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In vitro Antioxidant and Cytoprotective Activities of the Extract of *Dangyuja* (*Citrus grandis* Osbeck) Leaves

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Abstract The antioxidant activities of the extracts of *dangyuja* (*Citrus grandis* Osbeck) leaves were evaluated. The highest phenolic content was obtained from the ethyl acetate fraction (EF) (202.1±0.8 mg GAE/g dried extract) and it exhibited the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. The cytoprotective effects of EF on oxidative damage induced by *tert*-butyl hydroperoxide (*t*-BHP) in a human hepatoma cell line, HepG2 cells, were investigated to understand the intracellular antioxidant mechanisms. Treatment of HepG2 cells with EF prior to oxidative stress was found to inhibit reactive oxygen species (ROS) generation, lipid peroxidation, and DNA damage in a dose-dependent manner. Gas chromatography-mass spectrometry (GC-MS) studies on EF resulted in tentative identification of 19 compounds representing 94.3% of the total content. Taken together, these results demonstrated that EF has excellent antioxidant activities and thus *dangyuja* leaves have great potential as a source for natural antioxidant which can be applied in food products.

Keywords: *Citrus grandis* Osbeck, antioxidant, DPPH radical scavenging, *tert*-butyl hydroperoxide, HepG2 cell

Introduction

Free radicals and reactive oxygen species (ROS) are normal oxidant by-products of aerobic metabolism, and about 2-5% of the O_2 consumed by mitochondria is converted to ROS under normal metabolic conditions (1). An overall increase in cellular levels of ROS above the cell's defenses results in oxidative stress that can cause destructive and irreversible damage to the components of a cell, including lipids, proteins, and DNA (2,3). ROS are involved in the etiology of several age-related and chronic diseases, such as cancer, diabetes, and neurodegenerative and cardiovascular diseases (4,5).

Antioxidants are substances that play a preventive role against those diseases by removing ROS from biological systems (6). Several synthetic antioxidants are commercially available but the use of common synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) has become controversial issue because of adverse toxicological data (7). Hence, there has been a growing interest in replacing them with natural ingredients. Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, scavenging of free radicals, and changes in intracellular redox potential (8) and they also have been proposed as therapeutic agents against liver diseases (9,10). Therefore, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumption (11-14).

Citrus grandis Osbeck belongs to the family of Rutaceae and widely cultivated all warm countries as a domestic fruit. The flesh is usually separated from the skin of the segments and is eaten with or without sugar whereas the leaves are used as food flavoring. *Dangyuja* is a local name of *C. grandis* Osbeck in Jejudo(Island), southernmost of South Korea, where the fruit has been used as a folk remedy for hangovers and the leaves are harvested throughout the year and the dried leaves are boiled or brewed in water as a drink.

Previous studies about *dangyuja* have focused mainly on changes in limonoid content during fruit growth and on the evaluation of antioxidant activity of fruit of this species (15-17). However, studies reporting upon the activities of *dangyuja* leaves are limited. In this study we propose to evaluate the potential antioxidant and cytoprotective effects of ethyl acetate fraction (EF) of *dangyuja* leaves against *tert*-butyl hydroperoxide (*t*-BHP) induced oxidative damages in HepG2 cells. This hepatoma cell line is considered to be a good tool to study the cytoprotective effects of compounds or natural antioxidants to liver cells (18,19). Therefore, we investigated the antioxidant properties of the EF in a series of assays such as intracellular ROS generation, lipid peroxidation, DNA damage, and cytotoxicity using this model of *in vitro* hepatotoxicity. Furthermore, because it is important to determine the constituents present in the extract which may be contributing to the activity, a compositional analysis of the EF was carried out using gas chromatography-mass spectroscopy (GC-MS).

Materials and Methods

Materials *Dangyuja* leaves were collected from the National Institute of Subtropical Agriculture, Jeju, Korea. Botanical samples were taxonomically identified by Kim (20) and a voucher specimen (No. SKC.070531) is deposited at the laboratory of Professor Somi Kim Cho in the Faculty of Biotechnology, College of Applied Life Sciences, Cheju National University. All chemicals and

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reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Invitrogen Gibco (Grand Island, NY, USA).

