Kaempferol and Quercetin Isolated from The Leaves of Atingia Excelsa to Arrest Cell Cycle in G0/G1 Phase Human Tongue Cancer SP-C1 Cell Lines

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ABSTRACT

The leaves of Atingia excelsa were found to strongly inhibit SP-C1 human tongue cancer cell lines. This study was focused on identifying the antiproliferative compound found in A. excelsa leaves and assessing its mechanism of action. The active compound was isolated using column chromatography and identified by the spectroscopic method and was also tested for its anti-proliferative properties and the cell cycle analysis in SP-C1 cells by flowcytometry analysis. This work resulted in the isolation of a flavonoid, which was identified to be kaempferol and quercetin. The compounds inhibited SP-C1 cell proliferation in a time- and dose-dependent manner with IC\textsubscript{50} values of 0.72 µg/mL and 0.70 µg/mL for the 24 hours treatments, respectively. Furthermore, the flowcytometry analysis suggested that the compounds exerted its anticancer activities by inhibiting cell cycle. These results suggested that compounds found in A. excelsa provide a basis for its potential use in cancer disease management.

Keywords: Atingia excelsa, cancer, cell cycle, kaempferol, quercetin.

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INTRODUCTION

Approximately 300,373 new cases of oral squamous cell carcinoma (OSCC) are annually reported around the world, what makes oral cancer the sixth most common cancer worldwide. The term oral cancer is referred to as a subgroup of head and neck malignant neoplasms affecting the lips, the anterior two-thirds of tongue, the salivary glands, the gingiva, the floor of the mouth, the oral mucosal surface and the palate, with the tongue being the most common location. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of this anatomic site, and in approximately 80% of cases, it is associated with extrinsic factors such as the use of tobacco, alcohol or both. OSCC is a very difficult disease to treat because of multidisciplinary and diverse treatment strategies and the varied natural behavior of the cancer. Local invasion and frequent regional lymph node metastases together with relative resistance to chemotherapeutic. Management of OSCC varies considerably; small cancers of the oral cavity are usually managed by surgery alone, whereas advanced oral cancers are usually treated with primary radical surgery followed by radiation or chemoradiation and targeted therapy. The poor outcome of chemotherapy to OSCC contributes to the poor prognosis for OSCC. Therefore, novel, effective therapy for OSCC treatment is still needed. Due to this high incidence, the identification of novel compounds that inhibit cancer development has become a crucial objective for scientists. Of the hundreds of chemicals that have been and are being evaluated for their anti-cancer activities, natural products derived from medicinal plants rank among the most promising. To identify novel agents that may inhibit cancer development, we have focused our investigations on discovering bioactive compounds from high plants level.

In our previous study, we found that the leaves of the Hammamellidaceae family, demonstrated anti-tumor properties. These preliminary studies suggest that A. excelsa may be further developed as a source of anti-cancer agents. Thus, in this study, we investigated and characterized the inhibited cell cycle activities of A. excelsa leaf extracts.

MATERIALS AND METHODS

Plant materials. A. Excelsa leaves were gatted from Wayang Windu Mountain, Pangalengan, West Java, Indonesia. The plant species were identified by plant taxonomy laboratorium of Biology Departement, FMIPA, Padjadjaran University, Indonesia.

Extraction and isolation. Simplicia leaves of A. excelsa (2.5 kg) were extracted with methanol (3x24 h) at 20-25°C (Room temperature). The solvent was subsequently evaporated under reduced pressure at 50°C to produce a concentrated extract. 280 g of methanol extract was fractionated by n-hexane and water to gained an 86 g n-hexane extract and the layer of water. The water layer then extracted with ethyl acetate to gained an 120 g ethyl acetate fraction and 90 g water fraction. Fractions cytotoxicity was assessed on SP-C1 tongue cancer cells using methyl thiazolyl tetrazolium (MTT) assay. Ethyl acetate fraction, which was the most active fraction, was chromatographed by Wakogel C200 (Wako Pure Chemical, Japan) with a mixture of n-hexane, ethyl acetate and methanol with raised the polarity. Main compounds were then isolated and purified by silica G80 with sulfuric acid-ethanol (1:9) and were identified by spectroscopic methods consist of mass spectroscopy (MS), ultraviolet (UV), infrared spectrometry (IR), and nuclear magnetic resonance (NMR).

