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Antimutagenicity of Supercritical CO_2 Extracts of *Terminalia catappa* Leaves and Cytotoxicity of the Extracts to Human Hepatoma Cells

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Natural antimutagens may prevent cancer and are therefore of great interest to oncologists and the public at large. Phytochemicals are potent antimutagen candidates. When the Ames test was applied to examine the antimutagenic potency of supercritical carbon dioxide (SC-CO_2) extracts of *Terminalia catappa* leaves at a dose of 0.5 mg/plate, toxicity and mutagenicity were not detected. The antimutagenic activity of SC-CO_2 extracts increased with decreases of temperature (60, 50, and 40 °C) and pressure (4000, 3000, and 2000 psi) used for extraction. The most potent antimutagenicity was observed in extracts obtained at 40 °C and 2000 psi. At a dose of 0.5 mg of extract/plate, approximately 80% of the mutagenicity of benzo[*a*]pyrene (B[*a*]P, with S-9) and 46% of the mutagenicity of *N*-methyl-*N*′-nitroguanidine (MNNG, without S-9) were inhibited. Media supplemented with SC-CO_2 extracts at a range of 0−500 μg/mL were used to cultivate human hepatoma (Huh 7) and normal liver (Chang liver) cells. The viability of the cells was assayed by measuring cellular acid phosphatase activity. A dose-dependent growth inhibition of both types of cells was observed. The SC-CO_2 extracts were more cytotoxic to Huh 7 cells than to Chang liver cells. The observation that SC-CO_2 extracts of *T. catappa* leaves did not induce mutagenicity at the doses tested while exhibiting potent antimutagenicity and were more cytotoxic to human hepatoma cells than to normal liver cells is of merit and warrants further investigation.

KEYWORDS: Antimutagenicity; *Terminalia catappa*; supercritical fluid extraction (SFE); Ames test; cytotoxicity; human hepatoma; Chang liver cells

INTRODUCTION

Known and unknown mutagens and antimutagens present in the environment may affect the incidence of human cancer. In addition to contact with skin, ingestion is the most frequent channel of human exposure. Interactions among bioactive compounds are complicated and ubiquitous, making the detection and identification of natural mutagens and antimutagens important (*1*, *2*). When people are aware of sources of natural antimutagens, they are more likely to make selections of food or drink containing substantial amounts of active compounds, thereby enhancing their health status. Intake of sufficient amounts of antimutagens and/or anticarcinogens is believed to confer a preventive effect on the initiation and development of human cancers (*3*). Phytochemicals are potent sources of antimutagens.

Terminalia catappa, belonging to the family Combretaceae, is a tree that commonly grows in tropical and subtropical regions. The fallen leaves, after drying and shredding, have been used as a folk medicine (*4*) and also as a drink after infusion with hot water. Water extracts of *T. catappa* leaves have been reported to effectively suppress CCl_4-induced hepatotoxicity of male Wistar albino rats and bleomycin-induced genotoxicity of Chinese hamster ovary cells (*4*, *5*). Punicalin and punicalagin, both anti-AIDS compounds, have been identified in the water extracts of *T. catappa* leaves (*6*, *7*). Tannin and flavonoid glycosides in *T. catappa* leaves, as free radical scavengers, have been reported to inhibit Cu^{2+}-induced low-density lipoprotein oxidation (*8*−*10*). Extraction and identification of squalene from *T. catappa* leaves by supercritical carbon dioxide (SC-CO_2) have been achieved in our laboratory (*11*). In addition to the potent antioxidative characteristics of SC-CO_2 extracts, further investigation of the antimutagenicity of extracts was of interest.

Supercritical fluid extraction (SFE) has been demonstrated to effectively extract natural and neutraceutical compounds (*12*). SFE is a powerful tool in the differential and efficient extraction of the target compounds from solid matrices at a fairly low

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temperature (*13*). SC-CO_2 is commonly used as a solvent due to its low critical point (P_c = 7.38 MPa, T_c = 31.1 °C), low cost, ease in elimination of residual solvent, nontoxicity, nonflammability, and status as generally recognized as safe (GRAS) (*14*). In addition, SC-CO_2 is efficient in extracting compounds with low polarity. In this study, in accordance with the fact that the Ames test is a short-term genotoxicity assay for the simultaneous detection of mutagens and antimutagens (*15*), the antimutagenicity of the SC-CO_2 extracts as affected by extraction temperature and pressure was assayed by the Ames test. From the viewpoint of further application, an examination of the differential cytotoxicity toward human hepatoma (Huh 7) and normal human liver (Chang liver) cells was made.

