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Camboginol and Morelloflavone from *Garcinia dulcis* (Roxb.) Kurz Flower Extract Promote Autophagic Cell Death against Human Glioblastoma Cells through Endoplasmic Reticulum Stress

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ABSTRACT: *Garcinia dulcis* is a tropical plant native to Southeast Asia that is traditionally used as a folk remedy to cure several pathological symptoms. Camboginol and morelloflavone have been revealed by previous studies as the principal bioactive compounds from the flower extract of *G. dulcis*. The disease-preventing properties of camboginol or morelloflavone, including anti-cancer, from various parts of *G. dulcis* have been revealed by recent studies. Glioblastoma is the aggressive malignant stage of brain cancer and suffers from chemotherapeutic resistance. This study aimed to test the anti-cancer effect of *G. dulcis* flower extract against the proliferation of A172 human glioblastoma cells. The extract had cytotoxic activity and promoted cell cycle arrest at the S and G2/M phases. Autophagic cell death was promoted by cytotoxic concentrations of the extract, as observed by enhancing autophagic flux and the expression of autophagic markers. Autophagic cell death induced by the extract might be associated with endoplasmic reticulum (ER) stress. Conclusively, it was indicated by this study that the extract from the flower of *G. dulcis* had a protective effect against the proliferation of A172 human glioblastoma cells through the induction of ER stress-mediated cytotoxic autophagy.

Keywords: autophagy, endoplasmic reticulum stress, *Garcinia dulcis*, glioblastoma

INTRODUCTION

Glioblastoma (GBM) is the most prevalent and severe brain cancer, with a high mortality rate. GBM is extremely resistant to conventional chemotherapeutics, due to its heterogeneity and the presence of cancer stem cells originating from and transforming GBM (Oliver et al., 2020). Characterization and evaluation of natural bioactive compounds are warranted.

Natural products from tropical plants are traditionally used as folk medicine to treat diseases and food supplements. *Garcinia dulcis* belongs to the Guttiferae family. *G. dulcis* is a tropical plant cultivated in Southeast Asia. Various parts of the *G. dulcis* have beneficial effects on the medication of several diseases. The leaves and seeds of *G. dulcis* have been used traditionally for treating lymphatitis, parotitis, and goiter (Phongpaichit et al., 2006). The fruit juice has been used as an expectorant, while the stem bark has been used as an antiseptic agent. *G. dulcis* contains several bioactive compounds, including flavonoids, benzophenone, xanthones, and benzophenone-xanthone, which can be found in several parts of *G. dulcis*, including fruit, flower, leave, stem, and bark (Khamthong and Hutadilok-Towatana, 2017). Camboginol (plant benzophenone) and morelloflavone (biflavonoids) are found in the insoluble hexane extract of *G. dulcis* flowers, as previously reported (Deachathai et al., 2006). The oral administration of extracts from the flower of *G. dulcis* has been found by previous studies to reduce hypertension and promote diuretics in renovascular hypertensive rats (Thongsepee et al., 2020; Thongsepee et al., 2022).

Camboginol (garcinol) is classified as a prenylated benzophenone. The critical compartment of the molecule is the terminal alkene in an unsaturated isopropenyl substituent that can undergo cyclization under acidic conditions to produce isogarcinol. This substance has been as-

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sociated with disease-preventing properties, including antitumorigenic, anti-inflammation, anti-bacteria, antivirus, and anti-obesity properties (Saadat and Gupta, 2012). Camboginol has robust anti-oxidative activity analyzed with a 2,2-diphenyl-1-picrylhydrazyl assay. Camboginol has various pathological roles, including anti-bacteria, anti-inflammation, and anti-cancer (Liu et al., 2015). Camboginol derived from *G. dulcis* had vasorelaxation in 2-kidneys-1-clip (2K1C) renovascular hypertensive rats, which might be involved with its anti-oxidative activity (Thongsepee et al., 2018).

Morelloflavone is a biflavonoid containing apigenin and luteolin, which occurs in most *Garcinia* spp., including all *G. dulcis* parts (Khamthong and Hutadilok-Towatana, 2017). Morelloflavone has potent antioxidant effects on human low-density lipoprotein oxidations (Hutadilok-Towatana et al., 2007). Morelloflavone has several health-prevention activities, including anti-inflammation (Gil et al., 1997) and hypocholesterolemic activities (Tuansulong et al., 2011). Morelloflavone derived from *G. dulcis* exerted hypotensive and diuretic effects in 2K1C hypertensive rats (Thongsepee et al., 2017).

