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Potential Anticarcinogenic Natural Products Isolated from Lemongrass Oil and Galanga Root Oil

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Guided by the glutathione *S*-transferase (GST) assay, fractionation of lemongrass oil and galanga root oil from the plants *Cymbopogon citratus* and *Alpinia galanga*, respectively, led to the isolation of four active compounds: *d*-limonene (**1**) and geraniol (**2**) from lemongrass oil; ethyl *trans*-cinnamate (**3**) and ethyl 4-methoxy-*trans*-cinnamate (**4**) from galanga root oil. Their structures were determined on the basis of spectral analysis. These compounds were tested for their capacity to induce the activity of the detoxifying enzyme, GST, in several tissues of female A/J mice. Compound **1** increased GST activity 2.4–3.0-fold higher than controls in the mouse liver and mucosa of the small intestine and large intestine. Compound **2** showed high GST-inducing activity only in the mucosa of the small intestine and large intestine (about 2.5-fold greater than controls). Compounds **3** and **4** exhibited significant activity in the mouse liver and intestines. Induction of increased GST activity, which is believed to be a major mechanism for chemical carcinogen detoxification, has been recognized as one of the characteristics of the action of anticarcinogens. Thus, these compounds show promise as potential chemopreventive agents.

Consumption of cruciferous plants has been linked by epidemiological studies to a decreased incidence of cancer in humans (Committee on Diet, Nutrition and Cancer, 1982). A number of naturally occurring compounds from edible plants have shown inhibitory activity in several types of tumorigenesis assays in animals (Lam and Hasegawa, 1989; Lam and Zheng, 1992; Wattenberg and Lam, 1984; Wattenberg, 1985). Preliminary studies have indicated that essential oils are a potential source of natural inhibitors of carcinogenesis (Lam and Zheng, 1991). Anticarcinogenic substances have been found in several essential oils such as orange oil, onion oil, and garlic oil, as well as their monoterpene components (Belman, 1983; Lam and Hasegawa, 1989; Wattenberg et al., 1989). These discoveries suggest that more inhibitors of carcinogenesis may occur in essential oils that have not yet been studied. As part of our continuing program to isolate chemopreventive agents from natural sources (Lam and Zheng, 1991; Zheng et al., 1992), a number of essential oils were tested in the preliminary screening. Lemongrass oil and galanga root oil exhibited high biological activity (Lam and Zheng, 1991) and were subjected to further fractionation guided by a bioassay. In this paper, we report the isolation of four active compounds from the two oils.

Lemongrass oil is generally produced by steam distillation from the freshly cut or partially dried leaves of *Andropogon citratus* DC. or *Cymbopogon citratus* Stapf. (Poaceae or Gramineae) (Formacek and Kubcezka, 1982). *C. citratus*, native of Sri Lanka and India, now grows only under cultivation in India, Africa, the Philippines, Madagascar, Central and South America, the West Indies, and southeast Asia. Lemongrass oil is used mainly in food flavorings and to a lesser extent in perfumery. Galanga root oil is prepared from fresh and dried rhizomes of *Alpinia galanga* Wild. or *Languas pyramidata* (Blume) Merr. (Zingiberaceae) (De Pooter, 1985). This plant is cultivated throughout Malaysia and the Philippines as a common spice, and the rhizomes are used for flavoring foods such as in the preparation of meat dishes and curries. Galanga root oil is also used for perfume and resinoid (Trabaud, 1964).

