

Cytotoxic and Mutagenic Effects of *Cinnamomum cassia* Bark-Derived Materials

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Abstract The cytotoxic activities of *Cinnamomum cassia* (Blume) bark-derived materials toward six human HeLa epithelioid cervix, A549 lung, SK-OV-3 ovarian, SK-MEL-2 melanoma, XF-498 central nerve system, and HCT-15 colon tumor cell lines were evaluated by using sulforhodamine B assay and compared to those of the anticancer agents, cisplatin and mitomycin C. The biologically active constituent of the *Cinnamomum* bark was characterized as *trans*-cinnamaldehyde by spectroscopic analysis. The cytotoxic activity of cinnamaldehyde against HeLa, SK-MEL-2, and HCT-15 cell lines was comparable to that of cisplatin and mitomycin C. The compound showed lower activity against A549, SK-OV-3, and XF-498 cell lines than the anticancer agents. Eugenol exhibited moderate activity against SK-OV-3, XF-498, and HCT-15 tumor cells, and *trans*-cinnamic acid, cinnamyl alcohol, α -pinene, and β -pinene showed little or no activity against model tumor cells. Cinnamaldehyde was not mutagenic against four strains (TA 98, TA 100, TA 1535, and TA 1537) of *Salmonella typhimurium* (Castel and Chalm). These results indicate at least one pharmacological action of *C. cassia*.

Key words: *Cinnamomum cassia*, cytotoxicity, tumor cell, mutagenicity, *Salmonella typhimurium*, *trans*-cinnamaldehyde

Chemical carcinogenesis can be divided into two defined stages; initiation and promotion. Both initiators and promoters are found in human environments, and it is well established that naturally occurring tumor promoters play a more crucial role in the development of human cancer than initiators.

Therefore, inhibitors of tumor-promotion are expected to be highly effective for cancer therapy.

Cancer therapy consists primarily of repeated administrations of synthetic anticancer agents. Although effective, their continued or repeated use may lead to development of widespread resistance to the agents in the tumor cells [28] and undesirable clinical effects on human health such as alopecia, leucopenia, sterility, and secondary malignancies [19]. Decreasing efficacy and increasing concern over possible adverse effects of the earlier chemotherapeutic agents have highlighted the need for the development of selective alternatives.

Plants may be an alternative to currently used anticancer agents, because they are a rich source of bioactive chemicals. Since many of them are largely free from adverse effects and have excellent pharmacological actions, they could lead to the development of new classes of possibly safer anticancer agents [16]. Much efforts have, therefore, been focused on plant materials for potentially useful products as commercial anticancer agents or as lead compounds. We have recently reported that, among 45 medicinal plant species, a methanol extract of *Cinnamomum cassia* bark showed potent cytotoxic activity against A549 lung, SK-MEL-2 melanoma, XF-498 central nerve system, SK-OV-3 ovarian, and HCT-15 colon tumor cell lines [13]. This plant species is not only important as a spice in East Asia, but it is also considered to have some medicinal properties, such as stomachic agent, astringent agent, and carminative agent [22]. It is rich in essential oils and tannins [5, 22].

In the laboratory study described herein, we assessed the cytotoxic activities of *C. cassia* bark-derived materials (*trans*-cinnamaldehyde, *trans*-cinnamic acid, cinnamyl alcohol, eugenol, α -pinene, and β -pinene) against six human tumor cell lines and the mutagenicity against four *Salmonella*

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typhimurium strains to develop potentially new safer antitumor agents. Additionally, the antitumor or anticarcinogenic action of plant-derived extracts and phytochemicals is also discussed in connection with human intestinal bacteria.

MATERIALS AND METHODS

Chemicals

9-Aminoacridine (9AA), 2-aminofluorene (2AF), cisplatin, mitomycin C, sodium azide (SA), and sulforhodamine B (SRB) were purchased from Sigma (St. Louis, MO, U.S.A.). Fetal bovine serum and RPMI 1640, and α - and β -pinene were obtained from Gibco (Gaithersburg, MD, U.S.A.) and Aldrich (St. Paul, WI, U.S.A.), respectively. Cinnamic acid, cinnamyl alcohol, and eugenol were supplied by Tokyo Kasei (Tokyo, Japan). All other chemicals were of reagent grade.

