Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation–promotion protocol and identification of procyanidin B5-3′-gallate as the most effective antioxidant constituent

Jifu Zhao1, Jiannong Wang2, Yingjie Chen2 and Rajesh Agarwal1,3,4

1Center for Cancer Causation and Prevention, AMC Cancer Research Center, Denver, CO 80214, USA, 2Shenyang Pharmaceutical University, Shenyang 110015, People’s Republic of China and 3University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver, CO 80262, USA

4To whom correspondence should be addressed at: AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214, USA

Email: agarwalr@amc.org

Procyanidins present in grape seeds are known to exert anti-inflammatory, anti-arthritic and anti-allergic activities, prevent skin aging, scavenge oxygen free radicals and inhibit UV radiation-induced peroxidation activity. Since most of these events are associated with the tumor promotion stage of carcinogenesis, these studies suggest that grape seed polyphenols and the proconanidins present therein could be anticarcinogenic and/or anti-tumor-promoting agents. Therefore, we assessed the anti-tumor-promoting effect of a polyphenolic fraction isolated from grape seeds (GSP) employing the 7,12-dimethylbenz[a]anthracene (DMBA)-initiated and 12-O-tetradecanoylphorbol 13-acetate (TPA)-promoted SENCAR mouse skin two-stage carcinogenesis protocol as a model system. Following tumor initiation with DMBA, topical application of GSP at doses of 0.5 and 1.5 mg/mouse/application to the dorsal initiated mouse skin resulted in a highly significant inhibition of TPA tumor promotion. The observed anti-tumor-promoting effects of GSP were dose dependent and evident in terms of a reduction in tumor incidence (35 and 60% inhibition), tumor multiplicity (61 and 83% inhibition) and tumor volume (67 and 87% inhibition) at both 0.5 and 1.5 mg GSP, respectively. Based on these results, we directed our efforts to separate and identify the individual polyphenols present in GSP and assess their antioxidant activity in terms of inhibition of epidermal lipid peroxidation. Employing HPLC followed by comparison with authentic standards for retention times in HPLC profiles, physiochemical properties and spectral analysis, nine individual polyphenols were identified as catechin, epicatechin, procyanidins B1–B5 and C1 and procyanidin B5-3′-gallate. Five of these individual polyphenols with evident structural differences, namely catechin, procyanidin B2, procyanidin B5, procyanidin C1 and procyanidin B5-3′-gallate, were assessed for antioxidant activity. All of them significantly inhibited epidermal lipid peroxidation, albeit to different levels. A structure–activity relationship study showed that with an increase in the degree of polymerization in polyphenol structure, the inhibitory potential towards lipid peroxidation increased. In addition, the position of linkage between inter-flavan units also influences lipid peroxidation activity; procyanidin isomers with a 4–6 linkage showed stronger inhibitory activity than isomers with a 4–8 linkage. A sharp increase in the inhibition of epidermal lipid peroxidation was also evident when a gallate group was linked at the 3′-hydroxy position of a procyanidin dimer. Procyanidin B5-3′-gallate showed the most potent anti-oxidant activity with an IC50 of 20 µM in an epidermal lipid peroxidation assay. Taken together, for the first time these results show that grape seed polyphenols possess high anti-tumor-promoting activity due to the strong antioxidant effect of procanidins present therein. In summary, grape seed polyphenols in general, and procyanidin B5-3′-gallate in particular, should be studied in more detail to be developed as cancer chemopreventive and/or anticarcinogenic agents.

Introduction

Cancer is a leading cause of mortality world wide (1) and, therefore, a major focus of research has been the chemoprevention of cancer (2,3). This approach is a means of cancer control where the induction of this disease can be totally prevented or the rate of development slowed or reversed partially or substantially by the administration of one or more naturally occurring or synthetic chemical agents (2,3). Fruits, vegetables and common beverages, as well as several herbs and plants with diverse pharmacological properties, have been shown to be rich sources of chemicals with potential for chemoprevention of various cancers (4–12 and references therein). Based on epidemiological data followed by animal studies, or vice versa, many new classes of chemical compounds are being evaluated in clinical trials as chemopreventive agents against several malignancies (2–12). At present, ~30 classes of chemicals with cancer preventive effects have been described; most of them have practical implications in reducing cancer incidence in human populations (13). Among these, naturally occurring polyphenolic antioxidants have received increasing attention in recent years (14–16 and references therein). Employing different long-term experimental tumorigenesis protocols, several studies have demonstrated the cancer preventive effects of polyphenolic antioxidants (14–18). The cancer chemopreventive effects of polyphenolic antioxidants is specifically important since environmental pollutants, radiation, pesticides, certain medications, contaminated water and deep fried and spicy foods and UV radiation, as well as physical stress, exhibit the ability to produce enormous amount of free radicals which cause many diseases, including tumor promotion and cancer (19–23).

