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Black tea polyphenols modulate xenobiotic-metabolizing enzymes, oxidative stress and adduct formation in a rat hepatocarcinogenesis model

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Abstract

The present study was designed to investigate the modulatory effects of black tea polyphenols (Polyphenon-B) on phase I and phase II xenobiotic-metabolizing enzymes and oxidative stress in a rat model of hepatocellular carcinoma (HCC). Liver tumours induced in male Sprague-Dawley rats by dietary administration of ρ-dimethylaminoazobenzene (DAB) increased cytochrome P450 (total and CYP1A1, 1A2 and 2B isoforms), cytochrome b_5, cytochrome b5 reductase, glutathione S-transferase (GST total and GST-P isoform) and gamma-glutamyltranspeptidase (GGT) with decrease in quinone reductase (QR). This was accompanied by enhanced lipid and protein oxidation and compromised antioxidant defences associated with increased expression of the oxidative stress markers 4-hydroxynonenal (4-HNE), anti-hexanoyl lysine (HEL), dibromotyrosine (DiBrY) and 8-hydroxy 2-deoxyguanosine (8-OHdG). Dietary administration of Polyphenon-B effectively suppressed DAB-induced hepatocarcinogenesis, as evidenced by reduced preneoplastic and neoplastic lesions, modulation of xenobiotic-metabolizing enzymes and amelioration of oxidative stress. Thus, it can be concluded that Polyphenon-B acts as an effective chemopreventive agent by modulating xenobiotic-metabolizing enzymes and mitigating oxidative stress in an *in vivo* model of hepatocarcinogenesis.

Keywords: *Antioxidants, black tea polyphenols, chemoprevention, dimethylaminoazobenzene, oxidative stress, hepatocellular carcinoma, xenobiotic-metabolizing enzymes*

Introduction

Hepatocellular carcinoma (HCC), the fifth most common cancer and the third leading cause of cancer death worldwide, accounts for the highest morbidity and mortality. The most prominent risk factors for the development of HCC include viral hepatitis, alcohol and metabolic liver diseases [1]. Furthermore, recurrence after curative resection limits survival of patients with HCC [2]. Chemoprevention is therefore a promising strategy to control HCC incidence. In this context, HCC induced by dietary administration of the azo dye ρ-dimethylaminoazobenzene (DAB) in Sprague-Dawley rats serves as a relevant animal model for investigating the mechanisms of tumour development and effects of intervention by chemopreventive agents [3].

Azo dyes classified as group 2B carcinogens by the International Agency for Research on Cancer (IARC)

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are procarcinogens that undergo metabolic activation by xenobiotic-metabolizing enzymes to generate toxic electrophiles and reactive oxygen species (ROS) [4]. ROS react with and damage lipids, DNA and proteins, leading to the formation of carcinogenic adducts. The most commonly occurring modifications are 4-hydroxynonenal (4-HNE), anti-hexanoyl lysine (HEL) and dibromotyrosine (DiBrY), reliable markers of lipid and protein oxidation, and 8-hydroxy 2-deoxyguanosine (8-OHdG), a sensitive marker of oxidative DNA damage. Reaction of ROS with membrane polyunsaturated fatty acids forms 4-HNE, an advanced lipid peroxidation product that can bind covalently to proteins or interact with DNA to form hepatic etheno (epsilon)-modified DNA bases [5,6]. ROS can also react with proteins to form HEL, a novel lipid hydroperoxide modified lysine adduct or DiBrY formed by oxidative modification of tyrosine residues [7,8]. 8-OHdG has been used as a biomarker of oxidative DNA damage by aflatoxin B1 and hepatitis B virus, risk factors for HCC as well as in patients with HCC [9–11].

Antioxidants play a protective role against ROS-induced carcinogenesis by inhibiting ROS generation as well as formation of ROS-induced adducts. In addition, antioxidants prevent metabolic activation of procarcinogens and stimulate detoxification of carcinogenic metabolites [12].

Of late, diet-derived antioxidants have assumed significance in cancer prevention and therapeutic regimens [13]. Tea made from the leaves of *Camellia sinensis*, one of the most popular beverages consumed worldwide, abounds in antioxidant polyphenols and other bioactive compounds with chemopreventive potential. However, black tea, which accounts for 80% of tea consumption, has not received as much research focus as green tea, despite its health benefits [14]. In previous reports from this laboratory, we demonstrated the inhibitory effects of black tea polyphenols (Polyphenon-B) in the hamster buccal pouch, rat mammary and gastric carcinogenesis models [15–17].

The present study was designed to evaluate the chemopreventive efficacy of Polyphenon-B on DAB-induced hepatocarcinogenesis based on modulation of xenobiotic-metabolizing enzymes and ROS-induced oxidative stress. Cytochrome P450 (total and CYP1A1, 1A2 and 2B isoforms), cytochrome b_5 and cytochrome b_5 reductase were used to assess carcinogen activation and the activities of glutathione S-transferase (GST) and quinone reductase (QR) to evaluate carcinogen detoxification. Oxidative stress was evaluated by estimating the extent of ROS-induced damage to lipids, proteins and DNA and the status of the antioxidants superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and GSH-dependent enzymes.

Materials and methods

Chemicals

Bovine serum albumin (BSA), 2-thiobarbituric acid (TBA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 2,4-dinitrophenyl hydrazine (DNPH), 1-chloro-2,4-dinitrobenzene (CDNB), 3,3′-diaminobenzidine, reduced glutathione (GSH), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate reduced (NADPH), 2,6-dichlorophenolindophenol (DCPIP), gammaglutamyl-p-nitroanilide, DAB, 7-ethoxyresorufin, methoxyresorufin, pentoxyresorufin, resorufin and sodium dithionite were purchased from Sigma Chemical Company (St. Louis, MO). Polyphenon-B was kindly provided by Mitsui Norin Co., Ltd. (Tokyo, Japan). The composition of Polyphenon-B is the same as described previously [15]. It is a mixture of epicatechin (0.4%), epigallocatechin-3-gallate (1.4%), epicatechin-3-gallate (0.1%), gallocatechin-3-gallate (0.2%), free theaflavins (0.32%), theaflavinmonogallate-A (0.14%), theaflavinmonogallate-B (0.15%), theaflavindigallate (0.24%), tannin (35.6%) and caffeine (4.9%). All other reagents used were of analytical grade.

