Inhibitory Effects of *Cassia tora* L. on Benzo[*a*]pyrene-Mediated DNA Damage toward HepG2 Cells

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The effects of water extracts from *Cassia tora* L. (WECT) treated with different degrees of roasting on benzo[*a*]pyrene (B[*a*]P)-induced DNA damage in human hepatoma cell line HepG2 were investigated via the comet assay without exogenous activation mixtures, such as S9 mix. WECT alone, at concentrations of 0.1−2 mg/mL, showed neither cytotoxic nor genotoxic effect toward HepG2 cells. B[*a*]P-induced DNA damage in HepG2 cells could be reduced by WECT in a dose-dependent manner ($P < 0.05$). At a concentration of 1 mg/mL, the inhibitory effects of WECT on DNA damage were in the order unroasted (72%) > roasted at 150 °C (60%) > roasted at 250 °C (23%). Ethoxyresorufin-*O*-dealkylase activity of HepG2 cells was effectively inhibited by WECT, and a similar trend of inhibition was observed in the order unroasted (64%) > roasted at 150 °C (42%) > roasted at 250 °C (18%). The activity of NADPH cytochrome P-450 reductase was also decreased by unroasted and 150 °C-roasted samples (50% and 38%, respectively). Furthermore, glutathione *S*-transferase activity was increased by treatment with unroasted (1.26-fold) and 150 °C-roasted (1.35-fold) samples at 1 mg/mL. In addition, the contents of anthraquinones (AQs) in WECT, including chrysophanol, emodin, and rhein, were decreased with increasing roasting temperature. Each of these AQs also demonstrated significant antigenotoxic activity in the comet assay. The inhibitory effects of chrysophanol, emodin, and rhein on B[*a*]P-mediated DNA damage in HepG2 cells were 78, 86, and 71%, respectively, at 100 μM. These findings suggested that the decreased antigenotoxicity of the roasted samples might be due to a reduction in their AQs content.

Keywords: *Cassia tora L.; comet assay; antigenotoxicity; DNA damage; benzo[a]pyrene; anthraquinones; HepG2*

INTRODUCTION

There is considerable evidence that DNA damage is an important factor in carcinogenesis (*1*). Therefore, in theory, the most effective way to avoid carcinogenesis is to reduce the mutation of genetic materials. The single-cell gel electrophoresis (SCGE, or comet) technique to detect the DNA damage of mammalian cells is simple, rapid, sensitive, and visible. This assay can reflect different types of DNA damage, such as DNA single-strand breakage, alkali-labile AP site, or incomplete DNA repairing (*2, 3*); and it shows high sensitivity in detecting carcinogens (*4*). In recent years, this short-term experiment has not only been applied to evaluation of genotoxic agents, but also to screening of antimutagens and anticarcinogens (*5, 6*).

Benzo[*a*]pyrene (B[*a*]P), a polycyclic aromatic hydrocarbon (PAH), is extensively present in the environment. It is often observed in cigarette smoke, the surface of meat roasted with charcoal, or the incompletely combusted exhaust gas emitted from mills or automobiles (*7*). Although B[*a*]P is known to have no genotoxicity, its bioactivation by CYP-450 1A1 or other CYP-450 isoenzyme produces a variety of mutagenic or carcinogenic electrophiles. For example, benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) is the most representative ultimate carcinogen which goes a step further to form a covalent bond with DNA, RNA, or protein, and induces cancers (*8*). Most people are exposed to extremely low dosages of B[*a*]P or other PAHs in the environment. Research shows that contacting even a low dosage of mutagen could result in a genotoxic effect for a bioorganism. With comet assay, severe DNA breaking was found when C57BL/6 rat was fed with B[*a*]P (*9*). Hanelt et al. (*10*) compared the genotoxicity of B[*a*]P and its metabolite, BPDE, toward human MRC5CV1 cells with ^{32}P-postlabeling, HPRT gene mutation, and comet assay, and found that these carcinogens have extreme capacity for inducing DNA adducts and DNA damage. Consequently, evaluating DNA damage with comet assay will be a good index for evaluating cancer risks for bioorganisms.

Jue-ming-zi, seeds of the legume *Cassia tora* L., has physiological functions as an antiseptic, diuretic, diarrheal, antioxidant, and antimutagen (*11*−*14*). The water extract of *C. tora* L. (WECT) has been a healthy beverage in China. Previous study displayed that WECT possesses antimutagenicity effects on mutagens in food, such as 2-amino-6-methyldipyrido(1,2-*a*:3′:2′-*d*)imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 3-amino-1,4-dimethyl-5*H*-pyrido(4,3-*b*)indole (Trp-P-1), and B[*a*]P, in both the Ames test and comet assay. The major mechanism of the antimutagenicity was through suppressing the CYP-450 activity in rat's livers (*15*). Nevertheless, it still cannot be proved whether this antimutagenicity or desmutagenic action has similar modulation in cells. The disadvantage of lacking precise prediction for in vitro test is mainly due to the mislead-

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ing of xenobiotics metabolism system (*16*, *17*). Aden et al. (*18*) pointed out that the HepG2 cell, derived from human liver cancer, retains the characteristics of general normal liver cells and also possesses a bioactivation enzyme of PAHs (*19*). HepG2 reflects the effect of xenobiotics in bioorganisms more appropriately than general cells that lack metabolizing ability and thus need addition of S9 mix. HepG2 could be used directly to explore the modulation between xenobiotics and CYPs, and thus becomes the most favorable tool for evaluating chemical carcinogens (*20*).

