Abstract. In a previous study, 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (ChalcEA) isolated from the leaves of Eugenia aquea was reported to inhibit proliferation of the breast adenocarcinoma MCF7 cell line and to promote apoptosis via activation of poly(adenosine diphosphate-ribose) polymerase protein. The present study aimed to evaluate the inhibitory effect of ChalcEA on the proliferation of A549 lung cancer cells using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, and to examine the ability of ChalcEA to induce apoptosis through activation of the caspase cascade signaling pathway in a western blotting assay. The results revealed that ChalcEA inhibited proliferation of the A549 lung cancer cell lines in a time- and dose-dependent manner with IC50 values of 25.36 and 19.60 µM for 24 and 48 h treatments, respectively. Western blot analysis indicated that ChalcEA exerted its anti-proliferative effects by promoting apoptosis via the activation of caspase-9 and caspase-3. Based on in silico results, ChalcEA with the binding energy of -6.53 kcal/mol could compete better than 4-methyl benzenesulfonamide (-6.43 kcal/mol) as an inhibitor of caspase-3 (PDB: 2XYG). ChalcEA has potential since it has three hydrophobic features. These results provided a basis for further study of ChalcEA as an active compound for anticancer therapeutics.

Introduction

Cancer is one of the most malignant diseases that may affect different parts of the body and remains an extremely serious life-threatening disease for humans (1). This disease is characterized by a rapid and uncontrolled growth of abnormal cells, which may mass together to form a tumor or proliferate throughout the body, initiating abnormal growth at other sites (2). Among the various types of cancer, lung cancer is known to be the most common cause of cancer-associated mortality worldwide (3). Central and Eastern Europe is the region with highest mortality rate (3).

In general, treatment of cancer in humans uses chemotherapeutic agents in addition to surgery and radiation (2). Chemotherapeutic agents for cancer, including breast cancer, often provide temporary relief of symptoms, prolongation of life and occasionally cures (2). However, they often result in serious side effects and can cause excessive damage to normal cells (4). This has prompted continuous efforts of researchers to identify novel anticancer compounds through chemical synthesis, as well as isolation from plant origins. A number of compounds derived from medicinal plants have potential cytotoxicity against several types of cancer.
cells in anticancer evaluation in vitro or in vivo (5-7). Plants consumed by primates are assumed to be a promising source of therapeutic agents for the management of human diseases and a series of investigations have been conducted and provided novel findings of their cytotoxicity against breast cancer cell lines (1.8-10). Kaempferol-3-O-rhamnoside isolated from the leaves of Schima wallichii, a plant commonly consumed by primates, exhibits inhibitory activity against MCF7 breast cancer cell proliferation through the activation of the caspase cascade signaling pathway (8). Subarnas et al (9) evaluated anti-proliferative effects of 42 species of primate-consumed plants against MCF7 human breast cell lines using an MTT bioassay and revealed that some plant extracts have strong inhibitory activity against MCF7 cell proliferation. Furthermore, 2',4'-dihydroxy-6-methoxy-3',5'-dimethylchalcone (ChalcEA), isolated from the leaves of Eugenia aquea (E. aquea), inhibits the proliferation of MCF7 cell lines and promotes apoptosis via the activation of poly(adenosine diphosphate-ribose) polymerase (PARP) protein (10). Additionally, a friedelanostane triterpenoid from Garcinia celebica leaves inhibits the growth of MCF7 cells through induction of apoptosis and downregulation of the oncogene Akt (1).

The present study was conducted to clarify the inhibitory activity of ChalcEA against cell proliferation and molecular pro-apoptotic activity through activation of the caspase cascade of A549 lung cancer cells. Furthermore, a molecular interaction of ChalcEA with caspase-3 was also evaluated using a molecular docking simulation.

