Conference Paper

Two Antibacterial Compounds: Velutin and 4-(Hydroxy(Oxiran-2-yl)Methyl)-2-Methoxyphenol from the Stem Bark of *Drimys arfakensis* Gibbs.

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Abstract

Antimicrobial-guided fractionation and isolation of the bioactive compounds from the stem bark of *Drimys arfakensis* Gibbs. were carried out. Two antibacterial compounds were isolated. The sructures of the antibacterial compounds were elucidated by spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, 2-D NMR, ESI-MS and EI-MS. Based on the spectroscopic data, the two antibacterial compounds were velutin, **1** and 4-(hydroxyl(oxiran-2-yl)methyl)-2-methoxyphenol, **2**. Compound **1** exhibited low activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (B-1823) with MIC value of 250 $\mu g \cdot mL^{-1}$, while compound **2** possesses strong activity against *S. aureus* (MRSA) (B-1823) with MIC value of 15.60 $\mu g \cdot mL^{-1}$.No activity of both compounds against *Escherichia coli* (B-1634) and *Candida albicans* (B-2219) was observed.

Keywords: 4-(hydroxyl(oxiran-2-yl)methyl)-2-methoxyphenol; *C. albicans; D. arfakensis*; *S. aureus* (MRSA); velutin

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1. Introduction

Drimys is a plant genus that is part of the Winteraceae family. It consists of about 14 species of evergreen flowering shrubs. *Drimys* plants feature lance-shaped leaves, fragrant flowers, and black fruits. Plants of this genus are commonly used as border shrubs. Most of the species are adaptable to different soil types, and they are generally free of pests and diseases. These plants are found in primary and secondary tropical forests, and usually grow in high altitudes [1]. The species of this genus found in Papua island of Indonesia are *Drymis arfakensis* Gibbs., *Drymis. piperita* Hook, and *Drymis. beccariana* Gibbs. [2]. *D. arfakensis*, called "akway" in Indonesia, is an upright evergreen shrub with very hot peppery leaves and barks, and attractive white flowers in terminal

head and grows in full sun up to the height of around 4 m to 5 m. The stem barks of *D. arfakensis* are used traditionally as stimulant by the indigenous people of Manokwari, West Papua, Indonesia. The tea prepared from the stem bark of *D. arfakensis* has been used by several tribes in West Papua Province to treat stomachache, diarrhea, dysentery and malaria [3]. Previous studies reported that methanol extract from the stem bark of *D. arfakensis* exhibit a wide variety of potent and interesting biological activities. Methanol extract from the stem bark of *D. arfakensis* had antimicrobial activities againts *S. aureus, E.coli* and *C. albicans* [4, 5]. Resistance to antimicrobial is recognized at present as a major global health problem. Infective diseases account for appropriately one-half of all deaths in tropical regions. These diseases are caused by microorganisms which are critically important to develop new antimicrobial compounds before entering into the post-antibiotic era. The candidates of new antimicrobial compounds might be isolated from medicinal plants; therefore investigation on the antimicrobial compounds from medicinal plants such as the stem bark of *D. arfakensis* needs to be conducted since there is no report on the isolation of bioactive compounds from this species.

2. Material and method

2.1. General

EI-MS and ESI-MS were obtained on a GCMS-QP2010S SHIMADZU and BRUKER Daltonic spectrometer, respectively. ^{1}H NMR, ^{13}C NMR, 2-D NMR spectra were determined on an AGILENT spectrophotometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). Vacuum liquid chromatography (VLC) and radial chromatography utilized silica gel 60 GF_{254} Merck. Analytical TLC used silica gel GF_{254} aluminum sheet. All reagents used were analytical grade.

2.2. Plant material

D. arfakensis was collected from Arfak mountains, Anggi District, Manokwari, West Papua Province, Indonesia. The *voucher specimen* was lodged earlier at the Herbarium Manokwariense, the State University of Papua, with identification code BW 297. The sample was air- dried, oven- dried at 40 °C and ground before extraction.

2.3. Extraction and isolation

Finely ground bark (1 500 g) was initially extracted with 5 000 mL of hexane for 48 h. The marc was allowed to dry and the process of extraction was repeated with DCM, acetone and finally methanol. The extracts were concentrated *in vacuo* giving 55.0

g, 42.5 g, 48.6 g, and 30.0 g of hexane, DCM, acetone and methanol black-syrupy residues, respectively. All four extracts collected from sequential fractionation were subjected to antimicrobial bioassay on *Staphylococcus. aureus, Eschericia. coli* and *Candida. albicans* using cylinder cup and minimum inhibitory concentration (MIC) assays. Based on both assays, the most active extract against *S. aureus* was the acetone extract. Therefore, this extract was subjected to fractionation and isolation to get the pure active isolates. The isolation process was guided by bioautography assay using *S. aureus*.

