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Antiproliferative Activity and Apoptosis Induction of *Eucalyptus Citriodora* Resin and Its Major Bioactive Compound in Melanoma B16F10 Cells

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ABSTRACT: Antiproliferative activity and apoptosis induction of ethyl acetate of *Eucalyptus citriodora* resin (EAEER), and its major bioactive compound in melanoma B16F10 cells were investigated. 6-[1-(*p*-Hydroxy-phenyl)ethyl]-7-*O*-methyl aromadendrin (HEMA), a flavanol derivative, was isolated from EAEER and identified on the basis of its mass and NMR spectra. The results from MTT assay showed high antiproliferative effects of EAEER and HEMA on B16F10 cells. Moreover, EAEER- and HEMA-induced cell apoptosis was association with the decrease in the mitochondrial transmembrane potentials ($\Delta\psi_m$), increase in Bax/Bcl-2 ratio, and activation of caspase-3. Cells treated with EAEER and HEMA generated intracellular reactive oxygen species (ROS) and nitric oxide (NO), indicating that ROS and RNS play important roles in the induction of apoptosis in B16F10 cells. Taken together, EAEER and its major bioactive compound, HEMA, inhibited the proliferation of B16F10 cells via apoptosis and may be a potential antimelanoma agent.

KEYWORDS: *B16F10 melanoma cell, Eucalyptus citriodora resin, oxidative stress, apoptosis, 6-[1-(p-hydroxy-phenyl)ethyl]-7-O-methyl aromadendrin, antiproliferation*

INTRODUCTION

Cancer is a major public health problem in the world. In Taiwan, cancer ranks first in the leading causes of deaths, accounting for 28.1% of deaths in 2008. Among different types of cancer, skin cancer is the ninth most common cancer, and 2610 new cases were diagnosed in 2007. With respect to skin cancer, melanoma, being the most dangerous type of skin cancer, is a malignant neoplasm of melanocytes type of skin cancer.[1] Cancer can be treated by chemotherapy, radiation, surgery, monoclonal antibody therapy, and other methods. However, the effectiveness of chemotherapy is often limited by their tendency to destroy healthy cells along with cancerous cells.[2] Radiation may also destroy normal tissue. In other words, no effective curative treatment on cancer has been established so far. Therefore, complete removal of cancer without damage to the rest of tissue is the major goal of treatment. Thus, development of novel agents and strategies for cancer removal is a priority and an important global medical issue.

Several epidemiological studies have shown that increased dietary high intake of smoked, salted, and nitrated foods, but a low intake of natural bioactive compounds (e.g., chemopreventive agents, phytochemicals, and antioxidants), are linked to cancer incidences. In recent years, there has been a substantial increase in studies on the effects of natural plant-derived compounds in cancer prevention and treatment.[2] Meanwhile, more and more people have paid great attention to finding novel anticancer drugs from natural organism.

Eucalyptus is a large genus of *Myrtaceae* family that includes 900 species and subspecies.[3] Leaf extracts of *Eucalyptus* have been applied as food additives.[3] Traditionally, the leaves and bark of *Eucalyptus* plants such as *Eucalyptus citriodora* have been widely used as folk medicines in many countries for the treatment of serious diseases and for enhancing physical strength.[4] In addition, the essential oils of *Eucalyptus citriodora* have demonstrated antimicrobial properties against microorganism and termites.[5] Apart from these, *Eucalyptus citriodora* resin is obtained from the tree by making incisions in the trunk of *Eucalyptus citriodora*. It is traditionally employed in the treatments of diarrhea and bladder inflammation and applied to cuts and abrasions. However, relatively less is known about the biological properties of the *Eucalyptus citriodora* resin, more specifically, its major bioactive compound. Now that the resin of *Eucalyptus citriodora* showed some biological effects, it is possible that the resin may possess chemopreventive effects. However, whether it has any chemopreventive effect remains unclear. Thus, the aim of this study was to explore the antiproliferative effect of *Eucalyptus citriodora* resin and its major bioactive compound on B16F10 melanoma cells, and the mechanism of action was also elucidated.

MATERIALS AND METHODS

Materials. Ethyl acetate, *N*-acetyl-L-cysteine (NAC), and 2′7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies of Bax, Bcl-2, and β-actin were provided by Cell Signaling Technology Inc. (Beverly, MA.

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USA). Dimethyl sulfoxide (DMSO) was purchased from Aldrich Chemical (Milwaukee, WI, USA). DMEM medium was purchased from GIBCO Co. (Grand Island, NY, USA). All chemicals were of analytical reagent grade.

