

Galangin, a dietary flavonol inhibits tumor initiation during experimental pulmonary tumorigenesis by modulating xenobiotic enzymes and antioxidant status

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Abstract The aim of present study was to elucidate anti-initiating efficacy of galangin against benzo(a)pyrene (B(a)P)-induced lung carcinogenesis in male Swiss albino mice. Therefore, the activities of xenobiotic metabolic enzymes such as phase I and II were examined in lung as well as liver tissues (to compare the effects between target and non-target organs). Besides, the activities/levels of tissue marker enzymes, antioxidants, lipid peroxidation (LPO), cytochrome P450 1A1 (CYP1A1) expressions and histological observation of lungs were also analyzed. B(a)P (50 mg/kg body weight) was administered to male Swiss albino mice (20–25 g) to experimentally induce lung cancer. B(a)P-induced animals showed increased activity of phase I (Cytochrome P450, Cytochrome b5, NADPH Cytochrome P450 reductase and NADH Cytochrome b5 reductase) drug metabolic enzymes, LPO levels, tissue marker enzymes and decreased activity of phase II metabolic enzymes (glutathione-S-transferase, DT-diaphorase and UDP-glucuronyl transferase) as well as antioxidant levels. Histological examination of lungs revealed severe alveolar and bronchiolar damages in B(a)P-induced mice. Immunohistochemical and western blot analysis of CYP1A1 increased significantly in lung tissues of B(a)P-induced animals. Treatment with galangin (20 mg/kg body

weight) efficiently counteracted all the above anomalies and restored cellular homeostasis. Our results demonstrate that galangin can modify xenobiotic enzymes in murine model of pulmonary tumorigenesis.

Keywords Galangin · Benzo(a)pyrene · Lung cancer · Xenobiotic metabolic enzymes · CYP1A1 and antioxidants

Introduction

Lung cancer is the most commonly diagnosed cancer as well as leading cause of death in the world (Jemal et al. 2011). Tobacco smoking is considered as a major etiological risk factor for lung cancer. Among the constituents of smoke, the polycyclic aromatic hydrocarbons (PAHs) like benzo(a)pyrene (B(a)P) plays a major role in lung carcinogenesis (Hecht et al. 2002). Cytochrome P450s (CYPs), in general, is regulated by a cytosolic protein, aryl hydrocarbon receptor (Ahr) (Rowlands and Gustafsson 1997). Upon ligand binding, Ahr translocates into the nucleus, where it heterodimerizes with Ahr nuclear translocator protein (Nebert et al. 1993; Barouki et al. 2007) and binds with the xenobiotic response elements (XRE) flanking phase I and phase II enzyme genes, thereby activating their transcription (Lamb et al. 1994). While B(a)P is relatively non-toxic, it is a potent ligand which activates the Ahr pathway through CYP1A1 enzyme (phase I enzyme) and finally transforms into a highly carcinogenic electrophilic (\pm)-B(a)P-r-7,t-8 dihydrodiol-t-9,10-epoxide (BPDE). Though phase II enzymes catalyze the detoxification of BPDE, some of reactive electrophiles covalently bind with hexocyclic nitrogen of deoxyguanosine in DNA via trans-addition of C-10 position in epoxide molecule and forms DNA adducts (Sticha et al. 2000; Baird et al. 2005).

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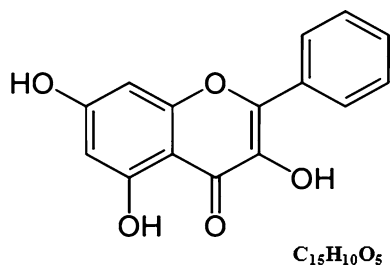


Fig. 1 Structure of galangin

These DNA adducts, if left unrepaired, can cause mutations in genes that regulate xenobiotic metabolism, apoptosis, tumor suppression and/or oncogenes leading to tumor development (Labib et al. 2012). This implies that the phase I and II enzymes play a vital role in carcinogenic metabolism and hence could be an important target for chemoprevention.

Chemoprevention is considered as the best way of inhibiting, delaying or reversing carcinogenesis (Bonnesen et al. 2001). A number of effective chemopreventive measures have been introduced substantially to reduce both the incidence and mortality due to lung cancer. The search for new compounds in foods or in plant medicines showing anti-cancer effects is one realistic and promising approach to prevention. Flavonoids, the naturally occurring polyphenolic compounds, that represent one major class of compounds found in vegetables, nuts, fruits, beverages such as coffee, tea and red wine (Hollman and Katan 1997; Martin and Appel 2010). The biological functions of flavonoids have been attributed to wide mechanisms, including modulating detoxifying enzymes and cytochrome P450 system (Walle et al. 2000; Conney 2003).

Galangin (3,5,7-trihydroxyflavone) (Fig. 1), a member of flavonol class of flavonoids, is present at high concentration in propolis, a natural material produced by honeybees and in *Alpinia officinarum*, a plant which has been used as a spice and a herbal medicine for a variety of ailments in Asia for centuries (Heo et al. 2001). Galangin has been demonstrated to possess anti-clastogenic (Heo et al. 1994), anti-mutagenic (So et al. 1997), anti-inflammatory (Kang et al. 2000), anti-viral (Meyer et al. 1997), antioxidative (Imamura et al. 2000) and metabolic enzyme modulating activities (Shih et al. 2000). Further, reports assert that galangin induces apoptosis in various cancer cells thereby exhibiting anti-cancer property (Gwak et al. 2011; Kim et al. 2012; Zhang et al. 2012). However, the role of galangin in modulating xenobiotic enzymes and tumor markers during B(a)P-induced lung carcinogenesis remains elusive. In this study, we have demonstrated that galangin exhibits anti-initiating mechanism by tuning phase I and phase II enzyme levels in swiss albino mice.

Materials and methods

Chemicals

Benzo(a)pyrene and galangin were purchased from Sigma chemical Company (St. Louis, MO, USA). The antibody used in this study was procured from Santacruz Biotech (USA). All other chemicals and reagents used were of the highest analytical grade commercially available.

Animals

Healthy male Swiss albino mice of 8–10 weeks old and 20–25 g of weight were procured from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India. Mice were housed in a controlled environmental condition of temperature (22–24 °C) and humidity (45–50 %) with a lightening schedule of 12 h light and 12 h dark. Mice were balanced with commercial mice diet (Hindustan UniLever Ltd., Mumbai, India) and water ad libitum. The protocols and procedures were approved by Institutional Animal Ethics Committee (University of Madras). The study was fully conformed to the ethical standards approved by the Ministry of Social Justices and Empowerment of India (IAEC No. 010/02/2011).

Experimental protocol

The experimental animals were divided into five groups, each group comprising of six animals.

- Group 1 Animals served as normal control
- Group 2 Animals were administered with B(a)P (50 mg/kg body weight dissolved in corn oil, orally) twice a week for 4 successive weeks to induce lung cancer (Wattenberg and Leong 1970; Selvendiran et al. 2003)
- Group 3 Animals were pre-treated with galangin (20 mg/kg body weight dissolved in corn oil was given twice a week, orally) (according to the optimum dose fixation study) two weeks before the first dose of B(a)P-induction (group 2) and continued for 16 weeks
- Group 4 Animals were post-treated with galangin (group 3) from 8th week of B(a)P induction till the end of experiment (16th week)
- Group 5 Animals were administered with galangin alone (group 3) for 16 weeks to analyze the cytotoxicity (if any) induced by galangin

The pre- and post-treated groups were used to study the chemopreventive as well as chemotherapeutic potential of galangin.

