Dietary phenolics as anti-mutagens and inhibitors of tobacco-related DNA adduction in the urothelium of smokers

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Human urine is known to contain substances that strongly inhibit bacterial mutagenicity of aromatic and heterocyclic amines in vitro. The biological relevance of these anti-mutagens was examined by comparing levels of tobacco-related DNA adducts in exfoliated urothelial cells from smokers with the anti-mutagenic activity in corresponding 24-h urine samples. An inverse relationship was found between the inhibition of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-mutagenicity by urine extracts in vitro and two DNA adduct measurements: the level of the putatively identified N-(deoxyguanosine-8-yl)-4-aminobiphenyl adduct and the total level of all tobacco-smoke-related carcinogen adducts including those probably derived from PhIP. Urinary anti-mutagenicity in vitro appears thus to be a good indicator of the anti-genotoxicity exerted by substances excreted in urine, that protect the bladder mucosal cells (and possibly other cells) against DNA damage. These substances appear to be dietary phenolics and/or their metabolites because (i) the anti-mutagenic activity of urine extracts (n = 18) was linearly related to their content in phenolics; (ii) the concentration ranges of these substances in urine extracts were similar to those of various plant phenols (quercetin, isorhamnetin and naringenin) for which an inhibitory effect on the liver S9-mediated mutagenicity of PhIP was obtained; (iii) treatment of urines with β-glucuronidase and arylsulfatase enhanced both anti-mutagenicity and the levels of phenolics in urinary extracts; (iv) urinary extracts inhibited non-competitively the liver S9-mediated mutagenicity of PhIP as did quercetin, used as a model phenolics. Several structural features of the flavonoids were identified as necessary for the inhibition of PhIP and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline mutagenicity. Fractionation by reverse-phase HPLC and subsequent analysis of two urinary extracts, showed the presence of several anti-mutagenic substances and phenolics; more lipophilic phenolics displayed the highest specific inhibitory activity. This suggests that enzymatic conversion of dietary flavonoids into their more lipophilic and anti-mutagenic O-methylcatechol derivatives, as noted for quercetin, may occur in vivo in man. Onion, lettuce, apples and red wine are important sources of dietary flavonoids which are probably responsible for the anti-mutagenicity associated with foods and beverages. After HPLC fractionation of urinary extracts, the distribution profile of anti-mutagenic activity corresponded roughly to that of onion and wine extract combined. Our study strongly suggests that smokersingesting dietary phenolics, probably flavonoids, are partially protected against the harmful effects by tobacco carcinogens within their bladder mucosal cells. This protective effect of dietary phenolics against the cancer of the bladder (and possibly other sites) should be verified and explored as a part of a chemoprevention strategy.

Introduction

Tobacco smoking causes a major fraction of urinary bladder cancer in humans (1). Previous studies have implicated primary aromatic and possibly heterocyclic amines as bladder carcinogens which are present in tobacco smoke (2). We have shown human urine from both smokers and non-smokers to contain substances that strongly inhibit the bacterial mutagenicity of aromatic and heterocyclic amines in vitro (3). The current report presents data on the characterization of the chemical nature and mode of action of these urinary anti-mutagens. The biological relevance was assessed by examining the relationship between the levels of tobacco-related DNA adducts in exfoliated urothelial cells and the anti-mutagenic activity in the 24-h urine of smoking volunteers. Based on the results, the role of various naturally occurring anti-mutagens in human diet is discussed.

Materials and methods

Materials

Bond Elut C-18 cartridges used to prepare urinary extracts were produced by Varian. Harbor City, CA. Phenolics were purchased from Extrasyntese, Lyon, France. β-glucuronidase (from E. coli type IX) were purchased from Sigma Chemical Company, St Louis, MO. Arylsulfatase (from Helix pomatia) was purchased from Boehringer-Mannheim, Germany. All other solvents and reagents were of analytical purity.

