Exceptional Activity of Tannic Acid among Naturally Occurring Plant Phenols in Protecting 7,12-Dimethylbenz(a)anthracene-, Benzo(a)pyrene-, 3-Methylcholanthrene-, and N-Methyl-N-nitrosourea-induced Skin Tumorigenesis in Mice

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ABSTRACT

Our recent studies have shown that naturally occurring dietary plant phenols such as tannic acid, quercetin, myricetin, and anthraflavic acid are capable of inhibiting polymeric aromatic hydrocarbon (PAH) metabolism and subsequent PAH-DNA adduct formation in epidermis of SENCAR mice (M. Das, et al., Cancer Res., 47: 760–766, 1987, and 47: 767–773, 1987). In this study these plant phenols were tested for their effects against PAHs and N-methyl-N-nitrosourea-induced skin tumorigenesis in mice. Each plant phenol was evaluated as a possible anticarcinogen in an initiation and promotion and a complete skin tumorigenesis protocol. In the two-stage tumor protocol in SENCAR mice using 7,12-dimethylbenz(a)anthracene, benzo(a)pyrene, and N-methyl-N-nitrosourea as the initiating agent followed by twice weekly applications of 12-O-tetradecanoylphorbol-13-acetate as tumor promoter each plant phenol afforded significant protection against skin tumorigenicity. The protective effects were verified both by prolongation of latency period and by subsequent tumor development. In the complete carcinogenesis protocol in BALB/c mice using 3-methylcholanthrene as a tumorigen the applications of each of the plant phenols 30 min prior to each PAH application afforded significant protection by delaying the onset and the subsequent development of skin tumors. Our results suggest that these plant phenols have substantial though variable potential for modifying the risk of skin tumorigenicity induced by a wide variety of chemicals and of these tannic acid was shown to have maximal chemoprotective effects.

INTRODUCTION

It is now widely accepted that the initiation of carcinogenesis by PAHs relates to the metabolic activation of the parent compound into highly reactive ultimate carcinogenic metabolites which covalently bind to DNA (1–5). This knowledge has led to a search for nontoxic inhibitors of these metabolic processes. For example there has been growing interest in the identification of naturally occurring dietary factors as potential anticarcinogens (6–9). The identification and characterization of such dietary compounds and the definition of their antitumor effects could lead to important strategies for reducing the risk of human cancer (10, 11).

In the recent past several naturally occurring plant phenols including tannic acid were shown to inhibit the mutagenicity of bey-region diol-epoxides of PAHs in the Ames mutagen assay using Salmonella typhimurium and in Chinese hamster V79 cells (8, 9, 12). Our recent studies have shown that plant phenols such as tannic acid, quercetin, myricetin, and anthraflavic acid were potent inhibitors of epidermal monooxygenases, BP metabolism, and the binding of [3H]BP, [3H]BP-7,8-diol, and [3H]-DMBA to epidermal DNA in SENCAR mice (13, 14). Our studies also indicate that topical application of each of these plant phenols inhibits in vivo epidermal and pulmonary BPDE-1-deoxyguanosine adduct formation in [3H]BP-treated SENCAR mice (14). It was therefore of interest to evaluate the anticarcinogenic activity of these polyphenols. We report that tannic acid, quercetin, myricetin, and anthraflavic acid also exhibit variable degrees of protection against DMBA-, BP-, MCA-, and MNU-induced skin tumorigenesis in mice.

MATERIALS AND METHODS

Chemicals. Tannic acid, myricetin, anthraflavic acid, DMBA, and BP were obtained from Aldrich Chemical Co., Milwaukee, WI. Quercetin, MCA, and TPA were purchased from Sigma Chemical Co., St. Louis, MO. MNU was a product of K&K Rare and Fine Chemicals. All other chemicals were obtained in the purest form commercially available.

Animals. Six-wk-old female SENCAR mice were obtained from National Cancer Institute-Frederick Cancer Research Facility, Bethesda, MD. Female BALB/c mice, aged 6 wk, were obtained from Charles River Laboratory (Wilmington, MA). The mice were shaved with electric clippers and Nair depilatory was applied 1 day prior to the beginning of the experiment. Only those mice that were not in the hair regrowth cycle were selected for studies.