Mice were weighed weekly once until the end of experimental period. After completion of the experimental period, animals from each group were anaesthetized with a combination of ketamine/xylazine (90 and 10 mg/kg body weight respectively) by intramuscular injection. The anaesthetized animals were sacrificed by cervical decapitation. Immediately after euthanizing, blood was collected and serum was separated by centrifugation at $2,500\times g$. The lung (target) and liver (non-target) tissues were immediately dissected out, washed in ice-cold saline to remove any extraneous matter, cleaned and blotted to dryness in filter paper. The weight of lungs and percentage of tumor incidence were observed. The tissues were homogenized with 0.1 M Tris-HCl buffer (pH 7.4). Total protein was estimated by the method of Lowry et al. (1951).

Biochemical analysis

Estimation of tissue marker enzymes was performed in lung homogenate and serum of control and experimental animals. The aryl hydrocarbon hydroxylase (AHH) was estimated by method of Mildred et al. (1981); gamma glutamyl transferase (γ -GT) was analyzed by method of Orłowski and Meister (1965); 5'-nucleotidase (5'-ND) was assayed by method of Luly et al. (1972); lactate dehydrogenase (LDH) was estimated by method of King (1965) and adenosine deaminase (ADA) was assayed by method of Baggott et al. (1986).

Phase I and II enzymes

The phase I and phase II drug metabolizing enzyme analyses were carried out in both lung and liver tissue homogenates. cytochrome P450 and cytochrome b5 content were estimated as described by Omura and Sato (1964); NADPH cytochrome P450 reductase was estimated by method of Phillips and Langdon (1962); NADH cytochrome b5 reductase was estimated by method of (Strittmatte and Velick 1956); glutathione-S-transferase (GST) was estimated by method of Habig et al. (1974); DT-diaphorase (DT-D) was estimated by method of Ernest et al. (1982) and UDP glucuronyl transferase (UDP-GT) was estimated by method of Bock et al. (1983).

Immunohistochemistry

Immunohistochemical analysis of CYP1A1 expression was carried out on paraffin embedded sections of lung tissue. Initially, the sections were deparaffinized with xylene and rehydrated with graded series of alcohol. The slides were incubated in citrate buffer (pH 6.0) for three cycles of five min each in a microwave oven for antigen retrieval. The sections were then allowed to cool at room temperature (RT)

and then rinsed with $1\times$ Tris buffered saline (TBS) and treated with 0.3 % H_2O_2 in methanol for 10 min to quench endogenous peroxidase activity. Non-specific binding was blocked with 3 % BSA in RT for 1 h. The sections were then incubated overnight with primary antibody (goat polyclonal antibody at a dilution of 1:500, at 4 °C). The slides were washed with TBS and then incubated with anti-goat horse radish peroxidase (HRP) conjugated secondary antibody (Genei, Bangalore, India) at a dilution of 1:500 for 1 h at RT. The peroxidase activity was visualized by treating the slides with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (SRL, Mumbai, India) and were counterstained with Meyer's hematoxylin. Quantitative analysis was made in a blinded manner to illustrate the total number of positively stained cells under a light microscope.

Western blot analysis

The expressions of CYP1A1 in the lung tissues of control and experimental group of animals were analyzed. About 30 μ g proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane, incubated with primary antibody (goat polyclonal antibody at a dilution of 1:100) overnight at 4 °C with gentle shaking. The membrane was subsequently washed with TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % Tween-20), conjugated with secondary antibody (anti-goat HRP conjugated antibody at a dilution of 1:500 for 1 h at RT) and washed again. By the addition of DAB as a substrate the protein antibody complexes were detected. We used the Image-J software to perform densitometric analysis to quantify target protein bands.

Histological evaluation

Autopsy samples taken from lung of mice in different groups were fixed in 10 % formalin and embedded in paraffin wax. Sections were cut at 4 μ m thickness, deparaffinized and stained by hematoxylin and eosin (H&E) stains for histological examination through the light microscope.

Lipid peroxidation (LPO) and antioxidants

The levels of lipid peroxidation (LPO) were measured in serum and lung homogenates using method of Ohkawa et al. (1979). From the lung tissue homogenates, the levels of enzymic and non-enzymic antioxidants such as SOD (Misra and Fridovich 1972), CAT (Takahara et al. 1960), GPx (Rotruck et al. 1973), GSH (Moron et al. 1979),

Vitamin C (Omaye et al. 1979) and Vitamin E (Desai 1984) were measured.

Statistical analysis

Results are presented as mean \pm standard deviation (S.D.) of six mice in each group. One-way analysis of variance was used to compare all the parameters of group 1–5 and least significant difference test was performed for all pair wise comparisons, when appropriate. Comparisons were considered significant for P values of 0.05 or less. Statistical analysis was performed using SPSS version 10.0.

Results

General observations

The body and lung weight, size, incidence as well as volume of tumor of different groups of mice were shown in Table 1. The final body weight of B(a)P-administered animals was found to be significantly ($P < 0.05$) decreased, while the lung weight, size, incidence and volume of tumor were significantly ($P < 0.05$) increased when compared to control animals. By contrast, administration of galangin to pre- and post-treated mice showed significant ($P < 0.05$) increase in the final body weight and reduced lung weight, tumor incidence, tumor size and volume when compared to B(a)P-induced animals. No significant changes were observed between the control and animals treated with galangin alone.

Evaluation of tissue marker enzymes

The effect of galangin on activities of marker enzymes in serum and lung tissue of control and experimental group of animals were shown in Supplementary Table 1. The marker enzymes AHH, γ -GT, 5'-ND, LDH and ADA were

significantly ($P < 0.05$) increased in B(a)P-administered animals and these were reverted to near normal levels in the animals pre- and post-treated with galangin. However, there was no significant difference between control and the animals treated with galangin alone.

Effect of galangin on cytochrome P450 and b5

Figure 2a represents the activities of lung and liver microsomal Cyt P450 and Cyt b5 in control and experimental groups of animals. There was a significant ($P < 0.05$) increase in lung and liver Cyt P450 and Cyt b5 activities in B(a)P-administered animals when compared to control animals. Galangin treatment (pre- and post-treatment) significantly ($P < 0.05$) decreased in Cyt P450 and Cyt b5 activities when compared to B(a)P-induced animals. There was no significant change was observed between control and the animals treated with galangin alone.

Effect of galangin on phase I enzymes

The activities of lung and liver microsomal phase I enzymes were represented in Fig. 2b, c. A significant ($P < 0.05$) increase in the activities of NADPH Cyt P450 reductase and NADH Cyt b5 reductase were observed in B(a)P-induced animals when compared to control animals. The enzyme activities were significantly ($P < 0.05$) reverse to near normalcy on treatment with galangin in both pre- and post-treated animals, when compared to B(a)P-induced animals. There was no significant difference between control and the animals treated with galangin alone.