Preparation of extracts Air-dried *dangyuja* leaves were pulverized using a milling machine and extracted with 80% methanol by stirring for 3 days at room temperature (RT). The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized. The methanol extract (ME) was suspended in water and further fractionated with 4 different solvents in a stepwise manner, as previously described (17). Each solvent was extracted 3 times at RT, evaporated and then freeze-dried. The resulting fractions were: *n*-hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), *n*-butanol fraction (BF), and water fraction (WF). The extract powder(s) were dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (PBS, pH 7.4) to give the final concentrations.

Determination of total phenolics and flavonoids Total phenolics were determined according to the method of Cheung *et al.* (21) with slight modification. Two mL of sample were mixed with 2 mL of Folin-Ciocalteu phenol reagent (1 :1 with water). After 3 min, 2 mL Na_2CO_3 (10%) were added to the mixture. The reaction was kept in the dark for 30 min, after which its absorbance was read at 725 nm using a microplate enzyme-linked immunosorbent assay (ELISA) reader (MRX II; DynexTechnologies, Chantilly, VA, USA). The results were expressed as gallic acid equivalents (GAE) in mg/g of dried sample. The flavonoids content was measured using a colorimetric assay developed previously (22). Absorbance was read at 510 nm against the blank (80% methanol) and flavonoid content was expressed as catechin equivalents (CE) in mg/g of dried sample. All analyses were done at least in triplicate.

Gas chromatography GC analysis of EF was carried out with Aligent 6850 system equipped with a flame ionization detector (FID) and HP-5MS capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness). Injector and detector temperature were maintained at 220 and 280°C, respectively. The column oven temperature was set at 100°C for injection (held for 5 min), then programmed at 5°C/min to 200°C (held for 5 min) then at 5°C/min to 300°C and finally held at 300°C for 10 min. Nitrogen was used as carrier gas with flow rate of 1.0 mL/min. Injection volume was 2 μL with split ratio of 5 :1. Quantitative data were obtained from FID area percent data.

GC-MS GC-MS analysis of EF was carried out with Agilent 6890N gas chromatography coupled to Agilent 5975N mass spectrometer. GC conditions and column were the same as those used in the GC analysis above. Helium was used as carrier gas with an inlet pressure of 10.48 psi corresponding to a flow rate of 1.0 mL/min. The MS instrument was operated in the electron impact (EI) mode with ionization energy of 70 eV. Transfer line was also set at 280°C, quadrupole temperature at 150°C, and source temperature at 230°C. Chemical constituents of the analyte were identified by comparing MS fragmentation pattern with corresponding reference data (Wiley 7th ed.). The experiment was replicated thrice.

Free radical scavenging activity DPPH radical scavenging activity was assessed according to the method of Blois (23) with minor modification. Initially, 10 μL of the test extracts in DMSO, yielding a series of extracts with different concentrations (50, 125, 250, 500 μg/mL) in each reaction, were mixed with 190 μL of 150 μM DPPH in ethanol. The resulting solutions were vigorously mixed and then left to stand in the dark at RT for 30 min. The absorbance of the remaining 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured at 517 nm using a microplate ELISA reader. BHT with different concentrations (50, 125, 250, 500 μg/mL), were used as positive controls. The negative control lacked the sample. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the A_{517} of DPPH without the sample (control), and A_1 is the A_{517} of DPPH with the sample.

Cell culture Human hepatoma HepG2 (KCLB No. 58065) cells were purchased from the Korea Cell Line Bank (Seoul, Korea). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10%(v/v) heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The culture medium was changed twice a week, and the cells were sub-cultured at a ratio of 1 :4 once a week.

Determination of ROS generation Cellular ROS were quantified using the dichlorofluorescein (DCFH) assay (24), in which the esterified form of DCFH-diacetate diffuses through the cell membrane and is enzymatically deacetylated by intracellular esterases. The resulting compound, DCFH, is reactive with ROS to give an oxidized fluorescent compound, DCF (25). HepG2 cultures were pre-treated with EF for 1 hr, and then the cultures were washed twice and incubated with 200 μM *t*-BHP for 3 hr. DCFH-diacetate was added to the culture plates at a final concentration of 25 μM, and DCF fluorescence was detected over a period of 30 min at 37°C at an excitation wavelength of 485 nm and emission wavelength of 530 nm, using a Genios multiwell fluorescence plate reader (Genios, Tecan, Salzburg, Austria).