Animals and diet

All the experiments were carried out with male Sprague-Dawley rats, aged 6–8 weeks, weighing 100–110 g, obtained from the Central Animal House, Annamalai University (India). They were housed four to a polypropylene cage and provided food and water *ad libitum*. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 h light/dark cycle. The animals were maintained in accordance with the guidelines of the Indian Council of Medical Research and approved by the ethical committee of Annamalai University. Experimental diet was prepared every day by mixing DAB (0.06%) and Polyphenon-B (0.05%) to pre-weighed standard pellet diet (Mysore Snack Feed, Mysore, India) containing crude protein (22.12%), crude oil (4.12%), crude fibre (3.18%), ash (5.17%) and sand silica (1.13%) with an energy value of 3625 kcal/kg. The diet was replenished every day and the food consumption was recorded.

Treatment schedule

The animals were randomized into experimental and control groups and divided into four groups of eight animals each. Rats in group 1 were given DAB (0.06%) in the diet for 3 months followed by normal diet [18]. Rats in group 2 administered DAB as in group 1, received, in addition, 0.05% Polyphenon-B in the diet. Group 3 animals were administered 0.05% Polyphenon-B alone in the diet as in group 2, but without DAB. Group 4 received basal diet and

tap water throughout the experiment and served as the untreated control. The dose for Polyphenon-B used in the present study corresponds to the daily dietary intake of four cups of tea (30–40 mg of tea polyphenols per kilogram body weight by humans) [19]. The experiment was terminated at the end of 24 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. The liver tissues were sub-divided and variously processed for distribution to each experiment. Biochemical estimations were carried out in the tissue homogenate and S9 fractions.

Histopathological examination

A portion of the tissues was immediately fixed in 10% neutral buffered formalin, embedded in paraffin and mounted on polylysine-coated glass slides. One section from each specimen was stained with haematoxylin and eosin. The remaining sections were used for immunohistochemical staining. Dysplasia and hepatocellular carcinoma were diagnosed. Irregular epithelial stratification, increased number of mitotic figures, increased nuclear-to-cytoplasmic ratio and loss of polarity of basal cells characterized the dysplastic lesions. HCC was diagnosed by the invasion of underlying tissues, nuclear pleomorphism and increased mitoses.

Preparation of tissue homogenate and S9 fraction

Tissue samples were weighed and homogenized using appropriate buffer in an all glass homogenizer with Teflon pestle and stored in ice until use. All the steps for the preparation of S9 fraction were carried out at 4°C, as described by Ames et al. [20]. The tissues after weighing were washed with cold 0.15 m KCl and homogenized in three volumes of 0.15 m KCl in an all glass homogenizer with Teflon pestle. The homogenate was centrifuged for 10 min at $9000 \times g$ and the supernatant so collected is the S9 mix fraction. The biochemical analyses were carried out immediately.

Biochemical estimations

Activities of aspartate and alanine transaminases (AST and ALT) were assayed by the method of King [21]. Cytochrome P450 and cytochrome b_5 content were assayed by the method of Omura and Sato [22]. Cytochrome P450 was determined by using the carbon monoxide difference spectra. Reduced cytochrome P450 combines with carbon monoxide to yield a pigment with an absorbance maximum at 450 nm. Cytochrome b_5 was measured from the difference spectrum between reduced and oxidized cytochrome b_5. The ethoxyresorufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD) and pentoxyresorufin-*O*-dependentylase (PROD) activities were assayed as described by Baer-Dubowska et al. [23] and Burke et al. [24]. The activity of GST was determined as described by Habig et al. [25] by following the increase in absorbance at 340 nm using CDNB as the substrate. Gamma-glutamyl transpeptidase (GGT) was assayed by the method of Fiala et al. [26] with gamma glutamyl *p*-nitroanilide as the substrate. The activity of quinone reductase was assayed as described by Ernster [27]. This method involves measurement of reduction at 550 nm using NADPH as the electron donor and DCPIP as the electron acceptor.

Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and conjugated dienes (CD). TBARS were assayed in the liver tissue by the method of Ohkawa et al. [28]. Lipid hydroperoxides were estimated by the method of Jiang et al. [29] and conjugated dienes by the method of Rao and Recknagel [30]. Protein oxidation was measured by the method of Levine et al. [31] based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. Total SOD and Mn-SOD activities were assayed as described by Oberley and Spitz [32] based on the half-maximal inhibition of nitrobluetetrazolium (NBT) reduction. Cu-Zn SOD activity was calculated by deducting the activity of Mn-SOD from total SOD activity. The activity of CAT was assayed by the method of Sinha [33] based on the utilization of hydrogen peroxide by the enzyme. GSH was determined by the method of Anderson [34] by measurement of the yellow colour that develops when DTNB is added to compounds containing sulphydryl groups. Selenium-dependent glutathione peroxidase (Se-GPx) activity was assayed by following the utilization of hydrogen peroxide according to the method of Rotruck et al. [35]. Se-independent GPx activity was assayed following the method described by Lawrence and Burk [36] using cumene peroxide as substrate. GR activity was assayed by the method of Carlberg and Mannervik [37] using GSSG as substrate and FAD as cofactor. The protein content was estimated by the method of Lowry et al. [38] with bovine serum albumin as the standard.

Immunohistochemistry

The tissue sections were deparaffinized by heat at 60°C for 10 min, followed by three washes in xylene. After gradual hydration through graded alcohol, the slides were incubated in citrate buffer (pH 6.0) for two cycles of 5 min in a microwave oven for antigen retrieval. The sections were allowed to cool for 20 min and then rinsed with Tris-buffered saline (TBS). The sections were treated for 15 min with 3% H_2O_2 in distilled water to inhibit endogenous peroxidase activity. Non-specific antibody binding was reduced

by incubating the sections with normal goat serum for 25 min. The sections were then incubated with CYP1A1 (generously provided by Dr Stegeman, Woods Hole Oceanographic Institute (WHOI), USA and Dr Gelboin, National Cancer Institute (NCI), USA), 4-HNE (generously provided by Dr Koji Uchida, Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan), 8-OHdG, HEL and DiBrY (JaICA, Shizuoka, Japan) mouse monoclonal antibodies and GST-P (BioGenex, San Raman, CA, USA) rabbit polyclonal antibody at room temperature for 1 h. The slides were washed with TBS and then incubated with anti-mouse and anti-rabbit biotin-labelled secondary antibodies (Dako, Carprinteria, CA) followed by streptavidin-biotin-peroxidase for 30 min each at room temperature. The immunoprecipitate was visualized by treating with 3,3′-diaminobenzidine and counterstaining with haematoxylin. For negative controls, the primary antibody was replaced with TBS. Positive controls were also processed simultaneously. The immunohistochemical data for CYP1A1, GXST-p 4-HNE, HEL, DiBrY and 8-OHdG were expressed as the number of cells with positive staining per 100 counted cells.

