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Anticancer and Antivirus Activities of two Biflavonoids from Indonesian *Araucaria hunsteinii* K Schum Leaves

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Abstract: *Araucaria* genera consist of 19 species, and three of them are grown in Botanical Garden, Bogor, Indonesia. These plants were reported to contain biflavonoids and are primarily found in leaves. Biflavonoids display an extensive range of biological properties such as anti-inflammatory, anti-oxidant, anti-tumor, antivirus, anti-microbial, anti-fungal, etc. However, no studies reported secondary metabolites, especially biflavonoids, from Indonesian *A. hunsteinii* leaves. Therefore, this research aims to isolate biflavonoid from *A. Hunsteinii* leaves and evaluate their anticancer and antivirus activities. *First, A. hunsteinii* leaves were macerated in acetone to give brownish-black crude extract (14.66%, w/w). Then, the natural extract was fractionated and purified using chromatographic techniques with silica gel and *Sephadex* LH-20 as a stationary phase to afford two isolated compounds. The acetone extract and two isolated compounds were examined for their cytotoxic activity against breast cancer MCF-7 cells and human immunodeficiency virus (HIV) SRV-2 viruses based on an assay of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). According to spectroscopic data, including IR, UV-Vis, LC-MS/MS, and NMR (^{1}H, ^{13}C, NOESY, HSQC, and HMBC), two compounds were successfully elucidated as 4',7,7"-tri-*O*-methylcupressuflavone (1) and 4"',7,7"-tri-*O*-methylagathisflavone (2). Both compounds were first isolated from *A. hunsteinii* leaves. The preliminary MTT assay of compounds 1 and 2 against MCF-7 cells showed IC_{50} of 91.74 and 314.44 μg/mL, respectively. They had a larger IC_{50} than an acetone extract of *A. hunsteinii* leaves (IC_{50} of 62.16 g/mL), indicating that all samples had lower activity than the positive control, epirubicin HCl (IC_{50} of 0.52 g/mL). Furthermore, both compounds were ineffective as antivirus agents against SRV-2 viruses.

Keywords: A549 cell, *Araucaria hunsteinii*, biflavonoids, MCF-7 cell, SRV-2 viruses.

印度尼西亚南洋杉 K 舒姆叶中两种双黄酮的抗癌和抗病毒活性

摘要：南洋杉属由 19 种组成，其中 3 种生长在印度尼西亚茂物植物园。据报道，这些植物含有双黄酮，主要存在于叶子中。双黄酮具有广泛的生物学特性，如抗炎、抗氧化、抗肿瘤、抗病毒、抗微生物、抗真菌等。然而，没有研究报道来自印度尼西亚南洋杉的次生代谢产物，尤其是双黄酮树叶。因此，本研究旨在从南洋杉叶子中分离出双黄酮类化合物并评估其抗癌和抗病毒活性。首先，将南洋杉叶子在丙酮中浸渍，得到棕黑色粗提物（14.66%，每重量重量）。然后，将天然提取物用硅胶和葡聚糖LH-20作为固定相的色谱技术进行分级和纯化，得到两种分离的化合物。基于 3-(4,5-二甲基噻唑-2-亚叉基)- 的测定，检测了丙酮提取物和两种分离的化合物对乳腺癌密歇根癌症基金会-7

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细胞和人类免疫缺陷病毒猴逆转录病毒血清型 2病毒的细胞毒活性。 2,5-二苯基溴化四唑。根据光谱数据，包括红外线的、紫外-可见光谱、液相色谱与串联质谱和核磁共振（^{1}H、^{13}C、核奥弗豪泽效应光谱、异核单量子相干和异核多重键相关），两种化合物被成功阐明为 4',7,7''-三邻甲基柏黄酮（1）和4''',7,7''-三-O-甲基阿加蒂斯黄酮（2）。这两种化合物都是首先从南洋杉叶子中分离出来的。化合物 1 和 2 对密歇根癌症基金会-7 细胞的初步 MTT 测定显示 IC_{50} 分别为 91.74 和 314.44微克/毫升。它们的 IC_{50} 大于南洋杉叶子的丙酮提取物（IC_{50} 为 62.16克/毫升），表明所有样品的活性均低于阳性对照盐酸表柔比星（IC_{50} 为 0.52克/毫升）。此外，这两种化合物作为抗猴逆转录病毒血清型 2病毒的抗病毒剂均无效。

关键词：A549 细胞、南洋杉、双类黄酮、粉刺基金会-7、猴逆转录病毒血清型 2病毒。

1. Introduction

Araucaria is one of the genera of the family Araucariaceae consists of 19 species. This genus contains anti-oxidant, antiproliferative, antiradical, antibacterial, anti-fungal, anti-insomnia, analgesic, anti-inflammatory, anti-tumor, anticancer, antidiabetic, and antivirus secondary metabolites such as phenols, flavonoids, biflavonoids, terpenoids, steroids, phenolic acids, and tannins [1]. Biflavonoids is one of the secondary metabolites found in *the Araucaria* Genus, which has excellent potential to be developed as an anticancer and antivirus activities [2,3]. Biflavonoids reported from 8 species, such as *A. angustifolia*, *A. araucana*, *A. bidwilli* Hook, *A. columnaris*, *A. cunninghamii* Mudie, *A. rulei* F. Muell, *A. cookii*, and *A. excelsa* [1,4]. Biflavonoids research in *Araucaria* has been widely carried out in India, especially in the leaves.