MATERIALS AND METHODS

***n*-Hexane and SC-CO_2 Extraction of *T. catappa* Leaves.** Collection and pretreatment of the abscisic leaves from *T. catappa* trees were done following a previously described procedure (*11*). For each extraction, an aliquot (15 g) of the dry, pulverized leaves was deposited into a 500 mL flask, mixed with 150 mL of *n*-hexane, sealed with a silicone stopper, and shaken in a shaker bath (150 rpm) at 25 ± 2 °C for 24 h. The solution was filtered through a filter paper (Whatman no. 1, Whatman International Ltd., Maidstone, U.K.), and the filtrate was concentrated to dryness using a rotary vacuum evaporator at 40 °C (RE 47, Yamato Scientific Co., Ltd., Tokyo, Japan).

For SFE of *T. catappa* leaves, the procedure of Ko et al. (*11*) was followed. Aliquots (1.0 g) of the pulverized leaves were loaded into a series of extraction vessels (10 mL) and subjected to SC-CO_2 extraction. A composite design of extraction conditions including temperatures of 40, 50, and 60 °C and pressures of 2000, 3000, and 4000 psi was used. Static and dynamic extraction times were both 15 min, and the flow rate was 3 mL/min. After extraction, the extracts were stored at −25 °C for further analyses.

Ames Test of SC-CO_2 Extracts. *Salmonella typhimurium* TA100 was obtained from the Food Industry Research and Development Institute (FIRDI), Hsinchu, Taiwan. The liquid preincubation method developed by Maron and Ames (*15*) was followed. Aliquots (0.1 mL) of a 14 h culture of *S. typhimurium* TA100 grown in nutrient broth (Oxoid Ltd., Hampshire, U.K.) were mixed with 0.1 mL of an aqueous solution containing B[*a*]P (Sigma Chemical Co., St. Louis, MO) and 0.5 mL of rat liver S-9 mixture (Organon Teknika Co., Durham, Switzerland) or *N*-methyl-*N*′-nitroguanidine (MNNG) (Sigma Chemical Co.) without S-9 in the presence or absence of 0.1 mL of *T. catappa* leaf extract. After preincubation of the culture for 20 min at 37 °C, the mixture was transferred to 2 mL of top agar containing 0.05 mM histidine, 0.05 mM biotin (Fluka Co., Gallen, Switzerland), and 0.09 M NaCl (kept warm at 45 ± 2 °C) and poured onto minimal glucose agar plates (Oxoid Ltd.). After the agar had solidified, these plates were incubated for 48 h at 37 °C, and then the number of his+ revertant colonies was counted. Antimutagenicity was estimated by determining the decrease in the number of mutants induced by a specific mutagen and expressed as a percentage of inhibition. Antimutagenicity was examined in terms of inhibition percentage (*I*) of mutagenicity and expressed as

$$I\,(\%) = 100 \times (A - B)/(A - C)$$

where *A* is the number of revertants for the positive control, *B* is the number of revertants per test plate, and *C* is the number of spontaneous revertants corresponding to the negative control without supplementation with mutagen.

Cytotoxicity of *T. catappa* Extracts Monitored by Acid Phosphatase Assay. Human hepatoma cells (Huh 7) and human normal liver cells (Chang liver) were obtained from the Medical College, National Taiwan University, Taipei, Taiwan. For determination of the viability of Huh 7 and Chang liver cells, the acid phosphatase assay described by Lin et al. (*16*) was followed. Huh 7 and Chang liver cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, and 100 μg/mL streptomycin sulfate (all obtained from Life Technologies Inc., Gaithersburg, MD) at 37 °C under 5% CO_2. The cells (3×10^3 cells/well, suspended in 180 μL of medium) were seeded and allowed to attach to 96-well plates and then cultivated for 24 h with supplementation of the extracts (20 μL/well to give final concentrations of 0, 31.3, 62.5, 125, 250, and 500 μg/mL). Prior to determining the number of viable cells, cells in each well were washed with 200 μL of phosphate-buffered saline (PBS; 1 × pH 7.4). After PBS was withdrawn and replenished with 100 μL of substrate solution containing 0.1 M sodium acetate, 0.1% Triton X-100, and 10 mM *p*-nitrophenyl-phosphate, cells were incubated at 37 °C for 2 h, and then the reaction was terminated by the addition of 10 μL of 1 N NaOH into each well. The contents of wells were subjected to absorbance determination at 410 nm with an ELISA reader (Dynex Technologies, New Lenox, IL).

