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

Risandy Anwar*, Puspita Hajardhini²

1Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Muhammadiyah Semarang, Semarang, Indonesia; ²Department of Oral Biology, Faculty of Dentistry, Universitas Muhammadiyah Semarang, Semarang, Indonesia

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 *Altingia 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 an 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* [9].

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, Cresophage, 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*.
The prospecting drugs from natural ingredients contain chemical compounds and pharmaceutically 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].

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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 using these medicinal herbs. One of the herbs is Altingia excelsa normha 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].

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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 of 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 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

<table>
<thead>
<tr>
<th>Extract</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>69.23</td>
</tr>
<tr>
<td>n-hexane</td>
<td>97.12</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>12.25</td>
</tr>
<tr>
<td>Water</td>
<td>98.03</td>
</tr>
</tbody>
</table>

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) λ maks nm 222, 271 and 404; IR (KBr) v maks 3422, 1649, 1408, 1020 cm⁻¹; 1H-NMR (CD3OD, 500 MHz) δ H (ppm) 7.15 (1H, s, H-2, H-6); 13C-NMR (CD3OD, 500 MHz) δ C (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 C₇H₆O₅, 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 λ max 271 and 222 nm. The absorption at λ max 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 λ max 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 v max 3422 cm⁻¹ and the C-O strain at v max 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 max 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 \(\delta C 167.6\) ppm and a CH sp\(^2\) signal resonating at \(\delta C 145.9\)–110.1 ppm. In compound 1, there are three oxygenated quaternary carbons, namely, \(\delta C 145.9\) ppm (C-3 and C-5) and \(\delta C 138.6\) ppm (C-4). Therefore, compound 1 showed a benzene framework with four substituents. Two carbon signals each contain two carbons (13CNMR data), thus compound 1 has a symmetrical structure [15], [16].

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

The molecular formula for compound 1 was designated as C\(_7\)H\(_6\)O\(_5\) based on the TOF-MS ES\(^{-}\)spectra (m/z 168.8390 [M + H]\(^{+}\), calculations for C\(_7\)H\(_6\)O\(_5\) 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>
<thead>
<tr>
<th>C position</th>
<th>Compound 1</th>
<th>3,4,5-trihydroxy benzoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>122.2</td>
<td>120.6</td>
</tr>
<tr>
<td>C-2</td>
<td>110.7</td>
<td>108.9</td>
</tr>
<tr>
<td>C-3</td>
<td>145.9</td>
<td>144.9</td>
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<tr>
<td>C-4</td>
<td>138.6</td>
<td>138.2</td>
</tr>
<tr>
<td>C-5</td>
<td>145.9</td>
<td>144.9</td>
</tr>
<tr>
<td>C-6</td>
<td>110.7</td>
<td>108.9</td>
</tr>
<tr>
<td>CO(_2)H</td>
<td>167.7</td>
<td>169.0</td>
</tr>
</tbody>
</table>

Table 2: Comparison compound 1 with gallic acid

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 anaerobic 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 \(\mu\)g/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 through destabilization, permeabilization, and inhibitory enzyme by oxidized products which perhaps through reaction with sulfhydryl groups or more
nonspecific interactions with proteins and inhibition of 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 respond 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 study its role in cytotoxicity gallic acid was 12.25 µg/mL which did not induce any toxicity, we suggest to study its role in cytotoxicity 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.

References

PMid:28770188

PMid:15015736

PMid:31448251


PMid:35048015

PMid:23349569

PMid:24932119


