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Ellagic acid peracetate is superior to ellagic acid in the prevention of genotoxicity due to aflatoxin B_1 in bone marrow and lung cells

Ajit Kumar, Yogesh K. Tyagi, Seema, Prija Ponnan, Vishwajeet Rohil, Ashok K. Prasad, Bilekere S. Dwarkanath, Virinder S. Parmar and Hanumantharao G. Raj

Abstract

Earlier observations carried out in our laboratory highlighted the mode of action of acetoxy 4-methylcoumarins and quercetin pentaacetate in preventing the genotoxicity of aflatoxin B_1 (AFB_1). We have extended the observation to an acetoxy biscoumarin i.e. ellagic acid peracetate (EAPA), which unlike ellagic acid (EA) has demonstrated time-dependent inhibition of liver microsomes catalysed AFB_1-epoxidation as measured by AFB_1 binding to DNA. EAPA was more potent than EA in preventing bone marrow and lung cells from AFB_1-induced genotoxicity. EAPA was acted upon by microsomal acetoxy drug:protein transacetylase (TAase) leading to modulation of the catalytic activity of certain functional proteins (cytochrome P450, NADPH cytochrome c reductase and glutathione S-transferase), possibly by way of protein acetylation.

Introduction

Polyphenols play an important role in human nutrition and are implicated with numerous biological properties including antioxidant, anti-inflammatory and anticancer activities (Bhalla et al 1992; Raj et al 1998a; Middleton et al 2000). Among the polyphenols, 4-methylcoumarins have come under some scrutiny regarding their ability to prevent chemical- and radiation-induced genotoxicity (Raj et al 1998b, 2001b). In general, methoxy/hydroxy derivatives of coumarin inhibit cytochrome P450-mediated formation of reactive intermediates of several classes of chemical carcinogens and mutagens, which damage cellular DNA by way of adduct formation (Raj et al 1999). Ellagic acid (EA; Figure 1) is a naturally occurring condensed biscoumarin found in a variety of plant species, especially fruits such as strawberries, raspberries, cranberries, walnuts, pecans, grapes and distilled beverages (Marwan & Nagel 1986; Chen et al 2001). Recent studies have indicated that EA possesses antimutagenic, antioxidant and anti-inflammatory activity in bacterial and mammalian systems (Wood et al 1982; Kaur et al 1997; Loarca-Pina et al 1998). EA was shown to act as a potent anticarcinogenic agent by modulating the metabolism of environmental toxins and therefore prevented the initiation of carcinogenesis induced by these chemicals (Zhang et al 1993). EA was found to inhibit the mutagenesis induced by aflatoxin B_1 (AFB_1) in salmonella tester strains TA 98 and TA 100 (Soni et al 1997). After oral administration, EA exhibited hepatoprotective activity against carbon tetrachloride, in-vitro and in-vivo. EA was also found to reduce the number of bone marrow cells with chromosomal aberrations and chromosomal fragments as effectively as α-tocopherol (Thresiamma et al 1998). Moreover, the administration of EA inhibited radiation-induced DNA strand breaks in rat lymphocytes and induced G1 arrest, inhibited overall cell growth and caused apoptosis in tumour cells (Narayana et al 1999). In our laboratory, we have demonstrated the inhibitory action of several acetoxy derivatives of 4-methylcoumarins on the microsomes-catalysed epoxidation of AFB_1 and related biological effects (Raj et al 1999, 2001a). These investigations revealed the unique action of the model compound 7,8-diacetoxy-4-methylcoumarin (DAMC; Figure 1) to cause a dramatic irreversible inhibition of microsomes-mediated AFB_1-epoxidation measured

Department of Biochemistry, V. P. Chest Institute, University of Delhi, Delhi-110007, India

Ajit Kumar, Yogesh K. Tyagi, Seema, Prija Ponnan, Vishwajeet Rohil, Hanumantharao G. Raj

Institute of Nuclear Medicine and Allied Sciences, Lucknow Road, Timarpur, Delhi, India

Bilekere S. Dwarkanath

Department of Chemistry, Bioorganic Laboratory, University of Delhi, Delhi-110007, India

