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Attenuation of genotoxicity, oxidative stress, apoptosis and inflammation by rutin in benzo(a) pyrene exposed lungs of mice: plausible role of NF-κB, TNF-α and Bcl-2

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Abstract

Background: Benzo(a)pyrene [B(a)P] is an environmental contaminant and potential carcinogenic agent that causes lung injuries which leads to lung cancer. Rutin, a well-known flavonoid present in various natural sources, possesses biological activities such as anti-oxidative and anti-inflammatory properties. The aim of this study was to evaluate the protective effects of rutin against B(a)P-induced genotoxicity, oxidative stress, apoptosis and inflammation in Swiss albino mice.

Methods: Pretreatment of rutin was given by oral gavage at doses of 40 and 80 mg/kg body weight (b.wt.) for 7 days before the administration of a single oral dose of B(a)P (125 mg/kg b.wt.). The ameliorative effect of rutin on oxidative stress, apoptotic and inflammatory markers in lung tissues and genotoxicity was studied using an alkaline unwinding assay and DNA fragmentation.

Results: B(a)P enhanced lipid peroxidation, xanthine oxidase, H_2O_2 generation and lactate dehydrogenase (LDH) activity; depleted activities of anti-oxidant enzymes and glutathione content; induced DNA strand breaks and fragmentation; disrupted normal histopathological architecture and also showed abnormal expression of NF-κB, COX-2, IL-6, TNF-α and Bcl-2. Rutin pretreatment caused a significant reduction in lipid peroxidation and LDH activity; increased glutathione content; restored antioxidant enzyme activity; reduced DNA strand breaks and fragmentation; modulated the expression of inflammatory, and apoptotic markers and restored the histopathological structure.

Conclusions: The findings of the present study supported the protective effect of rutin against B(a)P-induced lung toxicity and genotoxicity.

Keywords: benzo(a)pyrene, genotoxicity, histopathology, inflammation, rutin

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Introduction

Benzo(a)pyrene [B(a)P], a polycyclic aromatic hydrocarbon (PAH), is produced in the process of incomplete combustion of organic matter such as garbage, plant decay and fossil fuel. It is one of the main components of tobacco smoke and plays an important role in lung carcinogenesis [1–3]. B(a)P is metabolized by cytochrome P450 1A1 (CYP1A1) that activates it into epoxide which can form DNA adduct and produce reactive oxygen species (ROS) in cells [4]. B(a)P exposure causes structural and physiological changes in the lung that possibly will lead to the development of different chronic lung disorders, including emphysema, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and lung cancer [3, 5–7]. It also causes pulmonary inflammation, edema and surfactant dysfunction in lungs [8]. In short-term exposure B(a)P has been used to find out detrimental effect on lungs and establish as a model to study adverse effects on a pulmonary system [3, 8].

Several studies have shown that the majority of the herbal drugs exhibit a significant range of activities, including those that may control the pathways that are imbalanced during cancer development. Therefore, many herbal drugs have the potential to inhibit cancer and are considered as a chemopreventive agent [9]. In recent years, researchers have shown their keen interest to combat toxicity using herbal drugs. The present study was designed to investigate the preventive aspects of rutin against B(a)P-induced lung toxicity and oxidative stress.

Rutin is a glycoside of quercetin (flavonol) and rutinose (disaccharide). It is one of the flavonoids that are commonly consumed in daily products such as fruits, vegetables and plant-derived beverages including tea and wine. Numerous studies have demonstrated the chemopreventive activity of rutin in animal models [10, 11].

Rutin has also shown anti-hypertensive, anti-inflammatory and anti-oxidative properties [12–15]. Animal studies have shown that dietary supplementation of rutin has useful effects against various organ injuries caused by UV radiation, ammonia, dexamethasone, cisplatin and carbon tetrachloride [16–20]. Based on the above background evidence of biological activities of rutin, we started our study.

Inflammation performs a significant role in the regulation of oxidative stress [21]. ROS plays an important role in the initiation of a large range of transcription factors such as nuclear factor kappa B (NF-κB), which results in the discharge of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which promotes the activation of cyclooxygenase-2 (COX-2) [21–23]. It has been investigated that activation of NF-κB may also occur by anti-neoplastic agents in addition to inflammation [24]. Bcl-2 is considered as an anti-apoptotic protein and known as an oncogene, which inhibits apoptosis [25]. Increase in the level of Bcl-2 is reported to stop the discharge of cytochrome c from mitochondria, which inhibits the process of apoptosis [26].

However, to the best of our knowledge, there are no such previous reports regarding the effects of rutin against B(a)P-induced lung toxicity. Therefore, the present study was designed to evaluate the protective effects of rutin targeting genotoxicity, apoptosis, oxidative stress and inflammatory markers like NF-κB, IL-6, TNF-α, COX-2 and Bcl-2.

Materials and methods

Chemicals

B(a)P, rutin, reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH, NADP, sodium pyruvate, ethylenediaminetetraacetic acid (EDTA), bisbenzamide, proteinase K, thiobarbituric acid, bovine serum albumin (BSA), pyrogallol, glutathione reductase (GR) peroxidase from horseradish, dextrose, xanthine and Mayer's hematoxylin were obtained from Sigma-Aldrich Chemical Co. All other reagents and chemicals were of the highest purity grade and standard commercially available.

Experimental animals

Healthy, male Swiss albino mice 8–10 weeks old, weighing 25–30 g were used for the experimental studies. In this study all the animals used were reviewed and approved (Approval ID/Project Number: 1045) by the Institutional Animal Ethical Committee, accredited by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The well-ventilated rooms and temperature were set at 25 ± 2 °C with 12 h light/dark cycle. All animals were housed in plastic (polypropylene) cages in groups of six mice per cage in animal house facility of Hamdard University. They were given free access to standard laboratory feed (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*.

