Antibacterial and radical scavenger activities of extract and compounds of Wualae (*Etlingera elatior*) stems from Southeast Sulawesi

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Abstract. Two known compounds, namely stigmast-4-en-6β-ol-3-one (1) and p-hydroxybenzoic acid (2) are firstly reported from stems of *E. elatior*. Isolation were carried out by chromatography technique including Thin Layer Chromatography (TLC), vacuum liquid chromatography (VLC) and radial chromatography (RC) with silica gel as adsorbent and mixture of solvents as eluent. Structure of isolated compounds were determined by spectroscopy methods i.e. FTIR, ¹H and ¹³C NMR and also by comparison the spectroscopies data with the same data from references. Biological activities of isolated compounds evaluated against some bacteria consist of *Bacillus subtilis* FNCC 0060, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *Staphylococcus aureus* ATCC 25923, dan *Streptococcus mutans* ATCC 25175 and also against DPPH (2,2-diphenil 1-pichylhydazyl) radical as antiradical scavenger activity. The result showed that generally, antibacterial potency those samples i.e. methanol extract, stigmast-4-en-6β-ol-3-one and p-hydroxybenzoic acid less active than positive control (chloramphenicol). Individually, antibacterial activity of p-hydroxybenzoic acid > methanol extract > stigmast-4-en-6β-ol-3-one. In addition, p-hydroxybenzoic acid is the most active against *S. mutans*. Radical scavenger activity of tested samples is less active than than positive control (ascorbic acid). Furthermore, the radical scavenger potency of p-hydroxybenzoic acid > methanol extract > stigmast-4-en-6β-ol-3-one.

Keywords: *Etlingera elatior*, stems, compounds, antibacterial, and radical scavengers

1. Introduction

*Etlingera* is a genus of Zingiberaceae which comprises 150-200 species in the world, and 48 species grow in Sulawesi [1]. Two of the 48 species have been reported both phytochemical and pharmacological aspects that are *E. elatior* and *E. callophrys*. The pharmacological aspect of *E. elatior* included the leaves as body odor removers, wound medicines, antioxidants, antibacterial and tyrosinase inhibitors [2,3]. *E. elatior* flowers are also active against bacteria, fungi and antioxidants [4-6], and their rhizomes as anti-bacterial and antioxidant [3,7]. Furthermore, methanol extracts of *E. callophrys* stems is active towards some bacteria and radical scavenger [8].
Phytochemical aspect of *Etlingera* of Southeast Sulawesi has been reported that are 3-O-caffeoylquinic acid, 5-O-cafeoylquinic acid (chlorogenic acid), and 5-O-cafeoylquinic acid methyl ester from leaf of *E. elatior* [4]. In addition, leaves of *E. elatior* also produced kaempferol-3-glucuronide, quercetin-3-glucuronide, quercetin-3-glucoside, and quercetin-3-rhamnoside [2]. Some volatile compounds have been identified form *E. elatior* that are β-pinene (52.6%), α-thujene (28.6%) dan α-cymene (7.8%) dari *E. brevilobrum* [9], and borneol (28.3%), L-calamene (18.0%), α-bisabolol (5.9%) from *E. elatior* [10]. Three secondary metabolites firstly reported from steams of *E. calophrys* i.e. yakuchinone A, p-hydroxybenzoic acid and stigmasterol [8].

The above information showed that there are not paper about compounds and biological activities of stems of *E. elatior*. So, this paper will report phytochemical and pharmacological aspects of the plant tissue.