Preparation of urine extracts

Twenty-four-hour urine samples from healthy male blood donors were collected in polyethylene bottles which were stored at 4°C during the collection period. Part of each sample was used to prepare exfoliated cells as previously described (4). The remaining urine samples were stored for up to 4 years at -30°C. Samples of working-day urine from healthy male and female volunteers from IARC laboratories were also collected and stored, as described above, and analysed within 1-3 weeks. Nicotine, cotinine and creatinine in urine samples were determined as described (5). The urine extracts were prepared using Bond Elut cartridges containing 500 mg C-18 sorbent according to a procedure established in our laboratory. Extractions were performed as follows: cartridges were first rinsed with 24 ml of a methanol/water mixture (50:50) and 40 ml of deionized water, then aliquots of urine samples containing 3 mg creatinine were applied, after adjustment, to 4 ml with deionized water. Cartridges were washed with 20 ml deionized water. Organic materials
Methane followed by acetone (5). After evaporation of the solvent, the residues were dissolved in 500 µl of DMSO and stored at -70°C for not more than 1 week. Some extracts were also prepared according to a procedure described previously by passing 200 ml of urine aliquots through a column of XAD-2 resin and elution with dichloromethane followed by acetone (5). After evaporation of the solvent, the residues were dissolved in 500 µl of DMSO and stored at -70°C for not more than 1 month.

DNA isolation and measurement of tobacco-related carcinogen-DNA adducts. DNA was extracted from exfoliated urothelial cells, hydrolyzed, [32P]post-labelled and carcinogen-DNA adduct levels measured as described (4).

Salmonella mutagenicity assay
Salmonella typhimurium TA98 strain was supplied by B.N. Ames (Berkeley, CA). Using a modified liquid incubation assay described by Malaveille et al. (5), the mutagenic activities were measured in Salmonella in the presence of 2.5 µl Aroclor 1254-treated male BDVI rat liver S9 (final volume 220 µl). Incubations were carried out for up to 45 min at 37°C with shaking. The inhibitory effect of each urine extract on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP*) mutagenicity was measured in assays containing 20 ng of PhIP (added as 10 µl 25% DMSO aqueous solution) and up to 10 µl DMSO solution of urine extract (5.7% final DMSO concentration in each assay). The inhibitory effect of each phenolics/high-performance liquid chromatography (HPLC) fraction/Bond-Elut C-18 extract of wine and aqueous food extracts (added as 10 µl DMSO solutions) on the mutagenicity of PhIP (20 ng) or 2-amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQx) (1 ng) was measured as indicated above. The inhibitory effects were expressed as either percentage decrease in revertants per plate per concentration/fraction assayed or as decrease in revertants per ml urine equivalent calculated from the linear part of inhibitory curves.

Assay for cytotoxic activity in Escherichia coli PQ 37
Escherichia coli PQ 37 strain was supplied by P. Quillardet (Paris, France). The SOS chromotest (6) was carried out with a metabolic activation system identical to that used for the Salmonella liquid incubation assay. Enzyme activity (indicating surviving fraction) was measured as described, after incubation with a DMSO solution of the urine extract or the phenolics and PhIP in concentrations identical to those used in the Salmonella assays (5).

Fig. 1. (A, B) Levels of 4-aminobiphenyl adducts and of tobacco-related adducts of DNA from exfoliated urothelial cells of smokers (adjusted for tobacco exposure) versus the anti-mutagenic activity of the corresponding urine extract, using PhIP as representative heterocyclic amine. Relative adduct labelings of DNA were taken from Talaska et al. (8). The anti-mutagenic activities were calculated from the linear part (comprising 2–4 concentrations) of the curve of the inhibition of the liver S9-mediated mutagenicity in S. typhimurium TA98 of PhIP (20 ng per assay) as a function of increasing concentration of the urinary extract (DMSO solution). Inhibitory curves were plotted using results from one or two series of duplicate experiments each involving four concentrations. The mutagenicity of 20 ng PhIP after 45 min liquid incubation in the absence of inhibitor ranged from 665 to 880 revertants per plate for nine series of experiments, each involving two or three duplicate determinations. The mean value ± SE was 770 ± 30.

