

BIOACTIVE COMPOUNDS OF FLAVONE DIMERS FROM INDONESIAN *Araucaria columnaris* LEAVES

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ABSTRACT

This study aimed to isolate the flavone dimers in the acetone extract of *Araucaria columnaris* leaves and evaluate their potential biological activities. Three flavone dimers, 1-3, were isolated and identified by spectroscopic analysis using UV, IR, NMR, and mass spectrometry. The acetone extract and isolated metabolites were examined for their antiangiogenic activity against the Calf Pulmonary Arterial Endothelial (CPAE) cell and antioxidant performance using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The results showed that 4',4'',7,7''-tetra-*O*-methylcupressuflavone (1) and acetone extract had IC₅₀ values of 272.95±7.05 and 239.36±13.50 µg/mL, respectively, against CPAE cell. The other two isolated metabolites, 4',7,7''-tri-*O*-methylcupressuflavone (2) and 4'',7-di-*O*-methylcupressuflavone (3), showed high activity with IC₅₀ values of 39.5±1.44 and 66.13±15.96 µg/mL, respectively. Although none of the individual components demonstrated any detectable antioxidant activity (IC₅₀ > 100 µg/mL), the acetone extract showed moderate antioxidant activity with an IC₅₀ value of 69.2 µg/mL.

Keywords: *Araucaria columnaris*, Biflavonoids, Antiangiogenic, Antioxidant Activity, CPAE Cell.

RASAYAN J. Chem., Vol. 16, No. 3, 2023

INTRODUCTION

Araucariaceae species are endemic to New Caledonia and Polynesia, where they thrive in warm climates with soils rich in schist and calcareous metamorphic rocks.^{1,2} The characteristics of this plant family are the main content of secondary metabolites, namely biflavonoids, and terpenoids.^{3,4} Biflavonoids are flavonoid dimers that occur naturally and are linked to each other through a covalent bond of C-C or C-O-C. They exhibit a range of pharmacology activities, including antibacterial,^{5,6} antifungal,⁶ antioxidant,^{5,7} antiplasmodial,⁸ anti-inflammation,⁹ antitumor,¹⁰ neuroprotection,¹¹ antiproliferative, anti-insomnia, analgesic, anticancer, antiviral,² antidiabetic,¹² and anti-arthritis.¹³ *Araucaria columnaris* is a species belonging to the genus *Araucaria* (Araucariaceae), which contains bioflavonoid compounds.² A total of 11 biflavonoid compounds were reported from Hawaii and India, namely hinokiflavone, kayaflavone, sciadopitysin, 7''-*O*-methyl amentoflavone, 7,4'-di-*O*-methyl amentoflavone, 7,4',7'',4'''-tetra-*O*-methyl amentoflavone, 7,4'''-di-*O*-methyl agathisflavone, mono-*O*-methyl cupressuflavone, 7,7''-di-*O*-methyl cupressuflavone, 7,4',7''-tri-*O*-methyl cupressuflavone, and 7,4',7'',4'''-tetra-*O*-methyl cupressuflavone.^{14,15} Biflavonoids such as procyanidin, fukugetin, amentoflavone, and podocarpus flavone were reported to have solid antioxidant abilities to scavenge free radicals of DPPH.¹⁶ Cupressuflavone had shown high radical scavenging activity against ABTS· and DPPH.¹⁷ Furthermore, amentoflavone and agathisflavone exhibited potent antioxidant ability in scavenging DPPH, ABTS, NO, superoxide, and hydroxyl radicals.^{18,19} Biflavonoids of robustaflavone and amentoflavone were also found to have anticancer activity as inhibitors of endothelial cell proliferation for HUVEC (Human Umbilical Vein Endothelial Cells) cells. Their mechanism was predicted through binding to VEGF (Vascular Endothelial Growth Factor), which inhibited VEGF-1 and -2 receptors interaction, the formation of endothelial cells, and capillary tubes induced by VEGF-A.^{20,21} VEGFR-2 is a vascular endothelial protein receptor that is the main effector of VEGF/VEGFR signal transduction in promoting tumor angiogenesis. The phosphorylation of VEGFR-2 results in the activation of the Raf-1/MAPK/ERK signaling cascade, which encourages angiogenesis, raises vascular permeability and aids tumour migration.²²

Amentoflavone also prevented SVEC cell (Murine endothelial cells, which were transformed by Simian virus-40 or SV40) displacement and tube formation.²³ The wood and resin of *A. columnaris* are economically precious was used in industries such as furniture and paper. This species also serves as the city's lungs in terms of ecology. However, there are currently no reports on secondary metabolites, particularly biflavonoids, from Indonesian *A. columnaris*. Biflavonoids are known to have antioxidant and anticancer activities, but their effects on Calf Pulmonary Arterial Endothelial (CPAE) proliferation have not been investigated. Therefore, this study aimed to extract biflavonoids from Indonesian *A. columnaris* leaves and to evaluate their antioxidant activity and influence on CPAE growth.