Treatment regimen

Thirty male Swiss albino mice were randomly allocated into five groups having six animals in each group (Figure 1). A group I (C) served as controls and received distilled water (vehicle of rutin) for 7 days and corn oil (vehicle of B(a)P) on day 7 only. Group II (T) served as a toxicant group and received an oral dose of distilled water daily for 7 days and a single dose of B(a)P (125 mg/kg body

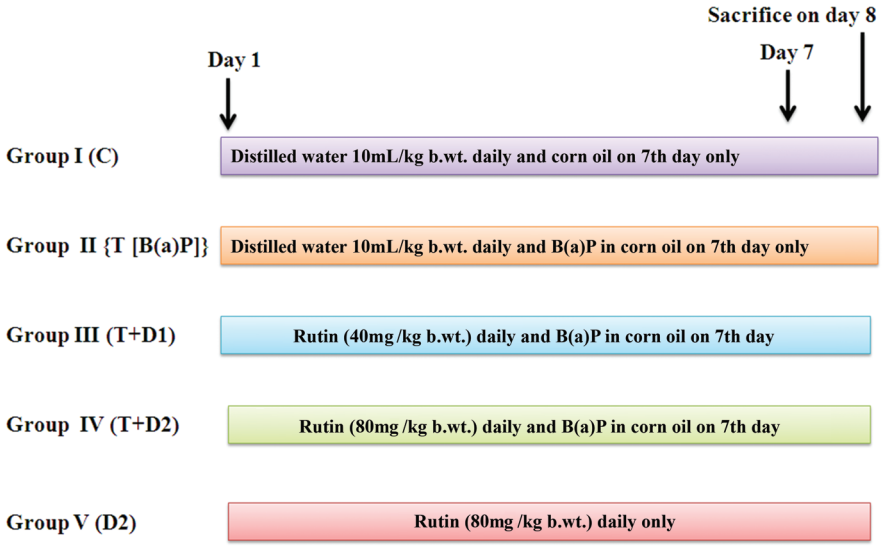

Figure 1: Schematic representation of experimental design.

weight (b.wt.) in corn oil) by oral gavage on the day 7 only. Groups III (TD1) and IV (TD2) were pretreated with an oral dose of rutin 40 mg/kg b.wt. (D1) and 80 mg/kg b.wt. (D2), respectively, for 7 days and a single dose of B(a)P (125 mg/kg b.wt. in corn oil) was administered on the 7th day only in both the groups. Group V (D2) received only 80 mg/kg b.wt. of rutin for 7 days continuously. The above-mentioned doses of rutin and B(a)P were selected on the basis of previous studies and reports [27, 28]. After 24 h, animals were anesthetized with mild anesthesia and sacrificed by cervical dislocation. Lung tissues and femur bone samples were taken for examination of various biochemical and other parameters. Before the sacrifice, mice underwent mild ether anesthesia. Later on, blood was drawn from the retro-orbital sinus and serum was obtained.

DNA isolation

DNA from lung tissue was extracted by a standard chloroform isoamyl method [29]. The quantity and quality of DNA extracted was measured by Thermo Scientific Nano drop spectrophotometer 2000 C (Wilmington, DE, USA) and 1% agarose gel electrophoresis. The amount of DNA was quantitated spectrophotometrically at 260 and 280 nm.

Gel electrophoresis and DNA fragmentation

The sample was mixed with 10 mL of loading solution (10 mm EDTA (pH 8.0), 1% (w/v) bromophenol blue and 40% (w/v) sucrose) preheated to 70 °C. The DNA samples were loaded onto a 1.8% (w/v) agarose gel and sealed with 0.8% (w/v) low melting point agarose. The DNA fragments were separated by electrophoresis at 25 V for 12 h at 4 °C in Tris Borate EDTA (TBE) buffer. The DNA was visualized using ethidium bromide and photographed using a digital camera.

Alkaline unwinding assay

The fluorescence of double-stranded DNA was determined by placing a 100-mmol DNA sample, 100 mL NaCl (25 mM) and 2 mL sodium dodecyl sulfate (SDS) (0.5%) in a prechilled test tube, followed by the addition of 3 mL potassium phosphate (0.2 M, pH 9) and 3 mL bisbenzamide (1 mg/mL). The contents were mixed and allowed to react in the dark for 15 min to allow the fluorescence to stabilize. The fluorescence of single-stranded DNA was determined as above, but using a DNA sample that had been boiled for 30 min to completely unwind the DNA. NaOH (50 mL, 0.05 N) was rapidly mixed with 100 mL of a DNA sample in a prechilled test tube. The mixture was incubated on ice in the dark for 30 min followed by rapid addition and mixing of 50 mL HCl (0.05 N). Immediately after the addition of 2 mL SDS (0.5%) the mixture was forcefully passed through a 21-G needle six times. Fluorescence of the alkaline unwound DNA sample was measured as described above. The ratio between double-stranded DNA to total DNA (F value) was determined as follows:

$$\mathrm{F-value} = (\mathrm{auDNA} - \mathrm{ssDNA})(\mathrm{dsDNA} - \mathrm{ssDNA})$$

where auDNA, ssDNA and dsDNA are the degree of fluorescence from the partially unwound, single-stranded and double-stranded DNA determinations, respectively [30, 31].

Biochemical estimation

The post-mitochondrial supernatant (PMS) was prepared by a previously described method with some modifications [32].

Estimation of lipid peroxidation

The assay for microsomal lipid peroxidation was done by the method of Wright [33].

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity has been estimated in serum by the method of Kornberg [34].

GR activity

GR activity was estimated by the method of Carlberg and Mannervik [35].

Xanthine oxidase activity

The activity of xanthine oxidase (XO) was assayed by the method of Stripe and Della Corte [36].

Superoxide dismutase activity

The activity of Superoxide dismutase (SOD) was measured by the method of Marklund and Marklund [37].

Quinone reductase activity

The Quinone reductase (QR) activity was determined by the method of Benson et al. [38].

Glutathione-S-transferase activity

Glutathione-S-transferase activity was assayed by the method of Habig et al. [39] and Athar et al. [40].

Glucose-6-phosphate dehydrogenase activity

The activity of glucose-6-phosphate dehydrogenase (G6PD) was determined by the method of Zaheer et al. [41].

Catalase activity

Catalase activity was measured by the method of Claiborne [42].

Glutathione peroxidase activity

Glutathione peroxidase activity was assayed by the method of Mohandas et al. [43].

Estimation of GSH

GSH was determined by the method of Athar and Iqbal [44] and Jollow et al. [45].

Measurement of hydrogen peroxide

H_2O_2 was assayed by H_2O_2-mediated horseradish peroxidase-dependent oxidation of phenol red dye by the method of Pick and Keisari [46].

