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Research Article

Isolation and Structural Characterization of Biflavonoids from *Araucaria hunsteinii* and *Araucaria columnaris*: Chemotaxonomic and Pharmacological Perspectives

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Abstract
Biflavonoids are a distinctive class of dimeric flavonoids known for their diverse biological activities and chemotaxonomic significance. In this study, two biflavonoids were isolated from the acetone extracts of *Araucaria hunsteinii* twigs and *Araucaria columnaris* leaves collected from Bogor Botanical Garden, Indonesia. Chromatographic techniques, including Sephadex LH-20 column chromatography and preparative thin-layer chromatography, were employed for purification, followed by structural elucidation using LC-MS/MS and 1D/2D NMR spectroscopy. The compounds were identified as 4',4''',7,7''-tetra-*O*-methylcupressuflavone (**1**) and 7-*O*-methylcupressuflavone (**2**). Notably, this is the first report of 7-*O*-methylcupressuflavone isolated from *A. columnaris* leaves, providing new chemotaxonomic insights into the genus Araucaria. A literature-based pharmacological analysis revealed promising cytotoxic and α-glucosidase-inhibitory activities of the isolated compounds. These findings contribute to the phytochemical profiling and highlight the pharmaceutical potential of Araucaria-derived biflavonoids.

Keywords: *Araucaria,* biflavonoids, chemotaxonomy 4′,4‴,7,7″-tetra-*O*-methylcupressuflavone, 7-*O*-methylcupressuflavone

1. INTRODUCTION

Biflavonoids, as a distinctive group of dimeric flavonoids, have attracted considerable attention due to their broad spectrum of biological activities, including antioxidants [1–9], antiviral [5–8,10], antidiabetic [11–15], anti-inflammatory [9,12,16–18], antitumor [19], anticancer [20–25], antibacterial [26], antimicrobial [10,27], and antifungal [4] effects. The genus Araucaria is recognized as a primary natural source of biflavonoids, yet comprehensive chemical profiling across its species remains incomplete.

Biflavonoids are unique dimeric flavonoids in which two flavone or flavanone units are covalently linked, resulting in higher structural rigidity, enhanced metabolic stability, and stronger biological activities compared to their monomeric analogs. The dimeric linkage and multiple hydroxylation or methoxylation patterns of biflavonoids influence their pharmacokinetic and pharmacodynamic properties,

thereby contributing to diverse bioactivities including antioxidant, anticancer, and enzyme-inhibitory effects [1,15,25]. Recent studies have highlighted that biflavonoids isolated from *Araucaria*, *Agathis*, and *Ginkgo* species exhibit promising pharmacological effects, making them valuable scaffolds for drug discovery and chemotaxonomic studies [24,28].

Previous phytochemical investigations have identified several biflavonoids in *A. hunsteinii* and *A. columnaris*, mainly from leaf extracts. These compounds are 4',4"',7,7"-tetra-*O*-methyl-cupressuflavone (**1**), 7-*O*-methylcupressuflavone (**2**), 4',7,7"-tri-*O*-methylcupressuflavone (**3**). 4"',7-di-*O*-methylcupressuflavone (**4**), 4"',7,7"-tri-*O*-methylagathisflavone (**5**), 7,7"-di-*O*-methylagathisflavone (**6**), and 4',4"'-di-*O*-methylamentoflavone (**7**) [24,29]. Compounds **1, 3**, and **4** have also been identified in the leaves of *A. columnaris* [3]. Both plants originate from the Bogor Botanical Garden, Indonesia. However, the biflavonoid composition of *A. columnaris* leaves has been poorly explored, and comparative data regarding different plant organs within the genus are still lacking.

The selection of cytotoxic and α-glucosidase inhibitory activities in this study was based on the pharmacological relevance of biflavonoids. Cytotoxicity was evaluated to assess the anticancer potential of these compounds, as several biflavonoids such as cupressuflavone and amentoflavone are known to induce apoptosis and suppress proliferation in breast and cervical cancer cells [21,24]. Meanwhile, α-glucosidase inhibition was chosen to explore the antidiabetic potential of the isolated biflavonoids, given their structural similarity to natural glucosidase inhibitors and their ability to interfere with carbohydrate metabolism [11,13]. Understanding these activities not only underscores the pharmacological importance of *Araucaria*-derived biflavonoids but also provides insights into the structure–activity relationships (SARs) associated with different substitution patterns of methoxy and hydroxyl groups.