Measurement of lipid peroxidation The extent of lipid peroxidation was determined by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a product formed by lipid peroxidation, according to the method reported by Ohkawa *et al.* (26) with slight modification. In our experiments, cells were exposed to the EF at various concentrations for 1 hr, followed by incubation with 200 μM *t*-BHP for 3 hr. The amount of MDA formed as a breakdown product was measured at 532 nm using a microplate ELISA reader. Hydrolyzed 1,1,3,3-tetramethoxypropane (TMP) was used as the standard and the protein concentration was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

All the measurements were conducted in triplicate and results were averaged.

Determination of DNA damage (Comet assay) The alkaline Comet assay was conducted according to Tice *et al.* (27) with slight modification. The cells in a 24-well plate were treated with the extract, as described above. The cell suspension was mixed with 100 mL 0.5% low-melting point agarose (LMPA) and added to slides precoated with 1.0% normal melting agarose (NMA). After solidification of the agarose, the slides were covered with another 100 mL of 0.5% LMPA and then immersed in lysis solution [2.5 M NaCl, 100 mM ethylenediamide tetraacetic acid (EDTA), 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO] at 4°C for 1 hr. The slides were then placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied at 4°C for 20 min. The slides were washed 3 times with a neutralizing buffer (0.4 M Tris-HCl, pH 7.5) at 4°C for 5 min, and then treated with ethanol for another 5 min before staining with 50 μL ethidium bromide (20 μg/mL). The percentage of fluorescence in the DNA tail of each cell (100 cells from each of triplicate slides) on the ethidium bromide stained slides were measured by image analysis (Kinetic Imaging, Komet 5.0, UK) and fluorescence microscopy (LEICA DMLB, Germany).

Cytotoxicity assay To evaluate cytotoxicity of the EF, a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay was performed (28). HepG2 cells were cultured at a density of 1×10^5 cells/mL on 96-well microplates for 16 hr, washed twice using PBS, and pretreated with EF. After 1 hr incubation, *t*-BHP solution was added to the wells, and the cells were re-incubated for 3 hr. MTT reagent (5 mg/mL) was added to each well, and the plate was incubated at 37°C for an additional 4 hr. The media were then removed, and the intracellular formazan product was dissolved in DMSO. Absorbance at 570 nm of the mixture was detected using a microplate ELISA reader. The results were determined for 3 independent experiments.

Statistical analysis All results were expressed as the mean±standard deviation (SD). One-way analysis of variance (ANOVA) using the SPSS v 12.0 software package was applied. A difference at $p<0.05$ was considered to be statistically significant. All assays were performed in triplicate.

Results and Discussion

Total phenolic content and total flavonoid content Many related polyphenols, commonly found in plants, have been reported to have several different biological activities, including antioxidant activity (29,30). There may be a causative relationship between total phenolic content and antioxidant activity (31,32). Thus, air-dried *dangyuja* leaves were extracted with 80% methanol and the fractions of *n*-hexane, chloroform, ethyl acetate, and *n*-butanol were prepared in a stepwise manner to measure total phenolic and flavonoids contents in each one of the extracts. Table 1 reports the results of total phenolics and total flavonoids analyses. Among the fractions tested, EF showed the highest total phenolic content, equivalent to 202.1 mg gallic acid, whereas the CF contained the highest amount of flavonoids (44.9 mg CE/g dried sample).

Table 1. Total phenolic and flavonoid contents of crude extracts and their derived fractions from the leaves of *dangyuja (Citrus grandis* Osbeck)

Sample	Total phenolic content (mg/g)[1)]	Total flavonoid content (mg/g)[2)]
ME	73.7±1.3[a]	2.1±0.7[a]
HF	72.3±0.2[a]	21.1±0.7[c]
CF	110.9±2.4[b]	44.9±1.8[f]
EF	202.1±0.8[d]	29.1±0.5[e]
BF	138.8±0.3[c]	26.2±0.9[d]
WF	75.8±1.2[a]	5.2±0.3[b]

[1,2)]Expressed as mg GAE/g and mg CE/g dried extract, respectively. Data represent mean±SD of 3 independent experiments performed in triplicate.