2.4. Acetone extract

VLC was done to fractionate the acetone extract on a 10 cm × 8.5 cm (diameter × height) column using increasing polarity hexane : ethyl acetate and finally ethyl acetate solvent systems. Twelve fractions labeled AF-1 to AF-12 were collected. TLC plate developed with 7:3 (v/v) hexane:EtOAc was subjected to bioautography assay against *S. aureus* which showed that fraction number 7 (AF-7) and 8 (AF-8) were active against *S. aureus*, exhibited good separation and high yield

2.5. AF-7 fraction

Active fraction AF-7 was subjected to VLC on a 6 cm × 16 cm (diameter × height) column using increasing polarity hexane:ethyl acetate solvent systems. Fourteen fractions were collected. Thin layer chromatography was performed on these fractions using solvent systems 6:4 hexane:EtOAc and chloroform. The TLC developed with solvent system 6:4 hexane:EtOAc was tested for activity by bioautography against *S. aureus*. Fraction number 13 (AF-7-13) was active against *S. aureus* and high yield. Therefore, this fraction was chosen for purification. Radial chromatography was performed to fractionate fraction AF-7-13 using solvent systems:chloroform, 9.5:0.5 chloroform:EtOAc, 9:1 chloroform:EtOAc giving 13 fractions. These fractions were developed on TLC using solvent system 8:2 chloroform:EtOAc. Fractions number 1 and 2 had only one spot with the same Rf, suggesting purity. Isolates number 1 and 2 were combined and labeled compound **1.** To prove the purity of compound **1**, it was developed on TLC using several solvent systems and showed only one spot. It melted at 210 °C to 214 °C, with melting point range of 4 °C. Based on these data, compound **1** was a pure isolate.

2.6. AF-8 Fraction

VLC of fraction AF-8 was perfomed on a 6 cm × 16 cm (diameter × height) column using increasing polarity hexane:EtOAc solvent systems. Fourteen fractions were collected. Then, all fractions were subjected to thin layer chromatography using developing solvent 6:4 hexane:EtOAc. The developed TLC plate was tested against *S. aureus* through bioautography assay which showed that fraction fourteen (AF-8-14) was active. Therefore, this fraction was then fractionated. Fraction FA-8-14 was subjected to radial chromatography using solvent systems:chloroform, 9:1 chloroform:EtOAc, and 8:2 chloroform:EtOAc, yielding compound **2**.

Compound **1** melted at 210^{o}C to 214 °C. It was a yellow amorphous powder and it exhibited moderate degree of inhibitory activity to *S. aureus*; no activity to *E. coli* and *C. albicans* was observed. ESI-MS m/z: 315 $(M+1)^+$, 300, 297, 282, 269, 167, 151, and 149.^{1}H NMR (500 MHz, $CHCl_3$-d1) δ_H ppm: 6.736 (1H, s, H-3), 6.324(1H, d, 2.0 Hz, H-6), 6.697(1H, d, 2.5 Hz, H-8), 7.648 (1H, d, 2.0 Hz, H-2′), 7.012(1H, d, 8.5 Hz, H-5′), 7.626 (1H, dd, 1.6, 8.0 Hz, H-6′), 12.984 (1H, s, 5-OH), 3.560 (3H, s, 7-OCH_3) and 3.54(3H, s, 3′-OCH_3), ^{13}C NMR (125 MHz, $CHCl_3$-d1). δ_C ppm: 165.2(C-2), 104(C-3), 183.2(C-4), 105.9(C-4a), 163.0(C-5), 98.6(C-6), 166.5(C-7), 93.3(C-8), 158.6(C-8a), 123.4(C-1′), 110.5(C-2′), 148.9(C-3′), 151.5(C-4′), 116(C-6′), 56.4 (7-OCH_3) and 56.6 (3′-OCH_3).

