BPDE-DNA adducts in P+C group is possibly due to following possibilities:

a) Increase in the newly non-adducted DNA formed by cell proliferation in the course of replication leading dilution effect of adducted DNA.
b) Selective increase in the apoptosis of the cells containing adducted DNA.
c) Increase in DNA repair process leading to decreased DNA damage

Various markers for proliferation, apoptosis and DNA damage were studied to understand the mechanism(s) involved in the decreased DNA adducts containing cells.

6.3.3.3 Post treatment with 1.5% black tea derived PBP rich extract decreased B(a)P induced cell proliferation:

To assess the cell proliferation as one of the possibility to decrease the cells containing BPDE-DNA adducts nuclei, PCNA proliferation index was measured in each study groups. Vehicle and polyphenol controls showed basal levels of cell proliferation in both liver and lung tissue. Carcinogen administration has increased cell proliferation as compared to vehicle

control group at all the three time points studied. Time related increase in proliferation index was observed in carcinogen group in both liver and lung **(Figure 6.9)**. Carcinogen induced proliferation was significantly decreased by polyphenols in P+C group in liver and lung at all the time points. Interestingly, time related decrease in proliferation index in P+C group was observed in liver as contrast to lung tissue.

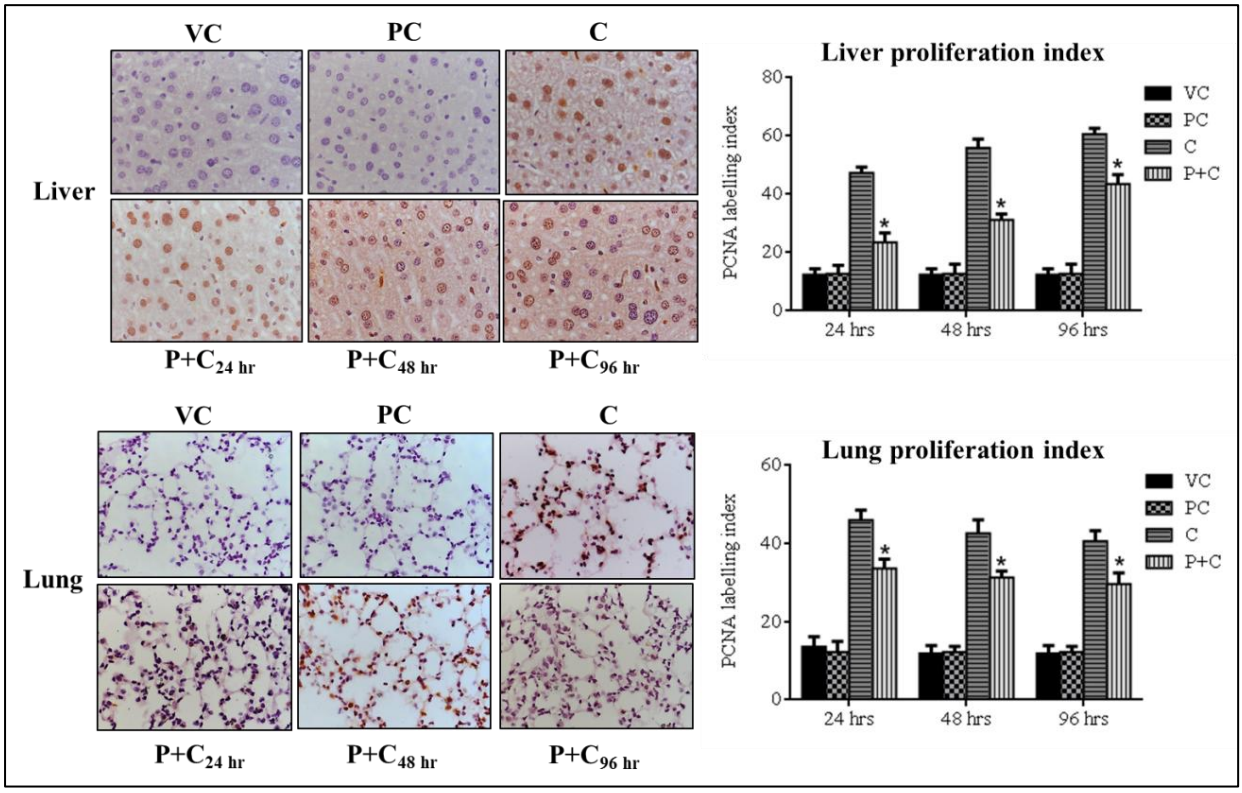


Figure 6.9 : Effect of post treatment with 1.5% black tea derived PBP rich extract on B(a)P induced cell proliferation in liver and lung

Representative photomicrograph showing immunohistochemical detection of apoptotic cells using TUNEL assay (magnification at 400x) in liver and lung tissue sections in all the groups studied at 24, 48 and 96 hr. post PBP treatment. Apoptotic index was calculated as number of positively stained cells × 100/ total number of cells. Analysis was conducted using Image J 1.43 (NIH) software by counting the positively stained nuclei in photomicrographs from at

least 10 different randomly selected fields. Data represented as mean±SD of three observations. *, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

6.3.3.4 Post treatment with 1.5% black tea derived PBP rich extract enhanced apoptosis:

Increase in cellular apoptosis can be one of the possible reasons for decrease in BPDE-DNA adducts, which was evaluated using TUNEL assay in both liver and lung. Apoptotic index was unaltered in vehicle and polyphenol control groups. In both the tissues, apoptotic index was found to be increased in carcinogen group as compared to its respective vehicle treated group. Polyphenol treatment in P+C group has further increased the apoptosis in both liver and lung. Time related polyphenol mediated increase in apoptosis was observed in both liver as well as lung **(Figure 6.10)**. Hence time dependent increase in apoptotic index was one of the reason for the observed decrease in BPDE-DNA adducts in both liver and lung.

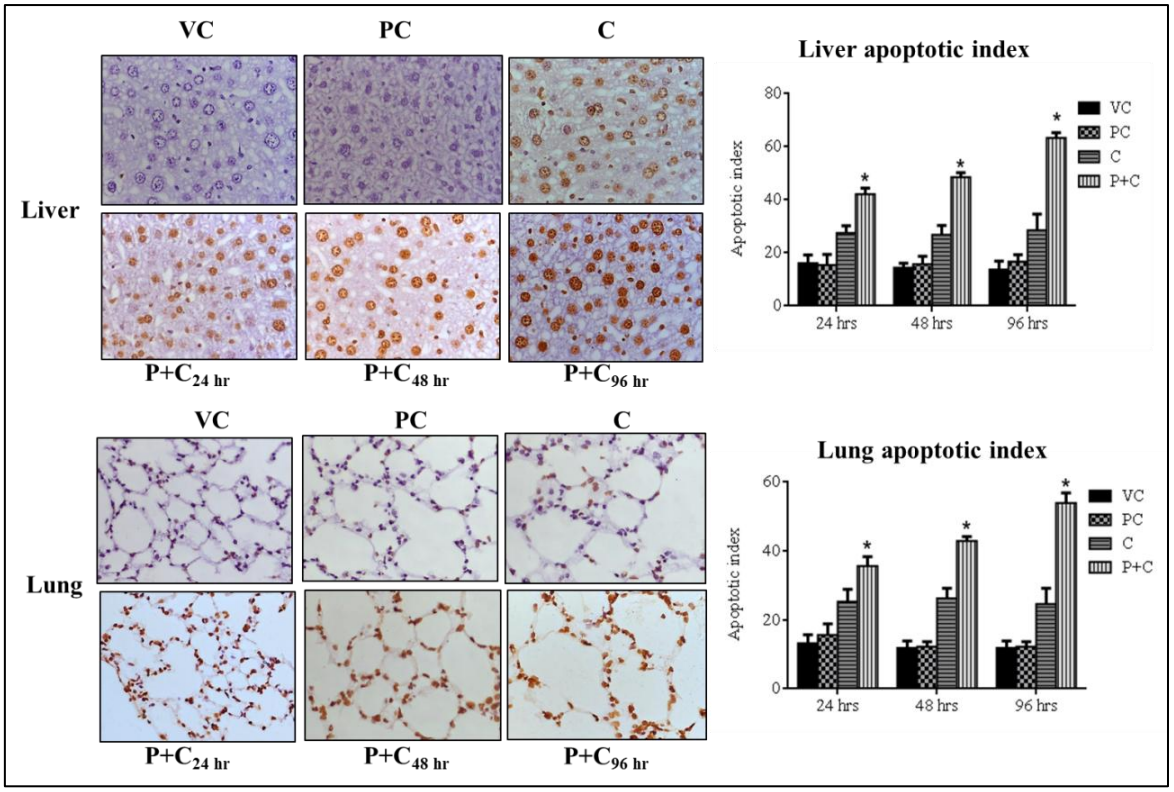


Figure 6.10 : Effect of pretreatment with 1.5% black tea derived PBP rich extract on apoptosis in liver and lung

Representative photomicrograph showing immunohistochemical detection of apoptotic cells using TUNEL assay (magnification at 400x) in liver and lung tissue sections in all the groups studied at 24, 48 and 96 hr. post PBP treatment. Apoptotic index was calculated as number of positively stained cells × 100/ total number of cells. Analysis was conducted using Image J 1.43 (NIH) software by counting the positively stained nuclei in photomicrographs from at least 10 different randomly selected fields. Data represented as mean±SD of three observations. *, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction)

6.3.3.5 Post treatment with 1.5% black tea derived PBP rich extract enhanced the DNA repair:

DNA damage proteins were analyzed to evaluate effect of PBP post treatment on DNA repair in the lung tissue. Phosphorylated forms of ATR and Chk1 protein were assessed. Expression of p-ATR and p-Chk1 was found to be unaltered in vehicle and polyphenol control groups. However, significant increase was observed in the phosphorylated forms of ATR and Chk-1 in carcinogen treated group from 24 hr. to 96 hr. treatment as compared to its respective vehicle control **(Figure 6.11)**. PBPs treatment resulted in decrease in the phosphorylated ATR and Chk1 when compared to only carcinogen treated groups from 24 hr. to 96 hr. In spite of PBPs-mediated decrease in phosphorylated ATR and Chk1, their levels were higher than those in vehicle controls. The observed differences in the phosphorylated forms of proteins involved in DNA damage suggest increase in DNA repair potential of PBPs.

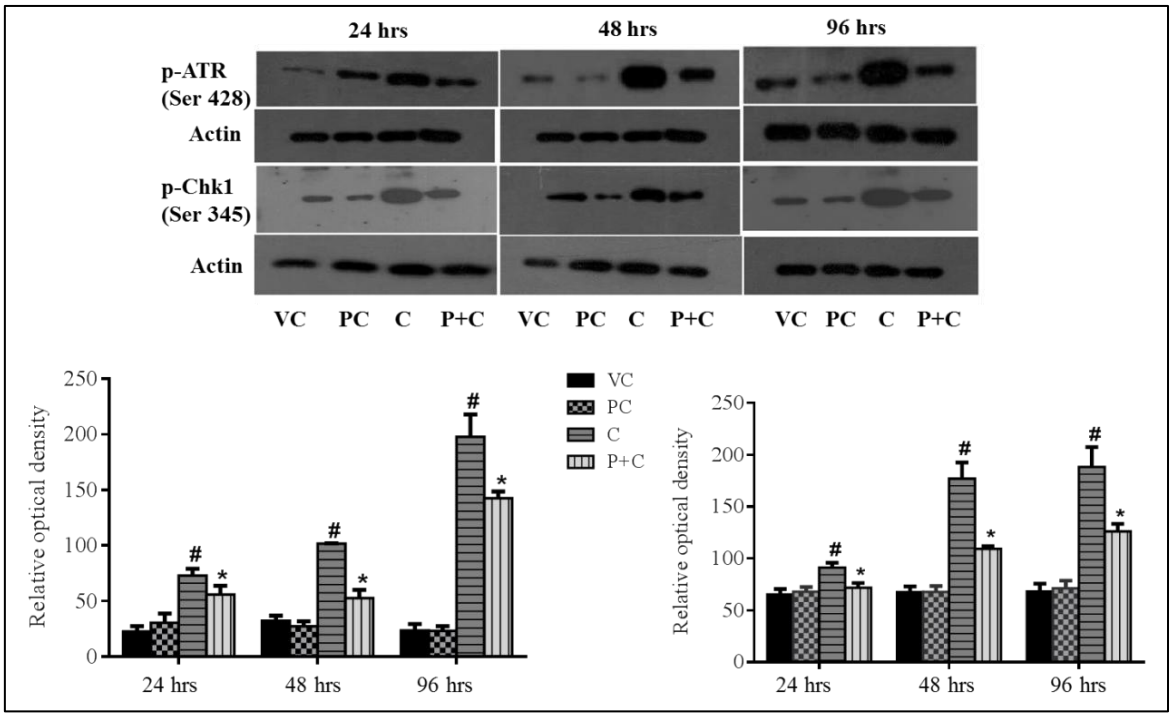


Figure 6.11 : Effect of pretreatment with 1.5% black tea derived PBP rich extract on DNA damage in lung

Representative blots showing the protein levels of phospho-ATR and phospho-Chk1 in lung total cell lysates. B-actin was used as loading control. Data represented as mean±SD of three observations. #, significantly different from VC;*, significantly different from C ($p<0.05$, ANOVA followed by Bonneferoni's correction).