Tumor Cell Lines and Culture Conditions

Six human tumor cell lines used in this study were HeLa epithelioid cervix, A549 lung, SK-MEL-2 melanoma, SK-OV-3 ovarian, XF-498 central nerve system, and HCT-15 colon tumor. They were maintained in the laboratory as stocks in RPMI 1640 supplemented with 10% fetal bovine serum. Cell cultures were passaged once or twice weekly using trypsin-EDTA to detach the cells from their culture flasks.

Isolation and Identification

The bark from *C. cassia* (3.6 kg) purchased as a commercially available product was dried in an oven at 60°C for 2 days, finely powdered, extracted twice with methanol (10 l) at room temperature, and filtered. The combined filtrate was concentrated *in vacuo* at 35°C to yield 10.1% (based on the weight of the bark). The extract (20 g) was sequentially partitioned into hexane (4.0 g), chloroform (4.4 g), ethyl acetate (2.0 g), butanol (0.5 g), and water-soluble (9.1 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 35°C, and the water portion was freeze-dried.

The hexane portion (10 g) was applied onto a silica gel column (Merck 70–230 mesh, 500 g, 5.5 i.d.×70 cm), and the column was successively eluted with a stepwise gradient of hexane/ethyl acetate (0, 10, 30, 50, 80, and 100%). The active 50% fraction was rechromatographed on a silica gel column and eluted with hexane/ethyl acetate (2:1). Fractions were collected and analyzed by TLC. Fractions with a similar TLC pattern were combined, and the active fraction was further fractionated on a preparative HPLC column [Waters Delta Prep 4000; 29 i.d.×300 mm Bondapak C₁₈ (Waters)] with methanol/water (3:7) at a flow rate of 10 ml/min and detection at 260 nm. Finally, a potent active principle (100 mg) was isolated. Structural

determination of the active isolate was made by spectroscopic analyses, including ¹H- and ¹³C-NMR spectra with a Bruker AM-500 spectrometer, UV spectra on a Waters 490 spectrometer, IR spectra on a Biorad FT-80 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer.

Cytotoxicity Test

SRB assay was used to measure the cytotoxic activity of test materials against model tumor cell lines [27]. The rapidly growing cells were harvested, counted, and inoculated at appropriate concentrations (1–2×10⁴ cells/well) into 96-well microtiter plates. After incubation for 24 h, test materials dissolved in culture medium were applied to the culture wells in triplicate, followed by incubation for 48 h at 37°C under 5% CO₂ atmosphere. The cultures fixed with cold trichloroacetic acid (TCA) were stained by 0.4% SRB dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered Tris base by gyratory shaker, absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). Fifty percent inhibitory dosage (ED₅₀) was defined as the dosage which reduced absorbance by 50% of untreated wells as the control in the SRB assay. It has generally been acknowledged that plant extracts with cytotoxic effect at <40 µg/ml (ED₅₀) may be useful for developing antitumor agents. Therefore, cytotoxic activity was classified as follows: very strong activity +++, ED₅₀ <10 µg/ml; strong ++, ED₅₀ 11–40 µg/ml; moderate +, ED₅₀ 41–100 µg/ml; weak +, ED₅₀ 101–200 µg/ml; and little or no activity -, ED₅₀ >200 µg/ml.

