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3. Permintaan untuk cek progress di OJS terlihat permintaan revisi naskah 19 September 2020
4. Bukti artikel “accepted” dan akan di muat di jurnal Desember 2021

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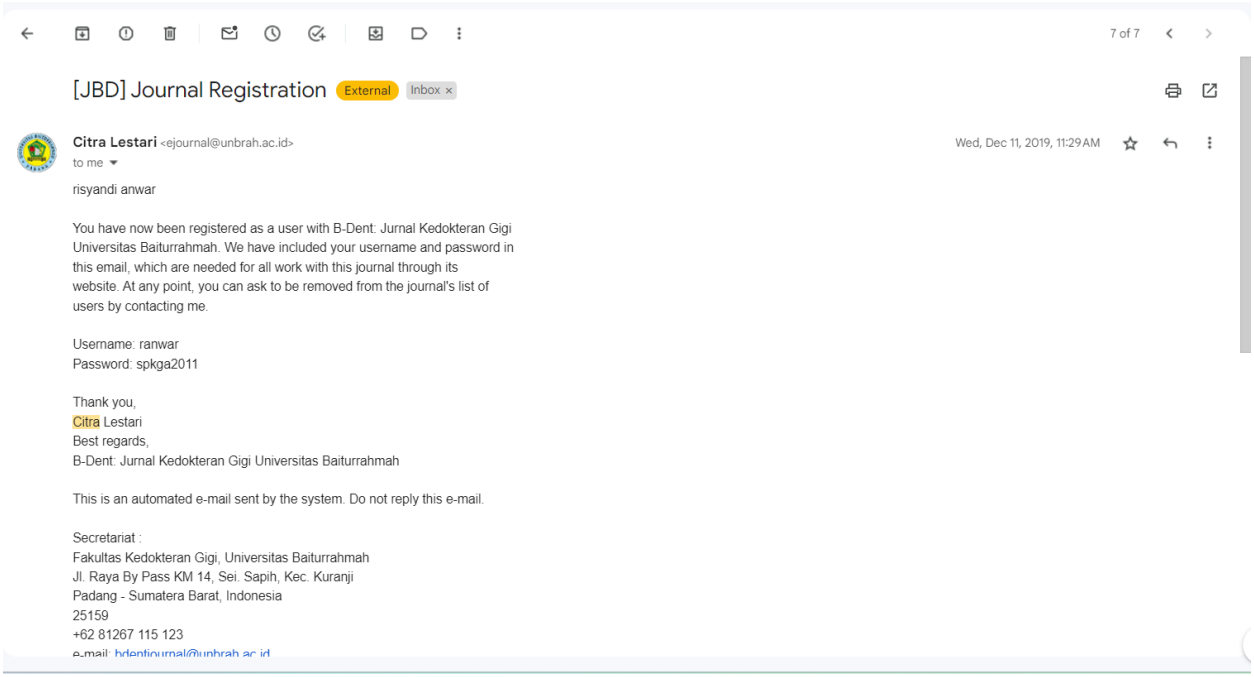

