Antioxidant compound from *Myristica fatua* Houtt Leaves

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**Abstract.** Antioxidant plays an important role in reducing the risk of chronic diseases. By using DPPH radical scavenging assay-guided fractionation, a bioactive compound has been isolated from the methanol extract of the leaves of *Myristica fatua* Houtt and subsequently identified as methyl 3,4-dihydroxybenzoic (MDB). The structure of MDB was confirmed by UV, FT-IR, MS, 1- and 2-D NMR spectroscopy. This compound exhibit antioxidant activity together with previous compounds *i.e.* (7S,8R,8′S,7′S)-7,7′-bis(3-hydroxy-5-methoxyphenyl)-8,8′-dimethylbuthane-7,7′-diol and 3′-hydroxy demethyldactyloidin with IC$_{50}$ values 7.96, 101.1 and 15.4 µg/mL, respectively. These findings demonstrate that MDB can be considered as a natural antioxidant.

1. Introduction

Antioxidant have been widely reported to plays an important role in the prevention of aging and degenerative diseases such as cancer, atherosclerosis, and Alzheimer’s, which are closely related with the production of reactive oxygen and nitrogen species [1]. Free radicals and other reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide are an entire class of highly reactive molecules derived from the normal metabolism of oxygen or from exogenous factors and agents [2]. Plants are believed to play a vital role in the prevention or progression of the degenerative diseases, which rich sources of natural antioxidants [3]. Therefore, there is a great deal of interest in natural antioxidants in view of their health implications such as *Myristica fatua* Houtt leaves from Indonesian medicinal plants.

*M. fatua* Houtt is a plant species originating from India, Borneo, Sulawesi, Maluku, Philippines, New Guinea, Solomon Islands, New Hybrid, Fiji, Samoa, Vanuatu and New Caledonia [4]. *M. fatua* extracts possessed several pharmacological activities, such as antioxidant, antibacterial, anti-parasitic and anti-proliferative against A549, DLD-1, DU145 and MCF-7 cancer cells [5-8]. The secondary metabolites of *M. fatua* mainly are lignan and polyketide such as malabaricon A, malabaricon C, licarin A, (7S,8R,8′S,7′S)-7,7′-bis(3-hydroxy-5-methoxyphenyl)-8,8′-dimethylbuthane-7,7′-diol and 3′-hydroxy demethyldactyloidin [8-9]. Essential oils (α-chopaene and caryophillene) are also found in *M. fatua* [10]. Our preliminary study from the methanol extract of *M. fatua* leaves showed a potency as an antioxidant (DPPH) with IC$_{50}$ 26.5 µg/mL. Thus, the present study was undertaken to isolate and identify methyl 3,4-dihydroxybenzoic (MDB) from *M. fatua* leaves, their antioxidant activity was also examined using DPPH method including (7S,8R,8′S,7′S)-7,7′-bis(3-hydroxy-5-methoxyphenyl)-8,8′-dimethylbuthane-7,7′-diol and 3′-hydroxy demethyldactyloidin from previous research [9].
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2. Research methods

2.1. Plant material
M. fatua leaves was collected from Mekongga forest, Southeast Sulawesi, Indonesia. The plant was identified at Research Center for Biology, Indonesian Institute of Sciences (LIPI) with voucher specimen UHA-55.

2.2. General experiments
The 1H, 13C, HMOC, and HMBC NMR spectra were measured JEOL JNM-ECA 500 MHz spectrometer using chloroform-d (CDCl3). Tetramethylsilane (TMS) was used as an internal standard. The molecular weight was obtained, using LC-MS Mariner Biospectrometry with ESI (Electrospray Ionisation) system. FTIR and UV/Vis spectra were recorded using Shimadzu Type IR Prestige-21 and Agilent Technologies (Cary 60 UV-Vis), respectively.

2.3. Extraction and Isolation
The dried powder of the leaves (1.37 kg) was extracted three times with methanol by maceration at room temperature for 3 X 10 L. The mixture was filtered and the filtrate was concentrated on a rotary evaporator under reduced pressure at 45 °C to afford 99.6 g of extract. A successive partition of methanol extract (45.6 g) was used with Vacuum Liquid Chromatography yielding n-hexane (66.1 mg), ethyl acetate (EtOAc) (35.7 g) and residue (2 g) fractions. Subsequently, these fractions were subjected to an assay of antioxidant activity.

The bioassay-guided fractionation was only carried out with ethyl acetate fraction because it was the most active antioxidant. The EtOAc fraction was subjected to silica gel chromatography (200-300 mesh, Merck). Elution was performed with a gradient elution of n-hexane, ethyl acetate and methanol to obtain 12 primary fractions (Fr. 1- Fr. 12). All the fractions were assayed for antioxidant activity by DPPH method (Table. 1). The most active fraction Fr. 5 (3.0 g) eluted with gradient elution (n-hexane-ethyl acetate-methanol) to obtain 10 sub-fractions (Fr. 5-1 until Fr. 5-10). Sub-fractions Fr. 5-3 and Fr. 5-4 further purified using centrifugal chromatography and Sephadex LH-20 to obtained (7S,8R,8'S,7'S)-7,7'-bis(3-hydroxy-5-methoxyphenyl)-8,8'-dimethylbuthane-7,7'-dial and 3''-hydroxy demethylactyloidin [9], but their antioxidant activity have not been done. Then, Fr. 5-5 (16.53 mg) was further purified by Sephadex LH-20 (Sigma) eluted with dichloromethane-methanol (1:1) to obtain compound 3 (4 mg). Compound 3 was further identified using spectroscopy methods and examined the antioxidant activity of compound 1-3.