A rapid enzyme assay for the screening of potential inhibitors of chemical carcinogenesis has been developed on the basis of the induction of the detoxifying enzyme

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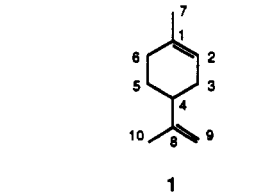
glutathione S-transferase (GST) (Habig et al., 1974; Jakoby and Habig, 1980; Chasseaud, 1979). GST catalyzes the reaction of glutathione (GSH) with electrophiles to form less toxic conjugates for excretion. Because most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, induction of increased GST activity is thus recognized as one of the characteristics of the action of chemopreventive agents (Lam and Hasegawa, 1989; Kensler et al., 1986; Talalay et al., 1987; Prochaska et al., 1985) and is belived to be a major mechanism for carcinogen detoxification (Chasseaud, 1979; Bensen et al., 1978; Sparnins et al., 1988). Compounds that induce an increase in the activity of this detoxifying enzyme system may be considered as potential inhibitors or carcinogenesis. Positive correlation has been established between the inhibitory activity of anticarcinogens and their ability to increase GST activity. Several classes of compounds that inhibit chemical carcinogenesis in laboratory animals have been found to elicit GST enzyme activity significantly (Lam and Zheng, 1992; Sparnins and Wattenberg, 1981; Lam and Hasegawa, 1989). Thus, examining the capacity to induce increased GST activity can be used as a method for detecting potential inhibitors of carcinogenesis.

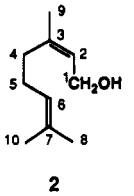
MATERIALS AND METHODS

General Procedure. Electron impact mass spectra (EI-MS) were obtained on Kratos MS-25 and AEI MS-30 mass spectrometers. Infrared (IR) spectra were taken on a Nicolet SX-170 FTIR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Nicolet NT-300 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H NMR spectra measured in $CDCl_3$. The solvent $CDCl_3$ was also used as the internal reference (δ 77.00) for ^{13}C NMR spectra. Preparative liquid chromatography was performed on a Waters PrepLC/system 500A instrument equipped with two connected PrepPak-500/silica cartridges purchased from Millipore Waters Associates (Milford, MA).

Chemicals. Lemongrass and galanga root oils were obtained from Berje Co. (Bloomfield, NJ). GSH was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade or better and purchased from Aldrich unless noted otherwise. d-Limonene (1) and geraniol (2) were isolated from lemongrass oil and ethyl



1



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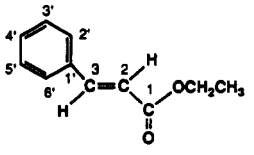

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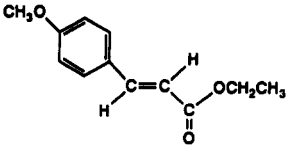

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trans-cinnamate (3) and ethyl 4-methoxy-*trans*-cinnamate (4) were isolated from galanga root oil by using GST-directed fractionation and identified by spectral analysis as described below.

Fractionation of lemongrass oil was carried out by using preparative liquid chromatography with the solvent system of hexane–ethyl acetate. A 20-g portion of the oil was subjected to separation on two connected silica gel cartridges eluted respectively with 1% (6 L), 5% (4 L), and 10% (4 L) EtOAc in hexane and finally EtOAc (2 L). Fractions were collected and analyzed by silica gel TLC. Fractions with the same R_f values were combined to yield 12 major fractions (A–L), which were tested with the GST assay. The pure fraction A (eluted with 1% EtOAc in hexane) showed high GST-inducing activity and was evaporated in vacuo to afford compound 1 (1.0 g, 5%). Fraction K (eluted with 10% EtOAc in hexane) also exhibited GST-inducing activity and yielded compound 2 (1.1 g, 5.5%). The rest of the fractions were inactive and thus not subjected to further investigation.

