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ANTIBACTERIAL ACTIVITY OF GALLIC ACID FROM THE LEAVES OF *ALTINGIA EXCELSA* NORONHA TO *ENTEROCOCCUS FAECALIS*

1.	Pembuatan akun di jurnal	Tanggal 21 April 2022
2.	Konfirmasi atau validasi balasan dari jurnal	Tanggal 6 Juni 2022
3.	Permintaan revisi sesuai reviewer	Tanggal 20 Juli 2022
4.	Permintaan pembayaran publikasi	Tanggal 20 Juli 2022
5.	Konfirmasi pembayaran biaya publikasi	Tanggal 12 Desember 2022
6.	Proses Keputusan editor	Tanggal 19 Desember 2022
7.	Proses final	Tanggal 19 Desember 2022
8.	Artikel publish	Tanggal 23 Desember 2022

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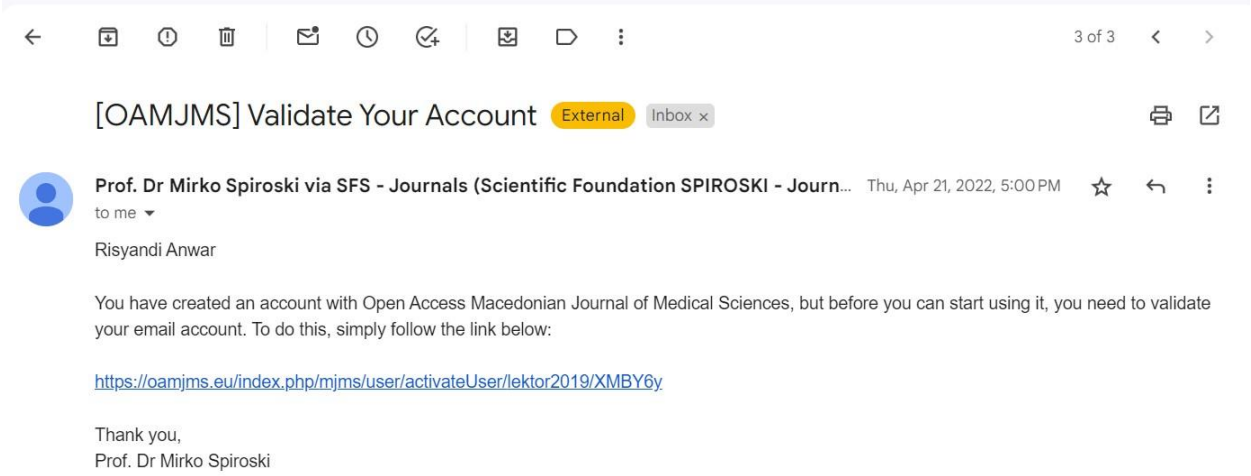

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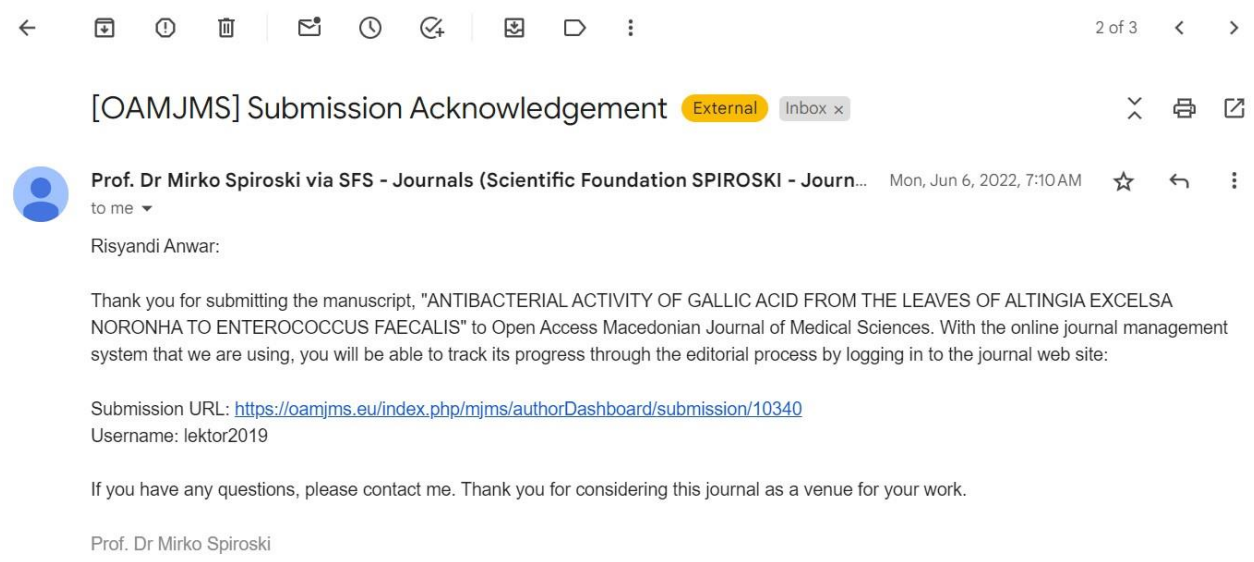

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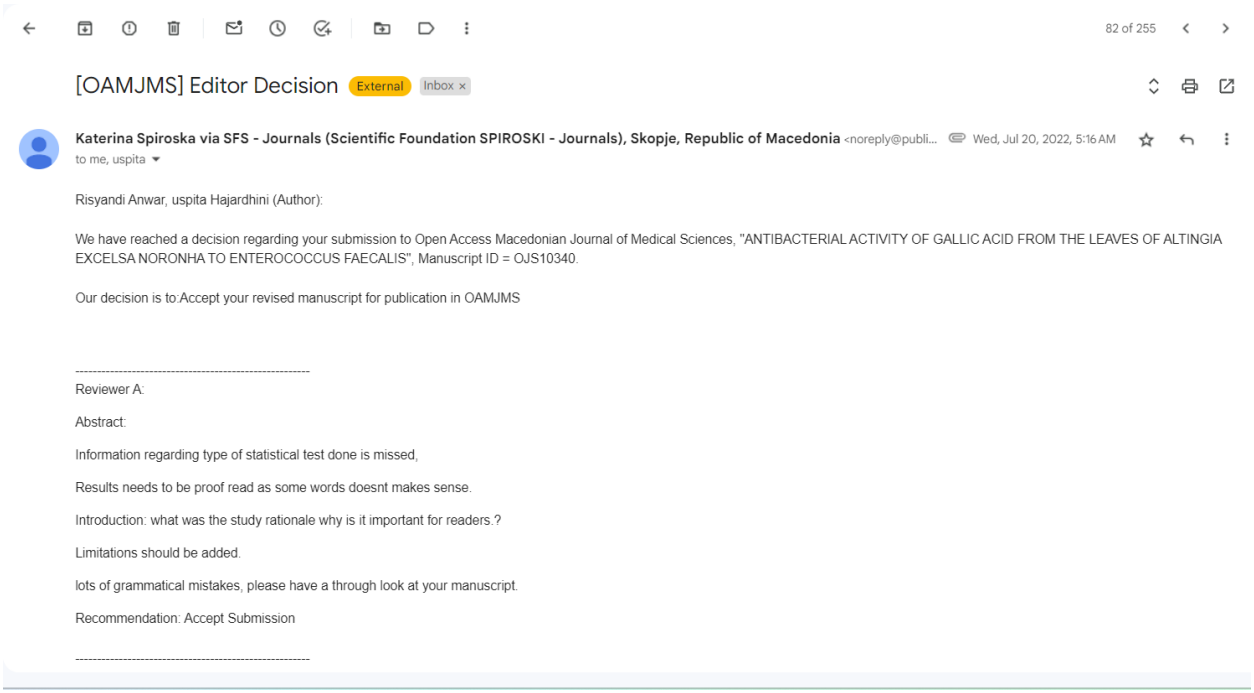

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Reviewer A:

Abstract:

Information regarding type of statistical test done is missed,

Results needs to be proof read as some words doesnt makes sense.

