Am J Transl Res 2023;15(9):5826-5834
www.ajtr.org /ISSN:1943-8141/AJTR0152169

Original Article

Thearubigins/Polymeric black tea polyphenols (PBPs) do not prevent benzo[a]pyrene (B[a]P) induced lung tumors in A/J mice

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Received July 12, 2023; Accepted September 15, 2023; Epub September 15, 2023; Published September 30, 2023

Abstract: Objectives: Previously we have demonstrated the chemopreventive effect of Thearubigins/Polymeric Black-tea Polyphenols (PBPs) upon pre-treatment to a combination of carcinogens, that is, Benzo[a]pyrene (B[a]P) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) which are present in Tobacco smoke (TS). However, the chemopreventive effect in response to B[a]P as a single carcinogen remains unexplored. B[a]P is a universal carcinogen and an important constituent of particulate matter 2.5 ($PM_{2.5}$) found in the environment and in TS. Methods: We investigated the pre-treatment of Thearubigins/PBPs as a chemopreventive agent at three doses (1.5, 5, 10%) against B[a]P-induced lung carcinogenesis at early & late time points. We also investigated the effect of PBPs at early time points to understand molecular changes by employing western blotting in xenobiotic metabolism pathways, DNA damage, inflammation, apoptosis, and proliferation as they are modulated in response to carcinogens. We used 6-8 weeks male A/J mice for tumorigenicty and western blotting to probe the molecular biomarkers. Results: We report no decrease in tumor incidence and multiplicity upon pre and concurrent treatment of Thearubigins/PBPs. Further, we also report no changes in molecular markers at early time points, in agreement with former observations. Conclusion: Our results suggest that chemopreventive agents need to be tested with different combinations of carcinogens and regimens to fully understand the complex interplay between carcinogenesis and the efficacy of chemopreventive agents. Studies like these will provide meaningful data before initiating large-scale clinical trials.

Keywords: Thearubigins, benzo[a]pyrene, A/J mice, experimental lung cancer, chemoprevention, CYP1A1/NQO1

Introduction

The two most extensively studied classes of carcinogens are Polycyclic Aromatic Hydrocarbons (PAHs) and tobacco-specific nitrosamines (TSNAs), which are involved in the manifestation of aerodigestive tract cancers, majorly lung cancers. Benzo[a]pyrene (B[a]P) belongs to the PAH class, which is formed chiefly because of incomplete combustion (of fuels, wood, plastic), high-temperature cooking, and also as a food contaminant [1]. Recently, the International Agency for Research on Cancer (IARC), U.S. Environmental Protection Agency (USEPA), and European Union (EU), in concordance, proposed a list of 16 priority PAHs to be frequently tested for occurrence and carcinogenic effects in food. B[a]P is the only PAH classified as a Group-1 carcinogen (carcinogenic to humans) by IARC and is used as an exposure marker for risk assessment [2]. In contrast, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-Nitrosonornicotine (NNN), which majorly constitute the TSNAs, are only found in the smoke and smokeless forms of tobacco and are also designated as Group-1 carcino-

gens [3]. The origin of PAHs is more from man-made sources (domestic, industrial, mobile, and agricultural) compared to natural ones like (forest/wildfires and volcanic eruptions) [4]. An aerosol-monitoring station from Japan reported that the seasonal averages of 15 priority PAH concentrations were highest in winter and lowest in summer [5], pointing to the trend that the exposure to these PAHs does not occur at a constant concentration but rather alternating between high & low PAHs concentration. Additionally, a recent U.S. study reported a stark correlation between the number of deaths and the intensity of the wildfire smoke particulate matter 2.5 ($PM_{2.5}$). Of all the reported deaths attributed to smoke $PM_{2.5}$, respiratory health ailments were among the top three causes [6]. Natural sources of PAH form the primary exposure route for those Non-smokers who are not exposed to passive smoking (which contains both PAHs & TSNAs). This is the major differentiating factor between a non-smoker exposed to environmental PAHs and one exposed to passive tobacco smoking. Our study aimed to test chemoprevention against these at-risk non-smokers who are not exposed to tobacco smoke but rather only to environmental PAHs, thus warranting the use of single and potent PAH carcinogen-B[a]P.

Earlier, we demonstrated the chemopreventive potential of Thearubigins/PBPs on B(a)P and NNK-induced lung tumors in AJ mice. We observed a significant reduction in tumor multiplicity upon pre and continued treatment with Thearubigins at three doses (0.75, 1.5 and 3%). We also reported that the molecular changes occurred in the p38 and pAKT (mitogen-activated growth) pathways and might be one of the key pathways in inhibiting tumorigenicity [7, 8]. In this study, we have tested the effect of pre-treatment of Thearubigins on B[a]P-induced early stages of lung carcinogenesis and the effect on lung tumors in A/J mice.

Materials and methods

PBP's (Thearubigins) extraction from black tea & analysis

Black tea powder was purchased from the regional brand (Red Label, Brooke Bond, Mumbai, India). Soxhlet extractor (Borosil Glass Works Ltd., Mumbai, India) was used for the extraction of Polymeric Black-tea Polyphenols (PBP's)/Thearubigins according to a previously published protocol [9]. Extracted Thearubigins were boiled in autoclaved distilled water to make a 10% stock, from which water dilutions prepared 1.5, 5 & 10% solutions. MALDI-TOF was performed to validate further that PBP's were free from Caffeine, EGCG, and Theaflavins as published previously [7]. The detailed methodology for the isolation and characterization of PBPs is provided in Supplementary Tables 1, 2 and Supplementary Figure 1, respectively.

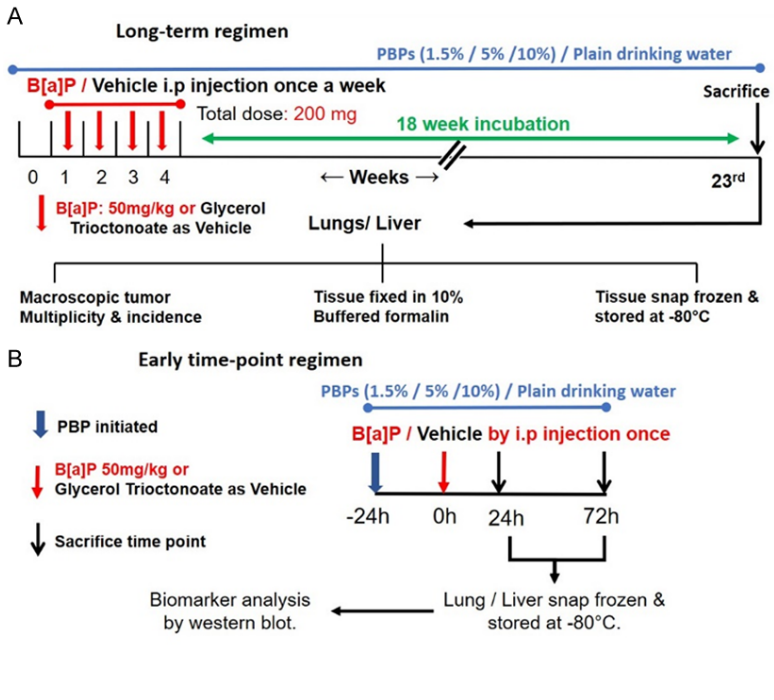
Reagents & consumables

Benzo[a]pyrene (B[a]P), Catalog No: B1760-1G (Purity >96%) and Glycerol Trioctonoate Catalog No: T9126 (Purity >99%) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Details for the primary antibodies are given in Supplementary Table 3. A secondary antibody, anti-rabbit HRP-conjugated, was purchased from GE Healthcare (Chicago, Illinois, USA). Clarity western ECL substrate for western blot visualization was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Animal experiments

Male A/J mice (also known as Strain A, RRID: IMSR_JAX:000646), 6-8 weeks old (~weight range 18-22 g), procured from the Jackson Laboratory, USA, were maintained at Laboratory Animal Facility of ACTREC under standard conditions of 12 h light/dark cycle and were used in all the animal experiments. All studies were conducted as per the IAEC guidelines (IAEC Project No: 02/2020).