Effect of galangin on phase II enzymes

Figure 2d, e depict the activities of phase II enzymes in lung and liver of control and experimental group of

Table 1 Effect of galangin on the body and lung weight of control as well as experimental animals

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
Body weight (g)	28.78 \pm 2.90	19.23 \pm 3.17 ^a	24.39 \pm 3.46 ^{b,d}	22.83 \pm 3.88 ^c	27.80 \pm 3.25 ^{ns}
Lung weight (mg)	281.23 \pm 35.07	357.94 \pm 37.71 ^a	291.69 \pm 24.18 ^{b,d}	296.42 \pm 21.96 ^c	270.02 \pm 35.52 ^{ns}
Incidence of tumor (%)	0	100 ^a	30 ^{b,d}	66 ^c	0 ^{ns}
Size (mm)	0	2.1 ^a	0.6 ^{b,d}	1.3 ^c	0 ^{ns}
Tumor volume (mm ³)	0	5.22 \pm 0.07 ^a	0.257 \pm 0.03 ^{b,d}	0.953 \pm 0.04 ^c	0 ^{ns}

Pathological grade of each tumor and total lung area was determined using pixel counts of digitized images. Each value is expressed as mean \pm SD for six mice in each group. Statistical significance $P < 0.05$

^{ns} Not significant

^a Group 2 compared with group 1

^b Group 3 compared with group 2

^c Group 4 compared with group 2

^d Group 3 compared with group 4

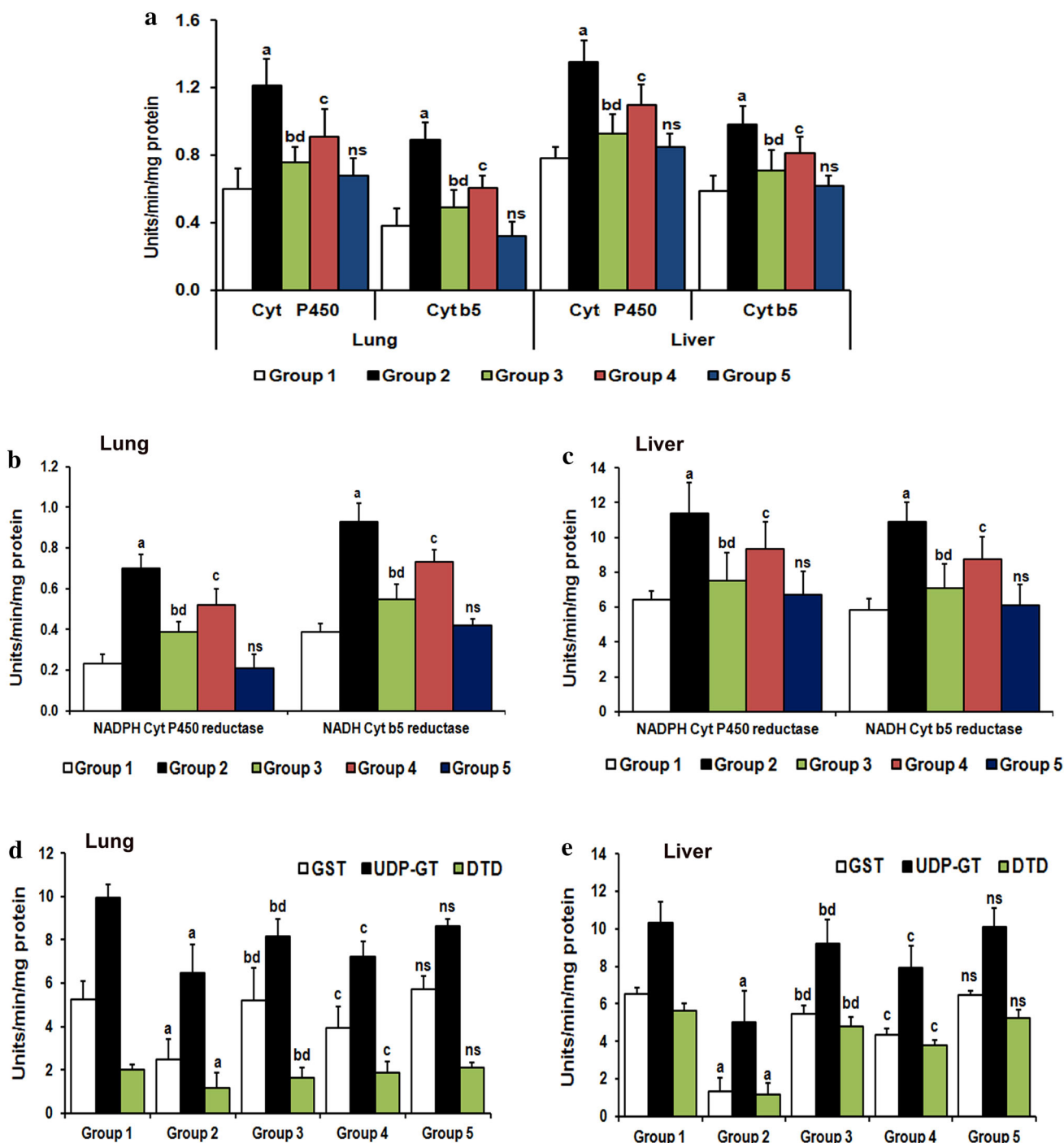


Fig. 2 Effect of galangin on the status of phase I and II metabolic enzymes of control and experimental groups. The levels of lung and liver microsomal Cyt P450 and Cyt b5 (a), the activities of lung (b) and liver (c) NADPH Cyt P450 reductase and NADH Cyt b5 reductase were found to be significantly higher in B(a)P-induced animals, whereas the activities of lung (d) and (liver) GST, UDP-GT and DTD were significantly lowered. Upon administration of galangin (pre- and post-treatment) the activities of these enzymes reverted to near normal levels. No significant difference was observed between control and the animals treated with galangin. Each value is expressed

as mean \pm SD for six mice in each group. Results are given as statistically significance at $P < 0.05$. a group 2 compared with group 1, b group 3 compared with group 2, c group 4 compared with group 2, d group 3 compared with group 4, ns not significant. Units Cyt P450 and Cyt b5-nmoles/min/mg protein, NADPH Cyt P450 reductase nmoles of Cyt c oxidized/min/mg protein, NADH Cyt b5 reductase nmoles of ferricyanide reduced/min/mg protein, GST μ mol of CDNB conjugated/min/mg protein, UDP-GT nmoles of p-nitrophenol conjugated/min/mg protein, DTD nmoles of glucuronides formed/min/mg protein