Ashok K. Prasad, Virinder S. Parmar

Correspondence: H. G. Raj, Department of Biochemistry, V. P. Chest Institute, University of Delhi, Delhi 110 007, India.
E-mail: rajhg@yahoo.com

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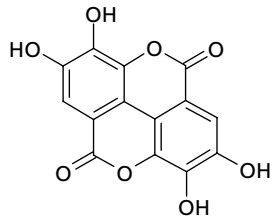

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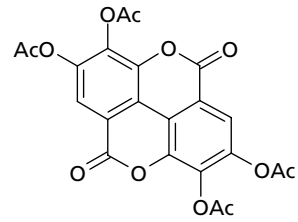

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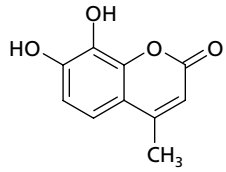
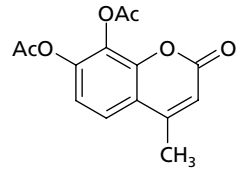


7,8-Dihydroxy-4-methylcoumarin (DHMC)



7,8-Diacetoxy-4-methylcoumarin (DAMC)

Figure 1 Structure of polyphenols and their peracetates.

as AFB_1 binding to DNA, while 7,8-dihydroxy-4-methylcoumarin (DHMC; Figure 1), the deacetylated derivative of DAMC, failed to elicit such effects of the acetoxycoumarin (Raj et al 1999). Those results prompted us to postulate the existence of a transacetylase enzyme in microsomes that could possibly catalyse the transfer of acetyl groups of DAMC to P450 apoprotein, and thereby cause the inhibition of microsomes-catalysed AFB_1–DNA binding both in-vivo and in-vitro (Raj et al 1999). Earlier work revealed the action of transacetylase on several classes of polyphenolic peracetates (PA) and hence this enzyme was termed as acetoxy drug:protein transacetylase (TAase) (Raj et al 2001a; Singh et al 2002; Kumar et al 2005). Recently, TAase has been purified to homogeneity from rat liver and human placenta (Raj et al 2006; Seema et al 2007). The N-terminal amino acid sequence analysis of TAase when aligned with non-redundant Swiss-Port Database sequence revealed a perfect match with N-terminal sequence of mature calreticulin, a prominent Ca^{2+} binding protein of endoplasmic reticulum (Michalak et al 1999). Further studies confirmed the identity of TAase with calreticulin and led to the designation of TAase as calreticulin transacetylase (CRTAase) (Seema et al 2007). In this study, we have compared meticulously the action of EA and EAPA (Figure 1) with special reference to CRTAase-mediated biological action and highlighted the superiority of EAPA over EA in the protection of AFB_1-induced genotoxicity.

Materials and Methods

Chemical reagents

$[^3H]AFB_1$-G was obtained from Moravek Biochemicals (Brea, CA). Ellagic acid, NADPH, calf thymus DNA, and AFB_1 were purchased from Sigma Chemical Co. (St Louis, MO).

Synthesis of ellagic acid peracetate (EAPA)

The organic solvents (acetic anhydride and pyridine) were dried and distilled before use. Analytical TLC was performed on pre-coated Merck silica gel 60 F_{254} plates; the spots were visualized under UV light. The ^1H NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer at 300 MHz using trimethyl silane (TMS) as internal standard. The chemical shift values were on δ scale and in Hz. Melting points were determined in a sulfuric acid bath and are uncorrected. The UV and IR spectra were recorded on a Cary Bio 100 and Perkin-Elmer model 2000 FT-IR spectrometer, respectively.

EAPA was synthesized according to Roshchin & Dzhumyrko (1972). The acetylation of EA (1.0 g) was carried out with acetic anhydride (2.5 mL) and pyridine (1.04 mL). The reaction mixture was stirred for 12 h and monitored on thin layer chromatography (TLC). On completion of the reaction, the compound was worked up by ice-cold water and the crude product was purified by crystallization from acetic anhydride and characterized by spectral data. It was obtained as a cream coloured solid: yield 70%; m.p. 340°C; UV (MeOH): λ_{max} 280, 265 nm; IR (KBR): V_{max} 3073, 1710, 1696, 1618, 1512, 1510, 1448, 1394, 1399, 1194, 1109, 1054, 921, 810, 757, 641, 577 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 2.68 (6H, s, $2 \times OCOCH_3$), 2.71 (6H, s, $2 \times OCOCH_3$), 7.99 (2H, s, C-4H and C-9H).