Biflavonoids are dimers of flavonoids such as flavones, flavonols, dihydroflavonols, flavanones, isoflavones, aurons, and auronols chalcones that generate C-C or C-O-C bonds by oxidative coupling [5]. One of the biflavonoids from the robustaflavone group, 7,5''-di-*O*-methyl-robusta flavone, has good potential as an anticancer MCF-7 breast thru the process of ferroptosis in the mitochondrial regulatory pathway [6]. Furthermore, robustaflavone and hinokiflavone have exhibited antivirus properties against the human immunodeficiency virus (HIV) by inhibiting HIV-1 reverse transcriptase [7]. Biflavonoids display many biological properties such as anti-inflammatory, anti-oxidant, anti-tumor, antivirus, anti-senescence, anti-microbial and anti-fungal effects, and therapeutic effects on the cardiovascular system and central nervous system, etc. [3,8,9]. *Araucaria* contains various types of biflavones, including amentoflavones, agathisflavones, cupresuflavones, robustaflavones, and hinokiflavones. Exploring the content of biflavonoids from this genus is fascinating because of the diversity of biflavonoids content and biological activity in this species.

Araucaria hunsteinii is one of the *Araucaria* species found in the Botanical Gardens, Bogor, West Java, Indonesia. This plant originally comes from the mountains of Papua New Guinea and can grow up to 40-90 m. However, research on *A. hunsteinii* (Araucariaceae), especially biflavonoids, and their biological activity had not been reported [10]. Furthermore, the content of secondary metabolites, particularly biflavonoids, and their biological activity in *A. hunsteinii* Indonesia has never been documented. As a result, a study on *A. hunsteinii* plants, particularly the leaves, is critical to learn more about secondary metabolites, particularly biflavonoid compounds, and their anticancer and antivirus properties.

2. Materials and Methods

2.1. Chemicals and Instrumentations

For isolation, the organic solvent used were acetone, methanol, *n*-hexane, ethyl acetate (EtOAc), dichloromethane (DCM), and chloroform. Silica gel 60 F_{254} was used for thin-layer chromatography (TLC) and visualized by ultraviolet (UV) light (254 and 366 nm). Fractionation was used with Sephadex LH-20 column chromatography (CC), and purification was used with silica gel 60 (0.063 – 0.2 mm) CC purchased from Merck. Two isolated fractions were characterized by Ultraviolet-Visible (UV-Vis) Spectrophotometer Thermo Scientific Genesys 10 in MeOH (Merck), Fourier Transform-InfraRed (FTIR) Bruker in KBr (Merck), NMR Agilent spectrometer operating at 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR) frequencies in acetone-d_6 and DMSO-d_6 solvent and Liquid Chromatography-Mass Spectrometry tandem Mass Spectrometry (LC-MS/MS) with the LC specification:

LC system of Ultra Performance Liquid Chromatography (UPLC), column C18 (1.8 µm 2.1 x 100 mm) HSS, a mobile phase of water + 5mM ammonium formic (A) and asetonitril + 0.05% formic acid (B), and flow rate of 0.2 mL/min (step gradient) running 23 min in acetone and DMSO solvent. The cytotoxic assay of anticancer used MCF-7 (ATCC TIB 22) cells, while the pre-antivirus assay used A549 (ATCC CCL 185) cells with an MTT reagent. The antivirus assay used A549/SRV-2 (PSSP IPB) by quantitative reverse transcriptase PCR (qRT-PCR). The assay has been done at the IPB Primate Research Center (PSSP-IPB).

2.2. Plant Material

Leaves samples of *A. hunsteinii* were collected and determined in the Botanical Garden, Bogor, West Java, Indonesia, in January and July 2020.

2.3. Extraction and Isolation of *A. hunsteinii* leaves

Extraction was carried out on 1.1 kg of dried leaves powder of *A. hunsteinii* with 5 L of acetone three times at room temperature. The acetone extract was carried out by liquid-liquid extraction using methanol and *n*-hexane for chlorophyll separation. The methanol fraction was partitioned with acetone for tannins separation. The acetone soluble fraction was fractionated by Sephadex LH-20 CC with methanol as eluent and obtained 31 (F1-F31) fractions [11]. Fractions F1-F14 are suspected of containing biflavonoids based on the results of TLC analysis with chloroform: methanol as eluent (19:1). TLC sprayed with $Ce(SO_4)_2$ solution, and yellow spots appeared, indicating biflavonoids compounds. The fraction F7 (107.4 mg) was purified by silica gel CC with DCM: methanol (100:1) as eluent, and eight fractions (F7.1 – F7.8) were obtained. Pure fraction F7.5 (17.7 mg) is a solid yellow powder soluble in acetone. The fraction F8 (160.4 mg) was purified by silica gel CC with DCM: methanol (65:1) as eluent, and five fractions (F8.1 – F8.5) were obtained. Pure fraction F8.2 (62.8 mg) is a solid yellow powder too but is difficult to dissolve in organic solutions. Fractions F7.5 and F8.2 were characterized by IR spectroscopy, UV-Vis spectrophotometer, LC-MS/MS, 1D NMR spectrometer (^{1}H-NMR, ^{13}C-NMR), and 2D NMR spectrometer (HSQC, HMBC, COSY, and NOESY) and known as were 4',7,7''-tri-*O*-methylcuppressuflavone (1) and 4'',7,7''-tri-*O*-methylagathisflavone (2).