Abbreviations: C, catechin; DMBA, 7,12-dimethylbenz[a]anthracene; EC, epicatechin; ECG, epicatechin 3-gallate; EGC, epigallocatechin; EGC, epigallocatechin 3-gallate; GSP, polyphenolic fraction isolated from grape seeds; MDA, malondialdehyde; MS, mass spectra; TBA, thiobarbituric acid; TPA, 12-O-tetradecanoylphorbol 13-acetate.
Grapes are one of the most widely consumed fruits in the world. Grapes are rich in polyphenols and -60–70% of grape polyphenols exist in grape seeds. The grape seed polyphenols are flavan-3-ol derivatives and are colorless in the pure state. Only 4% of grape polyphenols exist in grape pulp. In grape skin there is another type of polyphenol, called anthocyanins, which usually have a purple color and amount to ~30% of total polyphenols in grapes (24). Some of the individual polyphenols in grape seeds are also present in tea, such as catechin (C) and epicatechin (EC) (25), but most of the grape seed polyphenols are quite different from tea polyphenols. Besides EC and C, grape seeds are rich in dimers, trimers and other oligomers of flavan-3-ols (26), whereas most tea polyphenols are monomers, such as C, EC, epigallocatechin 3-gallate (EGCG), epigallocatechin (EGC) and epicatechin 3-gallate (ECG) (25). The combined name for monomers of the derivatives of flavan-3-ol is catechins and for dimers and other oligomers is procyanidins (or proanthocyanidins).

Recent studies have shown that procyanidins in grape seeds possess anti-inflammatory, anti-arthritic and anti-allergic activities and prevent heart disease and skin aging (27). In other studies, grape seed polyphenols have been shown to exert a much stronger oxygen free radical scavenging effect than vitamins C and E (28,29) and to prevent UVC-induced peroxidation (30). Oral administration of grape seed procyanidins at a dose of 2 mg/kg three times daily for 6 days inhibits peroxidation (30). Oral administration of grape seed procyanidins is procyanidins is a source of grape seed polyphenols. For example, in red wine the capillary wall and prevents the increase in capillary permeability caused by local cutaneous application of xylene (31). Oral administration of grape seed polyphenols also significantly improves visual performance in humans (32). In addition to these studies, wine consumption has been reported to have many beneficial health effects (33,34). Wine may also be a source of grape seed polyphenols. For example, in red wine production the alcohol generated from sugars in the grapes can extract some of the grape seed polyphenols into the wine. With regard to cancer chemopreventive effects of grape seed polyphenols, the only study we have come across was an abstract presented at the Annual Meeting of the American Association for Cancer Research showing that oral administration of 1% grape seed extract in the diet inhibits APC mutation-associated intestinal adenoma formation in Min mice (35). Regarding epidemiology, a case–control study showed that increased consumption of grapes is associated with reduced cancer risk (36).