Materials and methods

Plant materials. The leaves of E. aquea were collected from Pangandaran Beach Conservation Area (Pangandaran, West Java, Indonesia). Determination of the plant species was performed by the Department of Biology of Padjadjaran University (Bandung, West Java, Indonesia). The leaves were dried in the open air for 4-5 days, away from direct sunlight.

Isolation of ChalcEA of E. aquea. ChalcEA was obtained from the leaves of E. aquea. The isolation of the compound has been reported previously by Subarnas et al (10) and its structure was shown in Fig. 1. This compound was named 2',4'-dihydroxy-6-methoxy-3',5'-dimethylchalcone (10).

Cell culture and treatment. The A549 lung cancer cell line was purchased from the American Type Culture Collection. The cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 100 µg/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA). Cells were cultured under standard culture conditions in a CO2 incubator with 5% CO2 at 37°C. The medium was replaced once every 2 days. Cell condition were checked using an Axio Vert A.1 for Biology (Zeiss AG) inverted microscope (magnification, x200).

Cell proliferation assay using Cell counting Kit-8. Cell proliferation analysis was performed using an MTS assay on cells in the presence of various concentrations of ChalcEA (1.9-1,000 µg/ml). Cultured cells (1x10^4/well) were plated into 96-well microtiter plates in a final volume of 100 µl/well. Subsequent to the initial cell seeding, various concentrations of ChalcEA (1.9, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1,000 µg/ml) were added and the cells were incubated for 24 to 48 h at 37°C. After ChalcEA treatment was halted by the replacing media, 10 µl/well of Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) were added into each well and incubated for 3 h at 37°C in standard culture conditions according to the manufacturer's protocol. The cell proliferation rate was determined by measuring absorbance at a wavelength of 450 nm (reference 620 nm) using an Infinite® 200 PRO microtiter plate reader (Tecan Group, Ltd.). All samples were tested in triplicate. CPI rate was calculated according to the method stated in the manufacturer's protocol.

Cell extraction and western blotting. A total of 1x10^6 A549 cells and 1x10^5 untreated control cells were treated with ChalcEA (25 µM) for 12, 24, 36 and 48 h. Cells were washed twice using a cold PBS buffer and the cell lysate was prepared using RIPA lysis buffer (EMD Millipore; Sigma-Aldrich; Merck KGaA). The Pierce™ Modified Lowry Protein assay kit (Thermo Fisher Scientific Inc.) was used to measured total protein that extracted from A549 cells according to the manufacturer's protocol. A total of 25 µg/lane A549 cell protein extracts were loaded on a 30% polyacrylamide gel (Invitrogen; Thermo Fisher Scientific, Inc.) and electrotansferred onto a Amersham™ Protran™ 0.45-µm nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked using 5% skimmed milk and agitated at 25°C for 30 min. Apoptosis-associated proteins were analyzed using immunoblot analysis with caspase-3 (cat. no. AF-605NA; 1:1,000; R&D Systems, Inc.) and caspase-9 antibodies (cat. no. AF-8301; 1:1,000; R&D Systems, Inc.) incubated in 4°C for 24 h. β-actin (cat. no. 4967; 1:15,000; Cell Signaling Technology, Inc.) served as the loading control for 1 h incubation in room temperature. Mouse anti-goat IgG horseradish peroxidase-conjugated antibodies (cat. no. sc-2354; 1:10,000; Santa Cruz Biotechnology, Inc.) served as secondary antibody for 90 min incubation in room temperature. Visualization of protein bands was conducted using chemiluminesence reagent (GE Healthcare). Bands on the membrane were detected and measured using a C-DigIt® Blot scanner (LI-COR Biosciences) with Image Studio Digits v. 5.2 (LI-COR Biosciences) for band density measurement. All samples were tested in triplicate.