Cell culture and treatment. SP-C1 human tongue cancer cell line was used in this research were used RPMI-1640 as cultured medium (Sigma, St. Louis, MO, USA) added with 10% FBS and antibiotics among them 100 U/mL penicillin and 100 μg/mL streptomycin. Cell’s treatments, several concentrations of the sample were added to the cell culture medium. After 24 h, the cells were released from treatment, the medium was replaced, and cells were subsequently gathered at the indicated times.

Cell cycle analysis using flow cytometry. The cells were grown in 24-well plates at 37°C with CO₂ from 5% until 80% encounter was
reached. Afterwards, medium was changed, and flavonoids were added to the indicated concentrations. Furthermore, cells were incubated for 48 h at 37°C. After incubation, the cells were harvested and rinsed three times with ice-cold PBS (pH 7.4). The supernatant was eliminated, and the cells were rinsed with 1 mL of PBS and centrifuged at 4 °C. Finally, the supernatant was eliminated, and 200 μL of 70% ice-cold ethanol and 200μL of PBS was added to the cells and stored at –20 ºC for further use. Utilization in the flow cytometry experiments, the cell pellet was rinsed twice with PBS. Cell pellet was suspended in 0.5 mL of staining reagent (50μg/mL PI, 50 U/mL RNase, 0.1 mM EDTA,0.1% Triton X-100, and PBS) and incubated for 30 min at 37 °C in the dark. Measuring DNA fluorescence was used Becton Dickinson (Franklin Lakes, USA) FACScanto II flow cytometer with 488 nm excitation wavelength and 585 nm emission wavelength. Pulse width area signals were used to discriminate between G2 cells and cell doublets. Data analyzed was used FACSDiva Software (Beckton Dickinson). Relative distribution of $10^4$ events for each sample was analyzed for background aggregates and debris, apoptosis indicator and the G0/G1-, S-, and G2/M-phases of the cell cycle. Control treatments consisted of a culture medium supplemented with FBS. Serum-deprivation treatment was used as an inducer of G0/G1 cell cycle arrest. \(^{11}\)

**RESULTS**

Fraction of Ethyl acetate *A. excelsa* leaves inhibits SP-C1 cell proliferation. Treatment MTT assay shown that *A. excelsa* methanol extract was inhibit the proliferation of SP-C1 human tongue cancer cells (IC\(_{50}\) 75.41 μg/mL) (Fig. 1). Methanol extract then fractionated based on polarity, using n-hexane, ethyl acetate and water. Afterward the fractions individually applied to SP-C1 cells and inhibit cell proliferation with an IC\(_{50}\) value of 44.85 μg/mL for the n-hexane fraction, 12.85 μg/mL for the ethyl acetate fraction and 18.02 μg/mL for the water fraction. Due to its low IC\(_{50}\) value, we then explored the ethyl acetate fraction for its anti-cancer potential.

![IC\(_{50}\) value some extracts A. excelsa Nornha](image-url)
Main compounds of the ethyl acetate fraction are Kaempferol and quercetin. Isolation and purification of the ethyl acetate fraction A. excelsa extract to gathered bioactive compounds. The compound exhibited a melting point at 152.7-153°C and a molecular ion peak at m/z 432 in the LC-MS spectrum. According to hydrogen and carbon content, the molecular ion peak and 1H and 13C NMR profiles indicated that the compound has a molecular formula of C15H10O7. Ultraviolet spectrum provides maximal absorbance peaks at λmax 265 and 342 nm, which were characteristic of a flavonoid with flavone skeleton. The addition of NaOH produced bathochromic shift in the absorption bands, indicating the presence of hydroxyl groups in the skeleton, one of which was attached to C-5, as indicated by further bathochromic shift following the addition of H3BO3. Absorption bands at 1,675 and 3,197 nm of the IR spectrum indicated where the molecule harbors conjugated carbonyl and hydroxyl groups, respectively.