There have been studies revealing the anti-cancer effects of camboginol or morelloflavone derived from several *Garcinia* spp. Garcinol from *Garcinia indica* dose-dependently suppressed viability, invasion, migration, and cell cycle progression and promoted cell apoptosis in cervical cancer cell lines, inhibiting tumor growth in a xenograft model through activation of T-cadherin (Zhao et al., 2018). Morelloflavone from *G. dulcis* has antiangiogenesis in human umbilical vascular endothelial cell by targeting the activation of Rho-GTPases and extracellular signal-regulated kinase signaling pathways (Pang et al., 2009). The isolated morelloflavone from the Gambogic tree trunk inhibited the growth of U87 and xenografted C6 glioma cells, which were associated with the formation of intramolecular hydrogen bonds in biflavone compounds (Li et al., 2016).

We hypothesized that combining two major bioactive components in *G. dulcis*, camboginol and morelloflavone, might exert anti-cancer properties. This study demonstrated the anti-cancer effects of the combination of camboginol and morelloflavone derived from the *G. dulcis* flower extract against A172 human GBM cells.

MATERIALS AND METHODS

Preparation of the *G. dulcis* flower extract

The extraction and purification of the *G. dulcis* flower were previously described (Deachathai et al., 2006). The hexane-insoluble fraction, composed of camboginol and morelloflavone, was used in this study as previously described (Deachathai et al., 2006; Thongsepee et al., 2020). The *G. dulcis* fraction was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Pty Ltd., Darmstadt, Germany) and warmed at 40°C before use.

Cell culture and treatment

A172 human GBM cells were purchased from the American Type Culture Collection (Manassas, VA, USA). OUMS-36 normal human embryo fibroblast cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and a 1% penicillin/streptomycin cocktail in an incubator with an atmosphere of 5% CO_2 at 37°C. The solidified *G. dulcis* extract was prepared in DMSO and dissolved in DMEM before cell treatment. Autophagy inhibitor chloroquine (CQ, Enzo Life Sciences Inc., Farmingdale, NY, USA) was dissolved in DMEM at a final concentration of 1 mM to evaluate autophagy correlation.

Cytotoxicity

A172 cells cultured in 96-well plates at about 70% confluency were incubated for 24 h with increasing concentrations of *G. dulcis* extracts diluted in serum-free DMEM. The final concentration of DMSO was 0.25%. The cytotoxicity following *G. dulcis* treatment was determined using methyl thiazolyl tetrazolium (MTT) assays. MTT solution (Bio Basic Inc., Toronto, Canada) was dissolved in treated wells for a final concentration of 30 μg/mL, then incubated for 3 h. After that, DMSO was added to dissolve the MTT formazan. The reaction was measured with a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA) at 562 and 630 nm absorbance wavelengths.

Cell cycle analysis

G. dulcis extracts at 20, 40, and 100 μg/mL were treated in A172 cells cultured on 6-well plates for 24 h. Treated cells were harvested with 0.1% trypsin-ethylenediaminetetraacetic acid and fixed in methanol for 30 min. Cells were incubated with propidium iodide (PI)/RNase (BD Biosciences, San Jose, CA, USA) according to the manufacturer's guidelines. The fluorescence intensity of the PI-stained cells was measured using a DxFlex flow cytometer (Beckman Coulter, Brea, CA, USA) at a minimum of 10,000 events/sample.

Autophagy induction

Autophagy in *G. dulcis*-treated A172 cells was evaluated by fluorescence microplate reader using the CYTO-ID® Autophagy Detection Kit (Enzo Life Sciences Inc.), a cationic amphipathic tracer that specifically labels autophagic vacuoles in living cells, following the manufacturer's guidelines. In brief, treated A172 cells in 96-well plates

for 24 h were incubated with green detection reagent in DMEM medium without phenol red for 30 min at room temperature. CQ (20 μM) was used as the positive control. Green fluorescence signals indicating the presence of autophagosomes were measured by a fluorescence microplate reader (Varioskan LUX multimode microplate reader, Thermo Fisher Scientific) at excitation and emission wavelengths of 480 and 530 nm, respectively. Fluorescence photomicrographs were qualitatively visualized using an AX R confocal microscope (Nikon Corp., Tokyo, Japan).

Immunoblotting

Whole proteins from treated cells were extracted using a radioimmunoprecipitation assay cell lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA). Protein concentrations were determined using Bradford protein assays (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equivalent amounts of whole proteins (20 μg each) were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked in tris-buffered saline and Tween-20 (TBST) containing 5% bovine serum albumin for 1 h. The samples were incubated with the primary antibodies, including rabbit anti-light chain 3 (LC3), rabbit anti-p62, rabbit anti-binding immunoglobulin-protein (BiP), rabbit anti-inositol-requiring enzyme 1α (IRE1α), rabbit anti-protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK), and rabbit anti-β-actin (Cell Signaling Technology Inc.) at 4°C overnight. The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology Inc.) diluted in 0.01 M TBST for 1:5,000 dilutions at room temperature for 1 h. The expression of specifically targeted proteins was visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific). The relative expression level of target proteins was normalized to β-actin expression and compared with the control group.

