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Antiproliferative and Anticarcinogenic Enzyme-Inducing Activities of Green Tea Seed Extract in Hepatoma Cells

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Abstract We investigated the catechin content in green tea leaf (GTL) and green tea seed (GTS), the antiproliferative and detoxifying phase II enzyme-inducing activities of the methanolic (80%, v/v) extracts from GTL and GTS. GTL and GTS contained 8,685±1,061 and 108±32 μg/g epigallocatechin gallate (EGCG), 11,486±506 and 116±72 μg/g epigallocatechin (EGC), 3,535±308 and 821±95 μg/g epicatechin gallate (ECG), and 1,429±177 and 37±44 μg/g epicatechin (EC), respectively. The methanolic extract of GTS showed a greater increase in quinone reductase activity and antiproliferation potential against mouse hepatoma cells than GTL extract did. GTS treatment resulted in the accumulation at sub-G1 phase of mouse hepatoma hepa1c1c7 cells as assessed by flow cytometry. Enhancement of phase II enzyme activity by GTS extract was shown to be mediated, directly or indirectly, via interaction with the antioxidant response element (ARE) sequence in the genes encoding the phase enzymes. As the catechin content in GTS was significantly lower than that in GTL, components other than catechins appear to be responsible for the anticarcinogenic activity of the seed. In summary, these results suggest that the 80% methanolic extract of GTS deserves further study to evaluate its potential as an anticarcinogenic agent and to investigate its mechanism of action.

Key words: green tea seed, antiproliferative, phase II enzyme, quinone reductase, cell cycle

Introduction

Tea (*Camellia sinensis* L.) and its constituents have been shown to inhibit tumorigenesis in many animal models, including those for cancers of the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, pancreas, bladder, breast, and prostate (1-3). The major bioactive constituents of green tea are catechins which belong to the polyphenol family. (-)-Epigallocatechin-3-gallate (EGCG) is the most abundant and biologically active catechin, and has been studied extensively. Other catechins, such as (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epicatechin (EC), as well as other known or unidentified tea constituents, may also contribute to the biological activities of tea (3). In contrast to green tea leaf (GTL), green tea seed (GTS) has not received proper attention and, therefore, its biological function and bioactive components remain to be identified. GTS was reported to be rich in saponins and oleic acid (4). In Korea, the seed has been used as a folk medicine for asthma and sonitus. Recently, GTS production has increased with the increasing popularity of green tea. As GTS contains many biologically active compounds such as saponins, flavonoids, vitamins, and oils, it is expected to have cancer preventive activity. Especially, it has been reported to contain kaempferol glycoside of which the aglycone form has potent inhibitory activity against 5-α-reductase and antioxidant activity (5, 6).

NADPH:quinone reductase (QR; EC Number 1.6.5.5) is an electrophile processing metabolic enzyme. It is primarily cytosolic and catalyzes the reduction of a wide variety of quinones. QR is coordinately induced with phase II detoxifying enzymes by a variety of compounds that protects rodents from the toxic, mutagenic, and neoplastic effects of carcinogens. There is a large body of evidence suggesting that monitoring the enzyme is a convenient screening method for anticarci-nogenic activity (7, 8).

In this study, we analyzed the catechin content and assessed the anticarcinogenic potential of GTS by analyzing effect of methanolic extract of GTS on cell cycle and QR-inducing activity.

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Materials and Methods

Chemicals Authentic catechin standards including (-) EC, (-) EGC, (-)ECG, and (-)EGCG were kindly provided by Dr. Yu, Si-Yong at the Korean Research Institute of Chemical Technology (Daejeon, Korea).

Preparation of green tea seed (GTS) extract One hundred g of GTS and GTL obtained from Hwagejeda Co. (Hadong, Korea) were powdered and defatted by hexane extraction, and then extracted with 10 volumes of 80%(v/v) methanol for 24 hr at room temperature. The amount of dry extracts recovered from 100 g of GTS and GTL was 9.45 and 12.6 g, respectively.