On the basis of the above considerations, this study was carried out to explore whether WECT without external enzyme would still have the inhibitory effect on DNA damage in HepG2 cells induced by B[*a*]P. The effect and mechanism of WECT on the major bioactivating enzyme CYP-450 1A1, as well as glutathione S-transferase of B[*a*]P in HepG2 cells, were also examined.

MATERIALS AND METHODS

Materials. The seeds of *Cassia tora* L. were obtained from a local market at Taichung, Taiwan. Benzo[*a*]pyrene, chrysophanol, emodin, rhein, *N*-lauroyl sarcosinate, ethidium bromidine, Triton X-100, 7,8-benzoflavone, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione, 7-ethoxyresorufin, cytochrome C and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO), sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride were purchased from the E. Merck Co. (Darmstadt, Germany). Normal melting point agarose (NMA), low melting point agarose (LMA), Dubeccos' Modified Eagle Medium, fetal bovine serum (FBS), trypsin-EDTA (T/E), penicillin-streptomycin, l-glutamine, and MEM sodium pyruvate solution were purchased from Gibco BRL Co (Grand Island, NY). Tris and the protein assay kit were purchased from Bio-Rad laboratories (Hercules, CA). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from Wako Pure Chemical Co. (Osaka, Japan).

Sample Preparation. To obtain *Cassia tora* L. seeds with different degrees of roasting, samples were washed and sundried and then left unroasted or roasted at 150 and 250 °C (internal temperature) for 5 min using a roasting machine (rate 20 rotation/min, Nankung Machine Co., Taiwan). Each unroasted and roasted sample (50 g) was extracted with boiling water (500 mL) for 5 min, and the filtrate was freeze-dried. The yields of extracts from unroasted, roasted at 150 °C, and roasted at 250 °C of *C. tora* L. were 5.92, 6.00, and 3.90%, respectively.

Cell Cultures. Human hepatoma cells (HepG2 cells; Food Industry Research and Development Institute, Hsin Chu, ROC) were grown in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.37% (w/v) $NaHCO_3$, 0.1 mM NEAA, 1 mM sodium pyruvate, and 0.03% l-glutamine. Cells were maintained at 37 °C in a humidified atmosphere at 5% CO_2.

Single Cell Gel Electrophoresis Assay (Comet Assay). *Genotoxicity of WECT, Chrysophanol, Emodin, and Rhein toward HepG2 Cells.* The comet assay was performed according to the methods of Singh et al. (*3*) and Musatov et al. (*21*) with slight modifications. For the genotoxicity studies, cells were treated with different concentrations of WECT (0.1−2 mg/mL) or anthraquinones dissolved in DMSO/chrysophanol, emodin, and rhein (1−100 μM) (the DMSO concentration in the incubation medium never exceeded 1%). Control incubations contained the same concentration of DMSO or PBS. Cells were incubated as mentioned above. After incubation, cells were trypsinized with 0.5 mL/plate trypsin-EDTA and centrifuged at 800*g* for 5 min at 4 °C. Cell number and viability (Trypan blue exclusion) were determined by using a Neubauer improved haemocytometer, before and after samples were treated. After centrifugation, cells were resuspended in 75 μL of low-melting poin-agarose (LMA, 1% in PBS without calcium and magnesium) and plated on fully frosted slides, which had been covered with 75 μL of normal-melting-point agarose (NMA, 1% in PBS without calcium and magnesium). The slides were kept on ice for 5 min. After solidification, a top layer of 75 μL of LMA was added, then allowed to solidify for 5 min. Slides were then immersed in freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% *N*-lauroyl sarsosinate, 1% (v/v) Triton X-100, and 10% DMSO) for 1 h at 4 °C. The slides were then left in electrophoresis buffer (0.3 M NaOH and 1 mM Na_2EDTA) for 20 min at 4 °C and placed in a gel electrophoresis tank. Electrophoresis was conducted at 4 °C for 20 min with 25 V and 300 mA current. After electrophoresis, the excess alkali was neutralized twice in Tris buffer (0.4 M Tris, pH 7.5) for 5−10 min. Finally, the slides were stained with 50 μL of ethidium bromide (20 μL/mL) and examined at a Nikon EFD-3 fluorescence microscope (Japan) with excitation filter BP at 543/10 nm and a 590 nm emission barrier filter. Objective measurements of the distribution of DNA were performed for a sample of cells by using a Komet 3.1 (Kinetic Imaging Ltd., Liverpool, UK). One hundred cells on each slide (scored at random) were classified according to the relative intensity of fluorescence in the tail. The degree of DNA damage was scored by tail moment (TM; tail moment = tail length × tail DNA% /100).

Effects of WECT, Chrysophanol, Emodin, and Rhein on B[a]P-mediated DNA Damage toward HepG2 Cells. As described above, HepG2 cells were treated with a series of concentrations of WECT (final concentrations 0.1−2 mg/mL) or chrysophanol, emodin, rhein (final concentrations 1−100 μM), or 7,8-benzoflavone (100 μM) and B[*a*]P (100 μM). The plates were incubated at 37 °C for 24 h in 5% CO_2. After incubation, the cells were washed with PBS and trypsinized with 0.5 mL/plate trypsin-EDTA during 5−10 min. Medium (1 mL) was added, cells were centrifuged at 800×*g* for 5 min at 4 °C, the supernatant was discarded, and the cells were resuspended in 75 μL of low-melting-point agarose (LMA, 1% in PBS without calcium and magnesium) for comet analysis.