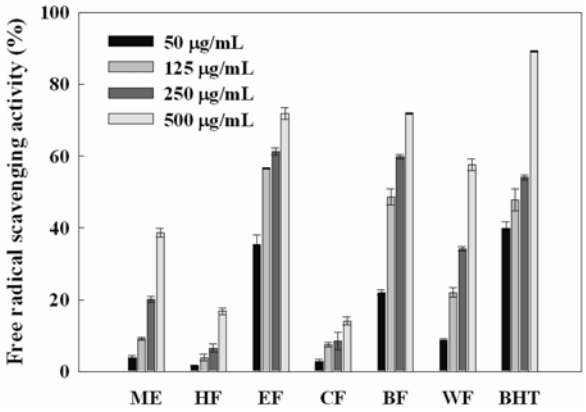

Fig. 1. DPPH radical scavenging activity of extracts of *dangyuja* leaves. All extracts were added at 50-500 μg/mL. ME, 80% methanol extract; HF, *n*-hexane; EF, ethyl acetate; CF, chloroform; BF, butanol; WF, water-extracted fractions; BHT, butylated hydroxytoluene. Values are means±SD (n=3).

DPPH free radical scavenging activity The DPPH assay has been widely used to test the ability of compounds as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity of plant extracts (33). The free radical scavenging activities of the various solvent-extracted fractions of *dangyuja* leaves were determined with the DPPH assay, and the results are shown in Fig. 1. The percentage DPPH radical scavenging activities of all the extracts were dose-dependent, although the HF showed minimum activity at almost all tested concentrations. At a concentration of 500 μg/mL, the 80% MeOH extracts of *dangyuja* leaves and their derived fractions decreased in the following order: BF (71.92%)>EF (71.86%)>WF (57.56%)>ME (38.68%)>HF (16.79%)>CF (14.04%). Both EF and BF showed good inhibitory effects, which were comparable to that of BHT, a well-known synthetic antioxidant. However, EF showed better performance

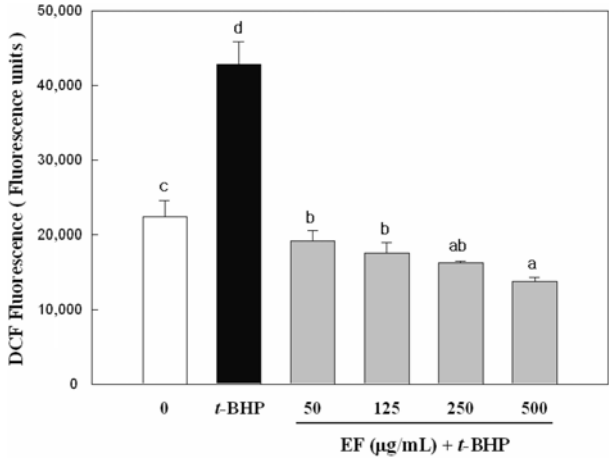

Fig. 2. Protective effects of EF treatment on *t*-BHP-induced intracellular reactive oxygen species (ROS) generation in HepG2 cells. Values are presented as means±SD (n=4).

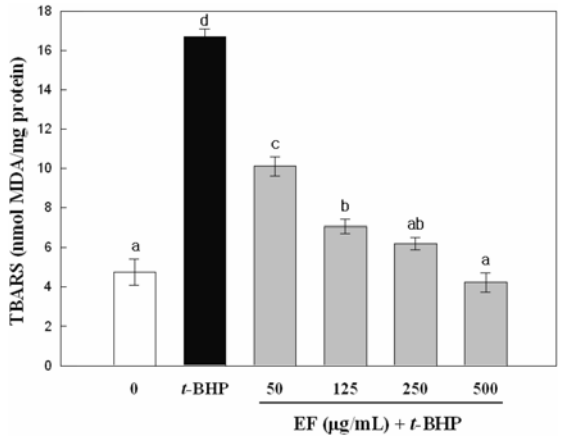

Fig. 3. The effects of EF treatment on lipid peroxidation in HepG2 cells after *t*-BHP-induced oxidative stress. Values are presented as means±SD (n=3).