Determination of catechins in leaf and seed of green tea The concentration of catechins in GTL and defatted seed and leaves was determined by the method described previously (9). Briefly, catechins were extracted from GTS and GTL by stirring 2.5 g dry sample with 20 mL 80%

methanol for 3 hr at room temperature. After the extraction was repeated 5 times, the filtrate from each extraction was pooled and used for catechin analysis. The aliquot of pooled extract was filtered through a 0.45 μm nylon filter (Nunc, Rochester, NY, USA). Four kinds of catechins, EC, GCG, EGC, and EGCG, along with caffeic acid and gallic acid, were analyzed by HPLC. A Jasco chromatograph with a Model AS 2055 autosampler, a Model PU 1580 dual pump, and a Model UV-2077 UV-visible detector (Jasco, Tokyo, Japan) was used. A Shiseido Capcell Pak C18 (5 μm, 4.6×250 mm) was employed for chromatographic separations. A linear gradient was composed of A (0.5% phosphoric acid in water) and B (50% CH_3CN in 0.5% H_3PO_4). After injection of a 20 μL sample, the system was increased from 90% A to 40% A in 50 min, and maintained at 40% A for another 10 min. The system was recycled to 90% A at the end of 65 min. The flow rate was 0.7 mL/min. The UV absorbance was monitored at 210 nm. UV spectra were recorded and peak areas were integrated using Young-Lin Autochro 2000 software (Young-Lin, Anyang, Korea).

Antiproliferative activity assay The antiproliferative activity of GTS extract and catechins on hepa1c1c7, BPRc1, and HepG2 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (10). Human hepatoma HepG2, mouse hepatoma hepa1c1c7 and its mutant, BRRc1 cells lacking aryl hydrocarbon receptor nuclear translocator (ARNT) (1×10^4 cells), were seeded into 96-well microtiter plates using either alpha-modified minimal essential medium (α-MEM; hepa1c1c7, BPRc1) or Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and left at 37°C 4 hr to allow the cells to attach, followed by incubating for another 72 hr in the presence of various concentrations of the sample. Cell survival was assessed by MTT assay.

Cell cycle analysis Cell cycle was analyzed by using flow cytometry with propidium iodide (PI) staining. HepG2 cells cultured in the presence of various concentrations of sample in DMEM with 10% FBS were trypsinized and washed twice with PBS and incubated in 100%(v/v) ethanol at 4°C for 1 hr or longer. After the incubation, the cells were washed with phosphate buffered saline (PBS) and stained with 12.5 mg PI with 8.75 U RNase A. The DNA contents of the cells were determined by flow cytometry. Data were stored as list-mode files of at least 10,000 single-cell events and analyzed by a flow cytometer (Becton Dickinson, FACS Calibur, NJ, USA).

Quinone reductase activity assay Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of 3×10^5 or 5×10^5 cells/plate in 10 mL α-MEM supplemented with 10% FBS. The cell culture was performed in a humidified incubator in 5% CO_2 at 37°C. Cells were cultured for 48 hr, followed by exposure to various concentrations of the sample for another 24 hr. QR activity was measured according to the method described by Benson *et al.* (11). Briefly, cells were plated, grown, and exposed to different concentrations of GTS extract for 24 hr before being harvested. The cells were washed with ice-cold 0.15 M KCl-10 mM potassium phosphate (pH 7.4), removed from the plates by scraping with a rubber policeman, and disrupted for 5 sec using an ultrasonic cell disrupter (50W; Kontes, Vineland, NJ, USA). Cell homogenates were centrifuged at 12,000×g for 5 min in a microcentrifuge (VS-15000CFN11; Vision, Seoul, Korea). QR activity was assayed by measuring the rate of oxidation of 2,6-dichlorophenolindophenol at 600 nm in the assay system containing 25 mM Tris-HCl (pH 7.4), 0.7 mg crystalline bovine serum albumin at pH 7.4, 0.01% Tween 20, 5 μM FAD, 0.2 mM NADH, 0 or 10 μM dicoumarol, and 200 μL cell extract in a final volume of 3.0 mL. The QR induction was expressed as 2,6-dichlorophenolindophenol reduced/min/mg protein.