All test anthraquinones (chrysophanol, emodin, and rhein) and 7,8-benzoflavone were dissolved in dimethyl sulfoxide (DMSO); DMSO concentration in the incubation medium never exceeded 1%. Control incubations contained the same concentration of DMSO or PBS.

Enzyme Assay. *Preparation of Whole Cell Homogenate.* All enzymic measurements of cultured cells were carried out using cell homogenates. HepG2 cells (1×10^6) were seeded onto 60-mm culture plates with 3 mL of DMEM complete medium in a humidified incubator at 37 °C with 5% CO_2. After 24 h of incubation, cells were treated with different concentrations of WECT (final concentrations 0.1−2 mg/mL) or 100 μM 7,8-benzoflavone at 37 °C for 24 h in 5% CO_2. After incubation, the cells were collected by trypsinization, washed with PBS and homogenized in PBS, pH 7.6, in a motor-driven Potter-Elvehjem homogenizer (800×*g*), equipped with a Teflon pestle. Cell homogenates were stored at −80 °C until analysis.

Determination of Ethoxyresorufin O-deethylase Activity. Ethoxyresorufin *O*-deethylase (EROD) activity was used as a measurement of CYP 1A1 activity as described by Grant et al. (*22*). EROD was measured by using 0.1 mL of cell homogenate with 0.3 mM NADPH, 10 μM dicumarol, and 5 μM ethoxyresorufin (substrate) in a final volume of 0.5 mL of 0.1 M sodium phosphate buffer, pH 7.6, at 37 °C in a shaking water bath for 20 min. Reaction was stopped by the addition of 0.25 mL of 5% ZnSO4 and 0.25 mL saturated barium hydroxide, and the sample was centrifuged at 2000×*g* for 15 min at 4 °C. The deproteinised supernatant (0.5 mL) was removed into a clean tube, and 1 mL of 0.5 M glycine-NaOH buffer, pH 8.5 was added. Fluorescence of the reaction mixture was read at an emission wavelength of 586 nm and an excitation wavelength of 522 nm. Enzyme activity was expressed as nmol/min/mg protein.

Determination of NADPH Cytochrome P-450 Reductase Activity. The activity of NADPH cytochrome P-450 reductase was spectrophotometrically measured by the reduction of

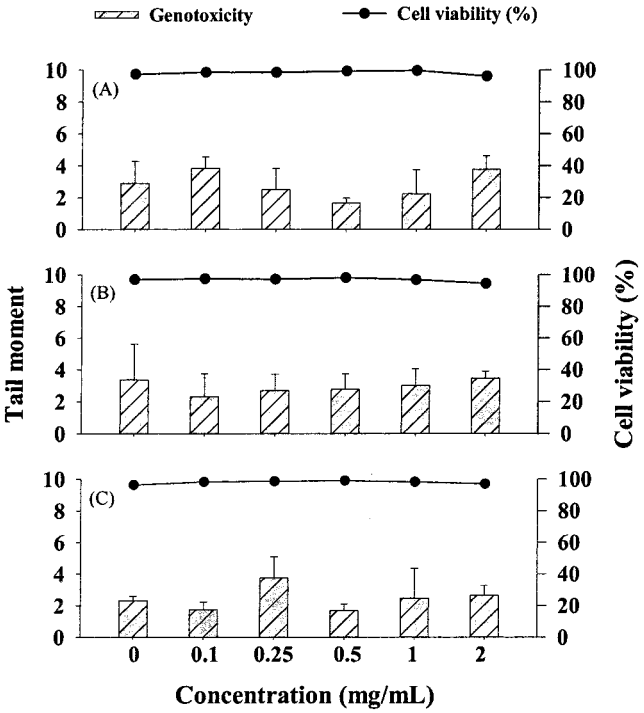

Figure 1. Cytotoxicity and genotoxicity of water extracts from *Cassia tora* L. prepared under different degrees of roasting toward HepG2 cells. (A) Unroasted; (B) roasted at 150 °C, 5 min; (C) roasted at 250 °C, 5 min. Results are mean ± SD for $n = 3$.

cytochrome *c* (125 μM) at 550 nm in the presence of 0.1 mL of cell homogenate, 5 mM $MgSO_4$, 10 mM NADPH, and 15 mM KCN to block nonmicrosomal enzyme activity due to possible mitrochondrial contamination. Enzyme activity was expressed as nmol/min/mg protein.

Determination of Glutathione S-transferase Activity. The glutathione S-transferase (GST) activity was determined spectrophotometrically according to Dierickx (*23*). GST activity was measured using 0.5 mL of cell homogenate, 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM glutathione as subtrate in a final volume of 1 mL of 0.1 M sodium phosphate buffer, pH 6.5, at 37 °C. Enzyme activity was calculated by the change of the absorbance value at 340 nm for 5 min. Enzyme activity was expressed as nmol/min/mg protein. The protein content of cell homogenates was determined using a Bio-Rad protein assay kit.

Statistical Analysis. All analyses were run in triplicate and averaged. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance were performed using the ANOVA procedure. Significant differences ($P < 0.05$) between the means were determined using Duncan's multiple range test.