Here, we report the isolation and structural characterization of two biflavonoids from the acetone extracts of *A. hunsteinii* twigs and *A. columnaris* leaves. Importantly, we identify 7-*O*-methylcupressuflavone for the first time from *A. columnaris* leaves, expanding the chemotaxonomic and pharmacological understanding of this conifer species. To further explore their biological significance, the study also discusses the pharmacological relevance of these biflavonoids from cytotoxic and α-glucosidase inhibition perspectives, highlighting their potential as promising pharmaceutical leads. Therefore, this study aimed to isolate, characterize, and evaluate the chemotaxonomic and pharmacological implications of biflavonoids from *Araucaria* species.

2. RESEARCH METHODS

Instruments and Materials

The instruments used in this study included a rotary evaporator (Buchi R-15, Germany), UV detectors at 254 nm and 366 nm (CAMAG), mass spectrometry instruments including an LC-MS system with an Ultra-Performance Liquid Chromatography (UPLC) system using electrospray ionization (ESI), and a GC-MS spectrometer (Agilent Technologies) with electron ionization (EI).

The chemicals used in this work contain: sephadex LH-20 (GE Healthcare), silica gel 60 PF_{254} (Merck), silica gel F_{254} TLC plates (Merck), cerium sulphate ($Ce(SO_4)_2$), and a variety of solvents of analytical grade from Merck, such as acetone, *n*-hexane, methanol (MeOH), chloroform ($CHCl_3$), dichloromethane (DCM), ethyl acetate (EtOAc), and ethanol (EtOH). Moreover, cotton and filter paper were employed in the purifying procedure.

Plant sample

Samples of *A. columnaris* leaves and *A. hunsteinii* twigs were gathered from the Bogor Botanical Garden in West Java, Indonesia. The acetone extracts from both samples were refined to exclude tannins and chlorophyll. The acetone extract of *A. hunsteinii* twigs (Ai) was obtained from Agusta *et al.* [29]. In contrast, the acetone extract of *A. columnaris* leaves (Ac) was sourced from Kurniawanti *et al.* [3]. Voucher specimens were deposited at the School of Life Sciences and Technology, Institut Teknologi Bandung (SITH–ITB), Bandung, Indonesia (No. FIPIA-DEP133 for *A. columnaris* and FIPIA-DEP134 for *A. hunsteinii*). The plant materials were collected at coordinates 6°35′31″S, 106°47′59″E, and were identified and authenticated by a botanist from SITH–ITB.

Fractionation of the Acetone Extracts from *A. hunsteinii* Twigs and *A. columnaris* Leaves Using Sephadex LH-20 Column Chromatography

Fractionation was performed using column chromatography with Sephadex LH-20 as the stationary phase. The Sephadex LH-20 resin was pre-soaked in methanol for 24 hours before being packed into a column. The acetone extracts from *A. columnaris* leaves and *A. hunsteinii* twigs were separately dissolved in methanol (2.15 g and 8.20 g, respectively) and filtered using a syringe filter. The extracts were then carefully loaded onto the column and eluted with methanol. The eluates were collected in numbered vials and analysed by thin-layer

chromatography (TLC) using a 5 % MeOH in $CHCl_3$ solvent system. The elution was monitored at 254 nm and 366 nm under UV light, followed by spraying with $Ce(SO4)_2$ solution. Fractions with similar Rf values were combined and further purified using preparative thin-layer chromatography (*p*-TLC).

The fractionation of the acetone extract from *A. columnaris* leaves (2.15 g) yielded five fractions (A–E). Among these, fraction E was suspected to contain biflavonoids based on the appearance of yellow spots after spraying with $Ce(SO_4)_2$ solution and heating. Further fractionation of fraction E (170.2 mg) using radial chromatography with a gradient elution system (starting from 100 % *n*-hexane, followed by increasing polarity using 50 % EtOH in DCM and 50 % H_2O in MeOH resulted in five fractions (E1–E5). Fraction E5 (78.9 mg) was further purified using *p*-TLC with 15 % EtOH in $CHCl_3$, yielding nine subfractions (E51–E59). Among these, subfraction E53 (21.8 mg) was confirmed as a single compound using three different solvent systems: 10 % EtOH in $CHCl_3$, 40 % EtOH in DCM, and 15 % EtOH in $CHCl_3$.

