Flavonoid Constituents of *Chorizanthe diffusa* with Potential Cancer Chemopreventive Activity

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An ethyl acetate-soluble extract of *Chorizanthe diffusa* was found to exhibit significant antioxidant activity, as judged by scavenging stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and inhibition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced free radical formation with cultured HL-60 cells. Bioassay-directed fractionation of this extract using the DPPH antioxidant assay as a monitor led to the isolation of five structurally related flavonoids (**1**−**5**), including the novel compound 5,8,3′,4′,5′-pentahydroxy-3,7-dimethoxyflavone (**1**). Isolates **1**−**5** demonstrated varying degrees of antioxidant or antimutagenic activity. Two of the compounds, 5,7,3′,4′-tetrahydroxy-3-methoxyflavone (**2**) and quercetin (**4**), were subsequently found to inhibit carcinogen-induced preneoplastic lesions in a mouse mammary organ culture model. Inhibitory activity of this type is known to correlate with cancer chemopreventive effects in full-term models of tumorigenesis.

Keywords: *Chorizanthe diffusa; Polygonaceae; flavonoids; 5,8,3′,4′,5′-pentahydroxy-3,7-dimethoxyflavone; antioxidant activity; antimutagenic activity; mouse mammary organ culture; cancer chemoprevention*

INTRODUCTION

Chemoprevention of cancer involves the use of dietary or pharmacological agents to enhance intrinsic physiological mechanisms that protect the organism against the development and progression of transformed cells (Sporn et al., 1976; Wattenberg, 1985). Chemopreventive agents can block initiation of the carcinogenic process, or arrest or reverse further progression of premalignant cells before they become invasive or metastatic, during the extensive latency period that is characteristic of most human cancers (Sporn, 1993). Compounds of this type are classified as either inhibitors of carcinogen formation, blocking agents, or suppressing agents. Most blocking agents are inhibitors of tumor initiation and can be assigned to one or more of five major categories: inhibitors of cytochrome P450 enzymes; inducers of cytochrome P450; inducers of phase II enzymes such as glutathione *S*-transferase (GST), uridine diphosphate (UDP)-glucuronyltransferase, and glutathione peroxidase; scavengers of electrophiles and free radicals; and inducers of DNA repair (Hong and Sporn, 1997; Stoner et al., 1997). Scavenging or trapping agents are compounds that physically react with the activated (electrophilic) forms of carcinogens and oxygen free radicals. Free radicals can be detrimental by reacting with, and sometimes by destroying, critical cellular components including the polyunsaturated fatty acids (PUFA) that comprise lipoprotein particles and plasma membranes. Damage by free radicals can result in loss of membrane fluidity, receptor misalignment, cell lysis, damage to sulfur-containing enzymes and other proteins (producing inactivation, cross-linking, and denaturation), and damage to carbohydrates (leading to altered cellular receptor functions, including those associated with cytokine activities and prostaglandin formation). Free radicals can also induce brain disorders, atherosclerosis, and colon cancer (Inserra et al., 1997). Several cancer chemopreventive agents exhibit antioxidant activity through their ability to scavenge oxygen radicals, inclusive of singlet oxygen, peroxy radicals, superoxide anion, and hydroxyl radicals (Pryor, 1988). Antioxidant defenses normally protect against DNA damage caused by reactive oxygen species from endogenous and exogenous sources, and reduced levels of antioxidants are associated with increased cancer risk (Carmia, 1997).

There are 50 species in the genus of *Chorizanthe* which occur in arid regions in the western part of North America (Mabberley, 1987). Previous work on a 50% ethanol extract of *Chorizanthe glabrescens* Benth. showed weak antimicrobial activity against *Sarcina lutea* and *Staphylococcus aureus* and cytotoxic activity with cultured KB cells (ED_{50} < 20.0 μg/mL) (Azarowicz et al., 1952; Bhakuni et al., 1974, 1976). However, there has been no detailed phytochemical work to date on any species in this genus, inclusive of *C. glabrescens*.