6.4 Discussion:

Exposure to complex mixtures of polycyclic aromatic hydrocarbons (PAHs), which have been implicated in inducing skin, lungs and breast cancer in humans. PAH induced carcinogenesis involves a number of steps including; (i) the enzymatic activation of the PAH into metabolites; (ii) the covalent binding of the PAH metabolites to DNA; and (iii) the induction of mutations that initiate the transformation process. Levels of DNA adducts observed at any point in time reflect tissue-specific rates of adducts formation which in turn depend upon carcinogen metabolism, DNA repair, adduct instability, tissue turnover time etc. The diet derived polyphenols can delay the onset of disease like cancer by delaying these processes is receiving increasing attention **(33)**. Dietary polyphenols like curcumin pretreatment has been demonstrated to decrease the formation of BPDE-DNA adducts in tissues of mice/rats as a result of decrease in B(a)P-induced phase I enzymes and/or induction of phase II enzymes **(114,138)**. However, the modulatory effect of PBPs pre and post treatment on BPDE-DNA adducts in lung and liver tissues remains elusive.

In our study, no BPDE-DNA adducts were observed in any of the vehicle and polyphenols control tissues (liver and lung) showing the specificity of the assay system. Importantly, after single i.p. administration of B(a)P, the number of DNA adducts observed in carcinogen treated liver was almost two times higher than that of lung. However, according to our previous study, suggests 8 weeks i.p administration of B(a)P induces pulmonary tumors

(127). This observed difference in the adducts formation and tumorigenesis is mainly attributed to the metabolic competency of the tissue. Liver, being metabolic hub, the rate of clearance/ turnover of the adducted DNA was found to be higher than that of lung which resulted in to pulmonary carcinogenesis **(127,139)**. Krishnan et al reported that pretreatment with 1.5% DBTE and PBPs decreases formation of BPDE-DNA adducts in skin **(126)**. In haddition, our study has shown that oral administration of 1.5% black tea derived PBPs pretreatment decreased the formation while post-treatment mediates enhanced loss of BPDE-DNA adducts in liver and lungs of mice. To our knowledge, chemoprotective effects of PBPs in terms of formation as well as enhanced disappearance of DNA-adducts repair/loss has not been reported till date. Formation of DNA adducts in a crucial genes (such as proliferation, apoptosis, DNA repair etc.) may potentially cause mutational event, resulting in subsequent alterations in gene expression leading to cell transformation. PBPs mediated increased disappearance/loss of DNA adducts, eventually is likely to affect the multiplicity and/or latency period of tumor development.

We have demonstrated that pretreatment with 1.5% black tea derived PBPs for two weeks prior to carcinogen administration has shown to decrease the formation of DNA adducts in both liver and lung. The probable reasons for the observed decrease in DNA adducts could be: a) pretreatment with PBP mediated decrease in the expression of Phase I enzymes (observation in Chapter 2) which results in the formation of less number of reactive metabolites b) PBP mediated increase in expression of Phase II enzymes (observation in Chapter 2) which will enhance the conjugation of reactive metabolites c) PBP mediated decrease in proliferation index/ increase in apoptosis selectively eliminating the adducts containing cell. We have further observed that the enhanced apoptotic index was observed to be more predominant than decrease in proliferation index by two weeks pretreatment with 1.5% black tea derived PBPs. These observations suggest that enhanced metabolism of

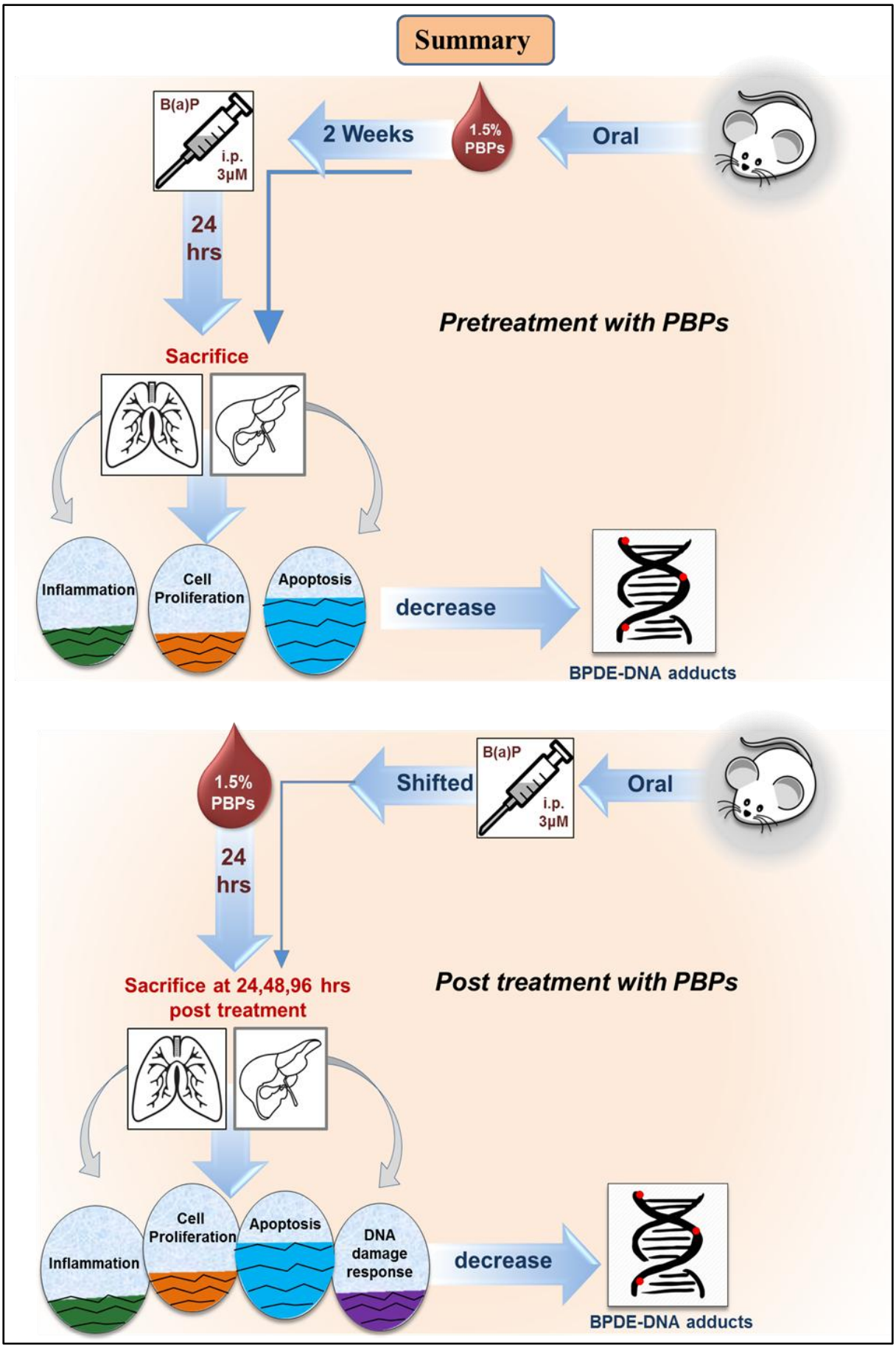
reactive intermediates along with increased apoptosis leading to loss of adduct containing cells is probably the major contributing factors for the observed decrease of BPDE-DNA adducts. We further observed that there is decrease in macrophage and lymphocyte count in BAL in P+C group suggestive of decreased inflammation after PBP treatment.

We have also demonstrated the time dependent loss of BPDE-DNA adducts upon administration of 1.5% black tea derived PBPs for 24, 48 and 96 hr. post carcinogen treatment. The probable reasons for the observed decrease in DNA adducts could be a) dilution of adducted DNA with newly synthesized DNA, b) loss or turnover of DNA adducts containing cells and/or c) increased repair of carcinogen-DNA adducts. To note, although PBP post treatment enhanced apoptosis and decreased cell proliferation, time dependent effect of PBPs on increasing apoptosis was found to be predominant. This was suggestive evidence that PBPs post treatment is effectively enhancing apoptosis of adducts containing cells. Black tea polyphenols induce apoptosis in cells that are either transformed and/or immortalized **(140-142)**. Earlier using same model system and under varying experimental conditions we have demonstrated that PBPs does not induce apoptosis in the normal cells **(127)**. We have reported for the first time that PBPs has apoptosis inducing ability in adducts bearing non transformed cells following exposure to DNA damaging agents *in vivo.* Further study highlights that PBPs post treatment decreases expression of active form of DNA damage markers like p-ATR and p-Chk1 depicting PBPs post treatment increases DNA repair in time dependent manner and potentially decreasing the loss of DNA adducts. Time related decrease in lymphocyte count in the post carcinogen PBP treated group further supports the PBP mediated decreased inflammation associated with DNA damage.

The observed decrease in BPDE-DNA adducts in pretreatment regimen is mainly attributed to PBP mediated enhanced adducts metabolism along with apoptosis. In PBPs post treatment regimen, loss of BPDE-DNA adducts may be attributed to reduced cell proliferation,

increased apoptosis of adducted cells and modulation of DNA repair. However, it is important to note that cell proliferation and apoptosis alone may not be sufficient to result in the extent of decrease which highlights potential contribution of DNA-repair.

In summary, our study helps to elucidate the anti-initiation mechanism of PBPs in liver and lung of B(a)P treated A/J mice model. Pretreatment with 1.5% PBP rich extract decreased the formation of B(a)P induced BPDE-DNA adducts in liver and lung tissue which was attributed to enhanced carcinogen metabolism, apoptosis while decreased cell proliferation and inflammation. While post treatment of PBPs showed time related loss of BPDE-DNA adducts containing cells which could be attributed to increased apoptosis of DNA adducts containing cells and enhanced DNA repair mechanism.


Summary
B(a)P
i.p.
3μM
2 Weeks
1.5%
PBPs
Oral
24
hrs
Pretreatment with PBPs
Sacrifice
Inflammation
Cell
Proliferation
Apoptosis
decrease
BPDE-DNA adducts
1.5%
PBPs
Shifted
B(a)P
i.p.
3μM
Oral
24
hrs
Sacrifice at 24,48,96 hrs
post treatment
Post treatment with PBPs
Inflammation
Cell
Proliferation
Apoptosis
DNA
damage
response
decrease
BPDE-DNA adducts

Chapter 7

Summary and conclusions

Chapter 7: Summary and conclusions

7.1 Summary of the work

Amongst number of dietary polyphenols, tea polyphenols have shown success as chemopreventive agents against variety of diseases including cancer. Monomeric tea polyphenols like epigallocatechin gallate (EGCG) and oligomeric polyphenols like theaflavins(TF) have shown promising chemopreventive effects in various pre-clinical studies and have now reached clinical trials for number of cancers **(36,103,143,144)**. Caffeine, one of the important modulator of various biochemical processes in the cells, has shown chemopreventive effects in various invivo systems. However knowledge about chemopreventive efficacy of most abundant polyphenols in black tea, known as polymeric black tea polyphenols (PBPs) or thearubigins (TR) is limited. Hence, aim of the present study was to investigate chemopreventive potential of this PBP fraction from black tea. These preclinical studies were carried out using A/J mice model and model carcinogens present in tobacco smoke to understand the following questions:

- To evaluate anti-initiation and anti-promotion mechanism of PBPs in B(a)P and NNK induced experimental lung tumors in A/J mice model
- To study chemopreventive efficacy of different doses of PBPs in experimental A/J mice model
- To decipher anti-initiation mechanism by pre and post treatment of PBPs in A/J mice model

Although several clinical trials have shown efficacy in cancer prevention, few have resulted in changes to medical practice. Some trials failed to show any benefit and some even failed miserably. These failures have highlighted the need to understand the mechanistic aspects of

a putative chemopreventive agent, before it is implemented in clinical trials. It should also be noted that exposure to various chemopreventive agents occur over time, potentially with different mechanisms on various target organs. Hence, interventions need to be tailored to the processes that occur at individual target organs over time. Also, there is need to develop models of clinical trials that can efficiently identify promising agents for cancer prevention in different target organs. This requires the identification of biomarkers that reflect clinical benefit, and eventually the validation of these markers if they are to be used as surrogates.

- **Chapter 1**

Chemoprevention with dietary phytochemicals is an evolving and promising approach for management of lung cancer. Various black tea polyphenols including EGCG, TF have showed promising chemopreventive potential against cancers including lung cancer. However the chemopreventive efficacy of polymeric black tea polyphenols (PBPs), one of the abundant polyphenols in black tea, in lung carcinogenesis remains to be investigated. In the present study the anti-promotion potential of PBPs on the process of sequential lung carcinogenesis measured as tumor incidence, multiplicity and latency period using a well-established B(a)P and NNK induced lung carcinogenesis model in A/J mice. We further studied the induction of phase I and phase II enzymes and its modulation by PBPs using same model system.

