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APOPTOSIS MEDIATED ANTI-PROLIFERATIVE ACTIVITY OF KAEMPFEROL AND QUERCETINE ISOLATED FROM THE LEAVES OF *ALTINGIA EXCELSA* AGAINST HUMAN TONGUE SP-C1 CELL LINES

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KEYWORDS

Altingia excelsa, cancer, apoptosis, kaempferol, quercetine

ABSTRACT

Introduction: *The leaves of Altingia excelsa were found to strongly inhibit SP-C1 human tongue cancer cell lines. This study was focused on identifying antiproliferative compounds found in A. excelsa leaves and assesment their action mechanism.* ***Methods:*** *Extracts of Methanol A. excelsa were fractionated based on their solvent polarity using n-hexane, ethyl acetate, and water. The anti-proliferative testing were tested in vitro against SP-C1 human tongue cancer cell lines using the MTT assay. Isolated the active compound used column chromatography and identified by the spectroscopic method. The isolated compound was also tested for its anti-proliferative testing and ELIZA method of apoptotic induction.* ***Results:*** *This work resulted in the isolation of a flavonoid, which was identified to be kaempferol and quercetin. The compound inhibited SP-C1 proliferation of cell in a time- and dose-dependent manner with IC50 values of 0.72 µg/mL and 0. 70 µg/mL for the 24 hours treatments, respectively. Furthermore, promoting apoptosis via the intrinsic pathway, thereby increasing the activity of caspase-8 and caspase-9.* ***Conclusions:*** *These results suggest that kaempferol and quercetin the anticancer compound found in A, excelsa provided a basic for its potential use in cancer disease treatment management.*

INTRODUCTION

Oral squamous cell carcinoma (OSCC) has increase morbidity and mortality rates across the world because it is frequently found in advanced stadium before therapy.[1,2] OSCC is now a global health problem with high incidence and mortality rates. The major environmental risk factors responsible for the development of OSCC include chewing of betel nut, smoking of cigarette, consumption of alcohol, and exposure to high-risk human papillomavirus. Treatment of OSCC is a very difficult because needs multidisciplinary and diverse treatment strategies and the varied natural behavior of cancer. Local invasion and frequent regional lymph node metastases together with relative resistance to chemotherapeutic. The treatment conventional of OSCC management still depend on surgery, radiotherapy, chemotherapy, and targeted therapy.[3] The bad outcome of chemotherapy to OSCC contributes to the bad prognosis for OSCC.[4] Therefore, origin, effective therapy for OSCC treatment is still needed. Due to this high incidence, the identification of origin compounds that inhibit cancer development has become a important objective for scientists. The hundreds of chemicals compound that have been and are being evaluated for their anti-cancer activities, products of natural derived from of medicinal rank among the most promising.[5]

To identify origin agents that may inhibit cancer development, we have focused our investigations on discovering compounds of bioactive from commonly high-level of plants.[6]

In our previous study, we found that the leaves of the Hammamelidaceae family, a high-level plant, demonstrated anti-tumor properties.[7,8] These preliminary studies suggest that *Altingia excelsa* Noronha. may be further developed as a source of anti-cancer agents. Thus, in this study, we investigated and characterized the inducted-apoptotic activities of *Altingia excelsa* Noronha leaf extracts.

LITERATURE REVIEW

Plant materials.

Altingia excelsa Noronha. leaves were collected from mountain of Wayang Windu, Pangalengan, kabupaten Bandung, West Java, Indonesia. Department of Biology, Faculty of Mathematics and Natural Sciences, University of Padjadjaran, Indonesia has indentified and determination this plant.

Extraction and isolation.

At room temperature, the dried leaves of *Altingia excelsa* Noronha. (2.5 kg) were extracted with methanol (3x24 h). The solvent was subsequently evaporated under reduced pressure at 50˚C to yield a concentrated extract. Extract of methanol (280 g) was fractionated between n-hexane solvent and water solvent to obtain an extract of n-hexan (86 g) and layer of water. The layer of water was then extracted with ethyl acetate solvent to obtain an fraction of ethyl acetate (120 g) and a fraction of water (90 g). The antiproliferative activity of the fractions was assessed on SP-C1 tongue cancer cells using the methyl thiazolyl tetrazolium (MTT) assay. The fraction of ethyl acetate, which was the most active fraction, was chromatographed on Wakogel C-200 (Wako Pure Chemical, Japan) with a mixture of n-hexane, ethyl acetate, and methanol with increased polarity. The major compound was then isolated and purified using silica G-60 with sulfuric acid-ethanol (1:9) and identified by spectroscopy methods including ultraviolet and infrared spectrometry (UV-IR), and nuclear magnetic resonance (NMR) (4).