Assay of reporter gene activity HepG2-C8 cells were plated in 6-well plates at a density of 10^5 cells/well (12). After overnight incubation, the cells were cultured in fresh F-12 containing 0.5% FBS for 12 hr before drug treatment. The luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI, USA). Briefly, after drug treatment, cells were washed twice with ice-cold PBS and harvested in reporter lysis buffer. The homogenates were centrifuged at 12,000×g for 2 min at 4°C. A 20 μL supernatant was assayed for luciferase activity using a Victor 3 Multilabel counter (Perkin Elmer, Wellesley, MA, USA). Luciferase activity was normalized against protein concentration.

Statistical analysis Statistical significance of enzyme activity data was tested by analysis of variance, followed by Duncan's multiple range test, using SPSS software (SPSS Inc, Chicago, IL, USA). The level of statistical significance for differences among the treatment groups was set at $p<0.05$.

Results and Discussion

Catechin contents in GTS and GTL The concentrations of catechins in GTS and GTL are shown in Table 1. The most abundant catechin present in GTS was ECG (821±95 μg/g), followed by caffeine (155±42 μg/g), EGC (116±72 μg/g), gallate (112±72 μg/g), EGCG (108±32 μg/g), and EC (37±40 μg/g). The concentrations of all kinds of catechin examined in the study were higher in GTL than GTS. In particular, EGCG, which has been reported to be a major compound contributing to anticarcinogenic activity in green tea, was 108±32 and 8,685±1061 μg/g in GTS and GTL, respectively.

Antiproliferative activity of methanol extracts of GTS and GTL, and catechins against mouse and human hepatoma cells The methanolic extracts of GTS and GTL were compared for their growth inhibitory activities against hepa1c1c7 cells. As shown in Fig. 1, GTS extract showed a stronger growth inhibitory activity than GTL extract against human and mouse hepatoma cells, with an IC_{50} of 40-80 μg/mL. In contrast, most of the catechins, except EC, showed a relatively stronger growth inhibitory activity than 80% methanolic GTL extract, and had a similar IC_{50} to GTS extract (Fig. 1). These results suggest the relatively minor contribution of catechins to the growth

Table 1. Contents of catechins[1], gallate, and caffeine in green tea seed and leaves (μg/g)[2]

	EGCG	EGC	ECG	EC	Gallate	Caffeine
Seed	108±32	116±72	821±95	37±44	112±72	155±42
Leaves	8,685±1061	11,486±506	3,535±308	1,429±177	97±2	2,722±302

[1]EGCG, epigallocatechin gallate; EGC, epigallocatechin; ECG, epicatechin gallate; EC, epicatechin.
[2]Green tea leaf and seed samples were extracted with aqueous methanol (80%, v/v) to determine the catechin contents. Values represent mean± SD (n=3).