In our continuing efforts to identify and define the cancer chemopreventive and/or anticarcinogenic potential of newer naturally occurring polyphenolic antioxidants (16,37–43) and in the light of the fact that grape seed polyphenols possess strong anti-inflammatory and antioxidant activities, studies were performed to assess the anti-tumor-promoting effect of a polyphenolic fraction isolated from grape seed (hereafter referred to as GSP) on 12-O-tetradecanoylphorbol 13-acetate (TPA) tumor promotion in the 7,12-dimethylbenz[a]anthracene (DMBA)-initiated SENCAR mouse skin two-stage initiation–promotion model. The selection of anti-promotion studies with GSP was based on the reasoning that it is possible a better approach to prevent cancer at the promotion stage since: (i) the promotion stage of multistage carcinogenesis is reversible at an early stage; (ii) the initiation stage of multistage carcinogenesis is irreversible and possibly unavoidable because of our continuous exposure to carcinogenic chemical and physical agents (43–45). It is important to emphasize that carcinogenesis is a multistage process in experimental models and possibly in human cancer induction, development and propagation (44,45). Human malignancies, however, also appear to involve a gradual accumulation of genetic changes over a period of years. Based on the results obtained showing that GSP affords significant protection against tumor promotion and that at much lower doses than those employed in most of anti-tumor-promotion studies in the same model system and under identical conditions, we proceeded to systematically separate, identify and define the antioxidant activity of polyphenols present in GSP. An effort was also made to determine a structure–activity relationship between the polyphenols present in GSP and their antioxidant potential in terms of inhibition of epidermal lipid peroxidation.

Materials and methods

Chemicals and equipment

DMBA and thiochloroactic acid (TBA) were purchased from Sigma Chemical Co. (St Louis, MO). TPA was from LC laboratories (Woburn, MA). All other chemicals and reagents used in the studies were obtained in the purest form available commercially. Proton nuclear magnetic resonance (1H-NMR) and carbon nuclear magnetic resonance (13C-NMR) were recorded on a JEOL GSX 400 spectrometer, with chemical shifts (δ) shown in p.p.m. with tetramethylsilane as internal standard. Mass spectra (MS) were measured on a JEOL JMS-SX 102 mass spectrometer. UV spectra were measured in a Shimadzu UV-260 spectrophotometer. Infrared spectra were recorded in a Perkin-Elmer IR-27G spectrometer. HPLC was performed using a Shimadzu LC-10AD liquid chromatography system equipped with a UV detector (Chelmsford, MA).

Preparation of GSP

Grapes, as large clusters with red berries, were bought from a local supermarket in the autumn and identified as Vitis vinifera. Grape seeds were removed from the grapes, air dried for 1 week and nilled to a particle size of <0.4 mm. The grape seed powder (100 g) was macerated for 12 h at room temperature three times with 800 ml of 100 mM acetate buffer, pH 4.8, in water/acetone (30:70 v/v), each time. The three macerates were combined and concentrated until no acetone was left using a rotary evaporator under reduced pressure and a water bath temperature <35°C. The concentrated solution was extracted four times with 200 ml of ethyl acetate each time. The four ethyl acetate extracts were combined, evaporated to remove ethyl acetate and GSP was obtained as a lyophilized powder. The total polyphenolic content of GSP in terms of catechins and procyanidins was determined by a vanillin–HCl colorimetric method (46) using catechin and procyanidin B2 as authentic standards. Briefly, 2 vol of reagent A (10% w/v vanillin in methanol) and 1 vol of reagent B (35% HCl in water) were mixed to generate reagent C. To 0.4 ml of sample solution in 50% aqueous methanol (10–75 µg/ml w/v) was added 1.25 ml of reagent C and the tubes were left at room temperature for 15 min. The absorbance of the solutions was then read at 500 nm and matched with a standard curve drawn using catechin and procyanidin B2 as authentic standards. The GSP preparation thus obtained contains 95% (w/w) polyphenols in terms of catechins and procyanidins and was highly soluble in 40% aqueous acetone (v/v), methanol, ethanol, acetone and ethyl acetate but insoluble in water and chloroform. This GSP preparation was used in anti-tumor-promotion and lipid peroxidation studies and was further fractionated into individual polyphenols for their identification and bulk preparation as detailed later for other studies.