In the current investigation, *Chorizanthe diffusa*

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Benth. (Polygonaceae) was selected for study, since the ethyl acetate-soluble extract of the entire plant was found to exhibit significant antioxidant activity based on scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (IC_{50}: 12.8 μg/mL) and inhibition of TPA-induced free radical formation with cultured HL-60 cells (IC_{50}: 22.0 μg/mL). In addition, a secondary biological analysis demonstrated the extract inhibited 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced preneoplastic lesion formation with a mouse mammary organ culture model (Mehta and Moon, 1991) (57% inhibition). Bioassay-guided fractionation of the active extract using the DPPH antioxidant assay led to the isolation of one novel (**1**) and four known (**2**−**5**) flavonoids, which were then evaluated for their individual biological activities. The initial screening biological data leading to the selection of *C. diffusa* for activity-monitored isolation have been published in a preliminary communication (Lee et al., 1998). At the onset of the present study, *C. diffusa* was considered a native species to the United States and of potential importance for its possible cancer chemopreventive effects.

MATERIALS AND METHODS

^{1}H NMR, ^{1}H−^{1}H COSY, and ^{13}C NMR spectra were measured on a Varian XL-300 instrument operating at 300 and 75.4 MHz, respectively. Compounds were analyzed in DMSO-d_6, with tetramethylsilane (TMS) as internal standard. ^{1}H−^{13}C HETCOR and ^{1}H−^{13}C FLOCK NMR spectra were run with standard pulse sequences. A General Electric Omega 500 NMR spectrometer, operating at 499.9 MHz, was used to perform ^{1}H−^{13}C HMQC and ^{1}H−^{13}C HMBC NMR experiments. Positive-ion electron-impact (EIMS), high-resolution EIMS (HR-EIMS), chemical-ionization (CIMS), and fast-atom-bombardment (FABMS) mass spectra were measured on a Finnigan MAT-90 (Bremen, Germany) double-focusing mass spectrometer. The CI reagent gas was methane, and glycerol was used as the FAB matrix. High-performance liquid chromatography−electrospray mass spectrometry (HPLC−ESMS) was carried out using a Hewlett-Packard (Palo Alto, CA) 5989B mass spectrometer equipped with a 1050 HPLC system. HPLC separation was carried out using a 60-min linear gradient from 12 to 90% methanol in water containing 0.1% formic acid on a Vydac (Separations Group, Hesperia, CA) C_{18} reversed-phase column, 5-μm packing, 150 × 2 mm, at a flow rate of 200 μL/min. Elution of compounds was monitored using negative-ion electrospray ionization and by scanning the mass range *m*/*z* 150−800 in 2 s. Negative-ion electrospray tandem mass spectra (MS−MS) were obtained using a Micromass (Manchester, U.K.) Quattro II mass spectrometer with argon collision gas and a collison energy of 50 V. IR spectra were taken on a Midac Collegian FT-IR spectrometer and UV spectra were measured on a Beckman DU-7 spectrometer. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected, and optical rotations were obtained on a Perkin-Elmer model 241 polarimeter.

Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) plates (silica gel, 0.25-mm layer thickness), with compounds visualized by spraying with 10% (v/v) H_2SO_4 followed by heating at 110 °C on a hot plate. Silica gel (Merck 60 A, 230−400 mesh ASTM), Sephadex LH-20 (25−100 μm; Pharmacia Fine Chemicals, Piscataway, NJ), and Sorbisil C_{18} reversed-phase silica gel (Phase Separations, Ltd., Deeside, Clywd, U.K.) were used for column chromatography. All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ) and distilled before use.

Plant Material. Whole plants of *Chorizanthe diffusa* Benth. were collected in California, in August 1978, under the auspices of Dr. Robert E. Purdue, Jr., Medicinal Plants Laboratory, USDA, Beltsville, MD, where voucher specimens are maintained. The dried plant material was stored at ambient temperature and milled just prior to the present investigation.

Antioxidant Potential of Test Compounds. *1. Assay for DPPH Free Radical Scavenging Potential.* This assay is based on the scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals (Fujita et al., 1988; Lee et al., 1998). Reaction mixtures containing test samples (dissolved in DMSO) and 300 μM DPPH ethanolic solution in 96-well microtiter plates were incubated at 37 °C for 30 min. Absorbances were then measured at 515 nm, and percent inhibition by sample treatment was calculated. IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH free radicals.