Mutagenicity Test

The mutagenic activity of the active isolate and reference compounds (2AF, SA, and 9AA) against four strains (TA 98, TA 100, TA 1535, and TA 1537) of *Salmonella typhimurium* (Castel and Chalm) was determined by the method of Maron and Ames [20]. Liver S-9 was prepared from Aroclor 1254-induced male rat as a metabolizing system, and S-9 mixture contained S-9 fraction (4%, 2.0 ml), 0.4 M MgCl₂-1.65 M KCl (1.0 ml), 1 M glucose-6-phosphate (0.25 ml), 0.1 M NADP (2.0 ml), 0.2 M phosphate buffer (pH 7.4, 25 ml), and distilled water (19.75 ml). These four strains were inoculated on stock culture plate and were incubated for 40 h at 37°C. The incubated strains were subcultured on the new plate and were incubated for 20 h at 37°C. Subsequently, they were transferred to nutrient broth agar and were grown at 37°C with shaking in a gyratory incubator. An aliquot (2 ml) of each strain was added to 2.5 ml of top agar. The strains were then introduced to minimal glucose medium plates which contained various concentrations of the active isolate in the presence or absence of S-9 mixture. Dimethyl sulfoxide-containing plates were used as controls. These colonies were determined after 48 h of incubation at 37°C. All tests were performed in triplicate.

Table 1. Cytotoxic activity of *C. cassia* bark-derived materials against human tumor cells, using sulforhodamine B assay.

Material	Cell line					
	HeLa	A549	SK-OV-3	SK-MEL-2	XF-498	HCT-15
Methanol extract	+++ ^a	+++	+++	++++	++++	+++
Hexane fraction	++++	+++	+++	++++	++++	+++
Chloroform fraction	-	-	-	-	-	-
Ethyl acetate fraction	++		++	+++	++	-
Butanol fraction	-	-	-	-	-	-
Water fraction	-	-	-	-	-	-

^a++++, ED₅₀ <10 µg/ml; +++, ED₅₀ 11–40 µg/ml; ++, ED₅₀ 41–100 µg/ml; +, ED₅₀ 101–200 µg/ml; -, ED₅₀ >200 µg/ml.

RESULTS

Identification

When fractions obtained from methanol extract of the *Cinnamomum* bark were assayed by the SRB method (Table 1), the hexane portion showed potent cytotoxic activity against all model tumor cell lines used. The ethyl-acetate portion revealed moderate activity against HeLa, SK-OV-3, SK-MEL-2, and XF-498 tumor cell lines, whereas little or no activity was found in the chloroform, butanol, and water portions. Therefore, purification of the biologically active compound from the hexane fraction was sequentially carried out by silica gel column and HPLC chromatography, and the isolates were bioassayed with human tumor cell lines. One active isolate showed strong cytotoxic activity, and the structure of the isolate was determined to be *trans*-cinnamaldehyde by spectral techniques. The compound was identified based on the following evidence: C₉H₈O (MW, 132); EI-MS (70 eV) m/z (% rel. int.): M+ 132 (3), 103 (2), 74 (83), 59 (100), 58 (75); IR (neat) max cm⁻¹: 2,920, 1,680, 1,630, 1,130; ¹H-NMR (CD₃OD, 400 MHz): 6.60 (dd, J=8 and 18 Hz), 7.35 (d, J=18 Hz), 7.1–7.7 (m), 9.52 (d, J=8 Hz); ¹³C-NMR (CD₃OD, 100 MHz): 195.6, 154.4, 135.0, 132.1, 129.9, 129.7, 129.5, 129.0, 128.9.

Cytotoxic Activity

The cytotoxic effects of *trans*-cinnamaldehyde and other components of the *Cinnamomum* bark against model tumor cells used are given in Table 2. Responses varied with both

the chemical and tumor cell line tested. All model tumor cells were highly sensitive to *trans*-cinnamaldehyde. Eugenol exhibited moderate cytotoxic activity toward SK-OV-3, XF-498, and HCT-15 tumor cells, but weak cytotoxic activity to HeLa and SK-MEL-2 tumor cells. However, *trans*-cinnamic acid, cinnamyl alcohol, α-pinene, and β-pinene showed little or no activity against all tumor cells.