Compound **2** melted at 110^{o}C to 114 °C. It was a yellow amorphous solid and it exhibited strong inhibitory activity to *S. aureus*, no activity to *E. coli* and *C. albicans* was observed. EI-MS m/z : 196 (M^+), 182, 178, 164, 153, 138, 135, 120, 91, 77, 43., ^{1}H NMR (500 MHz in $CDCl_3$) δ_H in ppm: 6.894 (1H, br d, H-3), 6.818 (1H, dd, 1.45, 8.15 Hz, H-5), 6.885 (1H, d, 8.15 Hz, H-6), 4.736 (1H, d, 4.0 Hz, H-7), 3.100 (1H, br q, 4.5, 6.5 Hz, H-8), 4.244 (1H, dd, 6.5, 9.5 Hz, H-9), 3.876 (1H, dd, 3.5, 9.5 Hz, H-9) 5.654 (1H, s, 5-OH), 3.480 (1H, s, 7-OH) and 3.901 (3H, s, 2-OCH_3) and ^{13}C NMR (125 MHz in $CDCl_3$) δ_C ppm: 145.2 (C-1), 146.7(C-2), 108.6 (C-3), 132.9 (C-4), 119.0 (C-5), 114.3(C-6), 85.9 (C-7), 54.2 (C-8), and 56.0 (C-9)

2.7. Antimicrobial assay

2.7.1. Test organisms

Microbial cultures used in the determination of MIC, bioautography and cylinder cup assays were obtained from the Philippine National Collection of Microorganims (PNCM) of BIOTECH, University of the Philippines Los Baños. The microbial cultures used in this project were: Methicillin-resistant *Staphylococcus aureus* (MRSA) (B-1823), *E. coli* (B-1634) and *C. albicans* (B-2219).

2.7.2. Cylinder cup assay

The assay procedure was adapted from Gatsin et al. [6]. A 24-h old *S. aureus* and *E. coli*, and 5 d old *C. albicans* were grown in Mueller Hinton Broth (MHB) and Potato Dextrose Agar (PDA) media, respectively. For each bacterial test organism, a loopful of culture was inoculated to a flask with MHB in 1 % agar. For the fungal test organism, isotonic saline containing 0.05 % Tween 80 was incorporated in the mycelial culture. The amount of 1 mL of mycelial culture in Tween 80 solution was then inoculated to a flask with PDA. MHB and PDA, 10 mL respectively were poured on a plate and overlaid by 5 mL seeded top agar. The sterilized assay cylinders were dropped on the plate from a height of 12 mm using a mechanical guide and the plates were covered to avoid contamination. After filling the cylinders on each plate with 0.1 mL 10 000 μg · mL^{-1} test extract or 0.1 mL 1 000 μg · mL^{-1} pure isolate and positive control solutions, the bacteria and fungi plates were incubated at 37 °C for 24 h and at 35 °C for 48 h, respectively. The cylinders were removed and measured to record the diameter of each zone of growth inhibition to the nearest 0.1 mm. Acetone as a solvent of the extracts and the pure isolates were used as negative control, and streptomycin and nystatin were used as positive control for bacteria and fungi, respectively. The diameter of the zone inhibition (ZOI) formed on triplicate plates were measured using a digital vernier caliper.

2.7.3. Minimum Inhibitory Concentration (MIC) against bacterial organisms

The assay procedure was adapted from Ellof [7]. With the use of serological pipette, 100 μL of Mueller Hinton Broth (MHB) were pipetted out to the sterile 96-well microtiter plate. Residues of the different extracts and the isolates were re-dissolved in acetone to a concentration of 1 mg · mL^{-1}. For each of the bacterium used, 100 μL aliquot of each plant extract and isolate tested in triplicate were two-fold serially diluted with 100 μL sterile distilled water in the sterile 96-well microtiter plates loaded with MHB. Then, 100 μL of bacterial inocula adjusted to 0.5 McFarland standard were introduced to each 96-well microtiter prepared above. Seven serial dilutions were prepared, ranging from 1 000 μg · mL^{-1} to 7.80 μg · mL^{-1}. A similar two-fold serial dilution of streptomycin (Sigma) of 1 mg · mL^{-1} was used as positive control against each bacterium, while acetone, as a solvent of the extracts and the isolates, was used as negative control, and distilled water was used as blank. The plates were covered and incubated overnight at 37 °C. Bacterial growth in the wells was indicated by the presence of turbidity, whereas clear wells indicated inhibition of the bacterial growth by the plant extracts or the pure isolates. The wells with the lowest concentration of the samples which did not exhibit

turbidity were reported as the MIC of the samples against the bacterial test organism. The tests were done in triplicate.