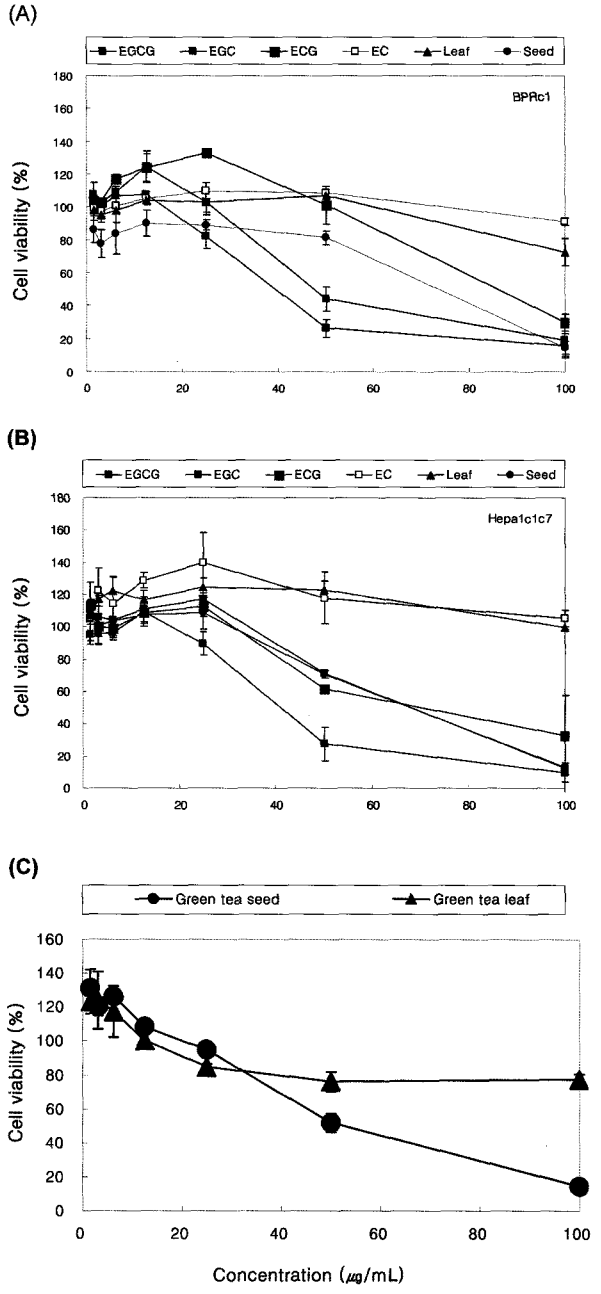

Fig. 1. Growth inhibition of mouse hepatoma cells (A and B) and human hepatoma cells (C) by green tea seed and leaf extracts. Curves represent the mean of triplicate measurements ± standard deviation.

inhibition activity of green tea extracts against tumor cells because the 80% methanolic extract of GTL contained much higher levels of catechins than that of GTS.

The active constituents and mechanisms of the cancer-preventive activities in GTS, however, are not clearly understood, although GTS has been used as a folk medicine for the treatment of asthma and sonitus (4). Many mechanisms have been proposed for the biological activities of tea polyphenols, including antioxidant activities, induction or inhibition of drug metabolism enzymes, inhibition of arachidonic acid metabolism, inhibition of cell proliferation, induction of apoptosis, and inhibition of DNA methyltransferase, dihydrofolate reductase, proteases, and telomerase (3). In addition, catechins, especially EGCG, have been found to affect various signal transduction pathways, such as the inhibition of many protein kinases, suppression of the activation of transcription factors, AP-1 and NF-κB, blocking growth receptor mediated pathways, and induction of cell cycle arrest or apoptosis (3).

Accumulation of cells at Sub-G1 by methanolic extract of GTS The cell cycle distribution of HepG2 cells treated with varying concentrations of methanolic extract of GTS (0, 10, 50, 100 μg/mL) is shown in Fig. 2. The cells harvested at 24 hr showed an accumulation at sub-G1 (Fig. 3), which is indicative of apoptosis. The treatment of HepG2 cells at the concentration of 10 μg/mL GTS extract increased the percentage of cells in sub-G1 at 24 hr (12% control vs. 20% treatment) and at the concentration of 100 μg/mL (12% control vs. 55% treatment). The percentage of cells in the S-phase was reduced by the methanolic extract of GTS at 24 hr, followed by a reduction of cells in G2/M. The cell cycle is regulated by a series of checkpoints monitoring genomic integrity and ensuring that DNA replication proceeds in a coordinated manner (13). Whereas cell proliferation and differentiation are specifically controlled in the G1 phase and the G1-S transition in the cell cycle, oncogenic processes exert their greatest effect by targeting particular regulators of G1 phase progression (14). In this study, we observed that the methanolic extract of GTS inhibited cell growth through the induction of cell cycle arrest at the sub-G1 phase, dependent on the concentration of the sample. The antiproliferative activity of GTS extract was higher than that of the leaves containing higher level of catechins, suggesting that cell growth inhibition by GTS extract is not associated with catechins.