Similarly, the fractionation of the acetone extract from *A. hunsteinii* twigs (8.20 g) obtained fifteen fractions. One of these fractions (Fraction C) was suspected to contain biflavonoids due to the presence of yellow spots after spraying with $Ce(SO_4)_2$ solution and heating. Further fractionation of Fraction C (1.2 g) using radial chromatography with a gradient elution system (starting from 100 % *n*-hexane, followed by 10 % to 90 % DCM in *n*-hexane, 100 % DCM, 50 % MeOH in DCM, and 100 % MeOH resulted in six fractions (C1–C6). Fraction C1 was further purified using $_P$-TLC with 10 % EtOAc in DCM, yielding a pure compound (C11) with a weight of 21.8 mg. The compound was confirmed to be a single substance through by TLC analysis with using three different solvent systems: 30 % EtOAc in n-hexane, 10 % DCM in n-hexane, and 20 % EtOAC EtOAc in $CHCl_3$.

Purification of Fractions from the Acetone Extracts Using Preparative Thin Layer Chromatography (*p*-TLC)

Preparative TLC (*p*-TLC**)** plates were prepared using silica gel Merck 60 GF_{254} as the stationary phase. The silica gel was mixed with distilled water to form a slurry, which was then spread onto glass plates and heated at 75 °C for 12 hours. The fractions to be purified were dissolved in acetone and applied onto the $_P$-TLC plates before elution. The plates were then analysed under UV light at 254 nm. and separated fractions were scraped off and macerated in acetone. The macerated fractions were filtered and concentrated using a rotary evaporator. The purity of the fractions was confirmed using TLC with three different solvents.

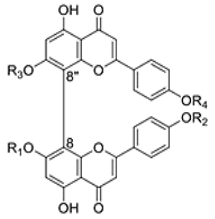
Structure elucidation

The structure of the isolated compounds were determined by spectroscopic measurement that includes: LC-MS/MS – C_{18} column (stationary phase particle size = 1.8 µM, internal diameter = 2.1 mm, and length = 100 mm), high strength silica, mobile phase A (water with 5 mM ammonium format), mobile phase B (acetonitrile with 0.05 % formic acid), eluent flow rate = 0.2 mL/min (gradient), and total elution time = 23 min; ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HMBC, and HSQC in acetone-d6 solvent.

3. RESULTS AND DISCUSSION

Biflavonoid isolation results

Two biflavonoids and their structures were discovered from the acetone extracts of *A. columnaris* leaves and *A. hunsteinii* twigs. Compound **1** (4',4''',7,7''-tetra-*O*-methylcupressuflavone) was consistent with previously reported structures, whereas compound **2** (7-*O*-methylcupressuflavone) was identified for the first time from *A. columnaris* leaves, expanding the known phytochemical diversity of the genus. Detailed spectroscopic analyses, including LC-MS/MS and 1D/2D NMR, confirmed the symmetric dimeric flavone structures characteristic of cupressuflavones. The comparative evaluation indicated structural variations potentially linked to distinct pharmacological activities, with compound **2** exhibiting superior bioactivity profiles according to the literature. The structures of these two biflavonoids are shown in **Figure 1.**



R_1=R_2=R_3=R_4=CH_3; 7.4′.7″.4‴-tetra-*O*-methylcupressuflavone (**1**)
R_1=CH_3; R_2=R_3=R_4=H: 7-*O*- methylcupressuflavone (**2**)

Figure 1: Structure of 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone (**1**) and 7-*O*- methylcupressuflavone (**2**)

One of the most prevalent types of biflavonoids found in Araucaria species is the cupressuflavone derivative. Numerous representatives of this genus, such as *A. hunsteinii* [24], *A. columnaris* [3,28,30], *A. cunninghamii* [25,26], *A. cookie*, and *A. excelsa* [31] have been reported to contain Compound **1** in significant amounts. This compound has also been isolated, albeit as a minor constituent, from several Agathis species outside of the Araucaria genus, including *A. robusta*, *A. australis*, *A. dammara*, and *A. ovata*, where it was identified as a minor constituent [32,33]. In contrast, the current investigation reports compound **2** as being new to *A. columnaris*. Nonetheless, it has been found in Agathis species, such as *A. robusta* [32–34] and *A. ovata* [33], as well as in Araucaria species, such as *A. hunsteinii* [24] and *A. bidwillii* Hook [28].