Animals and anti-tumor-promotion study

Four- to six-week-old female SENCAR mice were purchased from Charles River Laboratories (Kingston, NY). The animals were housed five per cage at 24 ± 2°C and 50 ± 10% relative humidity and subjected to a 12 h light/12 h dark cycle. They were acclimatized for 1 week before use and fed a Purina chow diet and water ad libitum. Prior to the study, the dorsal side of the skin was shaved using electric clippers and mice with the hair cycle in the resting phase were used in the experiment. The animals were randomly divided into five groups of 20 animals each. Mice in groups I–III were treated topically with a single 10 µg/mouse dose of DMBA in 0.1 ml of acetone as tumor initiator on the dorsal shaved skin. One week later, animals in group I were re-treated topically with standard dose of acetone and mice in group II 0.5 and 1.5 mg/mouse doses of GSP per application, respectively, in 0.1 ml of acetone. Thirty minutes following these treatments, animals in groups I–III were treated topically with a 2 µg dose of TPA in 0.1 ml acetone/mouse/
application. The pre-treatment regimen for GSP was based on our studies with other antioxidants showing that their topical application 30 min prior to that of TPA produces the maximum inhibitory effect against TPA tumor promotion in identical protocols (16,25,37,43,47). The TPA alone or two doses of GSP plus TPA treatments were repeated twice per week up to the termination of the experiment at 20 weeks from the start of DMBA. The animals in group IV were treated with 0.1 ml of acetone alone and served as a negative control for any spontaneous tumor induction. To assess whether GSP alone produces tumor promoting effects, the animals in groups V were initiated with a 10 µg dose of DMBA and 1 week later treated with 1.5 mg GSP/mouse/application twice a week up to the end of the experiment at 20 weeks. Animals in all the groups were watched for food and water consumption and any apparent signs of toxicity, such as weight loss or mortality, during the entire period of the study. Skin tumor formation was recorded weekly and tumors >1 mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Latent periods for the onset of tumors in various groups were computed and the tumors were diagnosed histologically at the termination of the experiment. At this point, the total tumor volume on the back of each mouse was also recorded.

**Results**

**Anti-tumor-promoting effect of GSP on TPA tumor promotion**

Topical application of GSP prior to TPA application resulted in a highly significant inhibition of TPA tumor promotion in DMBA-initiated SENCAR mouse skin. The preventive effect of GSP was dose dependent and was evident as a significant reduction in tumor incidence, tumor multiplicity and tumor volume (Figure 1A and B and Table I). In terms of any toxic effects of topical application of GSP, as monitored by weight gain profile, no noticeable difference was observed between the two doses of GSP and non-GSP-treated animals throughout the experiment (Figure 1C). In addition, the mean water consumption per animal per day was also comparable between the two GSP-treated and non-GSP-treated groups of animals (data not shown). Together, these observations suggest that topical application of GSP, at least at the doses employed in the present study, does not produce any apparent toxicity during the entire period of the experiment.

In terms of its anti-tumor-promoting effects, when the data were analyzed for tumor incidence, as shown in Figure 1A, topical application of GSP prior to that of TPA to DMBA-initiated SENCAR mouse skin resulted in a dose-dependent protection throughout the experiment. Compared with the non-GSP-treated positive control group of mice, the time of appearance of the first tumor was delayed by 1 and 2 weeks in the 0.5 and 1.5 mg GSP-treated animals, respectively. When these data were assessed in the middle of the experiment at 10 weeks (Figure 1A), compared with 100% of mice with skin tumors in the non-GSP-treated group, only 35 and 20% of
Table I. Topical application of GSP prior to TPA results in a significant protection against tumor volume (mm$^3$) in TPA tumor promotion in DMBA-initiated SENCAR mouse skin

<table>
<thead>
<tr>
<th>Treatment protocol</th>
<th>Total no. of tumors</th>
<th>Total tumor volume</th>
<th>Tumor volume per tumor-bearing mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA + TPA</td>
<td>384</td>
<td>20736</td>
<td>1037 ± 261</td>
</tr>
<tr>
<td>DMBA + (0.5 mg GSP + TPA)</td>
<td>148 (61.4%)$^a$</td>
<td>6956 (66.5%)</td>
<td>535 ± 103 (48.4%)$^a$</td>
</tr>
<tr>
<td>DMBA + (1.5 mg GSP + TPA)</td>
<td>66 (82.8%)$^a$</td>
<td>2640 (87.3%)</td>
<td>330 ± 83 (68.2%)$^a$</td>
</tr>
</tbody>
</table>

$^a$The data shown in each column are those obtained at the end of the tumor experiment at 20 weeks. The values in parentheses in columns 2–4 represent percent inhibition.

