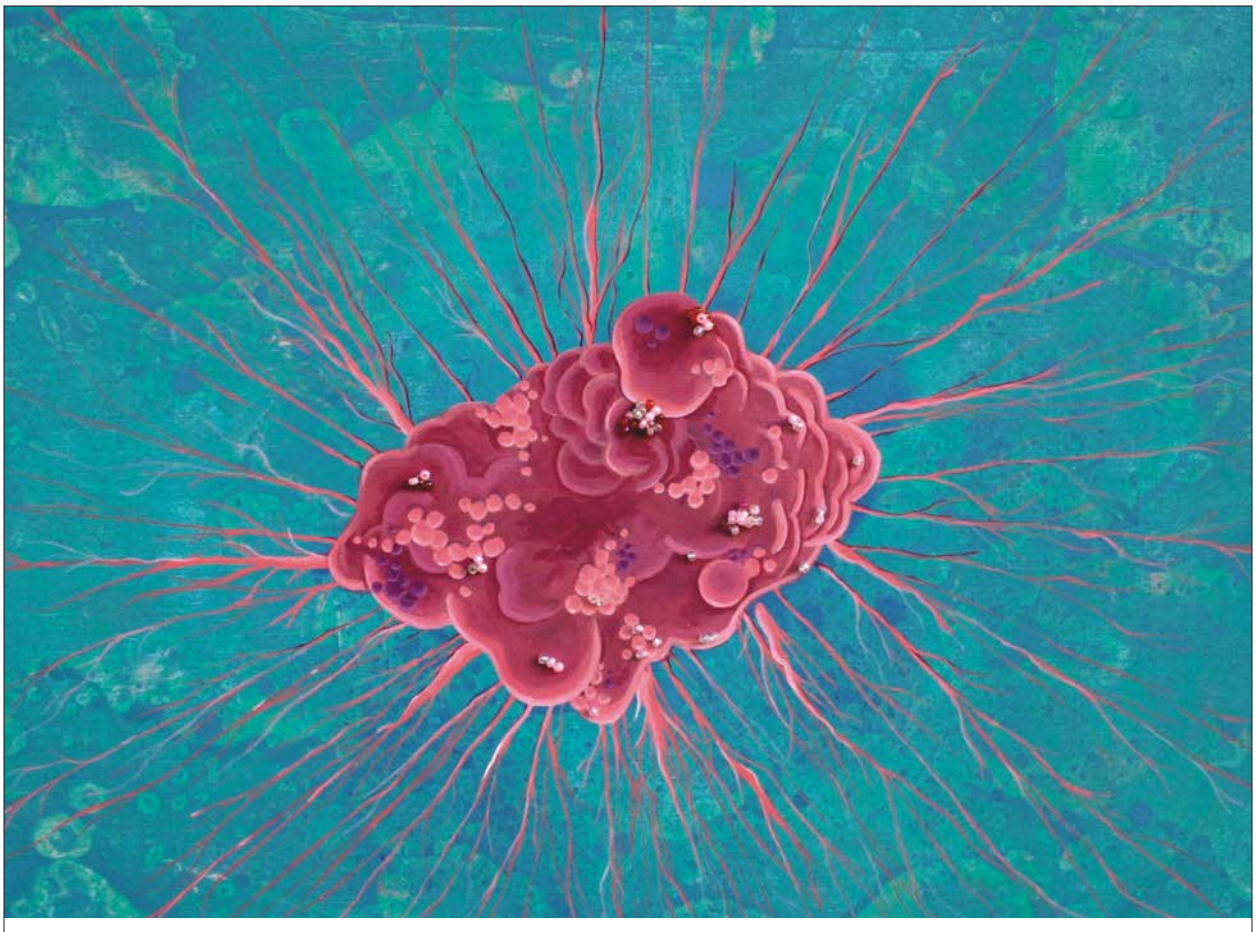

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Effect of Crocetin on Benzo (a) Pyrene Induced Lung Carcinogenesis in Swiss Albino Mice

