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Inhibitory effect of apigenin on benzo(a)pyrene-mediated genotoxicity in Swiss albino mice

Tajdar Husain Khan, Tamanna Jahangir, Lakshmi Prasad and Sarwat Sultana

Abstract

Apigenin, a bioflavonoid, is abundantly present in fruits and vegetables and possesses potential chemopreventive properties against a wide variety of chronic diseases. In this study we investigated the anti-genotoxic effects of apigenin against a known genotoxicant, benzo(a)pyrene (B(a)P) (125 mg kg^{-1} orally) toxicity in Swiss albino mice. B(a)P administration led to induction of cytochrome P-450 (CYP), aryl hydrocarbon hydroxylase (AHH) and DNA strand breaks ($P < 0.001$), which was suppressed by apigenin (2.5 and 5 mg kg^{-1} orally) dose dependently ($P < 0.001$). B(a)P-induced depletion in the level of reduced glutathione (GSH), quinone reductase (QR) and glutathione-S-transferase (GST) was also shown to be restored by apigenin pre-treatment ($P < 0.001$). A simultaneous significant and dose-dependent reduction was noted in DNA strand breaks and in-vivo DNA damage ($P < 0.001$), which gives some insight into restoration of DNA integrity in modulator groups. These results strongly support the protective nature of apigenin against B(a)P-induced toxicity.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) have become widespread environmental contaminants due to their occurrence in petroleum, coal and coal tars, soot, air pollutants and cutting oils (Hardin et al 1992; Mudzinski 1993). About 80% of human malignancies are due to exposure to environmental genotoxicants and mutagens. Individuals are exposed to PAH via inhalation of smoke from coal, wood, diesel fuel and tobacco and by ingestion of roasted, smoked or charbroiled foods (NTP 1998). Among the carcinogenic PAHs, B(a)P is the best studied and has in several studies served as a model for the carcinogenic and mutagenic effects of PAH. B(a)P is an established genotoxicant, neoplastigen, teratogen and environmental pollutant (Warshawsky 1999; Sparfel et al 2006). It requires metabolic activation to exert its genotoxicity (Shaw & Connell 1994). Cytochrome P450 (CYP) is one of the major enzyme systems mainly involved in the activation of carcinogens (Moorthy et al 2003). Once taken up by the organism, B(a)P can be metabolized to reactive intermediates through CYP1A1 to the proximate toxicant B(a)P-7,8-diol (Hayes et al 1996; Mitchelmore & Chipman 1998). Another part of the metabolic profile of B(a)P includes the B(a)P-quinones (BPQs), which may be produced biologically by peroxidases, and by CYP isozymes in combination with dihydrodiol dehydrogenases (Penning et al 1999; Reed et al 2003). BPQs have been established in chemical systems to undergo one electron redox cycling with their semiquinone radicals, resulting in the formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) (Penning et al 1999), collectively called reactive oxygen species (ROS). These ROS, can form alkali-labile sites on DNA and cause DNA damage (Shugart 1988; Cahill et al 2006). A major class of DNA damage concerns DNA strand breaks, divided into single-strand breaks (ssBs) and double-strand breaks (dsBs) (Niida & Nakanishi 2006). The DNA fragmentation or breaks are characteristic features of apoptosis. Antioxidants have been used to inhibit apoptosis because apoptosis was initially thought to be mediated by oxidative stress (Hockenbery et al 1993). Studies have advocated that a high intake of fruits and vegetables is linked with a reduced risk of many diseases, including breast, colon, lung, larynx, pancreas, oral and prostate cancer (Lin et al 2005; Michels et al 2005; van Dijk et al 2005).

Section of Chemoprevention and Nutrition Toxicology, Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi 110062, India

Tajdar Husain Khan, Tamanna Jahangir, Lakshmi Prasad, Sarwat Sultana

Correspondence: S. Sultana, Department of Medical Elementology and Toxicology, Faculty of Science, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi 110062, India. E-mail: sarwat786@rediffmail.com

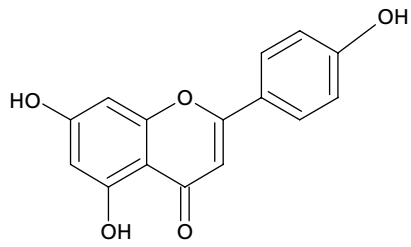

Figure 1 Chemical structure of apigenin.