We evaluated the chemopreventive efficacy of PBPs to inhibit benzo(a)pyrene B(a)P and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (i.p., 3μM/0.1ml each) induced lung adenoma in A/J mice. Administration of 1.5% black tea derived PBPs throughout the experimental period significantly decreased the multiplicity of surface tumors as well as number and area of microscopic lung lesions like hyperplasia and adenoma. Although, tumor incidence and latency period remains unaffected, sequential histopathological evaluation of

lungs at 4, 6, 10 and 18 weeks post-carcinogen treatment period showed decrease in tumor multiplicity which was also correlated with different molecular markers. Anti-inflammatory action of PBPs was demonstrated by reduced Cox-2 expression using western blotting and immunohistochemical analysis. PBPs further down-regulated the B(a)P and NNK induced cell proliferation (diminished PCNA expression and decreased PCNA labeling index in lesion compared to nonlesion area, Bcl-2 expression). It further enhanced apoptosis (increased Bax expression and TUNEL apoptotic index) potentially through phosphorylation of p38 and Akt. However, non-phosphorylated forms are unaltered. The study further highlights the anti-initiation potential of PBPs by inhibition of CYP isoforms (CYP1A1 and1A2) while induction of GST isoforms (GST mu, alpha and pi) decreasing the B(a)P induced BPDE-DNA adducts in both liver and lung tissue at week 1 and 8 of carcinogen treatment. In conclusion, the protective effects of PBPs could be attributed to modulation of XMEs, inhibition of cellular proliferation and induction of apoptosis. Some of these biomarkers are likely to be helpful in monitoring clinical chemoprevention trials.

- **Chapter 2**

The mechanisms of action of PBPs, one of the most abundant polyphenols in black tea, have been already studied in various carcinogenesis model system including skin, colorectal and lung. However, chemopreventive efficacy at different doses of PBPs remains to be elucidated which we investigated in mice employing benzo(a)pyrene B(a)P as a model carcinogen. Initially, we studied the effect of oral administration of various doses of black tea derived PBP rich extract (0.75, 1.5 and 3%) on biochemical parameters B(a)P induced A/J mice model. Pretreatment with various doses of PBPs showed dose related decrease in B(a)P induced expression and activity of CYP1A1 in liver while CYP1A2 in lung. Dose dependent significant increase in PBPs mediated over expression and activity of GST isoforms (alpha in

liver while pi in lung; mu in both liver and lung) were observed in polyphenol treated groups. The observed dose dependent decrease in expression and activity of XMEs further correlated with decrease in number and intensity of BPDE-DNA adducts containing nuclei were observed in liver and lung.

Our study further highlights the effect of administration of 1.5% and 3% black tea derived PBP rich extract on well-established B(a)P and NNK induced lung carcinogenicity in A/J mice model. We for the first time demonstrated /showed dose dependent significant decrease surface tumor incidence and multiplicity without affecting latency period in week 6 and 18 post carcinogen treatment. It further showed significant decrease in number and area of microscopic lung lesions including hyperplasia and adenoma in dose dependent manner. The observed decrease in tumorigenesis was further attributed to different molecular biomarkers like cell proliferation (diminished PCNA labeling index) and apoptosis (increased TUNEL apoptotic index) in well-established B(a)P and NNK model. In conclusion, dose dependent protective effect of PBPs could be attributed to a) induction of phase II and inhibition of phase I enzymes by decreasing BPDE-DNA adducts b) decreasing cellular proliferation and inducing apoptosis.

- **Chapter 3**

In the present study mechanism of 1.5% black tea derived PBP mediated anti-initiation were investigated in A/J mice by pre and post treatment regimen. In the pretreatment regimen, mice were pretreated for 2 weeks with 1.5% PBPs/ water followed by single i.p. injection of B(a)P /vehicle and sacrificed 24 hr. post carcinogen treatment. Pretreatment with PBPs decreased formation of B(a)P induced BPDE-DNA adducts containing cells in both liver and lung tissues. The potential reasons for the decrease could be; a) PBPs down regulated the carcinogen induced expression and activity of phase I enzymes while induced phase II

enzymes which ultimately less number of reactive intermediates available for adducts formation b) PBPs mediated to decrease the inflammation (lowered macrophage and lymphocytic count in BAL) c) PBPs induced apoptosis (increased TUNEL apoptotic index) while decreased cell proliferation (diminished PCNA labeling index).

In the post treatment regimen, mice administered B(a)P injection by i.p., were shifted to PBPs/water after 24 hr. and sacrifice was carried out at 24, 48 and 96 hr. respectively. A time dependent loss of B(a)P induced BPDE-DNA adducts containing cells were observed in both liver and lung. To understand the enhanced decrease was because of dilution effect due to newly synthesized DNA attributed to enhanced cell turnover, selective apoptosis of BPDE-DNA adducts containing cells and/or enhanced DNA repair mechanism. The comparative evaluation of all these parameters showed that time dependent enhanced apoptosis of adducts containing cells (increased TUNEL apoptotic index) along with decreased inflammation (decreased macrophage and lymphocyte count in BAL) and enhanced DNA repair (lowered p-ATM and p-Chk1 expression) together attributed to observed disappearance of BPDE-DNA adducts.

PBPs pretreatment decreases the formation of reactive intermediates required for DNA adducts formation and enhanced apoptosis of adducts containing cells ultimately suggesting lowered BPDE-DNA adducts containing cells. While post treatment with PBPs showed time dependent enhanced apoptosis (increased TUNEL apoptotic index) along with decreased inflammation and DNA damage resulting in enhanced disappearance of BPDE-DNA adducts.

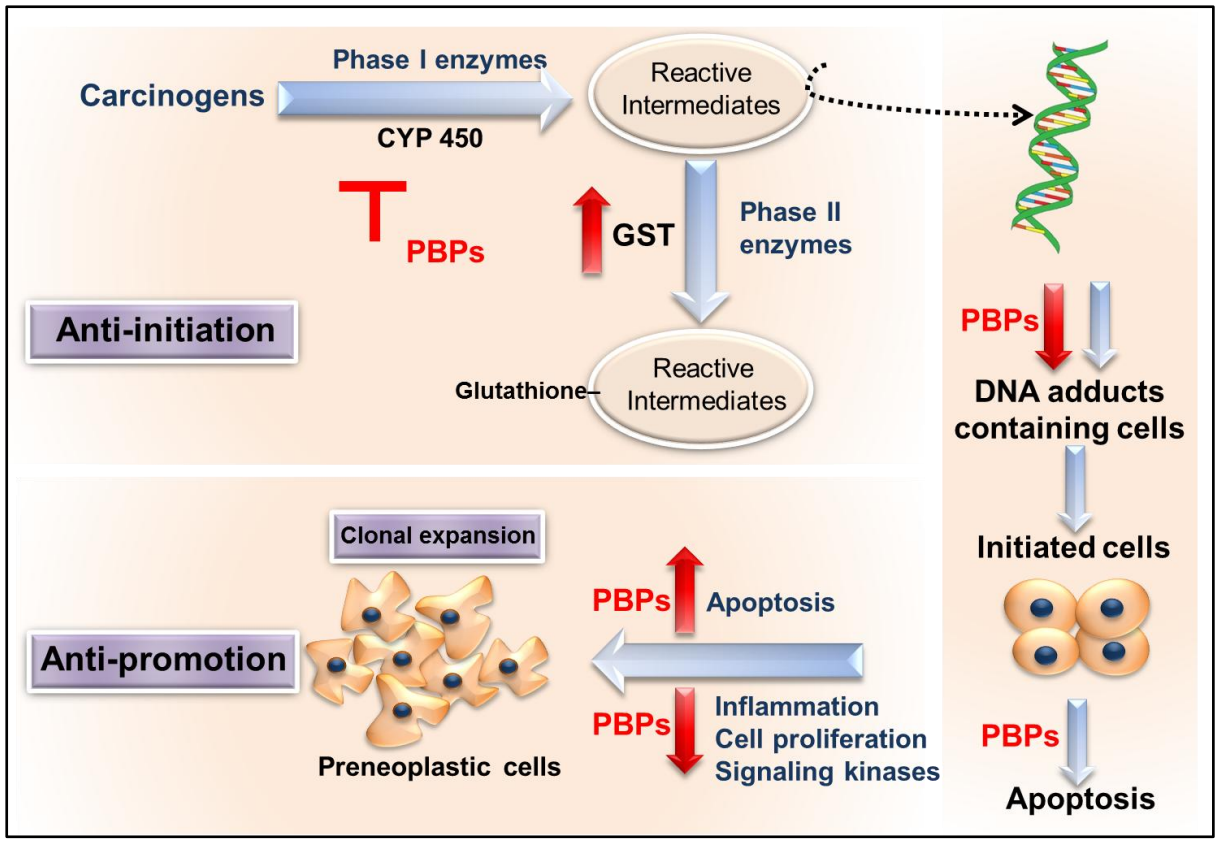


Figure 7.1 : Summary of mechanism of action of polymeric black tea polyphenols (PBPs) in the carcinogen induced lung carcinogenesis A/J mice model

In summary, black tea derived PBPs exert chemopreventive action potentially as anti-initiating agents and anti-promoting agent in the experimental lung carcinogenesis model.

Concluding remarks and future directions:

Dietary chemopreventive compounds offer great potential in the fight against degenerative diseases like cancer by modulating process of carcinogenesis through regulation of cell-defensive and cell death machinery. To modulate process of carcinogenesis, enhancement of the detoxifying and antioxidant enzymes for efficient elimination of carcinogenic species provides the basis, especially for the high risk population exposed to environmental carcinogens. While in the late stages of carcinogenesis, induction of apoptosis and cell cycle

arrest in adenoma and carcinoma cells is an important goal in the process of chemoprevention. Ultimately, to successfully convert a potent dietary chemopreventive compound to a clinically viable drug requires detailed consideration of invivo studies. Our study highlights the chemopreventive efficacy of one of the major polyphenolic fraction in the black tea, polymeric black tea polyphenols (PBPs). It is noteworthy from our study, that only PBPs fraction is having the large potential for the chemopreventive action against various cancers including lung cancer. Hence together, chemopreventive potential of complete polyphenolic fractions (monomeric, oligomeric and polymeric) and caffeine rich black tea provides new avenues in the field of chemoprevention.

Considering the limited progress in the black tea research as a cancer chemopreventive agent, further studies on

- Detailed characterization of major black tea polyphenols (BTP)
- Comparative evaluations of their chemopreventive efficacy, bioavailability and pharmacokinetic
- Mechanisms of chemopreventive actions of BTP and black tea extract derived monomeric, oligomeric and polymeric polyphenols
- Chemopreventive efficacy of BTPs combined with other chemopreventive agents
- Analysis of chemopreventive efficacy of BTP using various cancer models systems are needed for taking to the clinical trials. A clear understanding in this area will provide inputs for future developments in basic and applied medicinal compounds, which are part of our daily diet.

Chapter 8

References

Chapter 8: References

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Polymeric Black Tea Polyphenols (PBPs) Inhibit Benzo(a)pyrene and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone-Induced Lung Carcinogenesis Potentially Through Down-Regulation of p38 and Akt Phosphorylation in A/J Mice

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The aim of our study was to evaluate chemopreventive efficacy and possible mechanism of most abundant polyphenolic fraction in black tea, polymeric black tea polyphenols (PBPs), in experimental lung carcinogenesis model. Effect of 1.5% black tea derived PBPs on benzo(a)pyrene [B(a)P] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung lesions were studied over 28 wks. Chemopreventive efficacy was studied using decrease in tumor incidence and/or multiplicity and/or delay in the latency period in A/J mice. Histopathological analysis of lung was carried out post-carcinogen treatment weeks to analyze the microscopic lung lesions. Inflammation, cell proliferation, and apoptosis markers along with signaling kinases like p38, Akt, and their phosphorylated forms were studied using immunoblotting and immunohistochemistry at 4th, 10th, and 18th wk post-carcinogen treatment. Administration of PBPs throughout the treatment period significantly decreased the multiplicity of surface tumors as well as microscopic lung lesions, including adenomas. Although tumor incidence and latency period remains unaffected, histopathological evaluation of lung at 6, 10, and 18 wks post-carcinogen treatment period showed decrease in tumor multiplicity which was also correlated with different molecular markers. Anti-inflammatory action of PBPs was demonstrated by reduced Cox-2 expression. PBPs down-regulated the B(a)P and NNK-induced cell proliferation (diminished PCNA expression, proliferation index, and Bcl-2 expression) and enhanced apoptosis (increased Bax expression and apoptotic index) potentially through phosphorylation of p38 and Akt. PBPs, most abundant polyphenolic component in the black tea, have chemopreventive effect through inhibition of inflammation, cellular proliferation, and induction of apoptosis possibly via modulation of signaling kinases.

Key words: tobacco carcinogens; experimental lung carcinogenesis; chemoprevention; polymeric black tea polyphenols; molecular markers

INTRODUCTION

Lung cancer associated with cigarette smoking is a leading cause of death in both men and women worldwide [1]. Cigarette smoke or environmental exposure to various carcinogens including major classes like polycyclic aromatic hydrocarbons (PAHs) and nitrosamines are major risk factors associated with lung carcinogenesis. Benzo(a)pyrene [B(a)P] from PAHs and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) from tobacco-specific nitrosamines are potent carcinogens present in the tobacco smoke which has proven to be carcinogenic in in vitro and in vivo systems independently or in combination [2]. Minimizing the exposure to environmental smoke and cessation of cigarette smoking has been demonstrated as one of the successful approach to reduce the lung cancer risk and prolong quality of life [3]. Chemoprevention using dietary phytochemicals to retard the process of

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carcinogenesis could be an effective approach to reduce the risk of developing this disease [4]. Number of studies using variety of bioactive compounds from several fruits and vegetables have shown strong evidence of chemoprevention against various cancers including lung cancer [5].