EXPERIMENTAL

Material and Methods

The isolation stage used organic solvents (Merck) and various types of stationary phases such as Sephadex LH-20, silica gel 60 PF₂₅₄ with gypsum (Merck), and silica gel 60 GF₂₅₄ (Merck). TLC was performed using a silica gel 0.25 mm 60 F₂₅₄ (Merck) plate with aluminum zinc as the support. The spot staining used a reagent in cerium sulfate 1.5% H₂SO₄ 2N, which was detected using ultraviolet (UV) light at 254 and 366 nm. The isolated compounds were characterized following to procedure of Agusta *et al.*²⁴ The cytotoxic assay of antiangiogenetic used endothelial cells CPAE (ATCC CCL-209) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent.

Plant Material

A. columnaris was collected in January and July 2020 at the Bogor Botanical Garden, and a voucher was further placed at the Herbarium Bogoriense, Bogor, West Java, Indonesia.

Extraction and Isolation

A. columnaris powdered leaves of 872.5 g were macerated by acetone in a ratio of 1:5 at room temperature and repeated the process three times. The acetone crude extract was partitioned by MeOH and *n*-hexane solvent for removing chlorophyll. The concentrated MeOH part was partitioned by acetone to remove tannins, obtained the yield of acetone extract was 48.3002 g (5.54%). Subsequently, 1.0 and 2.0 g of acetone extract were separated using Sephadex LH-20 with MeOH eluent, which yielded 11 fractions (1A-1K) and nine fractions (2A-2I) in the first and second fractionations, respectively. The 1F (343 mg) fraction was separated using radial chromatography and a gradient eluent system (*n*-hexane, DCM, and MeOH), yielding compound 1 as a yellow powder, soluble in acetone, and weighing 67.4 mg. The 2G fraction of 110.5 mg was eluted by a gradient eluent system of *n*-hexane, DCM, and ethanol, afforded two compounds, a solid yellow powder soluble in methanol, namely compound 2 (13.8 mg) and compound 3 (27.6 mg), respectively. Compounds 1-3 were clarified by 1D and 2D NMR, UV-Vis, IR, and LC-MS/MS spectrophotometers, as described by Agusta *et al.*²⁴ These compounds were investigated *in vitro* for their antiangiogenesis against CPAE cells and antioxidants.

Antioxidant Assay

The antioxidant assay was conducted following the protocol of Salazar-Aranda *et al.*²⁵ and Rahayu *et al.*²⁶ Each fraction and acetone extract was dissolved in ethanol at a 1000 ppm concentration and diluted to 500, 250, 125, 62.5, and 32.5 ppm. Subsequently, 100 μ L of 125 μ M DPPH was mixed with 100 μ L of solution from each concentration. In a light-free room, the solution was stirred and given a 30-min incubation at 37°C. The scavenging ability of compounds was shown by decreasing the concentration of DPPH from purple to yellow due to the reaction between hydrogen from the sample and unpaired electrons in DPPH. The absorbance at 517 nm was measured using ethanol as a baseline correction. DPPH in ethanol solution was prepared without using a test sample as a control and ascorbic acid as a positive control. The antioxidant activity in DPPH scavenging was calculated using the equation below:

$$\text{Inhibition (\%)} = \frac{(\text{Blank absorbance} - \text{Sample absorbance})}{\text{Blank absorbance}} \times 100$$

A relationship between sample concentration and antioxidant activity was established, and the IC₅₀ was calculated using interpolation. The antioxidant capacity is reflected in the IC₅₀ value.

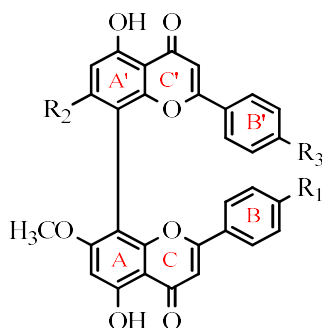
Cytotoxicity Testing *In Vitro*

The protocol²⁷ was followed to evaluate the ability of the samples to inhibit CPAE cell proliferation. CPAE was cultured at a concentration of 5000 cells in a 100 μ L growth media, containing DMEM, 20% FBS, and mixed antibiotics (penicillin at 100 U/mL and streptomycin at 100 μ g/mL). The carbon dioxide level in the ambience is set at 5% and the extract was added after 24 hours when the cells had attained 50% confluency. On the third day, 10 μ L MTT 5 mg/mL reagent was added and incubated for 4 hours at 37°C. The principle of this method was the change in the colour of the MTT reagent from yellow to orange due to the presence of living cancer cells. There was no color change when the cancer cells died and the produced formazan crystals were dissolved in ethanol. Subsequently, the number of cells inhibited by the sample was measured its absorption using a spectrophotometer microplate reader at 595 nm wavelength. The activity test results were reported as IC₅₀, which was the strength of the sample expected to inhibit the growth of CPAE cells by 50% with units of μ g/mL. The IC₅₀ value was calculated by extrapolating the 50% absorption blank line on the absorption curve for various sample concentrations. Doxorubicin-HCl was used as a positive control.