Animals

Male albino Wistar rats (150–200 g) were fed on rat chow supplied by Hindustan Lever Ltd, Mumbai (India) and water was freely available. The animal experiments were performed at the Animal Experiment Center at V. P. Chest Institute (Delhi, India). Ethical permission No. 00170 was provided by the Animal Research Committee of V. P. Chest Institute, University of Delhi, (India).

Preparation of liver microsomes and cytosol

Male rats were killed, liver excised and microsomes prepared as described by Raj et al (1999).

Assay of acetoxy drug:protein transacetylase (TAase)

TAase activity was assayed in rat liver microsomes. The TAase assay using PA and cytosolic glutathione S-transferase (GST) as the substrates was described in detail by Raj et al (2001a). Briefly, the assay mixture consisted of 0.25 M phosphate buffer (pH 6.5), liver microsomes (25 μg protein), test compound (50 μM) added in 50 μL dimethyl sulfoxide (DMSO), liver cytosol (10–15 μg protein) and water to make up 0.5mL. The contents of the tube were scaled up as per requirement and pre-incubated at 37°C for various periods. The samples were removed periodically into a spectrophotometer cuvette containing GSH and 1-chloro-2,4-dinitrobenzene (CDNB) to make up their concentration to 1 mM in a total volume of 1mL, and the progress of the GST activity was followed at 340 nm using a Cary spectrophotometer (Cary Bio100) as described by Habig et al (1974). In control samples, polyphenols and PA were replaced by DMSO. The unit of TAase was expressed in terms of % inhibition of GST under the conditions of the assay and ensured that the reaction was linear with respect to enzyme concentration and pre-incubation time.

TAase-mediated biological action of EAPA

Activation of NADPH cytochrome c reductase

The assay mixture consisted of 0.05 M phosphate buffer (pH 7.7), 0.1 mM EDTA, liver microsomes (25 μg protein), 36 μM cytochrome c and 1 mM NADPH. Accordingly, polyphenols and PA (5 μM) were separately pre-incubated along with liver microsomes at 37°C in shaking water bath followed by the addition of EDTA, cytochrome c and NADPH in a total volume of 1 mL. The progress of the reaction of NADPH cytochrome c reductase assay was followed by monitoring absorption at 550 nm (Masters et al 1967). In the control samples DMSO replaced PA. The increment in reductase activity due to test compound over the control was expressed as per cent activation.

Effect of test compounds on rat liver microsomes catalysed AFB_1-epoxidation measured as AFB_1 binding to DNA

The test compound (100 μM) was separately pre-incubated with rat liver microsomes (1 mg protein), phosphate buffer (100 mM, pH 7.4) and water to make the volume 0.8 mL. The contents (scaled up as per requirement) were pre-incubated at 37°C in a shaking water bath. The samples (0.5 mL) were removed periodically into another set of tubes containing [^{3}H]AFB_1 (250 μci μM^{-1}) in 20 μL DMSO, 0.1 mg calf thymus DNA, 2 mM NADPH, made to a final volume of 1 mL and incubated for different time of intervals (10, 20 and 30 min) at 37°C. At the end of incubation, 2 mL of the extraction mixture (phenol/chloroform/isoamyl alcohol 50:50:1, v/v/v) and 0.9 mg calf thymus DNA as the carrier were added. The DNA was isolated by the procedure of Wang & Cerutti (1979) and the recovery of DNA was 50–70%. Isolated DNA was dissolved in 0.1 M NaCl and one portion (0.2 mL) was added to a vial containing 8 mL Bray's scintillation fluid for determination of radioactivity using a liquid scintillation counter (Beckman Model LS 6000). Result of [^{3}H]AFB_1 binding to DNA was corrected for DNA recovery and expressed as [^{3}H]AFB_1 bound/mg DNA/10, 20 or 30 min. In this assay procedure samples with DMSO substituting for the test compound served as the control.