2.7.4. MIC against fungal test organism

The assay procedure was adapted from Fattouch et al. [8]. To determine the MIC against *C. albicans*, the macrobroth dilution assay was used. A 5 d old culture of *C. albicans* was suspended in isotonic saline containing 0.05 % Tween 80. The mycelial culture was then diluted with more saline until the desired concentration was reached (approximately 10^6spores/mL. Using a serological pipette, 100 μL of Sabourated Dextrose Broth (SDB) (Sigma) was pipetted out to the sterile 96-well microtiter plate. Residues of the different extracts and the isolates were re-dissolved in acetone to a concentration of 1 000 $\mu g \cdot mL^{-1}$. A 100 μL aliquot of each plant extract and isolate, tested in triplicate, were two-fold serially diluted with 100 μL sterile distilled water in the sterile 96-well microtiter plates loaded with SDB. Then, 100 μL of diluted fungal inoculum were introduced to each 96-well microtiter prepared above. Seven serial dilutions were prepared, ranging from 1 000 $\mu g \cdot mL^{-1}$ to 7.80 $\mu g \cdot mL^{-1}$. A similar two-fold serial dilution of nystatin (Sigma) of 1 000 $\mu g \cdot mL^{-1}$was used as positive control against fungi, while acetone as a solvent of the extracts and the isolates were used as negative control, and distilled water was used as blank. The plates were covered and incubated at 35 °C for 24 h. Fungal growth in the wells was indicated by the presence of turbidity, whereas clear wells indicated inhibition of the fungal growth by the plant extracts or the pure isolates. The wells with the lowest concentration of the samples which did not exhibit turbidity were reported as the MIC of the samples against the fungal test organism.

2.7.5. Bioautography assay

The assay procedure was adapted from Hamburger and Cordell [9]. Chromatograms of TLC plates prepared were left for 4 d under an air stream to allow the TLC solvent to evaporate before being overlayed with an actively growing culture of bacteria or fungi. The chromatograms were then incubated for 24 h at 37 °C for bacteria and 48 h at 35 °C for fungi under 100 % relative humidity to allow the microorganisms to grow on the plates. After incubation, the bioautograms were sprayed with an aqueous solution of 2 mg $\cdot mL^{-1}$ tetrazolium dye MTT Sigma M2128. The clear zones against dark purple background indicated inhibition of microbial growth by bioactive compounds in the samples.

3. Result and Discussion

Compound **1** was obtained as a yellow amorphous solid. The NMR spectra of **1** indicated the presence of two methoxy groups. Two overlapping peaks at δ 3.921 (s) and δ 3.916 (s) of the proton NMR spectrum were assigned to two methoxy protons. This was supported by the signals at δ 56.40 and δ 56.60 of the carbon-13 NMR which were assigned to the two methoxy carbons. The presence of a signal at δ 12.984 of the proton NMR of compound **1** obviously suggested the location of hydroxyl group at C-5. This characteristic signal was due to the hydrogen bonding between hydroxyl hydrogen of C-5 and carbonyl oxygen of C-4 thereby lowering the peak proton signal. The singlet signal at δ 6.736 was assigned to H-3. The doublets at δ 6.324 with a meta coupling constant of J = 2 Hz and at δ 6.697 with a meta coupling constant of J = 2.5 Hz were assigned to H-6 and H-8, respectively. The multiplicity and coupling constants of aromatic protons at the δ 7.600 to 700 region suggested a 3′, 4′-subtitution pattern in ring B. H-2′ appeared as a doublet at δ 7.648 with a meta coupling constant of J = 2.0 Hz, while H-5′ appeared as doublet at δ 7.012 with an ortho coupling constant of J = 8.0 Hz. H-6′ appeared as a doublet of a doublet at δ 7.627 since it coupled to both H-5′ (J = 8.0 Hz) and H-2′ (J = 1.6 Hz). The proton signal of 4-OH was not detected; it is expected because hydroxyl proton is an exchangeable proton [10].

Mass spectrum of compound **1** using electrospray ionization (ESI-MS/MS) showed ion peaks at m/z 315, 300, 282, 269, 167, 151, and 149. The ion peak at m/z 315 corresponded to the molecular ion ($M^+ + 1$) of protonated **1,** $C_{17}H_{14}O_6$+H^+. The molecular ion corresponds to the compound with a molecular formula of $C_{17}H_{14}O_6$. On the basis of the spectroscopic data presented above and comparison with the previous report [10], compound **1** was identified 3′, 7-dimethoxy-4′, 5-dihydroxyflavone or velutin (Figure 1).