The separation of galanga root oil (20 g) was performed on two silica gel cartridges. The oil was eluted with hexane (6 L), 1% (4 L) and 5% (4 L) ethyl acetate in hexane, and EtOAc (3 L) to afford 14 major fractions (A–N). The active fractions G and J (both eluted with 5% EtOAc in hexane) yielded compounds 3 (2.6 g, 13%) and 4 (0.8 g, 4%), respectively, upon removal of the solvent.

d-Limonene (1): colorless oil; ^{1}H NMR ($CDCl_3$) δ 1.47 (m, 2 H, CH_2-5), 1.64 (s, 3 H, CH_3-10), 1.72 (s, 3 H, CH_3-7), 1.85–2.15 (m, 5 H, H-4, CH_2-3 and CH_2-6), 4.69 (br s, 2 H, CH_2-9), 5.39 (m, 1 H, H-2); ^{13}C NMR ($CDCl_3$) δ 20.7 (C-10), 23.4 (C-7), 27.9 (C-5), 30.6 (C-3), 30.8 (C-6), 41.1 (C-4), 108.4 (C-9), 120.6 (C-2), 133.5 (C-1), 150.0 (C-8); EI-MS *m/z* (relative intensity) 136 (M^+, 25), 121 (32), 107 (28), 105 (13), 95 (11), 94 (35), 93 (83), 92 (38), 91 (31), 81 (13), 80 (15), 79 (33), 77 (14), 68 (100), 67 (60), 53 (18), 41 (21).

Geraniol (2): colorless oil; FT-IR 3330 (OH), 2970, 2920, 2860, 1670 (C=C), 1445, 1378, 1002 (C—O), 830, 780, 740 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.56 (s, 3 H, CH_3-9), 1.63 (s, 3 H, CH_3-10), 1.64 (s, 3 H, CH_3-8), 2.01 (m, 4 H, CH_2-4 and CH_2-5), 2.20 (s, 1 H, OH), 4.09 (d, 2 H, J = 6.8 Hz, CH_2-1), 5.06 (t, 1 H, J = 6.6 Hz, H-6), 5.36 (t, 1 H, J = 6.8 Hz, H-2); ^{13}C NMR ($CDCl_3$) δ 16.1 (C-9), 17.6 (C-10), 25.6 (C-8), 26.3 (C-5), 39.5 (C-4), 59.1 (C-1), 123.4 (C-2), 123.8 (C-6), 131.5 (C-7), 139.1 (C-3); EI-MS *m/z* (relative intensity) 154 (M^+, 3), 139 (2), 136 (6), 123 (16), 121 (8), 111 (10), 93 (27), 84 (15), 81 (16), 80 (11), 69 (100), 68 (51), 67 (32), 55 (32), 53 (34), 43 (38), 41 (98).

Ethyl trans-Cinnamate (3): white wax after stored in refrigerator; FT-IR 2980, 1710 (C=O), 1640 (C=C), 1580, 1500 (phenyl), 1310, 1175 (C—O), 1040, 980, 765, 710, 680 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3 H, J = 7.1 Hz, CH_3), 4.24 (q, 2 H, J = 7.1 Hz, CH_2), 6.41 (d, 1 H, J = 16.0 Hz, H-2), 7.26–7.50 (m, 5 H, phenyl), 7.67 (d, 1 H, J = 16.0 Hz, H-3); ^{13}C NMR ($CDCl_3$) δ 14.3 (CH_3), 60.3 (OCH_2), 118.2 (C-2), 127.9 (C-2′ and C-6′), 128.8 (C-3′ and C-5′), 130.1 (C-4′), 134.4 (C-1′), 144.4 (C-3), 166.8 (C-1); EI-MS *m/z* (relative intensity) 176 (M^+, 28), 148 (11), 147 (13), 131 (100), 104 (20), 103 (50), 102 (20), 77 (42), 51 (28), 40 (12).