Immunohistochemical staining for the detection of Bcl-2, NF-κB, IL-6, TNF-α and COX-2

The lung tissue sections of 5 μm were sliced from formalin-fixed, paraffin-embedded tissue blocks and mounted on poly-L-lysine-coated slides. The deparaffinized sections were dewaxed three times (5 min) in xylene followed by dehydration in graded ethanol and finally rehydrated in running tap water. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 5 min for antigen retrieval. Then sections were incubated with hydrogen peroxide for 15 min to reduce nonspecific staining and then rinsed three times with 1× phosphate buffered saline Tween 20 (PBST) (0.05% Tween-20) 5 min each. Tissue sections were stained with anti-Bcl-2 (1:400; Santa Cruz), anti-COX-2 (dilution 1:200, Santa Cruz), anti-NF-κB (p65) (1:300, Biolegend), IL-6 (1:200, Ebioscience) and TNF-α (1:200, Ebioscience) overnight at 4 °C in a humid chamber. After rinsing in the buffer, further processing was done according to the instructions of Ultra Vision plus Detection System staining kit (Thermo Scientific system). The peroxidase complex was visualized with 3, 3′-diaminobenzidine (DAB). Lastly, the slides were counterstained with hematoxylin and dried. Finally, the sections were mounted with distyrene plasticizer xylene (DPX) and covered with cover slips. The slides were ready to be observed under the microscope (BX 51 Olympus). The analysis was done at 40× magnification.

Histopathological analysis

After the mice were killed, the lungs were quickly removed and preserved in 10% neutral buffered formalin for histopathological processing. Sections were stained with hematoxylin and eosin before being observed under an Olympus microscope at 40× magnification.

Estimation of protein

The protein concentration in all samples was determined using BSA as standard following the method of Lowry et al. [47].

Statistical analysis

The data from individual groups were presented as mean ± SD. Differences between groups were analyzed using analysis of variance followed by Tukey–Kramer multiple comparison tests, and minimum criteria for statistical significance was set at $p<0.05$ for all comparisons.

Results

Effect of pretreatment of rutin on MDA formation

Malondialdehyde (MDA) production was calculated as a toxicity marker of membrane damage in B(a)P-induced toxicity of lungs in mice. MDA level significantly increased ($p<0.001$) in the B(a)P administrative mice (group II) when compared with control mice (group I). Both the doses of rutin (40 mg/kg b.wt. and 80 mg/kg b.wt.) pretreatment in groups III and IV respectively, significantly decreased ($p<0.01$, $p<0.001$) MDA formation which inhibits membrane damage while compared to B(a)P-treated group II (Figure 2). There was no significant difference between control and only rutin-treated group observed.

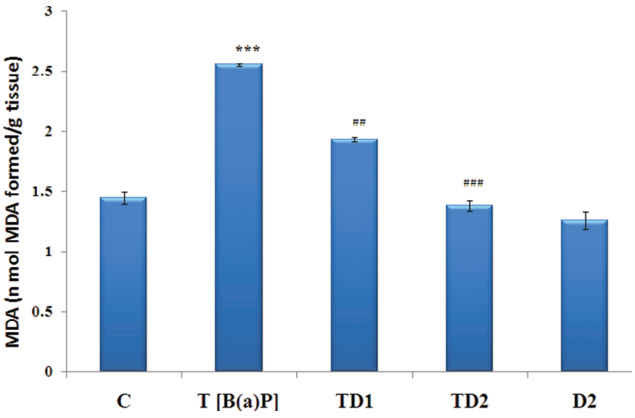

Figure 2: The effect of rutin on the lung toxicity marker LPO. The results represent mean ± SE of six animals in each group. Effect of rutin and B(a)P on lipid peroxidation in Swiss albino mice. Values are measured as nmol MDA formed/h/g tissue. B(a)P treatment increases the values of MDA in group II significantly (***$p<0.001$) as compared with vehicle-treated group I. Rutin pretreatment attenuates the value of MDA (##$p<0.01$ and ###$p<0.001$) in groups III and IV. There was no significant difference in the level of MDA in groups I and V. C, control (only vehicle); T [B(a)P], toxicant [benzo(a)pyrene (125 mg/kg b.wt.)]; TD1, toxicant + rutin (40 mg/kg b.wt.); TD2, toxicant + rutin (80 mg/kg b.wt.); D2, only rutin (80 mg/kg b.wt.).

Effect of pretreatment of rutin on LDH activity

The activity of Lactate dehydrogenase (LDH) was significantly ($p<0.001$) increased in B(a)P administrative mice (group II) as compared to control mice (group I). However, it was found that pretreatment of rutin in groups III ($p<0.01$) and IV ($p<0.01$) shows decreased

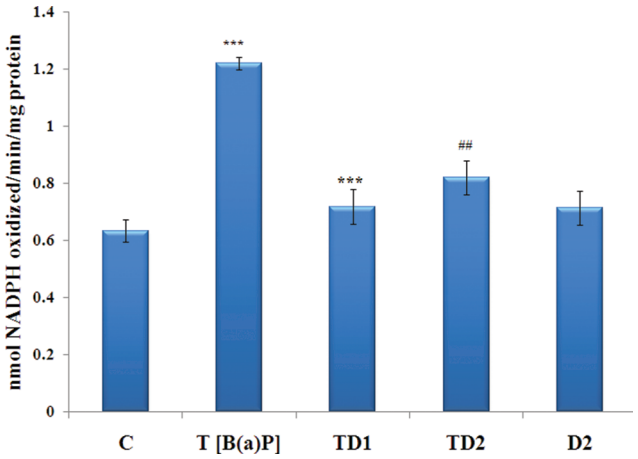

Figure 3: The effect of rutin pretreatment of LDH in lung injury induced by B(a)P.
The results represent mean ± SE of six animals in each group. Effect of rutin and B(a)P on the activity of LDH in mice serum. Activities were measured as nmol NADH oxidized/min/mg protein. B(a)P treatment increases the activity of LDH in group II significantly (***p<0.001) as compared with vehicle-treated group I. Rutin pretreatment attenuates the activity of LDH (###p<0.001 and ##p<0.01) in groups III and IV. There was no significant difference in the activity of LDH in groups I and V. Abbreviations as in Figure 2.

activity of LDH as compared with group II. No significant difference was observed between control and only rutin-treated group (Figure 3).