Velutin was first isolated from *Ceanothus velutinus* (Dougl. ex Hook). [11]. It was also isolated from *Piper clarkii* (H.Luther). Previous studies reported that velutin isolated from acai (*Euterpe oleracea* Mart.) showed strong anti-inflammatory effect and antioxidant activity [10, 12]. No previous work reported the antimicrobial activity of velutin, but similar flavonoid compounds such as apigenin, kaemferol, quercetin, rhamnazin and 5-hydroxy-7,4′-dimethoxy flavone isolated from *Combretum erythrophyllum* (Burch.) Sond.had good activity against *Vibrio cholera* (Pacini) and *Enterococcus faecalis* (Andrewes and Horder) [13]. Velutin has never been reported to be isolated from *D. arfakensis* before. In this study, velutin showed moderate activity against *S. aureus* (MRSA) on cylinder cup assay with inhibition zone of 14.61 mm and possessed a MIC value of 250 $\mu g \cdot mL^{-1}$. It did not exhibit activity against *E. coli* and *C. albicans* (Table 1 and Table 2).

Velutin is a flavone with two phenolic groups. Even though the exact antimicrobial mechanism of phenolic compounds is not clear, phenolic compounds are commonly

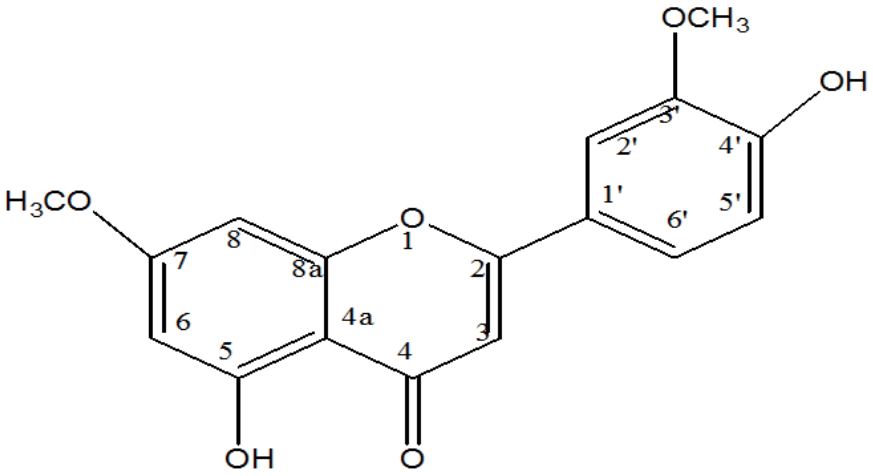

Figure 1: Chemical structure of compound **1**.

known for their antimicrobial effects, especially for gram positive bacteria [14, 15]. The melting point of velutin was not reported before. This study found that melting ;point was 210 °C to 214 °C.

Compound **2** was obtained as yellowish amorphous solid**.** The NMR spectra indicated that an aromatic group was part of compound **2** with several substitutions. This was shown by the multiplicity and coupling constants at δ 6.500 to.8.000 region of the ^{1}H NMR spectrum. It was revealed that the aromatic ring has a hydroxy proton shown by the singlet signal at δ 5.654 and substituted by a methoxy group indicated by the appearance of a singlet signal at δ 3.901 of the proton NMR spectrum. This was supported by the signal at δ 56.0 of the carbon-13 NMR spectrum which was assigned to the methoxy carbon atom.

The broad doublet at δ 6.894 was assigned to H-3, while H-6 appeared as a doublet at ð 6.885 with an ortho coupling constant of J = 8.15 Hz. H-5 appeared as a doublet of a doublet at δ 6.818 since it coupled to both H-6 (J = 8.15 Hz) and H-3 (J = 1.45 Hz). The singlet signal at δ 3.480 was assigned to hydroxy hydrogen located at C-7 observed by long range correlations to C-4 and C-8. The doublet signal at δ 4.736 with a coupling constant of J = 4.0 Hz was assigned to C-7, while H-8 appeared as a broad quartet signal at δ 3.100 with coupling constants of J = 4.5 Hz and J = 6.5 Hz.

Finally, both signals at δ 3.944 and δ 3.876 appearing as doublet of a doublet were assigned to H-9. Each signal has a geminal and a vicinal coupling constant. This indicated that each proton of H-9 was coupled to a geminal proton and to a vicinal proton. Signal at δ 3.944 has a geminal coupling constant of J = 9.5 Hz and *cis* vicinal coupling constant of J = 6.5 Hz, while signal at δ 3.876 has a geminal coupling constant of J = 9.5 Hz and *trans* vicinal coupling constant of J = 3.5 Hz.