2. Material and Methods

2.1. Materials

Spectroscopy instruments include Ultraviolet (UV) spectra were obtained by using Cary Varian 100 conc., Infrared (IR) spectra were acquired from Perkin-Elmer Spectrum One FT-IR Spectrophotometer. Spectrum 1H NMR, 13C NMR and NMR 2D (2-Dimension) were attained by JEOL ECP 500 spectrometer, operated at 500 MHz (1H) and 125 MHz (13C). The chemicals include methanol, ethylacetate, n-hexane, chloroform, aquades, acetone, thin layer chromatography plate: Kieselgel 60 F254, 0.25 mm (Merck), silica gel 60 GF254 p.a (Merck®), silica 60 G (Merck®), cerium sulphate (CeSO4) (Merck®), *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* and *Streptococcus mutans*, DPPH (2,2-diphenyl-1-picrylhydrazyl), *Nutrient Agar* (NA) (Merck®), and *Nutrient Broth* (NB) (Merck®). Stems of *Etlingera elatior* was collected at Wolasi Forest, South Konawe, Sulawesi Tenggara in October 2017. The specimen was identified and stored in Herbarium Bogoriense, Indonesia.

2.2. Extraction and Isolation

The powder (230-270 mesh) of stems of *E. elatior* (5.0 kg) was macerated by methanol (MeOH) 3 x 10.0 L for 3 x 24 h. The methanolic extract was concentrated by vacuum rotary evaporator at low/reduced pressure, giving a dark green gum (100 g). The extract was fractionated by VLC using a column of Φ 10 cm, adsorbent: Silica gel (150 g) and a mixture of ethylacetate:n-hexane (ethylacetate 20-100%, MeOH 100%) as eluent, to give 5 fractions, i.e. F1 (1.0 g), F2 (4.0 g), F3 (7.3 g), F4 (10.5 g) and F5 (47.3 g), respectively. F2 was refractionated using VLC with a column of Φ 5 cm, adsorbent: silica gel (50 g) and a mixture of ethylacetate:n-hexane (ethylacetate 30-100%, MeOH 100%) as eluent, providing 5 fractions, i.e. F21 (0.1 g), F22 (0.4 g), F23 (0.5 g), F24 (0.8 g), and F25 (1.2 g). F24 (0.8 g) was purified by RC, adsorbent: silica gel and eluent: chloroform: MeOH (95%: 5%, MeOH 100%), to give compound 1 (0.1 g) with white needle crystal. F3 was refractionated by conducting VLC with a column of Φ 5 cm, adsorbent: silica gel (50 g) and a mixture of ethylacetate:n-hexane (ethylacetate 40-100%, MeOH 100%) as eluent, to yield 4 fractions, i.e. F31 (0.1 g), F32 (0.6 g), F33 (0.8 g), and F34 (1.2 g). F33 (0.8 g) was purified by RC, adsorbent: silica gel and eluent: a mixture of n-hexane:ethylacetate (80% : 20%, MeOH 100%), to give compound 2 (0.05 g). Fractionation and purification of F34 (1.2 g) were carried out by using RC with a mixture of chloroform: MeOH (90%: 10%, MeOH 100%) as eluent, giving compound 3 (0.05 g).

2.3. Structure Determination

Structure of isolated compounds were determined by spectroscopy methods including FTIR, 1H and 13C NMR and also by comparison the spectroscopies data with the same data from references.
2.4. Antibacterial Activity

Antibacterial activity of the methanol extract and isolates from the rhizome was assayed using well agar diffusion method as described in Wahyuni [11]. The bacteria used were *Bacillus subtilis* FNCC 0060, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *Staphylococcus aureus* ATCC 25923, and *Streptococcus mutans* ATCC 25175. The bacterial cultures with OD_{625} 0.5 McFarland were grown in semisolid medium containing nutrient agar and nutrient broth. The samples (100 μg/ml) were pipetted into the well and incubated at 37°C for 24 h. Chloramphenicol and DMSO (10%) were used as positive and negative controls, respectively. The inhibition zones around the well were measured.