Statistical analysis

Data were expressed as mean±standard deviation on triplication. Statistical variations of all experiments were analyzed with GraphPad Prism statistical analysis software (GraphPad Software Inc., San Diego, CA, USA) using a one-way ANOVA test. Statistical testing comparing CQ and its co-treatment was analyzed using the Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Cytotoxicity

The cytotoxic effects of the *G. dulcis* extract in A172 cells were measured after treatment for 24 h using MTT assays. The viability of A172 cells decreased successively after treatment with the *G. dulcis* extracts at a final concentration of 25 to 200 μg/mL. Viability was dramatically reduced from 250 to 1,000 μg/mL (Fig. 1). The half-maximal inhibitory concentration value was 29.03±0.18 μg/mL. Additionally, treatment of the *G. dulcis* extract in the OUMS-36 normal human embryo fibroblast cell line for 24 h had no toxicity at a concentration between 10 and 100 μg/mL. Still, it reduced its viability after treatment between 500 and 1,000 μg/mL. This implies that the *G. dulcis* extract is safe for fibroblasts as a model of normal human cells.

Cell cycle arrest

A172 cell cycle progression following *G. dulcis* treatment was detected by PI/RNase assay using flow cytometry. After 24 h of treatment, *G. dulcis* at 40 and 100 μg/mL significantly increased the A172 cell cycle distribution percentage in the S and G2/M phases. This was accompanied by a decrease in cell distribution in the G1 phase

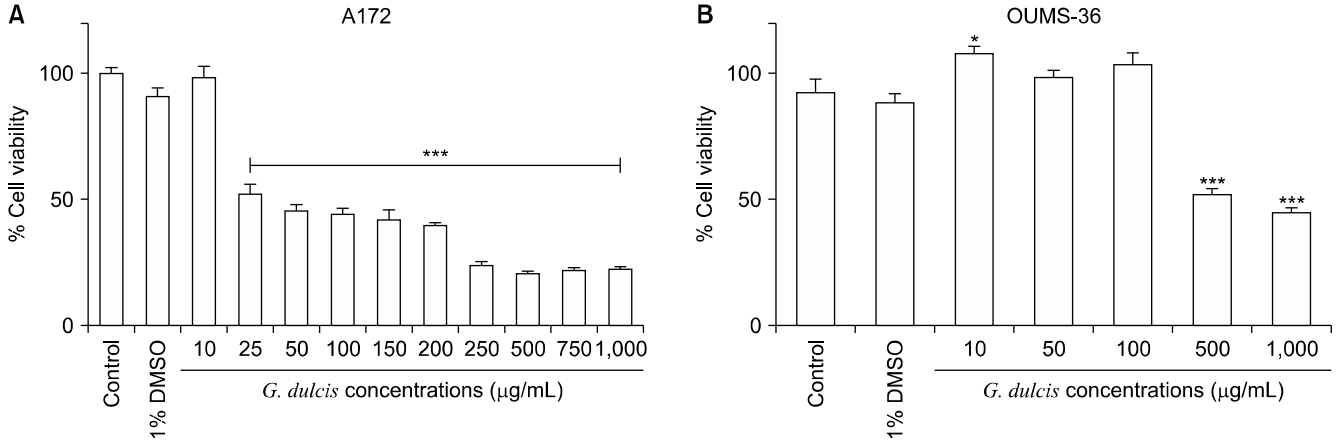

Fig. 1. Cytotoxic effects of *Garcinia dulcis* on A172 cells (A) and OUMS-36 cells (B) after incubation for 24 h, measured by methyl thiazolyl tetrazolium assays and compared with 1% dimethyl sulfoxide (DMSO). **P*<0.05 and ****P*<0.001.