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Abstract: In recent years, considerable emphasis has been focused on identifying new cancer chemopreventive agents, which could be useful for the human population. The present study was aimed to investigate effect of crocetin on Xenobiotic enzymes and glutathione metabolizing enzymes in Benzo(a)pyrene (B(a)p) induced lung cancer in Swiss albino mice. The lung cancer bearing mice shows elevated level of Phase I enzymes (cytochrome P_{450}, cytochrome b_5 and NADPH cyto.C reductase), glutathione-metabolizing enzymes (glutathione peroxidase, glutathione reductase and glucose-6-phosphoate dehydrogenase) and decreased level of Phase II enzymes (UDP-glucuronyl transferase, glutathione S-transferase, quinone reductase). Oral administration of crocetin (50 mg kg^{-1} body weight) to B(a)p administered animals altered the enzymes level near to normal during initiation and post-initiation treatment. Based on the results, our finding suggests that crocetin have protective role against B(a)P induced lung carcinogenesis.

Key words: Crocetin, Benzo(a)pyrene, lung cancer, xenobiotic enzymes, glutathione metabolizing enzymes

INTRODUCTION

Lung cancer is a major cause of morbidity and mortality worldwide both in men and women accounting for 29% of all other cancers. The incidence of lung cancer still remains very high. Tobacco smoke contains over 60 established carcinogens. Among the constituents of smoke the Polycyclic Aromatic Hydrocarbons (PAHs) such as benzo(a)pyrene, play a major role in lung carcinogenesis (Stephen *et al*., 2002). Benzo(a)pyrene is bioactivated by cytochrome P_{450} (CYP) enzymes to acquire its mutagenic and carcinogenic properties. The first step of activation is the formation of B(a) P-7,8-epoxide, followed by hydrolysis by epoxide hydrolase (EH) to the B(a)P-trans-7,8-dihydrodiol (7,8-diol), which is further metabolized by CYP enzymes to the ultimate genotoxic (±)-B[a]P-r-7,t-8-dihydrodiol t-9,10-epoxide (BPDE). BPDE isomers then bind to the exocyclic nitrogen of deoxyguanosine in DNA via trans addition of the C-10 position in the epoxide molecule. This adduct may furthermore cause activation of protooncogenes (Kristina *et al*., 2000).

An extremely promising strategy for cancer prevention today is chemoprevention, which is clearly defined as the use of synthetic or natural agents to block the development of cancer in human beings. Plants, vegetables, herbs and spices used in folk and traditional medicine has been accepted currently as one of the leading sources of cancer chemopreventive drug discovery and development (Abdullaev, 2001). A large and increasing number of patients in the world use medicinal plants and herbs for health purposes. Crocetin (*Crocus sativus* L.) a red stigmatic lobe is a constituent of

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saffron and its medicinal properties has been tested since ancient times in Siddha and Ayurveda (Magesh *et al*., 2006) and Crocetin do not exhibit any genotoxicity (Chang *et al.*, 1996). A wide variety of naturally occur substances including spices (Unnikrishnan and Kuttan, 1990) have been shown to inhibit chemical carcinogenesis in animal models (Williams, 1984; Wattenberg, 1985; Boone *et al*., 1990). Crocetin at nontoxic doses lowered genotoxic effect and neoplastic transformation in $C_3H_{10}T1/2$ cells induced by benzo(a)pyrene B(a)p (Chang *et al*., 1996). Oral administration of saffron also reduced tumors in mice (Nair *et al*., 1991).

Phase I metabolism (detoxification) involves oxidative, reductive and/or hydrolytic reactions that cleave substrate molecules to produce a more polar moiety. Phase II reactions (synthetic reactions) involve conjugation of certain endogenous molecules to the products of phase I reaction (Reen and Singh, 1991). Cytochrome P_{450} enzymes are responsible for the metabolic conversion of many drugs to the polar metabolites via Phase I and Phase II reactions to earlier excretion. The chemopreventive mechanisms are thought to involve multiple biochemical and biological mechanisms include enzyme induction and anti-oxidation (Unnikrishnan and Kuttan, 1990). Xenobiotic metabolizing enzymes play a major role in regulating toxic, oxidative damaging, mutagenic and neoplastic effect of chemical carcinogen (Gonzalez and Yu, 2006). In the present study these enzymes have been evaluated in lung and liver of mice as most reactions with xenobiotics are carried out both in lung and liver. 50 mg kg^{-1} body weight of crocetin was used to evaluate the induction pattern of phase II and I carcinogen metabolizing enzyme status in mice.