Introduction: what was the study rationale why is it important for readers.?

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lots of grammatical mistakes, please have a through look at your manuscript.

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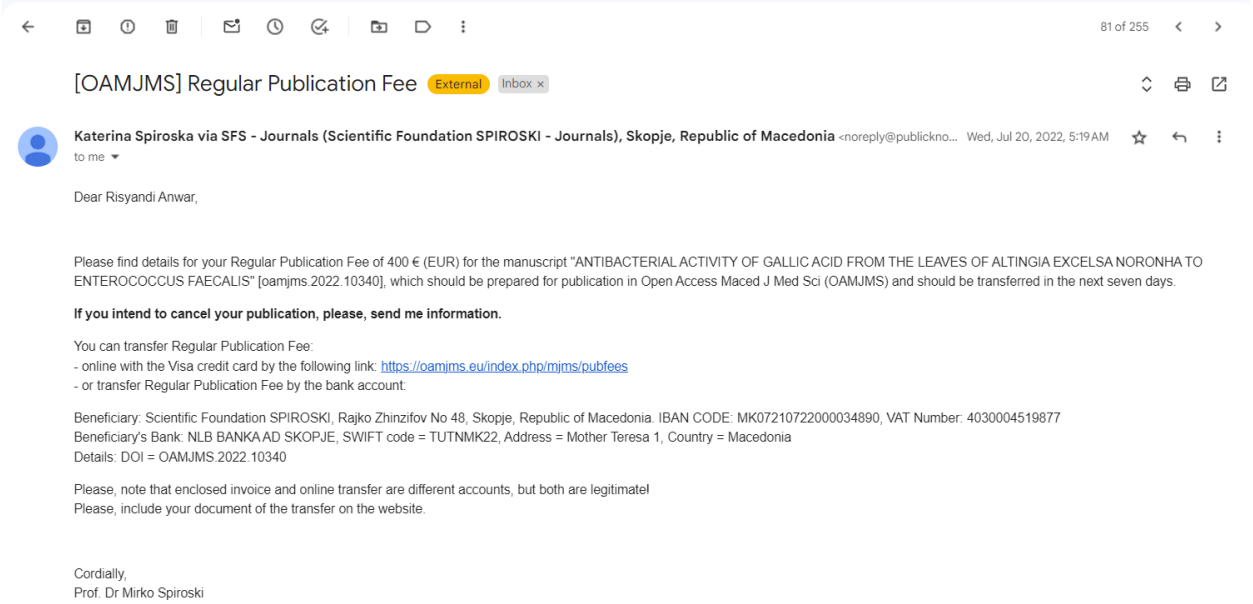

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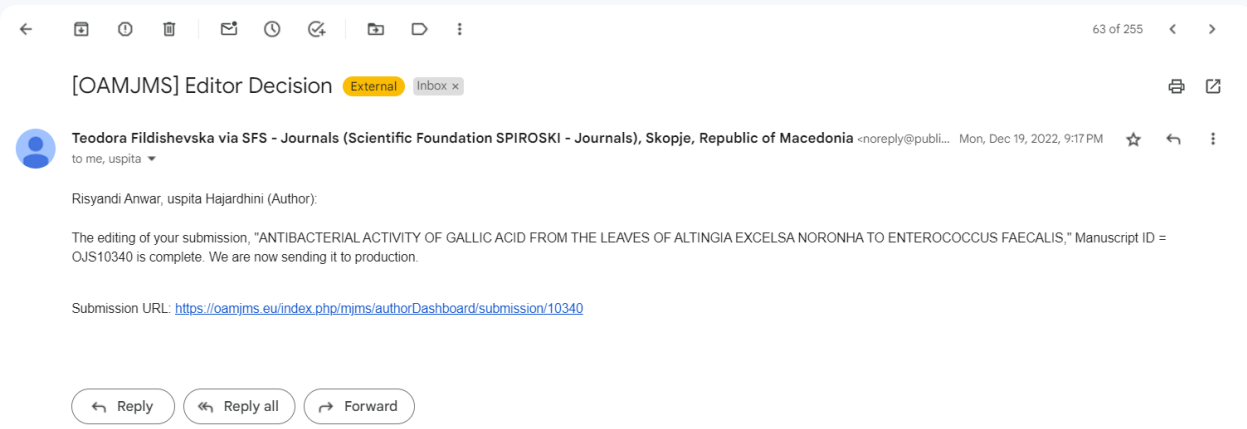

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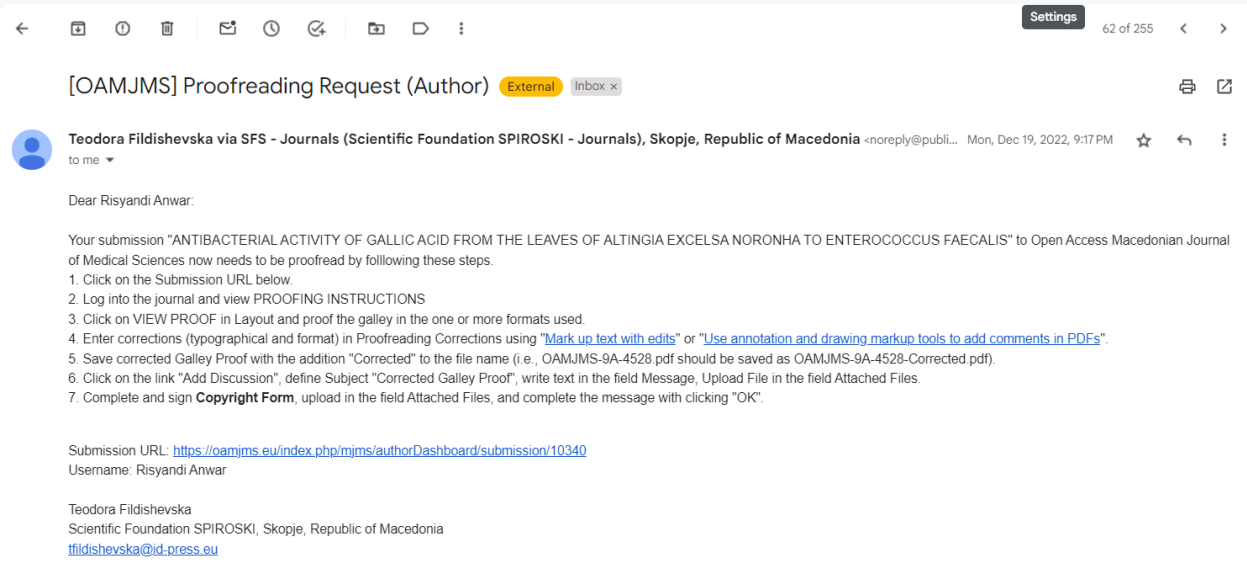