Effect of pretreatment of rutin on H_2O_2 activity

There was significantly increase in H_2O_2 formation in the B(a)P administrative mice as compared to control mice (p<0.001). However, it was found that pretreatment with low (40 mg/kg b.wt.) and high (80 mg/kg b.wt.) doses of rutin in groups III (p<0.01) and IV (p<0.01) significantly decreased when compared to B(a)P-treated group II (Figure 4). Only rutin-treated group showed no significant difference as compared to control.

Effect of rutin pretreatment on the XO activity

B(a)P administration significantly increased the activity of XO in group II as compared to the control group (p<0.001). However, it was found that pretreatment of rutin in groups III and IV (p<0.001) restored the activity of XO to the normal level. There was no significant difference observed in group V as compared to group I (Table 1).

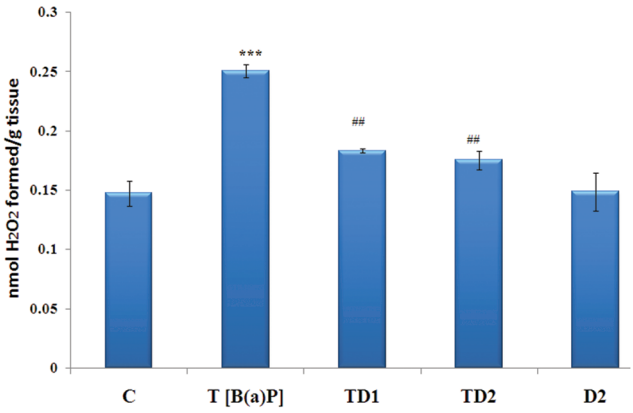

Figure 4: The effect of rutin pretreatment on H_2O_2.
The results represent mean ± SE of six animals in each group. Effect of rutin and B(a)P on the H_2O_2 level in mice PMS. B(a)P treatment elevated the level of H_2O_2 in group II significantly (***p<0.001) as compared with vehicle-treated group I. Rutin pretreatment attenuates the level of H_2O_2 (##p<0.01 and ##p<0.01) in groups III and IV, respectively. There was no significant difference in the level of H_2O_2 in groups I and V. Abbreviations as in Figure 2.

Effect of rutin supplementation on SOD activity

The level of SOD was significantly decreased in the B(a)P administration mice (group II) as compared to group I (p<0.001). SOD level significantly enhanced in both the doses of rutin pretreatment groups III (p<0.05) and IV (p<0.001) as compared with group II. There was no significant difference observed between groups I and V (Table 1).

Effect of rutin supplementation on catalase activity

The activity of catalase (CAT) was significantly depleted (p<0.001) in B(a)P-treated group as compared to the control group. However, it was found that pretreatment with low (40 mg/kg b.wt.) and high (80 mg/kg b.wt.) doses of rutin in groups III (p<0.01) and IV (p<0.05) significantly increased when compared to B(a)P-treated group II. No significant difference in the activity of CAT was found between groups V and I (Table 1).

Effect of pretreatment of rutin on G6PD enzyme

There was significant decrease in G6PD activity in the B(a)P administrative mice as compared to control mice (p<0.001).

Table 1: Effect of rutin on the activities of catalase (CAT), superoxide dismutase (SOD), xanthine oxidase (XO) and glucose-6-phosphate dehydrogenase (G6PD) on benzo(a)pyrene-induced lung toxicity in the lungs of mice.

Treatment groups	CAT, nmol H_2O_2 consumed/min/mg protein	SOD, units/mg protein	XO, µg of uric acid formed/min/mg protein	G6PD, nmol NADP reduced/min/mg protein
Group I (Vehicle only)	31.80 ± 3.73	191.26 ± 3.32	0.87 ± 0.024	244.53 ± 02.39
Group II (B(a)P only)	09.80 ± 0.86[a]	142.2 ± 4.35[a]	1.45 ± 0.029[a]	104.26 ± 02.40[a]
Group III (Dose 1 + B(a)P)	23.27 ± 2.179[b]	161.5 ± 3.43[c]	1.05 ± 0.009[d]	126.87 ± 05.89[b]
Group IV (Dose 2 + B(a)P)	19.78 ± 0.416[c]	168.03 ± 2.75[d]	0.87 ± 0.297[d]	135.39 ± 02.995[d]
Group V (Dose 2)	30.36 ± 0.416	185.6 ± 4.09	0.86 ± 0.013	234.70 ± 02.352

Results represent mean ± SEM of six animals per group. **Group I** (control); **Group II** (toxicant) – B(a)P (125 mg/kg b.wt); **Group III** – B(a)P (125 mg/kg b.wt) + rutin (40 mg/kg b.wt); **Group IV** – B(a)P (125 mg/kg b.wt) + rutin (80 mg/kg b.wt); **Group V** – rutin (80 mg/kg b.wt). Significant differences were indicated ([a]$p<0.001$) when compared with group I and ([d]$p<0.001$, [c]$p<0.05$ and [b]$p<0.01$) when compared with group II.

G6PD level significantly increased in rutin pretreatment groups III ($p<0.01$) and IV ($p<0.001$) when compared with B(a)P-treated group II. No significant difference was observed between groups I and V (Table 1).

Effect of rutin supplementation on QR activity

The level of QR was significantly ($p<0.001$) decreased in the B(a)P-treated group II as compared to group I. QR level significantly increased in rutin pretreatment groups III ($p<0.05$) and IV ($p<0.001$) when compared with group II. No significant difference was observed between groups I and V (Table 2).

Effect of rutin on glutathione-dependent enzymes

It was found that glutathione-dependent enzymes were significant depleted [GPx ($p<0.001$), GST ($p<0.001$) and GR ($p<0.001$)] (Table 2) in B(a)P group as compared to control group. Rutin pretreatment at the low dose (Dose 1) of 40 mg/kg b.wt. significantly increased the activities of GPx ($p<0.05$), GST ($p<0.05$) and GR ($p<0.05$) in group III as compared to group II. When compared to higher dose (Dose 2) of rutin (80 mg/kg b.wt.) it also exhibits significant increase in the activities of GPx ($p<0.05$), GST ($p<0.05$) and GR ($p<0.05$) in group IV as compared to group II. Only rutin-treated group showed no significant difference as compared to control.