Cell culture and treatment.

In this study we used the SP-C1human tongue cancer cell line, we was cultured in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). For treatments of cell, some concentrations of the sample were added to medium culture of the cell. After 24 h, the cells were released from treatment, the medium was replaced, and cells were subsequently collected at the firts times.

***Caspase-8 and -9 activity*.**

Caspase-8 and caspase-9 activity SP-C1cells were treated with 0.39, 0.78, and 1.56 μg/mL kaempferol and quercetin for 24 hours to detect caspase -8 and -9 activity. Caspase -8 and caspase-9 activity was assessed according to the manufacture's instructions (Caspase colorimetric kit R&D system Inc., MN, USA). Cells were harvested and lysed in 50 mL lysis buffer containing 2 mM DTT for 10 min. After centrifugation, the supernatant containing 200 mg protein was incubated with caspase-8 and caspase-9 substrate in a reaction buffer. Samples were then incubated in a 96-well flat-bottom microplate at 370C for 1 hour. Levels of released pNA were measured with an ELISA reader (Anthos 2001) at 405 nm wavelength.

RESULT

Ethyl acetate fraction of Altingia excelsa Noronha *leaves inhibit* SP-C1 *proliferation of cell.* Treatment with the *Altingia excelsa* Noronha. Extract of methanol was found to inhibit the proliferation of SP-C1 human tongue cancer cells (IC50 75.41µg/ml) (Fig. 1). The extract of methanol was fractionated based on polarity, using n-hexane, ethyl acetate, and water. The fractions were then individually applied to SP-C1 cells and were found to inhibit proliferation of the cell with an IC50 value of 44.85 µg/ml for the fraction of n-hexane, 12.85 µg/ml for the fraction of ethyl acetate, and 18.02 µg/ml for the fraction of water. Due to its low IC50 value, we then explored the fraction of ethyl acetate for its anti-cancer potential.

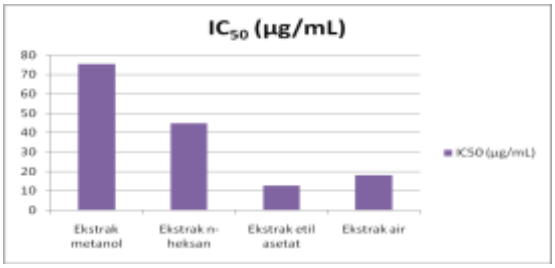


Figure 1. IC_{50} value some extracts *A. excelsa* Noronha

Kaempferol and quercetin are major compounds of the ethyl acetate fraction. The principle active compound of the ethyl acetate fraction of *Altingia excelsa* Noronha. the extract was isolated and purified. The compound exhibited a melting point of 152.7-153˚C and a molecular ion peak at *m/z* 432 in the LC-MS spectrum. Based on proton and carbon content, the molecular ion peak and 1H and 13C NMR profiles indicated that the compound had a molecular formula of flavonoids. The UV spectrum produced maximal absorbance peaks at λmax 265 and 342 nm, which were characteristic of a flavonoid with a flavone skeleton. The addition of NaOH produced a 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 a 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.

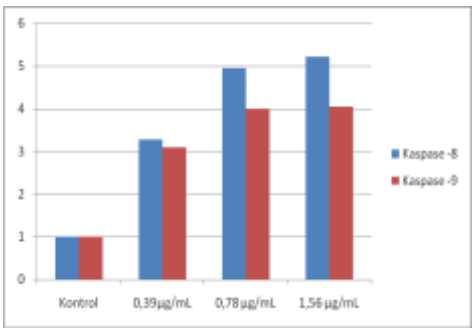
The proton NMR spectrum of the compound showed two aromatic hydrogen signals with ‘meta coupling’ at δ 6.35 (1H, *d*, J= 2.2 Hz) and 6.18 (1H, *d*, J=2.2 Hz), which was predicted by the H at C-6 and C-8 of the A ring of the flavone skeleton. Accordingly, this compound was suggested to have a hydroxyl group at C-5 and C-7. Furthermore, its proton 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 signals of which were approximated from the H at C-2', C-3', C-5' and C-6' of the B ring. The absence of a specific signal for olefinic H at C-3 and the presence of an anomeric H signal at δ 5.37 (1H, *d*, J=7.2 Hz) suggested that the compound was a flavonol glycoside. The appearance of an anomeric C signal at δ 94.9 in the carbon NMR spectrum indicated the presence of a sugar moiety. Due to a correlation between the anomeric H signal (δ 5.37) and the anomeric C signal (δ 94.9) that was revealed by analysis of the HMBC spectral data, the position of the sugar moiety was assigned to the C-3 hydroxyl group the methyl signal.