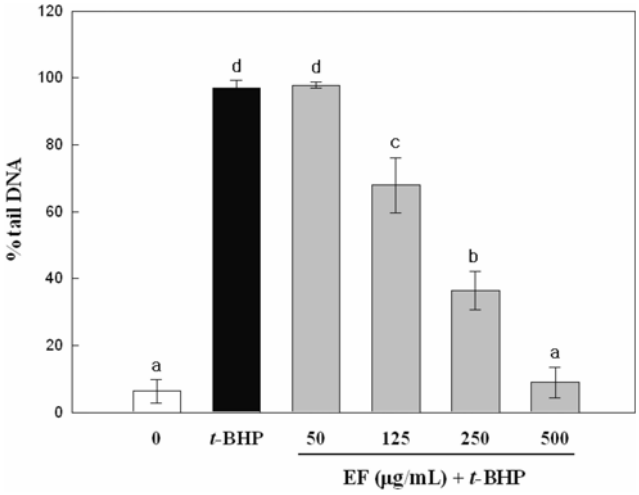

Fig. 4. Graphical representation of the Comet assay. One hundred cells were analyzed per experimental point in each of the 3 independent experimental cultures. DNA damage is expressed as the mean±SD (n=3) of the % DNA migrated in the tail of the comet (% DNA in tail).

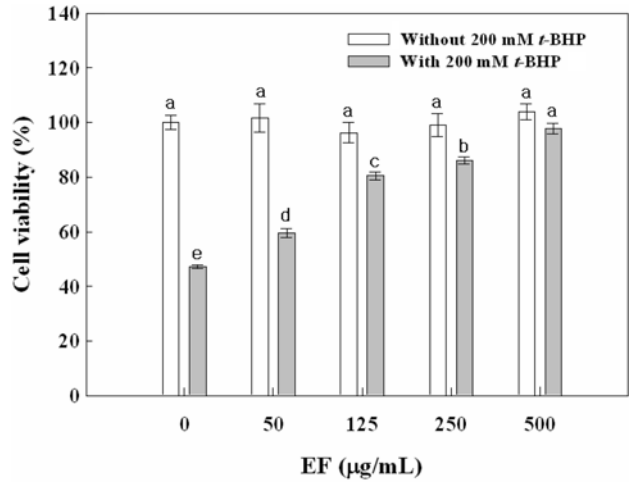

Fig. 5. Protective effects of EF on *t*-BHP-induced cytotoxicity in HepG2 cells. Values are presented as means±SD (n=3).

against the DPPH radical than BF, at a concentration of 50 μg/mL. Since these results indicate that the EF of *dangyuja* leaves might be a good potential source of antioxidants, it was further evaluated for its antioxidant and cytoprotective activity.

Effect of EF on *t*-BHP-induced intracellular ROS generation Direct evaluation of intracellular ROS levels is a very good indication of the oxidative damage to living cells (24). A prooxidant, such as *t*-BHP, can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus increasing fluorescence (19). As shown in Fig. 2, increased ROS generation in cultured HepG2 cells submitted to an oxidative stress by *t*-BHP was completely inhibited by pre-treatment with 50 μg/mL of EF for 1 hr. These results suggest that the natural antioxidant EF strongly inhibits the generation of ROS induced by *t*-BHP in cultured HepG2 cells.

Prevention of lipid peroxidation As *t*-BHP induces an array of cellular dysfunctions, including peroxidation of membrane lipids (34), the ability of EF to inhibit lipid peroxidation was determined. In our experimental conditions, treatment of HepG2 cells with 200 μM *t*-BHP induced a remarkable increase in the formation of lipid peroxidation products, as reported previously (19,35-37). However, incubation with EF prior to the oxidative challenge led to a decrease in the formation of lipid peroxidation products, as shown in Fig. 3. The protective effect exerted by the EF was observed after a short pre-incubation period (1 hr), suggesting that molecular features might aid this rapid absorption of bioactive components from the EF. More than 50% of the inhibitory activity was observed at a concentration of 50 μg/mL, which is similar to the degree of protection with tea catechins in the same cell line (38,39).