Oxidative stress usually implicates in all the stages of the development of cancer as well as in the genesis of other diseases (Li *et al*., 2000). It are widely accepted that induction of phase II detoxification enzymes [e.g., QR, GST and UDP-GT] and glutathione-metabolizing enzymes (e.g., GPx, GR and G6PDH) are a major strategy for protecting cells against a variety of endogenous and exogenous toxic compounds, such as Reactive Oxygen Species (ROS) and chemical carcinogens (Ozen and Korkmaz, 2003). So, further lung and liver glutathione-metabolizing enzymes glutathione peroxidase (GPx), Glutathione Reductase (GR) and reduced glutathione (GSH) have also been evaluated. Further more, to prove the efficacy of Crocetin, to afford protection against chemically induced carcinogenesis in appropriate animal model system.

MATERIALS AND METHODS

Chemicals

Benzo(a)pyrene was provided by MRI, MO, cytochrome C, 1-chloro 2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), glutathione reductase, oxidized glutathione, glucose- 6-phosphate, reduced nicotinamide adenine dinucleotide (NADPH) and crocetin were acquired from Sigma Chemical, U.S.A. All other chemicals used were of analytical grade.

Animals

Healthy male Swiss albino mice (7-8 weeks old) were used throughout the study. They were maintained in a controlled environment condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Gold Mohor rat feed, Ms Hindustan Lever Lt., Mumbai) and water *ad libitum*.

Experimental Design

The animals were divided into 5 groups and each group consisted of 6 animals. Group I served as control animals and were given corn oil orally for 16 weeks. Group II animals were treated with benzo(a)pyrene ((B(a)p) 50 mg kg^{-1} body weight dissolved in corn oil orally) weekly twice for 4 weeks. Group III animals were treated with crocetin (50 mg kg^{-1} body weight dissolved in DMSO i.p.,). For 16 weeks after they were treated with first dose of B(a)p. Group IV animals were treated

with crocetin (50 mg kg^{-1} body weight dissolved in DMSO ip.,) for 10 weeks after completion of B(a)p induction. Group V animals were treated with crocetin (as in group III) alone for 16 weeks to study the cytotoxicity (if any) induced by crocetin. The initiation and post-initiation treatment of crocetin were used to study the chemopreventive and/or chemotherapeutic efficacies of crocetin in the experimental animals.

Sample Preparation

Animals were sacrificed by cervical dislocation and the entire lung and liver was then perfused immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free of extraneous tissue and rinsed in chilled 0.15 M Tris-KCl buffer (0.15 M KCl + 10 mM Tris-HCL, pH 7.4). The lung and liver were then blotted dry, weighed quickly and homogenized in ice cold 0.15 M Tris-KCl buffer (pH 7.4) to yield 10% (w/v) homogenate. An aliquot of this homogenate (0.5 mL) was used for assaying glutathione peroxidase (Rotruck *et al.*,1973), glutathione reductase (Staal *et al.*, 1969), Glucose-6-phosphate dehydrogenase (Zaheer *et al.*, 1967), while the remaining portion was centrifuged at 10,000 rpm for 20 min. The resultant supernatant was transferred into pre-cooled ultracentrifugation tubes and centrifuged at 105, 000×g for 60 min in a Beckman ultracentrifuge (Model-L870M). The supernatant was discarded whereas the pellet representing microsomes was suspended in homogenizing buffer and used for assaying cytochrome P_{450} (Omura and Sato, 1964), cytochrome b_5 (Omura and Sato, 1964), NADPH cyto.C reductase (Phillips and Langdon,1962), UDP-glucuronyl transferase (Issalbacher *et al.*, 1962; Hollman and Touster, 1962), glutathione S-transferase (Habig *et al.*, 1974), quinone reductase (Benson *et al.*, 1980) and protein (Lowry *et al.*, 1951).

Statistical Analysis

Values are expressed in mean±SD for 6 rats in each group. For statistical analysis, one-way analysis of variance (ANOVA) was used, followed by the Newman-Keuls multiple comparison test. The levels of significance were evaluated with p-values.