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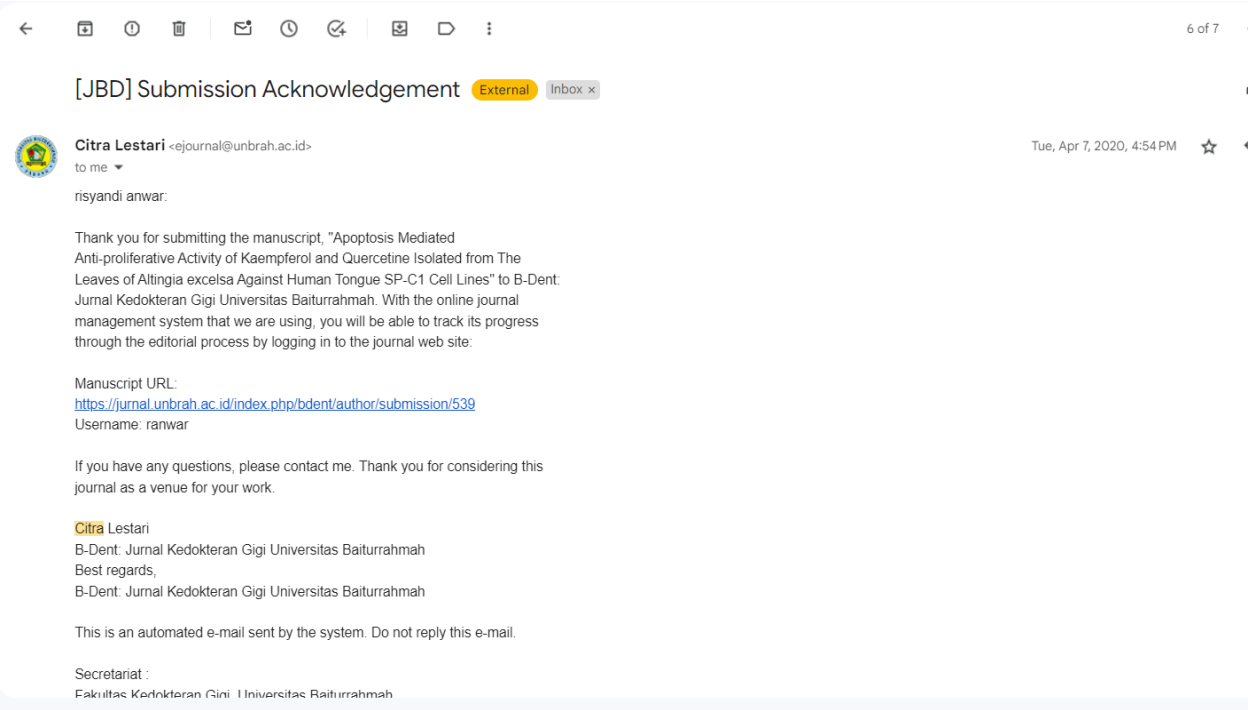

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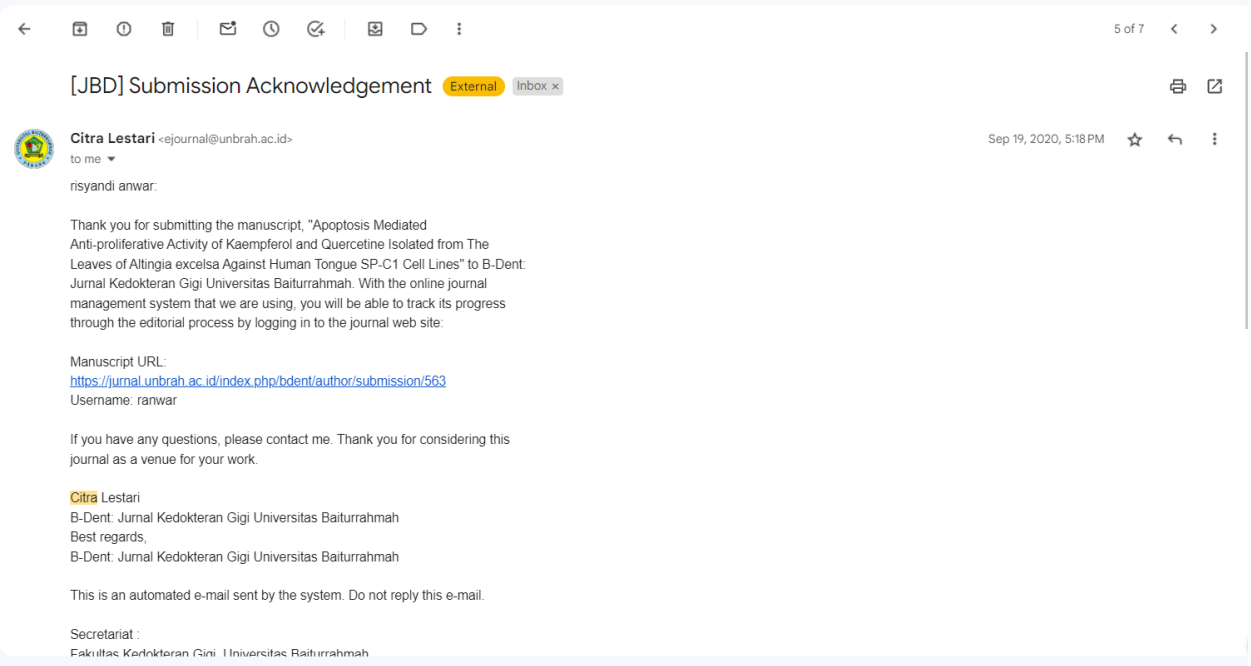