Apigenin (4′,5,7-trihydroxyflavone) (Figure 1), a flavone subclass of citrus bioflavonoid widely distributed in many herbs, fruits and vegetables, is a substantial component of the human diet and has been shown to possess a variety of biological actions, including tumour growth inhibition and chemoprevention. Flavonoids have been shown to possess both pro-oxidant and antioxidant activity (Sugihara et al 1999; Lebeau et al 2000; Burda & Oleszek 2001). Burda & Oleszek (2001) reported that the aromatic OH group is very important to the antioxidative effects of flavonoids, and flavonoids with 4′-OH groups in the B ring and a 2,3-double bond in conjugation with a 4-oxo group in the C ring, such as apigenin, exhibited antioxidant activity. The pro-oxidant nature of apigenin is shown by its apoptotic properties in the elevated level of H_2O_2 and depleted activity of catalase (CAT) and superoxide dismutase (SOD). The activity of antioxidant enzymes is responsible for the anti-apoptotic and pro-apoptotic nature of apigenin.

Various experimental data have shown that apigenin has antioxidant, anti-inflammatory, anti-apoptotic and anti-tumour properties (Chiang et al 2005; Zheng et al 2005; Khan & Sultana 2006). Apigenin, being a non-toxic dietary flavonoid with anti-tumour properties, possesses special interest for the development of a novel chemopreventive or chemotherapeutic agent for cancer (Chen et al 2005; Zheng et al 2005). However, its mechanism of action has not so far been elucidated. In this study we have investigated the effect of apigenin on B(a)P metabolizing enzymes, alteration in DNA integrity and DNA damage due to B(a)P treatment.

Materials and Methods

Chemicals

EDTA, Tris, reduced glutathione (GSH), oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate reduced (NADPH), bovine serum albumin (BSA), 1,2-dithio-bisnitrobenzoic acid (DTNB), 1-chloro-2,4-dinitro benzene (CDNB), B(a)P, sodium dithionate, bisbenzamide, EDTA, SDS, Trisma, phenol, chloroform, isoamyl alcohol, proteinase K, RNase and apigenin were purchased from Sigma (St Louis, MO). Other reagents and solvents were of a high analytical grade.

Animals

Eight-week-old adult male Swiss albino mice, 20–25 g, were obtained from the central animal house facility of Hamdard University, New Delhi and were housed in a ventilated room at 25 ± 2°C under a 12-h light–dark cycle. The mice were acclimatized for one week before the study and had free access to standard laboratory feed (Hindustan Lever Ltd, Bombay, India) and water. The study was approved from the Committee for the purpose of control and supervision of experimental animals (CPCSEA). Registration number and date of registration was: 173/ CPCSEA, 28th Jan 2000.

Experimental design

Twenty-five male Swiss albino mice were divided into five groups with five mice in each group. B(a)P dissolved in corn oil and apigenin in saline were administered orally. Group I served as control and was given saline only. The mice of group II served as positive control and were administered a single oral dose of B(a)P (125 mg kg^{-1}) dissolved in corn oil on the 8th day. Mice of group III were pre-treated with 2.5 mg kg^{-1} apigenin while groups IV and V were given 5 mg kg^{-1} apigenin for seven consecutive days. On day 8, the mice of group II, III and IV were given a single oral dose of B(a)P (125 mg kg^{-1}), while group I and V were given only corn oil.