2.5. Radical Scavenger Activity

The potency of isolated compounds as radical scavengers was evaluated against DPPH (2,2-diphenyl-1-picrylhydrazil) radical. The inhibition of DPPH radical was analyzed qualitatively and quantitatively. The qualitative analysis was conducted using TLC autographic spray. The procedures of the TLC autographic assay were as follow. After developing and drying, TLC plates (amount of samples ranging 0.1 –100 μg) were sprayed with 0.2 % (2 mg/mL) of DPPH solution in methanol. The plates were examined for 30 minutes after being sprayed. Active compounds appeared as yellow spots with a purple background. Bios method was adapted for quantitative analysis. One mL of DPPH (500 μM, 0.2 mg/mL) in methanol was mixed with the same volumes of the tested compounds at various concentrations. The mixture was kept in the dark for 30 minutes. The absorbance of each mixture was measured at 517 nm by using UV spectrophotometer after which the absorbance of the blank was measured. The concentration of sample at which the absorbance at 517 nm decreased to a half of its initial value was used as the IC_{50} value of compounds. IC_{50} values are expressed as μg/mL. The analysis was done in triplicate for standard and compounds [8].

3. Result and Discussion

Stigmaster-4-en-6β-ol-3-one (1), \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \textsuperscript{δ}H (ppm): 5.69 (1H, s, H-4), 4.30 (1H, d, \textit{J}=8.6 Hz, H-6), 0.79 (3H, s, H-18), 1.41 (3H, s, H-19), 0.98 (3H, d, \textit{J}=6.5 Hz, H-21), 0.87 (3H, d, \textit{J}=6.9 Hz, H-26), 0.85 (3H, d, \textit{J}=6.9 Hz, H-27), 0.88 (3H, d, \textit{J}=7.3 Hz, H-29). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \textsuperscript{δ}C (ppm): 37.2 (C-1), 33.9 (C-2), 200.7 (C-3), 125.4 (C-4), 168.6 (C-5), 72.3 (C-6), 39.6 (C-7), 29.7 (C-8), 53.9 (C-9), 37.9 (C-10), 20.8 (C-11), 39.7 (C-12), 42.9 (C-13), 55.9 (C-14), 24.0 (C-15), 28.1 (C-16), 56.1 (C-17), 11.5 (C-18), 18.8 (C-19), 36.0 (C-20), 18.3 (C-21), 33.8 (C-22), 25.9 (C-23), 45.8 (C-24), 29.1 (C-25), 19.2 (C-26), 18.5 (C-27), 22.9 (C-28), and 11.4 (C-29).

\textit{p}-Hydroxybenzoic acid (2); white amorphous powder. Spectra of \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \textsuperscript{δ}H (ppm): 9.43 (1H, s), 7.91 (2H, d, \textit{J}=8.6 Hz, H-2/H-6), 6.92 (2H, d, \textit{J}=8.4 Hz, H-3/H-5). Spectra of \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \textsuperscript{δ}C (ppm): 166.7 (C-7), 161.8 (C-4), 131.8 (C-2/C-6), 121.8 (C-1), and 115.1 (C-3/C-5).

Extraction, separation, and purification of isolates of Wualaei (*E. elatior*) stems using chromatography technique and structure determination of the isolates by spectroscopy methods produced two compounds. Based on comparison of NMR data (\textsuperscript{1}H and \textsuperscript{13}C NMR) between samples and references, can be concluded that compound 1 is stigmaster-4-en-6β-ol-3-one [12], and compound 2 is indentic with \textit{p}-hydroxybenzoic acid [13]. The structures of compounds are displayed at Figure 1.

Antibacterial potency of methanol extract, stigmaster-4-en-6β-ol-3-one, \textit{p}-hidroksibenzoicacid and positive control (chloramphenicol) towards some tested bacteria including *Bacillus subtilis* FNCC 0060, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, *Staphylococcus aureus* ATCC 25923, and *Streptococcus mutans* ATCC 25175 displayed in Table 1. Generally, data in Table 1 showed that the methanol extract of *E. elatior* stems and its isolated compounds were less active against the tested bacteria when compared to the positive control of chloramphenicol. However, rather than the isolated compounds, the methanol extract showed higher activity against the tested bacteria.
This result indicates that the presence of antibacterial active compounds in methanol extract which, at that moment, have not been successfully isolated yet. The compound 1, stigmast-4-en-6β-ol-3-one, showed no activity, whereas the compound 2, p-hydroxybenzoic acid, is active to all tested bacteria, and the most active against S. mutans. One of the factors that might affect the ability of a compound to inhibit the bacterial growth is the structure of the compound. Stigmast-4-en-6β-ol-3-one is not active as antibacterial probably due to its structure has no resemblance to the structure of chloramphenicol. In contrast, of p-hydroxybenzoic acid showed antibacterial activity since the structure of p-hydroxybenzoic acid is more similar to chloramphenicol [14]. The similar structure of p-hydroxybenzoic acid and chloramphenicol probably lies in the presence of the aromatic ring. The structure of aromatic ring is able to interact with the peptidyl transferase, an important enzyme in the bacterial chromosome, and therefore can inhibit the work of this enzyme [15].