RESULTS

Table 1 depicts an increase in the activities of enzymes viz. Cyt. P_{450}, Cyt. b_5 and NADPH Cyt. P_{450} reductase in group II ($p<0.001$) cancer bearing animals when compared with control animals. Crocetin treatment resulted in significantly ($p<0.001$) decreased activities of these enzymes in group III and IV animals when compared with group II animals. There seems to be no significant difference between crocetin alone treated animals and control animals.

Table 2 shows the activities of phase-I enzymes in liver and lung of control and experimental animals. The increased activities ($p<0.001$) of enzymes were observed in B (a) P induced cancer bearing animals when compared with control animals. These changes were significantly ($p<0.001$, $p<0.01$ and $p<0.05$) reduced in group III and IV animals on treatment with crocetin. Group V animals showed no significant changes in the activities of these enzymes when compared with group I control animals.

Table 1: Effect of Crocetin on phase-I enzymes in lung of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Cytochrome P_{450}	0.73±0.06	0.89±0.05^{a}**	0.78±0.04^{b}**	0.84±0.05$^{b@cNS}$	0.71±0.04
Cytochrome b_5	0.42±0.02	0.65±0.05^{a}**	0.52±0.05^{b}**	0.58±0.04$^{b@c@}$	0.43±0.05
NADPH Cyt C reductase	165.5±17.4	213.2±20.4^{a}**	178.6±18.6^{b}**	185.2±7.8$^{b\#cNS}$	167.2±15.8

Each value is expressed as mean±SD for six mice in each group. Cytochrome P_{450} and Cytochrome b_5 -nmol/mg protein. NADPH Cytochrome C reductase-nmol of cytochrome C reduced/min/mg protein, a: as compared with group-I; b: a s compared with group-II ; c: as compared with group-III. Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS-Not significant

Table 2: Effect of crocetin on phase-I enzymes in liver of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Cytochrome P_{450}	0.77±0.06	0.98±0.078^{a*}	0.81±0.07^{b*}	0.87±0.07$^{b@cNS}$	0.74±0.06
Cytochrome b_5	0.46±0.03	0.72±0.06^{a*}	0.50±0.04^{b*}	0.62±0.05$^{b@c@}$	0.45±0.03
NADPH Cyt C reductase	168.92±15.2	217.03±19.1^{a*}	179.43±15.3^{b*}	183.34±16.1$^{b#cNS}$	165.20±14.2

Each value is expressed as mean±SD for 6 mice in each group. Cytochrome P_{450} and Cytochrome b_5 -nmol/mg protein. NADPH Cytochrome C reductase-nmol of Cytochrome C reduced min mg^{-1} protein a: as compared with group-I; b: a s compared with group-II; c: as compared with group-III. Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS-Not significant

Table 3: Effect of crocetin on phase-II enzymes in lung of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
UDP-GT	5.48±0.49	2.81±0.26^{a*}	5.29±0.48^{b*}	4.70±0.39$^{b*c@}$	5.43±0.52
GST	0.47±0.03	0.21±0.01^{a*}	0.43±0.03^{b*}	0.34±0.02$^{b*c\#}$	0.50±0.04
QR	2.20±0.19	1.12±0.09^{a*}	1.89±0.01^{b*}	1.55±0.12$^{b@c@}$	2.81±0.19

Each value is expressed as mean±SD for 6 mice in each group. UDP Glucuronyl transferase-Units min mg^{-1} protein. GST-µmoles of CDNB conjugated min mg^{-1} protein. QR-nmol 2,6-dichlorophenol-indophenol induced min mg^{-} þprotein. a: as compared with group-I; b: a s compared with group-II; c: as compared with group-III. Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS-Not significant

Table 4: Effect of crocetin on phase-II enzymes in liver of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
UDP-GT	7.88±0.67	4.81±0.46^{a*}	7.29±0.68^{b*}	6.70±0.66$^{b*c@}$	7.43±0.72
GST	0.87±0.06	0.41±0.02^{a*}	0.73±0.06^{b*}	0.69±0.06$^{b*c\#}$	0.84±0.07
QR	3.20±0.19	1.72±0.10^{a*}	2.89±0.21^{b*}	2.55±0.23$^{b@c@}$	3.17±0.29