1H NMR spectrum of the compound shown two hydrogen of aromatic signals with ‘meta coupling’ at δ 6.35 (1H, d, J = 2.2 Hertz) and 6.18 (1H, d, J=2.2 Hertz), which was predicted by hydrogens at C-6 and C-8 of the A ring of the flavone skeleton. Hence, this compound was suggested to have a group of hydroxyl in C-5 and C-7. Furthermore, 1H NMR spectrum revealed two signals with ‘ortho coupling’ at δ 6.92 (2H, d, J = 6.7 Hz) and 7.74 (2H, d, J = 6.7 Hz), the approximation signals from the hydrogens at C-2', C-3', C-5' and C-6' of the B ring. Lack of a specific signal for olefinic hydrogen at C-3 and existence of an anumeric hydrogen signal at δ 5.37 (1H, d, J = 7.2 Hz) suggested that the compound was a flavonol glycoside. Appearance of anumeric carbon signal at δ 94.9 in the 13C NMR spectrum indicated the availability of a sugar moiety. In consequence to a correlation between the anumeric hydrogen signal (δ 5.37) and the anumeric carbon signal (δ 94.9) that revealed by analysis of the HMBC spectral data, sugar moiety position was established to the C-3 hydroxyl group, signal of methyl.

Quercetin and Kaempferol inhibits SP-C1 cell proliferation in a concentration-dependent manner. Kaempferol and quercetin effects on the viability of SP-C1 cells were evaluated. Treatment of cancer SP-C1 cell lines with kaempferol and quercetin shown that concentration-dependent inhibit of cell growth, as demonstrated by the MTT assay. 24 hours of treatment with kaempferol and quercetin inhibited the proliferation of SP-C1 cells with IC_{50} values 0.72 and 0.70 μg/mL, respectively. Subsequent cell cycle analysis, based investigation applying the IC_{50} concentration of 0.39, 0.78, and 1.56 μg/mL kaempferol and quercetin was applied on SP-C1 cells.

Kaempferol and quercetin inhibited the SP-C1 cell cycle in the G0/G1 phase. To determinate the percentage of SP-C1 cells present in different phases of the cell cycles G0/G1, S, and G2/M, first cells were synchronized with serum deprivation before
treatment with or without quercetin and kaempferol at several concentrations (C, K 0.39, K 0.78, K 1.56, Q 0.39, Q 0.78, and Q 1.56 μg/mL) for 24 h period, serum-deprived cells were progressively accumulated in the G0/G1-phase, whereas serum-supplied cells were present in the S- and G2/M-phases (Figure 2). Treatment with K (all concentrations) and Q (all concentrations) significantly increased the percentage of cells in the G0/G1-phase and decrease in S and G2/M-phase as compared with the control. Particularly, Cell percentage in G0/G1-phase increased an appropriate increase concentration compared to the control after K treatment. Percentage of cells in the G0/G1 – phase increase compared to the control after Q treatment but the percentage of cell decrease a long increase in concentration.

![Figure 2. Flowcytometry analysis SP-C1 cancer cell using treated some concentration of kaemperol and quercetin.](image)

**DISCUSSION**

The 'H- and ¹³C-NMR spectra of compounds from *A. Excelsa* exhibited resonances due to aromatic systems. The 'H-NMR spectrum of them showed the presence of two doublet signals corresponds to four aromatic protons in ring B, characteristics for the 1′,4′-disubstituted flavone. The ¹³C-NMR signals of them were assigned with the help of a DEPT experiment. A total of fifteen carbon signals were observed in the ¹³C-NMR spectrum. The degree of unsaturation was accounted for eight out of the total eleven double bond equivalents. The ¹³C-NMR spectrum of them showed the presence of 15 aromatic carbon signals. So, from 'H and ¹³C NMR profiles indicated that the compound had a molecular formula of C₁₅H₁₀O₇.

Rural medicinal plants since a long time as sources of potential therapeutic medication, and find out of novel medication or leads compound are usually based on that approach. In drug invention, researcher have recently applied a new approach to selecting plants based on the Hamamelidaceae family. In previous research, we found that the *A. excelsa* leaves extracts were strongly cytotoxic to the SP-C1 Human tongue cancer cell lines. Therefore, these extracts potential for further investigation. Present study focused on identifying anti-
proliferative compound from the A. excelsa leaves. This study was isolated of two flavonoids, kaempferol and quercetin, which strongly inhibited proliferation of SP-C1 cell lines in a time- and concentration-dependent manner. There is no compound that reported before in relation with its cytotoxicity in these cancer cell lines.