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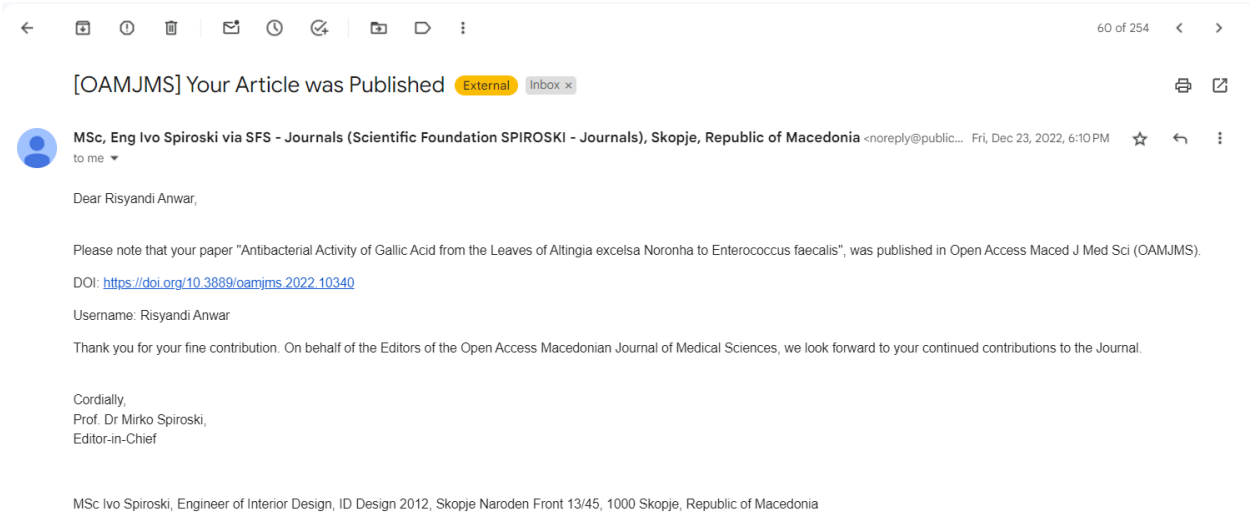

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DOI: https://doi.org/10.3889/oamjms.2022.10340

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Cordially,
Prof. Dr Mirko Spiroski,
Editor-in-Chief

MSc Ivo Spiroski, Engineer of Interior Design, ID Design 2012, Skopje Naroden Front 13/45, 1000 Skopje, Republic of Macedonia

ANTIBACTERIAL ACTIVITY OF GALLIC ACID FROM THE LEAVES OF *ALTINGIA EXCELSA* NORONHA TO *ENTEROCOCCUS FAECALIS*

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ABSTRACT

AIM : The aim of this study was to determine the antibacterial compound of *Altingia excelsa* leaves ~~to~~ that helps inhibit the growth of *Entrococcus faecalis*.
METHODS : Four extracts from *A. excelsa* leaves was obtained by the maceration method. The highest inhibitory effect from those extracts was then continued to be isolated until one compound was obtained. This extract via their compounds was separated using various chromatographic techniques. Chemical structure of compounds was determined by using UV spectra, infrared (IR), core magnetic resonance (NMR), and compared with spectra data.
RESULTS : The highest inhibitory effect was identified from ethyl acetate extract. The chemical structure of compound 1 was identified as an acidic compound 3,4,5 -trihydroxy benzoate, namely as the gallic acid which had inhibitory effect against *Enterococcus faecalis.* Antibacterial test for *Enterococcus faecalis* was done to determine inhibitory effect by its compound and MIC values showed of 12.25 µg/mL.
CONCLUSION : Gallic acid as the compound of *Altingia excelsa* leaves had a strong inhibitory effect to *Enterococcus faecalis.*

Keywords: *Altingia excelsa*, *Enterococcus faecalis*, *antibacterial*, *gallic acid*

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Introduction

Dental caries is a common oral dental disease which is commonly found in children [1]. Based on the WHO global oral health survey, global dental caries index in 12 years old children was 1.6 which means that on average a person can experience tooth decay on more than 1 tooth [2]. Ministry of Health Republic Indonesia also conducts oral health survey on preschoolers every 5 years. The average score of DMFT are 19.0 and 9.9 for 3-4 years old and 5 years old, respectively [3].

Untreated primary dental caries can rapidly spread and can lead to exposure of the pulp. The pulp which has been exposed becomes an entry pathway for microorganism that cause inflammation and if it continues, it will lead to non vital pulp. primary teeth with caries infection that reaches the pulp tissues will require an endodontic treatment. Clinical manifestation of teeth with periapical lesion or root canal infection usually gives symptoms of pain at night, with or without stimulation. Clinically, a periapical abscess or fistula and abnormal tooth mobility would give sensitive response to percussion and pressure examination [4]. There are many bacteria that inhabit within root canal of primary tooth such as aerob, anaerob and facultative. But, mostly found bacteria is *Enterococcus faecalis* [5].

Various antibacterial agent are available that can help with root canal sterilization and have been used to eliminate bacterial growth which still persists after the biomechanical preparation and also induces periradicular healing [6]. There are most common used root canal antibacterial such as Rockle's, Tricresol Formalin, Cresophane, and CHKM. Degradation of bacteria inside the root canal can be examined with bacterial culture [7]. However, the bacteria can be resistant to antibiotic causing primary failure of endodontic treatment in children [8]. Therefore, new antibacterial agent which explore natural ingredients originated from medicinal plants is required to overcome this problem in root canal treatment.

Mostly some medicinal plants posses antibacterial or antioxidant derived from herbs, spices, and their compounds which are composed of secondary metabolites such as phenolics, phenolics acids, quinones, saponins, flavonoids, tanins, coumarins, terpenoids, and alkaloids [9]. Study from de Castilho et al. reported that these chemical compounds obtained from plants extract at Amazon forest showed antibacterial activity against planktonic *E. faecalis* compared to chlorhexidine [10]. Indonesia, as the largest forest in the world with more than 30 thousand species of plants, has medicinal properties and potential for treating various diseases traditionally by utilizing these medicinal herbs. One of the herbs is *Altingia excelsa* nornha leaf which comes from Altingia genus and including Hammamelidaceae family. Traditionally, Altingia is useful as a anti-pyretic, vitality enhancer, anti-inflammatory, cough and stomach medicine [11]. Scientifically, previous study reported to aim the benefits of *A. excelsa*, as potentially anticancer and antibacterial [12].

The prospecting drugs from natural ingredients contain chemical compounds and pharmacologically biological agent? which need to be determined associated with their efficacy and usefulness. Isolation guided by biological tests is a chromatographic method to isolate chemical compounds which monitor its purity. The identification of antibacterial agents is directed not only based on ethnobotany and phytochemical studies but also in vitro antibacterial test. Therefore, further research to explore the potential of *A. excelsa* as antibacterial drugs is very important. Here, we show that antibacterial of *A. excelsa* leaves inhibit the growth of *Enterococcus faecalis* by its chemical compounds.

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Methods

Preparation of the natural extracts

Preparation of Altingia excelsa leaves extracts

Altingia excelsa leaves which obtained from the Wayang Windu mountains, Pangalengan, Bandung was used and analyzed at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University. This Iin vitro laboratory experimental research which which consisteds of extraction and isolation of active compounds from *A. excelsa* leaves was carried out throughout April 2018 until June 2020 at the Organic Chemistry Laboratory of Natural Materials, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Garut University, West Java. All research procedures have been approved by the Ethics Committee of the Faculty of Medicine, Universitas Muhammadiyah Semarang, Central Java (No. 041/EC/FK/2018). *Enterococcus faecalis* ATCC™ 19433 (Thermoscientific) were used in this study.

Maceration was done for dry leaves of *A. excelsa* using methanol for 24 hours and carried out by mass storage three times. The maserat concentrated with a rotary evaporator until 225 g of concentrated methanol was achieved. The concentrated methanol maserat was dissolved into water and split using n-hexane to produce n-hexane extract and water. The n-hexane extract was divided and concentrated using rotary evaporator to achieve 167 g concentrated n-hexane extract. The obtained water layer was then divided using ethyl acetate to produce ethyl acetate extract and water. Ethyl acetate extract was divided and concentrated using rotary evaporator to obtain 145 g of concentrated ethyl acetate extract. These extracts were tested for antibacterial against *Enterococcus faecalis.*

Characterization

Thin Layer Chromatography (TLC) (Merck, Darmstadt, Germany) analysis was used to obtain five main fractions (A-E) and determine the antibacterial activity. The chemical structure of compounds was determined ~~based on~~using the spectroscopic data including (UV), infrared (IR) (CAMO Software, Norway), core magnetic resonance (NMR), and compar~~ed~~ison with spectra data obtained from the literature. NMR spectrum measurements were carried out at the LIPI Serpong Chemical Research Center, and antibacterial tests were carried out at the Microbiology Laboratory, University of Muhammadiyah Semarang. The results was read by ELISA microplate reader (BioRed-Japan).