Table 1. Inhibition Zone of methanol extract and compounds from E.elatior stems against tested bacteria.

| Bacteria       | Inhibition Zone (mm). [sample]=100 µg/mL |
|----------------|------------------------------------------|
|                | Methanol extract of E. elatior stems     | stigmast-4-en-6β-ol-3-one | p-hydroxybenzoic acid | Chloramphenicol |
| B. subtilis    | 0.60±0.20                                | 0.00±0.00                 | 4.80±0.80              | 15.30±0.58     |
| E. coli        | 1.20±0.40                                | 0.00±0.00                 | 2.80±0.20              | 10.60±0.34     |
| P. aeruginosa  | 1.60±0.40                                | 0.00±0.00                 | 2.60±0.20              | 9.71±0.38      |
| S. enterica    | 1.60±0.60                                | 0.00±0.00                 | 3.80±0.60              | 12.70±0.22     |
| S. aureus      | 1.00±0.20                                | 0.00±0.00                 | 2.20±0.40              | 6.25±0.78      |
| S. mutans      | 1.80±0.60                                | 0.00±0.00                 | 6.20±0.60              | 15.60±0.66     |

In evaluating of radical scavenger potency such as displayed in Table 2, qualitative experiments showed that the methanol extracts, p-hydroxybenzoic acid and ascorbic acid bleached the purple DPPH color to pale yellow when the TLC plate on which they were sprayed by 0.2% of DPPH in methanol. However, stigmast-4-en-6β-ol-3-one can’t bleach the purple DPPH color. It is indicated that the methanol extract and p-hydroxybenzoic acid have potency as radical scavengers. In the quantitative DPPH radical scavenger assay, the methanol extracts and p-hydroxybenzoic acid were able to neutralize the DPPH free radicals but less active than positive control (ascorbic acid). The ability to reduce the effect of radicals can be associated with the number of aromatic units in the compounds [16]. Structure of p-hydroxybenzoic acid similar to ascorbic acid, has aromatic unit. So p-hydroxybenzoic acid is more active than stigmast-4-en-6β-ol-3-one as radical scavenger.
Table 2. Activity of methanol extracts and isolated compounds against DPPH.

| Sample (s)                        | Methanol extract of E. elatior stems | stigmast-4-en-6β-ol-3-one | p-hidroksibenzoic acid | Ascorbic Acid |
|-----------------------------------|--------------------------------------|--------------------------|------------------------|--------------|
| IC₅₀ (mg/mL)                      | 206.6                                | 219.95                   | 109.0                  | 25.18        |

4. Conclusion

Two known compounds, namely stigmast-4-en-6β-ol-3-one (1) and p-hydroxybenzoic acid (2) are firstly reported from stems of *E. elatior*. Generally, antibacterial potency those samples i.e. methanol extract, stigmast-4-en-6β-ol-3-one and p-hydroxybenzoic acid less active than positive control (chloramphenicol). Individually, antibacterial activity of p-hydroxybenzoic acid > methanol extract > stigmast-4-en-6β-ol-3-one. In addition, p-hydroxybenzoic acid is the most active against *S. mutans*. Radical scavenger activity of tested samples is less active than positive control (ascorbic acid). Furthermore, the radical scavenger potency of p-hydroxybenzoic acid > methanol extract > stigmast-4-en-6β-ol-3-one.

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