Molecular docking simulation. The X-ray crystallography derived caspase-3 (CAS329306) complex with 4-methyl-benzensulfonyamide (MB) was obtained from Protein Data Bank (PDB ID: 2XYG; https://www.rcsb.org/structure/2XYG) (11). The macromolecule and ligand structures were extracted using LigandScout version 4.2 Advanced (InteLigand GmbH). The molecular docking simulation methods were modified according to a previous study (12). All ligands (Met61, Arg64, Ser120, His121, Gly122, Glu123, Phe128, Ala162, Cys163, Thr166, Tyr204 and Arg207) and the estrogen receptor α (ERα) receptor were prepared for docking using AutoDockTools version 1.5.6. (The Scripps Research Institute) The ligands and the receptor were protonated. The default charges energy parameters were allocated to the protein and ligand atoms. A grid box comprised 40x40x40 points
spaced by 0.375 Å that was centered on the androgen receptor (AR) active site (x=36.357; y=38.829; and z=32.088). Autogrid was used to calculate grid box of binding affinity of each of the ligand atom types. The resulting docked conformations were clustered using a root-mean-square deviation tolerance of 1.0 Å. The ligand conformation with the lowest free energy of binding, selected from the most favored cluster as sorted by scores and by binding position, was selected for further analysis. The ligand-interaction features for each pose within the binding pocket were determined automatically using LigandScout Advanced version 4.2.

Structure-based 3D-pharmacophore modeling. A 3D structure-based pharmacophore model was derived automatically from the X-ray derived structure of PDB ID: 2AX6 in complex with hydroxyflutamide (HF), using Ligandscout version 4.2 Advanced based on a previous study (13).

Statistical analysis. Quantitative data was obtained using the band scanner Image Studio Digits v. 5.2 (LI-COR Biosciences). Data are presented as mean ± standard deviation. After calculation of the normality of the data using a stem and leaf plot, one-way ANOVA followed with Tukey’s post hoc test was performed to assess the statistical significance of more than two groups. The measurements were assessed in triplicate. SPSS version 26 (IBM Corp.) was used for all statistical analyses. *P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitory activity of ChalcEA against proliferation of A549 cells. ChalcEA was evaluated for its effect on the proliferation of A549 lung cancer cells in a proliferation assay. The evaluation revealed a dose- and time-dependent inhibition of cell proliferation by the compound. The compound strongly inhibited the proliferation of A549 cells in 24 and 48 h examinations with an IC_{50} value of 25.36 and 19.60 µM, respectively (Fig. 2).

Proapoptotic activity of ChalcEA. The proliferation assay results revealed strong inhibitory activity of cell proliferation by ChalcEA against A549 cells demonstrated morphologically, for example cells broke into small pieces (Fig. S1). Therefore, caspase-inducing activity of the compound was examined in the A549 cell line. As shown in Fig. 3, expression levels of caspase cascade-related proteins in A549 lung cells treated with ChalcEA (A) Changes in the expression levels of caspase-9 and -3 were determined in A549 cells treated with ChalcEA at 0, 12, 24, 36 and 48 h. Expression levels of cleaved caspase-9 and -3 were increased at 12 h, followed by elevated caspase-3 expression levels suggesting that apoptosis was occurring. (B) Expression levels of caspase-9, cleaved caspase-9, caspase-3 and cleaved caspase-3. Quantitative data of band density measured using Image Studio Digits v. 5.2 (LI-COR Biosciences). Data are presented as mean ± standard deviation. One-way ANOVA followed by Tukey’s post-hoc test was performed. *P<0.05. ChalcEA, 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone.
Table I. Molecular docking and pharmacophore modelling results.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Free binding energy (kcal/mol)</th>
<th>Hydrogen bond interaction</th>
<th>Pharmacophore features</th>
</tr>
</thead>
<tbody>
<tr>
<td>4'-dimethylchalcone</td>
<td>-6.53</td>
<td>His121, Tyr166</td>
<td>1 HBD, 2 HBA, 3 Hy</td>
</tr>
<tr>
<td>2',4'-dihydroxy-6'-methoxy-3' , 5'-dimethylchalcone</td>
<td>-6.53</td>
<td>His121, Cys163, Tyr166, Arg207</td>
<td>3HBA, 2Hy, NI</td>
</tr>
</tbody>
</table>