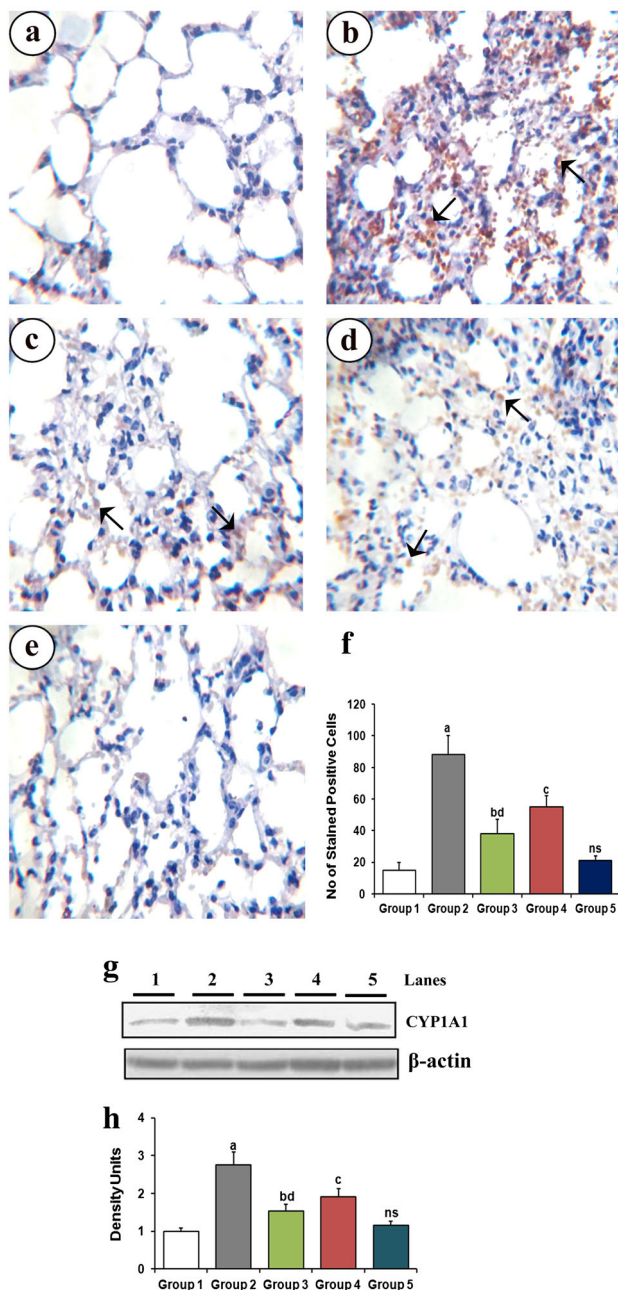


Fig. 3 The representative photo micrographs show immunohistochemistry and western blot expression of CYP1A1 in the lung tissues of control as well as experimental group of animals. **(a)** CYP1A1 expression in control, **(b)** B(a)P-induced group, **(c)** galangin pre-treated group, **(d)** galangin post-treated, **(e)** CYP1A1 expression of galangin alone group. Arrows indicate CYP1A1 positive cells and magnification $\times 40$, **(f)** quantitative analysis of CYP1A1 expression, the number of stained (positive) cells was averaged across 10 fields/section. **(g)** Immunoblots of CYP1A1 and β -actin protein expression: Lane 1 control, Lane 2 B(a)P-induced group, Lane 3 galangin pre-treated group, Lane 4 galangin post-treated group and Lane 5 galangin alone group. **(h)** Quantitative data expressing the corresponding protein levels were measured using Image J software and expressed as relative density. Hypothesis testing method included one way ANOVA followed by least significant difference. Values are given statistically significance at $P < 0.05$. *a* group 2 compared with group 1, *b* group 3 compared with group 2, *c* group 4 compared with group 2, *d* group 3 compared with group 4, *ns* not significant

animals. A significant ($P < 0.05$) decrease in the activities of GST, UDP-GT and DTD was observed in B(a)P-induced animals, when compared to control animals. Treatment with galangin (pre- and post-) significantly ($P < 0.05$) increased the above enzyme activities when compared to B(a)P-induced animals. No significant difference was observed between control and the animals treated with galangin alone.

Effect of galangin on the protein expression on cytochrome P450 1A1

Figure 3 depicts the expression of CYP1A1 by immunohistochemical and Western blot analysis in the lungs of control and experimental animals. There was a significant increase of CYP1A1 expression in B(a)P-induced animals when compared to control animals. Pre- and post-treatment with galangin showed significantly ($P < 0.05$) reduced expression of CYP1A1 when compared to B(a)P-administered animals. There was no significant changes were observed between control and the animals treated with galangin alone.

Histological observations

Figure 4 shows the histological examination of lungs section of control and experimental groups of animals. Lung tissue sections of control animals showed a normal architecture with uniform nuclei (Fig. 4a). B(a)P-administered animals (Fig. 4b) showed loss of architecture and alveolar damage as seen from increased number of hyper chromatic nuclei in the cells of alveolar wall. Animals pre-treated with galangin (Fig. 4c) exhibited reduced alveolar damage with near normal architecture and animals post-treated with galangin showed slightly reduced alveolar damage (Fig. 4d). Animals treated with galangin alone (Fig. 4e) showed no appreciable changes in histopathological features when compared to control animals.

Effect of galangin on the B(a)P-induced alteration in LPO and antioxidants levels

The extent of LPO in serum and lungs of control and experimental groups of animals was analyzed for oxidative stress (Fig. 5). In B(a)P-induced animals, there was a significant ($P < 0.05$) increase in the level of lipid peroxides when compared to normal control animals, whereas in the animals pre- and post-treated with galangin the levels decreased significantly ($P < 0.05$) when compared to B(a)P-induced animals. No significant changes between control and the animals treated with galangin alone. The effect of galangin on enzymic and non-enzymic antioxidants in lungs of control and experimental groups of animals is shown in Table 2. The enzymic and non-enzymic antioxidants such as

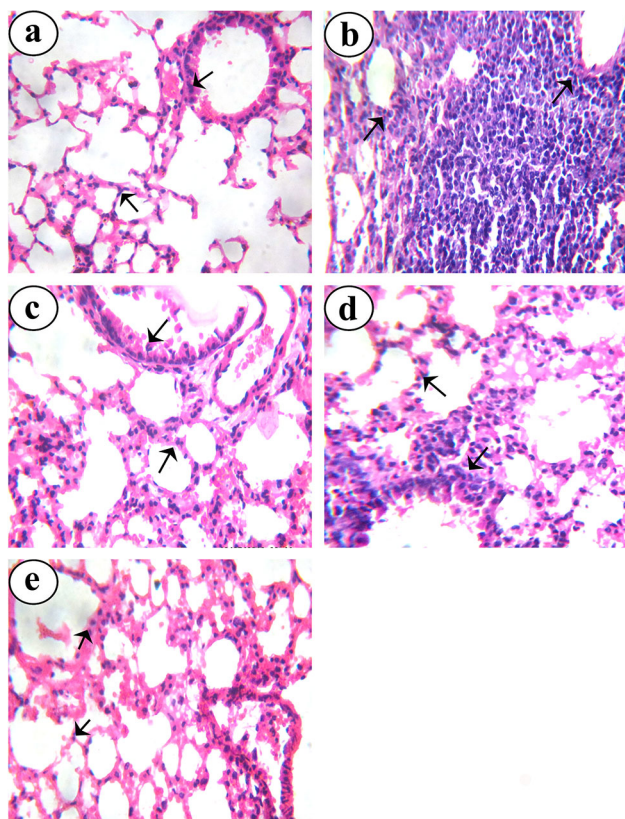


Fig. 4 The representative photo micrographs show the histological analysis on the effect of galangin on B(a)P-induced lung cancer (Hematoxylin & Eosin staining; $\times 40$). (a) Lung section of control mice showing normal architecture of alveolar and bronchiolar regions indicated by arrows. (b) Lung section of B(a)P-administered mice showing severe alveolar and bronchiolar damage with nuclear overcrowding verified its histological appearance of pulmonary carcinoma. (c) Lung section of mice pretreated with galangin showing significantly reduced damage in alveolar and bronchiolar architecture appearance near normal. (d) Lung section of mice post treated with Galangin showing slightly reduced alveolar and bronchiolar damage. (e) Section from the lung of mice administered with galangin alone showing normal architecture of alveolus and bronchioles similar to control mice

SOD, CAT, GPx, GSH, Vit. C and Vit. E were found to be significantly ($P < 0.05$) reduced in B(a)P-induced animals, whereas the animals treated (pre and post) with galangin showed significantly ($P < 0.05$) restored antioxidant levels to near normal when compared to B(a)P-induced animals. No significant change was observed between control and the animals treated with galangin alone.