2. Assay for Inhibition of TPA-Induced Free Radical Formation in Cultured HL-60 Cells. This assay method is based on inhibiting TPA-induced free radical formation in HL-60 cells by measuring cytochrome *c* reduction (Sharma et al., 1994). HL-60 cells were grown in RPMI 1640 containing 5% heat-inactivated fetal calf serum and penicillin/streptomycin. Cells were differentiated with culture medium containing 1.3% DMSO for 7 days and then harvested and washed twice with and resuspended in HBSS (Hank's balanced salt solution) (without phenol red; Cellgro, Mediatech, DMSO-HL60). Cells were suspended in HBSS and added to 96-well plates (1×10^6 cells/well). Free radical formation was induced by addition of TPA (8 μM). This was followed by the addition of 5 half-log concentrations of test agents together with cytochrome *c* (160 μM). After an incubation period of 1 h at 37 °C, cytochrome *c* reduction was measured at 515 nm using an ELISA reader. Relative to incubations performed with TPA alone, the percent inhibition of radical formation in TPA plus agent-treated samples was determined. Blank reaction mixtures were prepared in which cell suspensions were omitted, and these values were subtracted from the treatment groups. Percent inhibition by sample treatment was determined by comparison with a solvent-treated control group. IC_{50} values were determined from the concentration of sample required to reduce cytochrome *c* oxidation by 50%.

Antimutagenicity Assay with *Salmonella typhimurium* Strain TM677. Test compounds were evaluated for potential to inhibit forward mutation to 8-azaguanine resistance with *S. typhimurium* strain TM677 (Grüter et al., 1990; Shamon et al., 1994; Ito et al., 1998). Reaction mixtures were prepared consisting of 0.77 mL of bacteria in minimal essential medium, 0.1 mL of S9 liver homogenate derived from Aroclor 1254-pretreated rats, and 0.11 mL of a NADPH-generating system. To monitor antimutagenic activity, test substances (100 μM final concentration in 10 μL of DMSO) were added to duplicate incubation mixtures 1 min prior to the addition of DMBA (80 μM final concentration in 10 μL of DMSO). Following incubation in a rotating dry-air incubator (2 h, 37 °C), the reaction mixtures were quenched by the addition of 4 mL of phosphate-buffered saline. The bacteria were recovered by centrifugation, resuspended in 5 mL of phosphate-buffered saline, diluted, and plated on minimal agar in the presence and absence of 8-azaguanine. The plates were then incubated (48 h, 37 °C) and scored with an automatic colony counter (Fisher model 600). Results were expressed as a mutant fraction, and percent inhibition was calculated relative to controls.

Mouse Mammary Gland Organ Culture Assay. BALB/c female mice (4 weeks old; Charles River, Wilmington, MA) were pretreated for 9 days with 1 μg of estradiol and 1 mg of progesterone. On the tenth day, the mice were sacrificed and the second thoracic mammary glands were dissected on silk and transferred to 60-mm culture dishes containing 5 mL of Waymouth's 752/1 MB medium supplemented with 100 units of streptomycin and penicillin and 35 μg/mL glutamine. The glands were incubated for 10 days (37 °C, 95% O_2 + 5% CO_2) in the presence of growth-promoting hormones (5 μg of insulin, 5 μg of prolactin, 1 μg of aldosterone, and 1 μg of hydrocortisone per mL of medium). Glands were exposed to 2 μg/mL DMBA between 72 and 96 h. After the exposure, glands were rinsed and transferred to new dishes with fresh medium. The fully

differentiated glands were then permitted to regress by withdrawing all hormones except insulin for 14 additional days. Test compounds were present in the medium during days 1−10 of culture (10 μg/mL); mammary glands were scored for incidence of lesions (Mehta and Moon, 1991).