RESULTS AND DISCUSSION

Structure Elucidation of Isolated Biflavonoids from *A. columnaris*

Three discovered compounds from the leaves of Indonesian *A. columnaris* were fully described using LC-MS/MS, UV, IR, and NMR spectrum data. Although compounds 1-3 have same the physical properties as the yellow powder, the solubility of 1 is different from 2 and 3 due to its non-polar nature. Figure-1 displayed three chemical structures, which were isolated from *A. columnaris* leaves.



(1) R₁ = R₂ = R₃ = OCH₃; (2) R₁ = R₂ = OCH₃, R₃ = OH; (3) R₁ = R₂ = OH, R₃ = OCH₃

Fig.-1: The Chemical Structures of Compounds 1, 2, and 3 Isolated from the Leaves of *A. columnaris* in Indonesia

Compound 1 was isolated as a yellow powder and soluble in acetone. The IR spectroscopy analysis of compound 1 revealed the presence of conjugated carbonyl and C=C aromatic (1565-1441 cm⁻¹) as well as C-O (1263 cm⁻¹) groups.²⁸ The UV absorption maxima in methanol were observed at 324 and 273 nm. According to the literature, most flavonoids have two main absorption wavelengths in the ultraviolet/visible region. This is referred to as band I (320-385 nm) and band II (240-290 nm), which correspond to cinnamon and benzoyl groups, respectively.²⁹ The ¹H NMR spectrum data of 1 showed the presence of 26 protons and is a compound was symmetry, as shown by the presence of the signal twice integrated, in Table-1. A total of four aromatic ring systems were observed in proton NMR. One singlet signal shown from ring A protons had two protons in each signal at shifts 6.72 ppm originating from H6 and H6". Ring B has an AA'BB' spin system, indicated by the presence of proton doublet signals at δ 7.61 ppm (J = 8.9 Hz) and 6.96 ppm (J = 9 Hz), where each signal indicated four protons from the aromatic system, which were substituted for H2'/6', H2'''/6''', H3'/5', and H3'''/5''', respectively. Another singlet proton signal with two protons was observed in the proton methine signal from H3 and H3" in ring C. Furthermore, the protons NMR spectrum indicated one signal singlets (2H) for signal hydroxyl, which chelated with the carbonyl protons at δ 13.36 and four methoxy units by two signal singlets of six protons, each at δ 3.82 and 3.87 ppm. The integration signal showed the equivalent of three hydrogens from the methyl signal. The ¹³C NMR spectrum showed 34 carbons, as presented in Table-1 consisting of 26 aromatic carbons, two carbonyls (δ_c 183.6 ppm), four methyls (δ_c 56 and 56.9 ppm), and two methines (δ_c 96,1 ppm). EI-MS confirmed proton and carbon NMR data at retention times of 13.01 minutes, which showed the molecular