Discussion

In our general observation, regression in the body weight and progression in the lung weight of B(a)P-administered animals showed the severity of pulmonary tumorigenesis

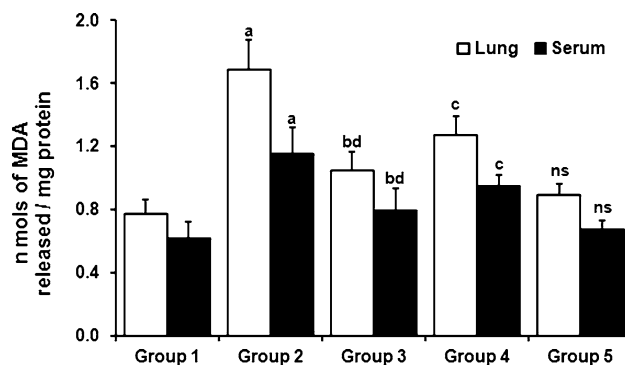


Fig. 5 The effect of galangin on the status of lipid peroxidation in lung and serum of control and experimental mice. Each value is expressed as mean \pm SD for six mice in each group. Results are given as statistically significance at $P < 0.05$. *a* group 2 compared with group 1, *b* group 3 compared with group 2, *c* group 4 compared with group 2, *d* group 3 compared with group 4, *ns* not significant. Units nmols of MDA released/mg protein

and as a common symptom of carcinogenesis as reported previously (Singh et al. 1998). The considerable weight loss in B(a)P-administered animals reportedly contributes to cancer cachexia, anorexia or malabsorption which leads to progressive wasting, notably in the skeletal muscle and adipose tissue (Magesh et al. 2006). An increased lung weight in B(a)P-induced animals could be uncontrolled proliferation of the cancer cells which leads to formation of tumor nodules. Galangin treatment showed gradual increase in the body weight and decrease in lung weight indicating its anti-tumor property against B(a)P-induced mice, tissue marker enzyme analyses also support this phenomenon. The marker enzymes such as AHH, ADA, GGT, 5'-ND and LDH are particular indicators of lung damage (Ferrigno et al. 1994). Their functions are mainly to metabolize some PAHs to protect plasma membrane from oxidative stress and also act as a marker for solid neoplasms (Buening et al. 1981; Kocic et al. 2002; Jean et al. 2002; Anbarasi et al. 2005). In the present study, there was a significant elevation in levels of these marker enzymes in B(a)P-induced animals. The activities of these enzymes were brought down to near normal level upon pre- and post-treatment with galangin. It reveals the anti-tumor effect of galangin against B(a)P induced mice.

The CYP system is the primary pathway for metabolic bioactivation of xenobiotics through the process of activation or deactivation and excretion of products. The phase I enzymes predominantly CYP450 metabolize the xenobiotics to more reactive electrophilic moieties, which in turn are detoxified by phase II enzymes (Dinkova-Kostova et al. 2001). Therefore, the inhibition of phase I enzymes and enhancement in the activity of detoxifying enzymes (phase II) by chemopreventives would play an important role in blocking the initiation process of tumorigenesis. In this

Table 2 Effect of galangin on the activities of antioxidant enzymes in lung of control and experimental mice

Particulars	Group 1	Group 2	Group 3	Group 4	Group 5
SOD	5.88 ± 0.44	3.18 ± 0.23 ^a	5.35 ± 0.28 ^{b,d}	4.19 ± 0.20 ^c	5.70 ± 0.39 ^{ns}
CAT	245.0 ± 28.57	118.33 ± 25.45 ^a	217.0 ± 33.85 ^{b,d}	171.0 ± 18.55 ^c	248.0 ± 31.05 ^{ns}
GPx	41.33 ± 4.76	20.33 ± 6.22 ^a	36.33 ± 3.98 ^{b,d}	34.67 ± 4.59 ^c	42.17 ± 3.66 ^{ns}
GSH	1.55 ± 0.07	1.05 ± 0.18 ^a	1.48 ± 0.07 ^{b,d}	1.11 ± 0.19 ^c	1.52 ± 0.10 ^{ns}
Vit C	0.50 ± 0.09	0.33 ± 0.07 ^a	0.39 ± 0.05 ^{b,d}	0.35 ± 0.08 ^c	0.52 ± 0.11 ^{ns}
Vit E	0.60 ± 0.10	0.35 ± 0.07 ^a	0.51 ± 0.12 ^{b,d}	0.41 ± 0.09 ^c	0.54 ± 0.04 ^{ns}

Each value is expressed as mean ± SD for six mice in each group. Results are given as statistically significance at $P < 0.05$

SOD inhibition of epinephrine autooxidation/min/mg protein, CAT μmol of H_2O_2 consumed/min/mg protein, GPx glutathione oxidized/min/mg protein, GSH $\mu\text{g}/\text{mg}$ protein, Vit C $\mu\text{g}/\text{mg}$ protein, Vit E $\mu\text{g}/\text{mg}$ protein, ns not significant

^a Group 2 compared with group 1

^b Group 3 compared with group 2

^c Group 4 compared with group 2

^d Group 3 compared with group 4

present study a pronounced increase in the expression of CYP1A1 together with elevations in the levels of Cyt P450, Cyt b5, and in the activities of phase I xenobiotic enzymes such as NADPH Cyt P450 reductase and NADH Cyt b5 reductase in B(a)P-administered animals, denotes carcinogenic processes. Simultaneously, a decrease in the above mentioned parameters were observed in the animals pre- and post-treated with galangin reflecting selective regulation of galangin against phase I enzymes. Result of the present study was further supported by reports documented that galangin inhibits various cytochrome P450s such as 1A1, A2, 1B1, 2C9 and 3A4 (Hamada et al. 2010; Shimada et al. 2010) and also acts as antagonist/blocker to Ahr for it inhibits the Ahr-B(a)P binding (Quadri et al. 2000).