HBD, hydrogen bond donor; HBA, hydrogen bond receptor; Hy, hydrophobic; NI, negative ionizable.

active fragments of caspase-9 and caspase-3 were increased in A549 cells at 12 h, including caspase-9 fragments with 46 kDa (P<0.0001) and 10 kDa (P=0.0001) and caspase 3 fragments with 34 kDa (P=0.0001) and 18 kDa (P=0.0001). These results suggested that the inhibition of A549 human lung cancer cell proliferation by ChalcEA was mediated by the induction of apoptosis through activation of caspase-9 and caspase-3.

Molecular docking and pharmacophore modelling of ChalcEA. Based on in vitro studies, molecular docking was used to demonstrate ChalcEA interaction with caspase-3 receptors by inhibiting ERα. As shown in Table I, ChalcEA had a lower binding energy affinity than 4-methyl-benzenesulfonamide (MB). ChalcEA formed a hydrogen bond interaction with amino acid residues of His121, Cys163, Tyr166 and Arg207 (Fig. 4B and D), whereas MB only had hydrogen bond interaction with His121 and Tyr166 (Fig. 4A and C). Furthermore, Fig. 4C revealed that MB induced conformation changes of Tyr204. ChalcEA also has hydrogen bond interaction at the Cys163 and Arg207 sites that not only conformationally changes Tyr204 but also stabilizes via interaction with Glu123 and Gly122. Moreover the interactions were stabilized by a π-π interaction between the aromatic rings of ChalcEA and Phe128-Met61 (Fig. 4B and D). The ChalcEA bond with caspase-3 can be seen in Fig. 4E. In the pharmacophore results, ChalcEA was mapped well in two hydrophobics (yellow ball) with pharmacophoric model of HF as indicated in Fig. 4D. However, ChalcEA has potential to bind amino acid residues from receptors since this compound has three hydrophobic regions, two hydrogen bond receptors (red) and one hydrogen bond donor (green).

Discussion

Previously, the compound ChalcEA obtained from the leaves of E. aquea has been shown to have antiproliferation activity in MCF7 cells and to promote proapoptotic activity via PARP protein activation (10). However, the present study investigated the effects of ChalcEA on the growth of A549 lung cancer cells as lung cancer is known as a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung (14). Worldwide, lung cancer is the most common cancer among men in terms of both incidence and cancer-associated mortality, and among women it has the third highest incidence in 2008 and 2012 (3,15). In 2012, there were 1.82 million new cases of lung cancer globally and 1.56 million cancer-associated deaths were due to lung cancer, representing ~19.4% of all cancer-associated deaths (15). ChalcEA is also hypothesized to be effective in inhibition of the growth of A549 lung cancer cells (13). The present study demonstrated that the compound showed significant inhibition of A549 cell proliferation with IC₅₀ values of 25.36 and 19.60 µM for 24 and 48 h treatments. The results of the present study suggested that the underlying molecular mechanism of this proapoptotic activity occurred through the activation of caspase-9 and caspase-3. Furthermore, a molecular interaction of ChalcEA with caspase-3 was evaluated using molecular docking simulation.

The present study showed that ChalcEA isolated from E. aquea leaves significantly inhibited A549 cell proliferation. This evidence was in line with a previous study which reported that the addition of ChalcEA isolated from the buds of Cleistocalyx operculatus resulted in inhibition of lung cancer GLC-82 xenografts (13). ChalcEA also significantly inhibits the growth of human liver cancer SMMC-7721 cells and may induce apoptosis of SMMC-7721 cells in vitro (16). The antitumor effects of this compound have also been demonstrated in vivo in a solid human tumor xenograft mouse model using human liver cancer SMMC-7721 cells (17). Additionally, ChalcEA inhibits subcutaneous tumor growth of human hepatocarcinoma Bel7402 cells (18); however, reports of ChalcEA IC₅₀ values in cancer cells remain limited.