Tumorigenicity experiment (long-term): This study was set up in a pre-treatment setting wherein 3 doses of PBP's/Thearubigins (1.5%, 5% & 10%) were given orally 1 week before carcinogen administration and continued until the sacrifice. Mice were injected with 0.1 mL (50 mg/kg) of B[a]P (carcinogen) or Glycerol Trioctonoate (vehicle control) intraperitoneally (i.p.) once a week for 4 weeks under the close supervision of a veterinarian. A total B[a]P dose of 200 mg/kg (50 mg/kg × 4 weeks) was administered for four weeks (**Figure 1**). There was a total of 8 groups (n=15/group). The following 4 groups were the control groups; Vehicle control (VC) and PBP's Control (3 groups at different doses): (1.5PC, 5PC, and



Groups (N=15/group)	Compound administered
Vehicle Control (VC)	Water + Vehicle
1.5 P	1.5% PBP + Vehicle
5 P	5% PBP + Vehicle
10 P	10% PBP + Vehicle
Carcinogen (C)	Water + B[a]P
1.5 P+C	1.5% PBP + B[a]P
5 P+C	5% PBP + B[a]P
10 P+C	10% PBP + B[a]P

Figure 1. Chemoprevention regimen for two different experiments. (A) Shows the experimental setup for Long-term chemoprevention regimen along with the tabulated groups on the right side. (B) Depicts the experimental design for the short-term chemoprevention experimental regimen tabulated groups on the right side.

10PC). The remaining were the treatment groups; Carcinogen group (C) and PBP's + Carcinogen (at 3 different doses viz. 1.5P+C, 5P+C, and 10P+C). Mice were given freshly prepared PBP's every week. PBP's and water consumption were recorded in the 1st week. The weight of the mice was monitored weekly, and they were sacrificed 18 weeks after the last injection of carcinogen. The total duration of the experiment was 23 weeks (**Figure 1A**).

Mice were anesthetized by isofluorane, followed by retro-orbital blood collection. Further, cervical dislocation was performed by following institutional animal ethics guidelines. Lungs/Liver were collected, tumors were counted and then fixed in 10% Neutral buffered formalin (NBF) and snap-frozen in liquid N_2 (and stored at -80°C). Tumor enumeration was done double-blinded by a veterinarian under the Stereo microscope (Model: Stemi Z-DV4, ZEISS, USA).

Early time point experiment (short-term): Male A/J mice (6-8 weeks) were administered 3 different doses of PBP (1.5%, 5% & 10%) orally 1 - the day before carcinogen administration & continued throughout till the sacrifice time point (24 h/72 h). B[a]P (carcinogen) (0.1 ml of 50 mg/kg) or Glycerol Trioctonoate (vehicle control) was administered by *i.p.* injection. There were 8 groups (n=5/group): VC, 1.5PC, 5PC, 10PC, C, 1.5P+C, 5P+C, 10P+C; the first four groups being control and the last four being treatment groups. Mice were sacrificed at two different time points: 24 hours and 72 hours after carcinogen treatment. Weights were recorded before and after the carcinogen injection. Lungs/Liver were collected, as mentioned above (**Figure 1B**).

Tissue lysis and protein estimation

Lungs (~120 mg) stored at -80°C were thawed on ice and minced by sterilized scissors or surgical blades. Minced tissue was added to 1 mL of lysis buffer and homogenized by a handheld homogenizer (Kinematica Polytron 1200E), keeping the lysates on the ice during the process. The lysates were incubated for 30 min on ice after homogenization to ensure proper lysis. The lysates were centrifuged at 15000 rpm (4°C for 20 mins) on a benchtop centrifuge (Eppendorf 5424R) to settle the cell debris and

nucleic acids. The supernatant was aliquoted and stored at -80°C for protein estimation and western blotting. Protein estimation was performed by the Folin-Lowry method for equal loading of proteins in western blotting.

Western blotting

The lung tissue lysates from the early time point (24 and 72 hrs) and late time point experiments were used in western blotting and the protocol was taken from Biorad laboratories (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6376.pdf). Approximately 20-30 ug of protein was loaded into each well of the polyacrylamide gel (Stacking: 4%, Resolving: 10%). Electrophoresis was done at 100 V for 100 mins at a constant voltage or until the tracking dye reached the end of the gel. Biorad Mini-protean cell was used for the wet transfer of all the proteins. Pre-chilled 1× transfer buffer was used for transferring proteins to the PVDF membrane (0.22 µm) at 90 V for 90 mins (4°C). Transfer efficiency was checked by Ponceau staining the membrane. Blots were cut and further blocked in the 5% Non-fat dry milk solution slowly on a rocker for 1 hr/Room temperature. They were then incubated overnight (4°C) in a primary antibody prepared in 3% BSA on a rocker (gentle shaking). Blots were then washed and incubated (25°C, 1 hr) with a secondary antibody prepared in 5% Non-fat dry milk solution, washed, and eventually developed with ECL (Biorad Clarity Max) as a substrate in Chemidoc Imager (Biorad). Following protein biomarkers were probed using primary antibodies: Beta-actin (internal control), proliferating cell nuclear antigen (PCNA: proliferation), cyclo-oxygenase-2 (COX-2: inflammation), poly ADP-ribose polymerase (PARP: DNA damage), Nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (KEAP1) both are antioxidant response biomarkers, Bcl-2 Associated X-protein (BAX) and B-cell lymphoma 2 (BCL2) both are apoptosis biomarkers, Cytochrome P450 Family 1 Subfamily A Member 1 (CYP1A1), NAD(P)H: Quinone Oxidoreductase 1 (NQO1) and Glutathione S-Transferase-isoform Mu (GST-Mu), all three are biomarkers of xenobiotic metabolism.

Statistical analysis

Data were analysed for normality using Shapiro Wilk normality test. One-way ANOVA was performed for multiple group comparisons, and a p-value <0.05 was considered as statistically significant. Power calculation was carried out using Balanced 1-way ANOVA, and threshold was set at 80% to ensure detection of minute differences. A paired sample t-test was used to check the differences between initial and final weights. Correlation analysis was performed using Pearson correlation. Statistical analysis was performed using SPSS v25.0 and graphs were plotted in GraphPad Prism 9.0. All values are represented as Mean ± S.D.

Results

Tumorigenicity experiment (long-term)

The control groups (VC, 1.5PC, 5PC, and 10PC) showed no macroscopic lung tumors/lesions. In contrast, the treatment groups which were administered B[a]P (C, 1.5P+C, 5P+C, 10P+C) showed lung tumors (Supplementary Figure 2). We did not observe any changes in fur coat, forestomach tumors, and mortality during the experiment (**Table 1**). We observed an excellent consistency in the consumption of PBPs similar to water in the 1st week of oral administration (Supplementary Table 4) and also remained similar throughout the experiments when measured weekly (Supplementary Figure 3). Additionally, all the groups showed a slight reduction in weight during the injection weeks but eventually recovered to be healthy (Supplementary Figures 4 and 5).