Ethyl 4-Methoxy-trans-cinnamate (4): yellowish wax after stored in refrigerator; FT-IR 2980, 2935, 1704 (C=O), 1630 (C=C), 1600, 1510 (phenyl), 1280, 1170 (C—O), 1025, 830, 780 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3 H, J = 7.1 Hz, CH_3), 3.79 (s, 3 H, OCH_3), 4.23 (q, 2 H, J = 7.1 Hz, CH_2), 6.28 (d, 1 H, J = 16.0 Hz, H-2), 6.86 (d, 2 H, J = 8.6 Hz, H-3′ and H-5′), 7.44 (d, 2 H, J = 8.6 Hz, H-2′ and H-6′), 7.61 (d, 1 H, J = 16.0 Hz, H-3); ^{13}C NMR ($CDCl_3$) δ 14.3 (CH_3), 55.2 (OCH_3), 60.3 (OCH_2), 114.2 (C-3′ and C-5′), 115.7 (C-2), 127.1 (C-1′), 129.6 (C-2′ and C-6′), 144.1 (C-3), 161.3 (C-4′), 167.1 (C-1); EI-MS *m/z* (relative intensity) 206 (M^+, 100), 161 (89), 134 (40), 89 (16), 77 (8), 63 (10), 39 (5).

The structures of these compounds were determined on the basis of spectral analysis and further by comparison of the spectral data with those published in the literature (Sadtler Research Laboratories, 1980; 1992; Breitmaier and Voelter, 1987; Von Sydow, 1963; Gavin and Tabacehi, 1975).

Animals. Female A/J mice, 7 weeks of age, were obtained from Harlan Sprague Dawley Co. (Indianapolis, IN). The animals were housed in temperature-controlled animal quarters with a 12/12 h light/dark cycle. Water was given ad libitum. They were acclimated for 1 week after arrival before they were fed semipurified diet (ICN Nutritional Biochemicals, Cleveland, OH) until the end of the experiment. This diet was similar in composition to that of AIN-76A except that the antioxidants were removed and the sucrose was replaced by a 1:1 mixture of starch and glucose. One week after the start of the semipurified diet, they were divided into control and experimental groups with four mice per group. The experimental groups were given

Table I. Effects of Compounds from Lemongrass Oil and Galanga Root Oil on the Activity of Glutathione *S*-Transferase in Target Tissues of Female A/J Mice

	liver		small bowel mucosa		forestomach		lung		large bowel mucosa	
compounds[a]	GST spec act.[b]	ratio[c]	GST spec act.[b]	ratio[c]	GST spec act.[b]	ratio[c]	GST spec act.[b]	ratio[c]	GST spec act.[b]	ratio[c]
control	0.85 ± 0.09		0.47 ± 0.05		0.47 ± 0.05		0.18 ± 0.03		0.18 ± 0.05	
d-limonene	2.57 ± 0.29[d]	3.02	1.11 ± 0.05[d]	2.36	0.55 ± 0.07	1.17	0.18 ± 0.03	1.00	0.28 ± 0.07[e]	1.56
geraniol	1.04 ± 0.09[e]	1.22	1.23 ± 0.01[d]	2.62	0.55 ± 0.04	1.17	0.19 ± 0.03	1.06	0.25 ± 0.05	1.39
control	1.13 ± 0.17		0.56 ± 0.08		1.04 ± 0.12		0.32 ± 0.06		0.26 ± 0.05	
ethyl *trans*-cinnamate	1.47 ± 0.27	1.30	0.80 ± 0.12	1.43	1.09 ± 0.16	1.05	0.33 ± 0.03	1.03	0.36 ± 0.05[e]	1.38
ethyl 4-methoxy-*trans*-cinnamate	1.90 ± 0.52[e]	1.68	1.21 ± 0.48[e]	2.16	1.55 ± 0.18[d]	1.49	0.41 ± 0.05[e]	1.28	0.30 ± 0.04	1.15

[a] Administered by p.o. intubation 20 mg of test compound in 0.3 mL of cottonseed oil per dose every 2 days for a total of three doses. The control mice were only given 0.3 mL of cottonseed oil. [b] Micromoles min^{-1} (mg of protein)$^{-1}$. [c] Test/control. [d] All P values are obtained by Student's t-test ($n = 4$), $P < 0.005$ vs control group. [e] $P < 0.05$ vs control group.

by gavage 20 mg per dose of the test compounds, dissolved in 0.3 mL of cottonseed oil, once every 2 days for a total of three doses. The control group was given cottonseed oil alone. Twenty-four hours after the last administration, the mice were killed by cervical dislocation. The liver, forestomach, lung, and mucosa of the proximal one-third of the small intestine and the large intestine including the cecum were removed for enzyme preparation. The tissues were homogenized in cold 1.15% KCl solution (pH 7.4) by means of a Brinkman homogenizer. The homogenate was centrifuged at 9000*g* for 20 min, and the supernatant was centrifuged at 100000*g* for 1 h. The cytosolic fractions were kept frozen at −80 °C until use. Each sample represents one tissue from each individual animal.