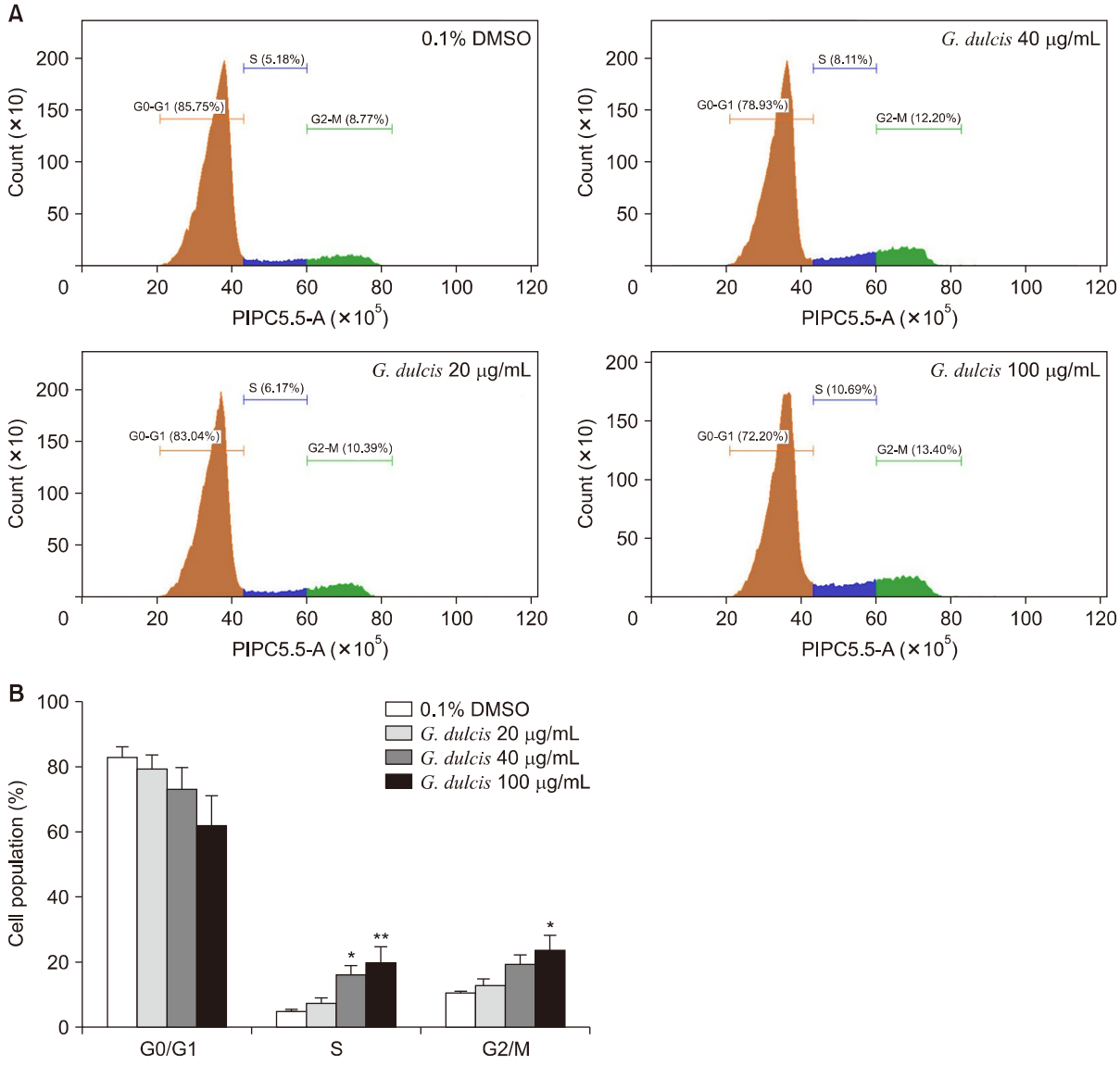

Fig. 2. (A) Cell cycle analysis of *Garcinia dulcis*-treated A172 cells after incubation for 24 h detected by propidium iodide staining. (B) The histogram represents the percentage of cell numbers in each cell cycle phase and compared with 1% dimethyl sulfoxide (DMSO). **P*<0.05 and ***P*<0.01.

(Fig. 2). This result indicated that *G. dulcis* at 40 and 100 μg/mL promoted cell cycle arrest at the S and G2/M phases in A172 cells.

Autophagy flux

The effect of *G. dulcis* extract on cellular autophagy was detected using the CYTO-ID® fluorescence probe. *G. dulcis*-treated A172 cells at 40 and 100 μg/mL had an increase in the green intracellular fluorescence intensity after treatment for 24 h compared with the DMSO-treated control group (Fig. 3). CQ treatment reduced the green fluorescence intensity but was further promoted by the co-treatment of *G. dulcis* at 100 μg/mL and CQ. It was indicated by the results that *G. dulcis* stimulated the formation of autophagosomes, representing the induction of autophagic flux in A172 cells.