The study was well-powered (89.6%) to detect minor differences, and outliers were detected in C and 1.5P+C groups. However, they were not excluded from statistical analysis as they resulted from experimental observations (Supplementary Table 5). However, we did not find any statistically significant differences when comparing lung tumor numbers between the following treatment groups: C, 1.5P+C, 5P+C, and 10P+C (**Table 2**). Furthermore, to understand if there are any trends in tumor numbers due to the influence of the mice weights, we conducted Pearson's correlation analysis. We found no correlation between the animal weights and tumor numbers, visual representation of weight vs tumor number is provided (Supplementary Figure 6).

Table 1. Comparison of incidence of lung and forestomach tumors in different experimental groups

Groups	n (Initial)	n (Final)	Mortality (%)	Incidence (%)	Forestomach tumors (Mean ± S.D.)
Controls					
Vehicle (VC)	15	15	0	100	Nil
1.5P	15	15	0	100	Nil
5P	15	15	0	100	Nil
10P	15	15	0	100	Nil
Treatment					
Carcinogen (C)	15	15	0	100	Nil
1.5P+C	15	15	0	100	Nil
5P+C	15	15	0	100	Nil
10P+C	15	15	0	100	Nil

Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5%, 5% and 10% PBPs), carcinogen (B(a)P + drinking water), polyphenol + carcinogen (1.5%, 5% and 10% PBPs + B[a]P).

Table 2. Comparison of macroscopic lung tumors between different experimental groups

Groups	PBP (P)	B[a]P 50 mg/kg ×4 (C)	Initial weight in grams	Final weight in grams	Tumor count (Mean ± S.D.)	Tumor range
Controls						
VC	-	-	19.7 ± 0.8	23.7 ± 1.3	0	
1.5P	+	-	20.0 ± 0.8	23.9 ± 1.4	0	
5P	+	-	20.2 ± 0.4	24.3 ± 0.4	0	
10P	+	-	20.2 ± 0.6	23.7 ± 0.3	0	
Treatment						
C	-	+	19.9 ± 0.8	23.7 ± 1.4	9.6 ± 4.7#	(3-24)
1.5P+C	+	+	20.4 ± 0.4	24.0 ± 0.5	7.4 ± 3.1#	(3-18)
5P+C	+	+	19.5 ± 2.5	24.8 ± 0.7	9.5 ± 4.6#	(5-18)
10P+C	+	+	20.3 ± 1.7	23.5 ± 1.0	10.1 ± 3.5#	(5-17)

Groups include Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5%, 5% and 10% PBPs), carcinogen (B(a)P + drinking water), polyphenol + carcinogen (1.5%, 5% and 10% PBPs + B[a]P). There were no statistically significant difference observed between the tumor count of Carcinogen vs P+C treatment groups (ANOVA test). Statistically significant when all carcinogen groups (carcinogen, 1.5P+C, 5P+C and 10P+C) vs compared to controls group (#, P<0.001).

Western blotting experiments of early and late time points

Additionally, we conducted a western blot analysis of each biomarker from inflammation, proliferation, and apoptosis pathways. The results indicate no significant differences observed upon intergroup analysis of all the groups. However, it is to be noted that although insignificant, only proliferation biomarker (PCNA) showed 2-fold increase in protein expression in the carcinogen-treated groups vs all the control groups (Supplementary Figure 7).

We conducted this experiment to understand whether molecular markers can predict long-term findings. We observed that the Phase I (CYP1A1) and Phase II (NQO1) enzymes were induced equally in all the treatment groups & there was no suppression or induction in the protein levels within C, 1.5P+C, 5P+C, 10P+C groups. We also analysed other relevant biomarkers, including inflammation (COX-2), proliferation (PCNA), xenobiotic metabolism (NRF2, GST-Mu (μ)), apoptosis (BAX), and DNA damage (PARP) at 24 hrs and 72 hrs. All biomarkers were done at n=5/group except PARP and GST-Mu at 72 hrs. KEAP1 was not analysed at 72 hrs. We did not observe any changes in these markers, confirming that the pleiotropic effects of PBP's are not seen in any of the pathways when challenged with B[a]P as a carcinogen (**Figures 2** and **3**). The overall power of the statistical analysis was 87%, and we report an overall significance in 2 biomarkers, CYP1A1 and NQO1. However, no statistically significant differences existed between the treatment groups in all the molecular markers analysed, including CYP1A1 and NQO1.

Discussion

For the first time, we have demonstrated that the PBP's/Thearubigins (1.5/5/10%), which are