formula is $C_{34}H_{26}O_{10}$ (m/z 595.1604 $[M+H]^+$). Compound 2 is a yellow powder and soluble in methanol. The IR spectroscopy of compound 2 revealed absorption bands at 3395, 1624, and 1260-1116 cm^{-1} , indicating the presence of hydroxy (-OH), conjugated carbonyl, and C=C aromatic groups, respectively.²⁶ The UV spectrum showed absorption maxima at 326 and 273 nm in methanol corresponding to aromatic moieties of a flavone.²⁷ Based on the NMR spectrum data of 2 in Table-1, compound 2 is not symmetrical, does not show a double integration signal, and has 24 protons and 33 carbons. The 1H NMR spectrum of compound 2 showed only three methoxy signals at δ_H 3.70, 3.75, and 3.79 ppm, with three protons at each chemical shift. Compound 2 also showed two singlet signals from rings A (H6 and H6'') and C (H3 and H3'') at δ_H 6.62 (2H) and 6.65 ppm (2H). Meanwhile, the four doublet signals, indicated two protons on each signal by ortho coupling ($J = 8.9-8.95$ Hz) of two para-substituted aromatic rings (B) at 6.8, 6.85, 7.3, and 7.45 ppm. The carbon signal of compound 2 represents 33 carbons, consisting of 10 oxyaryl carbon signals, two carbonyls (δ_C 183.8 and 184 ppm), three methoxy carbon (δ_C 55.7, 55.9, and 56.5 ppm), and 13 signals, representing 18 sp^2 carbons in the aromatic ring. Furthermore, the difference in the carbon signal of compound 2 at C4''' showed a more up-field chemical shift than compound 1. This was because compound 2 was more unshielded by the release of methoxy. EI-MS confirmed proton and carbon NMR data at retention times of 11.63 minutes, which showed the molecular formula is $C_{33}H_{24}O_{10}$ (m/z 581.1448 $[M+H]^+$). Compound 3 was a yellow solid that was soluble in methanol. Hydroxy (-OH), conjugated carbonyl, and C=C aromatic groups were present, as indicated by the IR vibrations at wavelengths 3600, 1581, and 1242-1106 cm^{-1} , respectively.²⁶ The UV spectra showed that in methanol, the highest absorption of flavone aromatic moieties occurred at 328 and 273 nm.²⁷ The spectrum of compound 3 in Table-1 showed 22 protons, of which there were two methyl signals (δ_H 3.72 and 3.81 ppm), 3 singlet signals from the aromatic ring equivalent to 4 protons (δ_H 6.5, 6.56, and 6.63 ppm), 3 signals with ortho coupling of 2 para-substituted aromatic rings and showing 8 protons in 6.64, 6.85, and 7.33 ppm. The ^{13}C NMR spectra revealed 28 signals. Moreover, compound 3 is not symmetrical because the number of carbons does not show the integrate signal twice. The carbon signal represents 32 carbons, consisting of 10 oxyaryl, two carbonyls (δ_C 183.5 and 184.1 ppm), 2 methoxy carbon (δ_C 55.8 and 56.7 ppm), and 13 signal signals representing the 8 sp^2 carbons on the aromatic ring. EI-MS evidence that the molecule had a molecular weight of 567.1291 m/z $[M+H]^+$ with the formula $C_{32}H_{22}O_{10}$ and a retention time of 10.31 minutes was consistent with the data presented. Based on the 1H and ^{13}C NMR spectrum in Table-1, compounds 1, 2, and 3 are identified as biflavonoid. This spectrum showed four aromatic proton signal spectrums, indicating the presence of four singlet proton signals on H3(3'') and H6(6''), and two sets of AA'BB' system signals with doublet proton signals at H2'(2'') as A ring with H6'(6'') as A' ring, and H3'(3'') as B ring with H5(5'') as B' ring. Furthermore, the spectrum of ^{13}C NMR showed that 1, 2, and 3 had 34, 33, and 32 carbons, consisting of 10 oxyaryl carbon signals, 2 carbonyl signals, and 13 signals representing 18 sp^2 carbons in the aromatic rings. The proton and carbon signals indicated that compounds 1, 2, and 3 are biflavonoid compounds consisting of 2 flavone units. The presence of 2 protons at H3 and H3'' and olefinic carbons at C3 and C3'' indicated that this flavone has an apigenin skeleton. The three isolated biflavonoid compounds belong to the cupressuflavone group. The flavone dimer bond of the cupressuflavone group is located at C8-C8'', which was indicated by the HMBC spectrum compounds 1, 2, and 3, as shown by the correlation between H6 and C8 as well as H6'' and C8''. The HSQC spectrum also confirmed that the C8-C8'' bond showed no protons. Because the carbon was quaternary, the flavone dimer bonds in the A ring were at C-8 and C-8''. A proton signal with J coupling on 8.5-9.2 Hz and the doublet multiplicity showed ortho coupling, indicating that the B ring is para-substituted and symmetrical, without a flavone dimeric bond. Furthermore, there is no flavone dimeric bond in the C ring, as shown by the absence of a proton signal at C3 and C3''.

Table-1: Displays 1H and ^{13}C -NMR Data for the Three Isolated Compounds

Carbon Position	1 (Acetone- d_6) ^a			2 (Methanol- d_6) ^a			3 (Methanol- d_6) ^a		
	δ_H (ppm), J (Hz)	δ_C (ppm)	H→C (HMBC)	δ_H (ppm), J (Hz)	δ_C (ppm)	H→C (HMBC)	δ_H (ppm), J (Hz)	δ_C (ppm)	H→C (HMBC)
2	-	164.8	-	-	165.4	-	-	166.4	-
3	6.72 (s)	104.1	C1', C2, C4, C10	6.62 (s)	103.6	C1', C2, C5	6.56 (s)	102.5	C1', C2, C4, C10
4	-	183.6	-	-	183.8	-	-	184.1	-