Treatment with test compound

Male albino Wistar rats (150–200 g) were administered separately the test compound (300 mg kg^{-1} dissolved in 0.1 mL DMSO) intraperitoneally (i.p.) and intratracheally (i.t.) for bone marrow and lung cells, respectively, followed by a second dose of the test compound along with a dose of AFB_1 (4 mg kg^{-1} in DMSO). A group of rats were injected with AFB_1 alone while control animals received DMSO alone. The animals were killed 26 h after the last injection.

Isolation of bone marrow cells

After the rats had been killed both femora were removed. Muscles and tissues were separated from the bone. Both ends of the femur were chopped off. A needle was inserted at the proximal end of the femur and the bone marrow was gently flushed out into a centrifuge tube by forcing 2 mL Hank's Balanced Salt Solution with the help of a syringe.

Isolation of bronchoalveolar lavage (BAL)

The isolation of bronchoalveolar lavage was carried out according to the method of Myrvick et al (1961). Rats were killed and the chest cavity was opened, cutting the diaphragm. The trachea was clamped with artery forceps to prevent any entry of blood into the lungs. The lungs were filled with 5 mL prewarmed (37°C) normal saline using a blunt tip of an 18 G needle. The lungs were gently massaged holding the trachea and the aspirate was collected; this was termed BAL. This procedure was repeated three times pooling the lavage containing the lung cells.

Preparation of smear and staining

For preparation of smear and staining, we adopted the method of Schmid (1975) followed in our earlier publications (Dwarkanath et al 1998; Raj et al 2001b). The bone marrow cells were stained with haematoxylin and eosin, and lung cells were stained with DNA specific fluorochrome, diamidino-2-phenylindole dihydrochloride (DAPI).

Microscopy

DAPI-stained slides were examined using a fluorescent microscope (Nikon) with UV mode using blue filter. Haematoxylin and eosin-stained slides were viewed under oil immersion lens of the light microscope (Nikon).

Counting of micronuclei

Air-dried slides of acetic acid/methanol (1:3, v/v) fixed cells were stained with DNA specific fluorochrome, DAPI (Dwarkanath et al 1998). Approximately 1000 cells were analysed

from slides. Counting 500–1000 cells is generally recommended for evaluating micronuclei. In the case of lung cells where cells were yielded from a single animal, the number was less and the results were expressed as number of micronuclei per 1000 cells, pooled from three to six animals. Micronuclei were recognized by their size, shape and fluorescence. They were found to be smaller than the adjacent main nucleus and emitted blue fluorescence of the same intensity as that of the corresponding nucleus. The micronuclei of bone marrow cells were best seen in polychromatic erythrocytes.

Statistical analysis

Differences between experimental factors were statistically determined using analysis of variance. In all cases $P<0.05$ denoted significance.

Results and Discussion

The aim of the study was to evaluate the effect of EAPA over EA on CRTAase-catalysed modulation of certain enzymes such as cytochrome P450-linked mixed function oxidase (MFO), NADPH cytochrome c reductase and glutathione S-transferase (GST). Special attention was focused on the CRTAase-mediated prevention of the clastogenic action of potent carcinogen AFB_1 in-vitro and in-vivo.

Evidence for the existence of a unique enzyme termed acetoxy drug:protein transacetylase (TAase) in rat liver microsomes, catalysing transfer of the acetyl group from PA to specific proteins, has been obtained previously (Raj et al 1999, 2001a; Singh et al 2002; Kumar et al 2005). We have conclusively established TAase catalysed transfer of acetyl groups from a model acetyl donor DAMC to a receptor protein (GST 3-3) by mass spectrometry studies (Kohli et al 2002b, 2004). The structure–activity relationship study of various classes of PA such as acetoxycoumarins, acetoxyflavones and acetoxyisoflavones revealed the following three features specific for TAase: firstly, the presence of acetoxy groups in proximity to the oxygen heteroatom; secondly, the absolute requirement of the presence of the pyran ring carbonyl group; thirdly, the inhibitory effect of the phenyl moiety on the pyran nucleus (Raj et al 2001; Singh et al 2002; Kumar et al 2005). We have purified TAase to homogeneity and established the identity of TAase with calreticulin, a prominent Ca^{2+} binding protein of endoplasmic reticulum. Hence TAase has been designated as calreticulin transacetylase (CRTAase) (Raj et al 2006; Seema et al 2006).