Black tea (*Camellia sinensis*, family Theaceae) is the most widely consumed beverage worldwide and has been associated with many health benefits including the prevention of cardiovascular diseases and cancer [6,7]. Crude black tea extract has been shown to have chemopreventive potential at various organ sites including skin, lung, oral cavity, and mammary glands [8]. Epigallocatechin-3-gallate (EGCG), most abundant monomeric catechin in green tea is known to modulate different cellular processes via signal transduction pathways, majorly through modulation of kinase function [8]. EGCG and theaflavins (oligomeric form in the black tea polyphenols) have reached clinical trials after their chemopreventive efficacies have been successfully established by various preclinical studies without any remarkable toxicity [9,10]. Thearubigins (TR) or polymeric black tea polyphenols (PBPs), which contribute as a most abundant constituents of the total polyphenolic fraction of the black tea, are polymeric fraction of polyphenols formed in the processing of black tea due to oxidation process. Despite being major polyphenolic component (>40%), studies on PBPs are limited. PBPs are pro-anthocyanidin, heterogeneous polymers of flavano-3-ols, and flavan-3-ol gallates with di- and tri- benzotropolone skeletons [11,12]. PBPs are structurally and chemically ill established because of its complexity due to number of isomeric compounds and matrix reactivity. Previously isolated and partially characterized PBPs fractions have shown to have anti-initiation activity by inhibiting formation of BPDE-DNA adducts in mouse skin and demonstrating decrease in liver microsomal CYP activity in vitro [13] and by negatively affecting the activity of metabolizing enzymes CYP1A1 and 1A2 [14]. Anti-promotion action of topically applied PBPs has already shown to inhibit the carcinogen-induced cell proliferation in the mouse skin through MAPK pathway and 1,2-dimethylhydrazine-induced colorectal carcinogenesis via Wnt/β-catenin pathway [15,16].

The objective of the present study was to investigate the effect of PBPs on the process of lung carcinogenesis measured as tumor incidence, multiplicity, and latency period using a well-established B(a)P and NNK-induced lung carcinogenesis model in A/J mice. We observed that PBPs treatment decreased B(a)P and NNK (i.p., 3 μM of each) induced microscopic lung lesions like hyperplasia, atypical adenomatous hyperplasia, and adenomas at 6th, 10th, and 18th wk post-carcinogen treatment. Administration of 1.5% black tea-derived PBPs ad libitum significantly decreased the multiplicity of carcinogen-induced lung adenomas by modulating the processes associated with inflammation, cell proliferation, and apoptosis with phosphorylation of signaling kinases like p38 and Akt during sequentially studied lung carcinogenesis process.

MATERIALS AND METHODS

Materials

Benzo(a)pyrene (B(a)P, ≥96% purity), and glyceryl trioctanoate was purchased from Sigma–Aldrich (St. Louis, MO) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 98% purity) was obtained from Eagle Picher Pharmaceutical services (Lenexa, KS). Antibodies for PCNA, Bax, Bcl-2, Cox-2, and β-Actin were purchased from Abcam (Cambridge, MA) and p38, phospho-p38, Akt and phospho-Akt, were from Cell Signaling Technology (Beverly, MA). The anti-rabbit or anti-mouse or anti-goat horseradish-peroxidase conjugated secondary antibodies were obtained from Abcam.

Preparation of PBPs Extract From Black Tea

Polymeric black tea polyphenols (PBPs) extract was prepared employing popular brand of black tea powder (Brooke Bond Red Label, Mumbai, India), using a Soxhlet extractor [17]. Briefly, 450 g of black tea powder was serially extracted in a Soxhlet extractor (Borosil Glass Works Ltd., Mumbai, India) with chloroform for decaffeination and then with ethyl acetate (which extracts PBP-1, catechins, and theaflavins). Dried ethyl acetate extract was reconstituted with acetone (200 ml) and to that diethyl ether (800 ml) was added to precipitate PBP-1 which was further separated by centrifugation, dried, and stored at −20°C till use. Residual tea powder was dried and stored at −20°C. Before use, residual tea powder was boiled in autoclaved miliQ water to get 1.5% black tea-derived extract which is known to contain mixture of PBPs-2, 3, 4, and 5. To this aqueous extract, PBP-1 was added back to obtain PBP rich extract (free of caffeine, catechins, and theaflavins). PBPs was aliquoted and fed to the animals after it was confirmed to be free of other biologically active components such as caffeine, theaflavins, and catechins using thin layer chromatography [17].

MALDI-TOF Analyses of PBPs Extract/Sera Samples

Detection of black tea-derived contaminants in PBPs extract

MALDI TOF analysis was employed additionally to confirm purity of PBPs. α-cyano-4-hydroxycinnamic acid (HCCA) matrix was used for sample analysis. Vacuum dried PBPs extract(s), catechin standard (EGCG), theaflavins, and caffeine standard were dissolved (1 mg/20 μl) in solvent, that is, the mixture of 0.1% trifluoroacetic acid (TFA) in 50% acetonitirle (ACN). Purity of PBPs was checked by analysis of five independently extracted samples which were mixed to the HCCA matrix solution (1:1 v/v). Aliquot of

this mixture (4 μl) was put on a spot of 396 array plate and dried at room temperature. MALDI-TOF mass spectra were acquired on a reflectron mode using Bruker Daltonics Ultraflex-II, (Instrumentation and calibration details described in supplementary text). The resulting MS data was analyzed using Flex analysis 3.0 (Brucker Daltonic, Bremen, Germany) software.

Detection of PBPs exposure related signals in sera of exposed animals and demonstration of biological activity of orally administered PBPs in lung

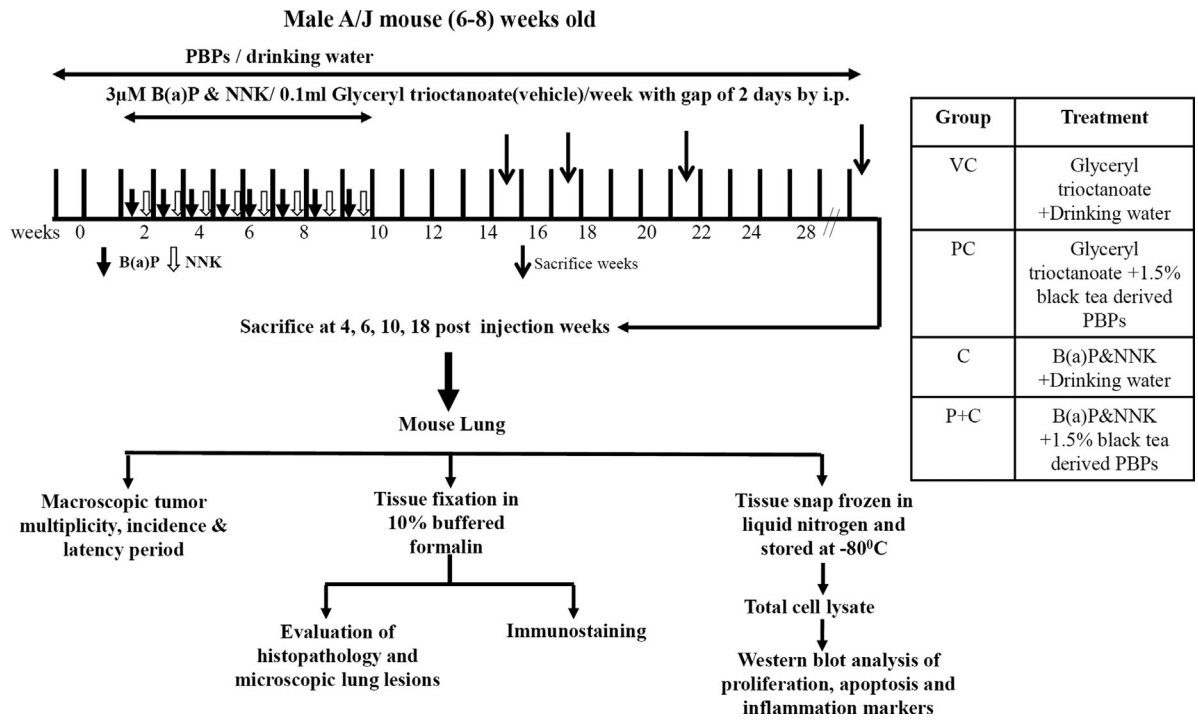
Five sera (50 μl) samples each from vehicle and PBPs exposed animals were extracted with methanol (500 μl, HPLC grade) and mixtures were incubated at −20°C overnight. Deproteinized supernatents were collected after centrifugation and vacuum dried. They were further reconstituted and processed for MALDI-TOF analysis for detection of PBPs/metabolites related signals as described above. To demonstrate the biological effects of orally administered PBPs if any in the lung, induction of GSTs isoforms (mu and alpha) was studied employing immunoblotting of lung cytosolic fraction at the end of 1st and 8th wk of carcinogen treatment in A/J mice belonging to various treatment groups, as described [18]. Total GST enzyme activity in lung cytosol was measured using Sigma GST assay kit CS0410, as per manufacturer's protocol using CDNB as a substrate. GST activity has been expressed as μmol of CDNB conjugate formed/ml/min.

Animal Treatment

All animal studies were conducted after approval from the institutional animal ethics committee (sanction no: 09/2011) endorsed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. A/J mice obtained from Jackson Laboratory, were inbred at Laboratory Animal Facility, ACTREC, India and they were housed under standard conditions: 22 ± 2°C, 55 ± 5% relative humidity, and 12 h light/dark cycle. Animals received a standard pellet diet ad libitum. Male A/J mice, 6–8 wks old were received from Laboratory Animal Facility, ACTREC and randomized into four different groups. Polyphenol control (PC) and polyphenol + carcinogen (P + C) groups were primed for 2 wks with PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen (C) groups received normal drinking water. Animals in vehicle control (VC) and polyphenol control (PC) groups were injected glyceryl trioctanoate (vehicle, i.p., 0.1 ml) and continued receiving drinking water and PBPs, respectively while carcinogen (C) and polyphenol + carcinogen (P + C) groups were injected B(a)P and NNK (i.p., 3 μM of each, 0.1 ml) and continued receiving drinking water and PBPs (ad libitum), respectively. Glyceryl trioctanoate or B(a)P and NNK injections were given with the gap of 2 d, once in a week for 8 wks while PBPs and drinking water were administered ad libitum, prior to (2 wks), during (8 wks) and after the carcinogen treatment (4, 6, 10, and 18 wks) till the end of the experiment (Figure 1). Fresh bottle of PBPs were employed every alternate day. Body weights were monitored after receiving the animal and before the sacrifice time points. Animals were sacrificed by cervical dislocation at 4, 6, 10, and 18 wks after administration of the last dose of carcinogen. Macroscopic/visible lung tumors were counted at each time point using magnifying glass. Lung tissues were perfused with sterile 0.15 M KCl and excised. All five lung lobes from three random animals per group were fixed in 10% buffered formalin and paraffin embedded blocks were used for histopathological evaluation of microscopic lung lesions and immunohistochemical analysis (IHC). Tissues from the rest of the animals were snap frozen in liquid nitrogen and stored at −80°C until preparation of the extract for immunoblotting.

Evaluation of Microscopic Lesions (Number and Area) and Their Histopathology

Evaluation of number and area of microscopic pulmonary lesions was carried out as described [19,20]. Briefly, formalin-fixed lung tissues from three independent mice were processed through a series of graded alcohols, embedded in paraffin, and four step sections (each 200 μm apart) having a thickness of 5 μm each were cut and stained with haematoxylin and eosin. Each stained section was tile scanned using LSM 510 Meta Carl Zeiss confocal microscope (Zen Software 2012, SP1, Zeiss, Jena, Germany) at final 100× magnification. Proliferative lesions in the lungs were classified as hyperplasia, atypical adenomatous hyperplasia (AAH), and adenoma independently by two pathologists based on recommendations published by the Mouse Models of Human Cancers Consortium [21]. Proliferative lesions were counted and recorded in each step section and the total number of each type of lesion per mouse ($n = 3$) was expressed as an average number of each lesion per mouse (sum of each lesion in three mice divided by three). Area of all proliferative foci in each tile scanned step section was marked independently by two pathologists and lesion size was evaluated using Image J software (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2015). *Image J* was precalibrated for conversion of pixels into square micrometer according to magnification used for image acquisition (100×). Individual lung lesion was outlined using free hand drawing tool and its size was measured as square micrometer. Area measurement was carried out by analyzing step sections from three independent mice and expressed as an average area of each type of lesion per mouse.



Group	Treatment
VC	Glyceryl trioctanoate +Drinking water
PC	Glyceryl trioctanoate +1.5% black tea derived PBPs
C	B(a)P&NNK +Drinking water
P+C	B(a)P&NNK +1.5% black tea derived PBPs

Figure 1. Experimental design for studying the effect of PBPs on B(a)P and NNK-induced lung carcinogenesis in A/J mice model. 6–8 wks old male A/J mice were randomized into four different groups, that is, vehicle control (VC), polyphenol control (PC), carcinogen (C), and polyphenol + carcinogen (P + C) as shown in the tabular format. Polyphenol control (PC) and polyphenol + carcinogen (P + C) groups were primed for 2 wks with PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen (C) groups received normal drinking water. After 2 wks of priming, B(a)P and NNK(3 μM each/0.1 ml) in glyceryl trioctanoate or only glyceryl trioctanoate were intraperitoneally injected with the gap to 2 d, once in a week for 8 wks (shown by solid and hollow arrows, respectively) while PBPs or drinking water were continued during (8 wks) and after the carcinogen treatment till the end of the experiment. Sacrifice was done at 4, 6, 10, and 18 wks after the last carcinogen treatment (shown by black arrow). All five mouse lung lobes were excised and screened for macroscopic tumor burden and either fixed in 10% buffered formalin or snap frozen in liquid nitrogen and stored at −80°C.