In this study, kaempferol and quercetin with some concentration to arrest G0/G1 phase cell cycle. Cell growth is an additional amount of cells from the cell cycle process. The cell cycle consists of 4 phases, that is G1, S, G2, and M. G1 (Gap 1) is a phase when the cell will synthesis the DNA or go out from cell cycle reversibly or irreversibly to differentiation. The cell that in the G1 phase will easily controlling cell cycle at a point, that is in the restriction point (R) that will determine the cell to come in back to the cell cycle, go out from cell cycle entering G0 phase or differentiating.

When the cell cycle phase flow in restriction point dan enter S phase controlled by cyclin-dependent kinase (Cdk) and D, E, and A cyclin. D cyclin function as a growth factor that the expression more depend on the extracellular signal than the cell position on the cycle. When the cell entering the G0 cycle, on or more of the D cyclin (D1, D2 and D3) were inducted as apart from the first response of a growth factor stimulation, protein synthesis and former complex with catalytic subunit (Cdk4, Cdk6) depend on the mitogenic stimulation. In reverse, if the mitogenic substance were removed, so the D Cyclin synthesis will stop. D cyclin is an unstable protein and the enzymatic activity quickly disappear, so that the cell quickly go out from the cell cycle. A specific inhibitor of Cdk4 and Cdk6 known as Ink4 can directly stop the activity of D cyclin/cdk 4/6 and causing G1 phase arrest (rest of G1 phase).

Cell cycle inhibition of tongue cancer (SP-C1 after kaempferol and quercetin intervention as the results of Rasmala leaves isolation. There is a system that controlled the cell cycle. The controlling system known as a checkpoint. There are 4 checkpoints that found on cycle cell, that is G1, S, G2, and M. Checkpoint in a cell cycle involving protein cyclin groups and cyclin-dependent kinase (cdk). Protein groups that involved on the cell cycle checkpoint determine cell cycle to stop, DNA repair, or apoptosis if the DNA repair doesn't occur. Kaempferol (4) can inhibit cell cycle on G0-G1 phase at 0.39 µg/mL, 0.78 µg/mL and 1.56 µg/mL concentration. This cell cycle inhibition suspected because of kaempferol (4)/ quercetin (5) that can inhibit the work of c-myc. We know that the function from c-myc transcription factor is increasing D cyclin and E cyclin, so that there are increasing activity of G1-CDK (D1-CDK cyclin) and G1/S-CDK (E-CDK6 cyclin). Thus, the quercetin in this research can inhibit tongue cancer SP-C1 cell cycle on G0-G1 phase at 0.39 µg/mL, 0.78 µg/mL dan 1.56 µg/mL concentration. This results analog with the research who is conducted by Mocanu et al, in 2013 years that shows quercetin can inhibit Epidermoid Cancer Cell line A-431 cell cycle at 5 and 10 µM concentration in 24 hours on G0/G1 phase and inhibit Mamary SK- BR-3 cell cycle at 50 and 75 µM concentration in 24 hours on G0-G1 phase. Research belonging to Chen et al, in 2011 years shows that quercetin can inhibit oral cancer OSCC SCC-25 cell cycle at 50 and 75 µM concentration in 12 hours incubation on the G1 phase. Cell cycle inhibition which is conducted by quercetin on G0-G1 phase possibly because quercetin will synergize with cyclin-dependent kinase inhibitor (CDK1) p21 that that function as inhibitor D-CDK4/6 cyclin activity. With inhibition of D-CDK4/6 cyclin activity so the cell will be obstructed to enter S phase, so that the cell will stop on the G0-G1 phase or p21 beside binding cdk and PCNA (proliferating cell nuclear antigen), that is a DNA polymerase α subunit, which needed for replication or DNA reparation. P21 will inhibit cell cycle on the G1 phase as a result of cell-contact inhibition and because of TGF β.

CONCLUSION

In conclusion, our results suggest that kaempferol and quercetin inhibited the growth of
SP-C1 cells through inhibited cell cycle in the G0/G1 phase.

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