Effect of rutin pretreatment on GSH level

The activity of Glutathione (GSH) was significantly ($p<0.001$) depleted in the B(a)P administrative mice (group II) as compared to control mice (group I). GSH activities significantly enhanced in rutin pretreatment groups III ($p<0.05$) and IV ($p<0.01$) when compared with control (group II). No significant difference was observed between groups I and V (Figure 5).

Table 2: Effect of rutin pretreatment on the antioxidant enzymes glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) and quinone reductase (QR) on benzo(a)pyrene-induced lung toxicity.

Treatment groups	GPx, µmol NADPH oxidized/min/mg protein	GST, µmol CDNB conjugate formed/min/mg protein	GR, nmol NADPH oxidized/min/mg protein	QR, µmol DCPIP reduced/min/mg protein
Group I (Vehicle only)	1.88 ± 0.098	952.9 ± 41.02	0.179 ± 0.0133	2.70 ± 0.115
Group II (B(a)P only)	0.66 ± 0.450[a]	658.0 ± 18.27[a]	0.040 ± 0.011[a]	0.98 ± 0.012[a]
Group III (Dose 1 + B(a)P)	1.15 ± 0.095[b]	873.9 ± 10.94[c]	0.093 ± .005[c]	1.72 ± 0.075[b]
Group IV (Dose 2 + B(a)P)	1.00 ± 0.0084[c]	879.96 ± 59.28[c]	0.098 ± 0.010[c]	2.10 ± 0.116[d]
Group V (Dose 2)	1.59 ± 0.039	938.08 ± 69.12	0.153 ± 0074	2.45 ± 0.078

Results represent mean ± SEM of six animals per group. **Group I** (control); **Group II** (toxicant) – B(a)P (125 mg/kg b.wt); **Group III** – B(a)P (125 mg/kg b.wt) + rutin (40 mg/kg b.wt); **Group IV** – B(a)P (125 mg/kg b.wt) + rutin (80 mg/kg b.wt); **Group V** – only rutin (80 mg/kg b.wt). Significant difference were indicated ([a]$p<0.001$) when compared with group I and ([d]$p<0.001$, [c]$p<0.05$ and [b]$p<0.01$) when compared with group II.

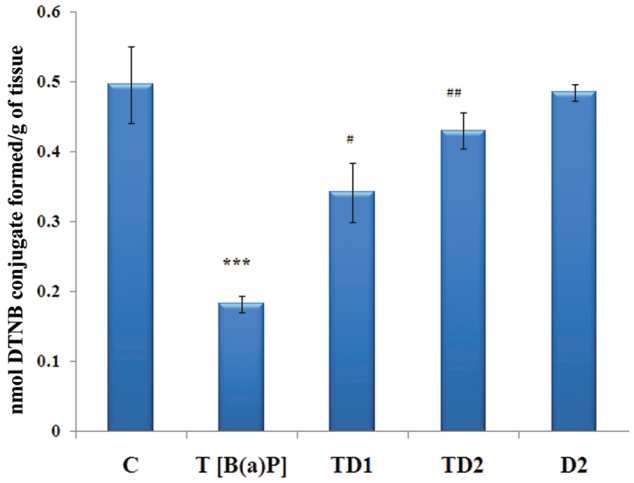

Figure 5: The effect of rutin on GSH level.
The results represent mean ± SE of six animals in each group. B(a)P treatment decreased the level of GSH in group II significantly (***p<0.001) as compared with vehicle-treated group I. Rutin pretreatment attenuates the level of GSH (#p<0.05 and ##p<0.01) in groups III and IV. There was no significant difference in the level of GSH in groups I and V. Abbreviations as in Figure 2.

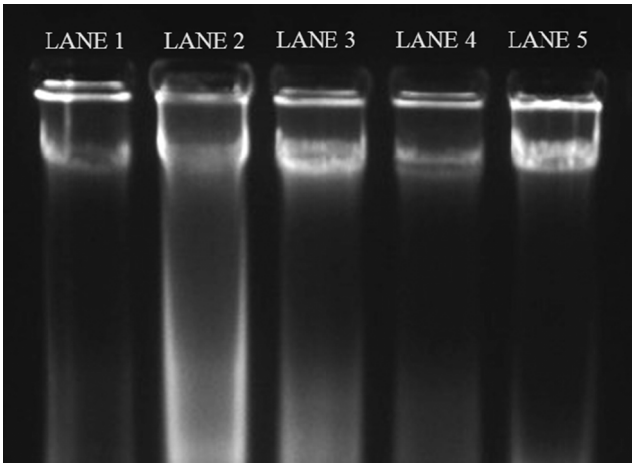

Figure 7: The effect of rutin on DNA damage.
Agarose gel electrophoresis of DNA obtained from mouse lung. Lane 1, control; lane 2, B(a)P only; lane 3, rutin (40 mg/kg) and B(a)P; lane 4, rutin (80 mg/kg) and B(a)P; lane 5, rutin acid only (80 mg/kg). B(a)P treatment caused DNA fragmentation as indicated by smearing of DNA compared with the control. There was a decrease in DNA smearing as a result of rutin pretreatment at both doses.

Effects of pretreatment of rutin on genotoxicity

The F value significantly (p<0.001) decreased in the B(a)P-treated group II as compared to group I (Figure 6). Whereas there was a significant increase (p<0.05, p<0.01) in the F value at both doses of rutin. DNA damage was also evaluated in terms of smearing pattern. Group II treated with B(a)P showed more smearing pattern as compared with group I (Figure 7), thus validating B(a)P-induced DNA damage, which may be due to ROS generated by B(a)P. Groups III and IV showed the protective effects of catechin against B(a)P-induced DNA damage. There was no significant DNA damage in groups I and V.

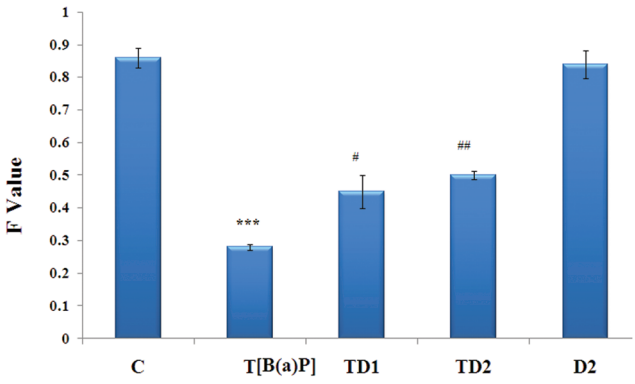

Figure 6: The effect of rutin on F value.
The results represent mean ± SE of six animals in each group. B(a)P treatment decreased the level of F value in group II significantly (***p<0.001) as compared with control group I. Rutin pretreatment attenuates the level of F value (#p<0.05 and ##p<0.01) in groups III and IV. There was no significant difference in the level of F value in groups I and V. Abbreviations as in Figure 2.