DNA adduct labelling and carcinogen-DNA adduct levels measured as described (4).

Fig. 2. Relationship between the anti-mutagenic activity of urine extracts (using PhIP as mutagen) and their content in phenolic substances (r = 0.58, P < 0.02; n = 18). The anti-mutagenic activities were calculated as described in the legend to Figure 1. The mutagenicity of 20 ng PhIP after 45 min liquid incubation in the absence of inhibitor ranged from 665 to 1100 revertants per plate for 15 series of experiments each involving two or three duplicate determinations, the mean value ± SE was 800 ± 30.

Enzymatic deconjugation of urinary glucuronides and sulfates
An aliquot of 24-h urine containing 3 mg creatinine (1.9–3.3 ml depending on the sample) was mixed with 1 ml 0.5 M Sorenson citrate buffer, pH 6, with or without up to 40 000 Roy units of arylsulfatase and 5000 Fishman units of β-glucuronidase. The final volume was adjusted to 6 ml with deionized water. The mixture was flushed for 30 s with nitrogen, incubated for 1 h at 37°C and extracted using a Bond Elut C-18 cartridge as described above for urine; the residue was taken up in 50 µl DMSO and stored at -70°C for not more than 1 week.

Spectrophotometric analysis of phenolics in urinary and food/beverage extracts
The amount of phenolics in DMSO solutions of extract or concentrate of urine, wine, HPLC fractions and aqueous food extracts, was measured by analysing the total phenol concentration by the Folin-Ciocalteu procedure of Singleton and Rossi (7) using gallic acid for the calibration curve. Briefly, 10 µl of DMSO solution of extract/concentrate or gallic acid were mixed with 190 µl deionized water, 1 ml of a 10-fold diluted aqueous Polin-Cicalete
Table I. Chemical structures of plant phenols assayed as anti-mutagens

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<tr>
<th>Chemical class/name</th>
<th>Substituent at position</th>
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<tr>
<td></td>
<td>3</td>
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<tr>
<td>Flavone</td>
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<td>luteolin</td>
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<td>diosmetin</td>
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<td>Flavonol</td>
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<td>quercetin</td>
<td>OH</td>
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<tr>
<td>isorhamnetin</td>
<td>OH</td>
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<tr>
<td>kaempferol</td>
<td>OH</td>
</tr>
<tr>
<td>quercitrin</td>
<td>O-Rham</td>
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<tr>
<td>Isoflavone</td>
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<tr>
<td>genistein</td>
<td>-</td>
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<tr>
<td>daidzein</td>
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<td>Flavanone</td>
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<td>naringenin</td>
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O-Rham, O-rhamnoglucoside.

Results

To investigate whether urinary anti-mutagens can reduce DNA adduction in the urinary bladder of smokers, arising from exposure to aromatic (and possibly heterocyclic) amines, we compared urinary anti-mutagenicity measured in 24-h urine samples of 10 smoking volunteers with the level of DNA adducts in exfoliated cells isolated from the same urine. The mean relative adduct labelling (RAL) of DNA from these urothelial cell preparations and the association with tobacco smoking have been reported previously (4,8). DNA adducts were detected in 10 out of 18 samples from smokers (4). Three adducts were related to cigarette smoking, one being the putative \( \text{A/-(deoxyguanosine-8-yl)-4-aminobiphenyl (ABP-dG) adduct, and thus were considered for our investigations. The level of the putative ABP-dG-adduct and the total of the three tobacco-related adducts were found to be linearly related to the number of cigarettes smoked per day. They were therefore expressed per the sum of nicotine plus cotinine contents in 24-h urine, to adjust for tobacco smoking. Both ABP-dG and total DNA adducts were found to be inversely related to} \)
the urine extracts contained phenolics, at concentrations of S. typhimurium TA98. All concentrations were then compared to the urinary antimutagenicity were linearly related, showing statistical significance (r = -0.81, P < 0.02, n = 18; Figure 1). One sample (not shown) exhibited antimutagenic activity about six times greater than that predicted from its content in phenolics.