RESULTS AND DISCUSSION

Cytotoxicity and Genotoxicity of WECT on Human Liver Cancer Cell HepG2. The cytotoxicity and genotoxicity of WECT after reacting with the HepG2 cell for 24 h were investigated. The results are shown in Figure 1. The viability of HepG2 cells was higher than 90%, and at a concentration of 0.1−2 mg/mL, the WECT showed no toxicity toward these cells. The DNA tail moment (TM) of the treated HepG2 cells was not significantly different ($P > 0.05$) from that of the control group.

Most of the short-term genotoxicity tests in vitro, using bacteria or mammal cells as assay models, lack xenobiotic-biotransformation enzyme systems. Therefore, S9 taken from the rat liver mitochondria has been

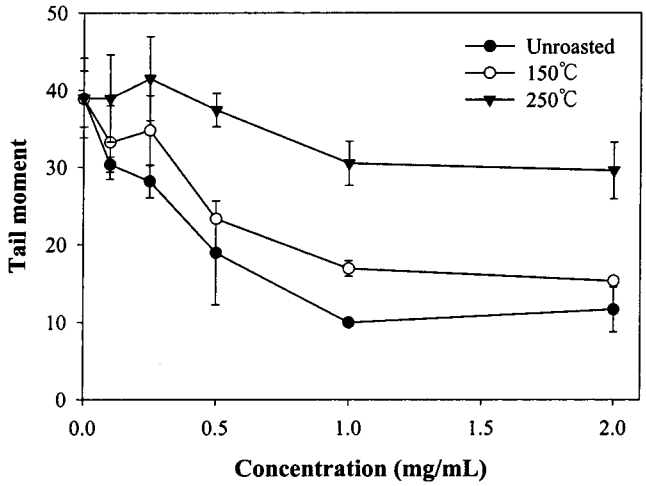

Figure 2. Inhibitory effect of water extracts from *Cassia tora* L. prepared under different degrees of roasting on the genotoxicity of B[*a*]P toward HepG2 cells. DNA tail moment that was treated with CYP-450 1A1 inhibitor 7,8-benzoflavone (100 μM) for 24 h was 6.3. Results are mean ± SD for $n = 3$.

widely used to simulate the metabolism reaction of the xenobiotics in bioorganisms. However, some scholars have expressed concern regarding whether test results from the metabolism system not belonging to the host itself could precisely predict in vivo results (*17*). Erroneous judgments resulting from external enzymes occur commonly. A typical example is safrole or hexamethylphosphoramide (HMPA) that could induce rat tumor even though a negative response was presented in the in vitro genotoxicity assay with added S9 mix. HepG2 cells, derived from human liver cancer, reserved the general characteristics of human liver cells and erroneous judgments would not happen when examining safrole, HMPA, and pyrene using these cells. Knasmuller et al. (*20*) indicated that HepG2 cells, containing various phase I and phase II enzymes, reflected the metabolism of xenobiotics in bioorganisms more than general cells that have no metabolism ability or the systems that need extra S9 mix. Exploring all modulation between xenobiotics and CYPs, and also extensively predicting the interaction between living forms and pharmaceuticals, have made HepG2 cells the most favorable system of evaluating chemical carcinogens.

Effects of WECT on HepG2 Cells DNA Damage Induced by B[*a*]P. As Figure 2 shows, *C. tora* L. unroasted, roasted with 150 °C, and roasted with 250 °C at 1 mg/mL had suppressing effects of 72, 60, and 23%, respectively, on DNA damage of HepG2 (TM ÷ 38.9) induced by B[*a*]P. However, the TM of samples did not decrease obviously ($P < 0.05$) with higher concentrations, possibly because the function of the system had come to its limit. Unroasted samples exhibited the best inhibitory effect. The higher degree of roasting resulted in less protecting effects. CYP-450 1A inhibitor 7,8-benzoflavone (BF, 100 μM) was added in the experiment as a negative control. The suppressing rate came to 84% after BF reacted with B[*a*]P/HepG2 under the same conditions for 24 h and decreased the DNA damage induced by the inducer to about TM ÷ 6.3 (triple experiments). These results have proved that the design of this experiment could truly reflect the possibilities of representing antigenotoxicity of a dismutagen after interfering internal activation enzymes.

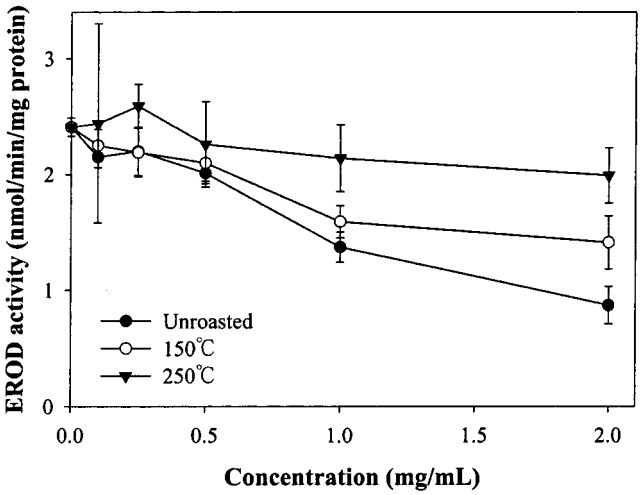

Figure 3. Inhibitory effect of water extracts from *Cassia tora* L. prepared under different degrees of roasting on ethoxyresorufin *O*-deethylase (EROD) activity in HepG2 cells. Results are expressed as the mean and standard deviation of three measurements from separate cultures. EROD activity that was treated with inhibitor 7,8-benzoflavone (100 μM) for 24 h was 0.54 nmol/min/mg protein.