$^b$The doses of DMBA and TPA used were 10 and 2 µg/mouse, respectively. The indicated doses of GSP per mouse were applied topically 30 min prior to TPA in each case.

$^c$Highly significant versus positive control, $P < 0.001$ (Wilcoxon rank sum test).

$^d$Highly significant versus positive control, $P < 0.001$ (Student's t-test).

animals in the 0.5 and 1.5 mg GSP-treated groups, respectively, had tumors, accounting for 65 and 80% inhibition ($P < 0.001$, $\chi^2$ test) of tumor incidence, respectively. In fact, 100% tumor incidence was observed in the positive control group as early as 9 weeks of the protocol. At the termination of the experiment at 20 weeks, with 100% of animals with skin tumors in the positive control group, only 65 and 40% of the animals in the 0.5 and 1.5 mg GSP-treated groups exhibited skin papillomas, accounting for 35 and 65% inhibition ($P < 0.001$, $\chi^2$ test) of tumor incidence, respectively (Figure 1A).

Similarly, when the tumor data were evaluated for tumor multiplicity (cumulative number of tumors per group or number of tumors per mouse), beginning with the first tumor appearance up to termination of the experiment, both the doses of GSP tested showed a highly significant protection against TPA tumor promotion in SENCAR mouse skin (Figure 1B). At the termination of the experiment, compared with 19.2 ± 3.3 (mean ± SD of 20 mice) tumors/mouse in the non-GSP-treated positive control group, only 7.4 ± 2.9 and 3.3 ± 3.3 (mean ± SD of 20 mice in each case) tumors/mouse were observed in the 0.5 and 1.5 mg GSP-treated groups, respectively, accounting for 61 and 83% protection ($P < 0.001$, Wilcoxon rank sum test), respectively (Figure 1B). When the tumor promotion data were analyzed in terms of tumor volume, compared with the positive control group, the total tumor volume and tumor volume per mouse were found to be significantly lower (50–87%, $P < 0.001$, Student's t-test) in the 0.5 and 1.5 mg GSP-treated groups (Table I). However, GSP treatment did not show a significant inhibition of tumor growth, as is evident in a marginal reduction (12 and 26%, $P < 0.1$, Student's t-test) in tumor volume per tumor in the 0.5 and 1.5 mg GSP-treated groups of mice, respectively, compared with the positive control group. The tumors in each group of mice were histologically identified as benign papillomas (data not shown). The animals initiated with DMBA and promoted twice weekly with 1.5 mg GSP were devoid of any skin tumors throughout the experiment (data not shown), suggesting that GSP itself is not a tumor promoter.

Composition of individual polyphenols in GSP

Based on the results showing that GSP affords a highly significant protection against tumor promotion, we focused our efforts on separating and identifying individual polyphenols present in GSP. Efforts were then made to isolate bulk amounts of the individual polyphenols from GSP by semi-preparative HPLC and perform studies to assess their antioxidant potential using the epidermal lipid peroxidation assay. Dry grape seeds (100 g) were extracted as described in Materials and methods, which resulted in 3.1 g (3.1% w/w of dry grape seeds) of total GSP preparation. The polyphenol quantification method (46) showed that this GSP preparation contained 95% polyphenols (w/w). The individual polyphenols were separated by analytical HPLC and then identified, followed by their quantification.