DNA isolation

DNA was extracted from approximately 500 mg of liver tissue by homogenizing the tissue in 5 mL TNE buffer (50 mM Tris, 100 mM EDTA, 0.5% SDS, pH 8.0) in a 2-mL ground-glass homogenizer. Each sample was homogenized with 10 standardized strokes of the pestle so as to minimize any potential effect on DNA integrity introduced by the homogenization procedure. An equal volume of buffered phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v, pH 8.0) (PCI) was then added to the sample. The sample was gently mixed and allowed to settle for 5 min. It was then centrifuged for 5 min at 13 000 rev min^{-1} at 4°C. The aqueous layer was transferred to a new micro centrifuge tube and PCI extraction was repeated. The aqueous layer was then digested by 5 μL of RNase (10 mg mL^{-1}) for 30 min at 37°C and the digest was extracted once by PCI and once by 500 μL of chloroform. DNA was precipitated from the resulting aqueous layer by adding 2 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2. The sample was then centrifuged (13 000 rev min^{-1}, 15 min), and the resulting pellet was rinsed with 500 μL of 70% ethanol and air-dried. The amount of DNA was quantitated spectrofluorometerically by using bisbenzimidazole (Cesarone et al 1979). A DNA sample (2 μg μL^{-1}) was dissolved in 1 mL of TE buffer (10 mM Trisma, 1 mM EDTA) and subsequently used in the DNA alkaline unwinding assay.

Gel electrophoresis and DNA fragmentation studies

The sample was mixed with 10 mL of loading solution (10 mM EDTA (pH 8.0), 1% (w/v) low-melting-point agarose, 0.25% (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 TBE buffer. The DNA was visualized using ethidium bromide and photographed by digital camera.

Alkaline unwinding assay

The procedure used for alkaline unwinding was essentially the same as that outlined by Shugart (1988) with slight modifications. For the fluorescence determination of dsDNA, ssDNA and partially unwound DNA (auDNA), three equal portions of diluted DNA sample were prepared. The amount of dsDNA was determined from the fluorescence of a sample without any treatment, while ssDNA was determined from a sample that had been boiled for 30 min. Fluorescence of the DNA sample that had been subjected to alkaline treatment (pH 12.2) on ice for 30 min provided an estimate of the amount of auDNA.

The fluorescence of initial or dsDNA was determined by placing 100 μmol DNA sample, 100 μL NaCl (25 mM) and 2 μL SDS (0.5%) in a pre-chilled test tube, followed by an addition of 3 mL 0.2 M potassium phosphate pH 9, and 3 μL bisbenzamide (1 mg mL^{-1}). The fluorescence of the sample was measured using a spectrofluorimeter (Ex: 360 nm, Em: 450 nm). The fluorescence of ssDNA was determined as above but using a DNA sample that had already been boiled for 30 min so as to completely unwind the DNA.

Fifty microlitres of NaOH (0.05 M) was rapidly mixed with 100 μL of DNA sample in a pre-chilled test tube. The mixture was incubated on ice in darkness for 30 min followed by rapid addition and mixing of 50 μl HCl (0.05 M) (Rao et al 1996). This was followed immediately by an addition of 2 μL SDS (0.5%) and the mixture was forcefully passed through a 21 G needle six times. Fluorescence of alkaline unwound DNA sample was measured as described above. Measurement of the alkaline unwounded sample was performed in triplicate and the average was reported.

The ratio between dsDNA to total DNA (F value) was determined as follows:

$$\text{F value} = (\text{auDNA} - \text{ssDNA})/(\text{dsDNA} - \text{ssDNA}) \quad (1)$$

Where auDNA, ssDNA and dsDNA were the degrees of fluorescence from the partially unwound, single-stranded and double-stranded determinations, respectively. The F value was inversely proportion to the number of strand breaks present and thus could be used as an indicator of DNA integrity.

Determination of aryl hydrocarbon hydroxylase (AHH) activity

Aryl hydrocarbon hydroxylase activity (AHH) was determined as described by Weibel & Gelboin (1975). The reaction mixture, in a total volume of 1 mL, contained 50 mol Tris-chloride buffer, pH 7.4, 0.36 mol NADPH, 3 mol $MgCl_2$ and 0.1 mL microsomes suspended in 0.1 M potassium phosphate buffer pH 7.4 (1 mg protein/mL). The substrate, B(a)P (100 nmol), was added in 0.05 mL methanol to start the reaction. After 10 min of incubation at 37°C, the reaction was terminated by the addition of 1.0 mL cold acetone and the mixture was shaken with 3.0 mL hexane for 10 min to extract the derivatives of B(a)P. A 1.0-mL volume of the organic layer was extracted with 2 mL 1 M NaOH. The fluorescence of the NaOH extract was measured immediately at 396 nm excitation and 522 nm emission using a spectrofluorometer. The amount of enzyme activity is defined as the picomoles of 3-hydroxy-B(a)P formed during the incubation per mg protein per min.