5-OH	13.36 (s)	163.7	-	-	163.4	-	-	163.5	-
6	6.68 (s)	96.1	C5, C7, C8, C9, C10	6.62 (s)	95.8	C7, C8, C10	6.63 (s)	96.1	C5, C7, C8, C10
7	-	164.5	-	-	163.6	-	-	164.9	-
8	-	100.4	-	-	100.5	-	-	100.8	-
9	-	155.5	-	-	155.5	-	-	155.7	-
10	-	105.7	-	-	105.3	-	-	105.6	-
1'	-	124.0	-	-	123.8	-	-	121.7	-
2' (A)	7.61 (d), 8.9	128.7	C3', C4', C6'	7.45 (d), 8.95	128.6	C4', C6'	7.33 (d), 8.5	129.3	C2
3' (B)	6.96 (d), 9	115.4	C1', C4', C5	6.85 (d), 8.9	115.2	C1'	6.64 (d), 9.2	117.6	C1', C5'
4'	-	163.7	-	-	164.0	-	-	162.7	-
5' (B')	6.96 (d), 9	115.4	C1', C3', C4'	6.85 (d), 8.9	115.2	C1'	6.64 (d), 9.2	117.6	C1'
6' (A')	7.61 (d), 8.9	128.7	C2', C4', C5'	7.45 (d), 8.95	128.6	C2', C4'	7.33 (d), 8.5	129.3	C2', C2
2''	-	164.8	-	-	166.1	-	-	165.1	-
3''	6.72 (s)	104.1	C1'', C2'', C4'', C10''	6.65 (s)	104.1	C2'', C5''	6.50 (s)	102.5	C2'', C10''
4''	-	183.6	-	-	184.0	-	-	183.5	-
5''-OH	13.36 (s)	163.7	-	-	163.3	-	-	165.6	-
6''	6.68 (s)	96.1	C5'', C7'', C8'', C10''	6.65 (s)	96.0	C7'', C8'', C10''	6.63 (s)	103.3	C8''
7''	-	164.5	-	-	164.9	-	-	162.3	-
8''	-	100.4	-	-	100.8	-	-	101.6	-
9''	-	155.5	-	-	155.6	-	-	156.8	-
10''	-	105.7	-	-	105.9	-	-	103.5	-
1'''	-	124.0	-	-	124.5	-	-	121.3	-
2''' (A)	7.61 (d), 8.9	128.7	C3''', C4''', C6'''	7.30 (d), 8.8	128.8	C6'''	7.33 (d), 8.5	128.9	C2''
3''' (B)	6.96 (d), 9	115.4	C1''', C4''', C5'''	6.9 (d), 8.6	115.0	C1'''	6.85 (d), 8.6	117.5	C1''', C4''', C5'''
4'''	-	163.7	-	-	161.6	-	-	163.8	-
5''' (B')	6.96 (d), 9	115.4	C1''', C3''', C4'''	6.9 (d), 8.6	115.0	C1'''	6.85 (d), 8.6	117.5	C1''', C4'''
6''' (A')	7.61 (d), 8.9	128.7	C2''', C4''', C5'''	7.30 (d), 8.8	128.8	C2'''	7.33 (d), 8.5	128.9	C2''
4'-OCH ₃	3.82 (s)	56.0	C4'	3.75 (s)	55.9	C4'	-	-	-
4'''-OCH ₃	3.82 (s)	56.0	C4'''	-	-	-	3.72 (s)	55.8	C4'''
7-OCH ₃	3.87 (s)	56.9	C6, C7	3.70 (s)	55.7	C7	3.81 (s)	56.6	C7
7''-OCH ₃	3.87 (s)	56.9	C6'', C7''	3.79 (s)	56.5	C7''	-	-	-

^a ¹H and ¹³C NMR data were recorded at 500 and 125 MHz, respectively.

Compounds 1-3 are derivatives of cupressuflavone, as confirmed by the presence of a methyl group signal. Compound 1 has four methyl groups, indicated by the presence of two methyl signals at δ_H 3.82 ppm (6H) and 3.87 ppm (6H) ppm, based on the HMBC spectrum. There was a correlation between protons at δ_H 3.82 ppm (s, 6H) and 3.87 ppm (s, 6H) with quaternary carbons at 163.7 and 164.5 ppm, indicating the presence of methoxy groups at C4', C4''', C7, and C7''. Compound 2 is a cupressuflavone derivative with three methyl groups. The HMBC spectrum confirmed the proton correlation at δ_H 3.7 (s, 3H), 3.75 (s, 3H), and 3.79 ppm (s, 3H) with quaternary carbons at δ_C 163.6, 164, and 164.9 ppm. Table-1 showed that the methoxy groups are located at C4', C7, and C7''. Compound 3 is a cupressuflavone compound with 2 methyl group substituents. Based on the HMBC spectrum, the location of the methyl group is indicated by the correlation of protons at δ_H 3.72 ppm (s, 3H) and 3.81 ppm (s, 3H) with the quaternary carbon at δ_C 163.8 and 164.9 ppm, as illustrated in Table-1. This indicated that the methoxy group is located at C4''' and C7. Meanwhile, Fig.-2 shows a selected 2D NMR (HSQC and HMBC) correlation of compound 3.