Effect of Plant Phenols in an Initiation and Promotion Protocol. Four hundred SENCAR mice were divided into four groups of 100 animals each and 20 animals from each group were topically treated with a single application of acetone, tannic acid, quercetin, myricetin, or anthraflavic acid (200 µg/animal in 0.2 ml acetone) daily for 7 consecutive days. At these test concentrations each plant phenol was soluble and the solutions were prepared fresh daily and were applied within 2 h of preparation. Twenty-four h after the last treatment with the plant phenols animals from each group received a single topical application of either DMBA (40 nmol), BP (400 nmol), MNU (20 µmol), or acetone (control) as the initiating agent. The dosages of PAHs used are standard for skin tumor induction in SENCAR mice as described earlier (15) while the MNU dose was chosen based on preliminary experiments and as described by Waynforth and Magee (16). Seven days following the single initiating dose of PAHs or MNU each animal then received TPA (3.24 nmol) twice weekly. The experiment was terminated only after 100% of the animals in each treatment group developed neoplasms. Skin tumor formation was recovered weekly and tumors greater than 1 mm in diameter were included in the cumulative total only if they persisted for 2 wk or more. No skin neoplasms occurred in any mice treated with acetone alone or with plant phenol alone. The latent periods were computed by the method of Shimkin and Andervont (17) as described earlier (18). Statistical significance was determined by a single-tailed Student's t test.

Effect of Plant Phenols in a Complete Carcinogenesis Protocol. Two hundred BALB/c mice were divided into five groups of 40 each.

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Animals in group 1 received skin applications of 0.2 ml of acetone daily for seven days whereas the mice of the remaining group received daily skin applications of 3.0 μmol per mouse of either tannic acid, quercetin, anthraflavic acid, or 0.75 μmol per mouse of myricetin, in 0.2 ml of acetone. After this pretreatment regimen 20 animals from each group received twice-weekly applications of the plant phenol followed 30 min later by 1.5 μmol of MCA in 0.2 ml of acetone whereas the remaining mice of each group received only plant phenol in acetone. This treatment was repeated in each group for 16 wk at which time the experiment was terminated. Skin tumor formation was recorded weekly, and tumors greater than 1 mm in diameter were included in the cumulative total only if they persisted for 2 wk or more. No skin neoplasm occurred in any mice treated with acetone alone or plant phenols alone.

RESULTS

Effect of Topical Applications of Plant Phenols on DMBA-induced Skin Tumorigenesis. The effect of pretreatment with plant phenols on skin tumor induction in DMBA-initiated and TPA-promoted SENCAR mice is shown in Fig. 1. The animals pretreated with each plant phenol showed considerable prolongation of the latent period prior to the onset of tumor development. The first tumor appeared after 6, 5, 5, and 4 wk of treatment with DMBA in the tannic acid-, quercetin-, myricetin-, and anthraflavic acid-pretreated animals, respectively, as compared to 3 wk in the corresponding control group without plant phenol pretreatment. At the termination of the experiment after 12 wk the cumulative number of tumors in 20 mice in the non-plant phenol-pretreated group was 680, whereas only 351, 368, 302, and 409 tumors were recorded in the groups pretreated with tannic acid, quercetin, myricetin, and anthraflavic acid, respectively (Fig. 1A). After 6 wk of topical application of DMBA only 30, 55, 25, and 55% of the mice in the tannic acid-, quercetin-, myricetin-, and anthraflavic acid-pretreated groups demonstrated any neoplasm as compared to 100% of the animals in the control group without plant phenol pretreatment (Fig. 1B). The data in Table 1 show skin tumors per mouse as a function of the number of weeks on test. At each time point highly significant protection against skin tumor formation was evident in animals pretreated with each plant phenol. By 12 wk of testing, the number of tumors per mouse in the non-plant phenol-pretreated group was 34.00, whereas the corresponding numbers in the groups pretreated with tannic acid, quercetin, myricetin, and anthraflavic acid were 17.55, 18.40, 15.10, and 20.45, respectively.

Effect of Topical Applications of Plant Phenols on BP-induced Skin Tumorigenesis. The data shown in Fig. 2 represent the effect of pretreatment with plant phenols on the cumulative number of tumors and percentage of mice with tumors in BP-initiated and TPA-promoted SENCAR mice. The animals pretreated with each plant phenol developed significantly fewer skin tumors and the latent period prior to the onset of tumor development was also considerably prolonged in these animals. The first tumor appeared after 9, 8, 8, and 7 wk of treatment with BP in the tannic acid-, quercetin-, myricetin-, and anthraflavic acid-pretreated animals, respectively, as compared to 5 wk in the corresponding control group without plant phenol pretreatment. At the termination of the experiment after 14 wk the cumulative number of tumors in 20 mice in the non-plant phenol-pretreated group was 243, whereas only 81, 124, 127, and 136 tumors were registered in the tannic acid-, quercetin-, myricetin-, and anthraflavic acid-pretreated groups, respectively (Fig. 2A). After 10 wk of test 40, 65, 70, and 95% of the mice in the tannic acid-, quercetin-, myricetin-, and anthraflavic acid-pretreated groups demonstrated neoplasms as compared to 100% of the animals in control group without plant phenol pretreatment (Fig. 2B). The data in Table 1 represent skin tumors per mouse as a function of the number of weeks on test. At each time point highly significant protection against BP-induced skin tumor formation was evident in animals pretreated with each plant phenol. By 12 wk of testing, the number of tumors per mouse in the non-plant phenol-pretreated group was 10.90, whereas the corresponding numbers in the groups pretreated with tannic acid, quercetin, myricetin, and anthraflavic acid were 2.90, 4.75, 5.25, and 5.60, respectively.