Assay of cytochrome P450 content

Ten percent (w/v) homogenate was prepared from the liver of mice and processed for the preparation of post-mitochondrial supernatant (PMS) and microsomes for CYP content. It was done by the method of Omura & Sato (1964). A pinch of sodium dithionate was added to 2 mL of sample. This was then divided equally between two matched cuvettes. The contents of the test cuvette were gently bubbled with carbon monoxide for about 1 min and then OD was measured at 450 nm and 490 nm simultaneously.

Assay for glutathione-S-transferase activity

Glutathione-S-transferase activity was assayed by the method of Habig et al (1974). The reaction mixture consisted of 1.475 mL phosphate buffer (0.1 M, pH 6.5), 0.2 mL reduced glutathione (1 mM), 0.025 mL CDNB (1 mM) and 0.3 mL PMS (10% w/v) in a total volume of 2.0 mL. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed per min per mg protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}.

Assay for quinone reductase activity

The activity of quinone reductase was determined by the method of Benson et al (1980). The 3-mL reaction mixture consisted of 2.13 mL Tris-HCl buffer (25 mM, pH 7.4), 0.7 mL BSA, 0.1 mL FAD, 0.02 mL NADPH (0.1 mM) and 50 μL (10%) PMS. The reduction of dichlorophenol indophenol (DCPIP) was recorded calorimetrically at 600 nm and enzyme activity was calculated as nmoles of DCPIP reduced per min per mg protein using a molar extinction coefficient of 2.1×10^4 M^{-1} cm^{-1}.

Estimation of reduced glutathione

Reduced glutathione was determined by the method of Jollow et al (1974). One-millilitre PMS was precipitated with 1.0 mL of sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h and then centrifuged at 1200 *g* for 20 min at 4°C. The assay mixture contained 0.1 mL filtered sample, 2.7 mL phosphate buffer (0.1 M, pH 7.4) and 0.2 mL DTNB (100 mM) in a total volume of 3.0 mL. The yellow colour developed was read at 412 nm on a spectrophotometer.

Protein estimation

Protein content in all samples was estimated by the method of Lowry et al (1964) using bovine serum albumin as standard.

Statistical analysis

The level of significance between different groups is based on analysis of variance test, followed by the Dunnett's *t* test.

Results

B(a)P, at the dose level of 125 mg kg^{-1} orally, caused modulation of several parameters of oxidative stress relative to control mice receiving corn oil only with concomitant enhancement of its metabolizing enzymes and loss of DNA integrity. Treatment with B(a)P alone decreased the hepatic GSH content, GST and QR activity significantly ($P < 0.001$) with a concomitant increase in CYP activity and AHH ($P < 0.001$), respectively, compared with the corn oil treated control. This increase in CYP activity and AHH was restored significantly back towards the normal control level by pre-treatment with apigenin. Also, pre-treatment of mice with apigenin at 2.5 and 5 mg kg^{-1} restored GSH content, GST and QR activity significantly ($P < 0.001$) (Table 1). To investigate the genotoxicity of B(a)P, the DNA was examined. As shown in Figure 2, agarose gel electrophoresis of soluble DNA showed DNA fragmentations, which are characteristic of apoptotic cells (DNA ladder). The results indicated that exposure to B(a)P led to DNA fragmentation and that apigenin pre-treatment protects against DNA fragmentation. DNA fragmentation was not observed in control and only apigenin treatment (Figure 2). DNA fragmentation is a visual parameter of DNA damage in which DNA smearing and lack of intact band are the main parameter. In Figure 2, lane 2 represents B(a)P + 2.5 mg kg^{-1} apigenin treatment in which intact band as well as smearing is seen. Lane 4 represents only B(a)P treatment in which smearing is seen but intact band is not prominent. So we can say that the lower dose of apigenin (2.5 mg kg^{-1}) slightly protects against DNA damage caused by B(a)P but our alkaline unbinding assay shows significant ($P < 0.001$) protection against it.