Extraction of RNA

Total RNA from the liver tissues was extracted using trizol reagent (Sigma) [39]. The RNA concentration was determined from the optical density at a wavelength of 260 nm (by using an OD_{260} unit equivalent to 40 μg/ml of RNA). In brief, 50 mg liver tissue was homogenized using (1 ml) trizol reagent. The homogenate was then treated with 0.2 ml of chloroform and shaken vigorously. The mixture was then centrifuged at 12 000 *g* for 15 min at 4°C. To the aqueous phase, 0.5 ml of isopropanol was added and centrifuged at 12 000 *g* for 8 min at 4°C. The supernatant was discarded gently and the precipitated RNA was rinsed twice with 1 ml of 75% ethanol and dried in air. The RNA was resuspended in 100 μl of diethylpyrocarbonate (Sigma) treated water at a final concentration of 1 μg/μl and stored at −80°C until further use.

Reverse transcriptase (RT) reaction: cDNA synthesis

Isolated total RNA (1 μg) was reverse-transcribed to cDNA in a reaction mixture containing 4 μl of 5X reaction buffer, 2 μl of dNTPs mixture (10 mm), 20 units of RNase inhibitor, 200 units of avian-myeloblastosis virus (AMV) reverse transcriptase and 0.5 μg of oligo(dT) primer (Promega, WI) in a total volume of 20 μl. The reaction mixture was incubated at 42°C for 60 min and the reaction terminated by heating at 70°C for 10 min. The cDNA was stored at −80°C until further use.

PCR amplification

The nucleotide sequences of the primers (Sigma Genosys, India) were: for amplification of GGT: 5′-CTCTGCATCTGGCTACCCAC-3′ (sense), 5′-GGATGCTGGGTTGGAAGAGG-3′ (antisense), generating a 418bp fragment and GST-P: 5′-TCATCTACACCAACTATGAG-3′ (sense), 5′-GCCACATAGGCAGAGAGCAG-3′ (antisense), generating a 226bp fragment. The PCR amplification reaction mixture (in a final volume of 25 μl) contained 1 μl of cDNA, 0.5 μl of forward primer, 0.5 μl of reverse primer and 10 μl of Hot Master Mix (2.5X) (Eppendorf, Hamburg, Germany). The PCR was carried out in a thermal cycler (Eppendorf). Thermocycling conditions included initial denaturation at 94°C for 5 min (one cycle), then denaturation at 94°C (30 s) for GGT or at 95°C (1 min) for GST-P, annealing at 59°C (45 s) for GGT or at 55°C (1 min) for GST-P and extension at 70°C (45 s) for GGT or at 72°C (1 min) for GST-P for 30 cycles and a final extension at 70°C (7 min) for GGT or at 72°C (7 min) for GST-P. Negative controls without cDNA were also performed. Amplification products were analysed by electrophoresis in a 2% agarose gel containing ethidium bromide with 100bp DNA ladder. The PCR products were visualized as bands with a UV-transilluminator and photographs were taken using gel documentation system (Gel Doc Mega, UK).

SDS-PAGE and Western blot analysis

Approximately 50 mg of each tissue sample was subjected to lysis in a sample buffer containing 62.5 mm Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. The protein concentration of lysates was determined by Bradford's [40] method. SDS-PAGE was performed using equivalent protein extracts (55 μg) from each sample according to Laemmli [41]. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Sartorius, Germany). The membranes were incubated in 1X PBS containing 5% non-fat dry milk for 2 h to block non-specific binding sites. The blots were incubated with 1:400 dilution of CYP1A1 and HEL overnight at 4°C. The blots were washed thrice with high salt buffer (2.18 g NaH_2PO_4, 7 g Na_2HPO_4, 23.37 g NaCl and 200 μl Triton X-100 in 400 ml distilled water) followed by low salt buffer (2.18 g NaH_2PO_4, 7.03 g Na_2HPO_4, 1.2 g NaCl and 200 μl Triton X-100 in 400 ml distilled water). The blots were then incubated with 1:1000 dilution of horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using enhanced chemiluminescence (ECL) detection reagents (Sigma). Densi-

Table I. Body weight, tumour incidence and histopathological changes in experimental and control animals (mean ± SD; $n = 8$).

Group	Treatment	Body weight (g)	Tumour incidence	Cirrhotic changes	Dysplastic nodules	Cholangiocarcinoma	HCC
1	DAB	145.23 ± 12.21**	8/8 (100)	+++	+++	–	8/8 (100%)
2	DAB + P-B	182.26 ± 16.23*	1/8 (12.5)[a]	+ to ++ (4/8)	+ (3/8)	1/8	–
3	P-B	195.31 ± 17.40		–	–	–	–
4	Control	191.46 ± 18.01		–	–	–	–

P-B: Polyphenon-B.
+ = mild; ++ = moderate; +++ = severe; – = no change; Parentheses, percentage of lesions.
**Significantly different from group 4 ($p < 0.05$), ANOVA followed by LSD test. *Significantly different from group 1 ($p < 0.05$), ANOVA followed by LSD test.
Tumour multiplicity = number of tumours per rat. Mean tumour burden was calculated by multiplying the mean tumour volume with the mean number of tumours (tumour volume was calculated using $4/3\ \pi r^3$, where r = ½ tumour diameter in mm)
[a]Significantly different from group 1 by χ^2-test ($p < 0.001$).

tometry was performed on IISP flat bed scanner and quantitated with Total Lab 1.11 software.

Statistical analysis

The data are expressed as mean ± standard deviation. Tumour incidence and tumour burden were analysed by χ^2-test and Student's *t*-test, respectively. The data for body weight, biochemical assays, immunohistochemical analysis and densitometric analysis were analysed using analysis of variance (ANOVA) followed by the least significance difference test (LSD). The results were considered statistically significant if the *p*-value was < 0.05.

Results

Tumour incidence and histopathological observations

Table I shows the mean body weight, tumour incidence and histopathological lesions in different groups. Rats in group 1 showed a tendency to be lower in body weight during the experiment and the mean final body weights were decreased significantly ($p < 0.05$) compared to control (group 4). No significant differences in the body weights were observed in groups 2–4. The amount of diet consumed in groups 1–4 was not significantly different.