In order to examine further the role of plant phenolics as urinary anti-mutagens, we assayed a series of representative flavonoids and other plant phenols to determine whether their inhibitory concentrations match those of the phenolics in urinary extracts. The chemical class, name and structure of representative naturally occurring phenolics are given in Table I. Inhibitory effects of various plant phenols on the liver S9-mediated mutagenicity of two heterocyclic amines, MelQx and PhIP are presented in Table II. The absence of bacterial toxicity by phenolics, which might interfere with their anti-mutagenicity, was verified by using SOS chromotest. This sensitive test for cytotoxicity, carried out under experimental conditions identical to those used for the Salmonella assay, showed that at the concentration range used the phenolics did not decrease bacterial survival, (expressed as alkaline phosphatase activity, see Materials and of about 10. A statistically significant linear relationship was found between the anti-mutagenicity of urine extracts and their content in phenolic substances (r = 0.58, P < 0.02, n = 18; Figure 2). One sample (not shown) exhibited anti-mutagenic activity about six times greater than that predicted from its content in phenolics.

bacterial anti-mutagenicity, expressed as the decrease in the number of revertants of S. typhimurium TA98 per ml urine equivalent. The latter was measured in liquid incubation assays, using PhIP as a representative mutagen for tobacco smoke-related heterocyclic amines. The logarithms of DNA adduct level versus anti-mutagenicity were linearly related, showing statistical significance (r = -0.81, P < 0.01 for both adduct measurements; Figure 1).

Since urinary excretion of plant phenolics, the human daily intake of which is up to 1 g (9,10), (i) has been reported (11,12) and (ii) some of them have been shown to inhibit the mutagenicity of various aromatic and heterocyclic amines (13-16) the amount of phenolics was measured in 19 human urine extracts using a spectrophotometric assay (7). The concentrations were then compared to the urinary anti-mutagenicity, using PhIP as mutagen in S. typhimurium TA98. All the urine extracts contained phenolics, at concentrations of 3.7-12.5 µg per 10 µl dimethylsulfoxide (DMSO) solution of extracts and had anti-mutagenic activities varying by a factor...
Dietary phenolics are partly excreted in urine as glucuronides and sulfates (11,12). Two urine samples were therefore treated with β-glucuronidase and arylsulfatase before the preparation of extracts. The effect of deconjugation on the anti-mutagenic activity of urine extracts and the liberation of free phenolics is shown in Figure 3A–C. After enzymatic treatment, anti-mutagenicity increased maximally 11-fold (based on the linear part of the dose–response curves Figure 3A, B) and the level of phenolics 2.5-fold. Based on combined results from two extracts, the increase in anti-mutagenicity was linearly related to the concentration of unconjugated phenolics (Figure 3C). These data strongly suggest that these free phenolic substances act as inhibitors of liver S9-mediated mutagenicity of PhIP.

To gain further evidence for the role of phenolics as urinary anti-mutagens, the mode of inhibition of liver S9-mediated mutagenicity of PhIP was determined for three urinary extracts and compared with that of quercetin. As inferred from double reciprocal plots (Figure 4), both the extracts and the pure flavonoid, quercetin, inhibited the mutagenicity of PhIP in a non-competitive fashion.

In order to identify the origin of anti-mutagenic substances in urinary extracts, chromatographic separations were performed using conditions reported to be efficient for separation of flavonoids (17,18). When two urinary extracts were subjected to reverse-phase HPLC, the UV absorption profiles revealed a complex elution pattern (Figure 5A, B). Subsequently, six fractions, covering the entire chromatographic profile, were analysed. All contained substances that inhibited liver S9 mutagenicity of PhIP, the most active fractions being 1 and 5. However, when the anti-mutagenicity of the fractions was expressed as a percentage inhibition per μg phenolics, fraction 6 in both urine extracts exhibited the highest specific inhibitory activity.