Effect of rutin on the histology of the lung

Oral administration of B(a)P caused disruptions of epithelium (Figure 8(B)) when compared with control group (Figure 8(A)). It also caused severe destruction of alveolar architecture and necrosis of the alveolar epithelium (Figure 8(B)). Infiltration of inflammatory cells can also be seen. These findings correlate with the cytotoxicity marker (LDH) level and support the results of the other parameters in the present investigation. Rutin at both the doses (40 and 80 mg/kg b. wt.) showed protection against B(a)P in terms of lung histology (Figure 8(C), 8(D)). Only the higher dose of rutin (Figure 8(E)) of 80 mg/kg b.wt. showed normal histology as observed in Figure 8(A). Original magnification 40×.

Effect of rutin on the expression of NF-κB, zIL-6, TNF-α, COX-2 and Bcl-2

Immunohistochemical analysis (original magnification 40×) of NF-κB, IL-6, TNF-α, COX-2 and Bcl-2 has been

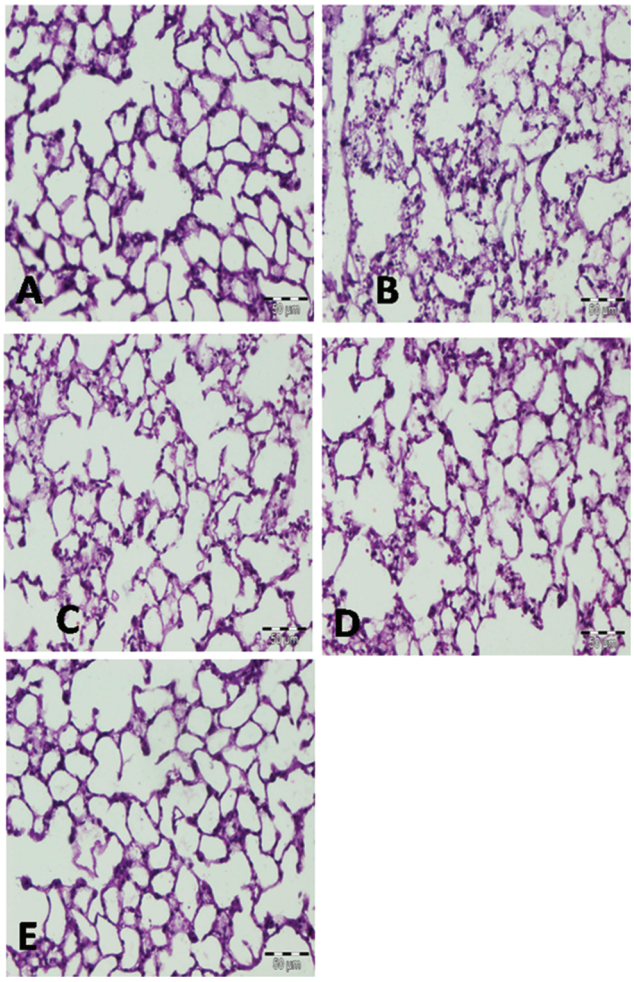

Figure 8: The effect of rutin pretreatment against B(a)P-induced histological alteration.
(A) Normal histology of control mice lung. (B) B(a)P (125 mg/kg b.wt.)-treated group shows severe destruction of alveolar architecture and necrosis of the alveolar epithelium of the lungs. (C and D) Rutin at both the doses (40 and 80 mg/kg b.wt.) showed protection against B(a)P-induced lung histopathology. (E) Only higher dose of rutin of 80 mg/kg b.wt. showing normal histology was observed as in (A). Original magnification 40×.

shown in Figures 9, 10, 11, 12 and 13, respectively. No detectable NF-κB, IL-6, TNF-α and COX-2 staining was observed in the lung of control mice. However, B(a)P-treated lung cell stained positive by antibodies against NF-κB, IL-6, TNF-α, and COX-2. Rutin pretreatment significantly reduced expression in a dose-dependent manner (Figures 9, 10, 11, 12). Immunostaining with anti-bcl-2 antibody showed moderate expression in the control mice lung (Figure 13). This immunoreactivity was found to be reduced in B(a)P-treated group. In contrast, rutin-administered mice showed positive staining and increased immunoreactivity.

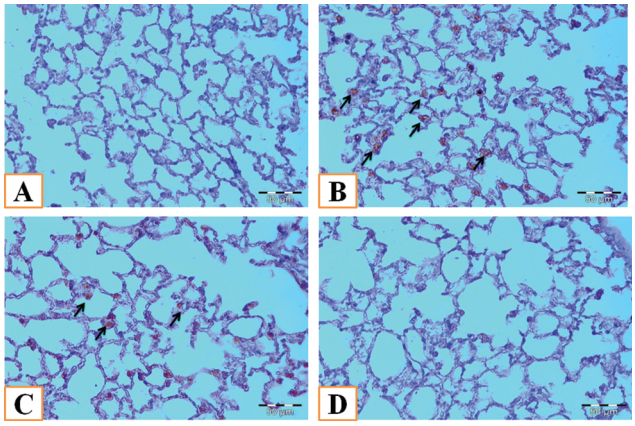

Figure 9: Photomicrographs depicting immunohistochemical staining of NF-κB.
For immunohistochemical analyses, brown color (arrows) indicates specific immunostaining of NF-κB and blue color indicates nuclear hematoxylin staining. The lungs of B(a)P-treated group (B) have more NF-κB immunopositive staining as indicated by brown color as compared to control group (A) while treatment with rutin in dose 40 and 80 mg/kg b.wt. (C and D, respectively) reduced NF-κB immunostaining as compared to toxicant group (B). Original magnification: 40×.