Statistics and Replicates. Triplicate experiments were conducted, and means of the determinations with standard deviation are reported. The paired-samples *t* test was applied for statistical analyses using SPSS 8.0 for Windows (SPSS Inc., Chicago, IL).

Table 1. Mutagenicity of *n*-Hexane and SC-CO_2 Extracts of *T. catappa* Leaves toward *S. typhimurium* TA100

extract (mg/plate)	revertants/plate[a]
spontaneous revertants (control)	130 ± 8 (100)
n-hexane extracts	
0.05	132 ± 11 (102)
0.1	130 ± 7 (100)
0.2	128 ± 8 (98)
0.5	125 ± 11 (96)
SC-CO_2 extracts	
0.05	123 ± 9 (95)
0.1	121 ± 10 (93)
0.2	119 ± 12 (92)
0.5	124 ± 7 (95)

[a] Values are means with standard deviation (n = 3); values in parentheses are the relative percentages of revertants in proportion to the number of spontaneous revertants as a control.

RESULTS AND DISCUSSION

The Ames test is a short-term genotoxicity assay that enables detection of mutagens and antimutagens simultaneously using specified strains of *S. typhimurium* as indicator microorganisms (*15*). When one is screening for antimutagens, determination of their tendency to cause cytotoxicity or mutagenicity to the indicator microorganisms should be considered as a prerequisite step. When the *n*-hexane and SC-CO_2 extracts of *T. catappa* leaves were applied to *S. typhimurium* TA100 plates at concentrations up to 0.5 mg/plate, no cytotoxic activity was observed (data not shown). Results of the Ames test for mutagenicity of *n*-hexane and SC-CO_2 extracts of *T. catappa* leaves toward *S. typhimurium* TA100 are shown in **Table 1**. Test doses ranging from 0 to 0.5 mg/plate caused no observed mutagenicity of *S. typhimurium* TA100. This indicates that *T. catappa* extract at these concentrations was not mutagenic.

The antimutagenic activity conferred by *n*-hexane extracts of *T. catappa* to *S. typhimurium* TA100 against MNNG and B[*a*]P is shown in **Table 2**. When media were supplemented with MNNG or B[*a*]P for the Ames test, the *n*-hexane extracts exhibited a dose−response antimutagenicity in the range of 0.05−0.5 mg/plate. Extracts exhibited a higher antimutagenic activity against B[*a*]P compared to MNNG. The antimutagenicities of *n*-hexane extracts at a concentration of 0.5 mg/plate against B[*a*]P and MNNG were 69.4 and 41.6%, respectively. Cancer chemoprevention can be defined as prevention, inhibition, or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or as naturally

Table 2. Antimutagenicity of *n*-Hexane Extracts of *T. catappa* Leaves against Mutagens of MNNG and B[*a*]P as Determined with the Ames Test Using *S. typhimurium* TA100

	inhibition[a] (%)	
sample (mg/plate)	MNNG	B[*a*]P
0.05	18.19a	10.05a
0.1	26.16b	23.86b
0.2	33.63c	53.61c
0.5	41.59d	69.37d

[a] Results are means (n =3); values in a column followed by the same letter are not significantly different ($p > 0.05$). Spontaneous: 130 ± 11 revertants/plate. Positive control: [MNNG (5 μg/plate)] = 1147 ± 62 revertants/plate; {B[*a*]P (2 μg/plate)}: 881 ± 40 revertants/plate. Sample treatment: {B[*a*]P (2 μg /plate) + sample}; [MNNG (5 μg/plate) + sample]. Inhibition (%) = [(positive control − sample)/(positive control − spontaneous)] × 100.