2.4. DPPH free radical scavenger activity assay [11]
The antioxidant assay was performed on the scavenging effect of DPPH. Briefly, 4 mg of sample was dissolved in 4 mL DMSO to obtain 1000 μg/mL as sample mother solution. The sample solution was diluted with ethanol to obtain various sample concentrations (10, 20, 50, 100, and 200 μg/mL). The test sample was mixed with the ethanol solution of 300 μM DPPH (Sigma) in 90-well microtiter plate and incubated at 37°C for 30 minutes. The absorption was measured with a spectrophotometer at λ 515 nm. Inhibition percentage of the sample was compared to control (DMSO). Gallic acid used as positive controls. Percentage of inhibition was calculated by the equation: [1-(B/A)] x 100%; whereas A is absorbance in the absence of sample (blank) and B is absorbance in the presence of sample. IC50 value denotes the concentration of sample required to scavenge 50% of the DPPH free radical.

3. Results and Discussion
A rapid, simple and inexpensive method to evaluate antioxidant activity involves the use of the free radical, 2,2-diphenyl-1-pirclylhydrazyl (DPPH). DPPH radical scavenging activity assay is commonly used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity [2]. It has also been used to quantify antioxidants in complex biological systems in recent years. The methanol extract of M. fatua leaves was screened for antioxidant activity with IC50
26.5 µg/mL. This extract was subjected to activity-guided repeated fractionation on Kieselgel 60G with n-hexane, ethyl acetate, methanol as mobile phase using Vacuum Liquid Chromatography technique. The ethyl acetate fraction exhibited the highest radical scavenging activity with IC$_{50}$ 58.73 µg/mL (Table 1). A further bio-guided fractionation of the ethyl acetate fraction on silica gel led to twelve fractions with fraction 5 (F-5) has the highest antioxidant activity (IC$_{50}$ 16.5 µg/mL, Table 1). Further purification using column chromatography technique afforded ten sub-fractions. Sub-fractions Fr. 5-3 and Fr. 5-4 had been purified on the previous report and obtained (7S, 8R, 8'S, 7'S) 7,7'-bis(3-hydroxy-5-methoxyphenyl)-8,8'-dimethylbuthane-7,7'-dil (I) and 3''-hydroxy demethyldactyloidin (2) [9], but their antioxidant activity have not been reported. Sub-fraction Fr. 5-3 further purified on Sephadex LH-20 and obtained compound 3. This compound identified using spectroscopy methods and evaluated its antioxidant activity.

**Table 1. Antioxidant activity of fractions of *M. fatua* leaves**

| No | Sample        | IC$_{50}$ (µg/mL) | No | Sample        | IC$_{50}$ (µg/mL) |
|----|---------------|-------------------|----|---------------|-------------------|
| 1  | n-hexane fraction | 80.88             | 9  | F-6           | 20.43             |
| 2  | Ethyl acetate fraction | 58.73             | 10 | F-7           | 19.24             |
| 3  | Residue       | >100              | 11 | F-8           | 35.87             |
| 4  | F-1           | >100              | 12 | F-9           | 77.79             |
| 5  | F-2           | >100              | 13 | F-10          | >100              |
| 6  | F-3           | 67.62             | 14 | F-11          | >100              |
| 7  | F-4           | 48.46             | 15 | F-12          |                   |
| 8  | F-5           | 16.05             |    |               |                   |

The compound 3 (4 mg) isolated from the subfraction Fr. 5-5 from ethyl acetate fraction of *M. fatua* leaves with a yellow light oil. Based on UV-Vis spectrophotometer measurement, compound 3 showed maximum wavelength (λ) at 205 and 224 nm similar to aromatic/benzene ring peaks [12]. In FTIR spectrum, compound 3 showed the absorbance peak at wave number 3352 cm$^{-1}$ as vibration of the -OH group supported by bending vibration at wave number 1300 cm$^{-1}$. Vibration of aliphatic group showed at 2939 and 2860 cm$^{-1}$. The wave number at v 1444 cm$^{-1}$ is the bending vibration of the aromatic C-H, while the C-O-C bond absorbs at 1228 and 1039 cm$^{-1}$. In addition, the sharp peak at v 1685 cm$^{-1}$ is the vibration of the carbonyl ester group. From LC-MS chromatogram, compound 3 showed peak (100%) [M + H]$^+$ = 169.33 with m / z 168.33 at retention time 5.3 min.