Isolation of the most active compound

MIC values of various fractions of the antibacterial test showed that Fraction C had~~s~~ potential as an antibacterial agent. Vacuum Liquid Chromatography (VLC) was used to analyze Fraction C on the G60 silica gel stationary phase with the mobile phase n-hexane-ethyl acetate methanol with a gradient of 10% (v/v). Thin Layer Chromatography (TLC) analysis was done further to obtain five fractions (C1-C5), and they were tested for antibacterial activity showed that the C3 fraction gave potential antibacterial activity.

Column chromatography (KK) was done to separate further C3 fraction in stationary silica gel (70-230 mesh) with n-hexane mobile phase ethyl acetate with 10% (v/v) gradient to obtain four fractions of C31 to C34 which was tested for antibacterial activity. From the results of the antibacterial test, which had the potential as an antibacterial was the C32 Fraction. The C32 fraction was further separated by gradient column chromatography (KK) using an n-hexane acetone mobile phase to obtain four fractions namely C321 to C324. One of the fractions, C321, which was an amorphous solid then further recrystallized with a mixture of benzene: methanol (8: 2), so that a white crystal (9 mg) compound 1 was obtained.

Determination of compound structure

Isolated compounds were determined by physical properties including color and melting point. The chemical structure of compounds was determined based on spectroscopic data including (UV), infrared (IR), core magnetic resonance (NMR), and compared with spectra data obtained from the literature [13–16].

Antibacterial assessment of the prepared extracts

Enterococcus faecalis antibacterial test

The extract and isolated compounds were diluted in DMSO 1% and carried out by the dilution method to antibacterial test against *Enterococcus faecalis* using 96-wells (Iwaki-Japan) [17]. We used the parameters turbidity that occurred due to the growth of bacteria at certain concentrations caused by the antibacterial activity of the extracts and isolates. The results was read by ELISA microplate reader at a wavelength of 630 nm.

Results

Minimum Inhibitory Concentration of various extracts against E. faecalis

To determine the antibacterial activity of various extracts against *E. faecalis* bacteria was carried out by the liquid dilution method [18]. Antibacterial activity of various extracts against *E. faecalis* ~~bacteria~~ was expressed by MIC value, referring to the criteria for the level of antibacterial extracts of natural ingredients in testing for antibacterial properties [19] as listed in Table 1. For the

antibacterial activity of the four extracts of *Altingia excelsa* leaves, they exhibited different antibacterial capacity. Ethyl acetate extract of *Altingia excelsa* leaves showed good antibacterial activity in this study at the concentration of 12.25 µg/mL followed by methanol extract, *n*-hexane extract, and water at the concentration of 69.23 µg/mL, 97.12 µg/mL and 98.03 µg/mL, respectively. *Enterococcus faecalis* was shown to be less sensitive to methanol extract, *n*-hexane extract, and water extract besides ethyl acetate extract exhibited stronger antibacterial activity than these three extracts. This result resumed that the inhibitory activity of the four extracts against *E. faecalis* was in the order of ethyl acetate> methanol>*n*-hexane>water.

The result of antibacterial test, showed that ethyl acetate extract ~~was~~ had the highest inhibitory effect, ~~so that the~~hence ethyl acetate was continued to be isolated.

Table 1. MIC value from various extract of *Altingia excelsa* leaves

Extract	MIC (µg/mL)
Methanol	69.23
n-hexane	97.12
Ethyl acetate	12.25
Water	98.03

With the guide of antibacterial test for ethyl acetate was done by separation and purification until it obtained one compound.

Commented [DF3]: Do you mean that separation and purification of ethyly acetate? Please write clearly

Compound 1

White solids, t.l. 258-260 °C, UV (MeOH) λmaks nm 222, 271 and 404; IR (KBr) vmaks 3422, 1649, 1408, 1020 cm-1; 1H-NMR (CD3OD, 500 MHz) δH (ppm) 7,15 (1H, s, H-2, H-6); 13C-NMR (CD3OD, 500 MHz) δC (ppm) 122,2 (C-1), 110,7 (C-2, C-6), 145,9 (C-3, C-5), 138,6 (C-4), dan 167,7 (C-7). TOF MS ES+ m/z [M+H]+ 168,6684 calculation for $C_7H_6O_5$, m/z 170,1195.

Chemical Compound Structure

Compound 1 was obtained as a white solid with a melting point of 258-260 ° C. The UV spectrum of compound 1 (Figure 1) showed~~s~~ the presence of two absorption bands at λmax 271 and 222 nm. The absorption at λmax 271 nm indicates a π→ π * transition which was thought to be derived from the B band of the benzene group. Meanwhile, the absorption at λmax 222 nm was thought to originate from the carbonyl group in the presence of the n→ π * transition (R band) [13,14].

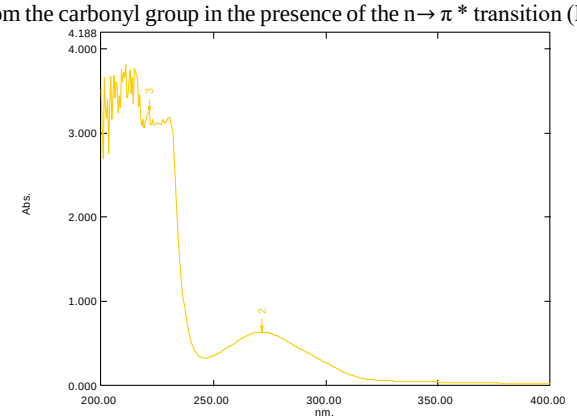

Figure 1. UV spectra compound 1 (methanol)

The IR spectrum of compound 1 (Figure 2) showed the presence of an -OH group as evidenced by the appearance of the O-H strain observed at vmax 3422 cm^{-1} and the C-O strain at vmax 1021 cm^{-1}.

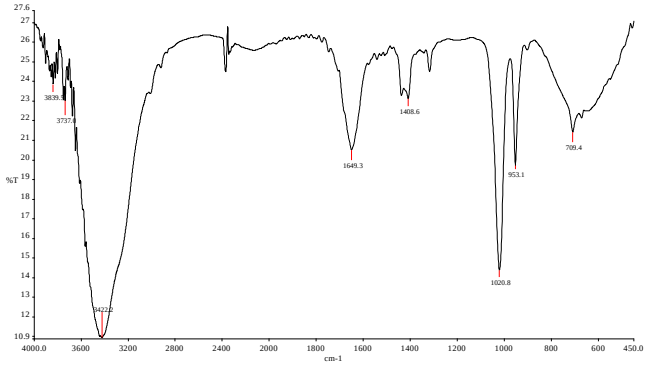

Figure 2. IR spectra compound 1 (KBr)

The carbonyl group which added ~~to~~ the degree of unsaturation in this compound ~~is~~ was indicated by strong absorption at v_{maks} 1649 cm-1. The vibration for this carbonyl group appeared at lower frequency due to conjugation between the carbonyl group and the benzene group. The existence of the C=C double bond was shown by the absorption at vmax of 1409 cm-1 [13,20].

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The 13C NMR spectrum (Figure 3) showed the presence of seven carbon signals consisting of one carbonyl carbon resonating at δC167.6 ppm and a CH sp^2 signal resonating at δC145.9-110.1 ppm. In compound 1, there are three oxygenated quaternary carbons, namely δC145.9 ppm (C-3 and C-5) and δC138.6 ppm (C-4). Therefore, compound 1 showed a benzene framework with four substituents. Two carbon signals each contain two carbons (^{13}CNMR data), thus compound 1 has a symmetrical structure [15,16].