Plant Material. *Eucalyptus citriodora* resin was collected in Yung Kang, Tainan, Taiwan, and identified by Prof. Chang-Sheng Kuoh, Department of Life Sciences, National Cheng Kung University. A voucher specimen (CNACNP0605) was deposited in the natural product laboratory of the Department of Medicinal Chemistry, Chia-Nan University of Pharmacy and Science, Tainan, Taiwan.

Sample Preparation. Dried powdered of *E. citriodora* resin was extracted and fractionated according to their polarity. The resin (520 g) was extracted with 1.5 L of 95% ethanol (EtOH) at room temperature for six days. After filtration, the residue was further extracted twice under the same condition. The filtrates were combined and concentrated under reduced pressure. The concentrated ethanol extract (455 g) was suspended in water. The suspension was extracted with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), successively. The EtOAc, *n*-BuOH, and aqueous fractions were separately combined and evaporated to dryness under reduced pressure. The three dried fractions, EtOAc (263 g), *n*-BuOH (132 g), and aqueous (38 g) fractions, were obtained and called EAEER, BEER, and WEER, respectively.

Spectral Methods and Chromatographic Materials. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker AV-500 spectrometer. EI-MS was recorded on a JMS-700 mass spectrometer. Column chromatography were performed on silica gel 60 (Merck, 70−230 mesh) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Thin layer chromatography (TLC) was performed on precoated silica gel plates (Merck, Kieselgel 60 F254, 0.25 mm).

Purification Producer and Identification of HEMA. The ethyl acetate (EtOAc) soluble fraction (263 g) was subjected to silica gel column chromatography and eluted with *n*-hexane/EtOAc/methanol (MeOH) mixtures of increasing polarities to afford 12 fractions (Fr1−Fr12). Each fraction was afforded to TLC using *n*-hexane/EtOAc/MeOH mixture as eluent. The developed TLC was sprayed with vanillin−sulfuric acid reagent and then the plate was heated under 100 °C for 10 min to detect the flavonoid components.[6] Fr6, rich in flavonoid, was afforded to silica gel column chromatography using *n*-hexane/EtOAc/MeOH (1:1:0.1, v/v/v) as eluent, followed by repeated Sephadex LH-20 column chromatography using EtOH/H_2O (1:1, v/v) as eluent to afford pale-yellow amorphous isolate (206 mg). The isolate was identified as 6-[1-(*p*-hydroxy-phenyl) ethyl]-7-*O*-methyl aromadendrin (HEMA, Figure 1) by comparing the MS and NMR spectral data of the isolate with those reported in the literature.[7]

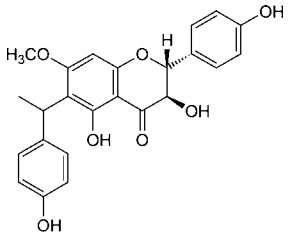

Figure 1. Chemical structure of HEMA. HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

6-[1-(*p*-Hydroxy-phenyl)Ethyl]-7-*o*-Methyl Aromadendrin (HEMA). EI-MS *m*/*z* (relative intensity, %): 422 (M^+, 77), 407 (37), 389 (5), 328 (10), 302 (10), 287 (36), 284 (21), 271 (32), 245 (27), 193 (100), 167 (22), 153 (41), 136 (33), 134 (47), 121 (15), 107 (88), 91 (12), 77 (20). ^{1}H NMR (500 MHz, CD_3OD): δ_H 7.37 (2H, d, *J* = 8.4 Hz, H-2′, 6′), 7.11 (2H, d, *J* = 8.4 Hz, H-3″, 7″), 6.85 (2H, d, *J* = 8.4 Hz, H-3′, 5′), 6.66 (2H, d, *J* = 8.4 Hz, H-4″, 6″), 6.09 (1H, s, H-8), 5.03 (1H, d, *J* = 11.8 Hz, H-2), 4.62 (1H, d, *J* = 11.8 Hz, H-3), 4.60 (1H, q, *J* = 7.2 Hz, H-1″), 3.72 (3H, s, 7-OC$\underline{H}_3$), 1.62 (3H, d, *J* = 7.2 Hz, H-8″). ^{13}C NMR (125 MHz, CD_3OD): δ_C 199.3 (C-4), 167.6 (C-7), 163.2 (C-9), 161.4 (C-5), 159.2 (C-4′), 155.8 (C-5″), 137.9 (C-2″), 130.5 (C-2′, 6′), 129.3 (C-1′), 129.3 (C-3″, C-7″), 116.3 (C-3′, 5′), 116.0 (C-6), 115.6 (C-4″, 6″), 102.5 (C-10), 92.8 (C-8), 85.1 (C-2), 73.8 (C-3), 56.4 (7-O$\underline{C}H_3$), 33.2 (C-1″), 18.1 (C-8″).