The dosage of BF in this experiment caused no cytotoxicity and DNA damage on HepG2 (data not shown).

Results of this experiment have proved that water extracts from *C. tora* L. prepared under different degrees of roasting possessed protective effect toward DNA damage induced by B[*a*]P without addition of exogenic enzyme S9 mix, and that WECT had the same antigenotoxicity effect (unroasted > 150 °C-roasted > 250 °C-roasted) shown previously by the Ames test in a bacterial system and the comet assay in a lymphocyte system (*15*). This shows the antigenotoxicity of WECT in the experiment system that needed S9 mix activation, and that it also had similar modulating functions on cells with related internal metabolism enzymes. To prove the possibilities of this assumption, it is necessary to explore the effects of WECT on the phase I and phase II enzymes in HepG2 cells. This will be discussed in the next section.

Effects of WECT toward Enzyme Activation of Ethozyresorufin *O*-deethylase (EROD, CYP-450 1A1) in HepG2 Cells. As Figure 3 shows, the control with PBS replacing the sample treatment group, EROD enzyme activity was 2.41 nmol/min/mg protein, and this enzyme activity clearly reduced to 0.54 nmol/min/mg protein (a suppressing effect of 78% after adding the CYP-450 1A inhibitor 7,8-BF (100 μM). WECT was added to the experiment groups. At concentrations of 0.5 to 2 mg/mL it possessed a dose-dependent inhibitory effect. Water extracts of unroasted *C. tora* L., and that roasted with 150 °C, and 250 °C had suppressing effects of 64, 42, and 18%, respectively.

There are many metabolism ways after B[*a*]P enters the bioorganism: the main metabolism way is through CYP-450 1A1 (EROD) activation to produce (−)-*trans*-7,8-dihydroxy-7,8-dihydro-benzo[*a*]pyrene, and then further through epoxidation to form the final carcinogens benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) (*8*). BPDE, a product of PAH metabolism with mutagenicity and carcinogenicity, would attack DNA directly to form DNA adduct, break DNA single strand, form sister chromosome exchange (SCEs), cause chromosome aberrations, or result in HPRT gene mutation (*10, 24*). Diamond et al. (*19*) pointed out that HepG2 possesses all the metabolism enzymes that activate B[*a*]P. If the enzyme activation of first generation cultivating rat liver cell CYP-450 1A1 could be induced by TCDD (2,3,7,8-tetrachloro-*p*-dioxin), production of the B[*a*]P−DNA adduct could be promoted (*25*).

Ferguson (*26*) indicated that the best way for antimutagenicity to suppress the toxicity or mutagenicity of a gene was to affect its metabolism and block its activation. Lee et al. (*27*) showed that the mechanism of extracts of pine cone to suppress B[*a*]P mutagenicity was to interfere with CYP-450 enzyme activation by S9 mix, and the key was suppressing NADPH CYP-450 reductase. Antimutagens such as green tea (black tea) were related to its suppressing activation enzymes in a similar system (*28, 29*).

This experiment showed that WECT had suppressing effects on EROD for activation of B[*a*]P. The correlation (*r*) between this suppressing effect and genotoxicity of samples was unroasted (0.88, $P < 0.05$), roasted at 150 °C (0.94, $P < 0.01$), and roasted at 250 °C (0.96, $P < 0.01$). Inferring from this, HepG2 cell DNA damage induced by B[*a*]P and suppressed by WECT may be through suppressing EROD. However, deserving of notice is that the unroasted samples had the best inhibitory effect on both antigenotoxicity and enzyme activity, but the correlation was less than that of the other two. It suggests that there are still other mechanisms of antimutagenicity of WECT.

First, the molecule complex showed that water extracts of unroasted *C. tora* L. could have direct interaction with B[*a*]P to reduce the biological function of mutagenicity (*15*). Second, more than just forming BPDE carcinogen through activation of CYP-450 for B[*a*]P, peroxide−B[*a*]P-7,8-dione (BPQ) also can be formed in liver cells through dihydrodiol dehydrogenase catalysis (*30*). The reaction process was proved to be with production of reactive oxygen species such as •OH, $O_2^{\bullet -}$, H_2O_2, and semiquinone anion radicals and followed with ϕ X 174 DNA single-strand breaking and of *S. typhimurium* TA97, TA100, TA102, and TA104 (*30*). Gútze et al. (*31*) indicated that B[*a*]P would be readily self-oxidized to produce H_2O_2. It would easily release the hydroxyl radical with existence of (Fe^{2+}) and induce 8-OH-dG in Sprague−Dawley rat liver and lung cells. WECT had the characteristics of chelating Fe^{2+} and scavenging hydroxyl radical, and, similarly, its activation was reduced with increasing degrees of roasting (*14*). Therefore, assuming that DNA damage induced by B[*a*]P in the comet assay is related to the free radicals produced in its activation process, antigenotoxicity of WECT may partly be from its antioxidant activity. Third, it has been known that the detoxification mechanism was mainly through a glutathione *S*-transferase (GST) effect (*32*). WECT could reduce the genotoxicity caused by B[*a*]P on HepG2 cells through promoting GST. This assumption will be discussed in the following experiments.