Expression of the autophagic marker

The expression level of autophagic markers LC3 and p62 in A172 cells was detected after incubation with *G. dulcis* for 24 h compared with cells treated with CQ, a late-stage autophagy inhibitor, by immunoblotting. *G. dulcis* treatment increased the LC3-II expression level, especially at 100 μg/mL. *G. dulcis* treatment degraded p62 expression during autophagy, as revealed by a decrease in p62 level at all selected concentrations after *G. dulcis* treatment. Co-treatment of CQ with 100 μg/mL of *G. dulcis* decreased LC3-II and p62 levels compared with the CQ-treated group (Fig. 4). These results indicate that *G. dulcis* treatment promoted autophagy in A172 cells.

Expression of molecules controlling ER stress

The expression level of ER stress-related proteins BiP, IRE1α, and PERK in A172 cells after incubation with *G.*

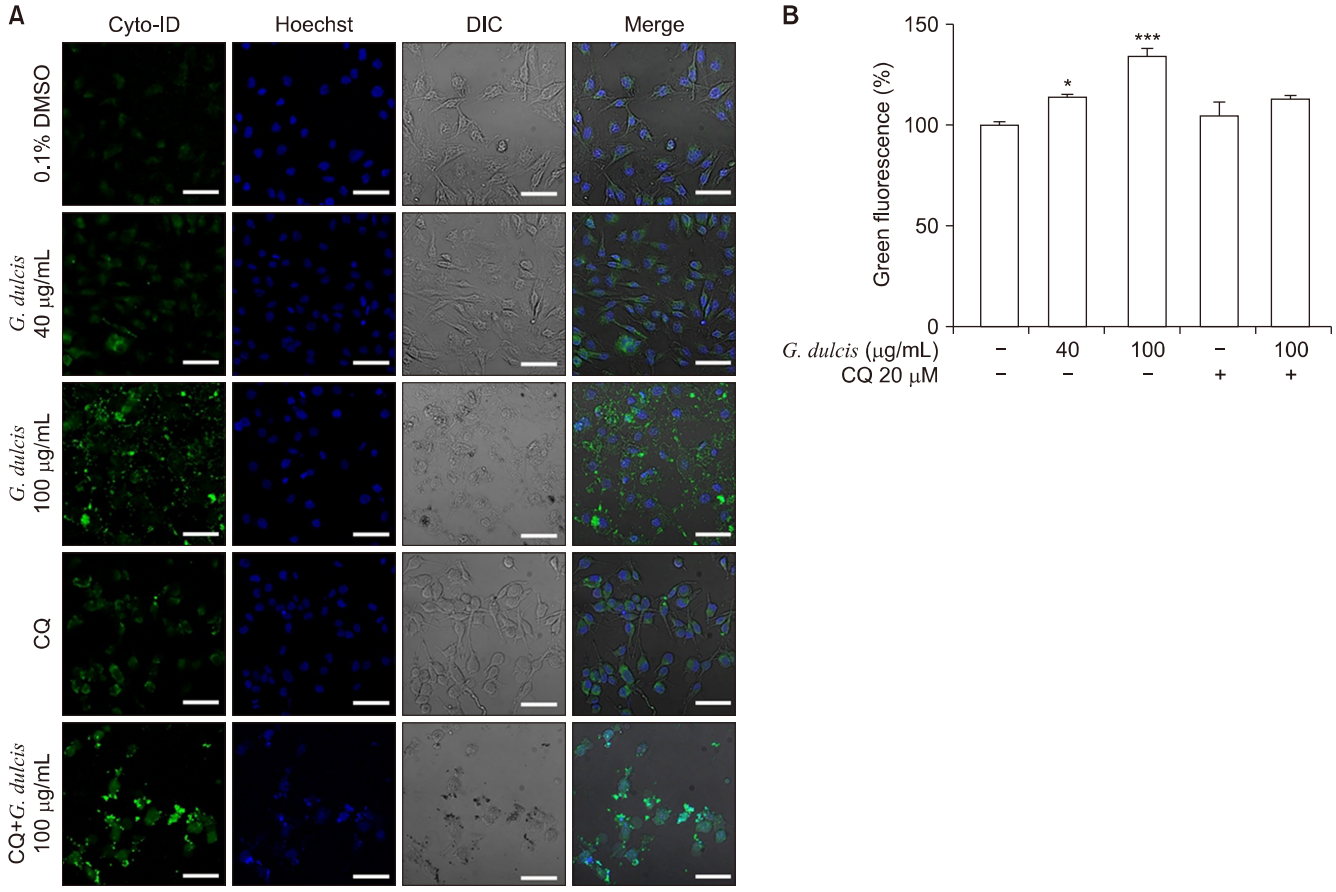

Fig. 3. Induction of autophagic flux on *Garcinia dulcis*-treated A172 cells after incubation for 24 h was detected by the CYTO-ID® autophagy assay staining. (A) Photomicrographs showing green fluorescence signals of CYTO-ID® and Hoechst33342 nuclear stains. Differential interference contrast (DIC) and merged micrographs were also revealed. (B) The bar graph shows the green fluorescence intensity detected in treated cells compared with the control. **P*<0.05 and ****P*<0.001. DMSO, dimethyl sulfoxide; CQ, chloroquine.