Extraction and Isolation Procedure. The milled, dried whole plants of *C. diffusa* (5 kg) were extracted with MeOH and then concentrated and partitioned sequentially with petroleum ether, EtOAc, and *n*-BuOH, producing petroleum ether (16.7 g), EtOAc (15.6 g), *n*-BuOH (23.5 g), and aqueous methanolic extracts. The EtOAc and *n*-BuOH extracts exhibited significant activity in the DPPH antioxidant assay (IC_{50} values of 33.4 and 39.9 μg/mL, respectively). The EtOAc-soluble (15.5 g) extract was adsorbed onto silica gel and separated over additional silica gel (750 g) by open column silica gel chromatography, using a gradient of 3−12% MeOH in $CHCl_3$, and eluates containing constituents with similar TLC profiles were combined to provide 12 pooled fractions (fractions 4−15). Fractions 13 and 14 (eluted with $CHCl_3$−MeOH, 93:7 and 90:10, respectively) were active in the DPPH antioxidant assay (IC_{50} values of 20.4 and 24.6 μg/mL, respectively) and yielded a pure flavone (**1**, 18.0 mg), which was recrystallized from MeOH. A pure flavonol (**4**, 14.7 mg) was obtained by further separation over a small column containing Sephadex LH-20, eluted with MeOH. Column chromatography of the *n*-BuOH-soluble extract (23.4 g) on silica gel with a gradient of 5−20% MeOH in $CHCl_3$ yielded 17 subfractions (fractions 26−42), with fractions 34 and 35 being moderately active in the DPPH antioxidant assay (IC_{50} values of 75.4 and 81.8 μg/mL, respectively). Further chromatography of fraction 34 using Sephadex LH-20 size-exclusion chromatography (MeOH) yielded two further pure flavones (**2**, 8.2 mg, and **3**, 17 mg), eluted from silica gel with 95% MeOH in H_2O. A flavonol glycoside (**5**, 12 mg) was obtained from fraction 35 using C_{18} reversed-phase silica gel, eluted with 85−90% MeOH in H_2O.

5,8,3′,4′,5′-Pentahydroxy-3,7-dimethoxyflavone (1): yellow-orange needles; mp 282 °C; positive reactions with the $FeCl_3$, Mg-HCl, and Zn-HCl tests; UV (MeOH) λ_{max} (log ϵ) 278 (4.53), 305 (3.07), 346 (3.35) nm; (+NaOMe) 278 (3.85), 372 (2.09) nm; (NaOAc) 275 (4.28), 303 (2.76), 348 (2.96) nm; (NaOAc + H_3BO_3) 263 (4.47), 307 (2.12), 387 (3.57) nm; ($AlCl_3$) 281 (4.84), 315 (2.05 sh), 464 (3.04) nm; ($AlCl_3$ + HCl) 285 (4.10), 323 (2.84), 370 (3.19) nm; IR (MeOH) ν_{max} 3270, 1657, 1607, 1507, 1454 cm^{-1}; ^1H NMR (DMSO, 300 MHz) δ 12.27 (1H, s, OH-5), 7.27 (2H, s, H-2′ and -6′), 6.57 (1H, s, H-6), 3.93 (3H, s, OMe-7), 3.81 (3H, s, OMe-3); ^{13}C NMR (DMSO, 75.6 Hz) δ 178.2 (s, C-4), 156.1 (s, C-2), 153.8 (s, C-7), 152.8 (s, C-5), 145.9 (s, C-3′ and -5′), 143.7 (s, C-9), 137.6 (s, C-3), 137.1 (s, C-4′), 126.2 (s, C-8), 119.9 (s, C-1′), 107.9 (d, C-2′ and -6′), 104.5 (s, C-10), 95.3 (d, C-6), 59.1 (q, OMe-3), 56.1 (q, OMe-7); EIMS *m/z* (rel int) $[M]^+$ 362 (100), 319 (36), 320 (6), 181 (4); HR-EIMS *m/z* found 362.0624, calcd for $C_{17}H_{14}O_9$ 362.0633.

5,7,3′,4′-Tetrahydroxy-3-methoxyflavone (2): pale-yellow powder; UV, ^1H NMR, and ^{13}C NMR data consistent with published values (Roitman and James, 1985); EIMS *m/z* (rel int) $[M]^+$ 316 (2), 302 (100), 273 (9), 161 (10), 137 (10), 108 (7).

5,8,3′,4′-Tetrahydroxy-3,7-dimethoxyflavone (3): yellow-orange powder; UV, ^1H NMR, and EIMS data consistent with published values (Sakakibara and Mabry, 1975); EIMS *m/z* (rel int) $[M]^+$ 346 (42), 345 (16), 315 (45), 303 (70), 287 (11).

Quercetin (4): yellow powder; mp 302 °C [lit. mp 313−314 °C (Wenkert and Gottlieb, 1977)]; UV, ^1H NMR, and ^{13}C NMR data consistent with published values (Wenkert and Gottlieb, 1977; Ulubelen and Timmermann, 1980); CIMS *m/z* (rel int) $[M + H]^+$ 303 (13), 286 (100), 229 (3), 133 (22), 154 (10).