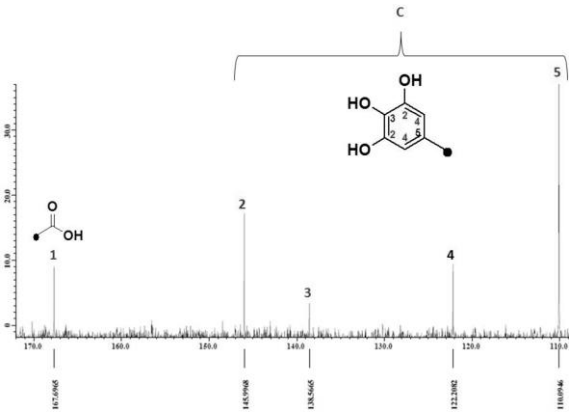

Figure 3. ^{13}C NMR Spectra compound 1 (aseton-d_6, 125 MHz).

The ^{1}H NMR spectrum of compound 1 (Figure 4) showed the presence of a singlet proton signal resonating at δH 7.14 ppm. Therefore, the singlet proton signal represented two equivalent protons thereby amplifying the benzene framework with four substituents [15,16,20].

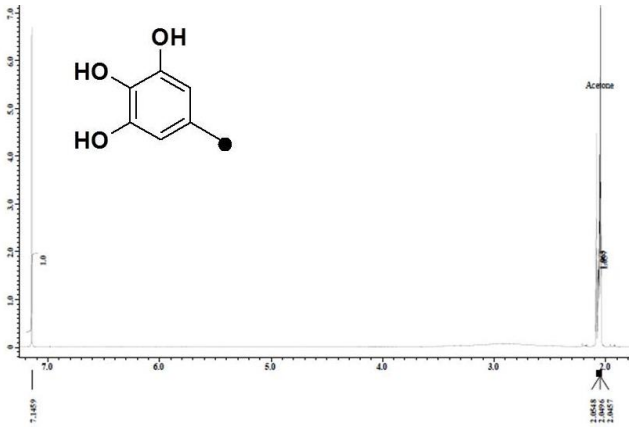

Figure 4. ^{1}H NMR Spectra compound 1 (aseton-d_6, 500 MHz).

The molecular formula for compound 1 was designated as $C_7H_6O_5$ based on the TOF-MS ES$^-$ spectra (m/z 168.8390 $[M + H]^-$, calculations for $C_7H_6O_5$ m/z 170.1195) and NMR data so that five degrees of unsaturation were obtained. Comparison of the NMR data of compound 1 with 3,4,5-trihydroxy benzoic acid compounds (Table 2), showed that the two compounds were very high compatibility, so that definitively, compound 1 was identified as an acidic compound 3,4,5 -trihydroxy benzoate (Figure 5) [15,16,20].

Table 2. Comparison compound 1 with gallic acid

C Position	Compound 1		3,4,5-trihidroksi benzoat acid	
	δ_C (ppm)	**δ_H (ppm), ∑ H, mult, *J* (Hz)**	**δ_C (ppm)**	**δ_H (ppm), ∑ H, mult, *J* (Hz)**
1	122,2	-	120,6	-
2	110,7	7,15 (1H; *s*)	108,9	7,07 (1H; *s*)
3	145,9	-	144,9	-
4	138,6	-	138,2	-
5	145,9	-	144,9	-
6	110,7	-	108,9	-
CO_2H	167,7	-	169,0	-

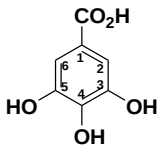

Figure 5. Chemical structure of 3,4,5 trihydroxy benzoate (gallic acid)

Discussion

Enterococcus faecalis has been known to be the most frequently isolated bacteria in root canal system which is associated in condition of previously or failure endodontically treated teeth followed

by secondary apical lesion rather than primary infection. It possesses some characteristics as anerobic facultative, Gram-positive bacteria, which enable this bacteria to live in low oxygen, high pH, at variable~~s~~ temperature from 10^{o}-60^{o}, and survives at low nutrient ~~so~~ that induces~~ing~~ resistance mechanism to cause opportunistic infections. Therefore, recently treatment ~~is~~ are used to decrease or disinfect it from colonizing biofilm in root canal system and periradicular area using biochemomechanical instrumentation combines with intracanal medicament and promising antibacterial agent from various sources [21].

To determine the antibacterial activity of compound 1 against *E. faecalis* bacteria was carried out by the liquid dilution method [18]. The antibacterial activity of compound 1 against *E. faecalis* were shown by the MIC value. Referring to the criteria for the level of antibacterial activity of natural compounds in testing for antibacterial properties [22], our result showed MIC of 12.25 µg/mL.

Gallic acid is a member of flavones (phenolic acid) and belongs to the subclass of flavonoids [23]. Gallic acid as derivative of cinnamic acid is formed through the pathway of shikimic acid with 3-dehydrosikimic acid as a base ingredient. Gallic acid has been tested as antibacterial agent, against *Staphylococcus aureus*, and *Helicobacter pylori* [22]. It showed antimicrobial activities associated with various pathways within cytoplasmatic membrane via destabilization, permeabilization, inhibitory enzyme by oxidized products which perhaps through reaction with sulfhydryl groups or more nonspecific interactions with proteins and inhibition the synthesis of nucleic acids for both Gram-negative and Gram-positive bacteria [22–24]. Previously conducted study reported that Gram-positive bacteria exhibited more resistance~~t~~ than Gram-negative bacteria due to additional number of hydroxyl groups followed by substitution of hydroxyl groups to methoxy groups resulting activity enhancement of gallic acid. Those studies revealed that antimicrobial efficacy of gallic acid was associated with the length of hydrophobic chain [23]. Gram-positive bacteria seemed to respond~~se~~ with higher concentration of MIC and MBC than Gram-negative bacteria. On the contrary result by Pinho et al., this study obtained higher MIC in *Klebsiella pneumoniae* (9,75 µg/mL) than *Staphylococcus epidermidis* (9,8 µg/mL) and *Staphylococcus aureus* (19,5 µg/mL) [22]. We like to confirm this phenomenon, thus we suggest to use other Gram-negative bacteria or different strains to determine antibacterial efficacy of gallic acid.

Gallic acid showed alteredation mechanism to bacterial hydrophobicity facilitated by its physicochemical surface properties. Alteration of bacterial cells was induced by gallic acid resulting in adjustment the polar, nonpolar, and electron acceptor (c +) of their components. It gave rise to differential ability for both increased electron acceptor as in Gram-positive and decreased electron acceptor as in Gram negative bacteria. It was also electrophilic and significantly depend on the bacterial surface components [24,25]. Although it ~~is~~ was due to characteristics of hydroxycinnamic acid via their propenoid side chain, it has antibacterial and less polar than the corresponding hydroxybenzoic acids [25]. However, other study reported gallic acid seemed to cause alteration more significantly to bacterial physicochemical than ferulic acid (hydroxycinnamic acid) [23]. Thus, cell membrane now easily could be penetrated enabled through its transport [25]. Facilitated by passive diffusion, gallic acid as phenolic acids could destroy the cell membranes, enter within cytoplasm, create intracellular acidification and lead to protein denaturation. This low pH compromised the bacteria to release ATP production via inhibition of H^{+}-ATPase enzyme [22].