Effect of WECT on Enzyme Activation of NAPPH CYP-450 Reductase in HepG2 Cells. As stated above, the major mechanism of forming antigenotoxicity of WECT is to suppress B[*a*]P activation. Because NADPH CYP-450 reductase is the carrier of electrons to CYP-450, the monooxygenase could function only while the cytochrome can accept electrons smoothly. Therefore, if NADPH CYP-450 reductase is inhibited, EROD activity would surely be affected. Effects of WECT on activation of NADPH CYP-450 reductase are

Table 1. Effects of Water Extracts from *Cassia tora* L. Prepared under Different Degrees of Roasting on NADPH Cytochrome P-450 Reductase in HepG2 Cells

concentration (mg/mL)	activity (nmol/min/mg protein)[a]		
	unroasted	150 °C	250 °C
0	245.8 ± 5.4^{ab}	245.8 ± 5.4^{a}	245.8 ± 5.4^{a}
0.1	198.3 ± 22.9^{ab}	196.1 ± 37.9^{ab}	247.3 ± 27.0^{a}
0.25	189.9 ± 31.0^{b}	189.8 ± 14.6^{b}	215.4 ± 47.1^{a}
0.5	167.7 ± 23.4^{bc}	185.7 ± 16.8^{bc}	230.5 ± 24.7^{a}
1	137.0 ± 18.9^{c}	169.1 ± 20.0^{c}	211.8 ± 37.8^{a}
2	124.2 ± 11.9^{c}	151.3 ± 11.0^{c}	198.1 ± 13.4^{a}

[a] Results are expressed as the mean and standard deviation of three measurements from separate cultures. [b] Values in column with different superscripts are significantly different ($P < 0.05$).

shown in Table 1. Compared to that of the control group (245.8 nmol/min/mg protein), reductase activity of HepG2 cells treated for 24 h with water extracts of unroasted and 150 °C-roasted *C. tora* L. was suppressed at concentration ≥ 0.25 mg/mL ($P < 0.05$). The suppressing effect was 50% and 38% when the reacting dosage was 2 mg/mL. The sample of 250 °C-roasted *C. tora* L. had no effects on reductase activity.

Sipes and Gandolfi (*33*) indicated that suppressing NADPH CYP-450 reductase was an effective mechanism for interfering with the progression of cancer. Many antimutagens, such as extracts of pine nuts (*27*), chlorophyll (*34*), menadione (*35*), and (+)-catechin (*36*), could interfere with electron transport. NADPH transfers electrons to cytochrome and makes CYP-450 incapable of activating mutagens through roles of electron acceptors. Therefore, suppressing the reducing activation could be defined as one of the causes of WECT's action on EROD. Some other reports pointed out that mutagens such as heterocyclicamine (*37*) or paraquat (*38*) could lead to oxidative damage of gene factors through activation of NADPH CYP-450 reductase to produce reactive oxygen substances. Consequently, results of WECT suppressing the enzyme could involve reacting process of breaking activation process of mutagen to produce reactive oxygen substances.

Effects of WECT on Enzyme Activation of Glutathione S-transferase (GST) in HepG2 Cells. Phase II biotransferase enzyme systems exist mainly in the cytoplasm of liver cells. After living forms contacted with xenobiotic substances, phase II enzymes would react with it to increase its hydrophilicity. This increases substantially the probability of the xenobiotic being excreted, so its metabolism is taken as a detoxification reaction. Halliwell and Gutteridge (*39*) pointed out that the detoxification mechanism of GST was to promote binding of the −SH group of glutathione with electrophilic compounds. This forces the conjugated compound to become more water-soluble and more easily excreted from the cells.

It was known that B[*a*]P exerted its detoxification functions through GST (*32*). Therefore, exploring the effects of WECT on GST activity of HepG2 cells might reveal their function in lessening the genotoxicity of B[*a*]P. As Table 2 shows, water extracts of only unroasted and 150 °C-roasted *C. tora* L. at 1 mg/mL promoted enzyme activation in HepG2 cells. These two water extracts increased the enzyme activity by 1.26 and 1.35 times, rescpectively.. The effect of the water extract of the 250 °C-roasted sample on GST activity was not significant ($P > 0.05$). Hu and Singh (*40*) showed that diallyl sulfide, diallyl trisulfide, and dipropyl sulfide in garlic enhanced the GST activity of A/J

Table 2. Effects of Water Extracts from *Cassia tora* L. Prepared under Different Degrees of Roasting on Glutathione *S*-transferase in HepG2 Cells

concentration (mg/mL)	GST activity (nmol/min/mg protein)[a]		
	unroasted	150 °C	250 °C
0	43.4 ± 0.7^{b}	43.4 ± 0.7^{b}	43.4 ± 0.7^{a}
0.1	43.9 ± 3.3^{b}	47.6 ± 5.4^{b}	60.8 ± 9.3^{a}
0.5	46.8 ± 0.4^{b}	47.5 ± 5.8^{b}	59.3 ± 2.9^{a}
1	54.8 ± 0.5^{a}	58.6 ± 4.9^{a}	54.6 ± 9.2^{a}

[a] Results are expressed as the mean and standard deviation of three measurements from separate cultures. Values in column with different superscripts are significantly different ($P < 0.05$).

rats and reduced the rate of lung cancer of rats under high dosages of B[*a*]P. There was 1.5 times as much promoting effect for enzyme activity in HepG2 cells treated with dially1 sulfide (*20*). Bu-Abbas et al. (*41*) indicated that three phase-II detoxicating enzymes (GST, glucuronosyl transferase, and epoxide hydrolase) were promoted in rats after four weeks of adding 2.5−7.5% (w/v) water extracts of green tea to their diets. Anticancer properties of extracts of rosemary were related to its promoting of GST activity (*42*). Thus, Prectera and Talalay (*43*) brought up that evaluating the ability to promote phase-II detoxification enzymes would be one of the indexes for detecting antimutagenicity and anticancer action of substances.