Structural Characterization

Based on mass spectrometry (MS) data, 4′,4‴,7,7″-tetra-*O*-methylcupressuflavone (**1**) and 7-*O*-methylcupressuflavone (**2**) exhibited $[M+H]^+$ peaks at m/z 595.1644 ($C_{34}H_{26}O_{10}$) and 553.1131 ($C_{31}H_{20}O_{10}$), respectively. Nuclear magnetic resonance (NMR) analysis of both compounds revealed characteristic features of cupressuflavones, as indicated by the observation of only half the number of NMR signals typically seen in biflavonoids. However, LC-MS/MS analysis confirmed that the molecular weights of both compounds were approximately twice that of a flavonoid monomer, supporting the conclusion that they possess dimeric flavonoid structures. Both compounds were further classified as flavones, a subclass of flavonoids.

The ^{13}C-NMR spectra of compounds **1** and **2** exhibited 15 and 13 signals corresponding to 34 and 31 carbon atoms, respectively. Key spectral features included a conjugated ketone carbonyl signal at δC ~182.0 ppm, indicative of sp^2-hybridized carbons at C-4 and C-4″; five oxyaryl carbon signals in the δC 153.0–164.0 ppm range; and methoxy group signals between δC 55.0–57.0 ppm. The ^{1}H-NMR spectra displayed two hydroxyl (-OH) proton signals at δH ~13.00 ppm, corresponding to the hydroxyl groups at C-5 and C-5″, which are chelated with adjacent carbonyl groups. Additionally, doublet signals with ortho-coupling constants (J = 8.4–8.9 Hz) were observed in the δH 6.69–7.96 ppm region, indicating that the B-ring is para-substituted and symmetric, as illustrated in **Figure 2**. These data suggest that dimerization does not occur on the B-ring.

On the A-ring, a singlet proton signal was observed at δH 6.68 ppm (compound **1**) and 6.63 ppm (compound **2**), consistent with a pentasubstituted benzene ring. Collectively, the MS and ^{13}C-NMR data confirm that both biflavonoids possess symmetric structures. Biflavonoids with such symmetric architectures found in Araucaria species are classified as members of the cupressuflavone group. A comparison of the ^{1}H-NMR and ^{13}C-NMR data of the isolated compounds with those reported in the literature is summarized in **Tables 1 and 2**.

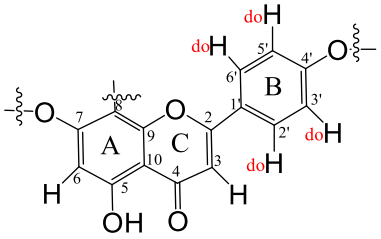


Figure 2: Structure of flavonoids with a symmetric B-ring

The hydrogen signals at δH 6.72 (compound **1**) and 6.79 (compound **2**) correspond to alkene protons which are correlated with C-3 at δC 104.1 (compound **1**) and 102.86 (compound **2**) in the HSQC spectrum providing evidence that both compounds have a flavone backbone. In the HMBC spectrum, these protons also exhibit correlations with C-2. C-4, and C-10. The Heteronuclear Multiple Bond Coherence (HMBC) spectra of compounds 1 and 2 show two- to three-bond correlations between protons and carbons**,** which can be used to identify the dimerization site (**Figure 3**). While H-6" correlates with C-5", C-7", C-8", and C-10", the proton at position H-6 correlates with C-5, C-8, and C-10.

The methoxy group is attached at C-7 as confirmed by correlations between the signals indicating methoxy (-OCH_3) groups at δH 3.87 (compound **1**) and 3.81 (compound **2**) and C-7 at δC 164.5 (compound **1**) and 162.67 (compound **2**). No proton signals were detected at C-8 and C-8", according to Heteronuclear Single Quantum Coherence (HSQC) analysis, which shows one-bond correlations between protons and carbons. This suggests the tertiary nature of these two carbons. Consequently, C-8 and C-8" are the dimerization sites of cupressuflavone.