The leaves of *Altingia excelsa* had been identified as antibacterial and antiproliferating agent which is reported in our previously conducted studies [26,27]. It was reported that the leaves of *Altingia excelsa* ~~owned~~ contained kaempferol, quercetin, 3,4 dihydroxybenzoic acid, gallic acid, and apigenin [27,28]. These secondary metabolites inhibited cell proliferation against Sp-C1 human tongue cancer lines via apoptotic activity. Apigenin-contained *Altingia excelsa* leaves also had been reported its antibacterial activity to *E. faecalis* [26]. However, there was still a few studies which revealed its secondary metabolites associated with antibacterial potency. Even though our results showed that MIC of gallic acid was 12.25 µg/mL which did not induce any toxicity, we suggest to further study its role in cytotoxicity determination. This concentration was still included within range

Commented [DF5]: Reference?

between 10 and 100 µg/mL without any toxicity in fibroblast cell which exhibited more than 70% viability of cells [22]. Further study is needed to confirm this safety concentration within human cell which may suggest the mechanism involved on the antibacterial efficacy.

Conclusion

It could be concluded that the highest inhibitory effect was ethyl acetate extract and then continued to be isolated. The chemical structure of compound 1 was identified as an acidic compound 3,4,5 -trihydroxy benzoate, namely as the gallic acid which had inhibitory effect against *Enterococcus faecalis.*

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Antibacterial Activity of Gallic Acid from the Leaves of *Altingia excelsa* Noronha to *Enterococcus faecalis*

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Abstract

AIM: The aim of this study was to determine the antibacterial compound of *Altingia excelsa* leaves to inhibit the growth of *Enterococcus faecalis*.

METHODS: Four extracts from *Amanita excelsa* leaves were obtained by the maceration method. The highest inhibitory effect from those extracts was then continued to be isolated until one compound was obtained. This extract through their compounds was separated using various chromatographic techniques. Chemical structure of compounds was determined by using UV spectra, infrared, core Nuclear magnetic resonance (NMR), and compared with spectra data.

RESULTS: The highest inhibitory effect was identified from ethyl acetate extract. The chemical structure of compound 1 was identified as an acidic compound 3,4,5-trihydroxy benzoate, namely, as the gallic acid which had inhibitory effect against *E. faecalis*. Antibacterial test against *E. faecalis* was done to determine inhibitory effect by its compound and MIC values showed of 12.25 μg/mL.

CONCLUSION: Gallic acid as the compound of *A. excelsa* leaves had a strong inhibitory effect to *E. faecalis*.

Introduction

Dental caries is a common oral dental disease which is commonly found in children [1]. Based on the WHO global oral health survey, global dental caries index in 12-year-old children was 1.6 which means that on average, a person can experience tooth decay on more than 1 tooth [2]. Ministry of Health Republic Indonesia also conducts oral health survey on preschoolers every 5 years. The average score of DMF-T is 19.0 and 9.9 for 3–4 years old and 5 years old, respectively [3].

Untreated primary dental caries can rapidly spread and can lead to exposure of the pulp. The pulp which has been exposed becomes an entry pathway for microorganism that causes inflammation and if it continues, it will lead to non-vital pulp. Primary teeth with caries infection that reaches the pulp tissues will require an endodontic treatment. Clinical manifestation of teeth with periapical lesion or root canal infection usually gives symptoms of pain at night, with or without stimulation. Clinically, a periapical abscess or fistula and abnormal tooth mobility would give sensitive response to percussion and pressure examination [4]. There are many bacteria that inhabit within root canal of primary tooth such as aerob, anaerob, and facultative. However, mostly found bacteria is *Enterococcus faecalis* [5].

Various antibacterial agent is available that can help with root canal sterilization and have been used to eliminate bacterial growth which still persists after the biomechanical preparation and also induces periradicular healing [6]. There is most common used root canal antibacterial such as Rockle's, Tricresol Formalin, Cresophane, and CHKM. Degradation of bacteria inside the root canal can be examined with bacterial culture [7]. However, the bacteria can be resistant to antibiotic causing primary failure of endodontic treatment in children [8]. Therefore, new antibacterial agent which explore natural ingredients originated from medicinal plants is required to overcome this problem in root canal treatment.

Mostly some medicinal plants possess antibacterial or antioxidant derived from herbs, spices, and their compounds which are composed of secondary metabolites such as phenolics, phenolics acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids [9]. The study from de Castilho *et al*. reported that these chemical compounds obtained from plants extract at Amazon forest showed antibacterial activity against planktonic *E. faecalis*

compared to chlorhexidine [10]. Indonesia, as the largest forest in the world with more than 30 thousand species of plants, has medicinal properties and potential for treating various diseases traditionally by utilizing these medicinal herbs. One of the herbs is *Altingia excelsa* nornha leaf which comes from Altingia genus and including Hammamelidaceae family. Conventionally, Altingia is useful as an anti-pyretic, vitality enhancer, anti-inflammatory, cough, and stomach medicine [11]. Scientifically, the previous study reported to aim the benefits of *Amanita excelsa*, as potentially anticancer and antibacterial [12].

The prospecting drugs from natural ingredients contain chemical compounds and pharmacologically biological which need to be determined associated with their efficacy and usefulness. Isolation guided by biological tests is a chromatographic method to isolate chemical compounds which monitor its purity. The identification of antibacterial agents is directed not only based on ethnobotany and phytochemical studies but also *in vitro* antibacterial test. Therefore, further research to explore the potential of *A. excelsa* as antibacterial drugs is very important. Here, we show that antibacterial of *A. excelsa* leaves inhibits the growth of *E. faecalis* by its chemical compounds.

Methods

Preparation of the natural extracts

Preparation of A. excelsa leaves extracts

A. excelsa leaves which obtained from the Wayang Windu mountains, Pangalengan, Bandung was used and analyzed at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University. This *in vitro* laboratory experimental research which consists of extraction and isolation of active compounds from *A. excelsa* leaves was carried out throughout April 2018 until June 2020 at the Organic Chemistry Laboratory of Natural Materials, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Garut University, West Java. All research procedures have been approved by the Ethics Committee of the Faculty of Medicine, Universitas Muhammadiyah Semarang, Central Java (No. 041/EC/FK/2018). *E. faecalis* ATCC™ 19433 (Thermoscientific) was used in this study.

Maceration was done for dry leaves of *A. excelsa* using methanol for 24 h and carried out by mass storage 3 times. The maserat concentrated with a rotary evaporator until 225 g of concentrated methanol was achieved. The concentrated methanol maserat was dissolved into water and split using n-hexane to produce n-hexane extract and water. The n-hexane extract was divided and concentrated using rotary evaporator to achieve 167 g concentrated n-hexane extract. The obtained water layer was then divided using ethyl acetate to produce ethyl acetate extract and water. Ethyl acetate extract was divided and concentrated using rotary evaporator to obtain 145 g of concentrated ethyl acetate extract. These extracts were tested for antibacterial against *E. faecalis*.

Characterization

Thin layer chromatography (TLC) (Merck, Darmstadt, Germany) analysis was used to obtain five main fractions (A-E) and determine the antibacterial activity. The chemical structure of compounds was determined based on spectroscopic data including (UV), infrared (IR) (CAMO Software, Norway), core Nuclear magnetic resonance (NMR), and comparison with spectra data obtained from the literature. NMR spectrum measurements were carried out at the LIPI Serpong Chemical Research Center, and antibacterial tests were carried out at the Microbiology Laboratory, University of Muhammadiyah Semarang. The results were read by ELISA microplate reader (BioRed-Japan).

Isolation of the most active compound

MIC values of various fractions of the antibacterial test showed that Fraction C has potential as an antibacterial agent. Vacuum liquid chromatography was used to analyze Fraction C on the G60 silica gel stationary phase with the mobile phase n-hexane-ethyl acetate methanol with a gradient of 10% (v/v). TLC analysis was done further to obtain five fractions (C1-C5), and they were tested for antibacterial activity showed that the C3 fraction gave potential antibacterial activity.