Glutathione *S*-Transferase Assay. The activity of cytosolic GST was determined according to the method of Habig et al. (1974) using CDNB as the substrate. Assays were performed at 30 °C in 0.1 M phosphate buffer (pH 6.5), in the presence of 5 mM GSH, 1 mM CDNB, and 20 μL of the cytosol. The reaction was monitored at 340 nm on a Beckman DU-65 spectrophotometer equipped with a temperature-controlled cell compartment. Complete assay mixture without the cytosolic enzyme was used as the control. Data were analyzed by Student's t-test, and P values were obtained in comparison to the control. The protein concentration of these samples was determined according to the method of Lowry et al (1951).

RESULTS AND DISCUSSION

Lemongrass oil and galanga root oil were fractionated by using GST assay as a guide to isolate active constituents. *d*-Limonene (1) and geraniol (2) were isolated from lemongrass oil, and ethyl *trans*-cinnamate (3) and ethyl 4-methoxy-*trans*-cinnamate (4) were from galanga root oil. *d*-Limonene and geraniol have been found in lemongrass oil previously (Formacek and Kubczeka, 1982). The effects of compounds 1–4 on the induction of glutathione *S*-transferase were determined in the liver, lung, forestomach, colon, and small intestinal mucosa of female A/J mice (Table I). *d*-Limonene increased GST activity 3.02- and 2.36-fold higher than controls in the mouse liver and small intestinal mucosa, respectively. A 56% increase in the GST activity in the large intestinal mucosa was observed with this compound. However, *d*-limonene showed no significant effect in the forestomach or lung. Geraniol showed high GST-inducing activity only in the mucosa of small intestine and large intestine (2.62 and 1.39 times over controls, respectively). Ethyl *trans*-cinnamate exhibited moderate activity in the mouse liver and intestines with 30–40% increases in GST activity. Ethyl 4-methoxy-*trans*-cinnamate is more active than its 4-demethoxyl analog in the mouse liver, small intestine mucosa, forestomach, and lung (T/C ratios ranging from 1.28 to 2.16) but less active in the large intestine (T/C = 1.15).

As major active components of lemongrass oil and galanga root oil, compounds 1–4 appear to be responsible for the high activity of the oils observed in the preliminary screening. *d*-Limonene has been reported to inhibit polycyclic aromatic hydrocarbon and nitrosamine-induced carcinogenesis in laboratory animals (Elson et al., 1988; Wattenberg, 1983; Wattenberg et al., 1989). *d*-Limonene is one of the most widely distributed monoterpenes present in large quantities in essential oils. For instance, orange oil contains over 50% *d*-limonene (Formacek and Kubczeka, 1982). The present results show that this compound can also elicit an increased activity of the detoxifying enzyme GST. The correlation between the induction of GST activity by natural anticarcinogens and their inhibitory activity against tumorigenesis has been well documented (Bensen et al., 1978; Chasseaud, 1979; Kensler et al., 1986; Lam and Hasegawa, 1989; Lam and Zheng, 1992; Prochaska et al., 1985; Sparnins and Wattenberg, 1981; Sparnins et al., 1988; Talalay et al., 1987). Thus, the GST assay can be used to direct the isolation of potential anticarcinogenic compounds. Since these essential oils are being consumed as flavorings and seasonings by humans on a daily basis, pure chemopreventive agents isolated from the same sources should have potential as diet supplements.

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