Each value is expressed as mean±SD for 6 mice in each group. UDP Glucuronyl transferase-Units min mg^{-1} protein. GST-µmoles of CDNB conjugated min mg^{-1} protein. QR-nmol 2,6-dichlorophenol- indophenol induced min/mg protein.
a: as compared with group-I; b: a s compared with group-II; c: as compared with group-III. Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS-Not significant

Table 5: Effect of Crocetin on glutathione-metabolizing enzymes in lung and liver of control and experimental animals

Parameters	Group I	Group II	Group III	Group IV	Group V
Liver- GPx	4.13±0.49	2.49±0.27^{a*}	3.77±0.40^{b*}	3.25±0.35^{b*c*}	4.06±0.38
GR	2.83±0.32	1.59±0.18^{a*}	2.55±0.31^{b*}	2.15±0.28^{b*c*}	2.85±0.24
G6PDH	311.0±32.5	166.0±15.5^{a*}	294.0±27.8^{b*}	269.0±27.2^{b*c*}	312.0±33.7
Lung-GPx	4.62±0.51	2.55±0.30^{a*}	4.34±0.48^{b*}	3.73±0.40^{b*c*}	4.56±0.48
GR	3.75±0.34	2.40±0.28^{a*}	3.49±0.38^{b*}	3.13±0.35^{b*cNS}	3.79±0.35
G6PDH	358.0±37.0	171.0±16.5^{a*}	342.0±33.5^{b*}	296.0±27.8^{b*c*}	360.0±35.3

Each value is expressed as mean±S.D. for 6 mice in each group. G6PDH-nanomoles NADPH reduced min(mg protein), GR-nanomoles NADPH oxidized min(mg protein, Gpx micromoles of GSH oxidized min(mg protein). a as compared with group I; superscript b as compared with group II; superscript c as compared with group III. Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS-Not significant

Table 3 and 4 show the activities of phase-II enzymes in lung and liver of control and experimental animals. The decreased activities of enzymes were observed in B (a) P induced cancer bearing animals (p<0.001) when compared with control animals (group I). These changes were significantly (p<0.001, p<0.01 and p<0.05) increased in group III and IV animals on treatment with Crocetin. There is no significant changes was observed in group V of these enzymes when compared with control animals.

Table 5 represents the changes in the activities of glutathione metabolizing enzyme in lung and liver tissues of experimental animals. The glutathione metabolizing enzymes such as glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase were found to be significantly reduced in group II animals. After Crocetin treatment, the altered glutathione metabolizing enzymes levels were significantly corrected to near normal.

DISCUSSION

We screened its potential to induce carcinogen metabolizing pase I and pase II enzymes. Assessment of enzymatic profile is the first step to examine the possibility of probable chemopreventive potentiality of any compound or material. Several evidence indicate that phase II

xenobiotic metabolizing enzymes, such as glutathione S-transferase (GST), UDP-glucuronyl transferase and quinone reductase, play a major role in the cellular detoxification of oxidative damaging, genotoxic and carcinogenic chemicals (Nakamura *et al*., 2003).

Cytochrome P_{450}, a terminal oxidase in the electron transport chain, metabolizes a number of xenobiotics (Guengerich *et al*., 1996) and is reported to be involved in the metabolic activation of chemical carcinogens such as polycyclic aromatic hydrocarbons- (Liu *et al*., 2002). Elevated levels of cytochrome P_{450} and b_5 were observed in lung cancer bearing mice. Murray (2000) has shown that an increase in the hepatic phase-I enzymes of cinnamaldehyde treated rats leads to liver nodule formation. In the present study, crocetin treated animals show reduced levels of phase- I enzymes. From the results it is likely that crocetin inhibits B[a]P-mediated attenuation of phase II enzyme activities. Inhibitors of cytochrome P_{450} have been shown to be anticarcinogenic. Evidences indicate that the neoplasms has been shown to have substantial effects on microsomal electron transport system- (Bharali *et al*., 2003). In the present study, the level of NADPH cytochrome- C-reductase was increased in cancer bearing animals. The increased levels of these enzyme has been brought back to near normal after crocetin supplementation thus providing data for the chemopreventive efficacy of crocetin through normalizing effects on these enzyme activities. In the present study we have revealed that during initiation and post initiation treatment of mice with crocetin leads to the recovery of the activities of glutathione-metabolizing enzymes. The antioxidant activity of crocetin is further strengthened by the inhibition of phase-I enzymes, which are generated by B(a)p. It was found in the present study that crocetin was more effective in initiation treated group (group-III) than the post initiation treated (group IV). This may be due to the inhibitory action of crocetin on the initiation of B(a)p activation/detoxification process.