At the end of the experimental period, the tumour incidence in group 1 was 100% with a multiplicity

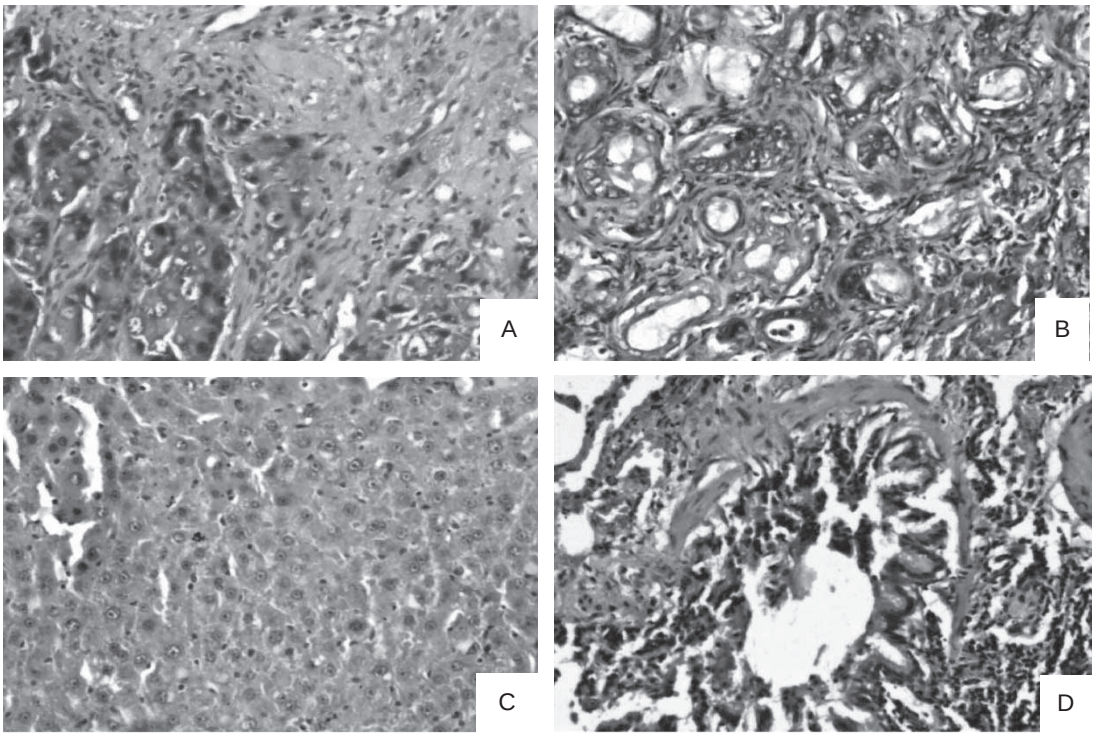

Figure 1. Photomicrographs of histopathological changes in the liver and lung tissues of control and experimental animals (Hematoxylin and eosin, ×10). (A) Well differentiated HCC with extensive infiltration into connective tissue in group 1 animals. (B) Cholangiocarcinoma and mild cirrhotic changes in the liver epithelium of rats in group 2. (C) Liver tissue showing normal histology in groups 3 and 4 animals. (D) Multiple metastatic deposits in the lung from a group 1 rat administered DAB.

Table II. Activities of AST, ALT and AST/ALT ratio in the liver and serum of control and experimental animals (Mean ± SD; $n = 8$).

		AST		ALT			
Group	Treatment	Liver (IU/g tissue)	Serum (IU/dL)	Liver (IU/g tissue)	Serum (IU/dL)	AST/ALT ratio	
1	DAB	10.96 ± 1.01**	9.94 ± 0.82**	9.02 ± 0.71**	8.90 ± 0.69**	1.22 ± 0.09**	1.12 ± 0.08**
2	DAB + P-B	5.92 ± 0.42*	5.62 ± 0.43*	6.38 ± 0.53*	6.41 ± 0.52*	0.93 ± 0.07*	0.88 ± 0.07*
3	P-B	3.90 ± 0.26	3.68 ± 0.21	4.54 ± 0.36	4.14 ± 0.39	0.78 ± 0.04	0.74 ± 0.05
4	Control	4.53 ± 0.29	4.42 ± 0.37	5.38 ± 0.42	5.41 ± 0.42	0.84 ± 0.05	0.81 ± 0.07

**Significantly different from group 4 ($p < 0.05$) ANOVA followed by LSD; *Significantly different from group 1 ($p < 0.05$).

of 4.90 per rat and tumour burden of 542.02 mm^3. Histopathological examination revealed cirrhotic changes, severe dysplastic nodules and HCC. Tumour cells showed an increased nuclear/cytoplasmic ratio, nuclear pleomorphism and hyperchromatism. Multiple metastatic lung deposits in addition to HCC were observed in one of the eight animals in group 1. Dietary administration of Polyphenon-B to DAB treated animals (group 2) decreased the tumour incidence to 12.5%, with a multiplicity of 1.56 per rat. Furthermore, the tumours were significantly smaller (mean tumour burden 15.85 mm^3) compared to group 1. In addition to reducing the incidence of HCC, Polyphenon-B also significantly decreased the incidence of cirrhosis, dysplastic lesions and cholangiocarcinoma. Of the eight animals, only one developed cholangiocarcinoma, while four animals showed mild-to-moderate cirrhotic changes, and the remaining three rats displayed only mild dysplastic nodules. No tumours were observed in groups 3 and