Onion, lettuce, apple and red wine are important dietary sources of flavonoids (9). Crude water extracts and wine (see Materials and methods) exhibited anti-mutagenic activity (Table III). When the percentage inhibition of liver S9-mediated mutagenicity of PhIP was expressed per 10 mg wet weight or...
was found between the inhibition by urine extracts of PhIP and the liver S9-mediated mutagenicity of these urinary putative bladder carcinogens. Extracts of 24-h human urines (stored at -30°C) that were collected for this multicentre investigation were shown to contain substances that strongly inhibited the mutagenicity in vitro and the level of two DNA adduct measurements: one concerns the putatively identified ABP-dG adduct and the second, the total of tobacco-smoke-related carcinogen adducts, including those probably derived from PhIP. Urinary anti-mutagenicity in vitro thus appears to be a good indicator of the anti-genotoxicity exerted by substances excreted in urine that protect the bladder mucosal cells (and possibly other cells) against DNA damage due to substances derived from tobacco smoke. Since extracts were prepared from urines of subjects who were also coffee drinkers, we first suspected caffeine and paraxanthine (a demethylated caffeine metabolite) as inhibitors. Both are known to be substrates for cytochrome P450 IA2-mediated reactions, as are aromatic and heterocyclic amines (19,20). Caffeine has been reported to inhibit the mutagenicity of various heterocyclic amines in Salmonella (15,21,22) However, analysis of urine extracts by HPLC revealed that the concentrations of these methylxanthines in urinary extracts were far too low to account for their inhibitory effect on PhIP mutagenicity, as reported previously (3). An inhibitory effect of unsaturated fatty acids on the mutagenicity of heterocyclic amines and other carcinogens has been reported (23) and their presence in human urine is well documented (24). We therefore measured the concentration of the most abundant, linoleic and oleic acid, in urine extracts by gas chromatography [after methylation, as described by Hayatsu et al. (25)]. The results (not shown) indicated again that the concentrations of these fatty acids were too low to account for the observed anti-mutagenicity.

We then focused on phenolics because (i) human dietary intake of flavonoids is up to 1 g/day (9,10), (ii) they are partly excreted in urine (12,26), and (iii) some had shown to inhibit the mutagenicity of aromatic and heterocyclic amines (13–15). The following pieces of experimental evidence suggest that much of the urinary anti-mutagenicity is attributable to dietary phenolics and their metabolites: (i) the anti-mutagenic activity found in human urine extracts (n = 18) was linearly related to their content in phenolics; (ii) the level of these substances in urine extracts (Figure 2) is similar to the concentration ranges of various plant phenols (quercetin, isorhamnetin and naringenin) for which an inhibitory effect on liver S9-mediated mutagenicity of PhIP was demonstrated (Table II); (iii) treatment of urine with β-glucuronidase and arylsulfatase enhanced both anti-mutagenicity of urinary extracts and their contents in phenolics, with a linear relationship between these two variables (Figure 3C); and (iv) three urinary extracts inhibited liver S9-mediated mutagenicity of PhIP in a non-competitive fashion, as did quercetin, used as a model compound (Figure 5).