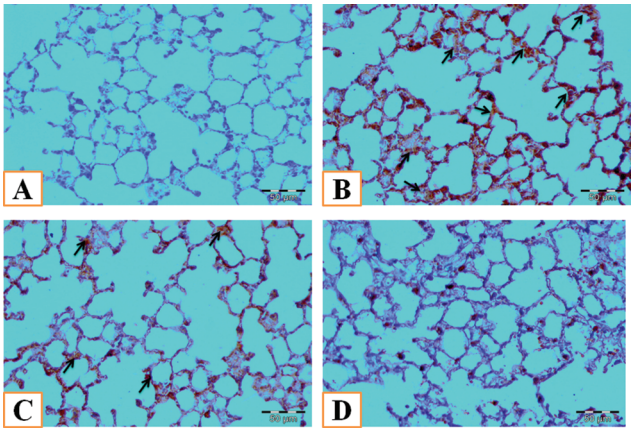

Figure 10: Photomicrographs depicting immunohistochemical staining of IL-6.
For immunohistochemical analyses, dark, intense brown color (arrows) indicates specific immunostaining of IL-6 and blue color indicates nuclear hematoxylin staining. The lungs of B(a)P-treated group (B) has more IL-6 immunopositive staining as indicated by dark, intense brown color as compared to control group (A) while treatment with rutin in dose 40 and 80 mg/kg b.wt. (C and D, respectively) reduced IL-6 immunostaining as compared to toxicant group (B). Original magnification: 40×.

Discussion

In the present study, we have shown that rutin may provide a significant protective effect against B(a)P-instigated

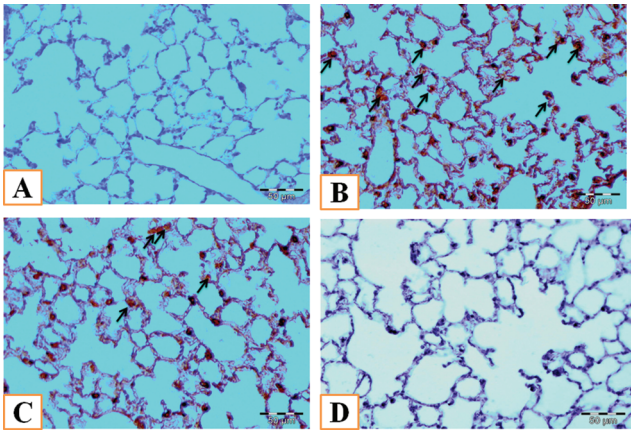

Figure 11: Photomicrographs depicting immunohistochemical staining of TNF-α.
For immunohistochemical analyses, dark, intense brown color (arrows) indicates specific immunostaining of TNF-α and blue color indicates nuclear hematoxylin staining. The lungs of B(a)P-treated group (B) have more TNF-α immunopositive staining as indicated by dark, intense brown color as compared to control group (A) while treatment with rutin in dose 40 and 80 mg/kg b.wt. (C and D, respectively) reduced TNF-α immunostaining as compared to toxicant group (B). Original magnification: 40×.

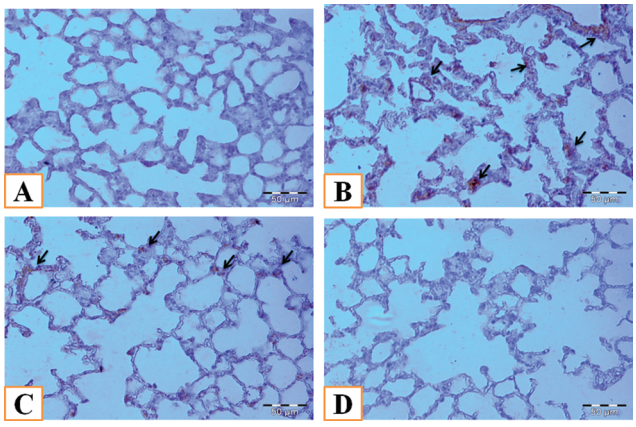

Figure 12: Photomicrographs depicting immunohistochemical staining of COX-2.
For immunohistochemical analyses, brown color (arrows) indicates specific immunostaining about COX-2 and light blue color indicates nuclear hematoxylin staining. The lungs of B(a)P-treated group (B) have more COX-2 immunopositive staining as indicated by brown color as compared to control group (A) while treatment with rutin in dose 40 and 80 mg/kg b.wt. (C and D, respectively) reduced COX-2 immunostaining as compared to toxicant group (B). Original magnification: 40×.

lung toxicity in Swiss albino mice. Although the mechanism underlying B(a)P-induced lung toxicity is still unclear, it may be due to DNA adduct formation with the B(a)P, which leads to the condition of free radical generation [3]. The present study was performed to interpret the outcome

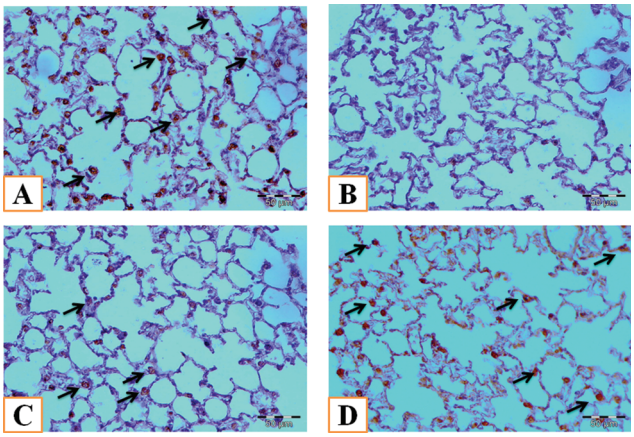

Figure 13: Photomicrographs depicting immunohistochemical staining of Bcl-2.
For immunohistochemical analyses, dark brown color (arrows) indicates specific immunostaining about Bcl-2 and light blue color indicates nuclear hematoxylin staining. The control group (A) has more Bcl-2 immunopositive staining as indicated by brown color as compared to B(a)P-treated group (A) while treatment with rutin in dose 40 and 80 mg/kg b.wt. (C and D, respectively) increased Bcl-2 immunostaining as compared to toxicant group (B). Original magnification: 40×.

of rutin on B(a)P-induced lung toxicity and to investigate its function in the genotoxicity, oxidative stress, apoptosis and inflammation.