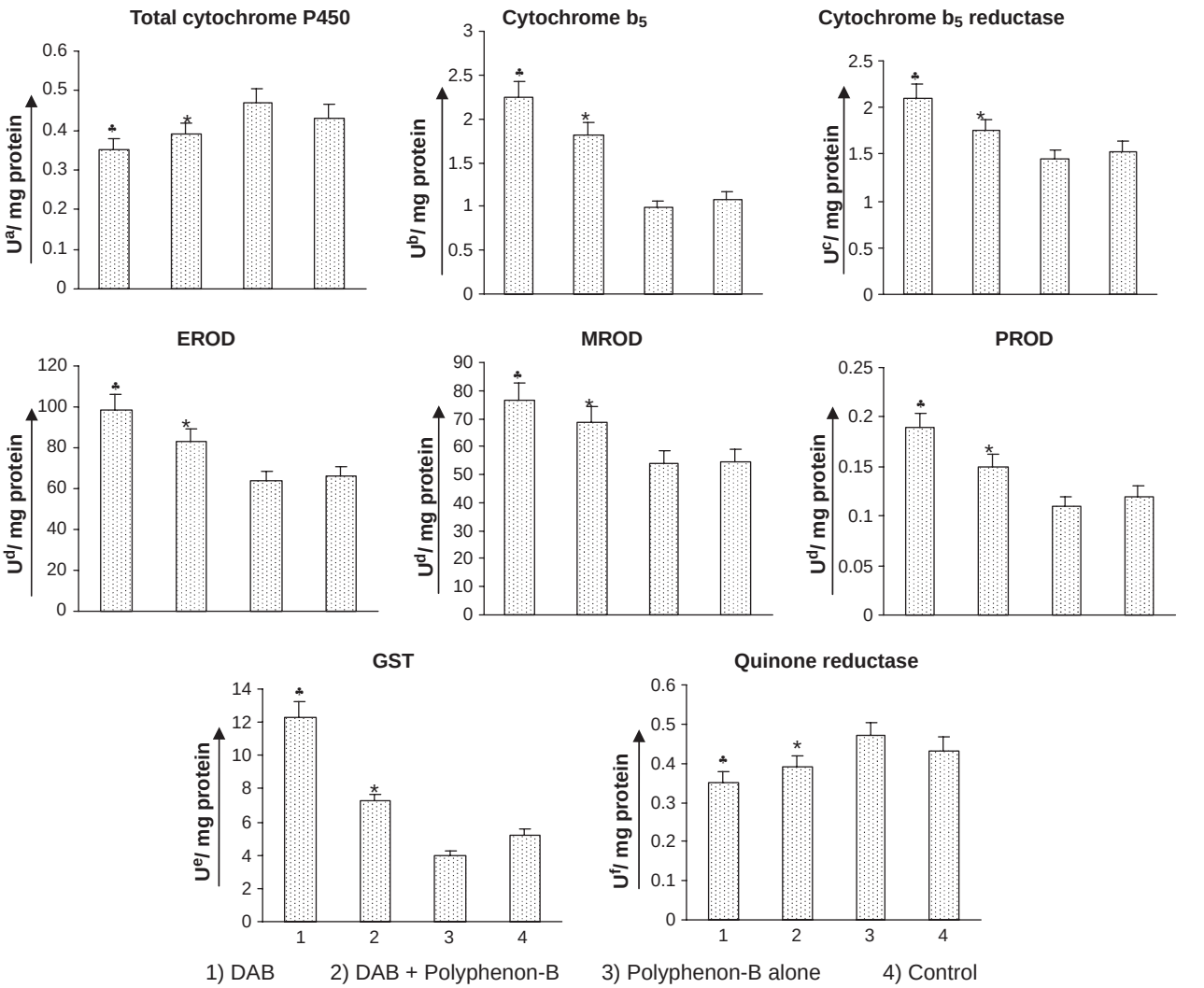

Figure 2. Activities of phase I and phase II enzymes in the S9 fractions of the liver of control and experimental animals (Mean ± SD; n = 8). § Significantly different from group 4 ($p < 0.05$) ANOVA followed by LSD. * Significantly different from group 1 ($p < 0.05$). a-µmoles of cytochrome P450; b-µmoles of cytochrome b_5; c-µmoles of cytochrome b_5 reductase; d-nmoles of resorufin formed/min; e-µmoles of CDNB conjugated with reduced glutathione per min; f-µmoles of DCPIP reduced per min.

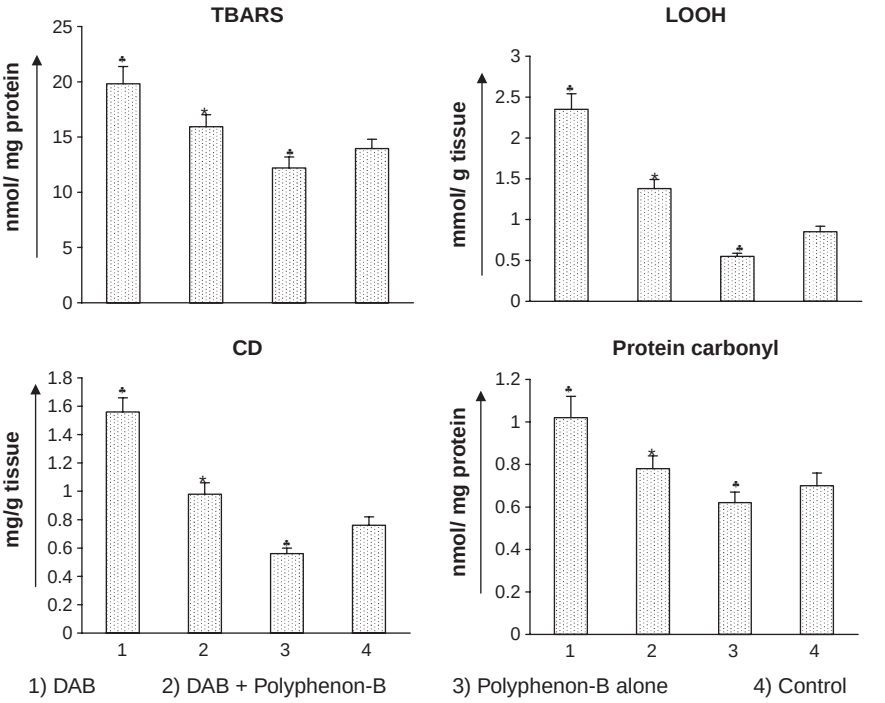

Figure 3. The levels of lipid and protein oxidation in the liver of experimental and control animals. (Mean ± SD; n = 8). § Significantly different from group 4 ($p < 0.05$) ANOVA followed by LSD. *Significantly different from group 1 ($p < 0.05$).