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Apoptosis Mediated Anti-proliferative Activity of Kaempferol and Quercetine Isolated from The Leaves of *Altingia excelsa* Against Human Tongue SP-C1 Cell Lines

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Abstract

The leaves of *Altingia excelsa* were found to strongly inhibit SP-C1 human tongue cancer cell lines. This study was focused on identifying antiproliferative compound found in *A. excelsa* leaves and assessing its mechanism of action. Methanol extracts of *A. excelsa* were fractionated based on their polarity using *n*-hexane, ethyl acetate, and water. The anti-proliferative properties were tested *in vitro* against SP-C1 human tongue cancer cell lines using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. The active compound was isolated using column chromatography and identified by spectroscopic method. The isolated compound was also tested for its anti-proliferative properties and ELIZA method of apoptotic induction. This work resulted in the isolation of a flavonoid, which was identified to be kaempferol and quercetine. The compound inhibited SP-C1 cell proliferation in a time- and dose-dependent manner with IC_{50} values of 0.72 µg/mL and 0. 70 µg/mL for the 24 hours treatments, respectively. Furthermore, promoting apoptosis through intrinsic pathway, thereby increasing activity of caspase-8 and caspase-9. These results suggest that kaempferol and quercetine the anticancer compound found in *A*, excelsa providing a basis for its potential use in cancer disease management.

Key words: *Altingia ecelsa*, cancer, apoptosis, kaempferol, quercetine

1. Introduction

Oral squamous cell cancer (OSCC) has high morbidity and mortality rates across the world because it is frequently found in advanced stages before therapy [1,2]. OSCC is now a global health problem with increasing incidence and mortality rates. The major environmental risk factors responsible for the development of OSCC include betel nut chewing, cigarette smoking, alcohol consumption, and exposure to high-risk human papillomavirus. 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. The conventional strategies of OSCC management still depend on surgery, radiotherapy, chemotherapy and targeted therapy [3]. The poor outcome of chemotherapy to OSCC contributes to the poor prognosis for OSCC [4]. 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 [5]. In an effort to identify novel agents that may inhibit cancer development, we have focused our investigations on discovering bioactive compounds from plants commonly high level plants [6].

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

II. **Materials and methods**

Plant materials. Altingia excelsa Nornha. leaves were collected from Wayang Windu Mountain, Pangalengan, West Java, Indonesia. The plant species was identified by the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Padjadjaran, Indonesia.

Extraction and isolation. The dried leaves of *Altingia excelsa* Nornha. (2.5 kg) were extracted with methanol (3x24 h) at room temperature. The solvent was subsequently evaporated under reduced pressure at 50˚C to yield a concentrated extract. The methanol extract (280 g) was fractionated between n-hexane and water to obtain an n-hexane extract (86 g) and a water layer. The water layer was then extracted with ethyl acetate to obtain an ethyl acetate fraction (120 g) and a water fraction (90 g). The cytotoxicity of the fractions was assessed on SP-C1 tongue cancer cells using the methyl thiazolyl tetrazolium (MTT) assay. The ethyl acetate fraction, 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 ultra violet and infrared spectrometry (UV-IR), and nuclear magnetic resonance (NMR) (4).