Protein Immunoblotting

Total cell lysates from the snap frozen entire lung tissues from each animal were prepared by previously described cell fractionation procedure [22]. The lysates were aliquoted, their protein content was determined [23] and stored at −80°C. Before analysis of analyte(s) proteins, concentration of total cell lysate and dilutions of primary and secondary antibodies were standardized and used for further detection of protein. Briefly, the total cell proteins (50 μg) were resolved on 8–12% SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Amersham Biosciences, Buckinghamshire, UK). After blocking with 5% non-fat skimmed milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween-20 (TBST), the membranes were probed with antibodies for Bax, Bcl-2, PCNA, Cox-2, β-actin, p38, Akt, phospho-p38, phospho-Akt overnight at 4°C. Blotting membranes were then washed three times with TBST and incubated with 1:4,000 dilutions of anti-rabbit, anti-mouse, or anti-goat HRP conjugated secondary antibodies. Immunoreactive bands were visualized using a chemiluminescence reagent (GE Amersham Biosciences). β- actin was used as the loading control. Densitometry of various analyte proteins and their respective loading controls from the same blot was performed using Image J 1.43 (NIH) software. Relative optical density was calculated by dividing the densitometric reading of analyte(s) protein with that of respective loading control.

Immunohistochemical Staining

For immunohistochemical (IHC) staining, 5 μm thick sections of paraffin embedded whole lung tissue were mounted on poly-L-lysine-coated glass slides. Sections were deparaffinized with xylene and rehydrated through a gradient series of alcohol and then rinsed with distilled water. Antigen retrieval was done using sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) or Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0). To quench the endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide in methanol for 30 min in darkness. Blocking with serum was done for 1 h. at 37°C in a humidified chamber. Sections were further incubated with primary antibodies for Cox-2, PCNA, Bax, and Bcl-2 overnight at 4°C. Detection was conducted using a Vectastain ABC system kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine was employed as the chromogenic

substrate and slides were counterstained with Mayor's haematoxylin. Images were captured with a Zeiss microscope (Imager Z1) to which was attached an Axiocam MRc5 digital camera. For negative or isotype controls, the primary antibody was replaced with PBS. In each batch, slides with positive control(s) were also processed simultaneously.

Proliferation Index in Lesion and Non-Lesion Areas

For PCNA nuclear protein staining, semi-quantitative analysis was conducted using Image J 1.43 (NIH) software. PCNA labeling index was calculated by counting the number of positively stained cells × 100/ total number of cells in photomicrographs of lung sections. At least 10 different randomly selected fields and/or minimum 1,000 cells were counted, at 400× magnification with at least three mice per group. Comparisons of non-lesion area in all the groups at 4th, 10th, and 18th wk were done with its respective controls. Proliferation labeling index in the lesion area of P + C was calculated and compared with its respective C group in all the weeks. Due to absence of any microscopic lesions, week 4 was not considered in the lesion area proliferation index analysis.

Terminal Deoxynucleotidyl Transferase Fluorescein-dUTP Nick End Labeling (TUNEL) Assay

Apoptotic index was assayed in formalin-fixed, paraffin embedded 5 μm tissue sections, according to the manufacturer's instructions (DeadEnd^{TM} Fluorometric TUNEL System, Promega, Madison, WI). The nuclei of the apoptized cells were stained fluorescent green and nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI). Slides were mounted by Vectashield (Vector Lab Cat.# H-1000, Vector Laboratories, Burlingame, CA). Images were captured using Zeiss confocal LSM 780 at 40× (oil immersion) magnification. The apoptotic index was calculated by counting the cells with positively stained nuclei × 100/total number of cells in photomicrographs of lung sections of at least 10 different randomly selected fields (~ 1,000 nuclei/ mice), with at least three mice per group.

Statistical Analysis

Statistical analysis was performed using SPSS 21.0 software. Initial and final body weights among the different groups were compared using non-parametric Kruskal–Wallis test followed by Mann–Whitney with Bonneferoni's correction. Surface lung tumors within the groups were compared using non-parametric Mann–Whitney Wilcoxon test while microscopic lung lesions were compared using Poisson regression, which represents the counts or number of events where few or no events occur. For microscopic tumor area analysis, data were log transformed and comparisons of area of each lesion between C and P + C groups were done using regression analysis. Mean of each analyte proteins were normalized with its respective loading controls in western blotting and data were compared with analysis of variance (ANOVA) with post-hoc testing. Cell counting was conducted for proliferation index (lesion and non-lesion areas) and apoptosis index using Image J (NIH) software. Data were represented as Mean ± SD and compared using ANOVA with Bonneferoni's correction. $P < 0.05$ was considered to be statistically significant.

RESULTS

Evaluation of Purity of PBPs Extract

Since black tea-derived contaminants such as caffeine, EGCG, and theaflavins are mobile in TLC analysis, while PBPs remain bound to matrix at the origin, even low levels of contamination is possible to detect by TLC. Absence of any mobile analyte in TLC based analysis of PBPs revealed that the extract was free of biologically active black tea-derived components. To confirm the absence of black tea-derived contaminants, PBP extract(s) were analyzed employing MALDI-TOF analysis. We analyzed the spectra of potential contaminants by using commercially available standards for EGCG (Sigma E4143), caffeine (Sigma C0750), and mixture of theaflavins (Sigma T5550) showing its *m/z* value 459.11, 195.13, and 565.10, respectively which matched with those reported (Figure 2). None of the PBP extracts showed the presence of any of these contaminants. However, all five independently extracted PBP samples showed two specific peaks of *m/z* value 854.8 and 876.7 (Figure 2) which confirms absence of black tea-derived other known biologically active contaminants in PBPs. Hence, the observed biological activity can be attributed to exposure to PBPs, the only variable.

Detection of PBPs Exposure Related Signals in Sera of Exposed Animals and Demonstration of Biological Activity of Orally Administered PBPs in Lung

MALDI-TOF analyses of five sera extracts each from vehicle and PBPs exposed (28 wks) animals did not show any PBPs/metabolites exposure related signals probably due to very low levels of analytes (data not shown). However, oral administration of PBPs resulted in enhanced expression of GST proteins (mu and alpha) as well as increase in activity of GST in lung cytosols from A/J mice demonstrating biological effects of PBPs and/or their metabolic products (Supplementary Figure S1). Observed PBPs/products-mediated biological effects in lung of A/J mice are in agreement with earlier reports [16,24].

General Observations and Body Weight

There were no signs of toxicity and no mortality was observed in any of the groups. At the time of sacrifice, no gross changes in the kidney, spleen, liver, stomach, or intestine were seen at varying treatment periods. Final body weights were found to be higher at the

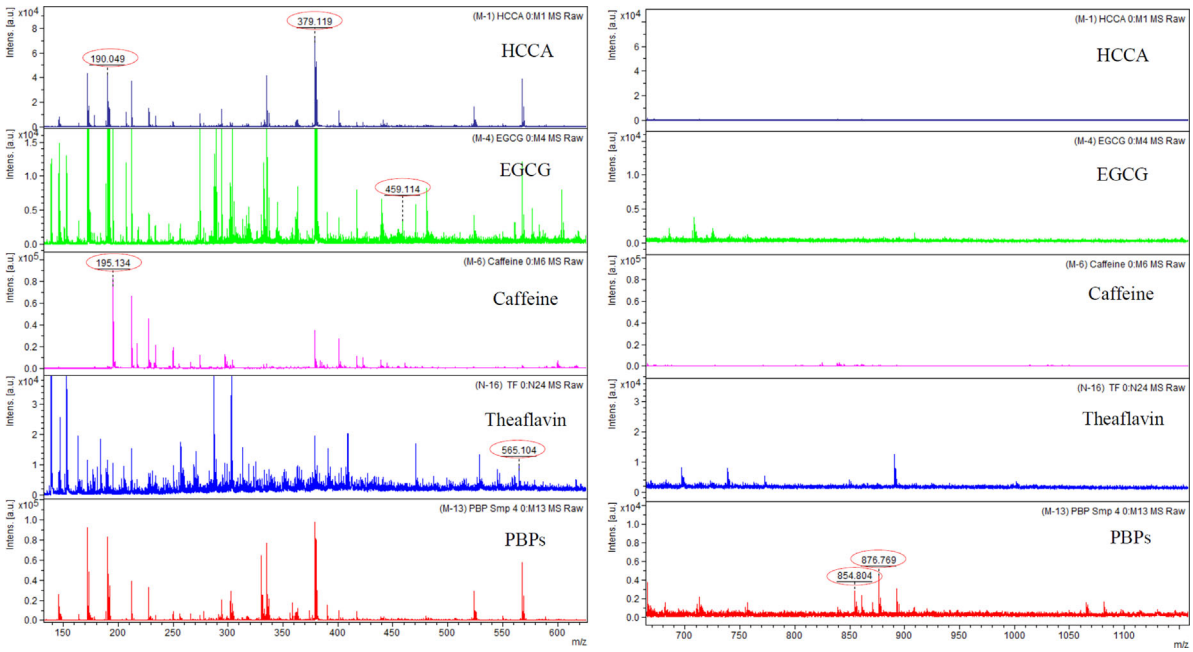


Figure 2. Evaluation of black tea-derived contaminants viz. EGCG, caffeine, theaflavins in PBPs extract by MALDI-TOF analysis. Representative MALDI-TOF mass spectra of EGCG (*m/z* 459.1), caffeine (*m/z* 195.1), theaflavin (*m/z* 565.1) using α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix. Absence of signals at those masses in PBPs extract(s) confirms that PBPs extracts were free from black tea-derived biologically active contaminants. Representative MALDI-TOF mass spectra of one of the five independently extracted PBPs extract showed signals at *m/z* 854 and 876, respectively.

termination time points in all the groups as compared to its initial probably suggesting lack of toxicity in the treated groups, although statistical significance was not attained (Table 1).

Analysis of Macroscopic Lung Tumor Multiplicity

PBPs reduced the pulmonary macroscopic tumor multiplicity

Mice were sacrificed at sequential time points, that is, 4th, 6th, 10th, and 18th wk after administration of last dose of carcinogen and macroscopic/visible lung tumors were counted. Both VC and PC showed no tumors at any of the time points studied. Macroscopic tumors were not observed at 4th and 5th wk post-carcinogen treatment. However, 100% tumor incidence was seen at 6th, 10th, and 18th wk post-carcinogen treatment in both C and P + C groups (Table 1). Latency period was found to be 6 wks post-carcinogen in both C as well as P + C group. Average tumor multiplicity was found to be increasing in C group from 6th to 18th wk post-carcinogen treatment. PBPs treatment resulted in significant decrease in average tumor multiplicity in P + C group as compared to its respective carcinogen treated animals at 6th, 10th, and 18th wk post-carcinogen treatment (Table 1). However, only decrease at 18th wk post-carcinogen treatment attained statistical significance, probably because of lower tumor number at 6th wk and high deviation at 10th wk.

Analysis of Microscopic Lung Lesions

PBPs reduced the number of microscopic pulmonary lesions

The microscopic lesions observed in lung tissues of mice were classified as hyperplastic foci, AAH, and adenoma based on established criteria [21]. Briefly, hyperplasia was assigned based on increased number of cuboidal, columnar, ciliated, or mucous cells without atypia with normal architecture of bronchioles and alveoli. AAH as focal and diffuse lesions involving alveoli and terminal bronchioles and consisting of relatively uniform atypical cuboidal to columnar cells with dense chromatin and cellular and nuclear atypia. Adenomas as small size well circumscribed and well demarcated lesion with typical features indicating benign character and absence of vascular invasion [21]. Tile scan image of representative lung tissue cross sections showing microscopic lung lesions in treated group are depicted in Figure 3A. VC and PC groups showed no microscopic lung lesion while C and P + C groups showed all three types of lesions. At weeks 4 and 5 post-carcinogen treatment no lesions in C as well as P + C group were observed. Microscopic foci were observed at 6th wk post-carcinogen treatment in both C and P + C groups in all the animals. Mice treated with polyphenol + carcinogen showed significant reduction in number of hyperplastic foci at both 6th and 10th wk post-carcinogen

Table 1. Effect of PBPs on B(a)P and NNK-Induced Macroscopic Lung Tumors

Group	Weeks	*n*	Body weight (g)		Lung tumors		*P*-value
			Initial†	Final†	Incidence (%)	Multiplicity†	
VC	4	10	21.78 ± 2.29	25.04 ± 1.98	0	0	
PC		9	22.01 ± 2.55	25.19 ± 2.15	0	0	
C		10	22.62 ± 2.54	24.98 ± 2.19	0	0	
P + C		10	22.16 ± 2.81	24.23 ± 2.12	0	0	
VC	6	14	20.73 ± 3.49	23.06 ± 3.65	0	0	
PC		13	21.21 ± 2.29	24.11 ± 2.39	0	0	
C		10	20.60 ± 2.38	22.81 ± 2.36	100	1.00 ± 1.18	0.606
P + C		10	19.33 ± 2.46	21.49 ± 2.55	100	0.64 ± 0.67	
VC	10	9	23.86 ± 3.12	26.80 ± 4.01	0	0	
PC		19	22.96 ± 2.44	25.51 ± 2.33	0	0	
C		9	23.33 ± 1.87	25.76 ± 1.98	100	8.20 ± 2.97	0.095
P + C		8	21.59 ± 1.83	23.91 ± 1.79	100	6.0 0 ± 1.76	
VC	18	8	21.61 ± 2.46	24.25 ± 1.38	0	0	
PC		9	22.06 ± 1.23	24.72 ± 1.01	0	0	
C		8	21.60 ± 2.27	24.47 ± 2.03	100	25.60 ± 1.51	0.001
P + C		8	22.29 ± 1.42	24.90 ± 2.54	100	20.00 ± 3.02*	

*Significantly different from C $P \leq 0.05$.
†Represented as Mean ± SD.
6–8 weeks old male A/J mice were randomized into four different groups, that is, vehicle control (VC), polyphenol control (PC), carcinogen (C), and polyphenol + carcinogen (P + C). Polyphenol control (PC) and polyphenol + carcinogen (P + C) groups were primed for 2 wks with PBPs as a sole source of drinking water while vehicle control (VC) and carcinogen (C) groups received normal drinking water. After 2 wks of priming, B(a)P and NNK (3 μM each/0.1 ml) in glyceryl trioctanoate or only glyceryl trioctanoate were intraperitoneally injected with the gap to 2 d, once in a week for 8 wks while PBPs or drinking water were continued during (8 wks) and after the carcinogen treatment till the end of the experiment. Sacrifice was done at 4, 6, 10, and 18 wks after the last carcinogen treatment. Initial and final body weights have been presented as Mean ± SD. Surface tumors were counted in each sacrifice weeks and compared with carcinogen treated group (*$P < 0.05$, Mann–Whitney Wilcoxson test).

treatment as compared to its carcinogen treated counterpart but not at 18th wk probably due to lower number of hyperplastic foci. However, mice treated with polyphenol + carcinogen showed significant reduction in adenomatous foci at 10th and 18th wk but not at 6th wk post-carcinogen treatment probably because of large intragroup variation (Figure 3B). On the contrary, although PBPs lowered the number of AAH lesions, it did not attain statistical significance because of lower number of AAH foci.