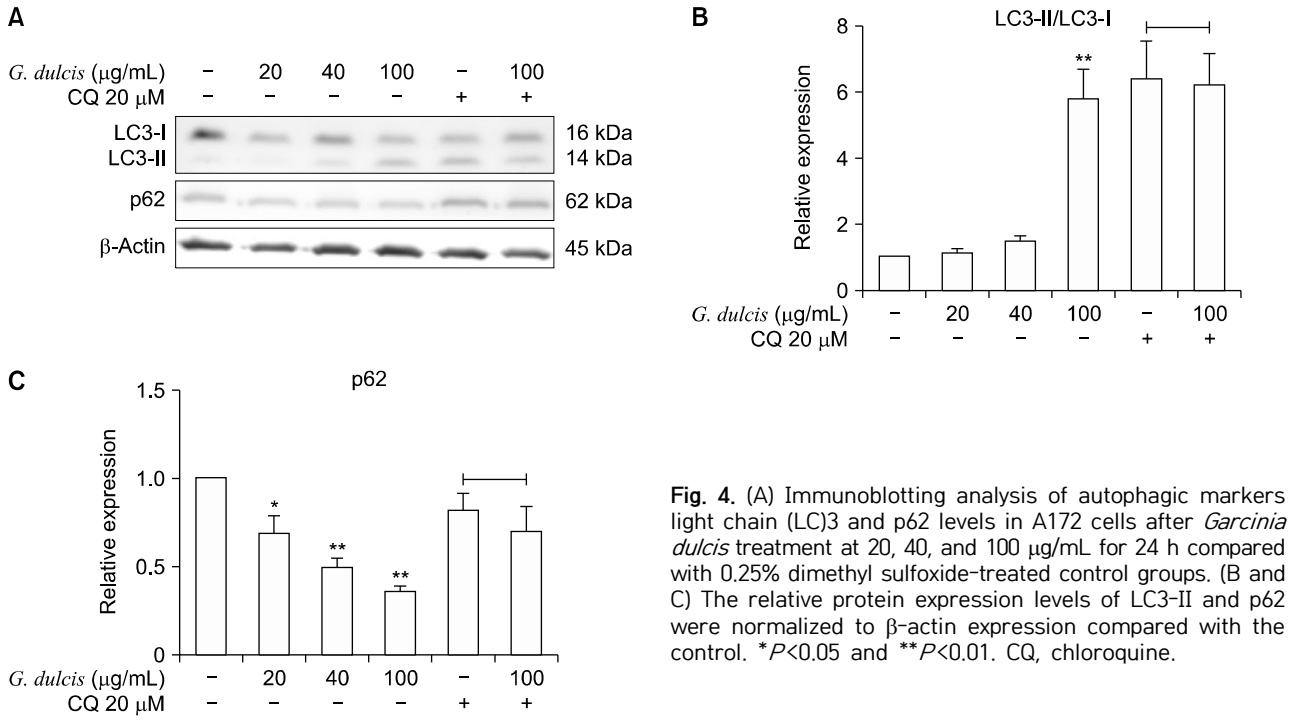

Fig. 4. (A) Immunoblotting analysis of autophagic markers light chain (LC)3 and p62 levels in A172 cells after *Garcinia dulcis* treatment at 20, 40, and 100 μg/mL for 24 h compared with 0.25% dimethyl sulfoxide-treated control groups. (B and C) The relative protein expression levels of LC3-II and p62 were normalized to β-actin expression compared with the control. **P*<0.05 and ***P*<0.01. CQ, chloroquine.