Cell Culture. B16F10 mouse melanoma cells obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). The cells were cultured in 9 mL of DMEM medium containing 1 mL of heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5%CO_2/95% air controlled incubator.[8]

Cell Viability Assay. 3-[4, 5-Dimethylthiazol-2-yl]-2,5-dephenyltetrazolium bromide (MTT), a tetrazolium salt, was converted to insoluble formazan by mitochondrial dehydrogenase of living cells. Briefly, cells were dispensed into 96-well plates and samples were added and cultured for 24 h.[8] Then 20 μL of MTT (5 mg/mL stock solution) were added to each well. After 1 h, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of dimethyl sulfoxide. The optical density of each well was measured with microplate reader at 570 nm.

Evaluation of ROS in B16F10 Cells. To determine the generation of ROS in B16 F10 melanoma cells, DCFH-DA was used when it penetrated the cell membranes and was hydrolyzed by intracellular esterase to form dichlorofluorescin (DCFH).[8] Subsequently, DCFH reacted with intracellular ROS to produce highly fluorescent DCF. B16F10 melanoma cells were pretreated with DCFH-DA (50 μM) for 30 min, and then samples were added to the medium. After incubation at 37 °C for 6 h, ROS produced from intracellular stress was detected using a Bio-Tek FLx800 microplate fluorescence reader (Winooski, VT, USA) with excitation and emission wavelengths of 485 and 528 nm, respectively.

Evaluation of NO in B16F10 Cells. The nitrite levels in the cultured media, which reflect the intracellular nitric oxide synthase activity, were determined by the Griess reaction.[9] Briefly, the B16F10 cells were cultured, then the growth medium was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in water), and then absorbance of the mixture at 550 nm was detected.

Evaluation of the Mitochondrial Membrane Potential. The mitochondrial membrane potential of cells was determined by JC-1 mitochondrial membrane potential assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). B16F10 cells were incubated in 100 μL of medium at 37 °C. After cells were pretreated with samples, with or without high glucose, were added to medium and incubated for 12 h. After incubation, the JC-1 dye staining solution was added to cells and incubated for 30 min. The fluorescence was detected using a Bio-Tek FLx800 microplate fluorescence reader (Winooski, VT, USA) with excitation and emission wavelengths of 560 and 595 nm, respectively.

Cytosolic Caspase-3 Activity Determination. The caspase-3 activity was determined by using a fluorometric assay kit (Promega-Corporation, Madison, WI, USA). After B16F10 cells were pretreated with samples for 30 min and incubated at 37 °C for 16 h. The substrate Ac-DEVD-AMC was cleaved by caspase-3, and fluorescence was detected using a Bio-Tek FLx800 microplate fluorescence reader (Winnoski, VT, USA) with excitation and emission wavelengths of 485 and 530 nm, respectively.

Western Blot. After B16F10 cells were cultured with samples for 16 h, the cells were washed with ice-cold PBS and then treated with lysis buffer. Cellular lysates were centrifuged at 10000*g* at 4 °C for 20 min. The supernatants were collected, and the protein contents were determined. Each sample, which contained 50 μg proteins, was separated on 12% SDS−polyacrylamide gels.[10] After electrophoresis, gels were transferred to nitrocellulose paper. After washing with distilled water, the membrane was incubated with 5% albumin in PBS (containing 0.1% Tween-20) and then immunoblotted as described with a 1:1000 dilution antirabbit Bax, Bcl-2, or β-actin (Cell Signaling Technology, USA). Blots were then incubated with antirabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz, CA, USA). Binding was detected by chemiluminescence and captured on Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA).

DAPI Nucleic Acid Staining. After B16F10 cells were cultured with EAEER and HEMA for 6 h, the cells were harvested, washed with phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde in PBS for 30 min. Fixed cells were washed with PBS and stained with DAPI (4′,6′ -diamidino-2-phenylindole). Evaluation was performed by a fluorescence microscope using a 350 nm excitation and a 461 nm filter for detection.

Statistical Analysis. All data were recorded as means ± SD. Statistical analysis involved use of the Statistical Analysis System software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$.