B(a)P also showed dose-dependent depletion in the F-value of DNA alkaline unwinding assay, which was however increased in apigenin pre-treated groups, and is a marker for alteration in DNA integrity as shown in Table 2 ($P < 0.001$). There was also a significant difference in both the treatments with apigenin ($P < 0.05$). There was also a concomitant amelioration of CYP and AHH activity ($P < 0.05$) in a dose-dependent manner (Table 2) and also a significant difference was shown by both the apigenin treatments ($P < 0.05$).

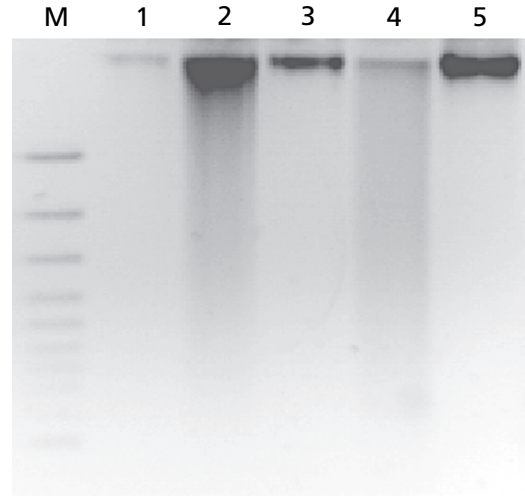

Figure 2 Agarose gel electrophoresis of DNA fragments in mouse liver. DNA was collected and assessed by agarose gel electrophoresis containing ethidium bromide. M, DNA marker; 1, only apigenin 5 mg kg^{-1} treated; 2, apigenin 2.5 mg kg^{-1} + B(a)P treated; 3, apigenin 5 mg kg^{-1} + B(a)P treated; 4, only B(a)P treated; 5, control.

Discussion

Antioxidant supplements have become popular because they reduce gene damage inflicted by free radicals (Khan et al 2005). Flavonoids are a class of polyphenolic compounds widely distributed in the plant kingdom, which display a variety of biological actions including chemoprevention and tumour growth inhibition. Various experimental studies indicate that they may reduce oxidative damage, inhibit the growth of human leukaemia cells and induce cell differentiation, inhibit cancer cell signal transduction, induce apoptosis and act as potent anti-inflammatory and anti-spasmodic agents (Chiang et al 2005; Fiorani & Accorsi 2005; Jeyabal et al 2005; Zheng et al 2005; Khan & Sultana 2006).

Table 1 Effect of pre-treatment with apigenin on B(a)P-induced toxicity on glutathione content, glutathione S-transferase and quinone reductase in mouse liver

Treatment regimen	Reduced glutathione (nmol GSH/g tissue)	Glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein)	Quinone reductase (nmol dichloroindophenol reduced/min/mg protein)
Corn-oil-treated control	0.586 ± 0.06	29.7 ± 0.09	198.0 ± 2.44
B(a)P alone	0.291 ± 0.009[a]	4.5 ± 0.29[a]	115.0 ± 1.19[a]
B(a)P + apigenin (2.5 mg kg^{-1})	0.317 ± 0.020[b]	9.8 ± 2.73[b]	165.1 ± 4.9[b]
B(a)P + apigenin (5 mg kg^{-1})	0.400 ± 0.04[b c]	13.9 ± 0.74[bc]	177.8 ± 1.7[b c]
Only apigenin (5 mg kg^{-1})	0.586 ± 0.01	47.17 ± 14.3	189.6 ± 5.7

Results represent mean ± s.e. of five mice/group. [a]$P < 0.001$, compared with corn-oil-treated group; [b]$P < 0.001$, compared with B(a)P-treated group; [c]$P < 0.05$, compared with group treated with B(a)P + apigenin 2.5 mg kg^{-1}.