Because of its potent cytotoxic activity against model tumor cells, *trans*-cinnamaldehyde was compared with the commercial anticancer agents, cisplatin and mitomycin C (Table 3). Cinnamaldehyde showed potently cytotoxic activity against model tumor cell lines HeLa (ED₅₀, 5.4 µg/ml), SK-MEL-2 (ED₅₀, 2.5 µg/ml), and HCT-15 (ED₅₀, 9.6 µg/ml), and the activity was comparable to those of cisplatin and mitomycin C. On the other hand, cytotoxic activity of the compound against XF-498, A549, and SK-OV-3 cells lines was lower than those of cisplatin and mitomycin C.

Mutagenic Activity

Effects of *trans*-cinnamaldehyde on *S. typhimurium* strains TA 98, TA 100, TA 1535, and TA 1537 mutagenesis were investigated in the presence or absence of S-9 for metabolic activation (Table 4). Mutant colonies of test strains could not survive at more than 300 µg/plate of *trans*-cinnamaldehyde in the presence or absence of S-9, thus indicating that the compound was lethal at >300 µg/plate, whereas no effect on survival of mutant colonies at 200 µg/plate was observed. Cinnamaldehyde at 200 µg/plate did not exhibit mutagenicity, when tested against four strains of *S. typhimurium*.

Table 2. Cytotoxic activity of *C. cassia* bark-derived compounds against human tumor cells, using sulforhodamine B assay.

Compound	Cell line					
	HeLa	A549	SK-OV-3	SK-MEL-2	XF-498	HCT-15
<i>trans</i> -Cinnamaldehyde	++++ ^a	+++	+++	++++	++++	++++
<i>trans</i> -Cinnamic acid	-	-	-	-	-	-
Cinnamyl alcohol	-	-	-	-	-	-
Eugenol	+	-	++	+	++	++
α-Pinene	-	-	+	-	-	-
β-Pinene	-	-	+	-	-	-

^aFor explanation, see Table 1.

Table 3. Cytotoxic activity of *trans*-cinnamaldehyde and anticancer agents against human tumor cells, using sulforhodamine B assay.

Compound	EC ₅₀ (µg/ml)					
	HeLa	A549	SK-OV-3	SK-MEL-2	XF-498	HCT-15
<i>trans</i> -Cinnamaldehyde	5.4	27.5	17.5	2.5	8.7	9.6
Cisplatin	4.2	2.3	5.8	4.5	1.3	6.0
Mitomycin C	5.1	1.9	2.4	1.8	0.8	3.5

DISCUSSION

In the laboratory study with six model tumor cell lines, the bark from *C. cassia* (Family Lauraceae) exhibited potent cytotoxic activity. An active principle of the *Cinnamomum* bark was identified as *trans*-cinnamaldehyde. It has been reported that a number of aliphatic and aromatic aldehydes and their derivatives have cytotoxicity against tumor cell lines [26] and some of them were clinically used as anticancer agents [11]. It has been well acknowledged that many of plant extracts and phytochemicals are potential alternatives to synthetic anticancer agents [3, 6, 14, 25]. Barclay and Perdue [3] suggested that the most promising botanical anticancer agents are in the families Annonaceae, Apocynaceae, Celastraceae, Cephalotaxaceae, Euphorbiaceae, Liliaceae, Menispermaceae, Podocarpaceae, Rutaceae, Simanubaceae, Taxaceae, and Thymelaeaceae.

Table 4. Mutagenicity of *trans*-cinnamaldehyde on *Salmonella typhimurium*.

Strain	Compound ^a	Dose (µg/plate)	-S-9	+S-9
			X±SE	X±SE
TA-98	CA	0		51±2
		25	35±3	48±3
		50	31±4	43±5
		100	29±2	50±5
		200	27±6	17±4
			32±4	46±4
TA-100	CA	0	104±7	115±10
		25	106±9	121±12
		50	112±10	119±8
		100	107±8	129±9
		200	109±7	128±10
			543±52	
TA-1535	CA	0	20±3	25±2
		25	19±1	24±1
		50	19±3	27±1
		100	21±2	28±4
		200	16±4	25±4
			496±43	
TA-1537	CA	0	14±1	20±2
		25	13±2	23±3
		50	10±2	19±3
		100	13±2	22±2
		200	14±3	17±2
			303±39	
	9AA	50		

^aCA, *trans*-cinnamaldehyde; 2AF, 2-aminofluorene; SA, sodium azide; and 9AA, 9-aminoacridine.