The $^1$H-NMR spectrum (500 MHz in CD$_3$OD) of compound 3 showed the presence of an ABX system aromatic ring at chemical shift $\delta$H 7.40 (1H, d, J = 1.53 Hz, H-6), 7.42 (1H, d, J = 1.95 Hz, H-2) and 6.80 ppm (1H, d, J = 7.79 Hz, H-5). The value of the J coupling constant of 1.95 and 7.79 Hz at $\delta$H 7.40 ppm (H-6) showed the meta position against the proton at a chemical shift of 7.42 ppm (1H, H-2, d, J = 1.53 Hz) and the ortho position against the proton at a chemical shift of 6.80 (1H, H-5, d, J = 7.79 Hz). Furthermore, a methoxy group also appeared at $\delta$H 3.3 ppm (3H, s, H-8).

$^{13}$C-NMR spectra of compound 3 (125 MHz in CD$_3$OD) showed eight carbons consist of a methoxy group at chemical shift $\delta$C 52.3 (C-8); three methine aromatics at $\delta$C 115.9 (C-2); 117.5 (C-5); 123.7 (C-6); and four quaternary carbons at $\delta$C 168.9 (C-7); 122.7 (C-1); 146.3 (C-3); 151.8 (C-4). A quaternary carbon at $\delta$C 168.9 ppm indicated the presence of a carbonyl ester [13].

Based on $^1$H-NMR, $^{13}$C-NMR and LCMS data, it could be assumed that compound 3 has a C6-C1 aromatic skeleton. This was supported by HMQC and HMBC correlation data. In the HMQC spectrum, compound 3 indicates the presence of a methoxy in a chemical shift of $\delta$H 3.3 ppm bound to carbon in C-8; and 3 aromatic protons ($\delta$H 7.42, 6.8 and 7.40 ppm) bounded to C-2, C-5 and C-6, respectively. From the HMBC spectrum, we can see the correlation of H-2 with C-1, C-3, and C-4; H-5 correlated with C-3 and C-4; and H-6 correlated with C-4, and C-5. A methoxy at $\delta$H 3.3 ppm correlated with a carbonyl at C-7 indicated a methyl ester group (Fig. 1).
Based on spectroscopic data of UV/Vis, FTIR, LCMS, FT-NMR, it can be concluded that compound 3 is a benzoate derivative, methyl 3,4-dihydroxybenzoate. This compound is first report from *M. fatua* leaves.

**Table 2.** NMR data of compound 3 and methyl 3,4-dihydroxybenzoic.

| No | ¹H-NMR (CD₃OD) (δH ppm, multiplicity, J (Hz)) | ¹³C-NMR (δC ppm) | Methyl 3,4-dihydroxybenzoic (Miyazawa *et al.*, 2003) (aseton δH ppm, multiplicity, J (Hz)) | (δC ppm) |
|----|---------------------------------------------|------------------|-------------------------------------------------------------------------------------------------|----------|
| 1  | -                                           | 122.7            | 7.39 (d, 2)                                                                                        | 117      |
| 2  | 7.42 (d, 2.60)                              | 115.9            | 6.80 (d, 8.3)                                                                                     | 115      |
| 3  | -                                           | 146.3            | 7.34 (dd, 2.0; 8.3 Hz)                                                                            | 123      |
| 4  | -                                           | 151.8            | 6.80 (d, 7.39)                                                                                     | 150      |
| 5  | 6.80 (d, 7.79)                              | 117.5            | 7.34 (d, 2.0; 8.3 Hz)                                                                              | 166      |
| 6  | 7.42 (d, 2.60)                              | 123.7            | 7.34 (dd, 2.0; 8.3 Hz)                                                                            | 166      |
| 7  | -                                           | 168.9            | 3.80 (s)                                                                                          | 51       |

**Figure 1.** HMBC correlation of compound 3.

The antioxidant activities of compounds 1-3 showed in Table 3. Compound 3 has highest antioxidant activity with IC₅₀ 7.94 µg/mL, but lower than gallic acid as positive control.

**Table 3.** Antioxidant activity of compounds 1-3.

| No | Sample                                      | IC₅₀ (µg/mL) |
|----|---------------------------------------------|--------------|
| 1  | methyl 3,4-dihydroxybenzoate (1)            | 7.96         |
| 2  | (7S, 8R, 8'S, 7'S) 7,7'-bis(3-hydroxy-5-methoxyphenyl)-8,8'-dimethylbuthane-7,7'-dioil (2) | 101.07       |
| 3  | 3''-hydroxy demethyldactylodin (3)          | 15.42        |
| 4  | Gallic acid (positive control)              | 2.30         |
Gallic acid was known as a strong radical scavenger compared to a range of phenolic acids. The number of electron donor hydroxy and methoxy substitutions will increase the stability of the phenoxy radicals so that it affected the radical-scavenging activity of phenolic acids. Therefore, gallic acid with three hydroxyl groups and a carboxyl group was observed to be more active than methyl 3,4-dihydroxybenzoate with two hydroxyl groups and a carboxylic methyl ester group. The methoxy group of methyl 3,4-dihydroxybenzoate may hinder the scavenging effect of the hydroxyl groups by intra- or intermolecular hydrogen bonding [14].

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