To clarify the biaryl linkage, long-range ^{1}H–^{13}C correlations was performed using HSQC spectra. The key correlations between H-6 and C-8, as well as between H-6″ and C-8″, confirm that dimerisation occurs through the C-8–C-8″ biaryl bond characteristic of cupressuflavone-type biflavonoids (**Figure 4**). This observation is consistent with previously reported data for related compounds [25,33]. The HSQC spectrum in ring A shows protons at C6 and C6", so there is no dimer bond, but no protons at C8 and C8", which indicates that these carbons are quaternary. The proton signal with a coupling frequency of 8–9 Hz and doublet multiplicity at H-2'(2'), H-6'(6'), H-3'(3'), and H-5'(5') shows that the positions of each hydrogen are ortho to each other,

indicating that ring B is para-substituted, symmetrical, and there is no dimer bond. Additionally, the presence of protons at C3 and C3″ suggests that there is no dimeric bond in the ring C. Although CD spectroscopy was not performed in this study, the symmetric signals observed in ^{1}H and ^{13}C NMR, along with literature comparison, suggest that compound **2** is an atropisomeric biflavonoid with a stable chiral axis at the 8–8″ linkage.

Table 1: Comparison of 1D ^{1}H-NMR and ^{13}C-NMR spectral data of compound C11 with 4',4''',7,7''-tetra-*O*-methylcupressuflavone reported by Ofman *et al.* [33] and Irfana *et al.* [25].

Carbon No.	Compound 1 isolated[a]		4',4''',7,7''-tetra-*O*-methylcupressuflavone[b] Ofman *et al.* [33]		4',4''',7,7''-tetra-*O*-methylcupressuflavone[c] Irfana *et al.*[25]	
	δ_H (*Intg.* mult. *J* Hz)	**δ_C (ppm)**	**δ_H (*Intg.* mult. *J* Hz)**	**δ_C (ppm)**	**δ_H (*Intg.* mult. *J* Hz)**	**δ_C (ppm)**
2 [2″]	-	164.8	-	162.4	-	162.94
3 [3″]	6.72 (2H; s)	104.1	6.97 (s)	102.3	6.71 (2H. *s*)	102.29
4 [4″]	-	183.6	-	181.2	-	181.73
5 [5″]	-	163.7	-	160.8	-	161.83
6 [6″]	6.68 (2H; s)	96.1	6.78 (s)	94.5	6.68 (2H. *s*)	94.24
7 [7″]	-	164.5	-	162.1	-	162.64
8 [8″]	-	100.4	-	-	-	98.55
9 [9″]	-	155.5	-	152.9	-	153.60
10 [10″]	-	105.7	-	103.3	-	103.83
1′ [1″]	-	124.0	-	113.6	-	122.16
2′/ 6′ 2‴/6‴	7.61 (4H; d; 8.9)	128.7	7.59 (d; 8.8)	126.7 126.7	7.60 (4H. *d*. 8.7)	126.78
3′/ 5′ 3‴/5‴	6.96 (4H; d; 9)	115.4	7.03 (d; 9)	113.6 113.6	6.96 (4H. *d*. 8.9)	113.53
4′ [4″]	-	163.7	-	161.3		161.86
5/5″-OH	13.36 (s)	-	13.22 & 13.32 (@ 1H. s)	-		
4′-OCH_3 4‴-OCH_3	3.82 (6H; s)	56.0	3.79 (s)	55.4 54.5	3.82 (6H. *s*)	54.08
7-OCH_3 7″-OCH_3	3.87 (6H; s)	56.9	3.81 (s) 3.80 (s)	55.4 54.5	3.88 (6H. *s*)	55.00

[a]NMR solvent using acetone-d_6 with ^{1}H-NMR frequency 500 MHz and ^{13}C frequency 125 MHz
[b]NMR solvent using DMSO-d_6 with ^{1}H-NMR frequency 500 MHz.
[c]NMR solvent using acetone-d_6 with ^{1}H-NMR frequency 500 MHz and ^{13}C frequency 125 MHz

Pharmacological Perspectives Based on Literature Studies

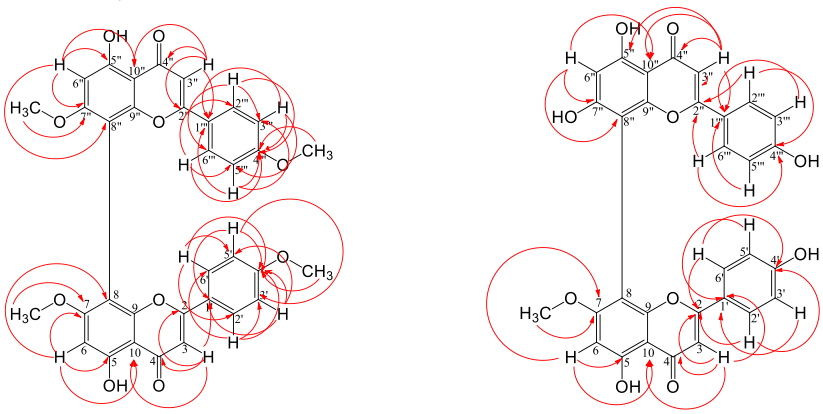
The pharmacological properties and structure-activity relationships (SAR) of the isolated biflavonoids were examined based on previously published data, given that no biological experiments were conducted in the present study. Compound **1** (4′,4‴,7,7″-tetra-*O*-methylcupressuflavone) and Compound **2** (7-*O*-methylcupressuflavone) have been reported to exhibit various bioactivities, including cytotoxic and α-glucosidase inhibitory effects. These literature-based insights provide valuable insights into the potential pharmaceutical applications of the isolated compounds.