Cell culture and treatment. The SP-C1human tongue cancer cell line used in this study were 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 cell treatments, various 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 collected at the indicated 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 kaemperol and quercetin for 24 hours to detect caspase -8 and -9

activity. Caspase -8 and caspase-9 activity were 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 reaction buffer. Samples were then incubated in a 96-well flat bottom microplate at 37^0C for 1 hour. Levels of released pNA were measured with an ELISA reader (Anthos 2001) at 405 nm wavelength.

III. Results

Ethyl acetate fraction of Altingia ecelsa Nornha. *leaves inhibits* SP-C1 *cell proliferation.* Treatment with the *Altingia excelsa* Nornha. extract methanol was found to inhibit the proliferation of SP-C1 human tongue cancer cells (IC_{50} 75.41 µg/ml)(Fig. 1). The extract 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 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.

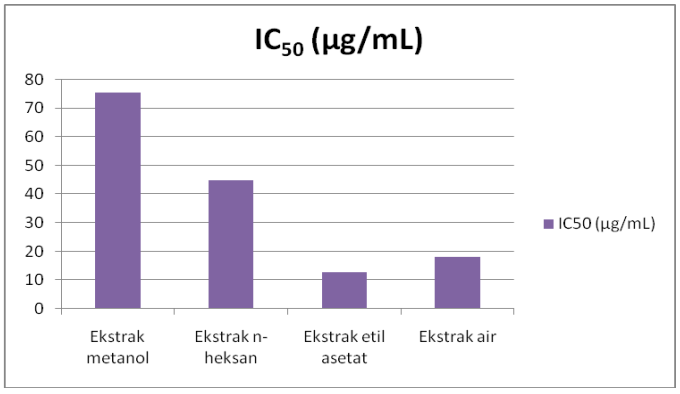


Figure 1. IC_{50} value some extracts *A. excelsa* Nornha

Kaemperol and quercetine are major compounds of the ethyl acetate fraction. The principle active compound of the ethyl acetate fraction of *Altingia excelsa* Nornha. 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 hydrogen and carbon content, the molecular ion peak and 1H and 13C NMR profiles indicated that the compound had a molecular formula of C21H20O10. 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.

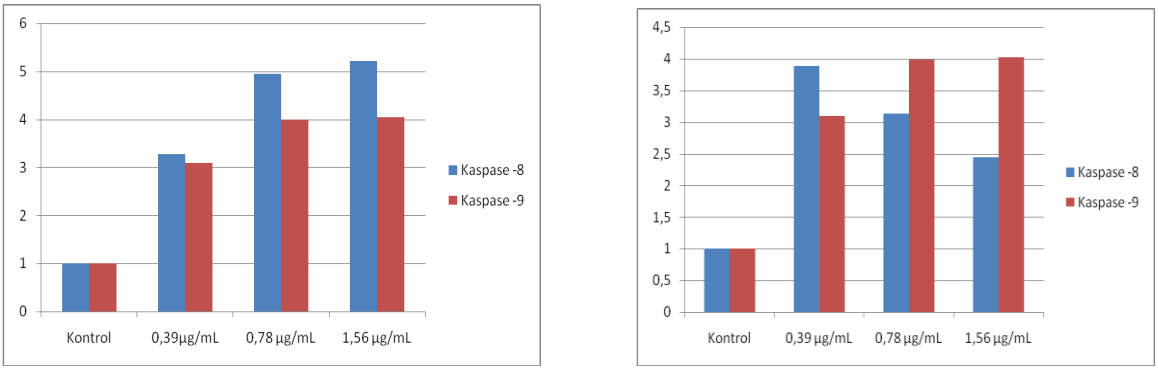
The 1H NMR spectrum of the compound showed two aromatic hydrogen signals with 'meta coupling' at δ 6.35 (1H, *d*, J= 2.2 Hz) and 6.18 (1H, *d*, J=2.2 Hz), which was predicted by the hydrogens at C-6 and C-8 of the A ring of the flavone skeleton. Accordingly, this compound was suggested to have a hydroxyl group at C-5 and C-7. Furthermore, its 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 signals of which were approximated from the hydrogens at C-2', C-3', C-5' and C-6' of the B ring. The absence of a specific signal for an olefinic hydrogen at C-3 and the presence of an anomeric hydrogen signal at δ 5.37 (1H, *d*, J=7.2 Hz) suggested that the compound was a flavonol glycoside. The appearance of an anomeric carbon signal at δ 94.9 in the 13C NMR spectrum indicated the presence of a sugar moiety. Due to a correlation between the anomeric hydrogen signal (δ 5.37) and the anomeric carbon 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 quercetine inhibits SP-C1cell proliferation in a dose-dependent manner. The effect of kaempferol and quercetine on the viability of SP-C1 cells was evaluated. The