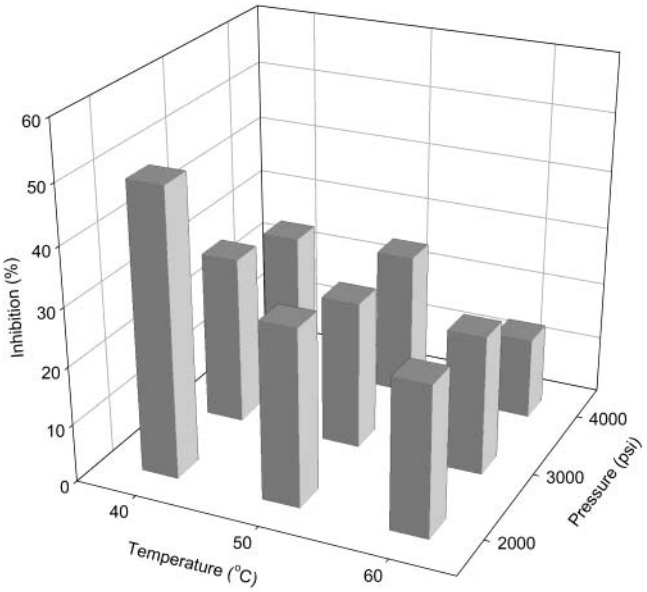

Figure 1. Antimutagenicity of SC-CO_2 extracts of the abscisic leaves of *T. catappa* against the mutagenicity of MNNG toward *S. typhimurium* TA100 (n = 3): spontaneous, 130 ± 11 revertants/plate; positive control [MNNG (5 μg/plate)], 1147 ± 62 revertants/plate; sample [MNNG (5 μg/plate) + sample (0.5 mg/plate)]; inhibition (%) = [(positive control − sample)/(positive control − spontaneous)] × 100.

occurring constituents of the diet (*17*). Initiation is the first step of carcinogenesis and usually results in genetic changes due to frequent attack by mutagens and/or mutagenic factors. It is apparent that the antimutagens against B[*a*]P and MNNG in the *T. catappa* leaves were extracted by *n*-hexane. *n*-Hexane extracts of *Psoralea corylifolia* fruit also exhibit a significant antimutagenic effect against B[*a*]P (*18*).

When media were supplemented with SC-CO_2 extracts of *T. catappa* leaves and MNNG or B[*a*]P and then used to culture *S. typhimurium* TA100 in the Ames test, antimutagenicity as affected by extraction temperature and pressure was evident (**Figures 1** and **2**). In general, the antimutagenicity of the SC-CO_2 extracts decreased with increases of extraction temperature and pressure. Among the various extracts, the highest antimutagenic activity was observed at 2000 psi and 40 °C. At a dose of 0.5 mg/plate, for extracts obtained at 2000 psi and 40 °C, 80% of B[*a*]P and 46% of MNNG mutagenicity were inhibited. In comparison, the SC-CO_2 extracts exhibited a higher antimutagenicity against B[*a*]P than against MNNG. This is in agreement with the observation that *n*-hexane extracts are more antimutagenic to B[*a*]P than to MNNG (**Table 2**). Alkylating

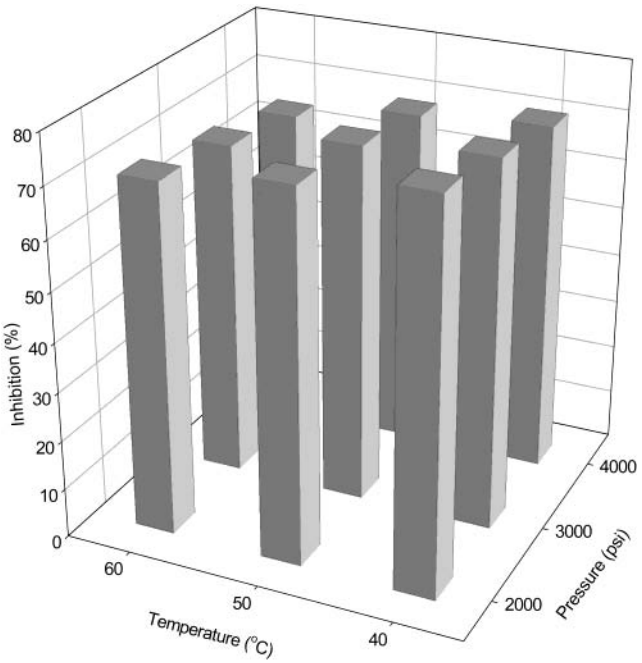

Figure 2. Antimutagenicity of SC-CO_2 extracts of the abscisic leaves of *T. catappa* against the mutagenicity of B[*a*]P toward *S. typhimurium* TA100 (n = 3): spontaneous, 146 ± 8 revertants/plate; positive control {B[*a*]P (2 μg/plate)}, 894 ± 32 revertants/plate; sample [B[*a*]P (2μg /plate) + sample (0.5 mg/plate)]; inhibition (%) = [(positive control − sample)/(positive control − spontaneous)] × 100.