Column chromatography (KK) was done to separate further C3 fraction in stationary silica gel (70–230 mesh) with n-hexane mobile phase ethyl acetate with 10% (v/v) gradient to obtain four fractions of C31 to C34 which was tested for antibacterial activity. From the results of the antibacterial test, which had the potential as an antibacterial was the C32 Fraction. The C32 fraction was further separated by gradient column chromatography (KK) using an n-hexane acetone mobile phase to obtain four fractions namely C321 to C324. One of the fractions, C321, which was an amorphous solid then further recrystallized with a mixture of benzene: methanol (8: 2), so that a white crystal (9 mg) compound 1 was obtained.

Determination of compound structure

Isolated compounds were determined by physical properties including color and melting point. The chemical structure of compounds was determined based on spectroscopic data including (UV), IR, core NMR, and compared with spectra data obtained from the literature [13], [14], [15], [16].

Antibacterial assessment of the prepared extracts

E. faecalis antibacterial test

The extract and isolated compounds were diluted in DMSO 1% and carried out by the dilution method to antibacterial test against *E. faecalis* using 96-wells (Iwaki-Japan) [17]. We used the parameters turbidity that occurred due to the growth of bacteria at certain concentrations caused by the antibacterial activity of the extracts and isolates. The results was read by ELISA microplate reader at a wavelength of 630 nm.

Results

Minimum inhibitory concentration (MIC) of various extracts against E. faecalis

To determine the antibacterial activity of various extracts against *E. faecalis* bacteria was carried out by the liquid dilution method [18]. Antibacterial activity of various extracts against *E. faecalis* bacteria was expressed by MIC value, referring to the criteria for the level of antibacterial extracts of natural ingredients in testing for antibacterial properties [19] as listed in Table 1. For the antibacterial activity of the four extracts of *A. excelsa* leaves, they exhibited different antibacterial capacity. Ethyl acetate extract of *A. excelsa* leaves showed good antibacterial activity in this study at the concentration of 12.25 μg/mL followed by methanol extract, *n*-hexane extract, and water at the concentration of 69.23 μg/mL, 97.12 μg/mL, and 98.03 μg/mL, respectively. *E. faecalis* was shown to be less sensitive to methanol extract, *n*-hexane extract, and water extract besides ethyl acetate extract exhibited stronger antibacterial activity than these three extracts. This result resumed that the inhibitory activity of the four extracts against *E. faecalis* was in the order of ethyl acetate> methanol>*n*-hexane>water.

Table 1: MIC value from various extract of *Altingia excelsa* leaves

Extract	MIC (μg/mL)
Methanol	69.23
n-hexane	97.12
Ethyl acetate	12.25
Water	98.03

MIC: Minimum inhibitory concentration.

The result of antibacterial test showed that ethyl acetate extract was the highest inhibitory effect, so that the ethyl acetate was continued to be isolated.

With the guide of antibacterial test for ethyl acetate was done by separation and purification until it obtained one compound.

Compound 1

White solids, t.l. 258–260°C, UV (MeOH) λmaks nm 222, 271 and 404; IR (KBr) vmaks 3422, 1649, 1408, 1020 cm-1; 1H-NMR (CD3OD, 500 MHz) δH (ppm) 7,15 (1H, s, H-2, H-6); 13C-NMR (CD3OD, 500 MHz) δC (ppm) 122,2 (C-1), 110,7 (C-2, C-6), 145,9 (C-3, C-5), 138,6 (C-4), dan 167,7 (C-7). TOF MS ES+ m/z [M+H]+ 168,6684 calculation for $C_7H_6O_5$, m/z 170,1195.

Chemical compound structure

Compound 1 was obtained as a white solid with a melting point of 258–260°C. The UV spectrum of compound 1 (Figure 1) shows the presence of two absorption bands at λmax 271 and 222 nm. The absorption at λmax 271 nm indicates a π→π* transition which was thought to be derived from the B band of the benzene group. Meanwhile, the absorption at λmax 222 nm was thought to originate from the carbonyl group in the presence of the n→π* transition (R band) [13], [14].

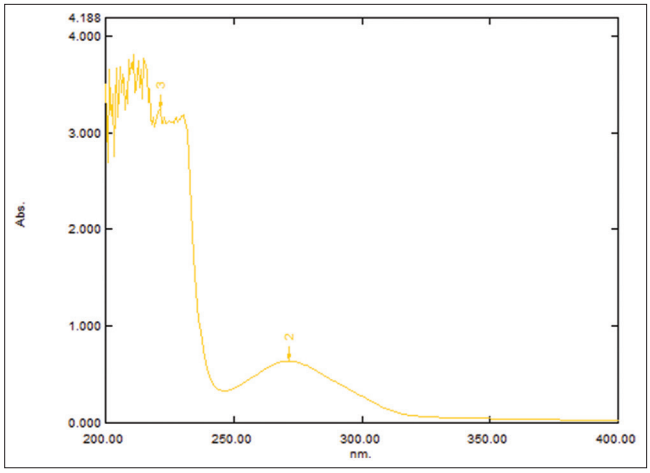

Figure 1: UV spectra compound 1 (methanol)

The IR spectrum of compound 1 (Figure 2) showed the presence of an-OH group as evidenced by the appearance of the O-H strain observed at vmax 3422 cm^{-1} and the C-O strain at vmax 1021 cm^{-1}.

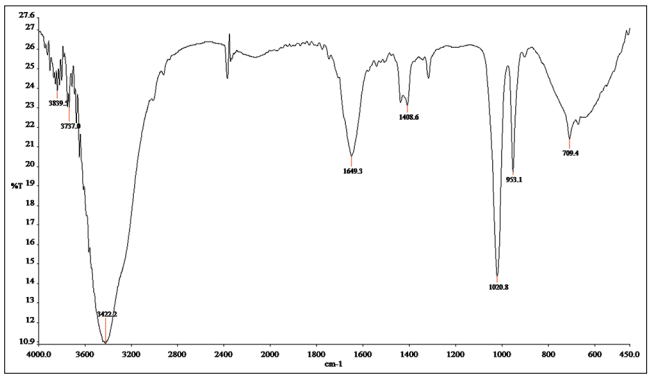

Figure 2: IR spectra compound 1 (KBr)

The carbonyl group which added to the degree of unsaturation in this compound is indicated by strong absorption at v_{maks} 1649 cm^{-1}. The vibration for this carbonyl group appeared at lower frequency due to conjugation between the carbonyl group and the benzene group. The existence of the C=C

double bond was shown by the absorption at vmax of 1409 cm^{-1} [13], [20].

The 13C NMR spectrum (Figure 3) showed the presence of seven carbon signals consisting of one carbonyl carbon resonating at δC167.6 ppm and a CH sp^2 signal resonating at δC145.9–110.1 ppm. In compound 1, there are three oxygenated quaternary carbons, namely, δC145.9 ppm (C-3 and C-5) and δC138.6 ppm (C-4). Therefore, compound 1 showed a benzene framework with four substituents. Two carbon signals each contain two carbons (^{13}CNMR data), thus compound 1 has a symmetrical structure [15], [16].

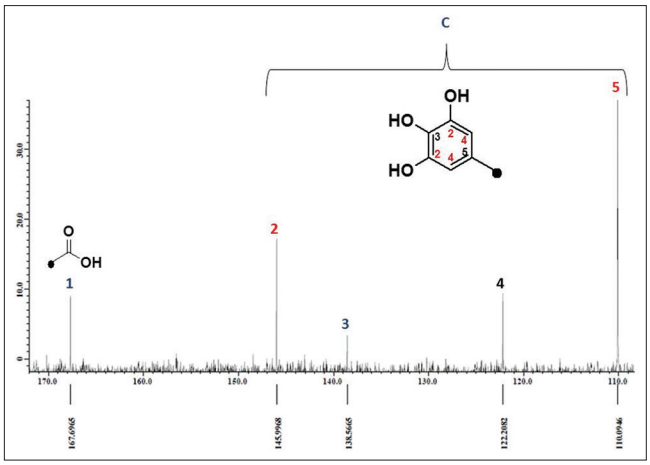

Figure 3: ^{13}C NMR Spectra compound 1 (aseton-d_6, 125 MHz)

The ^{1}H NMR spectrum of compound 1 (Figure 4) showed the presence of a singlet proton signal resonating at δH 7.14 ppm. Therefore, the singlet proton signal represented two equivalent protons thereby amplifying the benzene framework with four substituents [15], [16], [20].