Unroasted *C. tora* L. reduced 30% of the DNA damage induced by Trp-P-1 after incubating with human lymphocyte cells for 30 min (*15*). The samples were thus inferred to contain activation that promoted correlative detoxification enzyme inside the cells. This study showed that promoting GST activity of water extracts from unroasted and 150 °C-roasted *C. tora* L. might be another reacting mechanism of antigenotoxicity more than suppressing EROD.

Cytotoxicity, Genotoxicity, and Antigenotoxicity of Chrysophanol, Emodin, and Rhein on HepG2 Cells. Natural herbs contain a large number of potential anticancer materials. For example, within *Rhei rhizoma*, *Scutellariae radix*, and *Rehmanniae radix*, there is antimutagenicity that suppresses B[*a*]P (*44*). The major active component is probably a derivative of anthraquinone (AQ); and its mechanism was considered as suppressing exogenic activation enzymes (*45*). Analyzed with HPLC, WECT contained three known 1,8-dihydroxyanthraquinone (1,8-HQ) chemical compounds: chrysophanol, emodin, and rhein (*15*). To infer the previous results of this study (Trp-P-1/comet assay), strength of the antigenotoxicity of WECT depended on the relative quantities of AQ. Therefore, this experiment used these three 1,8-HQ compounds to explore antigenotoxicity in cells.

As Figure 4 shows, the TM of chrysophanol was not statistically different ($P > 0.05$) on DNA damage from that of the control group, suggesting that it had no genotoxicity. Adding B[*a*]P or not did not affect the toxicity of chrysophanol on cells. The cell viability was all ≥ 90% and would not pose interference on genotoxicity experiments. As for DNA damage induced by B[*a*]P (TM≒28), there were suppressing effects of 15−78% in selected concentrations (1−100 μM) of chrysophanol. Emodin (Figure 5) that suppressed B[*a*]P the most possessed 44% protective effect at 10 μM. The highest suppressing effect reached 86% and even had similar activation performance to 7,8-benzoflavone (BF) in the same concentration (88%). Emodin (1−100 μM) showed neither cell toxicity nor genotoxicity on HepG2 cells.

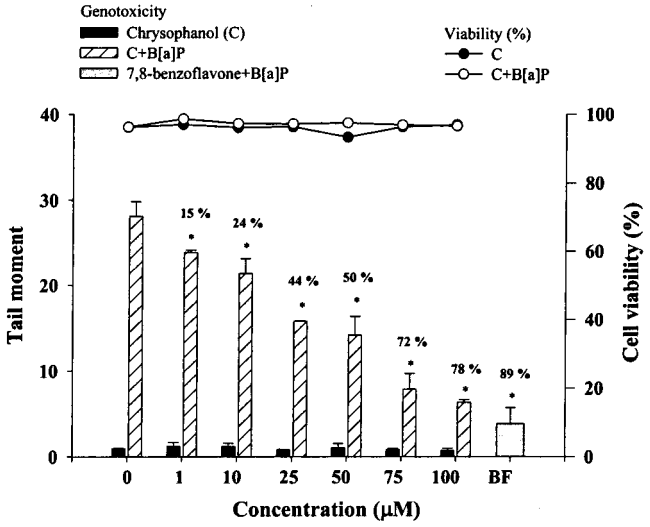

Figure 4. Inhibitory effect of chrysophanol on HepG2 DNA damage induced by B[*a*]P. Results are mean ± SD for $n = 3$. *$P < 0.001$ is significantly different by comparison with the control.

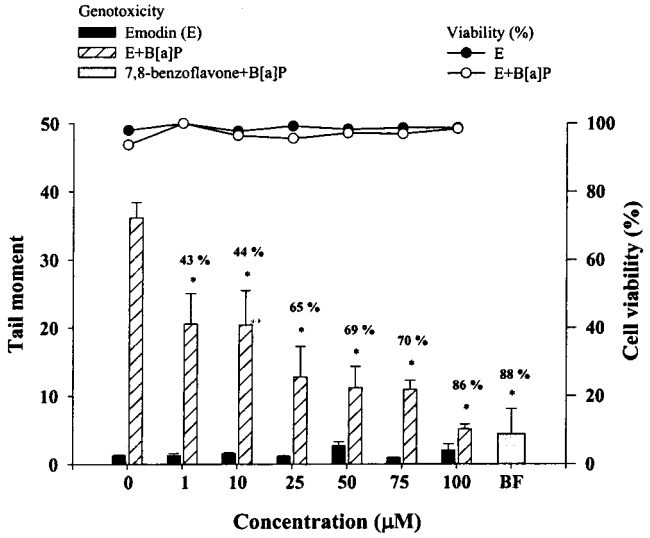

Figure 5. Inhibitory effect of emodin on HepG2 DNA damage induced by B[*a*]P. Results are mean ± SD for $n = 3$. *$P < 0.001$ is significantly different by comparison with the control.