Effect of methanol extracts of GTS and GTL, and catechins on quinone reductase activity of mouse hepatoma cells Methanolic extract of GTS (100 μg/mL) increased QR activity by 2- and 1.9-fold in hepa1c1c7 and BPRc1 cells, respectively, and its QR induction potential was higher than that of GTL extract (Fig. 3). Among catechins,

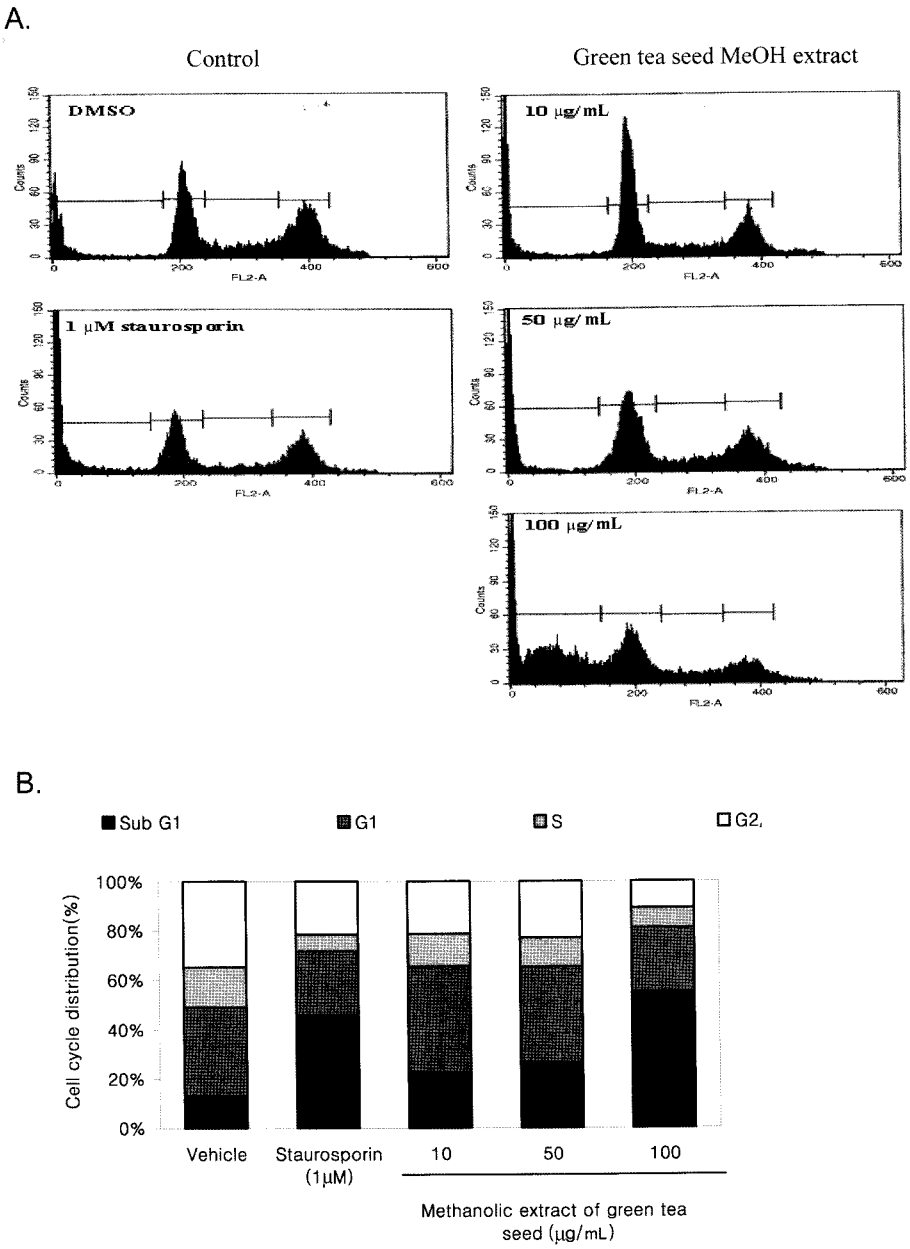

Fig. 2. Flow cytometric analysis of the effect of 80% methanolic extract of green tea seed on HepG2 cells. (A) analysed frequency histograms of DNA content in HepG2 cells after 24 hr exposure to 100 μg/mL GTS extract. (B) relative percentage of each cell cycle. Effect of green tea catechins on four different phases of the cell cycle, as measured by flow cytometry, was observed. Data are expressed as means±SD of triplicate determinations. For clarity, SD bars and statistical significances are not shown.

EGC showed the strongest QR induction, followed by EGCG. In particular, the methanolic extract of GTS (100 μg/mL) had a similar QR-inducing potential to 20 μM tert-butylhydroquinone (TBHQ), a known QR inducer, in both cell lines used in this study. Elevated tissue levels of detoxification enzymes are associated with decreased susceptibility to chemical carcinogenesis (15-17). These