The results of the present study suggested that ChalcEA triggered cell death intrinsically in A549 cells via the mitochondrial caspase-9 signaling pathway and activation of caspase-3 at 12 h. These results were in agreement with those of a previous study which stated that ChalcEA activates Akt before 12 h which then triggers cell death intrinsically through caspase-9 activation, in a xenograft model (13). Activation of caspase-3 marks the occurrence of apoptosis and cell death (19). This evidence of the underlying molecular mechanism may explain the effect of ChalcEA against cancer angiogenesis and tumor growth in a solid tumor xenograft in mouse model (13,17).

Based on previous in vitro studies, ChalcEA interaction with caspase-3 receptors has been identified by inhibiting ERα which then inhibits Akt to activate caspase-9 (12,17). Caspases function as mediators of programmed apoptosis (19). Caspase-3 is an activated death protease, catalyzing the specific cleavage of a number of cellular proteins, for example PARP, DFF40, DFF45, α-Fodrin and Gelsolin, that trigger cell DNA fragmentation and blebbing (19). Caspase-9 can directly cleave and activate caspase-3 and caspase-7 (20).

In the present study, ChalcEA was investigated as a prospective drug candidate which should be further evaluated and
Figure 4. Pharmacophore models. Pharmacophore models of (A) MB and (B) ChalcEA in 2D and (C) MB and (D) ChalcEA in 3D. (E) Corresponding confirmation of ChalcEA that was observed in the caspase-3 structure by AutoDockTools version 1.5.6. ChalcEA, 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone; MB, 4-methyl-benzensulfonamide.
applied against caspase receptors for the treatment of lung cancer, and it was revealed that ChalcEA serves as a caspase-3 inducer. The 3D structure of PDB ID: 2XYG was obtained from RCSB PDB (Fig. 4A). In a previous study, Ganesan et al (21) clarified that 4-methyl-benzensulfonamide (MB) (PDB ID: 2XYG) is an inhibitor against caspase-3. MB was a rotation of the Tyr204 side chain, which blocks the S2 subsite. S2 subsite is stabilized by hydrophobic contacts with Cys163, Trp206 and Phe25. ChalcEA was docked well toward caspase-3. Notably, ChalcEA (-6.53 kcal/mol) could compete better than MB (-6.43 kcal/mol; Table 1). That means that the bond between ChalcEA and caspase-3 is stronger than MB, which functions as a caspase-3 inhibitor (21). ChalcEA is stabilized by water and accommodated by Gly122 and Gly165 as well as MB (19). As shown in Fig. 4B and D, ChalcEA formed hydrogen bond interaction with His121, Cys163, Tyr166 and Arg207. The interactions were stabilized by π-π interaction between aromatic rings of ChalcEA and Phe128-Met61. Therefore, it can be concluded that the hydrogen bonds formed with ChalcEA are stronger than MB, so MB is unable to block the process of induction of apoptosis by ChalcEA and cancer cells going to enter apoptotic process.

Based on the current results, ChalcEA functions as a potential activator of caspase-3. This conclusion was supported by a previous study which indicated that four flavonoids (considering ChalcEA is itself a flavonoid compound) were estimated to be promising candidates for further evaluation for lung cancer prevention (22). Furthermore, Cui et al (23) reported that consumption of vegetables, tea and wine, all of which are rich sources of flavonoids, are associated inversely with lung cancer.

The present study suggested that ChalcEA obtained from the leaves of E. aequa may be a compound with potential for development as an anticancer treatment for lung cancer therapy. The influence of ChalcEA on other pathways, such as malignancy, and the ability of cells to invade to other organs, needs to be further investigated.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YEH, NC, MM, RL and AS designed and performed the experiments, analyzed the data and wrote the manuscript.

TRu, AYC, IS and TRo analyzed data and modified the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References