RESULTS

The cell viability of B16F10 after 24 h of treatment with EER extracted from three solvents, including water (WEER), ethyl acetate (EAEER), and *n*-butanol (BEER). The results showed a dose-dependent decrease in the cell growth in three extract-induced cells (data not shown). The three extracts at 500 μg/mL reduced cell viability by 24.2, 60.3, and 46.8% for WEER, EAEER, and BEER, respectively, compared with untreated cells, indicating that the antiproliferative effect of EAEER on B16F10 cells was more effective than that of BEER and WEER. In addition, the yield of EAEER is higher than WEER and BEER. Therefore, we focused on the EAEER in this study.

Next, both isolation and identification of bioactive compound from EAEER were conducted. According to the basis of MS and NMR spectra data, 6-[1-(*p*-hydroxy-phenyl) ethyl]-7-*O*-methyl aromadendrin (HEMA) was isolated and identified. Thus, HEMA was selected as a major compound in this series of experiments, along with EAEER.

Table 1 shows the effects of EAEER and HEMA on cell growth in B16F10 cell lines. The inhibition was dose-dependent, and a suppression of 60.2% was observed at the highest concentration of EAEER of 500 μg/mL, indicating that EAEER affected significantly survival of B16F10 cells. In addition, HEMA at 20 μg/mL (47.4 μM) and 40 μg/mL (94.8 μM) inhibited B16F10 cell growth. These results indicated that EAEER and its purification compound inhibited B16F10 cell growth with various degrees of potency. However, EAEER in the range of 1−100 μg/mL shows no cytotoxicity on B16F10 cell growth. On his basis, our finding provides a strong evidence for potent antiproliferation by EAEER ranged from 100 to 500 μg/mL. Therefore, concentrations ranging from 100 to 500 μg/mL were used for the subsequent series of experiments.

Table 1. Effects of EAEER and HEMA on B16F10 Melanoma Cell Viability Incubated for 24 h

sample	(μg/mL)	cell viability (% of control)[a]
EAEER[b]	1	98.8 ± 0.5 a,b
	10	98.6 ± 0.3 a,b
	50	97.4 ± 1.4 a,b
	100	93.9 ± 1.9 a
	200	75.9 ± 1.7 e
	500	39.8 ± 1.9 c
HEMA[c]	20	84.8 ± 3.2 f
	40	58.1 ± 3.6 d

[a]Results are displayed with mean ± SD ($n = 3$). Values with different letters are significantly different ($p < 0.05$). [b]EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin. [c]HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

To investigate the morphological changes of nuclear of B16F10 cells induced by EAEER and HEMA, DAPI staining was conducted to investigate the effect of EAEER and HEMA on the cells apoptosis. As shown in Figure 2, the normal cells displayed round and stained uniformly dispersed chromatin and intact cell membranes. However, the cells exposed to EAEER and HEMA for 6 h exhibited condensed and fragmented nuclei, indicative of apoptosis.

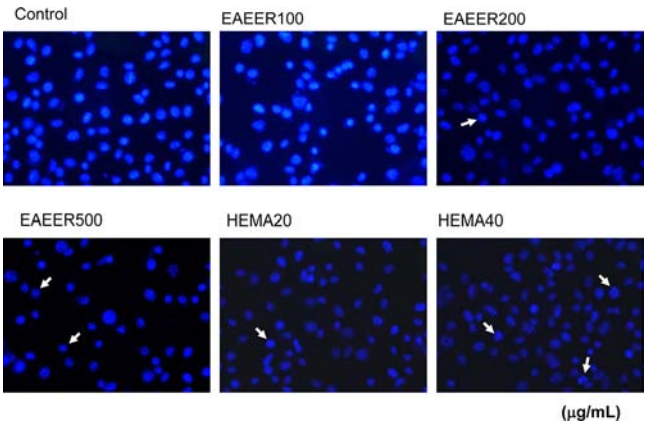

Figure 2. Effects of EAEER and HEMA on morphological changes in nuclear of B16F10 cells. The cells were exposed to EAEER and HEMA for 6 h, and oxidative DNA damage was evaluated using DAPI staining. The DAPI-stained cells were evaluated using fluorescence microscopy (200×). One representative result is presented from triplicate experiments that yielded similar results. EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin; HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

Figure 3 shows the effects of EAEER and HEMA on caspase-3 activity in B16F10 cell lines, revealing dose results showing a dose-dependent increase in the activity of caspase-3 in EAEER- or HEMA-treated cells. Caspase-3 activity increased 2.08−3.99-fold after treatment with EAEER in the range of 100−500 μg/mL. Similarly, significant increase in caspase-3 activity was observed after the addition of HEMA. These results indicated