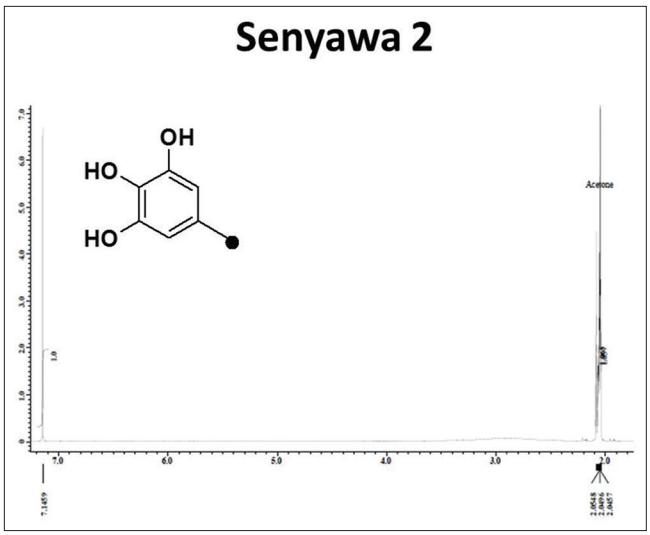

Figure 4: ^{1}H NMR Spectra compound 1 (aseton-d_6, 500 MHz)

The molecular formula for compound 1 was designated as $C_7H_6O_5$ based on the TOF-MS ES^- spectra (m/z 168.8390 $[M + H]^-$, calculations for $C_7H_6O_5$ m/z 170.1195) and NMR data so that five degrees of unsaturation were obtained. Comparison of the NMR data of compound 1 with 3,4,5-trihydroxy benzoic acid compounds (Table 2) showed that the two compounds were very high compatibility, so that definitively, compound 1 was identified as an acidic compound 3,4,5-trihydroxy benzoate (Figure 5) [15], [16], [20].

Table 2: Comparison compound 1 with gallic acid

C position	Compound 1		3,4,5-trihidroksi benzoat acid	
	δ_C (ppm)	δ_H (ppm), ∑ H, mult, *J* (Hz)	δC (ppm)	δ_H (ppm), ∑ H, mult, *J* (Hz)
1	122.2	-	120.6	-
2	110.7	7.15 (1H; *s*)	108.9	7.07 (1H; *s*)
3	145.9	-	144.9	-
4	138.6	-	138.2	-
5	145.9	-	144.9	-
6	110.7	-	108.9	-
CO_2H	167.7	-	169.0	-

Discussion

E. faecalis has been known to be the most frequently isolated bacteria in root canal system which is associated in condition of previously or failure endodontically treated teeth followed by secondary apical lesion rather than primary infection. It possesses some characteristics as anerobic facultative, Gram-positive bacteria, which enable this bacteria to live in low oxygen, high pH, varies temperature from 10° to 60°, and low nutrient so that inducing resistance mechanism to cause opportunistic infections. Therefore, recently treatment is used to decrease or disinfect it from colonizing biofilm in root canal system and periradicular area using biochemomechanical instrumentation combines with intracanal medicament and promising antibacterial agent from various sources [21].

To determine the antibacterial activity of compound 1 against *E. faecalis* bacteria was carried out by the liquid dilution method [18]. The antibacterial activity of compound 1 against *E. faecalis* was shown by the MIC value. Referring to the criteria for the level of antibacterial activity of natural compounds in testing for antibacterial properties [22], our result showed MIC of 12.25 μg/mL.

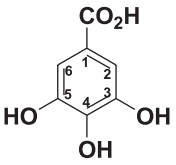

Figure 5: Chemical structure of 3,4,5 trihydroxy benzoate (gallic acid)

Gallic acid is a member of flavones (phenolic acid) and belongs to the subclass of flavonoids [23]. Gallic acid as derivative of cinnamic acid is formed through the pathway of shikimic acid with 3-dehydrosikimic acid as a base ingredient. Gallic acid has been tested as antibacterial agent, against *Staphylococcus aureus*, and *Helicobacter pylori* [22]. It showed antimicrobial activities associated with various pathways within cytoplasmatic membrane through destabilization, permeabilization, and inhibitory enzyme by oxidized products which perhaps through reaction with sulfhydryl groups or more

nonspecific interactions with proteins and inhibition the synthesis of nucleic acids for both Gram-negative and Gram-positive bacteria [22], [23], [24]. Previously, the study reported that Gram-positive bacteria exhibited more resistant than Gram-negative bacteria due to addition number of hydroxyl groups followed by substitution of hydroxyl groups to methoxy groups resulting activity enhancement of gallic acid. Those studies revealed that antimicrobial efficacy of gallic acid was associated with the length of hydrophobic chain [23]. Gram-positive bacteria seemed to response with higher concentration of MIC and MBC than Gram-negative bacteria. On the contrary result by Pinho *et al*., this study obtained higher MIC in *Klebsiella pneumoniae* (9,75 μg/mL) than *Staphylococcus epidermidis* (9,8 μg/mL) and *S. aureus* (19,5 μg/mL) [22]. We like to confirm this phenomenon, thus we suggest to use other Gram-negative bacteria or different strains to determine antibacterial efficacy of gallic acid.

Gallic acid showed alteration mechanism to bacterial hydrophobicity facilitated by its physicochemical surface properties. Alteration of bacterial cells was induced by gallic acid resulting adjustment the polar, nonpolar, and electron acceptor (c+) of their components. It gave rise to differential ability for both increased electron acceptor as in Gram-positive and decreased electron acceptor as in Gram-negative bacteria. It was also electrophilic and significantly depend on the bacterial surface components [24], [25]. Although it is due to characteristics of hydroxycinnamic acid through their propenoid side chain, it has antibacterial and less polar than the corresponding hydroxybenzoic acids [25]. However, other study reported gallic acid seemed to cause alteration more significantly to bacterial physicochemical than ferulic acid (hydroxycinnamic acid) [23]. Thus, cell membrane now easily could be penetrated enabled through its transport [25]. Facilitated by passive diffusion, gallic acid as phenolic acids could destroy the cell membranes, enter within cytoplasm, create intracellular acidification, and lead to protein denaturation. This low pH compromised the bacteria to release ATP production thorugh inhibition of H^+-ATPase enzyme [22].

The leaves of *A. excelsa* had been identified as antibacterial and antiproliferating agent which is reported in our previously studies [26], [27]. It was reported that the leaves of *A. excelsa* owned kaempferol, quercetin, 3,4 dihydroxybenzoic acid, gallic acid, and apigenin [27], [28]. These secondary metabolites inhibited cell proliferation against Sp-C1 human tongue cancer lines through apoptotic activity. Apigenin-contained *A. excelsa* leaves also had been reported its antibacterial activity to *E. faecalis* [26]. However, there was still a few studies which revealed its secondary metabolites associated with antibacterial potency. Even though our results showed MIC of gallic acid was 12.25 μg/mL which did not induce any toxicity, we suggest to study its role in cytotoxicity determination. This concentration was still included within range between 10 and 100 μg/mL without any toxicity in fibroblast cell which exhibited more than 70% of viability of cells [22]. Further study is needed to confirm this safety concentration within human cell which may suggest the mechanism involved on the antibacterial efficacy.

Conclusion

The highest inhibitory effect was ethyl acetate extract and then continued to be isolated. The chemical structure of compound 1 was identified as an acidic compound 3,4,5-trihydroxy benzoate, namely, as the gallic acid which had inhibitory effect against *E. faecalis.*

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