PBPs Reduced the Area of Microscopic Pulmonary Lesions

Average area of hyperplasia in polyphenol + carcinogen treated mice was reduced at 6th, 10th, and 18th wk post-carcinogen treatment as compared to its respective carcinogen treated mice; however, statistical significance was observed only at 10th and 18th wk post-carcinogen but not in 6th because of small lesion area to detect the changes, although differences in the numbers were significant (Figure 3B). Similarly, average area of adenoma in P + C group mice was significantly reduced at 10th and 18th wk as compared to carcinogen alone group but no statistical difference was observed in 6th wk due to low number and small area of lesions (Figure 3B). PBPs treatment decreased average area of AAH however, these differences were not statistically significant.

Analysis of Molecular Markers

To ascertain the contribution of cell proliferation and apoptosis along with inflammation in observed decrease in number and size of pulmonary lesions with PBPs administration, these markers were studied at 4th, 10th, and 18th wk post-carcinogen treatment. A 4th wk post-carcinogen treatment was selected to understand the modulation of processes, if any, before the formation of the tumors.

PBPs Decreased the B(a)P and NNK-Induced Inflammation During Lung Carcinogenesis

Levels of Cox-2, a well-known marker of inflammation, were evaluated during the sequential, that is, 4th, 10th, and 18th wk post-carcinogen treatment period. Cox-2 protein levels in vehicle and polyphenol controls were found to be similar while in carcinogen treated groups, they were significantly higher compared to respective vehicle controls at all the time points studied. In carcinogen treated groups there was time-related decrease in the levels of Cox-2 which was observed after cessation of carcinogen treatment, that is, 4th–18th wk. Time-related decrease in Cox-2 levels was further enhanced by PBPs at 4th

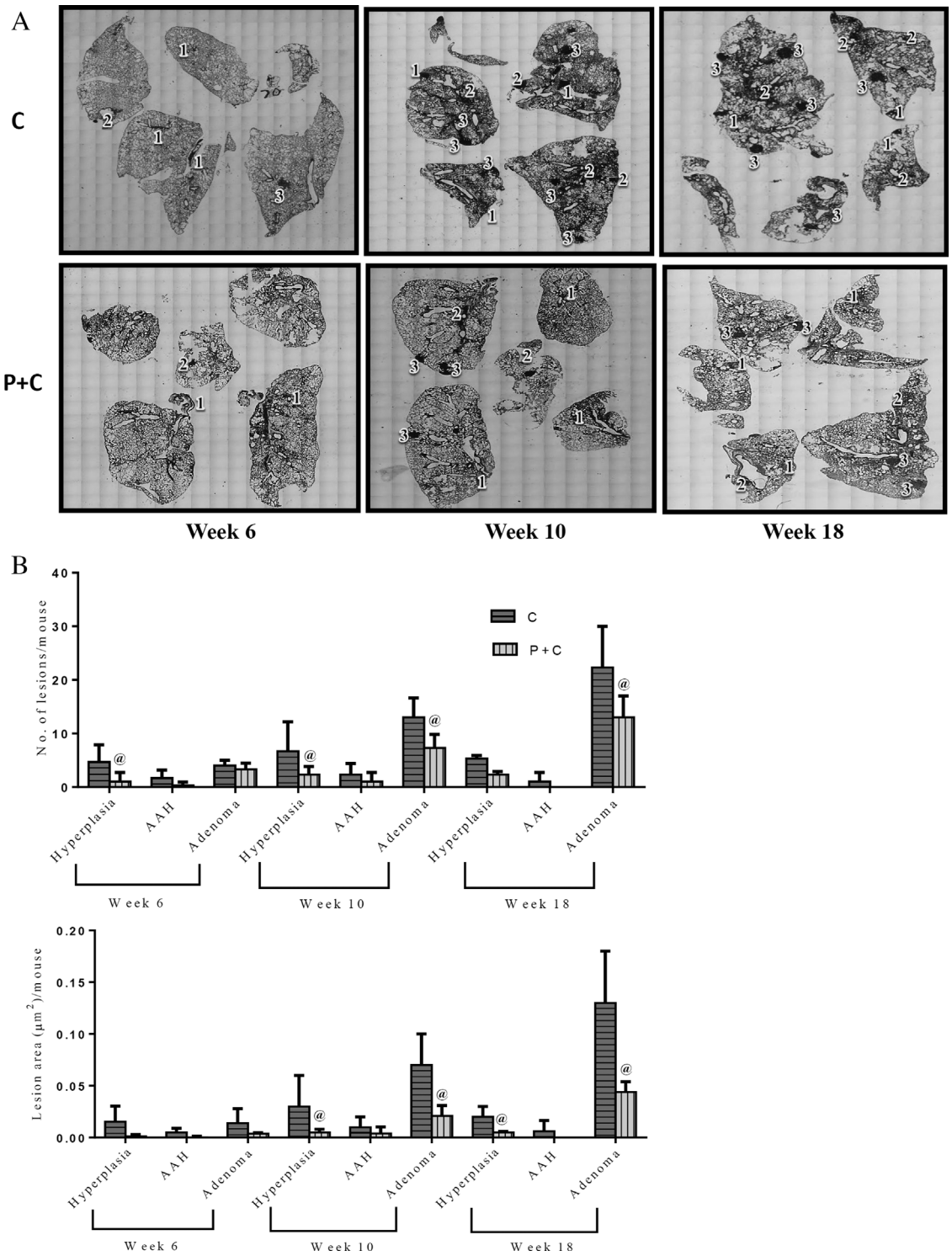


Figure 3. Effect of PBPs on multiplicity and area of microscopic lung lesions. A) Represntative tile scan images showing microscopic lung lesions in C and P + C group at 6th, 10th, and 18th wk post-carcinogen treatment (magnification 100×). The number 1, 2, 3 represents pulmonary hyperplastic lesion, atypical adenomatous hyperplasia, and adenoma, respectively. B) Number and area of each proliferative lesion assigned by pathologist were counted in each step section (200 micron apart) and the total number of each type of lesion per mouse was expressed as an average number of each lesion per section (sum of each lesion in three step sections divided by three) in three independent animals in C and P + C group and plotted in a graph (upper panel, @ significantly different from C, $P < 0.05$, Poison regression). The areas of lesions were counted using *Image J* software in each step section and total area of each type of lesion per mouse was expressed as average area of each lesion per section (lower panel, @ significantly different from C, $P < 0.05$, log transformation and regression analysis). Data represented as Mean ± SD of three observations.

and 10th wk but not in 18th wk in P + C group as compared to its time matched carcinogen group (Figure 4A). In spite of time-related and polyphenol mediated decrease in levels of Cox-2, it never attained the levels observed in respective vehicle control groups (Figure 4A). Observed relative differences in Cox-2 expression by immunoblotting were in good agreement with the expression levels observed in immunohistochemical staining of tissue sections. PBPs mediated decrease in carcinogen-induced Cox-2 levels suggests anti-inflammatory activity of PBPs (Figure 4B).

PBPs Inhibited B(a)P and NNK Mediated Cell Proliferation

To assess the effect of PBPs on B(a)P and NNK mediated cell proliferation in sequential lung carcinogenesis, lung tissues were analyzed for the cell proliferation marker, proliferating cell nuclear antigen (PCNA) at 4th, 10th, and 18th wk post-carcinogen treatment. Vehicle and polyphenol controls showed no significant difference in PCNA levels at any time point studied.

Carcinogen treatment resulted in significant increase in levels of PCNA when compared to respective vehicle control and it was significantly higher with passage of time (18th > 10th > 4th wk). PBPs treatment resulted in significant decrease in PCNA levels in P + C groups at all time points as compared to its respective carcinogen group, showing higher decrease at later time points (18th > 10th > 4th wk). In spite of PBPs mediated decrease in PCNA; the levels were relatively higher in P + C group as compared to vehicle control group at respective time points. Relative differences in protein expression were further substantiated by immunohistochemical staining in lung tissue sections depicting significant decrease in PBPs mediated enhancement in PCNA during B(a)P and NNK-induced lung carcinogenesis (Figure 4B). PCNA index was unaltered in vehicle and polyphenol control groups at all the three study weeks. In C and P + C groups, PCNA labeling index was calculated in both non-lesion and lesion(s) (hyperplasia, AAH, and adenoma) areas. In non-lesion areas carcinogen-induced proliferation was observed by increase in PCNA index at 4th, 10th, and 18th wk. PBPs reduced the carcinogen-induced cell proliferation which was evident by decrease in proliferation index in non-lesion area at all the time points (Figure 4C). In lesion areas, carcinogen-induced increase in PCNA index was observed in hyperplasia, AAH, and adenoma at 10th and 18th wk. PBPs significantly decreased carcinogen-induced proliferation by observed decrease in PCNA index in all the lesions, however, decrease in PCNA index in AAH was not found to be statistically significant at 10th wk (Figure 4C). Time-related increase in proliferation index was observed in the lesion area at 10th and 18th wk in carcinogen treated group (adenoma > AAH > hyperplasia) as compared to vehicle control, which is notably higher than that of the non-lesion areas. It is important to note that observed increase in proliferation index in non-lesion area in carcinogen group was twofold higher than that in vehicle control. However, in lesion areas the increase in proliferation index in carcinogen group was 4–5 fold higher than that of its respective vehicle control. Interestingly, treatment of PBP significantly reduced proliferation in both lesions as well as non-lesion areas compared to its time matched carcinogen group, however, observed reduction was pronounced in lesion areas. Hence, PBPs mediated decrease in PCNA levels in P + C group demonstrate anti-proliferative activity of PBPs.

PBPs Activated B(a)P and NNK Mediated Apoptosis

The levels of proapoptotic protein, Bax, and antiapoptotic protein Bcl-2 were evaluated at 4th, 10th, and 18th wk post-carcinogen treatment. Levels of Bax in vehicle and polyphenol control groups were not significantly different at any of the time points. Bax expression in carcinogen treated animals showed increase from 4th to 18th wk as compared to respective vehicle control group. Like PCNA, Bax expression in carcinogen treated animals showed time-dependent increase from 4th to 18th wk. PBPs treatment further enhanced carcinogen-induced Bax expression at all time points studied as compared to only carcinogen treated mice (Figure 5A). Levels of Bcl-2 were similar in vehicle or polyphenol control groups at various time points studied. Bcl-2 expression was higher in carcinogen treated group as compared to its respective vehicle control at all the time points. Similar to Bax, time-dependent overexpression of Bcl-2 was observed in carcinogen treated animals as compared to respective vehicle control. Bcl-2 expression showed significant polyphenol mediated decrease in P + C group as compared to only carcinogen treated group at all the time points studied (Figure 5A). As relative difference cannot be accurately judged by measurement of individual proapoptotic Bax or antiapoptotic Bcl-2, Bax/Bcl-2 ratio was calculated. Ratio of Bax/Bcl-2 was similar in vehicle or polyphenol control groups. Carcinogen treated group showed significant decrease in the ratio compared to respective vehicle control while PBPs treatment showed significant increase compared to only carcinogen treated group at various time points studied. PBPs mediated increase in the Bax/Bcl-2 ratio was the effect of relatively more pronounced increase in proapoptotic Bax expression consistently at all the time points (Figure 5A). Concomitant to immunoblotting, relative differences in levels of Bax and Bcl-2 were further substantiated by immunohistochemical staining of lung tissue section confirming the proapoptotic activity of PBPs (Figure 5B). Apoptotic index was unaltered in vehicle and polyphenol control groups at all the three study weeks. Time-dependent increase in apoptotic index was significantly high in carcinogen treated group (week 4 < week 10 < week 18)