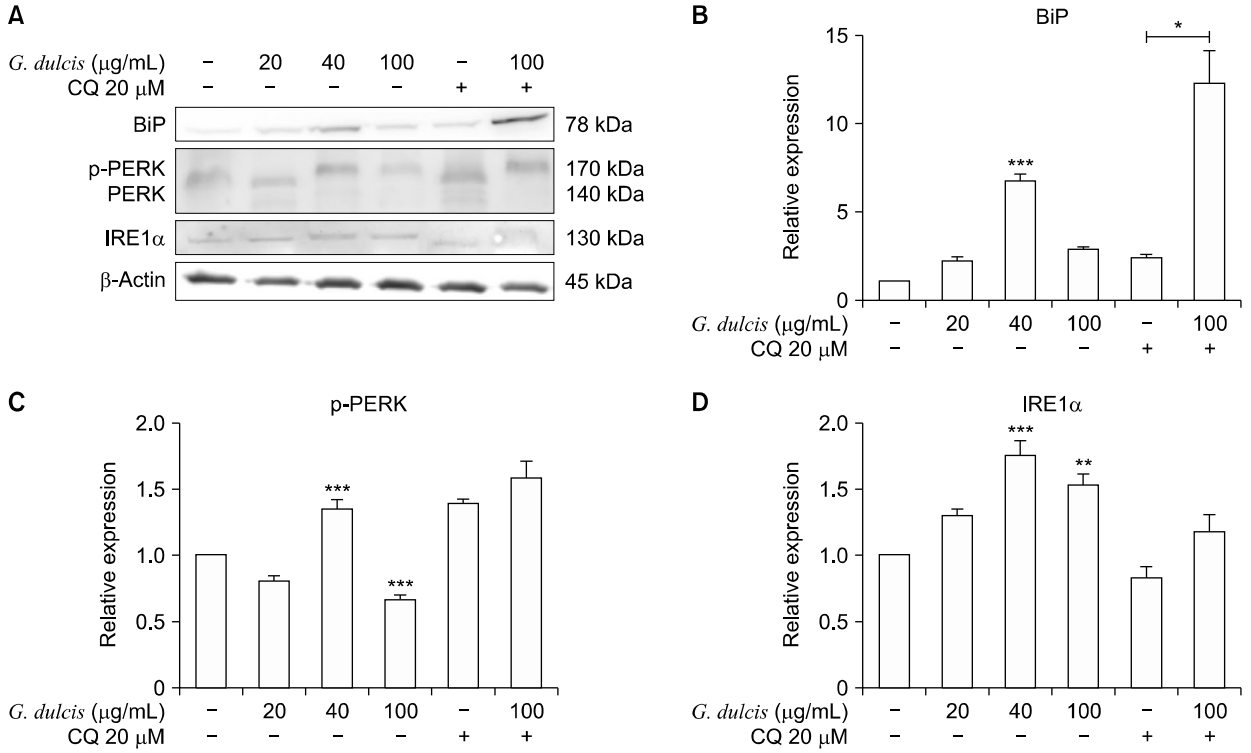

Fig. 5. (A) Immunoblotting analysis of ER stress markers binding immunoglobulin-protein (BiP), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1α (IRE1α) levels in A172 cells after *Garcinia dulcis* treatment at 20, 40, and 100 μg/mL for 24 h compared with 0.25% dimethyl sulfoxide-treated control groups. The relative protein expression levels of BiP (B), phosphor-PERK (C), and IRE1α (D) were normalized to β-actin expression compared with the control. *P<0.05, **P<0.01, and ***P<0.001. CQ, chloroquine.

dulcis for 24 h was detected by immunoblotting and compared with cells treated with CQ to investigate whether autophagy induction is adaptive to the ER stress response. BiP expression levels increased after *G. dulcis* treatment for 24 h. In particular, IRE1α and PERK hyperphosphorylation are present in A172 cells following increasing concentrations of *G. dulcis* treatment, prominently at 40 μg/mL, shown as the upper band. CQ inhibited BiP and IRE1α expression levels and re-promoted after co-treatment of CQ with 100 μg/mL of *G. dulcis* (Fig. 5). The band shift of p-PERK also appeared in the co-treatment condition. It was indicated by these results that *G. dulcis* might enhance the ER stress response in A172 cells.

DISCUSSION

The anti-cancer effects of the *G. dulcis* flower extracts against A172 GBM cells were demonstrated in this study with the promotion of autophagic cell death. Camboginol and morelloflavone were identified as major bioactive components in several parts of *Garcinia* spp. (Thongsepee et al., 2020). Morelloflavone was presented as a higher proportion in the extract than camboginol (unpublished data). This result implies that the anti-cancer effect of the extract might be synergistically influenced by both substances.

The cytotoxic effects of the *G. dulcis* extract might have resulted from its impaired proliferation effect. The induction of cell cycle arrest is essential to diminish the growth and progression of cancer cells following exposure to stressful substances. Treatment with *G. dulcis* extract enhanced cell cycle arrest at the S and G2/M phases. The arrest of the S and G2/M cell cycle phases reflects that damaged cells could inhibit their DNA synthesis and mitosis from stopping progression. The proliferation of endometrial cancer cells was inhibited by garcinol from *G. indica* by inducing cell cycle arrest at the S and G2/M phases in two different cell lines (Zhang et al., 2021). Garcinol also promoted the S phase cell cycle arrest of oral cancer cells (Aggarwal and Das, 2016). There was limited evidence of the role of morelloflavone in cell cycle control. Morelloflavone exerted anti-angiogenic action by arresting endothelial cell proliferation in the G2/M phase, with reduced migration ability (Pang et al., 2009). This suggests that camboginol and morelloflavone might serve as anti-cancer actions to block cell cycle progression and lead to cancer cell death.