3″-*O*-Acetylquercitrin (5): yellow powder; mp 172 °C [lit. mp 167−175 °C (Nobutoshi et al., 1978)]; UV, ^1H NMR (Tanaka et al., 1978), and ^{13}C NMR data consistent with published values (Markham et al., 1978); FABMS *m/z* (rel int) $[M + H]^+$ 491 (52), 303 (100), 245 (33), 189 (41), 129 (31), 165 (15).

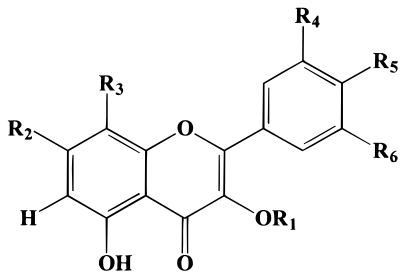
Methylation of Compound 1. To a solution of compound **1** (3 mg) in acetone (1 mL) were added $(CH_3)_2SO_4$ (0.6 mL) and K_2CO_3 (4 mg), and the mixture was stirred for 5 h at 40 °C. After being diluted with water the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water, dried with Na_2SO_4, evaporated, and applied to preparative TLC to obtain the pure methylated compound **1a** (3 mg).

5-Hydroxy-3,7,8,3′,4′,5′-hexamethylflavone (1a): yellow needles; mp 167 °C; UV (MeOH) λ_{max} (log ϵ) 247 (4.22), 352 (3.31) nm; unchanged by shift reagents; ^1H NMR 3.96 (6H, s, OMe), 3.95 (6H, s, OMe), 3.93 (3H, s, OMe), 3.90 (3H, s, OMe), 6.94 (1H, s, H-6), 7.52 (2H, s, H-2′ and -6′), 12.38 (1H, s, OH-5).

RESULTS AND DISCUSSION

The dried and ground whole plant of *C. diffusa* was extracted with methanol and partitioned between petroleum ether and 90% methanol in water, with the more polar layer then partitioned with EtOAc and *n*-BuOH. The dried EtOAc and *n*-BuOH extracts were subjected individually to a series of activity-guided chromatographic fractionation steps to afford, in turn, compounds **1** and **4** and compounds **2**, **3**, and **5**. All of these pure isolates gave characteristic flavonoid color reactions (purplish brown with $FeCl_3$, yellow with NaOH, yellowish orange with Mg-HCl, pale pink with Zn-HCl) (Markham, 1975). The UV, ^1H NMR, and ^{13}C NMR spectral data of the aromatic parts of compounds **1**−**3** and **5** were characterized by major bands that resembled those of 3-*O*-substituted flavones and flavonols (Agrawal et al., 1989; Mabry et al., 1970). The previously known compounds **2**−**5** were established as 5,7,3′,4′-tetrahydroxy-3-methoxyflavone, 5,8,3′,4′-tetrahydroxy-3,7-dimethoxyflavone, quercetin, and 3″-*O*-acetylquercitrin, respectively, by comparison with literature data (Roitman and James, 1985; Sakakibara and Mabry, 1975; Wenkert and Gottlieb, 1977; Ulubelen and Timmermann, 1980; Tanaka et al., 1978; Markham et al., 1978).

Compound **1** was assigned a molecular formula of $C_{17}H_{14}O_9$ from its HR-EIMS (*m/z* 362.0624), which was in accord with a flavone containing five hydroxyl and two methoxyl groups. Comparison of its UV, ^1H NMR, and ^{13}C NMR data with literature values indicated that this compound was a flavone (Agrawal et al., 1989). In addition, it was apparent that **1** contained five OH groups (δ_C 126.2, 137.1, 145.9 (double intensity), 152.8) and two OMe groups (δ_C 56.1, 59.1; δ_H 3.81, 3.93). On methylation of **1** using dimethyl sulfate under standard conditions, the hexamethoxylated product **1a** was produced. The relative positions of the functional groups in **1** were established from the following observations. The ^1H NMR spectrum of **1** suggested that the A ring was trisubstituted from the appearance of a singlet at δ_H 6.57, and an aromatic proton singlet integrating for two protons at δ_H 7.27 was assigned for H-2′ and H-6′ in ring B, respectively. After sodium acetate was added to compound **1**, the resultant UV spectrum did not exhibit any bathochromic shift, thus indicating the absence of any free hydroxyl group at the C-7 position (Mabry et al., 1970). However, a large bathochromic shift was observed upon addition of sodium methoxide, indicating the presence of a hydroxyl group at C-4′ in compound **1** (Markham and Mabry, 1975). The presence of a chelated hydroxyl group at C-5 was indicated in the UV spectrum by the observed bathochromic shift with $AlCl_3$, and the bathochromic shift observed with $AlCl_3$-HCl indicated the presence of *o*-dihydroxyl groups in ring B (Mabry et al., 1970). The UV shifts observed for compound **1** were consistent with data obtained for the model compound 5,8,3′,4′-tetrahydroxy-3,7-dimethoxyflavone (Voirin, 1983).