agents (e.g., MNNG) and polycyclic aromatic hydrogencarbons (e.g., B[*a*]P) are known mutagens and widely distributed in the environment. These compounds tend to interact with DNA to form DNA adducts and eventually transform to induce cancer cells. An increased risk of lung cancer has been identified in occupational cohorts frequently exposed to B[*a*]P in petroleum factories (*19, 20*). Forestomach and glandular stomach carcinogenesis promoted by MNNG was inhibited by phenolic compounds in rats (*21*). Chemoprevention of cancer can be achieved by administration of one or more chemical compounds, either as individual drugs or as natural food components in the diet (*22*). A comparison of *n*-hexane and SC-CO_2 extracts indicates that antimutagenicity of the former was lower than the latter at the same applied dose. In addition to considering post-treatment of solvents, SC-CO_2 extraction represents more perspective than a conventional extraction method when *n*-hexane is used as a solvent to extract natural antimutagens from *T. catappa* leaves.

The viability of Chang liver and Huh 7 cells treated with SC-CO_2 extracts of *T. catappa* leaves using 2000 psi at 40 °C is shown in **Figure 3**. At doses ranging from 0 to 100 μg/mL, the viability of Chang liver cells decreased with an increase of dosage. For Huh 7 cells, a dose-dependent growth inhibitory effect was observed. At a dose range of 31.3−500 μg/mL, effects were 20−30% more viable Chang liver cells than Huh 7 cells. Apparently, the SC-CO_2 extracts were more cytotoxic to Huh 7 than to Chang liver cells. The more pronounced cytotoxicity of the SC-CO_2 extracts to hepatoma cells than to normal cells suggests a potential for developing cancer-preventive phytochemicals.

In conclusion, antimutagenic activity was observed in *n*-hexane and SC-CO_2 extracts of *T. catappa* leaves. By comparison, SC-CO_2 extraction renders advantages of efficiency, time required for extraction, low cost, and less environmental pollution. Because *T. catappa* trees are popular and produce tremendous amounts of abscisic leaves each year, considerable

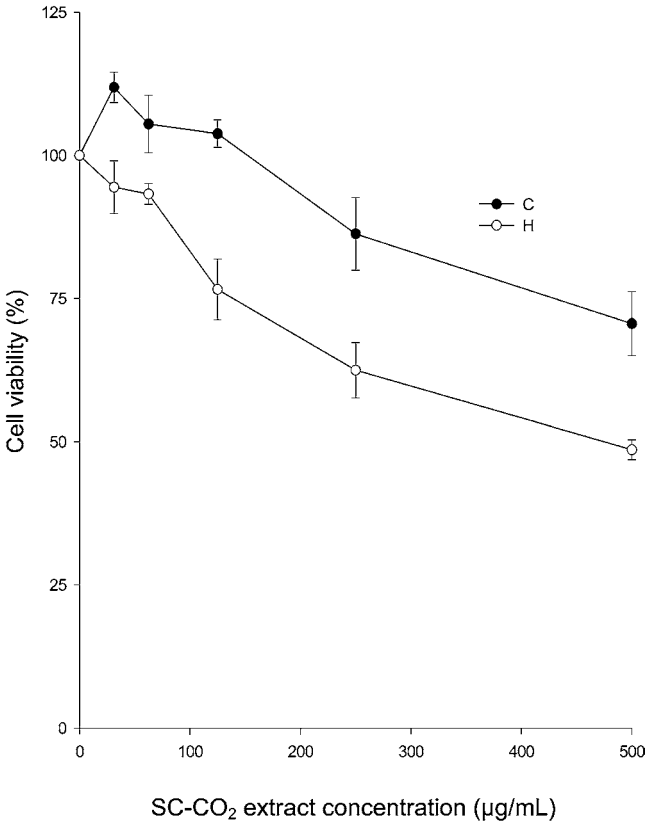

Figure 3. Cell viability of Chang liver (C) and Huh 7 (H) cells exposed to $SC\text{-}CO_2$ extracts (extracted at 2000 psi and 40 °C) of the abscisic leaves of *T. catappa* for 24 h ($n = 3$).

amounts of antimutagens could be extracted, in particular by $SC\text{-}CO_2$ extraction in a scale-up process. In addition, the observation that $SC\text{-}CO_2$ extracts are more cytotoxic to human hepatoma cells than to normal cells suggests the possibility of development of cancer preventive phytochemicals. This is in agreement with a report that $SC\text{-}CO_2$ extracts of *T. catappa* leaves have potent antioxidative activity (*11*). *T. catappa* leaves might be a novel source of biomedicinal phytochemicals. Further and intensive investigations focused on the identification of the active compounds in the extracts and the mode of action of specific constituents are needed.

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