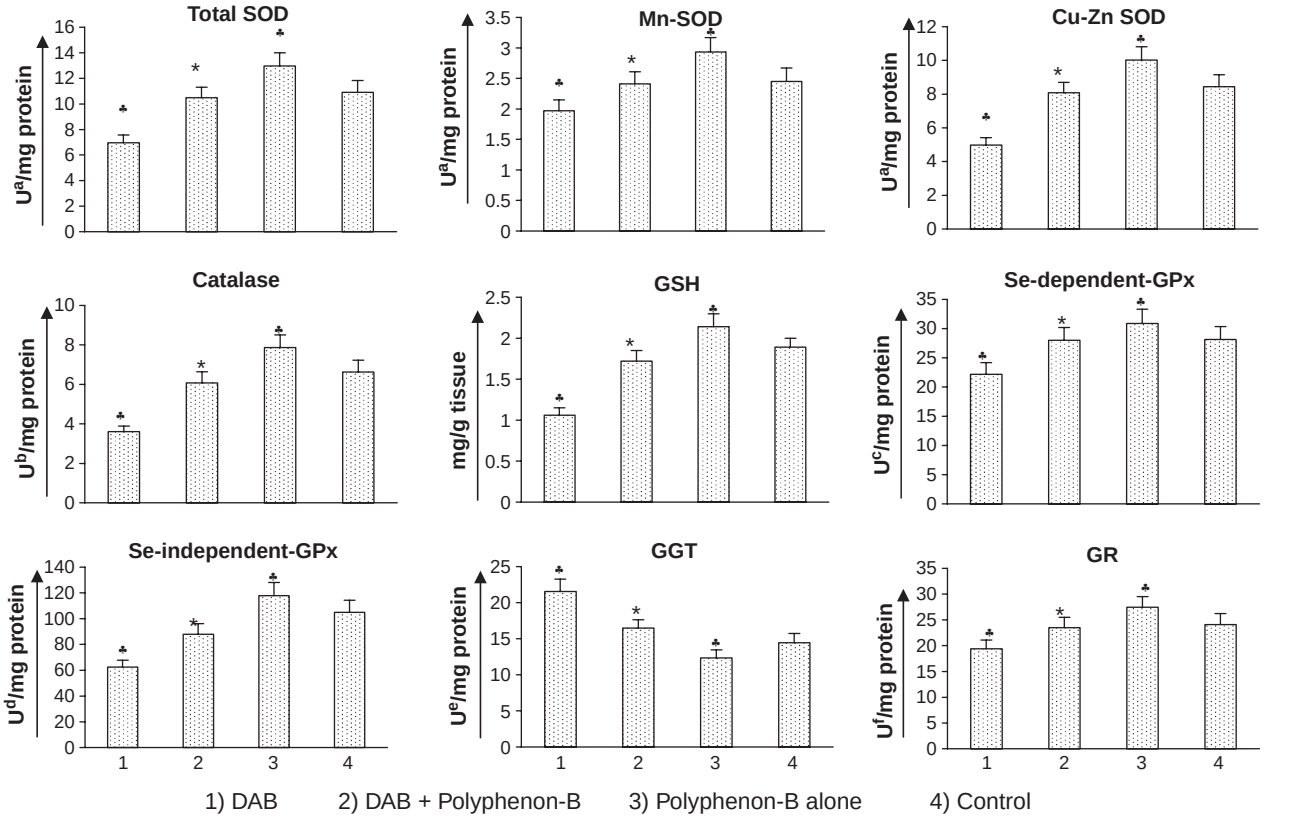

Figure 4. The activities of SODs, catalase, and GSH-dependent enzymes and GSH levels in the liver of experimental and control animals (Mean ± SD; n = 8) §Significantly different from group 4 ($p < 0.05$) ANOVA followed by LSD. *Significantly different from group 1 ($p < 0.05$). a-Amount of enzyme required to give 50% inhibition of NBT reduction; b-μmoles of H2O2 utilised per second; c-μmoles of GSH utilized per minute; d-μmoles of NADPH utilised per minute; e-μmoles of p-nitroaniline formed per hour; f-μmoles of NADPH oxidized per hour.

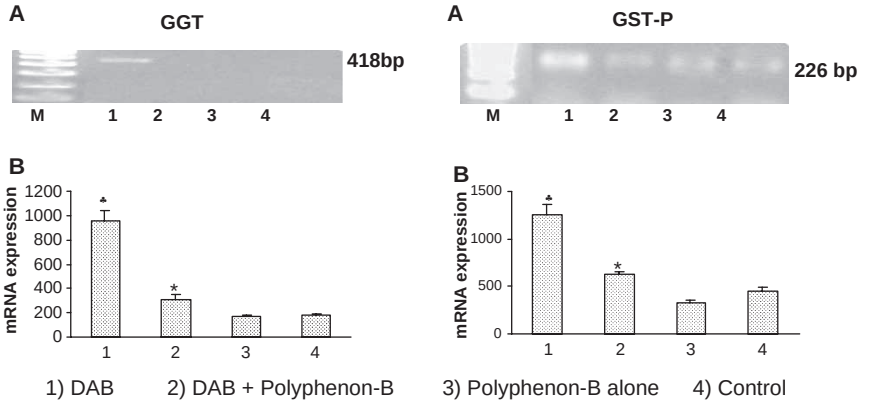

Figure 5. The effect of Polyphenon-B on the mRNA expression of GGT and GST-P in the liver tissue of control and experimental animals (Mean ± SD; n=8). (A) RT-PCR analyses of GGT and GST-P. (B) Densitometric analysis. § Significantly different from group 4 animals ($p < 0.05$). *Significantly different from group 1 animals ($p < 0.05$).

4 and histologically the epithelium was found to be normal, intact and continuous. Representative photomicrographs of histopathological changes in each group are shown in Figure 1.

Biochemical findings

Table II shows the AST, ALT enzyme activities and AST/ALT ratio in the liver and serum of control and experimental animals. The activities of AST, ALT and AST/ALT ratio were significantly increased in the liver of DAB-administered animals (group 1) compared to control (group 4). Dietary administration of Polyphenon-B to DAB treated animals significantly decreased the activities of AST, ALT and AST/ALT ratio compared to group 1. Treatment with Polyphenon-B alone did not significantly alter the activities of liver marker enzymes in group 3 animals compared to control (group 4).

Figure 2 shows phase I and phase II xenobiotic-metabolizing enzyme activities in the S9 fraction of the liver of control and experimental animals. The activities of total cytochrome P450, CYP1A1, 1A2, 2B, cytochrome b_5, cytochrome b_5 reductase and GST were significantly increased, whereas the activity of QR was significantly decreased in the liver of DAB-administered animals (group 1) compared to control (group 4). Dietary administration of Polyphenon-B to DAB treated animals significantly decreased the activities of phase I enzymes and GST and elevated QR enzyme activity compared to group 1. Treatment with Polyphenon-B alone did not induce any significant changes in xenobiotic-metabolizing enzyme activities compared with control (group 4).

Figure 3 shows the extent of lipid and protein oxidation in the liver of control and experimental animals. The extent of lipid and protein oxidation was significantly increased in the liver of DAB administered animals (group 1) compared to control. Administration of Polyphenon-B (group 2) significantly modulated DAB-induced changes in lipid and protein oxidation compared to group 1. Dietary administration of Polyphenon-B alone significantly reduced lipid and protein oxidation in group 3 animals compared to control (group 4).