	R_1	R_2	R_3	R_4	R_5	R_6
1	CH_3	OCH_3	OH	OH	OH	OH
1a	CH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
2	CH_3	OH	H	OH	OH	H
3	CH_3	OCH_3	OH	OH	OH	H
4	H	OH	H	OH	OH	H
5	rha-3-Ac	OH	H	OH	OH	H

Figure 1. Structures of antioxidant flavonoids from *C. diffusa* (rha, α-L-rhamnopyranosyl).

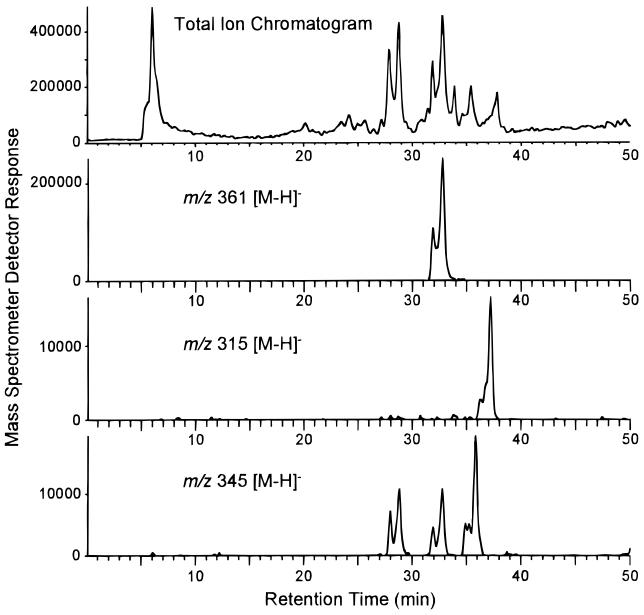


Figure 2. LC−MS analysis of the ethyl acetate extract of *C. diffusa* using C_{18} HPLC separation and negative-ion electrospray ionization. Ions of *m/z* 361, 315, and 345 correspond to the deprotonated molecules of compounds **1**, **2**, and **3**.

LC−MS and LC−MS−MS with electrospray ionization were used to analyze the ethyl acetate-soluble extract of *C. diffusa*. The negative-ion electrospray LC−MS analysis in Figure 2 shows the total ion chromatogram and computer-reconstructed mass chromatograms for the deprotonated molecules at *m/z* 361, 315, and 345, which correspond to compounds **1**, **2**, and **3**. Compound **1** eluted at 32.5 min and formed a deprotonated molecule of *m/z* 361 (Figure 2, second trace). The peak for this compound was 20 times more abundant than the peaks of compounds **2** or **3**. The mass spectrum corresponding to **1**, which eluted at 32.5 min, is shown in Figure 3A and indicates a high degree of purity (the ion of *m/z* 346 is probably a fragment ion). The MS−MS