In silico docking data from Sugita *et al.* [14] were assessed to strengthen the pharmacological analysis further. In that investigation, the binding affinities of 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone (**1**) and 7-*O*-methylcupressuflavone (**2**) were both greater than that of acarbose (−6.3 kcal mol^{-1}). Compound **2** showed the best experimental inhibition (IC_{50} = 68.75 μM), despite compound **1** exhibiting a slightly stronger theoretical affinity (more negative ΔG). This suggests that lower methoxylation can enhance enzyme-binding effectiveness and promote more favourable conformational interactions within the catalytic region. Compound **2** forms hydrogen bonds with Asp327, Thr205, and Tyr605, whereas compound **1** mainly interacts through Tyr605. Both compounds display hydrophobic and π-interactions with key residues Ala576, Asp203, Met444, Phe450, Tyr299, Asp542, Asp443, Arg526, Trp406, Phe575, and His600. These amino acids are essential components of the maltase–glucoamylase catalytic site, which governs catalysis and substrate binding. Consistent with the stronger biological activity of 7-*O*-methylcupressuflavone, the overlap of these binding interactions with known active-site residues indicates that the α-glucosidase inhibitory activity of Araucaria-derived biflavonoids results from a synergistic combination of hydrogen-bonding and hydrophobic stabilisation.

Table 2: Comparison of 1D ^{1}H-NMR and ^{13}C-NMR spectral data of compound C11 with 7-*O*-methylcupressuflavone reported by Sugita *et al.* [24] and Ofman *et al.* [33].

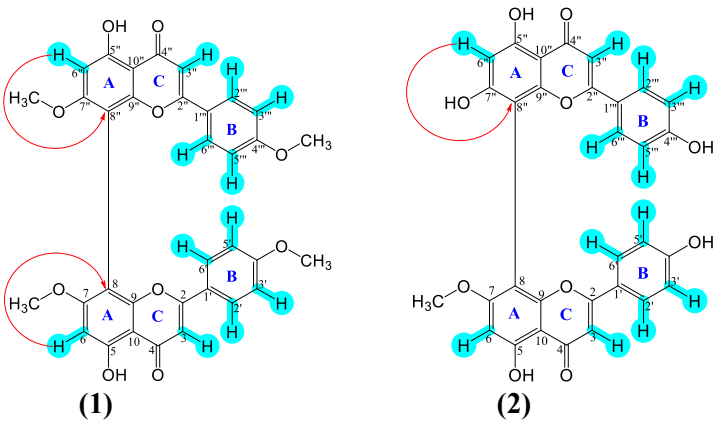
Carbon No.	Compound 2 isolated[a]		7-*O*-methylcupressuflavone[b] Sugita *et al.* [24]		7-*O*-methylcupressuflavone[c] Ofman *et al.* [33]	
	δ_H (*Intg.* mult. *J* Hz)	δ_C (ppm)	δ_H (*Intg.* mult. *J* Hz)	δ_C (ppm)	δ_H (*Intg.* mult. *J* Hz)	δ_C (ppm)
b2 (2")	-	163.62 (164.14)	-	165.3 (164.8)	-	163.3 (163.2)
3 (3")	6.79 (*s*.1H) [6.77 (*s*.1H)]	102.86 (102.45)	6.68 (*s*.1H) [6.67 (*s*.1H)]	103.7 (103.6)	6.82 *s* [6.78 *s*]	102.5 (102.5)
4 (4")	-	182.34 (181.59)	-	183.6 (183.4)	-	182.3 (181.9)
5 (5")	-	161.40 (161.33)	-	163.8 (163.1)	-	161.7 (161.3)
6 (6")	6.63 (*s*.1H) [6.41(*s*.1H)]	95.43 (94.14)	6.68 (*s*.1H) [6.53(*s*.1H)]	96.1 (99.6)	6.74 *s* [6.45 *s*]	99.5 (98.6)
7 (7")	-	162.67 (165.05)	-	164.8 (162.9)	-	164.0 (163.2)
8 (8")	-	105.07 (99.04)	-	100.1 (99.5)	-	95.4 (98.1)
9 (9")	-	153.62 (157.41)	-	155.8 (156.2)	-	154.7 (153.8)
10 (10")	-	104.14 (103.37)	-	105.7 (105.5)	-	114.3 (103.5)
1'(1"')	-	121.07 (120.86)	-	123.0 (123.0)	-	121.1 (121.1)
2' & 6' (2"' & 6"')	7.54 (*d*.*J*=8.45 Hz. 2H) [7.96 (*d*.*J*=8.85 Hz. 2H)]	128.29 (127.88)	7.60 (*d*.*J*=8.8 Hz. 2H) [7.53 (*d*.*J*=8.8 Hz. 2H)]	129.0 (128.8)	7.51 *d* (8.8) [7.43 *d* (8.8)]	128.1 (127.7)
3' & 5'(3"' & 5"')	6.69 (*d*.*J*=8.53 Hz. 2H) [7.13 (*d*.*J*=8.45 Hz. 2H)]	115.83 (116.22)	6.71 (*d*.*J*=8.53 Hz. 2H) [7.15 (*d*.*J*=9.09 Hz. 2H)]	116.8 (116.7)	6.75 *d* (8.7) [6.75 *d* (8.7)]	115.9 [115.9]
4'(4"')	-	161.45 (159.72)	-	161.9 (161.8)		161.1 (160.9)
5-OH (5"-OH)	13.23(*s*.1H) [12.92(*s*.1H)]		13.38 (*s*.1H) [13.22(*s*.1H)]		-	-
7-OCH_3	3.81 (*s*.3H)	56.41	3.88(*s*.3H)	56.8	3.80 *s*	56.4