GST is a crucial detoxification enzyme that functions primarily in conjugating 'functionalized P450 metabolite' with endogenous ligands (GSH) favoring their elimination from body of the organism (Hartman and Shankel 1990). Glucuronidation, catalyzed by UDP-GT a microsomal detoxification enzyme, that detoxified endogenous steroids, bile acids, drugs and carcinogens (King et al. 2001). DTD is a flavoprotein that catalyses two-electron reduction of quinones, quinone imines and nitrogen oxides. Reduction of quinones and nitrogen oxide might also make them available for conjugation with UDP-glucuronic acid, facilitating their excretion. Hence, DTD acts as an early cellular defense against tumorigenesis (Begleiter et al. 1997). In our present study, the activities of these phase II enzymes were significantly decreased in lung and liver of B(a)P-induced animals which denote that tissues were more susceptible to carcinogenic effect of B(a)P. The damaged liver cells are no longer capable of synthesizing these enzyme proteins. The observed increase in levels of these enzymes in the animals treated with galangin when compared to the control group. This may be due to

inhibitory action on the interaction of metabolites with cellular DNA. Thus, treatment with galangin was effective in inducing phase II metabolic enzyme activities. Though B(a)P is a lung specific carcinogen, it does not lead to any hepatocarcinogenicity which is concordant with previous studies (Garg et al. 2008; Office of Environmental Health Hazard Assessment (OEHHA) 2010). Primarily, the liver is an organ with the highest complement of cytochrome P450 in terms of quantity as well as number of isoenzymes and also it is the first site for metabolism of xenobiotics absorbed from GI tract. It is known that the types, amounts and location of carcinogen-DNA adducts and cell turnover in a tissue may be significant factors in tumor development and determining the target organ.

Histopathological observation also supports the above phenomenon, B(a)P-induced mice shows presence of adenocarcinoma, that could arise from progenitor cells of bronchioles and alveoli or from mucin-producing cells (Minna et al. 2002). The inhibitory effect of galangin was noticeable through delayed progression of the tumorigenic changes from anaplasia to adenocarcinoma. Hence, we suggest a protective role of galangin in restricting appearance of early lesions upon treatment and thus altering/delaying the progression of lung carcinoma.

ROS and organic free radical intermediates formed from BPDE mainly involve in the initiation and progression of carcinogenic transformation by reacting with lipids, causing release of LPO (Panandiker et al. 1994). The products of LPO include malondialdehyde, hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) that have been reported to be involved in formation of tumors (Mikhail et al. 1996; Kim et al. 2000). Antioxidants, the downstream of Nrf2 gene effectively scavenge these ROS. So, drugs having the potentiality to elevate these antioxidants enzymes play a vital role to scavenge these ROS and prevent LPO.

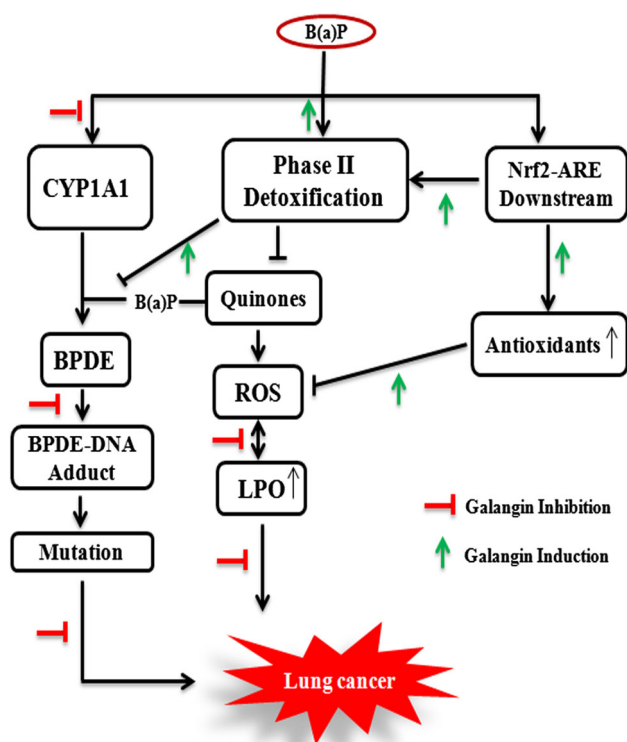


Fig. 6 Schematic representation of possible mechanism of action of Galangin during B(a)P-induced lung carcinogenesis

SOD vigorously catalyze the dismutation of superoxide (O_2^-) into oxygen and H_2O_2 . This H_2O_2 is broken down into water and oxygen molecules by catalase and GPx enzymes (Van Driel et al. 1997). So, these SOD, CAT and GPx constitute a mutually supportive team of defense against ROS which have been found to be decreased in B(a)P-induced animals. Neoplastic cells may sequester essential antioxidants from circulation to supply the demands of growing tumor (Ruddon 1995). GSH, vitamin C and E comprise the non-enzymic antioxidant system that protects the cells against free radicals. GSH acts directly as a free radical scavenger by donating a hydrogen atom and thereby neutralizing the hydroxyl radicals (Sies 1986). Vitamin E is the major lipid soluble peroxy radical scavenger by donating its labile hydrogen atom from phenolic hydroxyl groups to propagation lipid peroxy and alkoxy radical intermediates of LPO, which is again neutralized by vitamin C (Freisleben and Packer 1993; Beyers 1994). The lowered levels of these non-enzymic antioxidants in B(a)P-induced animals might be due to excessive utilization of this antioxidant for quenching enormous free radicals produced in this condition. Galangin supplementation significantly increased all the above enzymic and non-enzymic antioxidants which may be due to its potent free radical scavenging activity.

To support the above data the level of LPO was observed to be significantly higher in B(a)P-induced

animals whereas it was lower in the animals treated with galangin. Hence, B(a)P is a very effective carcinogen with a capability to induce enormous amounts of free radicals, which in turn reacts with lipids causing LPO (Kim et al. 2000). Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system, which quenches or scavenges free radicals and protect body against their deleterious effects. Hence, the decreased levels of LPO in the animals treated with galangin might be due to its ability to increase levels of antioxidants.

Conclusion

To conclude (Fig. 6), the dietary flavonol galangin inhibits B(a)P-induced CYP450 isozymes by modulating the transcriptional regulator CYP1A1 and induces phase II and antioxidant enzymes constitutively to enhance the detoxification process, which aids to eliminate the carcinogen from the body. Further, reduced levels of LPO and tissue marker enzymes with increased levels of antioxidant status in the animals pre- and post-treated with galangin reveal anti-carcinogenic efficacy of galangin. Hence, this preliminary study suggests that galangin is a possible candidate for the chemoprevention of cancer.

References

- Anbarasi, K., K.E. Sabitha, and C.S.S. Devi. 2005. Lactate dehydrogenase isoenzyme patterns upon chronic exposure to cigarette smoke: protective effect of bacoside A. *Environmental Toxicology and Pharmacology* 20: 345–350.
- Baggott, J.E., W.H. Vaughn, and B.B. Hudson. 1986. Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5'-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide. *Biochemical Journal* 236: 193–200.
- Baird, W., L. Hooven, and B. Mahadevan. 2005. Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environmental and Molecular Mutagenesis* 45: 106–114.
- Barouki, R., X. Coumoul, and P.M. Fernandez-Salguero. 2007. The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. *FEBS Letters* 581: 3608–3615.
- Begleiter, A., M.K. Leith, T.J. Curphey, and G.P. Doherty. 1997. Induction of DT diaphorase in cancer chemoprevention and chemotherapy. *Oncology Research* 9: 371–382.
- Beyers, R.E. 1994. The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. *Journal of Bioenergetics and Biomembranes* 26: 349–358.
- Bock, K.W., B. Burchell, G.J. Ditton, O. Hanninen, G.J. Mulder, I.S. Owens, G. Siest, and T.R. Tephly. 1983. UDP-glucuronosyl transferase activities: guidelines for consistent interim terminology and assay condition. *Biochemical Pharmacology* 32: 953–955.
- Bonnesen, C., I.M. Eggleston, and J.D. Hayes. 2001. Dietary indoles and isothiocyanates that are generated from cruciferous

- vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Research* 61: 6120–6130.
- Buening, M.K., R.L. Chang, M.T. Huang, T.G. Fortner, A.W. Wood, and A.H. Conney. 1981. Activation and inhibition of benzo(a)pyrene and aflatoxin B1 metabolism in human liver microsomes by naturally occurring flavanoids. *Cancer Research* 41: 67–72.
- Conney, A.H. 2003. Enzyme induction and dietary chemicals as approaches to cancer chemoprevention: the seventh DeWitt S, Goodman Lecture. *Cancer Research* 63: 7005–7031.
- Desai, I.D. 1984. Vitamin E analysis method for animal tissues. *Methods in Enzymology* 105: 138–147.
- Dinkova-Kostova, A.T., M.A. Massiah, R.E. Bozak, R.J. Hicks, and P. Talalay. 2001. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proceedings of the National Academy of Sciences of the United States of America* 98: 3404–3409.
- Ernest, L., L. Danielson, and M. Ljunggren. 1982. DT-diaphorase-purification from the soluble fraction of rat liver cytoplasm. *Biochemistry and Biophysics Acta* 58: 171–188.
- Ferrigno, D., G. Buccheri, and A. Biggi. 1994. Serum tumour markers in lung cancer: history, biology and clinical applications. *European Respiratory Journal* 7: 186–197.
- Freisleben, H.J., and L. Packer. 1993. Free-radical scavenging activities, interactions and recycling of antioxidants. *Biochemical Society Transactions* 21: 325–330.
- Garg, R., S. Gupta, and G.B. Maru. 2008. Dietary curcumin modulates transcriptional regulators of phase I and phase II enzymes in benzo(a)pyrene-treated mice: mechanism of its anti-initiating action. *Carcinogenesis* 29(1022): 1–32.
- Gwak, J., J. Oh, M. Cho, S.K. Bae, I.S. Song, K.H. Liu, Y. Jeong, D.E. Kim, Y.H. Chung, and Oh Sangtaek. 2011. Galangin suppresses the proliferation of β -catenin response transcription-positive cancer cells by promoting adenomatous polyposis coli/Axin/glycogen synthase kinase-3 β -independent β -catenin degradation. *Molecular Pharmacology* 79: 1014–1022.
- Habig, W.H., M.J. Pabst, and W.B. Jakoby. 1974. Glutathione-S-transferase. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* 249: 7130–7139.
- Hamada, M., H. Satsu, H. Ashida, Y. Sugita-Konishi, and M. Shimizu. 2010. Metabolites of galangin by 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P450 1A1 in human intestinal epithelial Caco-2 cells and their antagonistic activity toward aryl hydrocarbon receptor. *Journal of Agriculture and Food Chemistry* 58: 8111–8118.
- Hartman, P.E., and D.W. Shankel. 1990. Antimutagens and anticarcinogens: a survey of putative interceptor molecules. *Environmental and Molecular Mutagenesis* 15: 145–182.
- Hecht, S.S., P. Upadhyaya, M. Wang, R.L. Bliss, E.J. McIntee, and P.M. Kenney. 2002. Inhibition of lung tumorigenesis in A/J mice by N-acetyl-S- (N-2-phenethylthiocarbonyl)-L-cysteine and myo-inositol, individually and in combination. *Carcinogenesis* 23: 1455–1461.
- Heo, M.Y., S.J. Lee, C.H. Kwon, S.W. Kim, Sohn, and W.W. Au. 1994. Anticlastogenic effects of galangin against bleomycin-induced chromosomal aberrations in mouse spleen lymphocytes. *Mutation Research* 311: 225–229.
- Heo, M.Y., S.J. Sohn, and W.W. Au. 2001. Anti-genotoxicity of galangin as a cancer chemopreventive agent candidate. *Mutation Research* 488: 135–150.
- Hollman, P.C.H., and M.B. Katan. 1997. Absorption, metabolism and health effects of dietary flavonoids in man. *Biomedicine & Pharmacotherapy* 51: 305–310.
- Imamura, Y., T. Migita, Y. Uriu, M. Otagiri, and T. Okawara. 2000. Inhibitory effects of flavonoids on rabbit heart carbonyl reductase. *Journal of Biochemistry* 127: 653–658.
- Jean, J.C., Y. Liu, L.A. Brown, R.E. Marc, E. Klings, and M. Joyce-Brady. 2002. Gamma-glutamyl transferase deficiency results in lung oxidant stress in normoxia. *American Journal of Physiology Lung Cellular and Molecular Physiology* 283: L766–L776.
- Jemal, A., R. Bray, M.M. Center, J. Ferlay, E. Ward, and Forman. 2011. Global cancer statistics. *CA: A Cancer Journal for Clinicians* 61: 69–90.
- Kang, S.S., J.S. Kim, K.M. Son, H.P. Kim, and H.W. Chang. 2000. Isolation of COX-2 inhibitors from *Alpinia officinarum*. *Korean Journal of Pharmacognosy* 31: 57–62.
- Kim, D.A., Y.K. Jeon, and M.J. Nam. 2012. Galangin induces apoptosis in gastric cancer cells via regulation of ubiquitin carboxy-terminal hydrolase isozye L1 and glutathione S-transferase P. *Food and Chemical Toxicology* 50: 684–688.
- Kim, H.S., S.J. Kwack, and B.M. Lee. 2000. Lipid peroxidation, antioxidant enzymes, and benzo[a]pyrene-quinones in the blood of rats treated with benzo[a]pyrene. *Chemico-Biological Interactions* 127: 139–150.
- King, C., W. Tang, J. Ngui, T. Tephly, and M. Braun. 2001. Characterization of rat and human UDP-glucuronosyltransferases responsible for the in vitro glucuronidation of diclofenac. *Toxicological Sciences* 6: 49–53.
- King, J. 1965. The Dehydrogenases or Oxidoreductases, Lactate dehydrogenase. In *Practical clinical enzymology*, ed. D. Van Nostrand, 83–93. London: Company Ltd.
- Kocic, G., V. Djordjevic, P. Vlahovic, R. Kocic, D. Pavlovic, and T. Jevtovic. 2002. Antioxidants modulate adenosine metabolism in rat mesangial cells cultured under high glucose conditions. *Renal Failure* 24: 691–701.
- Labib, S., C. Yauk, A. Williams, V.M. Arlt, D.H. Phillips, P.W. White, and S. Halappanavar. 2012. Subchronic oral exposure to benzo(a)pyrene leads to distinct transcriptomic changes in the lungs that are related to carcinogenesis. *Toxicological Sciences* 129: 213–224.
- Lamb, J.G., P. Straub, and R.H. Tukey. 1994. Cloning and characterization of cDNAs encoding mouse Ugt1.6 and rabbit UGT1.6: differential induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochemistry* 33: 10513–10520.
- Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin's phenol reagent. *Journal of Biological Chemistry* 193: 265–276.
- Luly, P., O. Barnabei, and E. Tria. 1972. Hormonal control in vitro of plasma membrane-bound (Na⁺-K⁺)-ATPase of rat liver. *Biochimica et Biophysica Acta* 282: 447–452.
- Magesh, V., J.P. Singh, K. Selvendiran, G. Ekambaram, and D. Sakthisekaran. 2006. Antitumour activity of crocetin in accordance to tumor incidence, antioxidant status, drug metabolizing enzymes and histopathological studies. *Molecular and Cellular Biochemistry* 287: 127–135.
- Martin, K.R., and C.L. Appel. 2010. Polyphenols as dietary supplements: a double-edged sword. *Journal of Nutrition and Dietary Supplements* 2: 1–12.
- Meyer, J.J., A.J. Afolayan, M.B. Taylor, and D. Erasmus. 1997. Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*. *Journal of Ethnopharmacology* 7(56): 165–169.
- Mikhail, F., K. Denissenko, P. Annie, T. Moon-shong, and P.P. Gerd. 1996. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in p53. *Science* 274: 430–432.
- Mildred, K., L. Richerd, G. Joseph, W. Alexander, and A. Conney. 1981. Activation and inhibition of benzo(a)pyrene and aflatoxin B1 metabolism in human liver microsomes by naturally accruing flavonoids. *Cancer Research* 41: 62–67.
- Minna, J.D., J.A. Roth, and A.F. Gazdar. 2002. Focus on lung cancer. *Cancer Cell* 1: 49–52.
- Misra, H.P., and I. Fridovich. 1972. The role of superoxide anion in the autooxidation of epinephrine and a simple assay of