Effect of Topical Applications of Plant Phenols on MNU-induced Skin Tumorigenesis. Fig. 3 shows the effect of pretreatment with plant phenols on cumulative number of tumors and percentage of mice with tumors in MNU-initiated and TPA-promoted SENCAR mice. The animals pretreated with each plant phenol developed significantly fewer skin tumors and the latent period prior to the onset of tumor development was also considerably prolonged in these animals. The first tumor ap-
Table 1 Effect of topical application of plant phenols on DMBA-, BP-, and MNU-induced skin tumorigenesis in SENCAR mice

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Carcinogen used</th>
<th>Tumors/mouse</th>
<th>6 wk</th>
<th>9 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>Tannic acid</td>
<td>DMBA</td>
<td>12.25±2.10</td>
<td>32.10±3.18</td>
<td>34.00±2.73</td>
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<td></td>
<td>Quercetin</td>
<td>DMBA</td>
<td>1.50±0.55</td>
<td>11.50±2.38</td>
<td>17.55±1.45</td>
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<td></td>
<td>Myricetin</td>
<td>DMBA</td>
<td>1.90±0.49</td>
<td>14.20±2.40</td>
<td>18.40±1.45</td>
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<tr>
<td></td>
<td>Anthraflavic acid</td>
<td>DMBA</td>
<td>0.85±0.35</td>
<td>10.95±2.18</td>
<td>15.10±2.04</td>
</tr>
<tr>
<td></td>
<td>Solvent</td>
<td>DMBA</td>
<td>4.05±1.17</td>
<td>16.95±2.66</td>
<td>20.45±1.99</td>
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Experiment 2

<table>
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<th>Pretreatment</th>
<th>Carcinogen used</th>
<th>Tumors/mouse</th>
<th>6 wk</th>
<th>9 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannic acid</td>
<td>BP</td>
<td>0.70±0.18</td>
<td>3.70±0.55</td>
<td>10.90±1.07</td>
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<tr>
<td>Quercetin</td>
<td>BP</td>
<td>0.35±0.15</td>
<td>2.90±0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>BP</td>
<td>NTF</td>
<td>6.05±0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthraflavic acid</td>
<td>BP</td>
<td>0.65±0.24</td>
<td>5.25±1.07</td>
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<td></td>
</tr>
</tbody>
</table>

Experiment 3

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Carcinogen used</th>
<th>Tumors/mouse</th>
<th>6 wk</th>
<th>9 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannic acid</td>
<td>MNU</td>
<td>2.00±0.53</td>
<td>4.80±1.16</td>
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<tr>
<td>Quercetin</td>
<td>MNU</td>
<td>0.35±0.13</td>
<td>1.00±0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myricetin</td>
<td>MNU</td>
<td>0.60±0.18</td>
<td>1.55±0.35</td>
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<tr>
<td>Anthraflavic acid</td>
<td>MNU</td>
<td>0.65±0.20</td>
<td>2.25±0.46</td>
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<td></td>
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</table>

* Statistically significant from the control (P < 0.01) (Student’s t test).
* NTM, no tumor found.

**DISCUSSION**

The naturally occurring plant phenols tannic acid, quercetin, myricetin, and anthraflavic acid are widely distributed in the plant kingdom (19–22) and many of these compounds are ingested in the human diet. It has been estimated that some individuals ingest as much as 1 g of the plant phenols per day in their diet (23). The data in this study demonstrate that the topical application of tannic acid, quercetin, myricetin, and anthraflavic acid affords significant protection against BP-, DMBA-, MNU-, and MCA-induced skin tumorigenesis. In general the order of anticarcinogenic potency of these plant
Phenolic plant constituents have been shown to possess several activities which may influence the tumorigenic effect of certain chemical carcinogens. Some plant phenols have been shown to inhibit the activity of cytochrome P-450-dependent enzymes that metabolize drugs and carcinogens (24, 25). For example, our studies (13) indicated that topical applications of tannic acid, quercetin, myricetin, and anthraflavic acid to SENCAR mice inhibit epidermal PAH metabolism. Some of the plant phenols have been shown to possess antioxidant activity and inhibit the formation of nitrosamines (26, 27). Our recent studies have also shown that tannic acid, quercetin, myricetin, and anthraflavic acid inhibit the binding of [3H]BP, [3H]BP-7,8-diol, and [3H]DMBA to epidermal DNA in SENCAR mice (14). In these studies it was shown that tannic acid was the most potent inhibitor of BPDE-I-deoxyguanosine adduct formation in epidermis and lungs of SENCAR mice (14).