Figure 4 shows the activities of antioxidant enzymes SODs (total SOD, Mn-SOD), catalase GSH and GSH-dependent enzymes GPx, GGT and GR in the liver of control and experimental animals. The activities of SODs (SOD, Mn-SOD and Cu-Zn SOD), catalase, GSH, GPx and GR were significantly decreased, whereas GGT was significantly increased in the liver of DAB administered animals (group 1) compared to control. Administration of P-B (group 2) significantly enhanced SODs (total SOD, Mn-SOD and Cu-Zn SOD), catalase, GSH, GPx and GR activities and decreased GGT activity in the liver of group 2 animals compared to group 1. Dietary administration of P-B alone significantly modulated the antioxidant status in group 3 animals compared to control (group 4).

RT-PCR analysis

Figure 5 shows the representative RT-PCR data for GGT and GST-P in the liver tissue of control and experimental animals. Quantification of band by densitometric scanning shows a significant increase in the expression of GGT and GST-P in DAB treated group 1 rats compared to untreated control. Dietary administration of Polyphenon-B decreased the expression of GGT and GST-P in liver tissue as compared to group 1. In animals administered chemopreventive agents alone, the mRNA expression of GGT and GST-P was not significantly different from that in untreated controls.

Immunohistochemical findings

Figure 6 and Table III shows the expression of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG in the liver of control and experimental

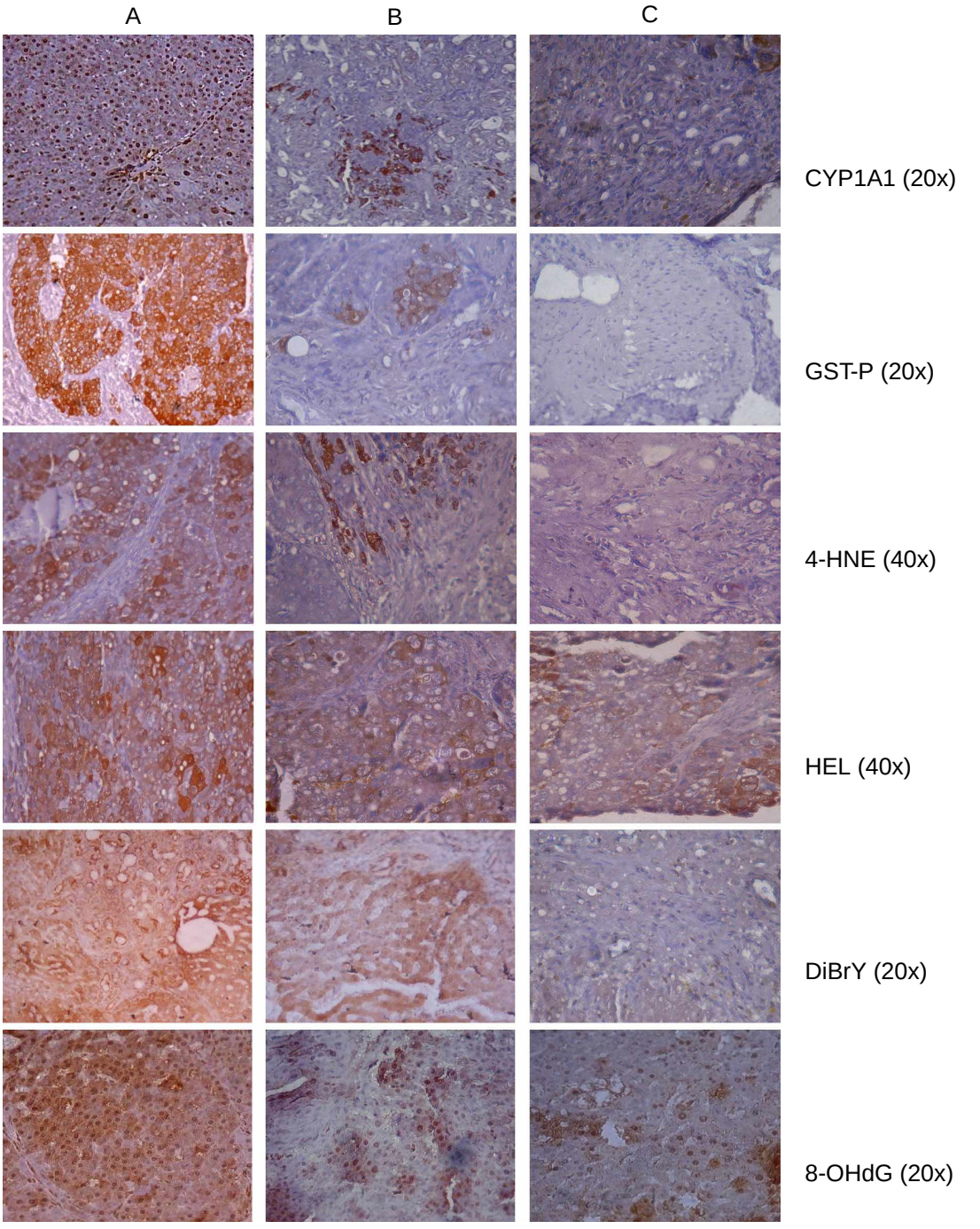

Figure 6. Photomicrographs of immunohistochemical staining of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG expressions in the liver of control and experimental animals (Mean ± SD; $n = 8$). (A) Over expression of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG in the liver tissues of DAB treated animals (Group 1). (B) Down regulation of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG in the liver tissues of DAB + Polyphenon-B treated animals (Group 2). (C) Normal expression of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG in the liver tissues of animals administered Polyphenon-B alone and control (groups 3 and 4, respectively).

Table III. Expression of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG in experimental and control animals (Mean ± SD; $n = 8$).

Group	Treatment	CYP1A1	GST-P	4-HNE	HEL	DiBrY	8-OHdG
1.	DAB	83.21 ± 5.43**	92.13 ± 7.82**	87.96 ± 6.08**	85.65 ± 5.67**	80.12 ± 6.23**	89.09 ± 6.78**
2.	DAB + P-B	54.53 ± 3.67*	58.32 ± 4.92*	65.76 ± 4.76*	60.10 ± 4.12*	57.63 ± 4.01*	56.96 ± 4.09*
3.	P-B	23.13 ± 1.67	26.35 ± 1.60	35.45 ± 2.60	30.10 ± 1.98	29.36 ± 1.25	32.21 ± 2.09
4.	Control	22.65 ± 1.50	24.98 ± 1.98	34.32 ± 2.06	29.01 ± 1.89	28.63 ± 1.56	31.03 ± 1.98

**Significantly different from group 4 ($p < 0.05$) ANOVA followed by LSD; *Significantly different from group 1 ($p < 0.05$).