enzymes, including QR and glutathione S-transferases, promote the conjugation of phase I products (principally cytochrome P450) with endogenous ligands such as glutathione and glucuronic acid, usually resulting in an increase in water-soluble products.

Dose-response of GTS extract on the expression of pARE-TI-Luciferase To explore further whether QR induction by GTS extract is mediated by the antioxidant response element (ARE) sequence in the promoter region of phase II enzymes, we investigated the effect of GTS extract on ARE-mediated gene expression via the activity of the luciferase reporter gene in the cytosol of the HepG2-C8 cells. Methanolic extract of GTS was added to HepG2-C8 cells at the indicated concentrations, as shown in Fig. 4. The luciferase activity was increased in a dose-dependent manner within the range of 10 to 100 μg/mL, i.e., the reporter enzyme activities in the transfectant cells exposed to 10, 50, and 100 μg/mL GTS for 24 hr were increased by 1.53, 2.88, and 3.73-fold, respectively. In the

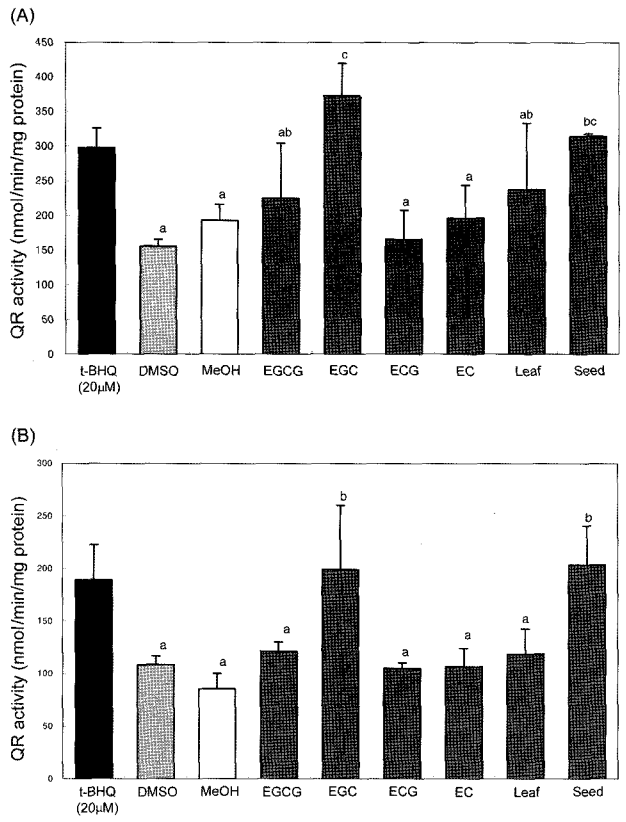

Fig. 3. QR-inducing activities of 80% methanol extracts of green tea seed and leaf and catechins (100 μg/mL) in mouse hepatoma hepa1c1c7 (A) and BPRc1 (B) cells. Data are means±SD (n=3).