Based on LC-MS/MS, the three compounds were identified at retention times of 13.01, 11.63, and 10.31 minutes. EI-MS assigned compounds 1, 2, and 3 the molecular formula of $C_{34}H_{26}O_{10}$ (m/z 595.1604 $[M+H]^+$), $C_{33}H_{24}O_{10}$ (m/z 581.1448 $[M+H]^+$), $C_{32}H_{22}O_{10}$ (m/z 567.1291 $[M+H]^+$), respectively. They were also confirmed to be biflavonoid compounds, with molecular weight twice that of flavonoids. The three compounds have varying numbers of methoxy units. The MS/MS fragmentation, as seen in Fig.-3, further supported the compounds' symmetrical substitution. Compounds 1, 2, and 3 showed two fragmentation pathways by passing through RDA (Retro-Diels-Alder) cleavage of the A ring of flavonoids at 1/3 position ($^{1,3}A^+$).³⁰ In phase I, the compounds passed through RDA cleavage at $^{1,3}A^+$ from m/z 595 to become 463, 581 to 449, and 567 to 449. This indicated the appearance of a methoxy group at C4' (compounds 1 and 2) and hydroxy group C4''' (compound 3).

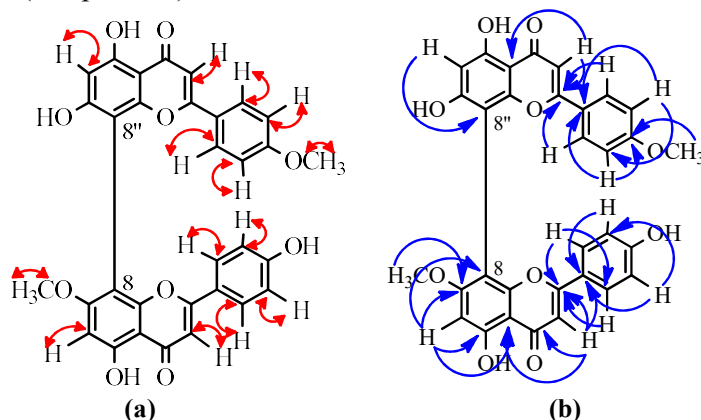


Fig.-2: Selected 2D NMR (HSQC (a) and HMBC (b)) Correlation of Compound 3

Phase II started with the release of the methoxy group at C7 and was shown in the fragmentation pattern of compound 3 at peak 567 to become 535. Furthermore, the compound experienced RDA cleavage $^{1,3}A^+$ at peak m/z 417 (3) and indicated a methoxy group at C4''' (for 3). The fragmentation pattern of compound 3 from m/z 567 became 391. This is a retrocyclization fragmentation involving the oxygen atom with four bonds in the C ring of the flavonoid, showing the existence of a methoxy at C4'''. The fragmentation pattern at m/z 121, 135, 153, and 167 is an essential ion because it indicated the existence of substituents on rings A and B. The peak of m/z 153 provided hydroxyl information at the C7'' position, and m/z 167 indicated the existence of a methoxy at C7 and C7''. The ion peak at m/z 121 and 135 related information about the hydroxyl group and a methoxy at C4 and C4''', respectively.^{30,31,32} Based on the elucidation results of the structure from the IR, UV, LC-MS/MS, and NMR (1D and 2D) data spectra, compounds 1-3 were identified as 7,4', 7'',4'''-tetra-*O*-methylcupressuflavone (1), 7,4',7''-tri-*O*-methylcupressuflavone (2), and 7,4''-di-*O*-methylcupressuflavone (3), respectively (Fig.-1). Compounds 1 and 2 have been found in other species in the family Araucariaceae. Compound 1 has been discovered in some species like *Agathis ovata*, *Araucaria rulei*, *A. cunninghamii*, *A. hunsteinii*, and *Wollemia nobilis*.^{2,24} Compound 2 has been reported in *Araucaria cunninghamii*, *A. hunsteinii*, *Agathis atropurpurea*, *A. australis*, *A. ovata*, and *Wollemia nobilis*.^{2,24} Meanwhile, 3 is the first time compound isolated from species *A. columnaris* and *A. hunsteinii*.³³ Compound 3 is estimated as a characteristic compound from *Araucaria* species that grow in Indonesia.

Antiangiogenesis and Antioxidant Activity of the Biflavonoids Isolated from *A. columnaris*

Acetone extract and three compounds isolated from *A. columnaris* were investigated in vitro for their antiangiogenetic against CPAE cells and antioxidants against DPPH. Doxorubicin HCl and ascorbic acid were used as a positive control for both assays, respectively. The IC_{50} value of inhibiting CPAE cell proliferation and antioxidants against DPPH for them are presented in Table-2.