Very few studies have assessed the ability of these plant phenols to protect against the development of tumors in experimental animals. Chang et al. (28) have shown that myricetin, quercetin, and ellagic acid were moderately effective inhibitors of the skin tumorigenic activity of BPDE-I but could not demonstrate statistically significant effects on the tumorigenicity of BP in CD-1 mouse skin. Prior studies have shown that quercetin inhibits the tumorigenicity of BP in mouse skin (29, 30) while some other plant phenols including ferrulic acid and caffeic acid have been reported to inhibit the formation of BP-induced forestomach tumors in the mouse (31). Recent studies have indicated that quercetin inhibits arachidonate-5-lipoxigenase activity in vitro (32) and the tumor-promoting activity of TPA on mouse skin (33). Tannic acid, quercetin, myricetin, and anthraflavic acid have been shown to be highly effective inhibitors of the mutagenicity of BP-7,8-diol-9,10-epoxide-2 (8, 2364)

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**Table 2** Effect of topical application of plant phenols in a complete skin carcinogenesis protocol using MCA in BALB/c mice

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Solvent</th>
<th>Tannic acid</th>
<th>Quercetin</th>
<th>Myricetin</th>
<th>Anthraflavic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumors/mouse</td>
<td>8 wk</td>
<td>12 wk</td>
<td>16 wk</td>
<td>8 wk</td>
<td>12 wk</td>
</tr>
<tr>
<td></td>
<td>0.05 ± 0.05</td>
<td>1.15 ± 0.28</td>
<td>11.61 ± 0.57</td>
<td>NTF*</td>
<td>NTF*</td>
</tr>
<tr>
<td></td>
<td>0.55 ± 0.20</td>
<td>5.00 ± 0.32</td>
<td>6.15 ± 0.32</td>
<td>NTF*</td>
<td>NTF*</td>
</tr>
<tr>
<td></td>
<td>0.40 ± 0.20</td>
<td>6.15 ± 0.32</td>
<td>8.30 ± 0.40</td>
<td>NTF*</td>
<td>NTF*</td>
</tr>
</tbody>
</table>

*NTF, no tumor found.

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* Statistically significant from the control (P < 0.01) (Student's t test).
tumorigenicity by PAHs is entirely related to their inhibitory effect on metabolism of carcinogens and their subsequent binding to DNA, then these plant phenols should have no antimutagenic effect against skin tumorigenicity by chemicals like MNU which do not require metabolism to elicit tumorigenicity. However, our results indicate that all of the plant phenols tested showed significant protection against MNU-induced skin tumorigenicity. MNU is a direct-acting carcinoma whose cancer-causing effect is related to alkylation of DNA and single strand breaks (34–36). Thus, the possible mechanism of protective effect of these plant phenols may be due to an inhibitory effect on the binding of the ultimate carcinogen to target tissue DNA. Teel (37) has shown that the anticarcinogenic and antimutagenic activity of plant phenols, for example, ellagic acid, is due to an interaction of the compound with target tissue DNA which in turn blocks the site(s) of DNA to electrophilic attack by reactive carcinogenic moieties. The studies of Dixit and Gold (38) have shown that ellagic acid can inhibit the activity of the direct-acting mutagen MNU in S. typhimurium TA 100. Furthermore, these workers have reported that the antimutagenic activity of ellagic acid against MNU is due to inhibition in methylation at the O-position of guanine in double stranded DNA (38).

In summary, our results indicate that plant phenols, particularly tannic acid, have antimutagenic activity against PAHs and MNU when applied in vivo and one possible mechanism may be an alteration in target tissue macromolecular binding of reactive carcinogenic species.

ACKNOWLEDGMENTS

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REFERENCES

17. Dixit, R. W. Ellagic acid binding to DNA as a possible mechanism for its antitumor activity of ellagic acid against MNU in S. typhimurium TA 100. Furthermore, these workers have reported that the antimutagenic activity of ellagic acid against MNU is due to inhibition in methylation at the O-position of guanine in double stranded DNA (38).

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