2.3.1. 4',7,7''-tri-O-methylcuppressuflavone (1)

Fraction F7.5 was identified as yellow amorphous powder with following properties: UV-Vis (MeOH) λ_{max} (nm): 273 (benzoyl chromophore) dan 330 (sinamoyl chromophore); FTIR (KBr) ν (cm^{-1}): 3429 (-OH), 1651 (C=O), 1442 (C=C, aromatic), 1241–1205 and 1177–1124 (C-O); ^{1}H-NMR δ (ppm, Asetone-d_6): 3.82 (*s*, 3H, -OC<u>H</u>$_3$, C-4'), 3,87 (s, 6H, -OC<u>H</u>$_3$, C-7 and 7''), 6.68 (*s*, 3H, H-3'', H-6, and 6''), 6.73 (*s*, 1H, H-3), 6.85 (*d*, *J*=8.9 Hz, 2H, H-3''' and 5'''), 6.96 (*d*, *J*=8.9 Hz, 2H, H-3' and 5'), 7.53 (*d*, *J*=8.8 Hz, 2H, H-2''' and 6'''), 7.61 (*d*, *J*=8.9 Hz, 2H, H-2' and 6'), 9.56 (*s*, 1H, -O<u>H</u>, H-4'''), 13.33 (*s*, 1H, -O<u>H</u>, H-5''), and 13.37 (*s*, 1H, -O<u>H</u>, H-5); ^{13}C-NMR δ (ppm, Asetone-d_6): 183.6 (C-4), 183.5 (C-4''), 165.2 (C-7), 165.1 (C-2''), 164.8 (C-2 and C-9''), 164.5 (C-9), 164.4 (C-7''), 163.7 (C-5), 163.6 (C-4' and 5''), 162.1 (C-4'''), 128.9 (C-2''' and 6'''), 128.7 (C-2' and 6'), 124.0 (C-1'), 122.8 (C-1'''), 116.8 (C-3''' and 5'''), 115.4 (C-3' and 5'), 105.6 (C-10 and C-10''), 104.0 (C-3), 103.5 (C-3''), 100.4 (C-8''), 100.3 (C-8), 96.0 (C-6 and 6''), 56.9 (-O<u>C</u>H$_3$, C-7), 56.8 (-O<u>C</u>H$_3$, C-7''), and 55.9 (-O<u>C</u>H$_3$, C-4'). LC-MS/MS (Acetone): LC rt 11.66 min, ESI/MS m/z 581.1458 $[M+H]^+$ (base peak), 566.1211, 535.1028, 520.0800, 463.1026, 446.0995, 431.0760, 403.0789, 359.0554, 297.0764, 284.0673, 255.0651, 135.0445 and 121.0289.

2.3.2. 4''',7,7''-tri-O-methylagathisflavone (2)

Fraction F8.2 was identified as pale-yellow amorphous powder with following properties: UV-Vis (MeOH) λ_{max} (nm): 273 (benzoyl chromophore) dan 330 (sinamoyl chromophore); FTIR (KBr) ν (cm^{-1}): 3486 (-OH), and 1650 (C=O); ^{1}H-NMR δ (ppm, DMSO-d_6): 3.76 (*s*, 3H, -OC<u>H</u>$_3$, H-4'''), 3,82 (s, 6H, -OC<u>H</u>$_3$, H-7 and 7''), 6.66 (*s*, 1H, H-6''), 6.94 (*s*, 1H, H-3''), 6.96 (*s*, 1H, H-3), 6.97 (*d*, *J*=8.5 Hz, 2H, H-3' and 5'), 6.98 (*d*, *J*=9 Hz, 2H, 3''' and 5''') 7.06 (*s*, 1H, H-8), 7.63 (*d*, *J*=8.9 Hz, 2H, H-2''' and 6'''), 8.05 (*d*, *J*=8.5 Hz, 2H, 2' and 6'), 10.44 (*s*, 1H, -O<u>H</u>, 4'), 13.20 (*s*, 1H, -O<u>H</u>, H-5''), and 13.24 (*s*, 1H, -O<u>H</u>, H-5); ^{13}C-NMR δ (ppm, DMSO-d_6): 182.3 (C-4''), 182.1 (C-4), 164.1 (C-2), 163.4 (C-2'' and 4'), 163.3 (C-7), 163.2 (C-7''), 161.6 (C-5''), 161.4 (C-4'''), 158.6 (C-5), 157.3 (C-9), 153.8 (C-9''), 128.7 (C-2''' and 6'''), 127.8 (C-2' and 6'), 122.7 (C-1'), 121.1 (C-1'''), 116.0 (C-3''' and 5'''), 114.7 (C-3' and 5'), 104.6 (C-10), 104.2 (C-10''), 103.4 (C-3''), 103.3 (C-3 and 6), 99.9 (C-8''), 95.6 (C-6''), 90.9 (C-8), 56.5 (-O<u>C</u>H$_3$, C-7 and 7''), and 55.6 (-OCH$_3$, C-4'''). LC-MS/MS (Acetone): LC rt 11.51 min, ESI/MS m/z 581.1464 $[M+H]^+$ (base peak), 535.1021, 449.0865, 431.0759, 405.0963, 375.0502, 361.0695, 297.0758, 285.0400, and 135.0450.