treatment of cancer SP-C1 cell lines with kaempferol and quercetine resulted in a dose-dependent inhibition of cell growth, as demonstrated by the MTT assay. Twenty-four hours of treatment with kaempferol and quercetine inhibited the proliferation of SP-C1cells with an IC_{50} value of 0.72 and 0.70 µg/mL. Subsequent cell cycle analysis, immunoblot and caspase activity-based investigation applying the IC_{50} dose of 0.39, 0.78, and 1.56 µg/mL kaempferol and quercetine 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 kaemperol and quercetin for all concentration was correlated with the processing of pro-caspases-8 and -9, respectively, as demonstrated in Figure 2.



a. Kaempferol b. Quercetine

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

IV. Discussion

Traditional medicinal plants traditionally have long been regarded as a source of potential therapeutic agents, and the search for new drugs or leads are usually based on that approach [9,10].. In drug discovery, we have recently applied a new approach of selecting plants based on Hammamelidaceae family [7,8]. In our previous study, we found that the extracts of the *A. excelsa* leaves were strongly cytotoxic to the SP-C1 Human tongue cancer cell lin. Thus, these extracts had the potential for further investigation.

The present study was focused on identifying an anti-proliferative compound from the *A. excelsa* leaves. This work resulted in the isolation of a flavonoid, kaempferol and quercetine, which strongly inhibited the SP-C1 cell lines proliferation 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 signalling 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 cell cycle and apoptosis [16].

In this study, kaempferol and quercetine with some concentration decrease the expression of c-myc, and then clearly decrease the activation of cyclin D/CDK4, so G0/G1 phase cell cycle arrest be happened. Thus, kaempferol and quercetine -induced apoptosis may be related to MAPK cascade to inhibited p90RSK activity. The function of p90RSK to inhibited pro apoptotic BAD. Therefore, the anticancer activity of kaempferol and quercetin may be useful for developing anticancer medicine.

V. Conclusion

In conclusion, our results suggest that kaempferol and quercetine 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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1. Introduction

Oral squamous cell cancer (OSCC) has high morbidity and mortality rates across the world because it is frequently found in advanced stages before therapy [1,2]. OSCC is now a

global health problem with increasing incidence and mortality rates. The major environmental risk factors responsible for the development of OSCC include betel nut chewing, cigarette smoking, alcohol consumption, and exposure to high-risk human papillomavirus. 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. The conventional strategies of OSCC management still depend on surgery, radiotherapy, chemotherapy and targeted therapy [3]. The poor outcome of chemotherapy to OSCC contributes to the poor prognosis for OSCC [4]. 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 [5]. In an effort to identify novel agents that may inhibit cancer development, we have focused our investigations on discovering bioactive compounds from plants commonly high level plants [6].