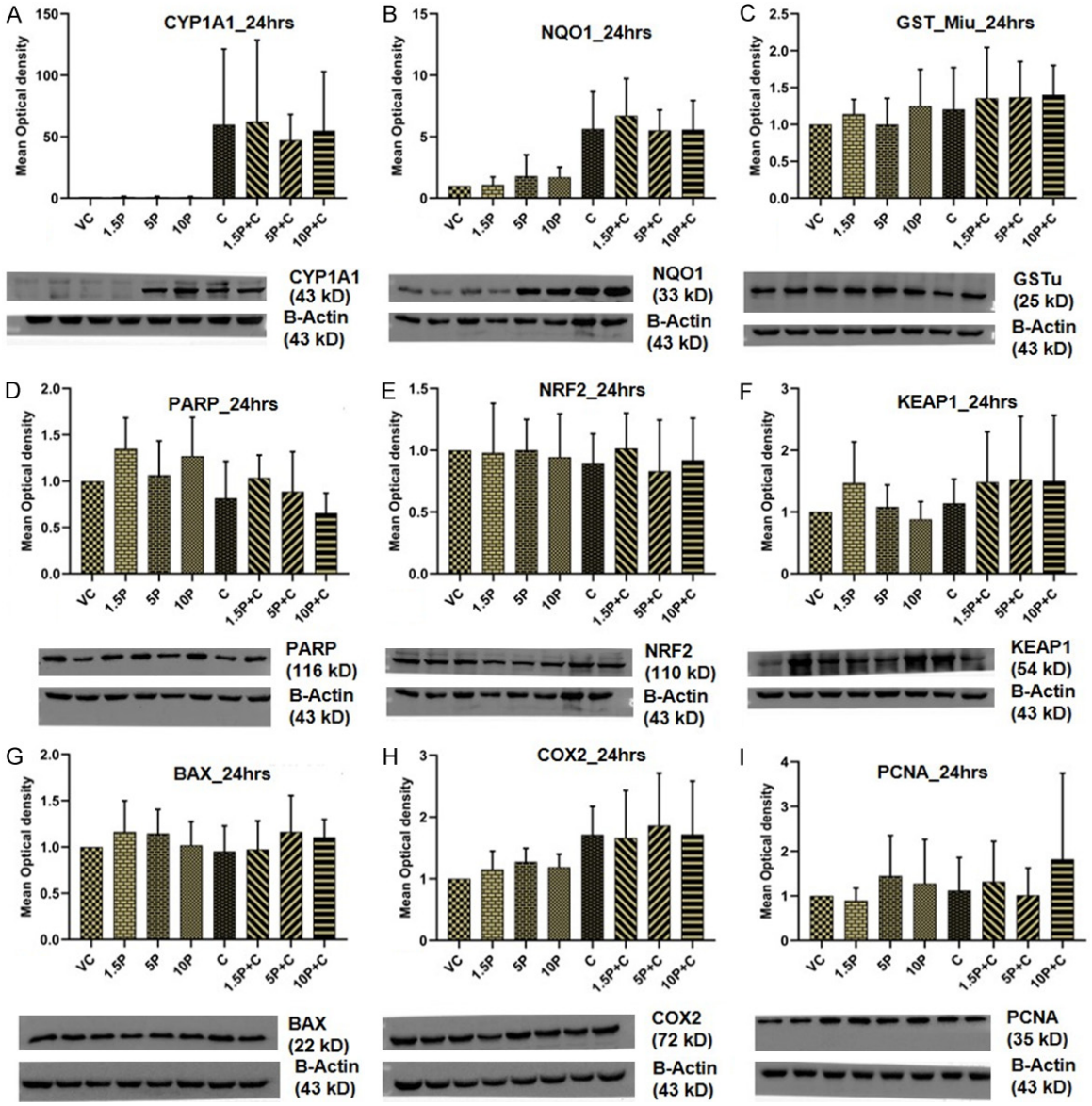


Figure 2. Representative images of the biomarkers at 24 hours timepoint. Blots and graphs are shown with their respective biomarker labels and loading control (B-Actin). Blots are to be read similar to the sequence of the graphs, viz, from left (lane 1) to right (lane 8). Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5%, 5% and 10% PBPs), carcinogen (B(a)P + drinking water), polyphenol + carcinogen (1.5%, 5% and 10% PBPs + B[a]P). A-C. Shows CYP1A1 (Phase-I), NQO1 (Phase-II) and GST-Mu (Phase-II) respectively, which are the biomarkers of xenobiotic metabolism. D-F. Shows PARP (DNA damage), NRF2 (oxidative stress) and KEAP1 (oxidative stress) respectively, which belongs to stress reponse pathways. G-I. Shows BAX (apoptosis), COX2 (inflammation) and PCNA (proliferation) biomarkers respectively. There was no statistically significant differences between the treatment groups, that is, Carcinogen, 1.5P+C, 5P+C and 10P+C.

the major component of Black tea, are ineffective against B[a]P-induced lung carcinogenesis when used as a single carcinogen in the pre-treatment setting. Also, the short-term experiments' biomarkers results were concordant with the long-term study results; however, they did not show any significant differences between the groups. Thearubigins could not modulate the expression of CYP1A1 and/or NQO1 in treatment (P+C) groups compared to the Carcinogen (C) group. However, both of these Phase I and Phase II enzymes, respectively, were induced when compared to the vehicle control (VC) and polyphenols control (PC)

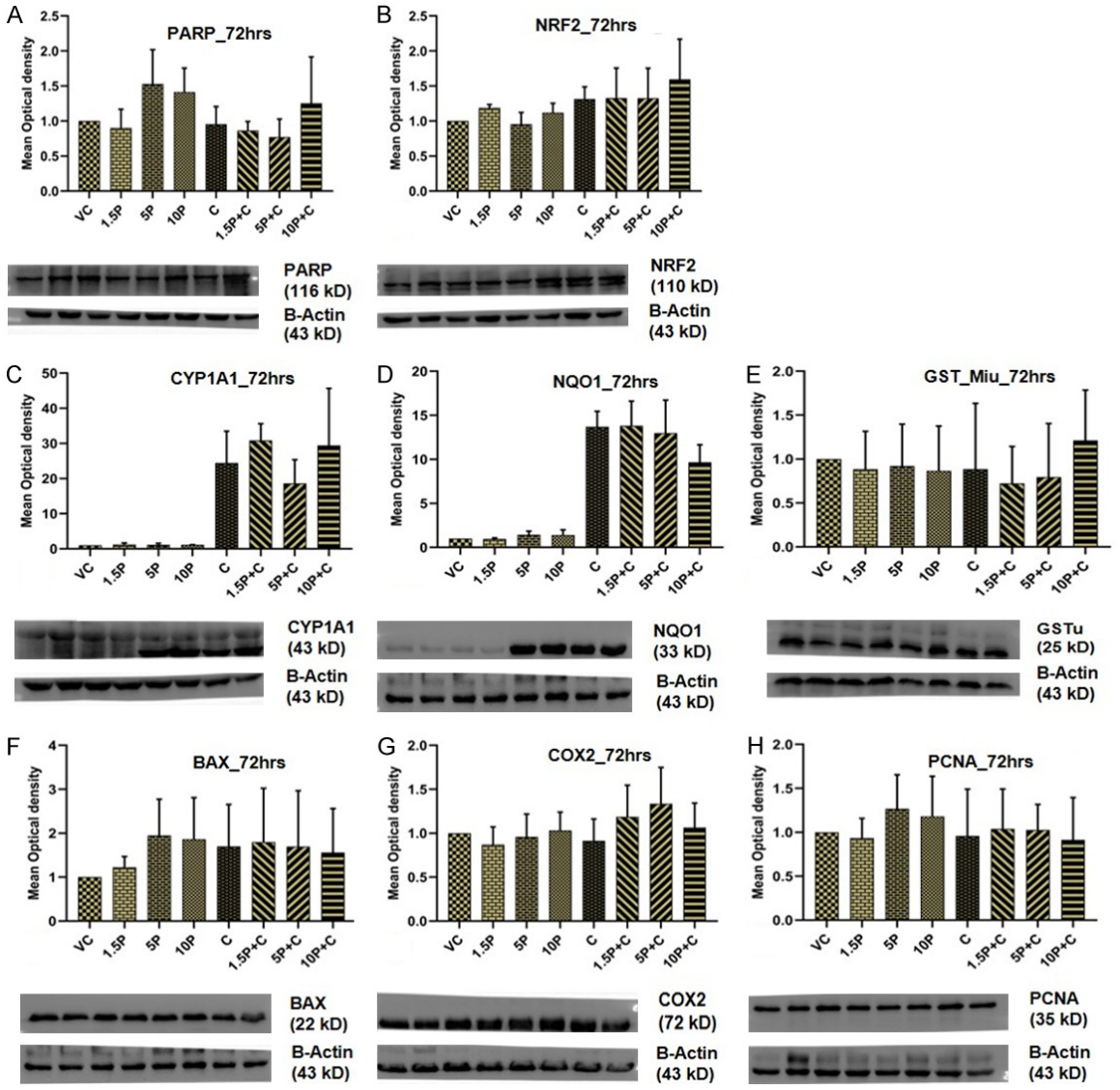


Figure 3. Representative images of the biomarkers at 72 hours timepoint. Blots along with graphs are shown with their respective biomarker labels and loading control (B-Actin). Blots are to be read similar to the sequence of the graphs, viz, from left (lane 1) to right (lane 8). Vehicle control (glyceryl trioctanoate + drinking water), polyphenol control (glyceryl trioctanoate + 1.5%, 5% and 10% PBPs), carcinogen (B(a)P + drinking water), polyphenol + carcinogen (1.5%, 5% and 10% PBPs + B[a]P). KEAP1 was not analyzed at 72 hrs. A. Shows PARP (DNA damage biomarker). B. Shows NRF2 (stress response) biomarker in whole cell lysate. C-E. Shows CYP1A1, NQO1 and GST-Mu respectively, the biomarkers of xenobiotic metabolism. F-H. Shows BAX (apoptosis), COX2 (inflammation) and PCNA (proliferation) biomarkers respectively. There was no statistically significant differences between the treatment groups, that is, Carcinogen, 1.5P+C, 5P+C and 10P+C.

groups suggesting that they are responsive when challenged with B[a]P. Furthermore, GST-Mu (another Phase-II enzyme) was not modulated in response to B[a]P demonstrating that only the NQO1 enzyme is specifically induced against B[a]P. The key strengths of our study are that it is highly powered (89%), no forestomach tumors were observed on the mice during experiments and the experiments were performed in 3 independent & overlapping batches along with water/PBP consumption and raw tumor number data. Additionally, this study is more relevant to non-smokers who are majorly exposed to PAH from the environment, wherein other tobacco smoke carcinogens are almost absent.