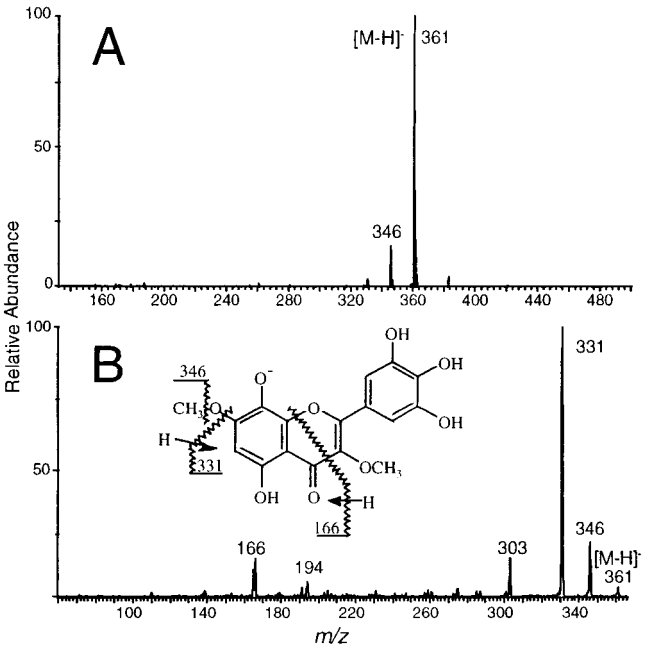


Figure 3. Negative-ion electrospray mass spectrum (A) and tandem mass spectrum (B) of compound **1** from *C. diffusa* eluting at 32.5 min in Figure 2.

spectrum of this compound is shown in Figure 3B. During MS−MS, the deprotonated molecule at *m/z* 361 fragmented to eliminate a methyl group at *m/z* 346 (also observed in the standard mass spectrum), or a molecule of formaldehyde, $[M - H - 28]^+$, to give a product ion of *m/z* 331. Of lower abundance are fragment ions at *m/z* 303 and 166, which correspond to elimination of two formaldehyde molecules or cleavage of the central ring, respectively (see structure and proposed fragmentation patterns in Figure 3B).

Diagnostic mass spectral retro-Diels−Alder (RDA) fragmentation is almost entirely absent in the spectra of flavones with four or more oxygen substituents (OH or OCH_3), and the spectra are dominated by ions such as $[M - 15]^+$, $[M - 28]^+$, and $[M - 43]^+$, along with the molecular ion (Kingston, 1971). Thus, the presence of a methoxyl group at C-3 was supported by the observation of a $[M - COCH_3]^+$ peak in the EIMS of compound **1**, which is characteristic of 3-methoxyflavones (Kingston, 1971). In the $^1H-^{13}C$ HETCOR NMR spectrum of **1**, the signal at δ_H 7.27 (H-2′ and H-6′) correlated to δ_C 107.9, and δ_H 6.57 (H-6) also showed a cross-peak with the resonance at δ_C 95.3. In addition, the methoxy peak at δ_H 3.93 correlated to δ_C 56.1, whereas that at δ_H 3.81 correlated to δ_C 59.1. The OCH_3 group (δ_H 3.93) could be assigned at C-7 based on 1D-NOE experiment, in which irradiation at δ_H 6.57 (H-6) resulted in a NOE for OCH_3-7 (δ_H 3.93). Analysis of the HMBC NMR spectrum allowed the complete assignments of the ^1H and ^{13}C NMR spectra of **1**. Thus, the δ_H 6.57 resonance also showed cross-peaks with signals at δ_C 104.5 (C-10), 152.8 (C-5), and 126.2 (C-8), which strongly supported this being the H-6 signal, rather than at H-7 or H-8. Furthermore, the δ_H 7.27 (H-2′ and H-6′) signal exhibited connectivities with signals at δ_C 145.9 (C-3′ and C-5′), 137.1 (C-4′), 107.9 (C-2′ and C-6′), and 156.1 (C-2). The HMBC data supported the location of an OH group (δ_H 12.27) at C-5, because correlations were observed with the δ_C 152.8 (C-5) and 104.5 (C-10) signals. A diagnostic feature of 3-methoxyflavones is the appearance of the OMe-3 group at δ_C 59.5 ± 0.5,

Table 1. Antioxidant and Antimutagenic Activities of Flavonoids from *C. diffusa* and Inhibition of DMBA-Induced Preneoplastic Lesions in Mouse Mammary Organ Culture

compound	antioxidant activity (IC_{50}, μg/mL) DPPH[a]	HL-60[b]	anti-mutagenicity (% inhibition)[c]	MMOC[d]
1	10.4	21.6	90.0	17
2	39.2	25.7	92.0	67
3	29.8	>50.0	nd[e]	45
4	15.0	9.3	39.0	67
5	12.0	18.4	nd[e]	0
ascorbic acid	22.0	25.5		
2(3)-*tert*-butyl-hydroxyanisole	21.0	50.0		
caffeic acid	12.0	6.7		
gallic acid	5.0	1.5		
nordihydro-guaiaretic acid	12.0	1.2		