The mass spectrum of compound **2** using Electron impact (EI) ionization, showed peaks at m/z 196, 182, 178, 164, 153, 138, 135, 120, 77, 43. The ion peak at m/z 196

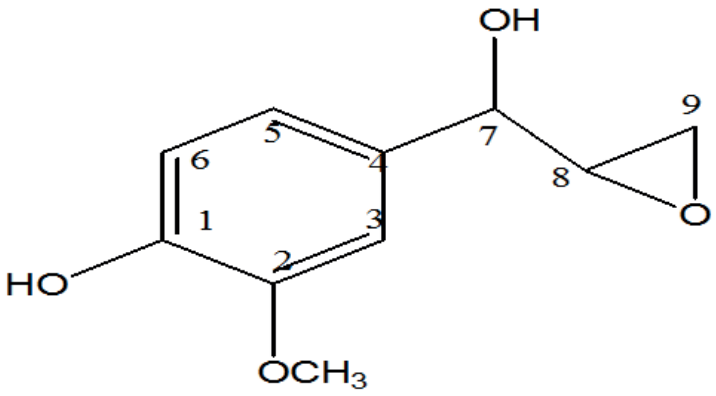

Figure 2: Proposed chemical structure of compound **2**.

Compound	Dose (μg)	Diameter of inhibition zones (mm)		
		S. aureus	*E. coli*	*C. albicans*
1	100	14.61 ± 1.32	0.00	0.00
2	100	29.10 ± 1.42	0.00	0.00
Streptomycin	100	18.73 ± 2.01	22.37 ± 0.21	NT
Nystatin	100	NT	NT	23.72 ± 1.30
NT = not tested				
Values are average of three replicates with standard deviation shown				

Table 1: Antimicrobial activity of compound 1 and 2 on cylinder cup assay.

corresponds to the molecular ion. The molecular ion corresponds to a compound with a molecular formula of $C_{10}H_{12}O_4$. Therefore, on the basis of the detailed analysis above, compound **2** is the most probably 4-(hydroxy (oxiran-2-yl) methyl)-2-methoxyphenol (Figure 2).

This is the first report on the isolation compound **2** from *D. arfakensis*. It possessed high activity against *S. aureus* with bigger zone of inhibition of 29.10 mm compared to streptomycin with zone of inhibition of 18.73 mm, had an MIC value of 15.60 μg · mL^{-1} against *S. aureus* (MRSA), and was not active against *E. coli* and *C. albicans*. The explanation of its activity is similar to compound 1, which might be phenolic group content [14, 15]. In addition, it possesses one oxirane ring (Figure 2), a functional group found in compounds with strong antimicrobial as well as an antitumor activities [16-18]. Moreover, a limonene epoxide found in many plants is a promising schistosomicidal agent [19]. Therefore, the strong antimicrobial activity of compound **2** is probably attributed to the phenolic, hydroxyl and oxirane groups.

Compound	Minimum inhibitory concentration (μg/ml)		
	S. aureus	*E. coli*	*C. albicans*
1	250.00	> 1 000	> 1 000
2	15.60	> 1 000	> 1 000
Streptomycin	7.80	7.80	NT
Nystatin	NT	NT	7.80
NT = not tested			

Table 2: Minimum inhibitory concentration of compound 1 and 2.

4. Conclusion

Two antimicrobial compounds were isolated from the stem bark of *D. arfakensis* and their structures were elucidated by spectroscopic analysis. This was the first time these two compounds were isolated from *D. arfakensis* and probably the first time were tested against *S. aureus* strain MRSA. Detailed analysis on spectral data of compound **1** was found to be 3",7-dimethoxy-4',5-dihydroxyflavone or velutin and compound **2** was found to be 4-(hydroxyl(oxiran-2-yl)methyl)-2-methoxyphenol. Compound **1** exhibited low activity against methicillin-resistant *S. aureus* (MRSA) (B-1823) with MIC value of 250 $\mu g \cdot mL^{-1}$, while compound **2** possessed strong activity against *S. aureus* (MRSA) (B-1823) with MIC value of 15.60 $\mu g \cdot mL^{-1}$. No activity of both compounds against *E. coli* (B-1634) and *C. albicans* (B-2219) was observed.

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