Table 2 Effect of pre-treatment with apigenin on B(a)P-induced toxicity on alkaline unwinding assay, aryl hydrocarbon hydroxlase activity and cytochrome P-450 content

Treatment regimen	Alkaline unwinding assay (F-value)	Aryl hydrocarbon hydroxylase (nmol (mg protein^{-1}))	Cytochrome P-450 content (n mol (mg protein)$^{-1}$)
Corn-oil-treated control	0.921 ± 0.018	57.52 ± 0.73	1.61 ± 0.07
B(a)P alone	0.467 ± 0.026[a]	93.64 ± 5.59[a]	3.39 ± 0.12[a]
B(a)P + apigenin (2.5 mg kg^{-1})	0.646 ± 0.035[b]	30.38 ± 1.89[b]	2.71 ± 0.24[NS]
B(a)P + apigenin (5 mg kg^{-1})	0.732 ± 0.032[b c]	23.35 ± 3.26[b c]	1.72 ± 0.21[b c]
Only apigenin (5 mg kg^{-1})	0.938 ± 0.042	22.24 ± 3.43	1.67 ± 0.04

Results represent mean ± s.e. of five mice/group. [a]$P < 0.001$, compared with corn-oil-treated group; [b]$P < 0.001$, compared with B(a)P-treated group; [c]$P < 0.05$, compared with group treated with B(a)P + apigenin 2.5 mg kg–^{1}; NS, not significant.

Decreasing the rate of DNA damage and enhancing antioxidant defence suppress several diseases, including cancer. ROS scavengers have therefore evolved as effective cancer chemopreventive agents (Shugart 1990; Kim & Lee 2004). There have been in-vivo studies in which CYP enzyme activity was reduced, resulting in lower cancer rates and longer life span (Celander et al 1996; Guengerich 1998). Here, in our study we have shown that apigenin suppresses the metabolism of B(a)P through inhibition of phase I metabolizing enzymes, i.e. AHH, and depletion of total CYP content. Apigenin concomitantly inhibits DNA strand breaks. The mechanism of the induction of phase II detoxification enzymes, such as NAD(P)H:(quinone-acceptor) oxidoreductase (quinone reductase (QR)) or glutathione S-transferase (GST), is fairly important. These inducible enzymes facilitate the metabolic detoxification of xenobiotics in mammalians and can achieve chemopreventive activity by modification of carcinogen metabolism through increased carcinogen excretion and decreased availability of carcinogen reactive metabolites capable of interacting with DNA (Talalay et al 1995). QR is responsible for conversion of quinones to less toxic and soluble hydroquinones. Several studies have shown a strong correlation between modulation of QR activity and chemoprevention of cancer (De Flora & Ramel 1988; Wilkinson & Clapper 1997). GST is one of the most important detoxification enzymes that functions to conjugate functionalized P450 metabolites with endogenous ligand (GSH), leading to their excretion from the organism (Hartman & Shankel 1990). Prophylactic treatment with apigenin before B(a)P administration significantly modulated GST- and QR-mediated inhibition of reactive electrophiles from reacting cellular target and production of thioether-linked glutathionyl conjugates.

Glutathione (GSH) plays an important role against oxidative DNA damage and provides defence against various xenobiotics (Halliwell et al 1992). Concomitant amelioration of tissue GSH content in pre-treated groups of mice suggests the antioxidant action of apigenin. Metabolic activation of B(a)P to ultimate carcinogen is considered essential for induction of its genotoxic and carcinogenic effects. In this study, pre-treatment in modulator groups significantly inhibited activity of AHH and CYP content when compared with the only toxicant group; hence there was inhibition of B(a)P metabolism and so generation of toxic metabolites of B(a)P was decreased. The single-strand DNA breaks assay detected sites on the DNA where one of the strand had been nicked: the more nicks, the more rapidly the DNA unbinds under alkaline conditions, and the lower the F value and agarose gel electrophoresis of mouse liver DNA.

In this study, therefore, it is concluded that the mechanism of action of apigenin is through: scavenging of reactive oxygen species and induction of antioxidant enzymes; detoxification through induction of phase II pathways (determination of specific isoforms of these could provide more information on the protective mechanisms of apigenin action); decrease in CYP and AHH content; and maintenance of DNA integrity. Thus our results show that apigenin is a potent antioxidant and antigenotoxic and an effective inhibitor of DNA strand breaks.

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