2.4. In-vitro Anticancer and Antivirus Assay

The anticancer and antivirus assay was carried out on acetone extract of *A. hunsteinii* leaves F7.5 and F8.2 fractions, which two-fold dilutions from 500 ppm to 31.25 ppm. Cytotoxic assay were performed for anticancer and antivirus by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium boride (MTT) method

against breast cancer MCF-7 cells and A549 cells [12,13,14]. Cells were grown with a concentration of 5000 cells in 100 µL of growth medium (RPM 1640, FBS 5%, penicillin 100U/mL, Streptomycin 100ug/mL). The pure extract and cells to be tested were added after the cells reached 50% confluency (24 h). The MTT assay was carried out on day 3 by adding 10 µL of MTT (5 mg/mL) per well, then incubated for 4 hours at room temperature (37 ºC). The formazan crystals formed and dissolved in ethanol. Absorbance readings were carried out at a wavelength of 595 nm using a spectrophotometric plate reader. The IC_{50} value was obtained from the linear regression equation between the sample concentration (x-axis) and % inhibition (y-axis). The A549/SRV-2 (PSSP IPB) were used for antivirus assay against the soluble fraction of *A. hunsteinii* leaf acetone and fractions F7.5 and F8.2 by qRT-PCR [14].

3. Results and Discussion

This study succeeded in isolating two biflavonoids from leaves of Indonesian *A. hunsteinii*. There are 4',7,7"-tri-O-methylcuppressuflavone (1) and 4",7,7"-tri-O-methylagathisflavone (2). Both compounds were isolated for the first time from the Indonesian *A. hunsteinii*. However, both compounds have been isolated from other Araucaria plants. Compound 1 has been discovered in *Agathis atropurpurea, Agathis australis, Agathis ovata, Araucaria columnaris, Araucaria cunninghamii, Wollemia nobilis*, while 2 have been found in *A. columnaris* and *A. excelsa* [1,15,16]. Both compounds are the oxidative coupling of apigenin monomers, but apigenin as a monomer is not found in this plant. However, apigenin monomer has been found in the leaves of *A. angustifolia* [4]. The chemical structures of 1 and 2 can be seen in Fig. 1.

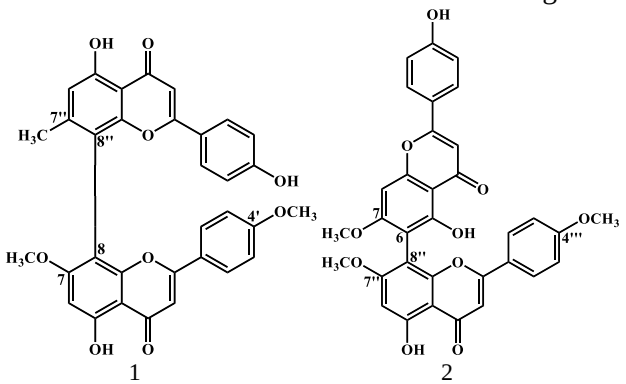


Fig. 1 Biflavonoids isolated from *A. hunsteiniii* leaves

3.1. Identification of Compounds 1 and 2

Compound 1 is a yellow amorphous powder and soluble in acetone, while compound 2 is a pale-yellow amorphous powder and is difficult to dissolve in organic solutions. UV-Vis studies revealed substantial absorption at 273 nm and 330 nm for both substances. The peak of 273 nm indicates the presence of a chromophore in the benzoyl group, and the height of 330 nm indicates the presence of a chromophore in the cinnamoyl group of flavonoid monomer. The FTIR 1 spectrum shows the presence of several OH groups at (ν) 3429 cm^{-1}, C=O at 1651 cm^{-1}, C=C aromatic at 1442 cm^{-1}, CO at 1241–1205 cm^{-1}, and 1177–1124 cm^{-1}; the FTIR 2 spectrum showed the presence of the -OH functional group at 3486 cm^{-1} and C=O at 1650 cm^{-1}.