[a]The NMR solvent used was DMSO-d_6 with a frequency of 500 MHz
[b]The NMR solvent used was Acetone-d_6 with a frequency of 500 MHz
[c]The NMR solvent used was DMSO-d_6



(1) **(2)**

Figure 3: HMBC correlation of 4'.4"',7,7"-tetra-*O*-methylcupressuflavone (**1**) and 7-*O*-methylcupresuflavone (**2**).



(1) **(2)**

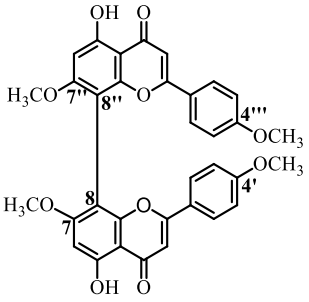
Figure 4: Correlation confirming the position of the biaryl bond at C-8–C-8″ of 4'.4'",7,7"-tetra-*O*-methylcupressuflavone (**1**) and 7-*O*-methylcupresuflavone (**2**).

Biflavonoids possess a unique structure, distinct characteristics, and a broad spectrum of biological activities. Based on literature reviews, the pharmacological activities of both compounds have been documented. Compound **1** was found to be inactive as an inhibitor of calf pulmonary artery endothelial (CPAE) cell proliferation (IC_{50} = 239.36 ± 13.50 µg/mL) [3], human umbilical vein endothelial cells (HUVEC) (IC_{50} > 100.0 µg/mL), and murine leukaemia P-388 cells (IC_{50} = 70.5 µg/mL) [32]. Furthermore, Compound **1** exhibited no significant inhibitory activity against MCF-7 and HeLa cancer cell lines, with IC_{50} values of 397.89 ± 28.6 µM and 528.78 ± 40.0 µM, respectively [24]. It was also inactive against the α-glucosidase enzyme, with an IC_{50} value greater than 10,000 ppm [14].