Previous findings from our lab have shown that pre-treatment with Thearubigins/PBP's has chemopreventive potential against B[a]P+NNK

Table 3. In-vivo pre-clinical studies using different chemopreventive agents demonstrating no chemopreventive effects in lung carcinogenesis

Sr No.	Chemopreventive agent	Agent type	Treatment setting	Results	Ref
1	Silibinin	Natural	Pre-treat/F	Ineffective	[14]
2	PEITC	Natural	Pre-treat/F	Ineffective	[15]
3	PEITC	Natural	Pre-treat/F	Increased tumor number	[16]
4	Dihydroxy-Myristicin	Natural	Parallel-treat/F	Ineffective	[17]

We have selected only those studies in which experimental lung tumors were induced by B[a]P in A/J mice. "F" refers to the female mice. "Pre-treat" refers to the agent administered before carcinogen treatment. "Parallel-treat" refers to agent administered during carcinogen administration.

induced lung tumors in a dose-dependent fashion [7, 8]. The carcinogen dose used in the previous study is splitted equally between B[a]P and NNK (biweekly); however, using the same dose of B[a]P biweekly we observed diarrhea and mortality onwards 5th week of injections out of total 8 injections indicating toxicity. Because of the widespread mortality, none of the animals survived till the 18-week endpoint from the last carcinogen treatment. In contrast in the present study, only a single carcinogen (B[a]P) was used once weekly for four weeks (keeping the total delivered dose similar to the previous study 200 mg/kg) to induce lung tumors and to test the chemopreventive efficacy of PBPs. The observations indicate that Thearubigins have a differential effect on lung tumors in the presence of a single vs combination of carcinogens. Interestingly, within all the carcinogen receiving groups, the levels of these enzymes remained similar between P+C and C groups showing it is not acting either chemopreventive nor tumor-promoting. Similar reports on chemoprevention of lung-cancer employing in vivo studies (**Table 3**) and clinical trials strongly highlights the current approach is not making headway [10, 11]. A shortfall of this study was the lack of B[a]P adsorbed to $PM_{2.5}$, which resides in the distal part of the lung for a longer period [12] and thus may help in the leaching of carcinogens in the alveoli. This can be addressed while designing future studies with respect to environmental carcinogens.

Our in vivo model tries to closely recreate the conditions wherein a non-smoker is exposed only to the environmental carcinogen (B[a]P), assuming the absence of other TS carcinogens; but not being able to recapitulate the exposure through inhalation. The limitations of the current models in lung chemoprevention studies are that these tumors are benign adenomas and do not truly represent the human lung tumors, and the tumor numbers exhibit wide variation. It is our understanding that the reason for this variability in lung tumor number might be inherent to the biological system or else might be a result of the combination of the following factors, viz, nutrition during the weaning period (immune system modulation), diet consumed during adult age, and differences in metabolism by gut microbiota [13] which play a key role in digestion and absorption.

This study sheds unique and novel insights into the chemopreventive potential of PBP's/Thearubigins with respect to B[a]P-induced vs B[a]P+NNK induced (combination) experimental lung carcinogenesis. We have also demonstrated that the change in tumor numbers and molecular markers at late time points can be predicted by visualizing the relevant biomarkers even at early time points, and they may act as surrogate markers to foresee the change in chemopreventive trials. However, these experimental settings need further validation in chemopreventive trials and experiments with positive and negative outcomes. However, pre and simultaneous treatment with Thearubigins before and during this exposure did not exert any anti-initiation/promotion effect. These studies show that combining carcinogens might give another different outcome than a single carcinogen. Additionally, if all the chemopreventive agents exhibit chemopreventive potential, people consuming a diet rich in these compounds must show complete prevention of cancer, which is not observed in real-life situations. In conclusion, chemopreventive agents need to be tested with different combinations of carcinogens to understand the complex interplay between carcinogenesis and chemopreventive agents.

Acknowledgements

We thank CRI-ACTREC, Tata Memorial Centre, for supporting this study (No. 1/3(7)/2020/TMC/R&D-II/8823 Dt.30.07.2021 & No. 1/3(6)/2020/TMC/R&D-II/3805 Dt.18.03.2021). We would also like to thank CSIR-HRDG, Government of India, for supporting the CSIR-NET JRF fellowship to Mr. Zaid M Shaikh (09/513(0105)/2018-EMR-I).

Disclosure of conflict of interest

None.

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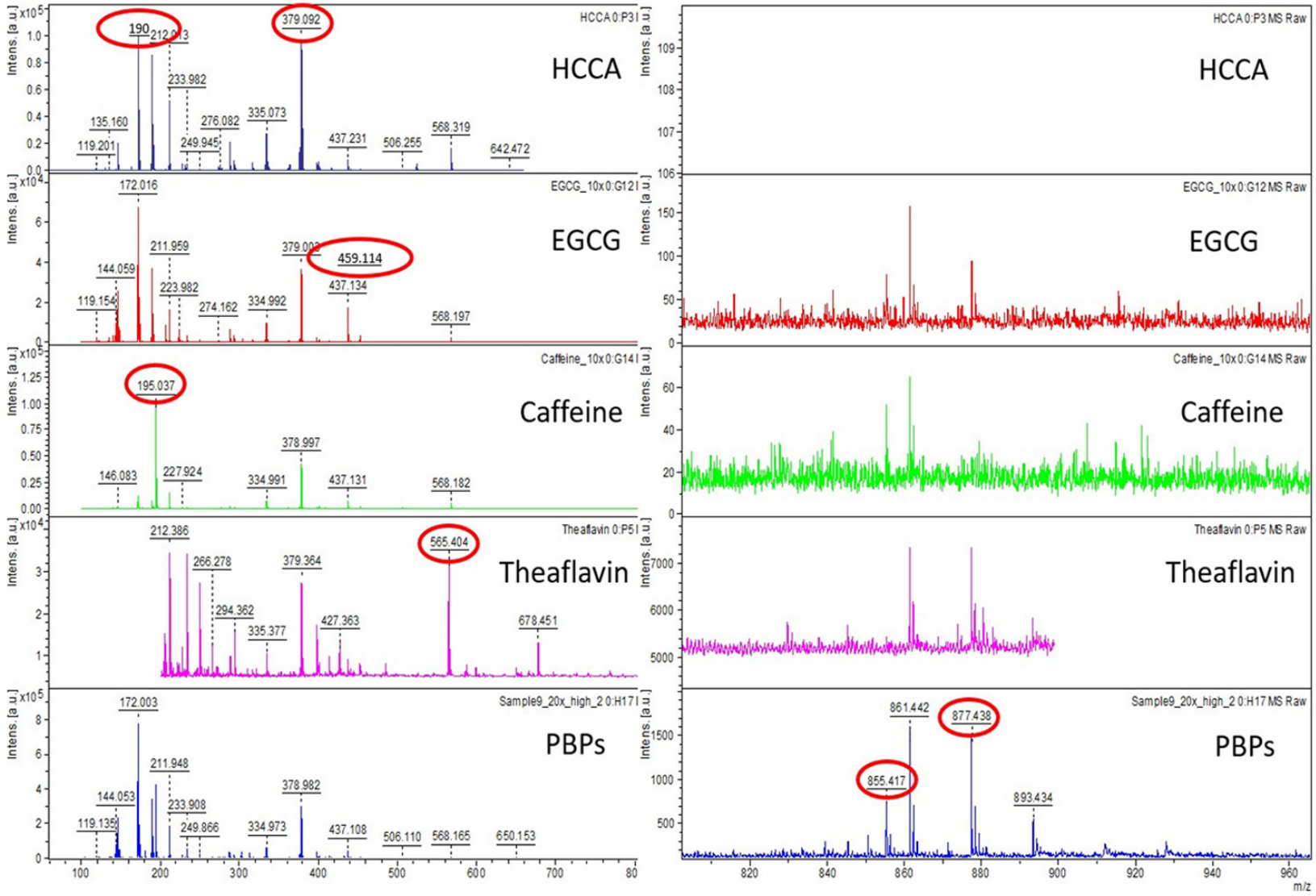
Supplementary Table 1. Batch-wise details and summary of the extraction of PBP's/TR by soxhlet extractor