The typical signals of biflavonoids in the chemical shift region (δH) of 13.00 ppm were found in both compounds, which indicated two hydroxyl groups chelated to the carbonyl groups. The ^{1}H-NMR spectra of 1 showed 11 signals, while 2 showed 13 signs of aromatic protons representing 24 protons. The ^{13}C-NMR spectra of both compounds displayed 29 signals representing 33 carbons with two typical signals of conjugated ketone carbonyl at the chemical shifts (δC) 182.1–183.6 ppm, ten oxyarylcarbon signals at δC 162.1–165.1 ppm in 1 and δC 153.8–164.1 ppm in 2. This signal indicates that the two compounds obtained are biflavonoids.

Based on heteronuclear multiple bond coherence (HMBC) and heteronuclear singular quantum coherence (HSQC), 1 has a dimer bond at C8 and C8" (Cupressuflavone), seen from the correlation between C8 (δ_C 100.3) with H6 (δ_H 6.68) and C8" (δ_C 100.4) with H6" (δ_H 6.68). Furthermore, C8 and C8" do not bind protons (quaternary carbon). HMBC and HSQC analysis of 2 showed the location of the coupling at C6 and C8" (Agathisflavone), seen from the correlation of C6 (δC 103.3 ppm) with H8 (δH 7.06 ppm) and OH-5 (δH 13.24 ppm) also C8" (δC 99.9 ppm) with H6" (δH 6.66 ppm). Correlation spectroscopy (COSY) spectra denote the correlation between H2' with H3' and H5' with H6'. That confirms B rings in both compounds are symmetrical, so a dimeric bond is not found in these rings.

The ^{1}H-NMR spectra showed two signals for 1 at δH 3.82 and 3.87 ppm and three signals for 2 in the δH 3.77–3.81 ppm region representing protons from three methoxy groups, while the ^{13}C-NMR spectra showed three carbons of methoxy signal. That is reinforced corroborated by the value of the carbon chemical shift at 55.9–56.9 ppm in 1 and 55.6–56.5 ppm in 2, which indicates the presence of three methoxy groups. HMBC analysis and Nuclear Overhanse Enhancement spectroscopy (NOESY) identified the position of methoxy substituents on both compounds, scilicet C4', C7, and C7" for 1 and C4'", C7 and C7" for 2. HMBC, HSQC, COSY, and NOESY correlations can be seen in Fig 2. ^{1}H-NMR data of 1 matched the report for 4',7,7"-tri-*O*-methylcupressuflavone (1), while 2 matched the report for 4'",7,7"-tri-*O*-methylagathisflavone (2) [15].

Based on LC-MS/MS analysis, both compounds are biflavonoids which can be seen from their molecular weight, 580 g/mol ($C_{33}H_{24}O_{10}$). The LC chromatogram of 1 showed a retention time of 11.66 min, while 2

showed a retention time of 11.51 min. According to the MS spectra, 1 has a molecular ion of 581.1458 m/z $[M+H]^+$, and 2 has 581.1464 m/z $[M+H]^+$ as its base peaks. The fragmentation of both compounds is proposed in this paper and is presented in Fig. 3. Compounds 1 and 2 are fragmented at m/z 581-535 peaks due to losing 2 methoxy groups at C7 and C7", while both carbons bond with oxygen as a bridge. Peaks at m/z 297 and 285 are fragments derived from the cleavage of the dimer bond. C ring structure cleavage in one of the monomers occurred in the fragments at the peaks of m/z 581-431, 581-403, and 446-359 of 1; 581-449, and 535-375 of 2. Carbonyl groups release occurs in the fragment's m/z 520 to 463 and 284 to 255 of 1, 449 to 405, and 431 to 361 of 2. In addition, hydroxyl groups (-OH) cleavage occurred in the peaks of m/z 520 to 463 of 1 and 449 to 431 of 2. Monomer fragments from 297 to 135 m/z undergo bond cleavage between the A and C rings. The methyl group of methoxy C4' of 1 cleavage at an m/z 535 to 520.

3.2. Cytotoxic Activity of Compound 1 and 2 as Anticancer MCF-7 Agent

The MTT test assessed the cytotoxic effects of acetone extract, 1 and 2 of *A. hunsteinii* leaves on MCF-7 cells. MCF-7 cells are breast cancer lines harboring the wild-type p53 gene that hasn't been altered, making them susceptible to chemotherapeutic treatments [17]. The IC_{50} values for the acetone extract, 1 and 2, were 62.16 g/mL, 91.74 g/mL, and 314.44 g/mL, respectively. Compound 2 is inactive, while acetone extract and 1 have considerable activity against MCF-7 breast cancer. These compounds, however, have lower activity than the positive control, epirubicin HCl (IC_{50} 0.52 µg/mL).

Compound 1 is a derivative of cupressuflavones, while 2 is a derivative of agathisflavones. Cupressuflavone has been reported to have activities such as lung cancer A549 and prostate cancer cells (PC3) with IC_{50} values of 65 and 19.9 µM, respectively [18]. This compound was isolated from *Juniperus phoenicea* in Libya. Agathisflavones are reported to be active against human cervical cancer (HeLa) cells (IC_{50} 10 ± 0.88 µM). In comparison, the derivative of 7''-*O*-methylagathisflavone has been reported to inhibit DNA topoisomerases II and exert cytotoxic effects (IC_{50} 24 ± 1.4 µM) in humans K562 leukemia cells [19].