Photodiode array detection has shown that GSP contains C, EC, gallic acid, EGC, dimers of C and/or EC (for example, procyanidin B1 (EC-4β-8-C), procyanidin B2 (EC-4β-8-EC)), procyanidin B3 (C-4α-8-C), procyanidin B4 (C-4α-8-EC) and procyanidin B5 (EC-4β-6-EC), trimers of C and/or EC (for example, procyanidin C1 (EC-4β-8-EC-4β-8-EC), procyanidin T2 (EC-4β-8-EC-4β-8-C) and procyanidin T3 (EC-4β-6-EC-4β-8-EC) and polyphenolic esters of gallate (for example, procyanidin B5-3'-gallate, EGCG, ECG and procyanidin B2 3'-gallate) (26). In the present study, we used UV detection to separate and identify individual polyphenols by HPLC. As shown in Figure 2, using a UV detector with absorbance at 270 nm, the analytical HPLC profile of GSP showed several peaks. To identify these polyphenols, they were collected as individual peaks and lyophilized for analysis. Based on the retention times with authentic compounds and physicochemical properties and spectral evidence, nine of these peaks were identified as procyanidin B3, procyanidin B1, catechin, procyanidin B4, procyanidin B2, epicatechin, procyanidin C1, procyanidin B5-3'-gallate and procyanidin B5 (Figure 2). For example, peak 5 in the HPLC profile (Figure 2), which was an amorphous powder, gave an orange-red color with anisaldehyde–sulfuric acid reagent and a dark-blue color with ferric chloride reagent; these are typical properties of procyanidins (51). In spectral analysis we observed: UV $\lambda_{max}$ nm (logε): 212 (4.8), 230sh (4.5), 281 (3.9); i.r. $\nu_{max}$ cm$^{-1}$: 3350 (OH), 1600, 1500, 1440 (aromatic ring); positive ion FAB-MS m/z: 579 [M+H]$^+$; $^1$H-NMR (methanol-d4) δ: 7.08 [1H, br s], 6.88 [1H, br s], 6.73–6.77 [4H, 5.09–6.05 [3H], 5.05 [1H, br s], 4.92 [1H, br s], 4.62 [1H, br s], 4.26 [1H], 3.90 [1H], 2.91 [1H], 2.78 [1H]; $^{13}$C-NMR (methanol-d4) δ: 155.2, 157.2 [2C], 158.5, 159.1 [2C], 146.4 [2C], 146.5 [2C], 132.8, 133.4, 120.1 [2C], 116.7 [2C], 116.1 [2C], 108.1, 102.1, 101.3, 97.1, 98.0, 98.1, 80.6, 77.9, 74.3, 67.8, 37.7, 30.5. All the spectral data are identical to those of procyanidin B2 (51) and, therefore, we identified peak 5 in the HPLC profile as procyanidin B2. Similar criteria were used for the identification of other individual polyphenols, detailed elsewhere (51). The structures of nine identified individual polyphenols present in GSP are shown in Figure 3 and their total quantities in GSP are listed in Table II. As shown in Table II, so far we have identified 60.2% (w/w) of total polyphenols present in GSP and identification of the remainder is in progress. Peaks 8 and 9 in the HPLC profile (Figure 2), procyanidin B5-3'-gallate and procyanidin B5, respectively, represent a relatively major portion of GSP (Figure 2 and Table II). As can be seen from the data provided in Table II, the amounts of individual polyphenols quantified are not consistent with the peak heights shown in the HPLC profile detected by UV detector (Figure 2). This is due to the fact that the monomers, dimers, trimers and other oligomer forms of C and/or EC have almost the same molar absorbances, but the molecular weight of the
trimer is three times greater than the monomer. This means that the trimer represents three times more weight than the monomer even though it has a comparable UV absorbance. It is important to mention here that the contents of individual polyphenols present in grape seeds may vary depending on the origin, season and climate, however, in all the grape seed extracts the concentration of procyanidins was much higher than that of catechins and/or epicatechins.

Inhibition of epidermal lipid peroxidation by GSP and individual polyphenols isolated from GSP

It has been well documented that oxidative stress caused by tumor promoters contributes largely to their tumor promoting potential (52,53). The phorbol ester TPA has been studied extensively in this regard and has been shown to cause oxidative stress as one of its multifactorial mechanisms of tumor promotion (52–54). If not scavenged and/or balanced...
Inhibitory effect of GSP on epidermal lipid peroxidation and its comparison with vitamin E. Epidermal microsomes were prepared from SENCAR mouse epidermis and a microsomal suspension (2 mg protein) in 0.1 M phosphate buffer, pH 7.4, containing 0.1 mM MgCl₂ was incubated for 1 h at 37°C in the presence of ferric ions (1 mM FeCl₃) and ADP (5 mM) in a total volume of 1 ml. The incubation mixtures were added with acetone alone or varying concentrations of GSP or vitamin E (40, 80 and 120 µg/ml) in acetone. The final concentration of acetone in an incubation mixture did not exceed 4% (v/v). The reaction was terminated with 10% trichloroacetic acid followed by 1.2 ml of 0.5% TBA. The generation of MDA measured at 532 nm absorbance was employed as a marker of lipid peroxidation and calculated using a molar extinction coefficient of 1.56×10⁴/M/cm as detailed in Materials and methods. The data shown as percentage inhibition are from means (± SD of <10%) of three independent assays, each done in triplicate.