Batch No.	Initial weight of Black tea powder ('g')	Weight after Decaffeination ('g')	Caffeine content ('g')	PBP fraction (2, 3, 4, 5) ('g')	PBP fraction-1 ('g')
Batch-1	450	441	9	412	3
Batch-2	450	440	10	407	8
Batch-3	450	440	10	406	2
Batch-4	450	438	12	422	2
Batch-5	450	441	9	420	5
Batch-6	450	444	6	420	5
Batch-7	450	446	4	422	2
Batch-8	450	441	9	404	3
Batch-9	450	438	12	404	2
Batch-10	450	447	3	415	2
Batch-11	450	447	3	410	3
Batch-12	450	440	10	420	4
Batch-13	450	441	9	415	3
Batch-14	450	439	11	424	4
Batch-15	450	440	10	417	4
Mean ± SD	450.0 ± 0.0	441.5 ± 3.0	8.5 ± 3.0	414.5 ± 7.0	3.5 ± 1.6

Summary represented as Mean ± SD.

Supplementary Table 2. Preparation of different doses of PBP's and their dry weight analysis

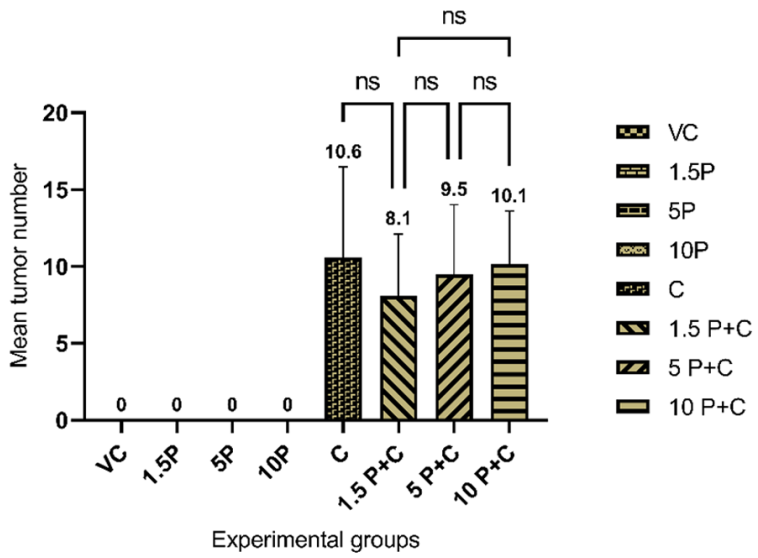
PBP dose concentration (%)	Weight of Residual Black tea (1, 2, 3, 4, 5) (grams)	Final Volume of water (mL)	PBP's Dry weight (Mean ± SD) (mg/mL)
1.5%	9.73	1000	1.18 ± 0.3
3%	19.46	1000	2.34 ± 0.1
5%	32.43	1000	3.64 ± 0.4
10%	64.86	1000	7.60 ± 0.1



Supplementary Figure 1. Analysis of black tea-derived PBPs by MALDI-TOF to evaluate black tea-derived contaminants such as EGCG, Caffeine, and theaflavins. Representative MALDI-TOF spectra with a-cyano-4-hydroxycinnamic acid (HCCA) as a matrix shows EGCG (m/z 459.11), caffeine (m/z 195.03) and theaflavin (m/z 565.40) specific peaks. Black tea-derived PBP sample spectra were free from any of these contaminant signals, which confirmed their purity. Further, PBPs showed the presence of two PBP-specific peaks with m/z values 855.41 and 877.43.

Supplementary Table 3. Details of western blotting antibodies and optimizations

Biomarker	Source, Catalog No, Lot No.	Host species, Clone	Molecular wght.	Protein loaded (ug)	Blocking	Primary antibody Dilution	TBST wash after Primary	Secondary antibody Dilution	Further TBST washes
B-Actin	Santa Cruz, SC-1616, F2111	Rabbit, polyclonal	43 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:3000	10 min × 3	1:3000	10 min × 3
PCNA	Abcam, ab18197, GR31923 41-1	Rabbit, polyclonal	35 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:3000	10 min × 3	1:3000	10 min × 3
PARP	Thermofisher PA5-34803	Rabbit, polyclonal	116 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:1000	10 min × 3	1:3000	10 min × 3
NRF2	Abcam, Ab137550	Rabbit, polyclonal	110 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:1000	10 min × 3	1:3000	10 min × 3
COX-2	Abcam, ab15191, GR320409-6	Rabbit, polyclonal	72 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:3000	10 min × 3	1:3000	10 min × 3
BAX	Abcam, ab7977, GR98755-2	Rabbit, polyclonal	22 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:3000	10 min × 3	1:3000	10 min × 3
CYP1A1	Abcam, Ab79819	Rabbit, polyclonal	58 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:3000	10 min × 3	1:3000	10 min × 3
GST-Miu	Abcam, Ab92369	Rabbit, polyclonal	25 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:1000	10 min × 3	1:3000	10 min × 3
NQO-1	Abcam, ab34173	Rabbit, polyclonal	33 kDa	25-30 µg	5% Milk (1 hr:25°C)	1:3000	10 min × 3	1:3000	10 min × 3

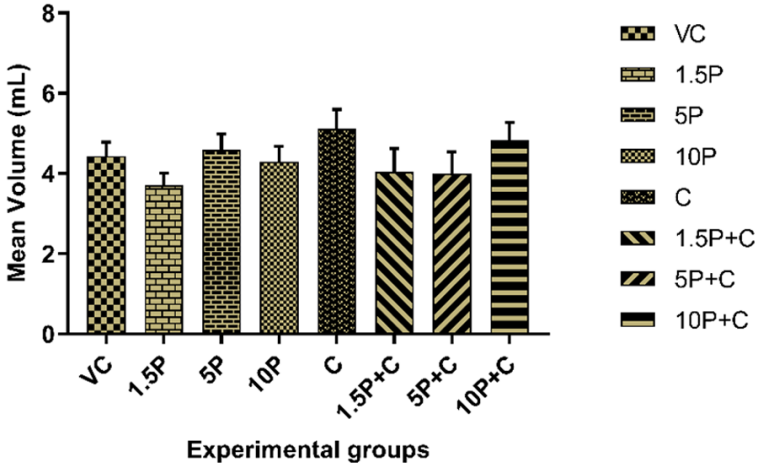


Supplementary Figure 2. Mean tumor count in different study groups. Represented as Mean ± SD (n=15/group).