The cytotoxicity test results in Table-2 demonstrated that compounds 2 and 3 were active against CPAE cell proliferation, with respectively IC_{50} values of 66.13 ± 15.96 and 39.5 ± 1.44 $\mu g/mL$. Compound 1 and acetone extract showed weak activity with IC_{50} of 239.36 ± 13.50 and 272.95 ± 7.05 $\mu g/mL$, respectively. Cupressuflavone and its derivatives had not been reported as inhibitors of CPAE cell proliferation. However, previous similar investigations reported that amentoflavone and its derivatives inhibited tubular structure formation and VEGF-induced migration of SVECs by 50% at 50 μM concentration.²⁰

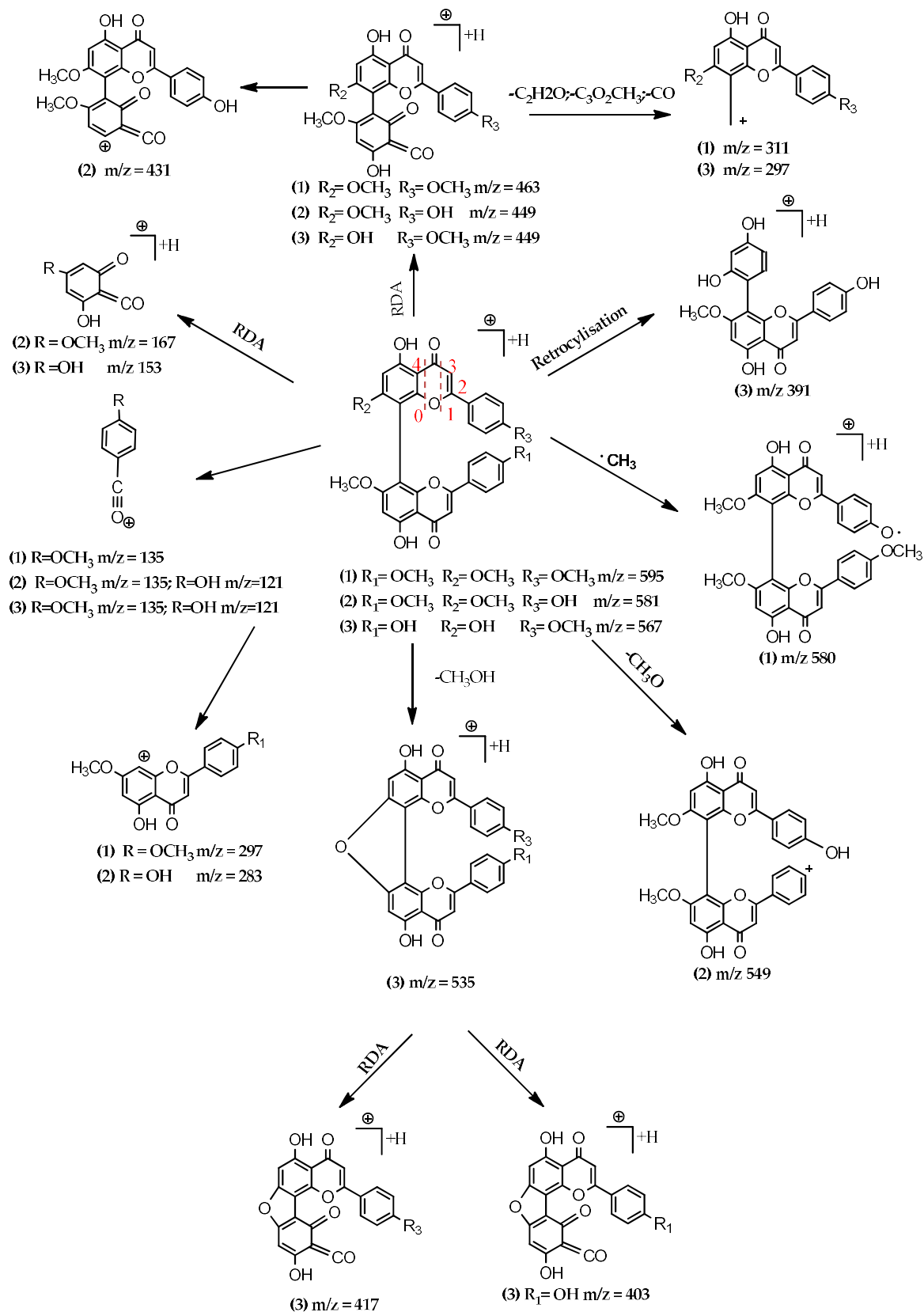
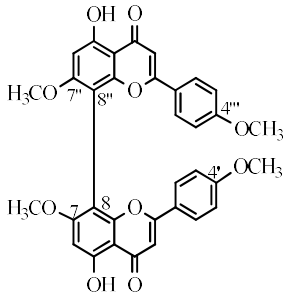
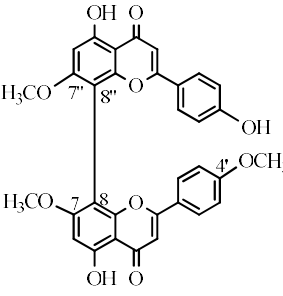
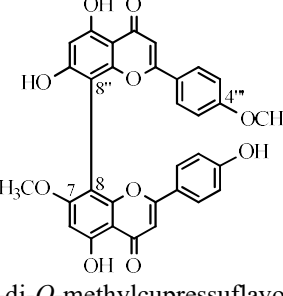