### Table II. Composition of individual polyphenols identified in GSP

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Compound</th>
<th>Percent of total GSP (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Procyanidin B3</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>Procyanidin B1</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>Catechin</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>Procyanidin B4</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>Procyanidin B2</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>Epicatechin</td>
<td>6.0</td>
</tr>
<tr>
<td>7</td>
<td>Procyanidin C1</td>
<td>6.0</td>
</tr>
<tr>
<td>8</td>
<td>Procyanidin B5-3'-gallate</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>Procyanidin B5</td>
<td>19</td>
</tr>
<tr>
<td>None</td>
<td>Total polyphenols</td>
<td>60.2</td>
</tr>
</tbody>
</table>

*GSP was subjected to reverse phase analytical HPLC as detailed in Materials and methods.

The identification of each polyphenol listed here is based on their retention time with authentic compounds in an HPLC run and their spectral analyses as detailed in Materials and methods. The quantification of each polyphenol was done using peak area under curve analysis and comparison with standards.

The calculations are based on considering total GSP as 100%.

by cellular antioxidant molecules and enzymes, oxidative stress ultimately leads to a highly reactive oxygen species, the hydroxyl radical, that attacks cellular targets such as DNA and protein as well as lipid-rich membranes (55). The formation of lipid peroxides via the lipid peroxidation process and their inhibition in biological membranes have been explored as a useful system to assess both oxidant and antioxidant activity of endogenous as well as exogenous agents (55,56). In the present study, we used the epidermal lipid peroxidation assay to: (i) evaluate whether the observed inhibitory effects of GSP on tumor promotion are associated with the potent antioxidant activity of GSP; (ii) assess and identify the most potent polyphenolic antioxidant present in GSP and derive a structure–activity relationship in terms of antioxidant activity for different polyphenols present in GSP; (iii) assess the magnitude of antioxidant activity of GSP and individual polyphenols present therein as compared with the well-known antioxidants vitamins C and E.

As shown in Figure 4, in vitro addition of GSP to lipid peroxidation incubation mixtures resulted in a highly significant to complete inhibition of epidermal lipid peroxidation in a dose-dependent manner. When these results were compared with those obtained for the inhibition of epidermal lipid peroxidation by vitamin E, GSP showed much stronger inhibition at all three doses tested (Figure 4). For example, compared with 44% inhibition by vitamin E, GSP showed 65% inhibition (P < 0.001, Student’s t-test) of lipid peroxidation in an epidermal microsomal suspension at 80 µg/ml (Figure 4). A higher concentration of GSP (120 µg/ml) showed complete inhibition (P < 0.0001, Student’s t-test) of epidermal lipid peroxidation, as compared with 70% inhibition by vitamin E (Figure 4).

With regard to individual polyphenols isolated from GSP, we selectively assessed the antioxidant activity of polyphenols based on an evident structural difference. For example, catechin, procyanidin B2, procyanidin B5, procyanidin C1 and procyanidin B5-3’-gallate were evaluated for their inhibitory activity on epidermal lipid peroxidation and compared with vitamins C and E. All these agents showed a highly significant (P < 0.01–0.0001, Student’s t-test) inhibition of epidermal lipid peroxidation in a concentration-dependent manner, albeit to different degrees (Table III). In each case, the observed inhibitory effect of individual polyphenols was much stronger than those with vitamins C and E (Table III). When a structure–activity relationship was sought between individual polyphenols, there was an obvious proportionality between inhibition of lipid peroxidation and the degree of polymerization (Table III). For example, catechin (monomer) < procyanidin B2 (dimer) < procyanidin C1 (trimer). In addition, the position of the linkage between inter-flavan units also influenced lipid peroxidation activity (Table III); 4–6 linkage isomers (e.g. procyanidin B5, structure shown in Figure 3) showed stronger inhibitory activity than 4–8 linkage isomers (e.g. procyanidin B2, structure shown in Figure 3). Moreover, when a gallate group was linked to the 3’-OH of a dimer (e.g. procyanidin B5-3’-gallate), the inhibitory activity against lipid peroxidation increased sharply (Table III). It is important to
Fig. 5. IC$_{50}$ (mM) of individual polyphenols present in GSP and vitamins C and E in the epidermal lipid peroxidation assay. The data shown are those derived from a concentration response tested with each compound and shown in Table III. Each bar represents the mean ± SD of three independent assays in concentration determination studies; each assay was done in triplicate (Table III).