The potential as a chemopreventive of chemotherapeutic agents was highlighted by the induction of autophagic cell death by some anti-cancer agents. Autophagy is an intracellular catabolic process in which dysfunctional or unwanted cytoplasmic components are degraded through the formation of autophagolysosomes. Autophagy has

been promoted as a tumor suppressor in the early stages of cancer, which might develop as a target for inhibiting cancer cell growth (Alvarez-Meythaler et al., 2020). Cell cycle progression could be regulated by autophagy induction with the corresponding selective degrading cell cycle-related proteins (Zheng et al., 2019). We speculated that the cytotoxic and cell cycle arrest effects of the *G. dulcis* extract might be modulated by autophagy. Autophagic cell death was promoted by *G. dulcis* treatment by the increased autophagic flux and enhanced conversion of LC3-I to active LC3-II and decreased p62 expression. The autophagic flux, represented by decreased CYTO-ID® fluorescence signals after treatment, could be inhibited by the autophagy inhibitor CQ. CQ inhibits lysosomal degradation by decreasing autophagosome fusion and lysosome (Mauthe et al., 2018). Therefore, CQ acts on the downstream autophagy pathway by inhibiting the degradation of LC3-II, which leads to the accumulation of LC3-II (Xin et al., 2019). *G. dulcis* might decrease the accumulation of LC3-II after CQ incubation, resulting from the utilization of LC3 in the progression of autophagolysosome fusion.

We hypothesized that autophagic cell death induced by *G. dulcis* treatment in A172 cells might be promoted by ER stress. Several physiological or pathological events might cause an imbalance between the ER protein folding load and capacity, leading to the accumulation of unfolded proteins in the ER lumen, called ER stress. Formation of the unfolded protein response (UPR) had a pivotal role in regulating ER stress by ameliorating the protein load on the ER to increase ER folding and maintain ER homeostasis. Accumulating misfolded proteins in the ER lumen activates BiP, thus enabling the release of ER stress signal molecules, including IRE1α and PERK. However, failure to maintain ER homeostasis may result in persistent ER stress and the induction of cell death, including autophagy (Almanza et al., 2019).

Autophagy could remove misfolded proteins or damaged organelles resulting from the ER stress response (Rashid et al., 2015). It has been suggested in several reports that ER stress can effectively induce the progression of autophagy in cancer cells and cause cell death (Verfaillie et al., 2010). The ER stress marker BiP was markedly increased following *G. dulcis* treatment, especially at 40 μg/mL. *G. dulcis* treatment also enhanced hyperphosphorylation of IRE1α and PERK, as shown by the mobility-shift bands in *G. dulcis*-treated cells at 40 and 100 μg/mL, reflecting autophosphorylation upon ER stress sensor activation (Amodio et al., 2019). Autophosphorylation of IRE1α and PERK is becoming active and is believed to initiate UPR events (Oslowski and Urano, 2011; Qi et al., 2011). It was speculated by these results that the *G. dulcis* extract promoted BiP release, causing activation of IRE1α and PERK through autophosphorylation, which might be associated with the ER stress response in A172 cells. Autophagic inhibitor CQ inhibited the expression of these ER stress markers and was promoted prominently after co-treatment with 100 μg/mL of *G. dulcis*. Together, it was indicated by these results that autophagic cell death induced by *G. dulcis* might be associated with the induction of ER stress in A172 cells. This was the first report on the effect of camboginol and morelloflavone on the correlation with the induction of mammalian autophagy and ER stress.

The research collectively presented the protective role of camboginol and morelloflavone in *G. dulcis* extracts against A172 human GBM cell proliferation through the induction of ER stress-mediated cytotoxic autophagy. Long-term administration of purified camboginol or morelloflavone from *G. dulcis* for GBM prevention is warranted. Additionally, medication combinations to treat GBM and reduce adverse drug effects are interesting (Surarak et al., 2021). A combination of temozolomide, a conventional chemotherapeutic drug against GBM with camboginol, and morelloflavone would also be further applied.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: TS, KS. Analysis and interpretation: TS, KS. Data collection: NT, PM, PS, PC, PP, JR, KS. Writing the article: TS, KS. Critical revision of the article: all authors. Final approval of the article: all authors. Statistical analysis: TS, PP, JR, KS. Obtained funding: PM, KS. Overall responsibility: KS.

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