[a] DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity. [b] HL-60, inhibition of TPA-induced free radical formation in cultured HL-60 cells. [c] Inhibition of DMBA-induced forward mutation with *S. typhimurium* strain TM677. [d] MMOC, inhibition percent of DMBA-induced preneoplastic lesion formation in mammary gland organ culture at the test concentration of 10 μg/mL. Based on historical controls, inhibition of ≥60% is classified as active at this test concentration. [e] Not determined.

whereas aromatic methoxyl groups resonate at δ_C 61.5 ± 1.5 (with di-*ortho*-substitution) and 55.5 ± 1.0 (with mono- or no *ortho*-substituents) (Agrawal et al., 1989). This inference was consistent with the observation of cross-peaks between δ_C 59.6 and 137.6 (C-3) and between δ_C 56.1 and 153.8 (C-7) in the HMBC experiment. The ring B ^{1}H and ^{13}C NMR chemical shifts of **1** were comparable with published values for those of a model compound with a symmetrical 4′-hydroxy-3′,5′-dimethoxy-substituted B ring, pumilaisoflavone D (Yenesew et al., 1989). From an analysis of all the data described above, the structure of **1** was assigned as the novel compound 5,8,3′,4′,5′-pentahydroxy-3,7-dimethoxyflavone.

It is known that many flavonoids are effective antioxidants (Bors and Saran, 1987; Robak and Glyglewski, 1988; Bors et al., 1990) and that radical scavenging activity is potentiated with 3,4-disubstitution or 2,3,4-trisubstitution in the B ring (Cotelle et al., 1996; van Aker et al., 1996). Flavonoids **1**, **4**, and **5** were significantly active as free radical scavengers in the DPPH assay, and their activity compared favorably with the activity observed with a number of standards in this same assay (Table 1). Compounds **2** and **3** were only moderately active in this assay, as well as in the free radical quenching assay utilizing HL-60 cells, possibly because of the lesser degree of hydroxylation compared with **1**. However, compounds **1** and **5** were not highly active in the HL-60 cell-based assay, but compound **4** mediated a significant response. Three of the isolates were also evaluated for antimutagenic potential in a DMBA-induced forward mutation assay with *S. typhimurium* strain TM677 (Shamon et al., 1994), and compounds **1** and **2** were found to be highly active. These two compounds are likely to either inhibit the metabolic activation of DMBA or scavenge the reactive metabolites of DMBA.

Finally, compounds **1**−**5** were evaluated for their potential to inhibit DMBA-induced preneoplastic lesions with mouse mammary glands in organ culture (MMOC). As noted previously (Mehta and Moon, 1991), compounds active in this model system are considered good candidates for full-term cancer chemopreventive studies. As shown in Table 1, compounds **2** and **4** mediated significant inhibitory activity and, as such, seem worthy of evaluation in experimental carcinogenesis models. Quercetin (**4**) is one of the most common and abundant flavonoids in the human diet, found in tea, wine, and onions (de Vries et al., 1997; Hertog and Katan, 1998), and has already been reported to exhibit pronounced antioxidant activity that enables it to scavenge active oxygen and electrophiles (Huang et al., 1992; Saija et al., 1995). It is believed able to exert beneficial effects on human health and has been shown to inhibit the incidence of papillomas and tumors induced by DMBA (Balasubramanian and Govindasamy, 1996), as well as mammary adenocarcinomas in a DMBA-induced rat mammary carcinogenesis model (Verma et al., 1988). A study of quercetin (**4**) as a chemopreventive agent in sarcoma-180-induced ascites tumors model has been also reported (Ravichandran et al., 1997). Compounds **1**, **3**, and **5** were not active in the MMOC model, irrespective of their potential to facilitate antimutagenic (compound **1**) or antioxidant responses. We have previously established a general correlation between antimutagenic (desmutagenic) activity in TM677 and inhibitory activity in the MMOC system (Shamon et al., 1994; Ito et al., 1998), so it is possible that this mechanism applies in the case of compound **2**.

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