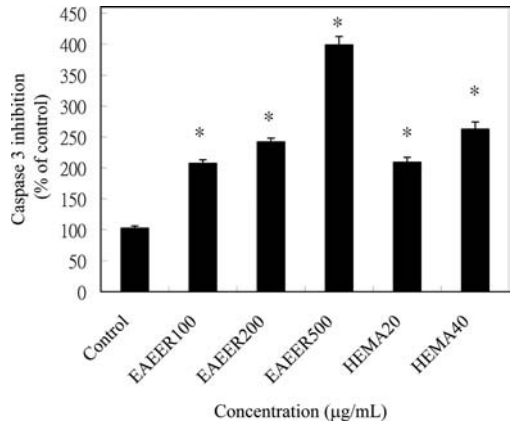

Figure 3. Effects of EAEER and HEMA on caspase 3 activity in B16F10 melanoma cell incubated for 16 h. Data are presented by means ± SD ($n = 3$). # ($p < 0.05$) compared with the control group and * ($p < 0.05$) compared with B16 melanoma cells alone. EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin; HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

that caspase-3 activity was involved in the apoptotic effects induced by EAEER and HEMA.

Figure 4 shows the effects of EAEER and HEMA on mitochondria in B16F10 cell lines with the mitochondrial

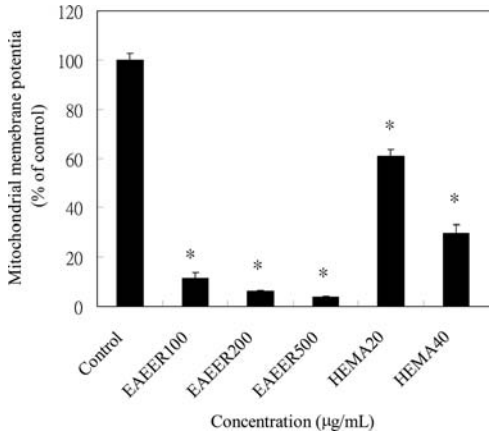

Figure 4. Effects of EAEER and HEMA on mitochondria membrane potential ($\Delta\psi_m$) in B16F10 melanoma cell incubated for 12 h. Data are presented by means ± SD (n = 3). # (p < 0.05) compared with the control group and * (p < 0.05) compared with B16 melanoma cells alone. EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin; HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

membrane potential ($\Delta\Psi_m$) determined. As can be seen, when treated with 100−500 μg/mL of EAEER and HEMA at 20 and 40 μg/mL for 12 h, the cells showed a concentration-dependent decrease in $\Delta\Psi_m$. Moreover, compared with untreated cells, those treated with 100−500 μg/mL of EAEER showed 11.4−3.7% red fluorescence, and addition of HEMA to cells led to a significant decrease in mitochondrial membrane potential. These findings reveal that mitochondrial membrane was depolarized (loss of $\Delta\Psi_m$) when cells were treated with EAEER and HEMA.

In view of the dose-dependent reduction in $\Delta\Psi_m$ in cells treated with EAEER and HEMA, it was necessary to explore the proapoptotic and antiapoptotic members that were related to mitochondrial control during apoptosis. Figure 5 shows the effects of EAEER and HEMA on the ratio of Bax/Bcl-2 in B16F10 cell lines. Cells treated with EAEER at 100 μg/mL and HEMA at 20 μg/mL showed a significant increase in the ratio of Bax/Bcl-2, suggesting that EAEER-induced apoptosis in B16F10 cells via alternation of the Bax/Bcl-2 ratio.

To elucidate whether the generation of reactive oxygen species (ROS)/nitric oxide (NO) played a crucial role in the induction of apoptosis, the DCF assay and determination of nitrite levels were conducted. Figure 6 shows the effects of EAEER and HEMA on the ROS/NO generation in B16F10 cell lines. Slight increases in ROS generation were observed after the addition of EAEER at 100 μg/mL and HEMA at 20 μg/mL but did not reach statistical significance (p > 0.05). However, a significant influence on the generation of ROS was observed for EAEER at 200 and 500 μg/mL and HEMA at 40 μg/mL. ROS/NO generation increased 649.5%/362.4% and 217.0%/103.4% after treatment with EAEER at 500 μg/mL and HEMA at 40 μg/mL, respectively, indicating that EAEER and HEMA showed an inductive effect on the generation of ROS/NO, except for low concentrations of EAEER and HEMA. Because EAEER- and HEMA-induced apoptosis was suggested to be regulated by ROS, we next used NAC (*N*-acetyl-L-cysteine), an