Various compounds, including phenolics, terpenoids, and alkaloids, are present in plants. Jointly or independently, they may contribute to the generation of a variety of biological activities. In recent years, much concern has been focused on selective plant-derived modulators on tumor cells, since many of the plant-derived materials are relatively nontoxic to human, act on various types of tumor complex, and may be applied to humans in the same way as other synthetic anticancer agents. Additionally, some plant extracts or phytochemicals have been found to be effective against cancer cells which are resistant to conventional chemotherapeutic agents [7, 29]. In our study, the cytotoxic effect of the *Cinnamomum* bark-derived *trans*-cinnamaldehyde varied with tumor cells used. This compound exhibited potent cytotoxic activity against HeLa, SK-MEL-2, XF-498, and HCT-15 tumor cell lines, and the activity of the compound was comparable to those of cisplatin and mitomycin C.

It may be of great interest to investigate relationships between carcinogenesis and intestinal microorganisms, because infectious agents such as bacteria play a large role in carcinogenesis. However, the information available is limited. It has been reported that populations at risk for gastrointestinal carcinoma had higher rates of clostridia caries [9, 21], suggesting that the organism may play a large role in the cause of gastrointestinal cancer, because of its ability to produce *N*-nitroso compounds or aromatic steroids which are highly carcinogenic [4, 12, 21]. Epidemiological investigations of gastric cancer have found a negative relationship between gastric cancer-related death and frequent intake of green tea [23]. Green tea constituents such as polyphenols may be responsible for the protective effect on the cancer, and impede gastric carcinogenesis by inhibiting the formation of carcinogens [15]. Ahn *et al.* [1] reported growth-inhibitory activity of polyphenols against *Clostridium perfringens* that has been associated with sudden death, aging, toxicity, Alzheimer's disease, and gastrointestinal disease in humans [10, 12, 17, 21]. Recent *in vivo* investigations with human volunteers have shown that intake of ginseng extract or green tea extract affects favorably fecal microbiota and biochemical aspects of feces [24], indicating that daily intake of these materials may normalize disturbed physiological functions which result in the prevention of diseases caused by pathogens in the gastrointestinal tract. In our study, the *Cinnamomum* bark-derived *trans*-cinnamaldehyde had a strong cytotoxic

activity against six human tumor cell lines. Lee and Ahn [18] have already reported that this compound has significant growth-inhibitory effect on harmful bacteria such as *Cl. perfringens*, *Cl. paraprutificum*, and *Bacteroides fragilis*. Our present results suggest that cinnamaldehyde may directly inhibit the formation of carcinogens or indirectly inhibit the formation of carcinogens by inhibiting growth of harmful intestinal bacteria mentioned above. It has also been reported that *Galla rhois*-derived methyl gallate and gallic acid have a potent cytotoxic activity toward five human tumor cell lines [13] and growth-inhibitory activity against various clostridia [2]. Therefore, more studies should be carried out on the relationship between cytotoxic activity of plant-derived materials against model tumor cells and their growth-inhibitory activity against intestinal bacteria.

In conclusion, *C. cassia* bark-derived *trans*-cinnamaldehyde has significant cytotoxic effect against model tumor cells used, without mutagenesis on four *Salmonella* strains tested. Cinnamaldehyde was approved by FDA/WHO with a conditional acceptable daily intake (ADI) for man of 1.25 mg/kg [8]. On the basis of our data and earlier findings, the cytotoxic activity of *trans*-cinnamaldehyde against model tumor cells may indicate at least one worthy pharmacological action of the *Cinnamomum* bark.

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