In this study, a comparison of relative specificities of CRTAase to EAPA was made and correlated with CRTAase-mediated biological effects, such as activation of NADPH cytochrome c reductase and prevention of AFB_1-induced genotoxicity in bone marrow and lung cells. The pre-incubation of rat liver microsomes and cytosolic GST along with EAPA resulted in time-dependent inhibition of GST, while EA failed to produce such effects (Table 1). The inhibition of GST under the conditions of the assay was considered to be proportional to the CRTAase activity as described in earlier publications (Raj et al 1999, 2000, 2001a; Kumar et al 2005). The irreversible activation of NADPH cytochrome c reductase and the inhibition of cytochrome P450 linked MFO by acetoxycoumarin and other PA were established (Raj et al 1998b, 2001b; Singh et al 2002; Kumar et al 2005). CRTAase catalysed activation of NADPH cytochrome c reductase by EAPA was found to be significantly higher than that caused by EA, which is the deacetylated product of EAPA (Table 2). This was taken as

Table 1 Specificity of ellagic acid peracetate (EAPA) and ellagic acid (EA) to rat liver microsomal calreticulin transacetylase (CRTAase)

Test compounds	Time of pre-incubation	GST activity (mean ± s.d.)	CRTAase catalytic activity[a]
Control		0.210 ± 0.0016[a]	
EAPA (50 μM)	10	191.60 ± 0.0022	9.23
	20	171.20 ± 0.0021	16.09
	30	152.25 ± 0.0028	27.50
	40	138.00 ± 0.0047	34.28
EA (50 μM)	10	192.25 ± 0.0022	8.45
	20	190.00 ± 0.0019	9.52
	30	187.00 ± 0.0023	10.95
	40	191.50 ± 0.0018	8.80

[a]There was no significant difference among control data upon pre-incubation for different periods (10, 20, 30 and 40 min). Values are an average of five observations. The numbers in the parentheses indicate the concentration of the test compound in the pre-incubation reaction mixture. The addition of DMSO (vehicle for test compound) served as a control. [a]CRTAase was expressed as percent inhibition of GST. The details are given in Materials and Methods.

Table 2 Influence of ellagic acid peracetate (EAPA) and ellagic acid (EA) on rat liver microsomal calreticulin transacetylase (CRTAase)-catalysed activation of NADPH cytochrome c reductase

Test compound	Time of pre-incubation	Activity of NADAH cytochrome c reductase (mean ± s.d.)	NADPH cytochrome c reductase (% activation)
Control		0.0486 ± 0.0020[a]	
EAPA (5μM)	5	0.0565 ± 0.0025	16.25
	10	0.0632 ± 0.0019	30.04
	20	0.0797 ± 0.0013	63.99
	30	0.0950 ± 0.0019	95.45
EA (5μM)	5	0.0517 ± 0.0020	6.48
	10	0.0511 ± 0.0013	5.14
	20	0.0521 ± 0.0019	7.20
	30	0.0519 ± 0.0025	6.79

[a]There was no significant difference among control data upon pre-incubation for different periods (5, 10, 20 and 30 min.). Values are an average of five observations. The numbers in the parentheses indicate the concentration of the test compound in the pre-incubation reaction mixture. In the control samples, DMSO replaced polyphenols the test compound. The increment in reductase activity due to test compound over the control was expressed as percent activation. The details are given in Materials and Methods.