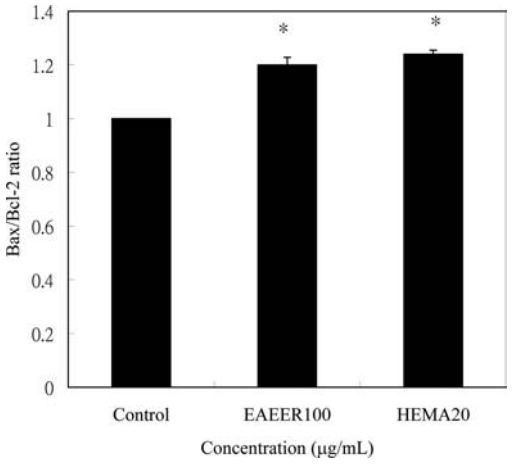

Figure 5. Effects of EAEER and HEMA on the protein levels on Bax and Bcl-2 in B16F10 melanoma cell incubated for 16 h. Data are presented by means ± SD (n = 3). # (p < 0.05) compared with the control group and * (p < 0.05) compared with B16 melanoma cells alone. EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin; HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

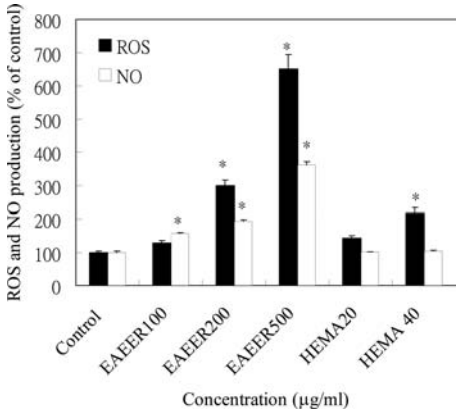

Figure 6. Effects of EAEER and HEMA on inducing intercellular ROS and NO production in B16F10 melanoma cell incubated for 6 h. Data are presented by means ± SD (n = 3). # (p < 0.05) compared with the control group and * (p < 0.05) compared with B16 melanoma cells alone. EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin; HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin.

antioxidant, for further experiments. Treatment of B16F10 cells with NAC suppressed significantly the generation of intracellular ROS (Figure 7A). Moreover, inhibitory effects of EAEER and HEMA on B16F10 cell growth were prevented significantly by NAC treatment (Figure 7B). These observations indicated that the modulation of molecules involved in the redox system may determine the sensitivity of B16F10 cells to EAEER and HEMA.[11]

DISCUSSION

In the present study, the antiproliferative activity of extracts from ethyl acetate was higher than that of extracts from butanol and water. Apparently, ethyl acetate had a higher efficiency on the extraction of antiproliferative compounds from *Eucalyptus citriodora* resin. Moreover, a flavanol derivative, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin, was isolated as a major compound from the *Eucalyptus citriodora* resin. Thus, HEMA and EAEER were used as substrates to evaluate their potential antitumoral effects on B16F10 cells. To