Discussion

The present work was conducted as a part of a series of molecular dosimetry and biochemical epidemiological studies with the aim of elucidating the mechanisms involved in tobacco smoking-related bladder cancer [reviewed in (2)]. The previous studies have reinforced the association between cigarette smoking and carcinogen-DNA adducts in exfoliated urothelial cells, and have implicated aromatic and possibly heterocyclic amines as bladder carcinogens. Extracts of 24-h human urines (stored at -30°C) that were collected for this multicentre investigation were shown to contain substances that strongly inhibited the liver S9-mediated mutagenicity of these urinary putative bladder carcinogens in bacteria. The inhibitory effect was not related to bacterial cytotoxicity and was similar in urine extracts from smokers and non-smokers, suggesting a dietary origin. The biological relevance of these bacterial anti-mutagens was examined by comparing the levels of tobacco-related DNA adducts in exfoliated urothelial cells from smoking volunteers (4) with the anti-mutagenic activity in the corresponding 24-h urine samples. A strong inverse relationship was found between the inhibition by urine extracts of PhIP and the level of two DNA adduct measurements: one concerns the putatively identified ABP-dG adduct and the second, the total of tobacco-smoke-related carcinogen adducts, including those probably derived from PhIP. Urinary anti-mutagenicity in vitro thus appears to be a good indicator of the anti-genotoxicity exerted by substances excreted in urine that protect the bladder mucosal cells (and possibly other cells) against DNA damage due to substances derived from tobacco smoke. Since extracts were prepared from urines of subjects who were also coffee drinkers, we first suspected caffeine and paraxanthine (a demethylated caffeine metabolite) as inhibitors. Both are known to be substrates for cytochrome P450 IA2-mediated reactions, as are aromatic and heterocyclic amines (19,20). Caffeine has been reported to inhibit the mutagenicity of various heterocyclic amines in Salmonella (15,21,22) However, analysis of urine extracts by HPLC revealed that the concentrations of these methylxanthines in urinary extracts were far too low to account for their inhibitory effect on PhIP mutagenicity, as reported previously (3). An inhibitory effect of unsaturated fatty acids on the mutagenicity of heterocyclic amines and other carcinogens has been reported (23) and their presence in human urine is well documented (24). We therefore measured the concentration of the most abundant, linoleic and oleic acid, in urine extracts by gas chromatography [after methylation, as described by Hayatsu et al. (25)]. The results (not shown) indicated again that the concentrations of these fatty acids were too low to account for the observed anti-mutagenicity.

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liver microsomes. This suggests that genistein does not inhibit P450 IA2-mediated activation of heterocyclic amines, while flavonoids inhibit P450 IA1, P450 3A4 and P450 IA2 that metabolize polycyclic aromatic hydrocarbons (19,20,28). None of the phenolics tested (except quercetin at the highest concentration assayed) exhibited mutagenic activity at concentrations required for the inhibition of liver S9-mediated mutagenicity of PhIP and MelQx. The mutagenicity of flavonoids carrying a catechol moiety (e.g. quercetin) has been attributed to metabolic activation by redox cycling involving these catechols and their corresponding quinones (29). These phenolics are rapidly O-methylated by catechol-O-methyltransferase (COMT) in vitro and in vivo into non-mutagenic methoxy derivatives (30). Interestingly, we found thatisorhamnetin, the 3’-O-methyl derivative of quercetin, is a much stronger inhibitor of PhIP mutagenicity than quercetin itself (see Table II), as reported previously for other heterocyclic amines (14). This O-methylation has been proposed as a reason for the absence of toxicity of these compounds in vivo (30). O-Methylation renders them more lipophilic than the parent flavonoid, allowing a stronger interactions with cytochrome P450 within microsomal membranes. Published data on the mechanism(s) by which flavonoids inhibit rat liver monooxygenase indicating that a combination of electrostatic and lipophilic interactions are required for membranal transport processes and the binding of flavanones to cytochrome P450 are compatible with this notion (31).

Analysis of two urinary extracts fractionated by reverse-phase HPLC indicates that all six fractions contained antimitagenic substances and phenolics (Figure 5A, B). Fraction 6, containing the most lipophilic substances, displayed the highest inhibitory activity when expressed per µg phenolics. It is likely that in the human body, dietary flavonoids are enzymatically converted into O-methylcatechols, which are both more lipophilic and, thus, more anti-mutagenic than the parent compounds, as noted for quercetin. Onion, lettuce, apple and red wine are important dietary sources of flavonoids (9) and these substances are probably responsible for the anti- mutagenicity observed in their extracts (Table III). The distribution of anti-mutagenic activity in the HPLC fractions of urinary extracts corresponds roughly to a combination of inhibitors present in onion and wine extracts (Figure 6A, B versus Figure 5A, B), suggesting that these phenolics and their metabolites comprise a major part of the complex mixture of urinary anti-

Dietary phenolics against cancers in humans as a part of a cancer chemoprevention strategy.

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