another yardstick to assess the activity and specificity of EAPA. AFB_1 is oxidized at the 8,9-vinyl ether bond by cytochrome P450 isoforms yielding exo- and endo-isomers of the highly reactive electrophile AFB_1 8,9-epoxide, of which exo-isomer is the most potent mutagen which reacts with cellular DNA forming N-7 guanine adduct (Raney et al 1992). The pre-incubation of rat liver microsomes with EAPA resulted in irreversible inhibition of liver microsomses–catalysed AFB_1-epoxidation (measured as AFB_1–DNA binding) in a time-dependent manner in-vitro (Table 3). EA was found to be ineffective. Previous results from our laboratory have described DAMC and quercetin pentaacetate as very promising inhibitors of AFB_1-induced genotoxicity in-vivo (Raj et al 2001a; Kohli et al 2002a). The results documented in Table 4 highlight a comparative account of EA and EAPA to prevent the AFB_1-induced clastogenic action in bone marrow and lung cells. The incidence of micronuclei per 1000 cells in the control rats receiving their vehicle (DMSO) alone was 1.5 ± 0.837 in bone marrow cells as compared with 2 ± 0.753 in lung cells (Table 4). The administration of EA and EAPA separately failed to cause micronuclei in bone marrow and lung cells (Table 4). Upon administration of AFB_1 (serving as positive control), the extent of micronuclei induction was significantly enhanced as compared with control, yielding 7.0 ± 1.41 and 7.5 ± 1.52 micronuclei per 1000 bone marrow and lung cells, respectively ($P < 0.05$). The pretreatment of rats with EAPA caused a 40% reduction of AFB_1 induced micronuclei in bone marrow as well as lung cells (Table 4), while for EA it was found to be only 20% as compared with positive control (rats treated with AFB_1 alone). It was interesting to note that the inhibition of micronuclei induction due to AFB_1 by EAPA was significantly higher as compared with EA ($P < 0.05$). Our results (Tables 1–4) denote an important observation that EAPA, like acetoxycoumarins (Raj et al 1998b, 2001b), is an irreversible inhibitor of AFB_1-epoxidation leading to AFB_1-induced clastogenic action, and modulation of catalytic activity of some other enzymes such as GST and NADPH cytochrome c reductase. These results confirmed our hypothesis that EAPA was acted upon by the microsome membrane-bound CRTAase resulting in the possible acetylation of enzyme proteins with altered catalytic activity. We have reported for the first time the potential of EAPA through the action of CRTAase in preventing AFB_1-epoxidation-induced genotoxicity in rat bone marrow and lung cells.

Table 3 Influence of ellagic acid peracetate (EAPA) and ellagic acid (EA) on liver microsomes-catalysed aflatoxin B_1 (AFB_1)-binding in-vitro

Test compound	Time of pre-incubation (min)	AFB_1–DNA binding (pmol AFB_1 bound/mg DNA per 10/20/30/ min) (mean ± s.d.)	% inhibition of AFB_1-epoxidation binding to DNA
Control		208.41 ± 2.55[a]	
EAPA	10	191.50 ± 2.38	8.11
	20	177.00 ± 2.58	15.55
	30	160.25 ± 2.50	23.10
EA	10	194.25 ± 2.53	6.79
	20	196.00 ± 2.94	5.95
	30	194.00 ± 3.36	6.91

[a]There was no significant difference among control values (nine observations) for different period of pre-incubation (10/20/30 min). Values are mean of three observations for the test compounds. Liver microsomes catalysed inhibition of AFB_1 binding to DNA under conditions of the assay. Concentration of the test compounds was 100 μM.

Table 4 Influence of ellagic acid (EA) and ellagic acid peracetate (EAPA) on aflatoxin B_1 (AFB_1)-induced micronuclei formation in rat bone marrow and lung cells

Group	Treatments	n	Micronuclei/1000 bone marrow cells (mean ± s.d.)	Micronuclei/1000 lung cells (mean ± s.d.)
1	Control	6	1.5 ± 0.837	2.0 ± 0.753
2	AFB_1	6	7.0 ± 1.41	7.5 ± 1.52
3	EA	6	1.5 ± 0.837	1.75 ± 0.753
4	EAPA	6	1.75 ± 0.758	1.25 ± 0.516
5	EA + AFB_1	6	5.50 ± 1.50 (21.42)[a]	6.0 ± 1.42 (20.00)[a]
6	EAPA + AFB_1	6	4.15 ± 0.853** (40.71)[a]	4.45 ± 1.05 (40.66)[a]

n, Number of animals in each group. Values are mean of six observations. **$P < 0.05$, significantly different compared with AFB_1. [a]Numbers in parentheses denote the percentage of inhibition of formation of micronuclei in AFB_1-treated bone marrow and lung cells due to test compound.

Conclusion

The biological significance of ellagic acid is known, but for the first time we have reported CRTAase-mediated biological action of ellagic acid peracetate. Ellagic acid peracetates was found to be superior to ellagic acid in the prevention of genotoxicity due to aflatoxin B_1 in bone marrow and lung cells.

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