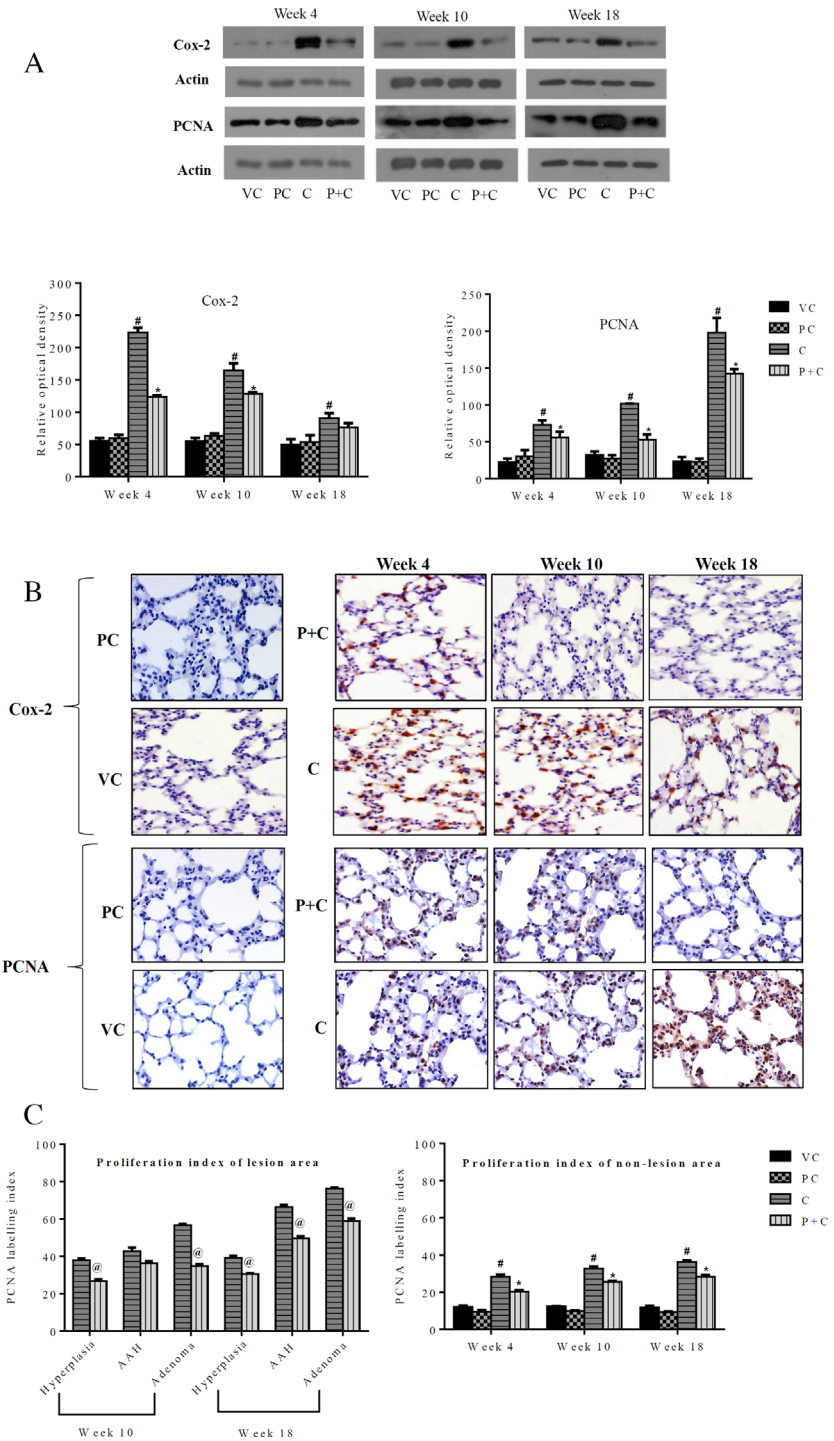


Figure 4. Effect of PBPs on B(a)P and NNK mediated alterations in inflammation and cell proliferation markers in A/J mice. A) Representative blots and relative densitometric levels of PCNA and Cox-2 proteins at various sacrifice weeks (4th, 10th, and 18th wk) in lung total cell extract analyzed by immunoblotting. Beta actin was used as a loading control. Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5% black tea-derived PBPs), carcinogen (3 μM, B(a)P, and NNK each + drinking water), polyphenol + carcinogen (3 μM B(a)P and NNK each + 1.5% black tea-derived PBPs); Data represented as Mean ± SD of three observations. #, significantly different from VC; *, significantly different from C ($P < 0.05$, ANOVA followed by Bonneferoni's correction). B) Representative photomicrographs showing immunohistochemical detection of PCNA and Cox-2 positive cells (magnification at 200×) at various sacrifice weeks (4th, 10th, and 18th wk) in formalin fixed, paraffin embedded lung tissue sections. C) PCNA index was calculated as number of positively stained cells × 100/total number of cells. Semi quantitative analysis was conducted by Image J 1.43 (NIH) software by counting the brown-stained nuclei in photomicrographs of at least 10 different randomly selected fields from lesion and non-lesion areas. Data represent Mean ± SD of three observations. #, significantly different from VC; *, significantly different from C ($P < 0.05$, ANOVA followed by Bonneferoni's correction).

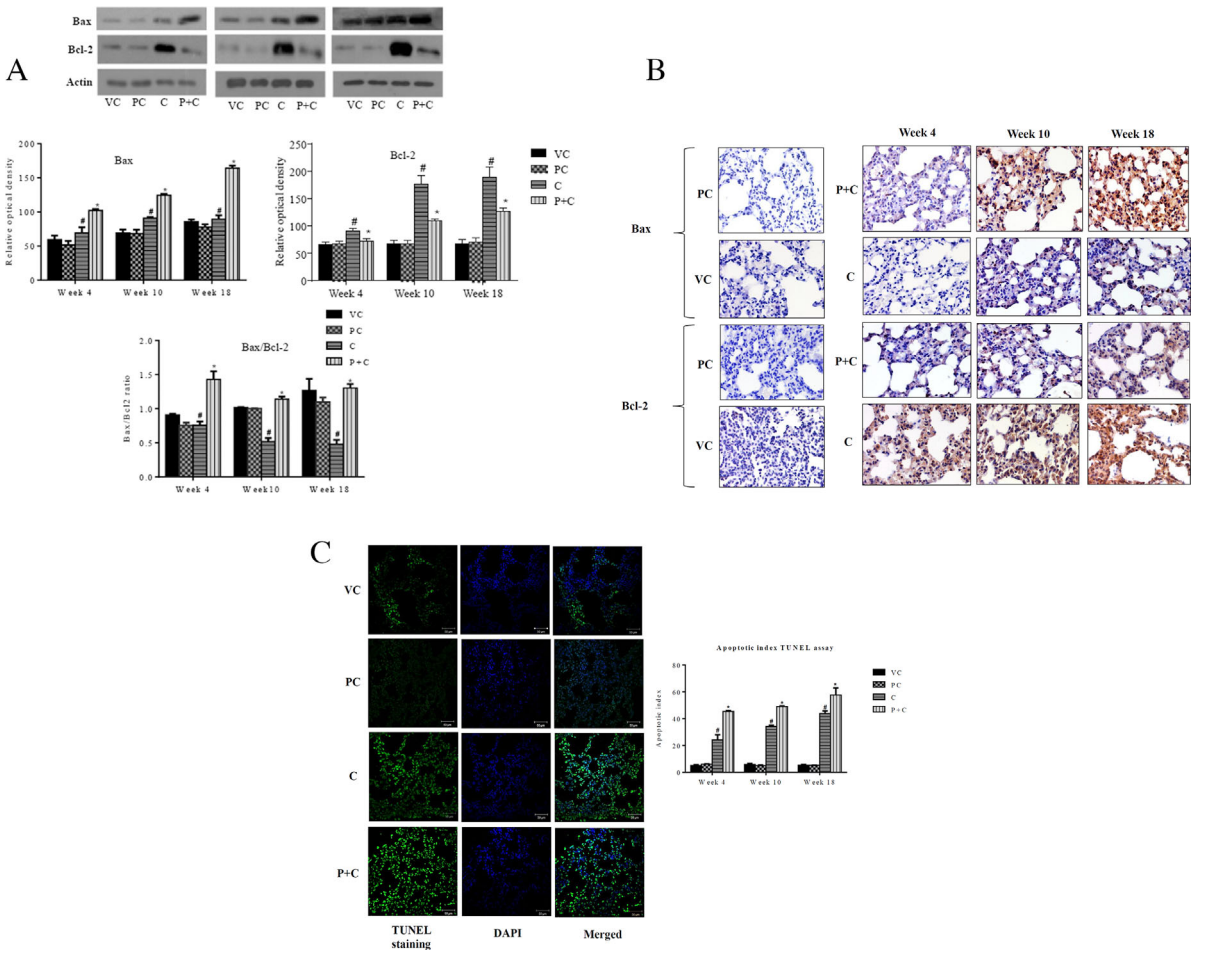


Figure 5. Effect of PBPs on B(a)P and NNK mediated up regulation in apoptosis in A/J mice. A) Representative blots and relative densitometric levels of proapoptotic marker, Bax, and antiapototic marker Bcl-2 at various sacrifice weeks (4th, 10th, and 18th wk) in lung total cell extract analyzed by immunoblotting. Beta actin was used as a loading control. Extent of apoptosis in lung tissues was determined by calculating the ratio of normalized band density of Bax and Bcl-2 in each treatment groups. Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5% black tea-derived PBPs), carcinogen (3 μM B(a)P and NNK each + drinking water), polyphenol + carcinogen (3 μM B(a)P and NNK each + 1.5% black tea-derived PBPs); Data represented as Mean ± SD of three observations. #, significantly different from VC; *, significantly different from C ($P < 0.05$, ANOVA followed by Bonneferoni's correction). B) Representative photomicrographs showing immunohistochemical detection of proapoptotic marker, Bax and antiapoptotic marker, Bcl-2 positive cells (magnification at 200×) at various sacrifice weeks (4th, 10th, and 18th wk) in formalin fixed, paraffin embedded lung tissue sections. C) Representative photomicrographs showing fluorescence detection of apoptotic cells using TUNEL assay in formalin fixed, paraffin embedded tissues of lung (magnification 400×). The apoptotic index was calculated by counting the cells with positively stained nuclei × 100/total number of cells in photomicrographs of lung sections of at least 10 different randomly selected fields (~1,000 nuclei/mice), with at least three mice per group. Apoptotic index represented graphically at all three study weeks (4th, 10th, and 18th wk). Data represent Mean ± SD of three observations. #, significantly different from VC; *, significantly different from C ($P < 0.05$, ANOVA followed by Bonneferoni's correction).

as compared to its respective vehicle control. The observed increase in apoptotic index in carcinogen group was 5–7 fold higher than its respective vehicle control. PBPs treatment further enhanced the apoptotic index in P + C group which was evident from statistically significant increase in apoptotic nuclei at all the time points. The increase in the apoptotic index in P + C group was about 1.5 fold higher than that of its respective carcinogen group (Figure 5C). Hence, PBPs mediated modulation in Bax and Bcl2 expression along with increase in apoptotic index in P + C group demonstrate apoptotic activity of PBPs.

PBPs Modulated Activation of p38 and Akt in NNK and B(a)P

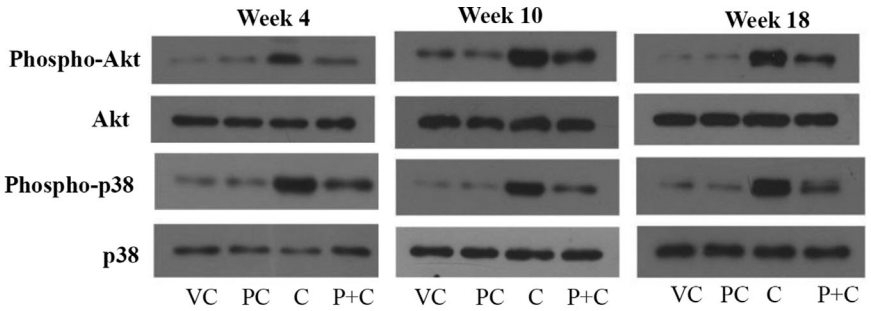
PBPs have been observed to modulate cell proliferation, apoptosis, and inflammation. In order to delineate the potential mechanism of PBPs-mediated effect on cell proliferation and apoptosis via modulation of cellular kinases in B(a)P and NNK-induced lung carcinogenesis, we assessed phosphorylated and non-phosphorylated forms of major downstream kinases, p38 and Akt. Expression of non-phosphorylated forms of p38 and Akt remained unchanged at all the time points in all the animal groups (Supplementary

Figure S2). However, significant increase was observed in the phosphorylated forms of p38 and Akt in carcinogen treated group from 4th to 18th wk post-carcinogen treatment as compared to its respective vehicle control (Figure 6). It is noteworthy, that time-dependent increase in phosphorylation of p38 and Akt was observed in post- carcinogen treatment groups from 4th to 18th wk similar to that observed in case of PCNA and Bax. PBPs treatment resulted in decrease in the phosphorylated p38 and Akt when compared to only carcinogen treated groups from 4th to 18th wk. In spite of PBPs-mediated decrease in phosphorylated p38 and Akt, their levels were higher than those in vehicle controls. The observed differences in the levels of proteins involved in cellular proliferation and apoptosis along with signaling kinases suggest anti-tumorigenic potential of PBPs via modulation of kinase function.