The suppressing effect of rhein in low concentration was obviously low (Figure 6). It possessed antigenotoxicity when its concentration was more than 18 μM and could reduce, at most, 71% of DNA damage (100 μM). Moreover, it did not affect the viability of HepG2 cells, which always came to more than 90%, whether the inducer B[*a*]P was added or not. Deserving of note is that, after electrophoresis, HepG2 cells came to 5.2 TM after being simply treated with 100 μM rhein. It had significant difference ($P < 0.05$) compared to, and had less DNA damage on, HepG2 cells.

The major active component of *C. tora* L. is the anthraquinone compounds (*12*, *13*, *46*). Yen and Chung (*14*) indicated that along with the increasing of roasting degrees of WECT, the quantity of AQ decreased. This study showed, from analyzing composition of AQ in WECT with HPLC, that unroasted *C. tora* L. contained the most rhein in 1,8-HQ, about 10.42 mg/g WECT. Secondary were 0.61 mg/g WECT of chrysophanol and 0.28 mg/g WECT of emodin. The quantity of 1,8-HQ similarly decreased along with the increasing of roasting

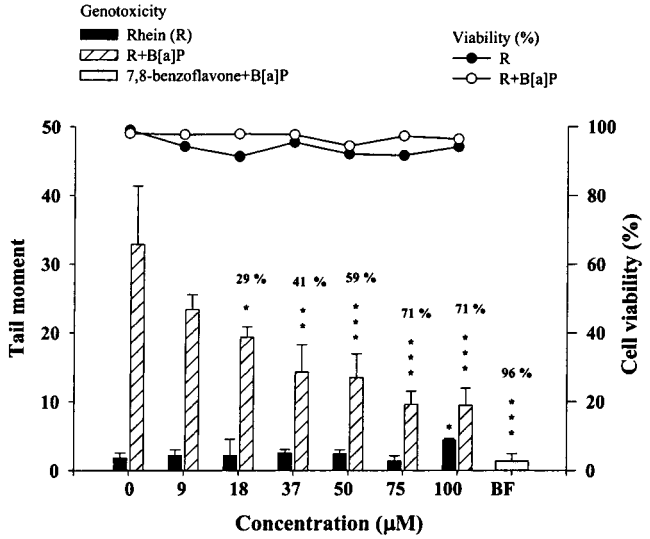

Figure 6. Inhibitory effect of rhein on HepG2 DNA damage induced by B[*a*]P. Results are mean ± SD for $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ are significantly different by comparison with the control.

degrees. The sample roasted at 250 °C could not even be analyzed for the above three components of 1,8-HQ. Water extracts of unroasted *C. tora* L. (2 mg/mL) contained chrysophanol 5 μM, emodin 2 μM, and rhein 75 μM.

On the basis of the above results, the intensity of antigenotoxicity of water extracts from *C. tora* L. might relate to the change of AQs quantity. Although rhein did not perform as well as the other two of the three 1,8-HQ compounds, it made up its activity difference because of its advantage of quantity, and supplied the most suppressing effect on DNA damage induced by B[*a*]P. At the same time, samples roasted at 250 °C contain no 1,8-HQ compounds, but still show protection of 23%, indicating that there may be other active components in WECT that are worthy of being explored.

Another part of this experiment worthy of discussion is that anthraquinones in *C. tora* L. showed genotoxicity in some in vitro assays. Chrysophanol, emodin, etc., upon metabolism by rat liver microsome enzyme (CYP-450), might give products with genotoxicity (*47*, *48*) and show mutagenicity in various bacterial and cell test systems (*49*−*51*). On the contrary, Meng et al. (*52*) in an internal experiment indicated that when feeding a rat with the highest suggested daily intake of 2000 mg/kg of emodin and then measuring the emodin amount in the rat's internal plasma, emodin reached 190 μg/mL, the highest in plasma (about 700 μM in this experiment). It is 10 times or more than the genotoxicity produced in the studies of Westendorf et al. (*50*) and Muller et al. (*51*). Nevertheless, no cytotoxicity or micronucleus caused by emodin were discovered in the study. Heidemann et al. (*53*) also pointed out that rhein and aloe-emodin have no mutagenic or carcinogenic reactions. The U.S National Toxicology Program conducted rat internal tests on subacute toxicity and mutagenicity of 30 suspected mutagenic chemical materials in 1996. Anthraquinone and emodin were categorized as probable noncarcinogens and possible noncarcinogens, i.e., they had no unfavorable mutagenic actions on developing animals (*54*).

As confirmed in this experiment, enzyme metabolism systems from different sources do give different results. Chrysophanol, emodin, and rhein possess antigenotox-

icity that suppresses DNA damage induced by B[*a*]P in cells. In the meantime, there was no DNA damage after reaction for 24 h in the activation of HepG2 cells enzyme system. However, a cell model is only the initial screening tool; clinical work is still necessary.

CONCLUSION

WECT reacted under HepG2 cell cultivating model of nonexogenic enzyme, possesses suppressing effects for DNA damage induced by indirect mutagen B[*a*]P. The mechanism involved in antigenotoxicity includes suppressing EROD, NADPH CYP-450 reductase in cells and promoting GST activity. The intensity of antigenotoxicity of WECT might be related to the quantity of AQs present.

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