3.3. Antiretrovirus Activity of Compounds 1 and 2 Against SRV-2

The cytotoxic activities of acetone extract, 1 and 2 against A549 cells were measured against lamivudine (positive control) (Table 1). Compound 2 has no inhibition against normal cells A549, seen from the negative % cells death values. Acetone extract and 1 also have no inhibition in 62.5 and 31.25 ppm concentrations, so the safest concentration to use is below 62.5 ppm. On the other hand, acetone extract has a high inhibition at 500 ppm. Hence, 1 and 2 were used for SRV-2 assay at a concentration of 31.25 ppm.

A549 cells infected with SRV-2 were utilized in the SRV-2 assay, which was evaluated using the qRT-PCR technique. Measurements were taken on days 5 and 7, as evidenced by the number of virus copies (Table 2). On both days, compound 2 had more viruses than the negative control, whereas positive control had fewer viruses. On day 5, Compound 1 had a higher number of viruses, but on day 7, it had a lower amount. Both compounds showed increased numbers of viruses compared to the positive control. So, it can be concluded that both compounds do not have activity as SRV-2 antivirus.

Table 1 The % cells death of A549 cells with acetone extract, compounds of 1 and 2

Sample	Concentration (ppm)	% cells death
Acetone extract	500	76.79
	250	52.12
	125	42.5
	62.5	-63.84
	31.25	-71.15
Compound 1	500	26.53
	250	21.20
	125	21.25
	62.5	-18.20
	31.25	-38.39
Compound 2	500	-50.26
	250	-55.36
	125	-74.25
	62.5	-84.79
	31.25	-68.46

Cupresuflavone was reported to show low activity against Herpes Simplex Virus (HSV-1). This compound was isolated from the leaves of *A. angustifolia* [20]. On the other hand, the cytotoxic assay of agathisflavone (IC_{50} 100 µM) against HIV-1 reverse transcriptase has a moderate inhibition [19].

Agathisflavone was known to have several antivirus activities, such as anti-influenza A, influenza B, measles virus, adenovirus type 5, parainfluenza type 3 virus, respiratory syncytial virus, herpes virus (HSV-1, HSV02, HCMV, and VZV), and dengue virus (DENV2 NS2B-NS3 and SENV3 NS2B-NS3). In addition, Agathisflavone suppressed neuraminidase (NA) activity of wild-type and OST-resistant influenza viruses, according to de Freitas *et al.* [21], with IC_{50} values ranging from 20 to 2.0 M, respectively. With an EC_{50} of 1.3 M, agathisflavone suppressed influenza virus replication.

Table 2 The number of viruses on days 5 and 7 against compounds 1 and 2

Day	Sample	Number of viruses
5	Compound 1	282.8
	Compound 2	390.0
	Lamivudine	76.0
	Negative control	114.0
7	Compound 1	122.2
	Compound 2	348.7
	Lamivudine	8.2
	Negative control	212.8