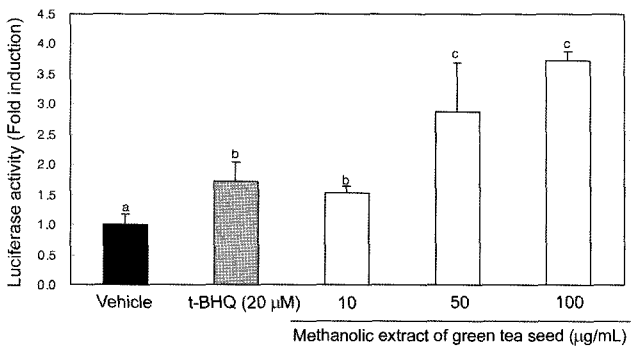

Fig. 4. Induction of ARE-luciferase by 80% methanolic extract of green tea seed in HepG2-C8 cells. Data are means±SD (n=3).

positive control, using TBHQ (20 μM), the induction activity of luciferase of HeG2-C8 cells exposed to 10 μg/mL GTS extract showed a similar trend. This result suggests that QR induction by the sample was mediated via the interaction between nuclear factor-E2-related factor 2 (Nrf2) and ARE-sequence (18). Induction of phase II enzymes by inducers involves ARE, which is found in the 5'-flanking region of the phase II and antioxidant genes (18, 19). Nrf2 has been identified as the ARE-binding transcription factor (20) and has been shown to play a critical role in the ARE-mediated gene expression. The small Maf protein forms a heterodimer with Nrf2, which binds the AREs of phase II genes (21). Overexpression of Nrf2 results in the up-regulation of ARE-regulated reporter genes, which are induced by a variety of chemopreventive agents (22-25).

Our study demonstrated that GTS has the potential to induce phase II enzymes, including QR, and that its activity is probably mediated by the interaction with Nrf2 and/or Keap1. In addition, the methanolic extract of GTS appeared to contain higher levels of QR inducers than GTL extract as it showed stronger QR-inducing potential than GTL extract. However, catechins may not be major contributors to cytosolic QR induction because the GTS extract containing low levels of catechins showed higher QR-inducing activity while the GTL extract with higher catechin content showed relatively low QR-inducing potential. Our preliminary experiment also indicated that the active component(s) belongs to flavonoids. It has been reported that GTS contains a reasonable amount of kaempferol glycosides, which mainly consist of kaempferol-3-O-[2-O-beta-D-galactopyranosyl-6-O-alpha-L-rhamnopyranosyl]-beta-D-glucopyranoside and kaempferol-3-O-[2-O-beta-D-xylo-pyranosyl-6-O-alpha-L-rhamnopyranosyl]-beta-D-glucopyranoside (6). Kaempferol has been reported to have antiproliferative activity and cause G2-M cell cycle arrest (26). The compound has also been reported to induce QR in BPRc1 cells lacking ARNT, suggesting that it is a monofunctional inducer of phase 2 enzymes (27). Therefore, it is possible that kaempferol contributes in some extent to the antiproliferative and QR-induction activities of the methanolic extract of GTS. However, as GTS was more potent in apoptosis induction than kaempferol it should contain compound(s), other than kaempferol, with strong cytotoxic activity (28). In conclusion, the anti-proliferative and phase 2 enzyme-inducing activities in mouse hepatoma cells exhibited by the methanolic extract of GTS demonstrated its potential as an anticarcinogenic agent which warrants further study to identify its anti-carcinogenic compound(s).

Acknowledgments

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