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>IC$_{50}$ (mM)</th>
</tr>
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<tbody>
<tr>
<td>Vitamin C</td>
<td>0.70</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.80</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.60</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>0.50</td>
</tr>
<tr>
<td>Procyanidin B5</td>
<td>0.40</td>
</tr>
<tr>
<td>Procyanidin C1</td>
<td>0.30</td>
</tr>
<tr>
<td>Procyanidin B5-3'gallate</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Discussion

The central finding of the present study is that a polyphenolic fraction isolated from grape seeds affords significant protection against tumor promotion in the mouse skin tumorigenesis model and that this effect of GSP may largely be due to the exceptionally high antioxidant activity of procyanidins present therein. A wide range of studies have shown that naturally occurring polyphenolic antioxidants, specifically those present in fruits and vegetables, common beverages such as green tea and several herbs and plants with diverse pharmacological activities, are promising classes of cancer chemopreventive agents, possibly due to their strong anti-tumor-promoting potential (43,47,57–59). Comparing the anti-tumor-promoting effects of GSP observed in the present study with those published for other agents, it is important to highlight here that the doses of GSP used (0.5 and 1.5 mg) are much lower than those (~3–18 mg) used in other studies, however, the anti-tumor-promoting effects are comparable. When compared with green tea polyphenols and the major component EGCG present therein, GSP contains unique procyanidins which are typical polyphenols with a larger molecular weight than tea polyphenols. Another characteristic of GSP is that it is a mixture of monomers, dimers, trimers and other oligomers of catechin and/or epicatechin, whereas tea polyphenols are only monomers of catechins and epicatechins with gallate substitution (25). It is plausible that dimerization and trimerization of catechins and epicatechins lead to a sharp increase in their anti-tumor-promoting potential, possibly due to a significant increase in their antioxidant activity. This suggestion is supported by our lipid peroxidation findings and the anti-tumor-promoting effects of GSP at much lower doses than those used in the case of green tea polyphenols reported earlier (47,57).

After establishing the cancer chemopreventive effect of GSP in terms of inhibition of tumor promotion, we continued our efforts to identify the individual polyphenols present therein and assess their antioxidant activity. Using reverse phase analytical HPLC, nine pure individual polyphenolic compounds were separated from GSP and their structures were identified by comparing HPLC profiles with standard compounds and interpreting the spectroscopic evidence. Based on the standardization of analytical HPLC conditions, semi-preparative HPLC was used as a more powerful and quick way to separate greater quantities of individual polyphenols in the pure form for further studies. These pure compounds are being used to further investigate the structure–activity relationship in continuing studies on the cancer chemopreventive effects of GSP and the polyphenols present therein. To the best of our knowledge, ours is the first study reporting the chemopreventive effects of GSP on tumor promotion in an experimental model of tumorigenesis and establishing the structure–activity relationship in terms of antioxidant activity of polyphenols present in GSP.

It is widely believed that inhibition of tumor promotion is a better strategy in cancer chemoprevention than inhibition of tumor initiation because initiation is a short irreversible event whereas promotion is a long cumulative process that is reversible during the initial stage (44,45). With the notion that long-term targeting of the tumor promotion stage could be a useful strategy for the prevention of cancer (11), daily consumption of vegetables and fruits has already been associ-
ated with a reduced risk for several human malignancies (4–16). Our results provide additional support for this strategy as well as for the already existing epidemiological and laboratory studies (35,36) reporting that grape seed polyphenols have beneficial effects against several diseases, including cancer. In summary, based on the results shown in the present study, grape seed polyphenols in general, and procyanidin B5-3'-gallate in particular, should be studied in more detail to be developed as cancer chemopreventive and/or anticarcinogenic agents.

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References


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