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II. **Materials and methods**

Plant materials. Altingia excelsa Nornha. leaves were collected from Wayang Windu Mountain, Pangalengan, West Java, Indonesia. The plant species was identified by the

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Identification of Caspase-8 and -9 activity. Caspase-8 and caspase-9 activity was identified according to the manufacture's instructions (Caspase colorimetric kit R&D system Inc., MN, USA) in SP-C1 culture cells ~~were~~ that treated before with 0.39, 0.78 and 1.56 µg/mL

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kaemperol and quercetin for 24 hours ~~to detect caspase -8 and -9 activity~~. ~~Caspase -8 and caspase-9 activity were assessed~~. Samples that have been incubated with antibodies in reaction buffer using a 96-well flat bottom microplate at 37^0C for 1 hour then levels of released pNA were measured with an ELISA reader (Anthos 2001) at 405 nm wavelength.~~was incubated with caspase-8 and caspase-9 substrate~~.

III. Results

Ethyl acetate fraction of Altingia ecelsa Nornha *leaves inhibits* SP-C1 *cell proliferation.* Treatment with the *Altingia excelsa* Nornha. extract methanol was found to inhibit the proliferation of SP-C1 human tongue cancer cells (IC_{50} 75.41 µg/ml)(Fig. 1). The extract 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 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.

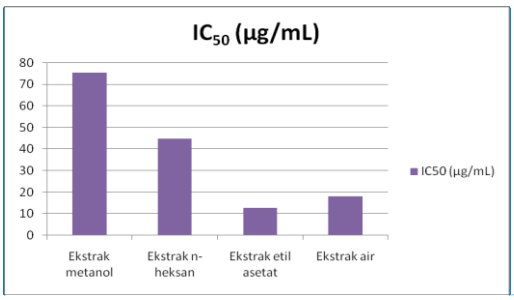

Figure 1. IC_{50} value some extracts *A. excelsa* Nornha

Kaemperol and quercetine are major compounds of the ethyl acetate fraction. The principle active compound of the ethyl acetate fraction of *Altingia excelsa* Nornha. 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 hydrogen and carbon content, the

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molecular ion peak and 1H and 13C NMR profiles indicated that the compound had a molecular formula of C21H20O10. 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 1H 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 hydrogens 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 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 signals of which were approximated from the hydrogens at C-2', C-3', C-5' and C-6' of the B ring. The absence of a specific signal for an olefinic hydrogen at C-3 and the presence of an anomeric hydrogen signal at δ 5.37 (1H, *d*, J=7.2 Hz) suggested that the compound was a flavonol glycoside. The appearance of an anomeric carbon signal at δ 94.9 in the 13C NMR spectrum indicated the presence of a sugar moiety. Due to a correlation between the anomeric hydrogen signal (δ 5.37) and the anomeric carbon 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 quercetine inhibits SP-C1cell proliferation in a dose-dependent manner. The effect of kaempferol and quercetine on the viability of SP-C1 cells was evaluated. The treatment of cancer SP-C1 cell lines with kaempferol and quercetine resulted in a dose-dependent inhibition of cell growth, as demonstrated by the MTT assay. Twenty-four hours

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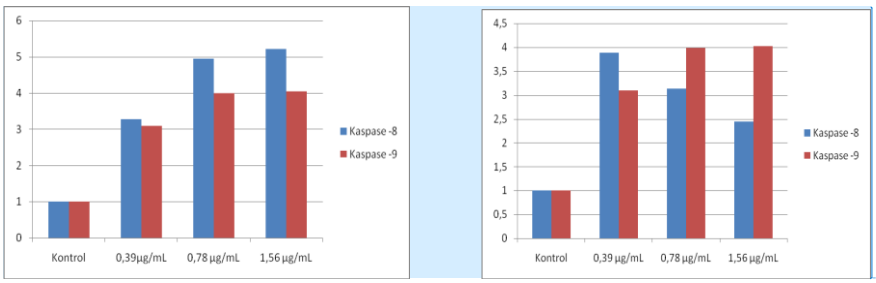
of treatment with kaempferol and quercetine inhibited the proliferation of SP-C1cells with an IC_{50} value of 0.72 and 0.70 µg/mL. Subsequent cell cycle analysis, immunoblot and caspase activity-based investigation applying the IC_{50} dose of 0.39, 0.78, and 1.56 µg/mL kaempferol and quercetine was 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 kaemperol and quercetin for all concentration was correlated with the processing of pro-caspases-8 and -9, respectively, as demonstrated in Figure 2.