Inhibition of DNA damage and cell death In a previous

Table 2. Chemical composition of the ethyl-acetate fraction from the leaves of *dangyuja (Citrus grandis* Osbeck)

RT[1)]	Compound[2)]	RC[3)] (%)	Homology (%)
11.61	Bezene, 1,3-bis(1,1-dimethylethyl)-	2.2	93
18.24	Phenol, 2,4-bis(1,1-dimethylethyl)-	5.5±0.7	96
19.58	Hexadecane	1.0±0.4	91
25.85	Isopropyl myristate	1.1±0.3	81
28.68	Hexadecanoic acid	14.1±3.2	97
34.01	Octadecanoic acid	21.1±4.6	99
34.47	Octadecane, 2-methyl-	4.8±0.7	72
38.59	9-Octadecenamide	1.7±1.0	99
48.11	Octadecane	1.3±0.6	95
48.88	Eicosane	1.4±0.1	93
49.65	Heptadecane, 3-methyl-	1.1±0.2	78
50.12	Eicosane, 2-methyl-	3.1±0.2	94
52.02	Demecolceine	1.0±0.2	38
53.64	Stigmasta-5,22-dien-3-ol	9.7±0.3	91
54.11	Ethylcholest-5-en-3β-ol	5.4±1.6	99
54.62	3',4',5,6,7,8-Hexamethoxyflavone	3.9±1.1	91
55.19	α-Amyrin	7.7±0.1	78
58.00	Cyclotrisiloxane, hexamethyl-	3.2±0.2	38
59.82	1-Phenyl-2,3-di(4-hydroxyphenyl)-2-propen-1-one	5.0±0.6	47

[1)]Retention time (as min).
[2)]Compounds listed in order of relative area percentage.
[3)]Relative area percentage (peak area relative to total peak area %).

study, incubation of HepG2 cells for 1 hr with 200 μM *t*-BHP induced significant DNA damage without cell death (18). Thus, we investigated the effects of EF on DNA damage using the Comet assay. As shown in Fig. 4, when cells were treated with EF before exposure to *t*-BHP, DNA damage significantly decreased following a dose-response ratio in concentrations larger than from 250 μg/mL. Considering that the increases in intracellular ROS levels have been associated with the induction of DNA strand breaks (40), our results suggest that the EF-treated cells were significantly protected against DNA damage induced by *t*-BHP.

Figure 5 shows the effect of pre-incubation with EF on HepG2 cell death caused by treatment of 200 μM *t*-BHP. In our MTT sassy, exposure of cells to 200 μM *t*-BHP increased cell death by up to 50% and pre-incubation of cells with EF for 1 hr inhibited the cell death induced by *t*-BHP in a dose-dependent manner. Cell viability was restored to about 90% by pre-treatment with EF at a concentration of 0.5 mg/mL, indicating that EF protected HepG2 cells against *t*-BHP-induced cytotoxicity.

Compositional analysis of EF The GC-MS analysis of the EF from the leaves of *dangyuja* revealed total 35 compounds. Nineteen compounds, representing 94.3% of the total content, of them are listed in Table 2. The fatty acids, octadecanoic acid and hexadecanoic acid, were found in high amounts (21.1 and 14.1%, respectively) in the EF. The fraction was also rich in stigmasta-5,22-dien-3-ol (9.7%), α-amyrin (7.7%), and ethylcholest-5-en-3β-ol (5.4%). Overall, the results obtained in the present study demonstrated that cells preincubated with the EF had increased resistance to oxidative challenge under *in vitro* conditions. Based on these results, it is suggested that the EF might be used as an easily accessible source of natural antioxidants. The broad range of activity of the EF suggests that multiple mechanisms are responsible for the antioxidant and cytoprotective activities. Since several candidate bioactive compounds were identified in the extract, the study on the contribution of these compounds to antioxidant properties of the EF is in progress. Further studies are needed to unravel the mechanism of their antioxidant and cytoprotective activities.

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