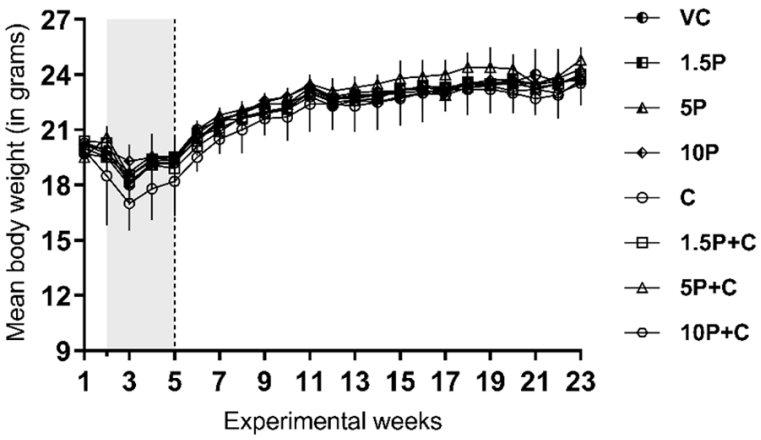
Supplementary Table 4. Day-wise consumption of water/PBPs in different study groups

Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Mean ± SD (mL)
VC	5.0	4.1	4.5	4.3	4.3	4.4 ± 0.3
1.5+ PC	4.2	3.5	3.5	3.5	3.8	3.7 ± 0.3
5 PC	5.0	4.2	4.3	5.0	4.5	4.6 ± 0.3
10 PC	4.6	4.6	3.8	4.0	4.5	4.3 ± 0.3
Carci (C)	5.5	4.4	5.1	5.0	5.6	5.1 ± 0.4
1.5P+C	4.9	3.8	3.3	4.0	4.2	4.0 ± 0.5
5P+C	3.1	4.4	4.4	3.9	4.2	4.0 ± 0.5
10P+C	5.4	4.3	4.6	4.8	5.1	4.8 ± 0.4

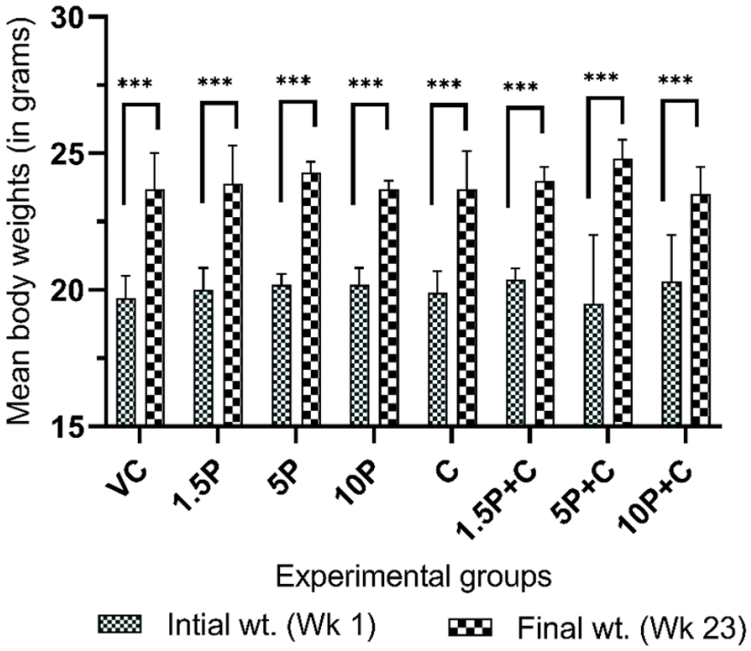
Total n=9 mice/group.



Supplementary Figure 3. Average daily consumption of water and polyphenols in different study groups. Each bar represents an average daily consumption for 23 weeks.



Supplementary Figure 4. Mean bodyweights of mice during the experimental study. Grey shaded area represents the weeks during intraperitoneal (i.p.) injections & reduced weight due to trauma of the i.p. injections.

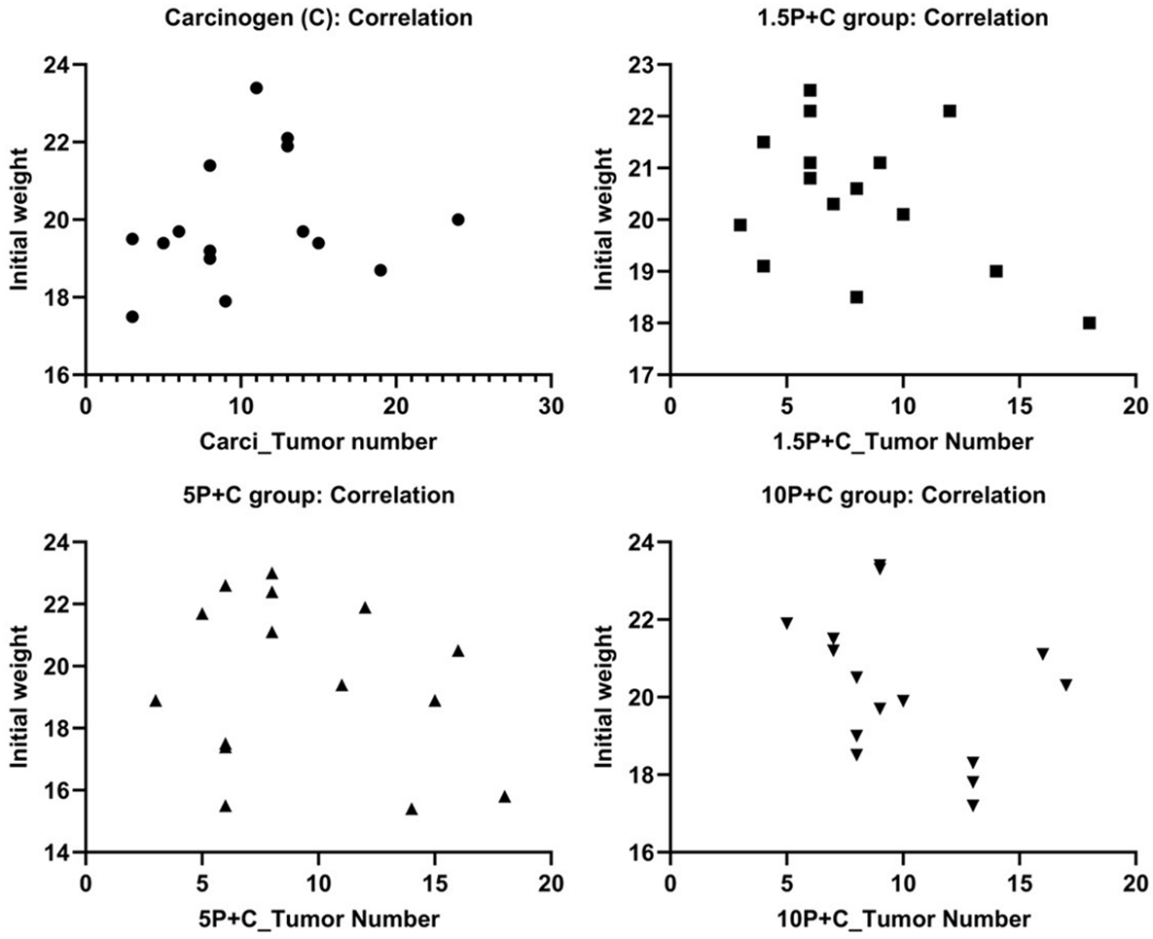


Supplementary Figure 5. Difference between Initial and final weights in different study groups (n=15/group).