DISCUSSION

In the present study, chemopreventive efficacy of orally administered PBPs was evaluated by measuring its effect on incidence/multiplicity and/or delay in the latency period of both macroscopic and microscopic lung tumors in B(a)P and NNK-induced lung carcinogenesis in A/J mice model. The number as well as area of the microscopic lung lesions like hyperplasia, AAH, and adenoma was analyzed as per the recommendations and guidelines by Nikitin et al. [21].

We demonstrated for the first time that PBPs (thearubigins), one of the major black tea polyphenols, when administered orally decrease macroscopic lung tumor multiplicity as well as multiplicity and area of microscopic lung lesions induced by B (a)P and NNK (tobacco carcinogens) at 10 and 18 wks post-carcinogen treatment. The observed decrease in tumor multiplicity and/or lesion areas have not attained the statistical significance at early time points possibly because of the: (i) low tumor numbers; (ii) very small tumor size; and (iii) possible variation in the doses of PBPs received by animals due to intra group variation in uptake of drinking water resulting in variable doses of PBPs. However, PBPs administration did not affect the incidence of B(a)P and NNK-induced lung tumors and latency period. Similar observations were reported previously in other studies



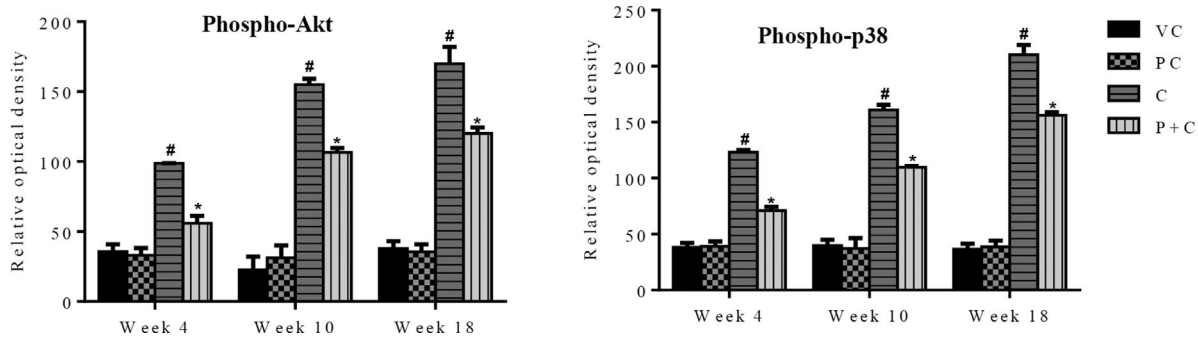


Figure 6. Effect of PBPs on B(a)P and NNK mediated activation of p38 and Akt kinases in A/J mice. Representative blots showing the protein levels of p38, Akt, and phospho-p38 and phospho-Akt in lung total cell lysates. Protein levels of phospho-p38 and phospho-Akt were normalized to that of respective total kinase. Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5% black tea-derived PBPs), carcinogen (3 μM B(a)P and NNK each + drinking water), polyphenol + carcinogen (3 μM B(a)P and NNK each + 1.5% black tea-derived PBPs); Data represented as Mean ± SD of three observations. #, significantly different from VC; *, significantly different from C ($P < 0.05$, ANOVA followed by Bonneferoni's correction).

using different chemopreventive agents like phenethyl isothiocyanate, butylated hydroxyanisole, myo-inositol, resveratrol, and lycopene which affect tumor multiplicity without affecting incidence [8,25]. Oral administration of 2% crude black tea extract and 0.6% decaffeinated black tea extract (mixture of free catechins, theaflavins, and PBPs) have resulted in the decrease in the tumor multiplicity by 31% and 65%, respectively, without affecting the tumor incidence in NNK-induced lung carcinogenesis model [26,27].

Earlier reports on crude black tea extract has been shown to be affecting various pulmonary microscopic lesions like hyperplasia while, Polyphenone-E, and caffeine have been shown to reduce progression from lung adenoma to adenocarcinoma in experimental model [4,28]. In our study, PBPs significantly decreased number and size of both hyperplasia and adenomas at all the time points studied indicating PBPs act on both early as well as late proliferative lesions in lung carcinogenesis process. However, observed decrease in AAH did not attain the statistical significance due to low occurrence and small size of the foci.

In our study, administration of 1.5% black tea-derived PBPs have decreased the lung tumor multiplicity by ~21%. This observation when compared to reported chemopreventive activity of various crude and decaffeinated extracts of black tea [26,27] suggest PBPs to be major contributory components in black tea mediated chemopreventive activity. PBPs have been reported to be heterogeneous polymers of flavano-3-ols and flavan-3-ol gallates with di- and tri-benzotropolone skeletons [11,12]. Ideally, demonstration of presence and evidence correlating circulating or tissue levels of PBPs and/or their metabolic products with biological activity is needed to attribute observed chemopreventive effects to PBPs, however, currently the information on structural/molecular characterization and methods for detection of PBPs and/or their products are not available due to complexity of oxidation product (PBPs) comprised of unknown numbers of isomeric structures [11,29,30]. Additionally our attempts to detect exposure related signals in MALDI-TOF analyses of sera from exposed animals were not successful probably because of very low levels of circulating PBPs/ metabolites. However, observed PBPs-mediated increase in expression of protein and activity of GST in lung suggest its exposure to parent PBPs and/or their metabolic products after oral administration of PBPs. Under the circumstances, since the exposure to PBPs was the only variable in our study and as MALDI-TOF analyses of PBPs extract used in our study has demonstrated them to be free of other biologically active black tea-derived contaminants (e.g., caffeine, catechins, theaflavins), observed chemopreventive activity could be attributed to PBPs.

In our experiment, oral administration of PBPs was 2 wks prior to carcinogen treatment, during 8 wks of carcinogen exposure and subsequent to the carcinogen treatments hence observed chemopreventive effects can be interpreted to be due to both anti-initiation as well as anti-promotion activity of PBPs. However, chances for predominant effects of anti-promotion activity of PBPs are more likely due to prolonged treatment of PBPs after cessation of carcinogen treatment. Similarly, observed chemopreventive effects can not only be attributed to to parent PBPs as contribution of metabolic products of PBPs cannot be ruled out, hence observed bioactivity is likely to be due to PBPs and/or their products. To the best of our knowledge, this is the first report demonstrating chemopreventive effects of major black tea polyphenols, PBPs/ thearubigins exerting the chemopreventive effects on both macroscopic as well as microscopic lung lesions by decreasing the tumor multiplicity.

As reported in the literature, A/J mice are known to develop spontaneous lung tumors [31]. One notable difference in our observation is none of the control groups developed visible or microscopic spontaneous lung tumors till the end of experiment, that is, 36 wks of age. A/J mice used for all the previously reported studies, including our study, were procured from Jackson Laboratory (Bar Harbor, ME). After procuring, A/J mice were bred in the Lab Animal Facility, ACTREC and 38–42 generation was used for the present study. Ruling out all the possibilities, the diet employed for the animals is the only potential factor which can influence absence of spontaneous lung tumors. In previous studies, AIN-76/AIN-93 synthetic diet was used wherein spontaneous lung tumors were observed even at 16 wks of age [31–35]. However, diet employed in our study is majorly plant derived (composition of both the diets is given in supplementary data). Plant-derived diets are known to induce the Phase II metabolizing enzymes which are responsible for conjugation reaction and delay the spontaneous or carcinogen- induced tumors [18]. Despite using plant-derived diet; demonstration of significant chemopreventive activity attributable to PBPs exposure suggest that PBPs mediated chemopreventive effect would have been pronounced if synthetic diet devoid of any chemopreventive agents was used.

To explain the observed decrease in lung tumor multiplicity and to understand the mechanism of chemoprevention by PBPs, we have also analyzed molecular markers of inflammation, cell proliferation, and apoptosis. In our study, Cox-2 expression was found to be modulated by PBPs at all the time points studied. Interestingly, anti-inflammatory effect of PBPs was more pronounced at early weeks as compared to late weeks. Previous studies have shown that dietary administration of crude black tea polyphenols modulates the AOM-induced Cox-2 and iNOS gene expression in tumors [36]. Similarly, black tea extract rich in theaflavins (TFs) and EGCG has been shown to inhibit B(a)P-induced Cox-2

expression in mouse lungs [37]. We have also observed modulation of process of proliferation and apoptosis by PBPs in sequential lung carcinogenesis. Decrease in PCNA along with Bcl-2 down regulation and Bax overexpression occurred progressively, during lung tumorigenesis with PBPs treatment. It is noteworthy that we observed decrease in PCNA expression/proliferation index as well as modulation of Bax/Bcl-2 ratio and apoptotic index even before the appearance of first microscopic tumor, which further reassured the anti-promotion efficacy of PBPs in B(a)P and NNK-induced lung carcinogenesis. However, it is important to note that despite of significant decrease in carcinogen-induced proliferation index by PBPs, it never matched with vehicle control. Thus, PBPs decreased carcinogen-induced cell proliferation and increased apoptosis which in turn is responsible for observed decrease in lung tumor multiplicity and lesion areas. Our results showing decrease in proliferative index and increase in apoptosis are consistent with previous reports on theaflavins and caffeine in NNK-induced lung carcinogenesis model [28]. Furthermore, dietary administration of black tea polyphenols decreased the DMBA-induced Bax/Bcl-2 ratio in hamster buccal pouch carcinogenesis model [38]. Thus, PBPs could be one of the potential contributing components in observed anti-promotion mechanism of whole black tea extract.

It has been reported that EGFR and K-ras mutations are mutually exclusive genetic events associated with development of lung carcinogenesis [39,40]. p38 mitogen-activated protein kinase (MAPK) family members function in a cell context-specific and cell type-specific manner to integrate signals that affect proliferation, differentiation, survival, and migration [41,42]. p38 MAPK plays a dual role as a regulator of cell death, and it can either mediate cell survival or cell death depending not only on the type of stimulus but also in a cell type specific manner [43]. Mutations in MAPK pathways are frequently affecting Ras and in p38 extracellular signal-regulated kinase pathway [44]. However, limited evidence is available about association between Akt and K-ras mutations in lung carcinogenesis.

Studies on EGCG and theaflavins in various model systems have demonstrated modulation of MAP kinases and Akt/PKB signaling pathway [45]. Previously, pre-treatment of mouse skin with PBPs has shown to decrease the TPA-induced phosphorylation of ERK and p38 during tumor development, while expressions of total ERK and p38 were unaltered indicating that signaling kinase functions are one of the targets for chemoprevention by PBPs [15]. Interestingly, our results showed significant decrease in phosphorylated forms of p38 and Akt at post-carcinogen treatment weeks which are in agreement with observed decrease in the various biomarkers of cell proliferation and enhancement of apoptosis described earlier, suggesting modulation by the signaling kinases. It gives suggestive evidence that PBPs either as a parent compound or its metabolic products with differences in structure and molecular weights (when compared to monomeric catechins) also retain ability to modulate carcinogen-induced signaling kinase functions.

B(a)P and NNK-induced lung carcinogenesis model in A/J mice has been extensively used to understand the complex interactions of carcinogens and to test the effect of various chemopreventive agents with varying dose, duration, and route [32–35]. Administering B(a)P and NNK by i.p. injections offered several advantages such as: (i) effectively delivers the complete dose of carcinogen; (ii) avoids direct interaction with PBPs which are administered orally; and (iii) prevents development of the fore stomach tumors as in case of gavage [46]. Despite the fact that defined doses of B(a)P and NNK were administered by i.p. route; while PBPs was given through drinking water ad libitum resulting in absorption and uptake of probably part or low levels of PBPs continuously over a period of time (resulting in very low levels of circulating PBPs and/or its products), detectable effects were observed at different time points studied. Future studies on measurement of circulating or tissue levels of parent (PBPs) or product (metabolites of PBPs) compounds will enhance our understanding about modulation of various biomarkers in the chemopreventive trials. Since, we have used single dose of PBPs, minimal effective dose cannot be ascertained from the given study. However, dose response study employing various doses of PBPs will further address the issues about absorption and bioavailability.

Thus by using B(a)P and NNK-induced lung carcinogenesis model, we deciphered the chemopreventive efficacy of PBPs evident by decrease in the tumor multiplicity as well as lower the number and size of the microscopic lesions (hyperplastic and adenomatous foci) at various time points studied. Our study further attempted to understand the molecular mechanisms underlying the given chemopreventive efficacy exhibited by PBPs which is apparent by marked reduction in the inflammation, cell proliferation, and increase in the apoptosis may be possibly governed by lowered phosphorylation of p38 and Akt. Our study further suggests that black tea interventions offered throughout the life are likely to present an effective approach in decreasing carcinogen-induced cell proliferation while increasing the probability of initiated cell being apoptosised. Hence, effect of PBPs administration through drinking water suggests that polymeric and relatively high molecular weight compounds or their products are getting absorbed/metabolized and exert their chemopreventive effect through various pathways including signaling kinase function. Similar mechanistic studies using various model systems with bioavailability and pharmacokinetics features will further help in

elucidating the cellular targets which can give the meaningful information for monitoring the clinical chemoprevention trials.

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AUTHORS' CONTRIBUTIONS

Conception and designing the experiments: RRH, GBM, and MBM. Development of methodology: RRH, SD, RK, ADI, GBM, MBM. Acquisition of data: RRH, VBV, RAT, SD, RK, ADI. Analysis and interpretation of data: RRH, VBV, SD, RK, ADI, SK, and MBM. Writing, review and/or revision of the manuscript: All authors. Administrative, technical, or material support: MBM. Study supervision: GBM and MBM.

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