Fig.-3: Proposed MS Fragmentation Scheme of Compounds 1, 2, and 3

Amentoflavone has also been found to inhibit HUVEC cell proliferation with IC_{50} $290 \pm 17.8 \mu M$ and HUVEC cell migration in reaction to VEGF-A and PIGF-1 at $10.0 \mu M$ for four hours.²³ Robustaflavone was reported to inhibit HUVECs cell proliferation at IC_{50} $8.7 \pm 0.6 \mu M$.²¹ Meanwhile, cupressuflavone derivative (1) showed inactive ($IC_{50} > 100.0 \mu g/mL$) activity on HUVEC cell proliferation.³⁴ Cupressuflavone showed vigorous activity against A549 lung cancer cells (IC_{50} $65 \mu M$), PC3 cancer cells (IC_{50} $19.9 \mu M$), MDA-MB 231 the breast adenocarcinomas cancer cell from human (IC_{50} $16.1 \mu M$), A375 malignant melanomas (IC_{50} $12.7 \mu M$), and HCT116 colon carcinomas (IC_{50} $19.3 \mu M$).^{17,35} Cupressuflavone derivative, 7-*O*-methylcupressuflavone demonstrated very strong inhibitory effects against HeLa and MCF-7 cells with IC_{50} of $1.42 \pm 1.1 \mu M$ and $3.40 \pm 0.3 \mu M$, respectively.³³

Table-2: The IC_{50} ($\mu g/mL$) Rate of Inhibitory on the CPAE Cell Proliferation and Antioxidant Three Isolated Compounds and Acetone Extract of *A. columnaris* Leaves

Samples	$IC_{50} \pm SD$ ($\mu g/mL$)	
	Inhibitory on the CPAE cell proliferation	DPPH scavenging capacity
Doxorubicine-HCl	0.75 ± 0.21	-
Ascorbic acid	-	7.44
Acetone extract <i>A. columnaris</i> leaves	272.95 ± 7.05	69.2
 7,4',7'',4'''-tetra- <i>O</i> -methylcupressuflavone (1)	239.36 ± 13.50	>100
 7,4',7''-tri- <i>O</i> -methylcupressuflavone (2)	66.13 ± 15.96	>100
 7,4'''-di- <i>O</i> -methylcupressuflavone (3)	39.50 ± 1.44	>100

The structure-activity relationship (SAR) study of biflavonoids and comparison of the CPAE cell inhibitory activities of cupressuflavone methylated derivatives 1, 2, and 3 in Table-2 indicated that the location and number of methoxy groups in the compounds influenced their pharmacological activities.

The presence of a hydroxy group at C4''' and the displacement of the methoxy group in compound 2 increased its activity compared to 1. However, it still has a lower activity than 3, with two hydroxy groups located at C4' and C7''. The hydroxy group at C4', C4''', and C7'' are essential in cupressuflavone derivatives

that inhibit CPAE cell proliferation. The differences in activity through the quantity and location of hydroxy groups can be linked to changes in polarity and the ability to form hydrogen bonds with CPAE cells.³⁶ The polarity increases in the order of 3 > 2 > 1. The antioxidant activities of biflavonoids 1, 2, and 3 were analyzed towards DPPH radicals, as presented in Table-2. Acetone extract *A. columnaris* leaves showed moderate scavenging activity. However, all isolated compounds showed weak antioxidant activity ($IC_{50} > 100$ ppm) due to the absence of some phenolic -OH in the molecule.³⁷ Cupressuflavone had a moderate scavenging activity with an IC_{50} of 59.24 $\mu\text{g/mL}$ but no information for cupressuflavone derivatives.¹⁶ Several biflavonoids such as amentoflavone, robustaflavone, agathisflavone, hinokiflavone, rhusflavanone, and potifulgene (PF-2) were also reported to have antioxidant activity.^{16-19,38}

CONCLUSION

The three biflavonoids, 7,4',7'',4'''-tetra-*O*-methylcupressuflavone (1), 7,4',7''-tri-*O*-methylcupressuflavone (2), and 7,4'''-di-*O*-methylcupressuflavone (3), were successfully isolated from the acetone extract of Indonesian *A. columnaris* leaves and their structures were determined by spectroscopic. Among these compounds, 2 and 3 exhibited vigorous activity against CPAE cell proliferation. However, all isolated compounds showed weak scavenging activity toward the DPPH radical.

ACKNOWLEDGMENTS

This study was sponsored by the Ministry of Education, Culture, Research and Technology Indonesia, through the “Penelitian Tesis Magister (PTM)” scheme under IPB University, Project No. 082/E5/PG.02.00.PT/2022, dated May 10, 2022. The authors are grateful to Center of Chemical Instrumentation Faculty of Mathematics and Natural Sciences Institut Teknologi Bandung, for recording the NMR data and The Primate Research Centre (PSSP) IPB University for cytotoxic testing.

CONFLICT OF INTERESTS

The authors stated that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

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