The collective action of both antioxidants and phase-II enzymes such as GST, UDP-GT and QR, besides small non enzymatic water soluble bio molecules, is to afford protection against the adverse effects of oxidants or reactive metabolites of pre carcinogens. In addition, the activities of antioxidant defense enzymes are also known to be lower in transformed cells and/or tumors (Heather *et al*., 2001). Depletion of these enzymes follows exposure to chemical carcinogens and/or tumour promoters is also known. On the contrary, cancer chemoprevention studies have shown that following administration of chemopreventive agents, the levels of phase II and antioxidant enzymes are elevated in lung cancer bearing animals (Reen and Singh, 1991; Selvendiran *et al*., 2004). The significant increase in the activity of the glutathione-metabolizing enzymes and phase-II enzymes in the lung cancer bearing mice treated with crocetin, suggests that it contributes to chemopreventive effects during lung cancer. A phase-II enzyme such as GST not only catalyses the conjugation of both hydroquinones and epoxide polycyclic aromatic hydrocarbons with reduced GSH for their excretion, but also shows low activity towards organic hydro peroxides for their detoxification from cells/tissues (Kensler, 1997; Song *et al*., 1999). It are therefore, reasonable to assume that increased activities of phase-II enzymes in lung cancer bearing animals after supplementation of crocetin play an important role in the chemoprevention of B(a)p induced lung carcinogenesis.

Mounting evidence has suggested a relevant mechanism between the induction of pase II detoxification enzymes and cancer chemoprevention. Among the Phase II enzymes, GSTs are a family of enzymes that catalyzes the conjugation of reactive chemicals with GSH and plays a major role in protecting cells. After generating conjugated GSH these are subsequently eliminated via a GSH conjugate-recognizing transporter. Recent studies hypothesize that GST affords protection against hydrogen peroxide induced oxidative stress and also protects cells against cancer by detoxifying various mutagenic xenobiotics. The inducers of GST were suggested to induce isoforms of GST, which specifically detoxify products of free radical damage (Fiander and Schneider, 2000). Moreover, many naturally occurring chemopreventive agents have been reported to convert the DNA damaging entities into excretable metabolites through induction of GST (Margaret *et al*., 1997;

Dasgupta *et al.*, 2001). There are ample evidence that phase II drug metabolizing enzymes, e.g., GST, quinone reductase and UDP-glucuronosyl-transferase, play important role in the detoxification of electrophilic toxicants and their induction protect against carcinogenesis and mutagenesis.

Crocetin is a plant carotenoid it acts in various ways on biological system, they are consistently linked to low the risk of cancer (Yay, 1979). GPx plays an important role in cellular defense as well as maintenance of cellular membranes from oxidative damage of free radicals by eliminating H_2O_2 (Apaja, 1980) Increased activity of GPx in lung and liver tissues from the mice treated with crocetin and decreased activity of these enzymes in the tissues from the mice treated with B(a)p suggest that crocetin could influence host detoxification processes.

In addition crocetin being an antioxidant (Papandreou *et al.*, 2006) and a carotenoid compound may also act as health promoting agent by enhancing gap junction intercellular communication (Yeh and Hu, 2003), act as anti-inflammatory (Bai *et al.*, 2005) and anti-tumour (Magesh *et al.*, 2006) promotes agents by inducing endogenous phase II detoxification enzymes. It was found in the present study that crocetin was more effective during the initiation treated group (Group III) than the post-initiation treated (Group IV).

CONCLUSION

Osur present studies indicates that Crocetin plays an important role against B(a)p induced lung carcinogenesis by protecting the glutathione-metabolizing enzymes, Phase I and Phase II xenobiotic enzymes. A beneficial effect of Crocetin in detoxifying free radical mediated damage in biological systems is apparently proving from the data.

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