a. Kaempferol b. Quercetine

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

IV. Discussion

Traditional medicinal plants traditionally have long been regarded as a source of potential therapeutic agents, and the search for new drugs or leads are usually based on that approach [9,10].. In drug discovery, we have recently applied a new approach of selecting

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plants based on Hammamelidaceae family [7,8]. In our previous study, we found that the extracts of the *A. excelsa* leaves were strongly cytotoxic to the SP-C1 Human tongue cancer cell lin. Thus, these extracts had the potential for further investigation.

The present study was focused on identifying an anti-proliferative compound from the *A. excelsa* leaves. This work resulted in the isolation of a flavonoid, kaempferol and quercetine, which strongly inhibited the SP-C1 cell lines proliferation 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~~ c-myc cascade was the first signalling 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 cell cycle and apoptosis [16].

In this study, kaempferol and quercetine with some concentration decrease the expression of c-myc, and then clearly decrease the activation of cyclin D/CDK4, so G0/G1 phase cell cycle arrest be happened. Thus, kaempferol and quercetine -induced apoptosis may be related to MAPK cascade to inhibited p90RSK activity. The function of p90RSK to inhibited pro

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apoptotic BAD. Therefore, the anticancer activity of kaempferol and quercetin may be useful for developing anticancer medicine.

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V. Conclusion

In conclusion, our results suggest that kaempferol and quercetine 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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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.

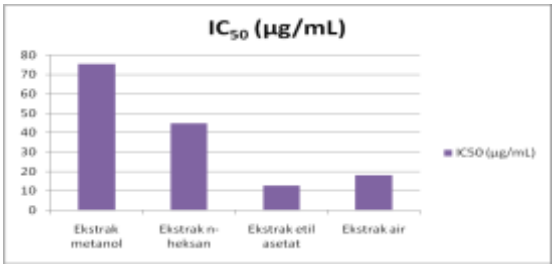

Figure 1. IC_{50} value some extracts *A. excelsa* Noronha

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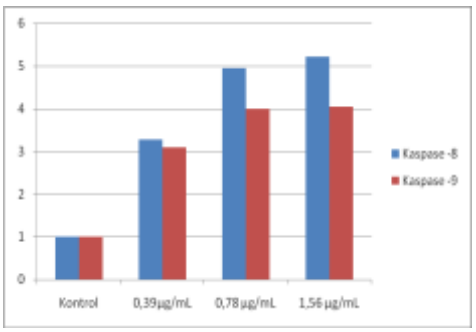
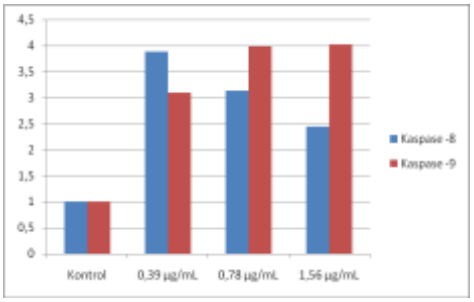
Kaempferol and quercetin inhibit SP-C1 proliferation of cell in a dose-dependent manner. The effect of kaempferol and quercetin on the citotoxycity of SP-C1 cells was evaluated. The treatment of cancer SP-C1 cell lines with kaempferol and quercetin resulted in a dose-dependent inhibition of cell growth, 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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