- superoxide dismutase. *Journal of Biological Chemistry* 247: 3170–3175.
- Moron, M.S., J.W. Depierre, and B. Mannervik. 1979. Level of glutathione, glutathione reductase and glutathione S-transferases activities in rat lung and liver. *Biochemistry and Biophysics Acta* 582: 67–68.
- Nebert, D.W., A. Puga, and V. Vasiliou. 1993. Role of the Ah receptor and the dioxin-inducible [Ah] gene battery in toxicity, cancer, and signal transduction. *Annals of the New York Academy of Sciences* 685: 624–640.
- Office of Environmental Health Hazard Assessment (OEHHA). 2010. Benzo(a)pyrene. In *Public Health Goals for Chemicals in Drinking Water*. <http://oehha.ca.gov/water/phg/pdf/091610Benzopyrene.pdf>.
- Ohkawa, H., N. Ohishi, and K. Yagi. 1979. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* 95: 351–358.
- Omaye, S.T., J.D. Turnbull, and H.E. Sauberlich. 1979. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods in Enzymology* 9(62): 3–11.
- Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *Journal of Biological Chemistry* 239: 2379–2385.
- Orlowski, M., and A. Meister. 1965. Isolation of γ -glutamyl transpeptidase from hog kidney. *Journal of Biological Chemistry* 240: 338–347.
- Panandiker, A., G.B. Maru, and K.V.K. Rao. 1994. Dose response effects of malachite green on free radical formation, lipid peroxidation and DNA damage in hamster embryo cells and their modulation by antioxidants. *Carcinogenesis* 15: 2445–2448.
- Phillips, A.H., and R.G. Langdon. 1962. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *Journal of Biological Chemistry* 237: 2652–2660.
- Quadri, S.A., A.N. Qadri, M.E. Hahan, K.K. Mann, and D.H. Sherr. 2000. The bioflavonoid galangin blocks aryl hydrocarbon receptor activation and polycyclic aromatic hydrocarbon induced pre-B cell apoptosis. *Molecular Pharmacology* 58: 515–525.
- Rotruck, J.T., A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman, and W.G. Hoekstra. 1973. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 179: 588–590.
- Rowlands, J.C., and J.A. Gustafsson. 1997. Aryl hydrocarbon receptor-mediated signal transduction. *Critical Reviews in Toxicology* 27: 109–134.
- Ruddon, R.W. 1995. *Cancer biology*, 3rd ed. New York: Oxford University Press.
- Selvendiran, K., J.P.V. Sing, K.B. Krishnan, and D. Sakthisekaran. 2003. Cytoprotective effect of piperine against benzo(a)pyrene induced lung cancer with reference to lipid peroxidation and antioxidant system in swiss albino mice. *Fitoterapia* 74: 109–115.
- Shih, H., G.V. Pickwell, and L.C. Quattrochi. 2000. Differential effects of flavonoid compounds on tumor promoter-induced activation of the human CYP1A2 enhancer. *Archives of Biochemistry and Biophysics* 373: 287–294.
- Shimada, T., K. Tanaka, S. Takenaka, N. Murayama, M.V. Martin, M.K. Foroozesh, H. Yamazaki, F.P. Guengerich, and M. Komori. 2010. Structure-function relationships of inhibition of human cytochromes P450 1A1, 1A2, 1B1, 2C9, and 3A4 by 33 flavonoid derivatives. *Chemical Research in Toxicology* 23: 1921–1935.
- Sies, H. 1986. Biochemistry of oxidative stress. *Angewandte Chemie (International ed. in English)* 25: 1058–1071.
- Singh, S.V., P.J. Benson, X. Hu, A. Pal, H. Xia, S.K. Srivastava, S. Awasthi, H.A. Zaren, J.L. Orchard, and Y.C. Awasthi. 1998. Gender-related differences in susceptibility of A/J mouse to benzo[a]pyrene-induced pulmonary and forestomach tumorigenesis. *Cancer Letters* 128: 197–204.
- So, F.V., N. Guthrie, A.F. Chambers, and K.K. Carroll. 1997. Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. *Cancer Letters* 112: 127–133.
- Sticha, K.R., M.E. Staretz, M. Wang, H. Liang, P.M. Kenney, and S.S. Hecht. 2000. Effects of benzyl isothiocyanate and phenethyl isothiocyanate on benzo[a]pyrene metabolism and DNA adduct formation in the A/J mouse. *Carcinogenesis* 21: 1711–1719.
- Strittmatte, P., and S.F. Velick. 1956. A microsomal cytochrome reductase specific for diphosphopyridine nucleotide. *Journal of Biological Chemistry* 221: 277–286.
- Takahara, S., B.H. Hamilton, J.V. Nell, T.Y. Kobra, Y. Ogura, and E.T. Nishimura. 1960. Hypocatalasemia, a new genetic carrier state. *Journal of Clinical Investigation* 39: 610–619.
- Van Driel, B.E., H. Lyon, D.C. Hoogenraad, S. Anten, U. Hansen, and C.J. Van Noorden. 1997. Expression of CuZn- and Mn-superoxide dismutase in human colorectal neoplasms. *Free Radical Biology and Medicine* 23: 435–444.
- Walle, T., Y. Otake, A. Galijiatovic, J.K. Ritter, and U.K. Walle. 2000. Induction of UDP glucuronosyl transferase UGT1A1 by the flavonoid chrysin in the human hepatoma cell line hep G2. *Drug Metabolism and Disposition* 28: 1077–1082.
- Wattenberg, L.W., and J.L. Leong. 1970. Inhibition of the carcinogenic action of benzo(a)pyrene by flavones. *Cancer Research* 30: 1922–1925.
- Zhang, H.T., J. Wu, M. Wen, L.J. Su, and H. Luo. 2012. Galangin induces apoptosis in hepatocellular carcinoma cells through the caspase 8/t-Bid mitochondrial pathway. *Journal of Asian Natural Products Research* 14: 626–633.