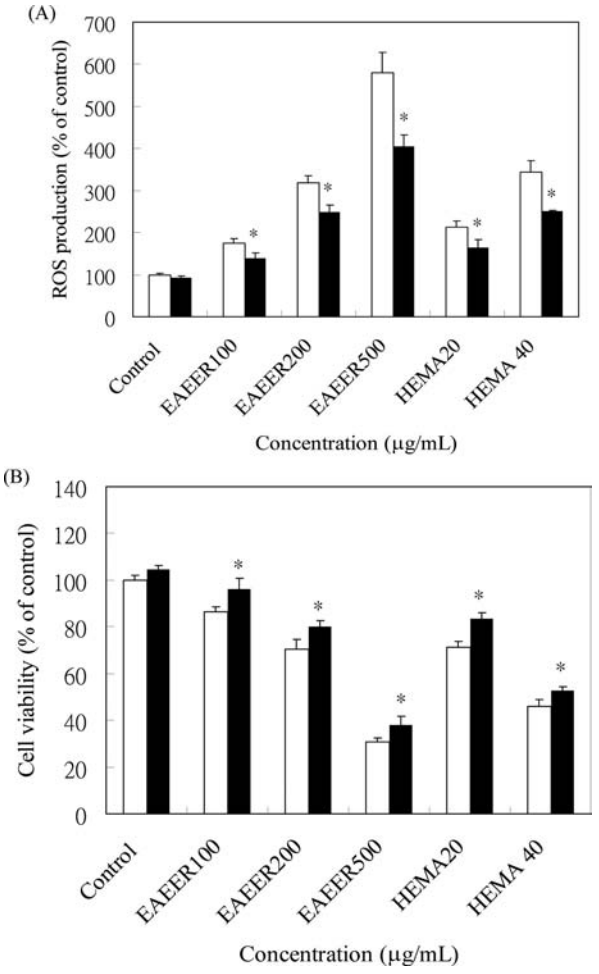

Figure 7. Effects of NAC on intercellular ROS production (A) and cell growth (B) in B16F10 melanoma cell exposed to EAEER and HEMA. The cells were exposed to EAEER and HEMA for 6 and 24 h with or without 20 μM NAC-pretreated and determination of ROS production (A) and cell growth (B) by DCFDA and MTT methods, respectively, as described in the Materials and Methods. Data are presented by means ± SD (n = 3). # (p < 0.05) compared with the control group and * (p < 0.05) compared with B16 melanoma cells alone. NAC, *N*-acetyl-L-cystein; EAEER, ethyl acetate extract of *Eucalyptus citriodora* resin; HEMA, 6-[1-(*p*-hydroxyphenyl) ethyl]-7-*O*-methyl aromadendrin. (□) EAEER or HEMA without NAC, (■) EAEER or HEMA with NAC.

investigate the effect of EAEER and HEMA on apoptosis in B16F10 cells in vitro, DAPI staining was used to study the morphological changes in nuclear of B16F10 cells. The DAPI results showed that nuclear morphology changed significantly in B16F10 cells induced by EAEER and HEMA, indicating that the DNA changes occurred in the cell apoptotic process.[12] These results support the conclusion that EAEER and HEMA induced B16F10 cells.

To confirm whether EAEER and HEMA induced apoptosis through a mitochondria-dependent pathway, the disruption of $\Delta\Psi_m$ in mitochrondria was conducted. Treatment with EAEER and its purified compound, HEMA modified the mitochondrial membrane potentials of B16F10 cells, indicating that both EAEER and HEMA induced reduction in $\Delta\Psi_m$ of B16F10 cells by disturbing their mitochondrial membrane permeability. In addition, the disruption of $\Delta\Psi_m$ leads to depolarization and, subsequently, an increase in mitochondrial outer membrane permeability, which is considered as one of the earliest indicators of induction of cellular apoptosis.[13] Hence, B16F10 cell lines treated with EAEER and HEMA showed a reduced mitochondrial membrane potential, suggesting that apoptosis was triggered by treatment with EAEER and HEMA.

The Bcl-2 family of proteins, comprising pro-apoptotic (e.g., Bax) and antiapoptotic (e.g., Bcl-2) members, are major regulators of the intrinsic mitochondria-mediated pathways.[14] Moreover, these proteins form ion channels in the biological membrane, which regulates apoptosis by influencing the permeability of the intracellular mitochondria membrane.[15] Indeed, down-regulation of Bcl-2 is indicative of apoptosis. Instead, Bax overexpression allows apoptosis.[14] Although Bax overexpression alone is indicative of accelerated apoptotic cell death, the ratio between Bax and Bcl-2 is more important in regulation of apoptosis than the level of individual Bcl-2 family protein.[16] As illustrated in Figure 5, under our experimental conditions, treatment with EAEER and HEMA markedly increased the ratio of Bax/Bcl-2, showing that balance between Bax and Bcl-2 was in favor of Bax. Hence, increase in Bax vs Bcl-2 triggered by EAEER and HEMA suggests both mitochondrial permeability transition pore opening and induction of apoptosis in B16F10 cells.[17]

The activity of caspase-3, which is one of the major executing enzymes in apoptosis, was determined to shed light on the effect of EAEER and HEMA on apoptosis. According to the data obtained from Figure 3, we verified that caspase-3 activity was activated in B16F10 cells incubated in the presence of EAEER and HEMA. The activation of caspase-3 induced by both EAEER and HEMA was likely to be responsible for the pro-apoptotic effect exhibited by EAEER and HEMA. Thus, increased activity of caspase-3 in cells may enhance the risk of apoptosis, which consequently promotes nuclear condensation and cell shrinkage and finally causes cell death.[18] Our data indicated that EAEER and HEMA activated caspase-3 activity, thereby facilitating the apoptosis of these cancer cells. In other words, EAEER and HEMA are able to induce cell death through apoptosis in B16F10 cells and the activation of caspase-3 is involved.