Early diagnosis and chemoprevention are essential and most important steps to slow down the process of cancer [48]. Although researches have been done to combat cancer in terms of natural therapy, no satisfactory and complete therapeutic agent has been found. Most of the natural products of food sources have been studied to reduce different diseases together with cancer [49]. Reports from our laboratory and also from other groups indicated that a variety of dietary compounds possibly take a necessary role in the alleviation of lung injury [8, 50].

B(a)P genotoxicity was confirmed by both *in vitro* and *in vivo* studies; the genotoxicity included chromosome damage, induction of genetic mutations and single strand breaks in DNA [51–53]. B(a)P also results in the formation of DNA strand breaks, which results in a DNA disintegration or smearing due to necrosis. It is evident from this study that rutin was not only able to reduce the F value, but also suppressed DNA strand breaks *in vivo*, which are the hallmarks of B(a)P-induced genotoxicity [54].

ROS formation causes oxidative stress that is linked to aging and various severe diseases such as neurodegenerative diseases, diabetes, atherosclerosis, arthritis and pulmonary fibrosis, which are usually accompanied by inflammation [55–58]. B(a)P produces ROS, viz., the

superoxide anion (O_2^{-2}), H_2O_2, hydroxyl radical (OH) etc., which also induce oxidative stress. The serum toxicity markers like LDH showed a noticeable increase in a B (a)P-retreated group of mice. However, mice given rutin by oral gavage showed a significantly restored serum toxicity marker level toward normal. Mice pretreated with rutin restored LDH level to normal as compared to those receiving only B(a)P. These results have shown that rutin may protect against B(a)P-induced lung toxicity. Peroxidation of lipid membrane results in the generation of MDA that results in the formation of ROS leading to oxidative stress. Previous studies showed that MDA formation causes mutagen and tumor promoter [59–61]. Therefore, we have evaluated the outcome of rutin on B(a)P induced MDA formation and found that rutin significantly ameliorate the level of MDA production.

In addition, we also measured other parameters such as XO, H_2O_2, GST, GSH, etc. to confirm the protective effect of rutin after B(a)P treatment. XO is an enzyme that converts O_2 to superoxide anion radical (O_2^-) which results in oxidative stress [62]. In this study, it was observed that B(a)P enhanced XO activity which results in the formation of ROS. Rutin treatment significantly attenuated XO activity. GSH is a tripeptide which has low molecular weight and it shows antioxidant properties. It prevents the peroxidation of a lipid membrane by binding with the intermediate such as 4-hydroxy-3-onenal (HNE), produced in lipid peroxidation and as a result, it gets exhausted in this conjugation reaction [63]. This binding of GSH via the sulfhydryl (-SH) group to electrophile is catalyzed by an antioxidant enzyme, i.e., GST, and thus the level of GST is depleted after B(a)P treatment [64]. In this study, it was shown that pretreatment of rutin significantly restored the GSH level and the activity of GST to the normal. Furthermore, it was also shown that the activities of antioxidant enzymes, namely GPx, CAT, SOD, G6PD, GR and QR (phase II enzymes), were decreased in the B(a)P-treated group, whereas rutin given to the mice at both the doses significantly ameliorated the activities of these antioxidants. The reduced activities of antioxidant and phase II detoxifying enzymes in the B (a)P-treated group validated the connection of oxidative stress in the pathophysiology of B(a)P-induced lung toxicity (Figure 6). H_2O_2 amount produced in the lung tissue is also linked with DNA damage by oxidative stress and it may play an important role in cancer progression. In these present findings, it is found that the basal level of H_2O_2 was significantly depleted in the lung tissue of rutin-treated mice because rutin increases the activity of antioxidant enzymes.

Inflammation is a physiological reaction generated by pathogens and injuries, removing the risk and re-establishing homeostasis. NF-κB, one of the ubiquitous redox-sensitive transcription factors, regulates various genes which involve many processes, including cellular proliferation, inflammation immunity, differentiation and survival [65]. The current findings of the study reflect the anti-inflammatory property of rutin, which attenuates B(a)P-induced activation of NF-κB. The enzyme NF-κB can modulate the expression of COX-2 [66]. COX is a key proinflammatory enzyme that plays a vital role in inflammation. In the present study, we hypothesized to measure the expression of COX-2 in order to evaluate whether rutin treatment has some effect on inflammation in the B(a)P-treated mice. We found that B(a)P induces activation of COX-2 in mice lung tissues which was actively suppressed by rutin pretreatment signifying the strong anti-inflammatory effect and hence, it shows antitumor promoting potential of rutin as well. B(a)P administered to the mice induced the expression of proinflammatory cytokines IL-6 and TNF-α which are under direct transcriptional regulation of NF-κB. Previous studies showed that the levels of TNF-α and IL-6 were increased in the mice lung administered with B(a)P [67]. Thus, inhibition in their activities by rutin seems to perform a significant role in its protective effect against lung toxicity. Our results are in conformity with the findings of previous studies [68]. Protective effects of rutin on proinflammatory cytokines may now be established as another important approach to control the toxicity. Bcl-2 is an important controller of the mitochondrial apoptotic pathway which impedes cell death by inhibiting molecules which are needed for the stimulation and cleavage of caspases [69–72]. It was found that there was negligible staining in Bcl-2 protein in B(a)P-treated group as compared with the rutin-treated group. Rutin treatment of both the doses significantly attenuated Bcl-2 expression.

The histological observations further support the above findings and show the protective effects of rutin against B(a)P-induced toxicity when compared with control mice (b.wt.). B(a)P administration induces serious damage to alveolar structural design and necrosis of the alveolar epithelium (Figure 8(B)). These results match up with the cytotoxicity marker like LDH and lipid peroxidation (LPO) levels in PMS and also support the findings of the other parameters in the present study. In our studies rutin at both the doses (40 and 80 mg/kg b.wt.) shows protection against B(a)P-induced toxicity in lungs of mice.

Conclusions

Our data strongly support the prophylactic treatment of rutin against B(a)P-induced upregulation of NF-κB, COX-2, IL-6 and TNF-α, and downregulation of Bcl-2 via alleviation of lung oxidative stress, genotoxicity, apoptosis and inflammatory response. The present study data together with reports available in the literature suggest rutin possibly as a promising candidate for use in chemoprevention or in an adjuvant therapy.

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