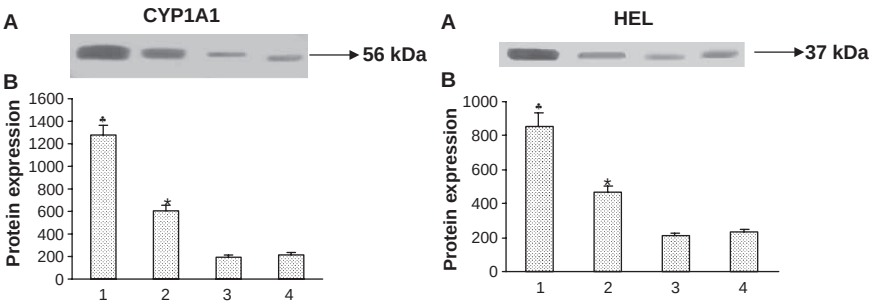

Figure 7. The effect of Polyphenon-B on CYP1A1 and HEL expression in the liver tissue of control and experimental animals (Mean ± SD; n=8). (A) Representative immunoblots of CYP1A1 and HEL. (B) Densitometric analysis. §Significantly different from untreated control ($p < 0.05$). *Significantly different from group 1 animals ($p < 0.05$).

animals. In DAB treated animals, the expression of CYP1A1, GST-P, 4-HNE, HEL, DiBrY and 8-OHdG in the liver tissues was significantly higher than in control animals (group 4). Dietary administration of Polyphenon-B (group 2) significantly decreased the expression of CYP1A1, GST-P and adducts in liver tissue as compared to group 1. No significant changes in the expression of markers were observed in animals administered Polyphenon-B alone (group 3) compared to control.

Western blot analysis

Figure 7 shows the representative western blot data for CYP1A1 and HEL in the liver tissue of control and experimental animals. Quantification of each band by densitometric scanning shows significant increase in the expression of CYP1A1 and HEL in DAB treated group 1 rats compared to untreated control. Dietary administration of Polyphenon-B decreased the expression of CYP1A1 and HEL as compared to group 1. No significant changes in the expression of CYP1A1 and HEL were observed in animals treated with chemopreventive agents alone as compared to control.

Discussion

In the present study, administration of Polyphenon-B significantly inhibited the development of DAB-induced hepatomas and pre-neoplastic lesions, as well as the elevation in serum hepatic marker enzymes. The inhibitory effects of Polyphenon-B on DAB-induced hepatocarcinogenesis was associated with decreased CYP activity and CYP1A1 expression, modulation of phase II enzyme activities, reduced lipid and protein oxidation, adduct formation and upregulation of antioxidants.

Azo dyes such as DAB are procarcinogens that undergo metabolic activation through N-oxidation and N-hydroxylation by CYP in the liver to form electrophilic intermediates N-hydroxy-N-methyl-4-aminoazobenzene (N-OH-MAB) and N-hydroxy-4-aminoazobenzene (N-OH AAB) that interact with tissues to generate lipid peroxides [4,42,43]. In addition, toxic ROS such as $O_2^{\cdot -}$ and H_2O_2 generated as byproducts during CYP mediated metabolism of DAB can cause oxidative damage to cellular macromolecules [43]. The decrease in CYP activity and CYP isoforms induced by Polyphenon-B can block the generation of toxic electrophiles and ROS. These findings are in line with observations by other workers. Krishnan and Maru [44] documented the inhibitory effects of polymeric black tea fractions on CYP isoenzymes in rat liver microsomes. Catterall et al. [45] reported inhibition of hepatic CYP enzymes by black tea theaflavins. EGCG, the major catechin present in black tea, has been found to modulate the activity of CYP1A1 and 1A2 [46].

Phase II detoxification enzymes function to eliminate electrophiles and ROS generated by phase I reactions, thereby preventing oxidative stress, adduct formation and malignant transformation [47]. Despite increased activities of GST and GGT, there was evidence of oxidative stress in DAB-induced hepatomas as reflected by increased expression of 4-HNE, HEL, DiBrY and 8-OHdG. This may be due to reduced activity of the detoxification enzyme QR that plays an important role in preventing the formation of semiquinones and ROS [48]. It is also conceivable that the increase in total GST activity reflects increased expression of GST-P, which together with GGT functions as a marker of chemical-induced hepatocarcinogenesis [49]. The most important cause for oxidative stress, however, is the compromised enzymic and non-enzymic antioxidant defences in DAB-induced hepatomas. Mauriz et al. [50] have also shown chaotic antioxidant and detoxification systems in diethylnitrosamine-induced rat hepatomas.

In contrast to its effect on CYP, Polyphenon-B showed a dual action on phase II enzymes with decrease in GST and GGT and increase in QR activities, thereby counteracting the influence of DAB. Black tea extract was shown to lower the number of GST-P and GGT-positive foci, hallmarks of hepatic preneoplasia and neoplasia [51]. Black tea infusion was found to increase QR activity in cell lines [52]. In a recent report, Patel and Maru [53]

demonstrated that polymeric black tea polyphenol extract activated Nrf2-antioxidant-responsive element (ARE)-mediated upregulation of hepatic phase II enzymes through phosphorylation of Nrf2 by PKC and PI3-kinases.

Ours is the first report on the effect of black tea preparations on expression of the oxidative stress markers 4-HNE, HEL, DiBrY and 8-OHdG. The protective effects of Polyphenon-B against oxidative stress can be correlated to its antioxidant properties. Recently, we reported the potent *in vitro* ROS scavenging properties of Polyphenon-B as well as its *in vivo* antioxidant enhancing effects in the hamster buccal pouch carcinogenesis model [54]. Black tea preparations have been demonstrated to ameliorate aflatoxin-induced lipid peroxidation by inducing enzymic and non-enzymic antioxidants and prevent cellular DNA damage in HepG2 cells by inhibiting oxidative stress [55,56]. The mechanisms by which black tea preparations exert their antioxidant effects include chelation of metal ions, prevention of ROS generation and inhibition of lipid and protein oxidation [57].

The results of the present study provide evidence that exposure to DAB causes imbalance in phase I and phase II metabolizing enzyme activities, decrease in antioxidant defence mechanisms resulting in oxidative stress and development of HCCs. Polyphenon-B offers promise as a potential chemopreventive agent for HCC owing to its ability to modulate xenobiotic metabolizing enzymes, prevent lipid and protein oxidation, reduce adduct formation and enhance antioxidants. Studies from this laboratory as well as by other workers have documented the anti-carcinogenic effects of Polyphenon-B in other animal tumour models, emphasizing its broad spectrum chemopreventive potential [15–17,58]. Since carcinogen activation and detoxification are early events in carcinogenesis, agents such as Polyphenon-B that modulate these reactions deserve considerable attention in chemoprevention of cancer.

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