Reactive oxygen and nitrogen species (ROS/RNS) are generated during detoxification, which leads to oxidative stress.[13] For example, apoptosis can be induced by ROS, leading to pathological cell death. NO reacts with superoxide and generates reactive nitrogen species, thereafter modifying biomolecules. Thus, enhancement of ROS/RNS generation causes lipid peroxidation in cell membranes, DNA damage, and decrease in a mitochondrial transmembrane potential, which can lead to cell death/injury. The present study showed that treatment with EAEER at 500 μg/mL and HEMA at 40 μg/mL stimulated ROS/RNS overproduction, which was accompanied by increases in reactive oxygen species of up to 649.5% and 217.4%, respectively, and in reactive nitrogen species of 362.4% and 103.4%, respectively, compared with untreated cells. These findings are in line with the reports of Pacheco-Palencia et al.,[19] which noted that polyphenolic-rich extracts from acai pulp and acai oil significantly inhibited cell proliferation and increased the generation in ROS in a concentration-dependent manner. In other words, the generation of ROS has been closely linked to apoptosis. In the present study, NAC, an antioxidant, significantly blocked ROS production and attenuated EAEER- and HEMA-induced cell death, suggesting that ROS played an upstream important mediator during EAEER- and HEMA-induced apoptosis in B16F10 cells.[11] Our data reveal that

EAEER and HEMA without direct ROS/RNS scavenging can inhibit B16F10 cell proliferation by relying just on their pro-oxidant activity and contributing to oxidative stress, which in turn leads to cell death/injury. This finding demonstrates that EAEER and HEMA act as promoters of ROS generation in B16F10 cells. Hence, exposing B16F10 cells to either EAEER or HEMA can induce the generation of ROS, which initiates apoptosis.

Past research has reported that polyphenols induce apoptosis, consequently contributing to cancer prevention.[20] In our previous study, the concentrations of total phenolic compounds and flavonoids in EER were 297.7 ± 0.14 mg/g and 86.2 ± 2.01 mg/g, respectively,[21] which are higher than 211.8 mg/g of phenolics in Oolong tea.[22] In the present study, EER had high total phenolic compounds. Among them, the major flavonoid detected in EAEER was HEMA. The content of HEMA in EAEER was 783 μg/g. Diets supplemented with a single or a few compounds do not have the same health benefits as a diet rich in fruits and vegetables. In addition, pure compounds may either lose their bioactivity or may not behave in the same way as the compound in whole foods.[20] However, according to the data obtained, HEMA and EAEER showed a similar trend in their antiproliferative activity in B16F10 cells (Table 1). It is important to note that the results of antiproliferation and apoptosis stimulated by HEMA are in good agreement with those by EAEER. Thus, we suggest that HEMA is one of the major components that inhibit B16F10 cell proliferation and induce apoptosis. However, the data obtained from Figures 4 and 6 showed HEMA displayed weak activity on $\Delta\Psi_m$ reduction and ROS/NO production. We speculated that other bioactive compounds present in EAEER may exhibit antiproliferative action on B16F10 cells along with HEMA.

Oxidative stress has been proven to be involved in many diseases and plays an important role in indirect genotoxicity. Previous studies on the pro-oxidant activity of phenolics such as EGCG have shown that EGCG could be converted to a phenoxyl radical after neutralizing the peroxy or other radicals.[23] The experimental data in the literature can be divided into two main groups: those showing cell protective effects of phenolics against various oxidative stress and those, mainly of tumor cells, demonstrating proliferation inhibition and cell death induced by phenolics.[24] Hadi, Asad, Singh, and Ahmad,[25] proposed a mechanism for the cytotoxic action of phenolic compounds against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action. In the present study, the inhibition of cancer cell proliferation in vitro by EAEER and its purified compound, HEMA, may be independent of their antioxidant activity. Potential mechanisms for this chemopreventive effect may be attributed to promotion of ROS/RNS, which in turn lead to alternation of Bax/Bcl-2 ratio, loss of $\Delta\Psi_m$, and activation of caspase, thereby initiating cell death/injury. Thus, we suggest that it is the pro-oxidant action of EAEER or HEMA, rather than their antioxidant activity, that contributes to their anticancer and apoptosis-inducing properties.[25]

In conclusion, our results found that EAEER could inhibit B16F10 proliferation and induce cancer cell apoptosis. The possible mechanism for their effect involves the excessive production of ROS/RNS after EAEER exposure in B16F10 cells, thus creating a situation of oxidative bursts and leading to cytotoxicity of B16F10 cells. In addition, HEMA isolated from EAEER displayed marked antiproliferation and apoptosis induction, making it an efficient chemopreventive agent. Thus, EAEER and HEMA may be potential agents for the prevention and/or treatment of cancer. However, further studies should be conducted in appropriate animal models of cancer, and ultimately, human cancer prevention trials should be performed.

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Notes

The authors declare no competing financial interest.

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