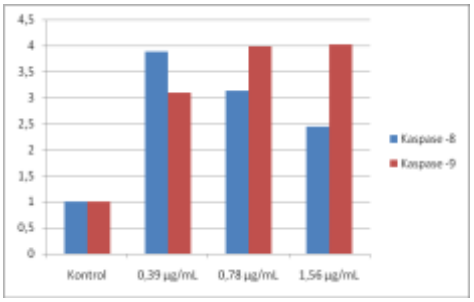
Kaempferol and quercetin inhibit SP-C1 proliferation of cell in a dose-dependent manner. The effect of kaempferol and quercetin on the citotoxycity of SP-C1 cells was evaluated. The treatment of cancer SP-C1 cell lines with kaempferol and quercetin resulted in a dose-dependent inhibition of cell growth, as evaluated by the MTT assay. Twenty-four hours of treatment with kaempferol and quercetin inhibited the proliferation of SP-C1cells with an IC50 value of 0.72 and 0.70 μg/mL. Subsequent caspase activity-based investigation applying the IC50 dose of 0.39, 0.78, and 1.56 μg/mL kaempferol and quercetin was performed on SP-C1cells.

Kaempferol and quercetin increase caspase-8 and -9 activity

Kaempferol and quercetin-activated caspase-8 and caspase-9 to determine whether caspases are involved in kaempferol and quercetin-induced apoptosis, we examined the enzymatic activity of caspases using two fluorogenic peptide substrates (Ac-IETD-AMC and Ac-LEHD-AMC), which are specific substrates for caspases-8 and -9, respectively. It can be seen in Figure 5 that kaempferol and quercetin induced a rapid rise in caspase-8 and -9 activity. The increase in activity of each of the 2 caspases by kaempferol and quercetin for all concentrations was correlated with the processing of pro-caspases-8 and -9, respectively, as demonstrated in Figure 2.



a. Kaempferol



b. Quercetin

Figure 2. Caspase-8 and -9 activity was treated some concentration of kaempferol and Quercetine.

DISCUSSION

Medicinal plants of traditional have long been regarded as a source of good therapeutic agents, and the search for new material for drugs or leads compound is usually based on that approach.[9,10] In new drug discovery, we have recently applied a new approach to selecting plants based on the Hammamelidaceae family.[7,8] In our preliminary study, we found that the extracts of the *A. excelsa* leaves were strongly anti proliferative to the SP-C1 Human tongue cancer cell line. Thus, these extracts had the potential for further investigation.

In this study was focused on isolated and identifying the anti-proliferative compound from the *A. excelsa* leaves. This work resulted in the isolation of a flavonoids, are kaempferol and quercetin, which strongly citotoxycity the SP-C1 cell lines in a time- and dose-dependent manner. This compound has not been reported before in connection with its cytotoxicity in these cancer cell lines. The Raf-MEK-ERK-C Myc cascade was the first signaling pathway to be entirely mapped from the cell membrane to the cell nucleus and its structure became the paradigm for MAP kinase modules in general.[11] The Raf cascade is initiated by the small G protein Ras, which recruits Raf from the cytosol to the cell membrane for activation. Activated Raf phosphorylates and activates MEK, which in turn phosphorylates and activates ERK.[12] ERK has many substrates both in the cytosol and the nucleus. It can affect gene expression directly by phosphorylating transcription factors, such as Ets, Elk, and Myc, as well as indirectly by targeting other substrates, such as p90-RSK (ribosomal S6 kinase) family kinases, which can modify transcription factors and histones.[13] Apoptosis is an important series of events leading to programmed cell death that is also essential for development and tissue homeostasis.[14] Recently, the regulation of apoptosis has been proposed as a promising target for cancer chemotherapy.[15] The downregulation of c-Myc mediates coordinated antiproliferative effects,

including effects on the cell cycle and apoptosis.[16]

In this study, kaempferol and quercetin-induced apoptosis may be related to MAPK cascade to inhibited p90RSK activity. The function of p90RSK is to inhibit pro-apoptotic BAD. Therefore, the anticancer activity of kaempferol and quercetin may be useful for developing anticancer medicine.

CONCLUSION

In conclusion, our results suggest that kaempferol and quercetin inhibited the growth of SP-C1 cells through the induction of apoptosis. Further evaluation of its toxicity and detailed mechanisms of its anti-proliferative action is required to provide a scientific basis for its chemopreventive and chemotherapeutic application in tongue cancer management.

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