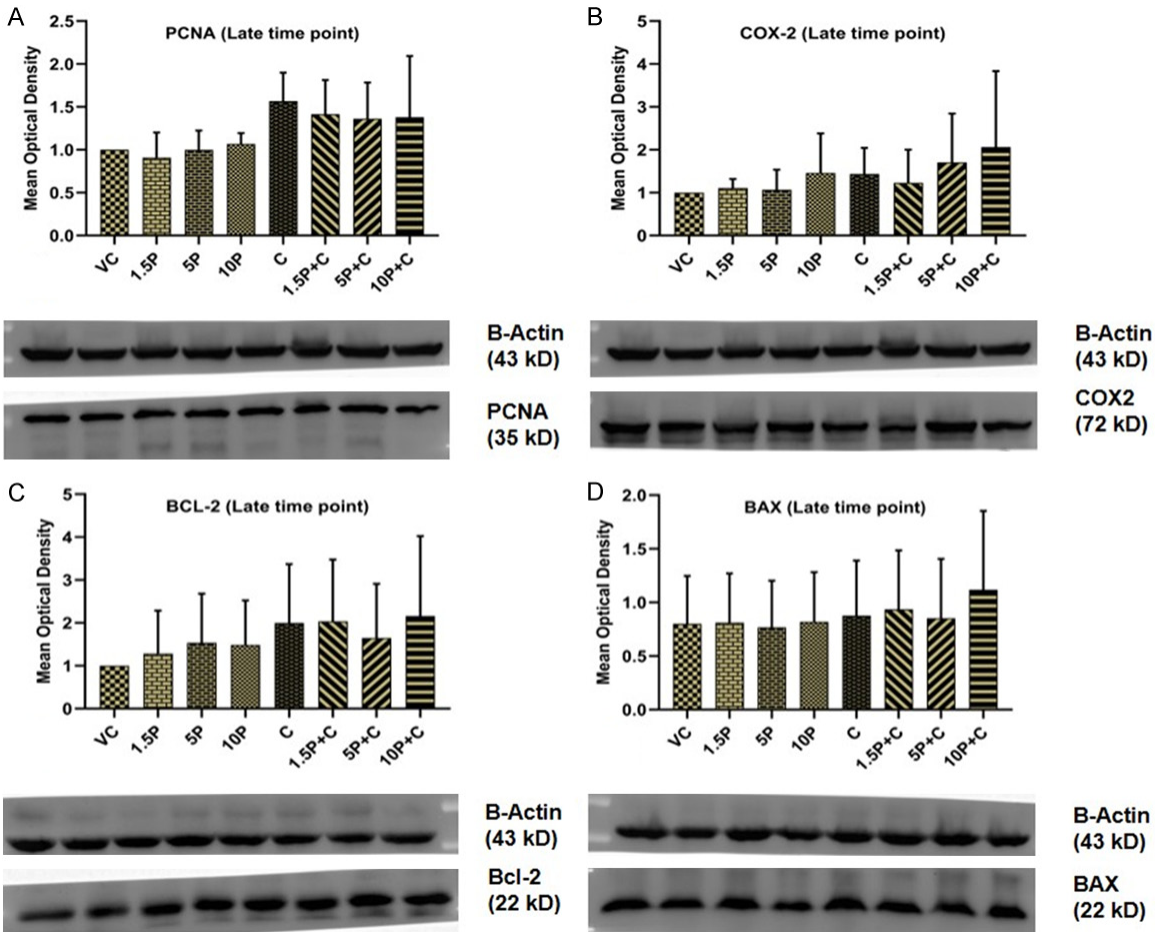
Supplementary Table 5. Count of lung tumors in different study groups

A. Control groups							
Animal Number	VC	Animal Number	1.5PC	Animal Number	5PC	Animal Number	10PC
L/2866	0	L/2849	0	L/2869	0	L/2848	0
L/2840	0	L/2852	0	L/2865	0	L/2836	0
L/2870	0	L/2858	0	L/2838	0	L/2847	0
L/2845	0	L/2860	0	L/2855	0	L/2856	0
L/2831	0	L/2851	0	L/2844	0	L/2837	0
Q/1251	0	Q/1240	0	Q/1254	0	Q/1269	0
Q/1265	0	Q/1263	0	Q/1236	0	Q/1252	0
Q/1255	0	Q/1264	0	Q/1233	0	Q/1267	0
Q/1266	0	Q/1270	0	Q/1256	0	Q/1250	0
Q/1249	0	Q/1258	0	Q/1247	0	Q/1242	0
Q/2124	0	Q/2115	0	Q/2123	0	Q/2121	0
Q/2131	0	Q/2125	0	Q/2126	0	Q/2127	0
Q/2145	0	Q/2149	0	Q/2140	0	Q/2117	0
Q/2119	0	Q/2152	0	Q/2146	0	Q/2153	0
Q/2132	0	Q/2142	0	Q/2147	0	Q/2135	0
Mean ± S.D.	0 ± 0		0 ± 0		0 ± 0		0 ± 0
Range	NA		NA		NA		NA
B. Treatment groups							
Animal Number	Carcinogen (C)	Animal Number	1.5P+C	Animal Number	5P+C	Animal Number	10P+C
L/2868	3	Q/2122	3	L/2839	3	Q/1237	5
Q/1767	3	Q/1253	4	Q/1259	5	Q/1232	7
Q/1768	5	Q/2129	4	Q/1257	6	Q/2134	7
Q/1770	6	Q/1235	6	Q/2130	6	Q/2120	8

Q/1766	8	Q/1241	6	L/2850	6	Q/2133	8
Q/1769	8	Q/1248	6	L/2832	6	L/2842	8
Q/2150	8	L/2834	6	Q/1243	8	Q/2139	9
Q/2128	9	L/2841	7	Q/1244	8	Q/1238	9
Q/2138	11	L/2867	8	Q/2137	8	Q/1245	9
Q/2136	13	Q/1262	8	Q/2116	11	Q/2118	10
L/2835	13	Q/2141	9	Q/2154	12	L/2861	13
Q/2145	14	Q/2143	10	V2853	14	L/2862	13
Q/2843	15	Q/2144	12	Q/1268	15	L/2863	13
L/2859	19	L/2833	14	Q/2151	16	Q/1261	16
L/2854	24	L/2846	18	L/2864	18	L/2857	17
Mean ± S.D.	10.6 ± 5.9		8.1 ± 4.1		9.5 ± 4.6		10.1 ± 3.5
Range	(3-24)		(3-18)		(5-18)		(5-17)



Supplementary Figure 6. Plots showing the correlation between individual animal weight and lung tumor number.



Supplementary Figure 7. Western blots and graphs indicating the biomarkers at the late time point experiments. Statistical analysis indicates no statistically significant differences between any of the groups.