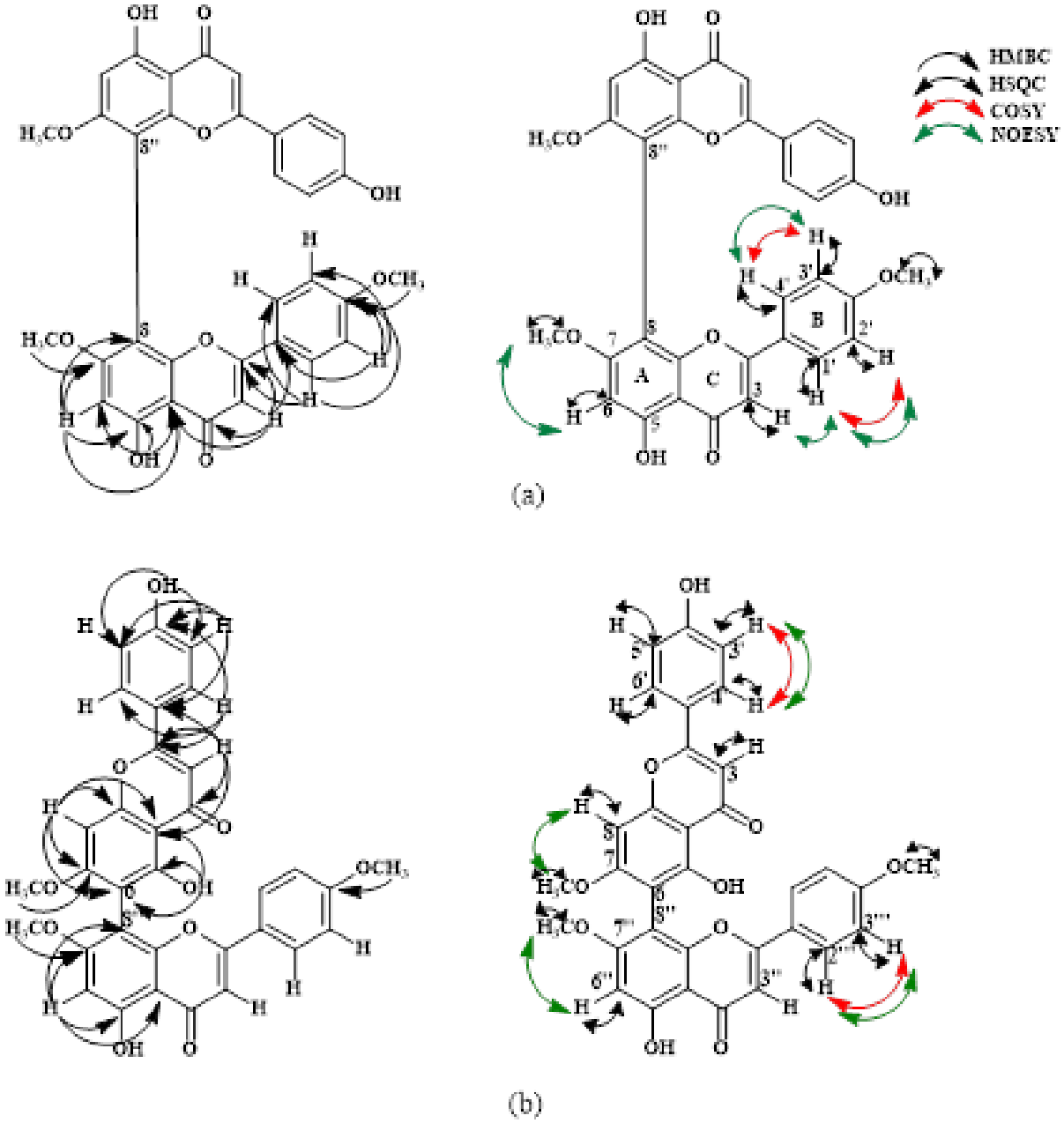
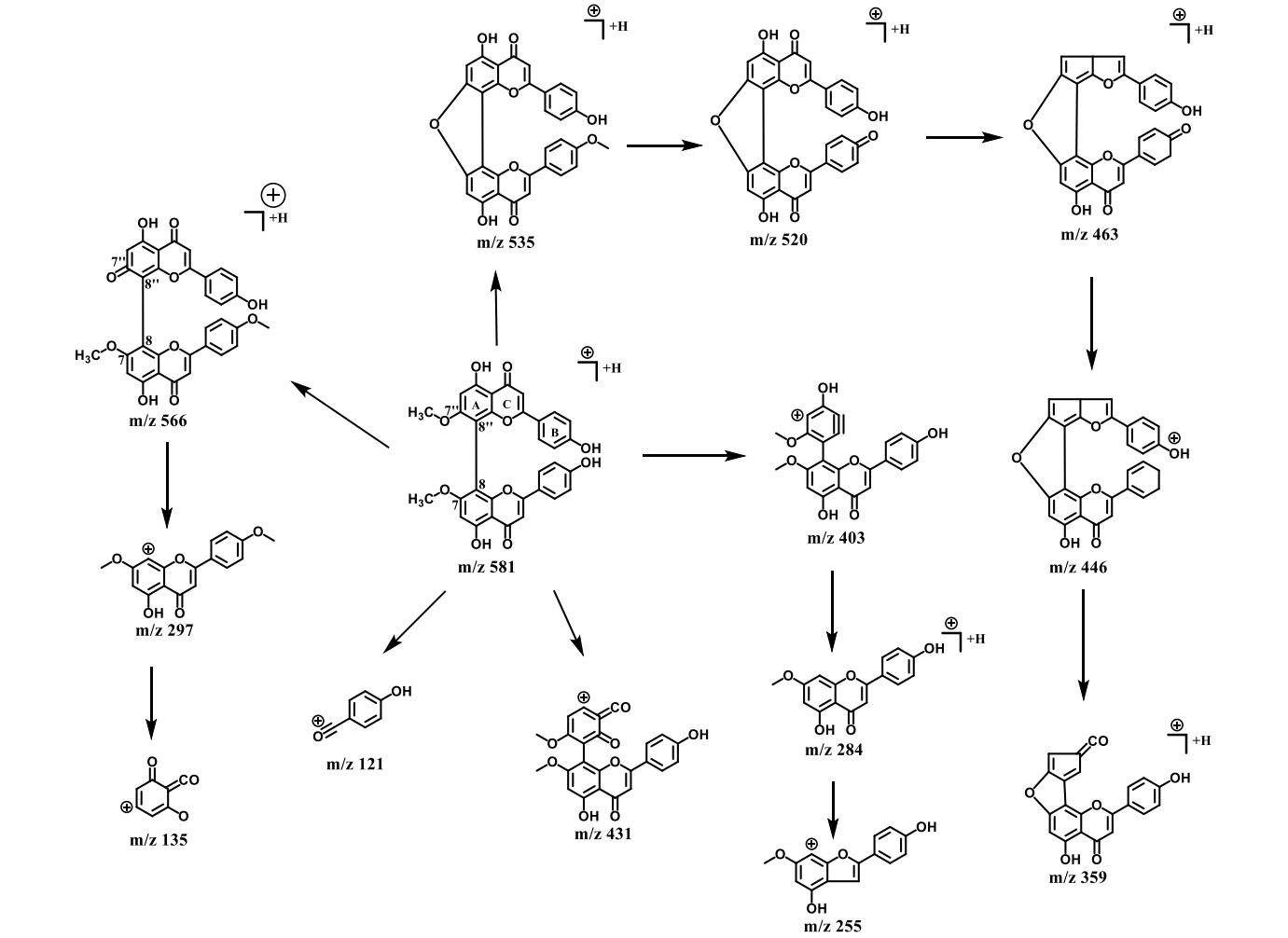
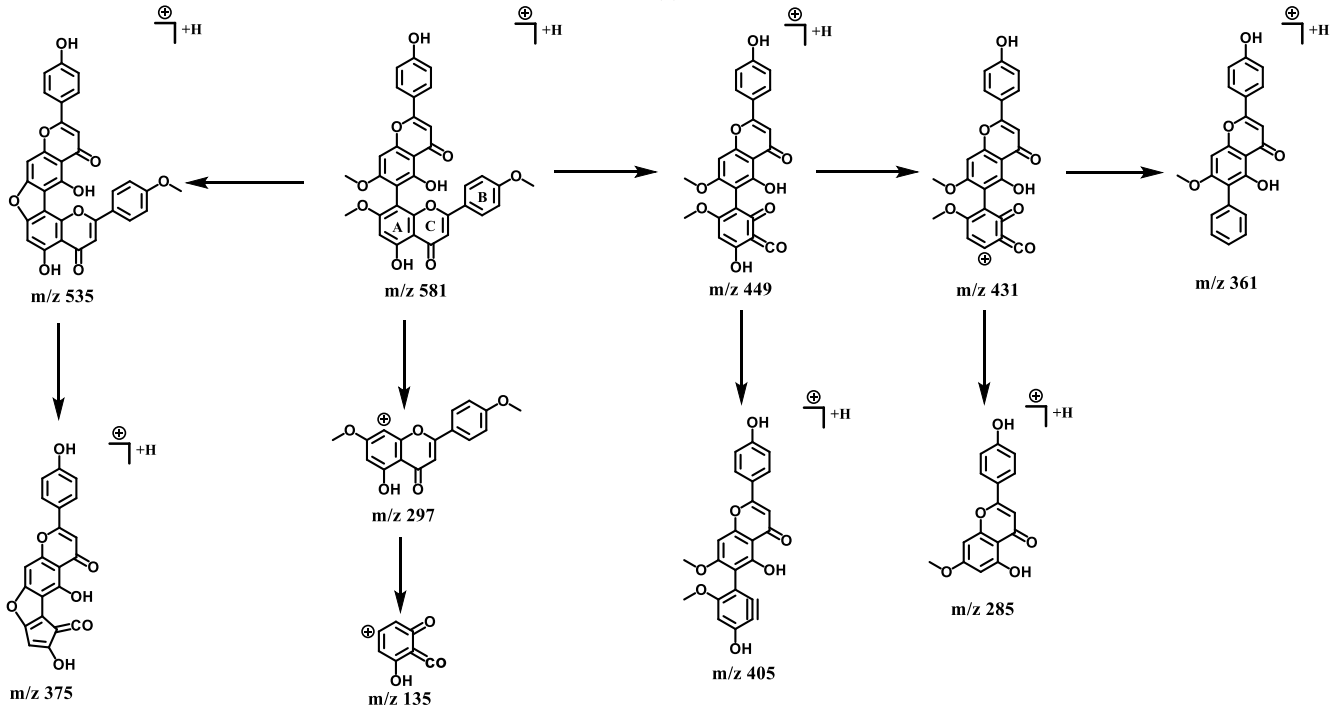


Fig. 2 HMBC, HSQC, COSY, and NOESY correlations of compound 1 (a) and compound 2 (b)



(a)



(b)

Fig. 3 Proposed MS fragmentations scheme for compounds (a) 1 and (b) 2

4. Conclusion

In this study, the authors succeeded in isolating two biflavonoid compounds. Both compounds were identified as 4',7,7"-tri-O-methylcupressuflavone (1) and 4"',7,7"-tri-O-methylagathisflavone (2) by IR spectroscopy, UV-Vis spectrophotometer, LC-MS/MS,

1D NMR spectrometer (^{1}H-NMR, ^{13}C-NMR), and 2D NMR spectrometer (HSQC, HMBC, COSY, and NOESY). Both compounds were isolated for the first time from the Indonesian *A. hunsteinii*. Based on the assay results, acetone extract and 1 have suitable activities as anticancer MCF-7 cells. However, both compounds were inactive as SRV-2 antivirus. Further research is needed to isolate biflavonoids from *A. hunsteinii* plants, especially on the leaves, and modify the structure to improve their biological activities.

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