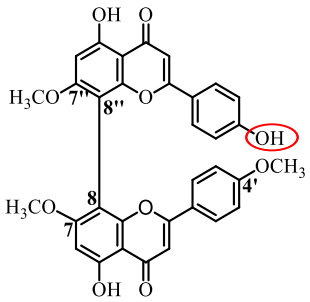
In contrast, compound **2** exhibited significant activity as an inhibitor of MCF-7 and HeLa cancer cells as well as the α-glucosidase enzyme, with IC_{50} values of 3.40 ± 0.3 µM. 1.42 ± 1.1 µM [24], and 78.32 ± 0.52 µM [14], respectively. According to Molfetta *et al.* [35], the difference in activity between the two compounds is likely due to the high number of methoxy (-OCH_3) groups, which reduce compound polarity. Compound **1** contains four -OCH_3 groups at positions C7, C4', C7", and C4"' while compound **2** has only one -OCH_3 group at position C7. The presence of methoxy groups at C4', C7", and/or C4"' appears to reduce bioactivity. Methylation at position C7 on the cupressuflavone framework seems to play a crucial role in inhibiting the proliferation of MCF-7 and HeLa cancer cells. A similar trend was also observed for the α-glucosidase enzyme. The in vitro results for compound **2** against α-glucosidase further support the in silico predictions [14].

The structure-activity relationships (SAR) of the isolated biflavonoids were also analyzed based on available literature. A key factor influencing bioactivity appears to be the number and position of methoxy (-OCH_3) groups on the biflavonoid scaffold. Compound **1**, possessing four methoxy groups at C-7, C-4', C-7", and C-4"', exhibited lower bioactivity, potentially due to reduced polarity and steric hindrance effects. In contrast, compound **2**, with only a single methoxy group at C-7, demonstrated significantly stronger cytotoxic and α-glucosidase inhibitory activities. These observations are consistent with prior studies indicating that free hydroxyl groups at positions C-4' and C-4" enhance hydrogen bonding to biological targets, thereby improving bioactivity [36]. Therefore, selective methylation patterns critically affect the biological properties of cupressuflavone derivatives. The structure-activity relationship of compounds **1** and **2** is illustrated in **Figure 5.**

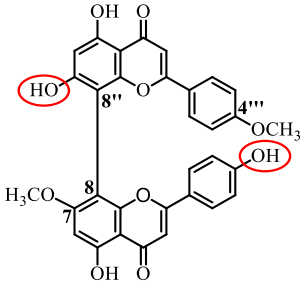
The presence of hydroxyl (-OH) groups at C4' and/or C4" on the B-ring of each monomer in biflavonoids influences their ability to inhibit the α-glucosidase enzyme, as observed in flavonoids from *Tinospora crispa* leaf extract [37]. The IC_{50} value of myricetin is lower than that of quercetin, kaempferol, and apigenin, indicating that myricetin exhibits more potent inhibition compared to the other three flavonoids. The key structural difference among these four flavonoids lies in the number of hydroxyl (-OH) groups present on the B-ring. These hydroxyl groups are suspected to play a direct role in the inhibitory activity of flavonoids against the α-glucosidase enzyme. In conclusion, the literature-based analysis highlights the noteworthy pharmacological potential of the isolated biflavonoids, despite the lack of direct biological experiments in this investigation. The results provide essential information for future drug development and chemotaxonomic research using Araucaria species, especially highlighting the significance of unique hydroxylation and methylation patterns in modifying biological activity.



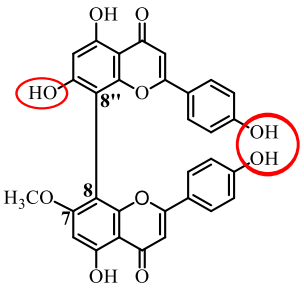
4',4''',7,7''-tetra-*O*-metilkupresuflavon (**1**)
IC_{50} 397,89 ± 28,6 µM (MCF-7)[a]
IC_{50} 528,78 ± 40 µM (HeLa)[a]
IC_{50} >10.000 ppm (α-glucosidase)[b]



4',7,7''-tri-*O*-metilkupresuflavon
IC_{50} 91,74 ± 5,6 µM (MCF-7)[a]



7,4'''-di-*O*-metilkupresuflavon
IC_{50} 11,54 ± 3,4 µM (MCF-7)[a]
IC_{50} 35,59 ± 1,3 µM (HeLa)[a]
IC_{50} = 537,98±2,35 µM (α-glucosidase)[b]



7-*O*-metilkupresuflavon (**2**)
IC_{50} 3,40 ± 0,5 µM (MCF-7)[a]
IC_{50} 1,42 ± 1,1 µM (HeLa)[a]
IC_{50} = 78.32 ± 0.52 (α-glucosidase)[b]

Figure 5: Structure-activity relationship of MCF-7 and HeLa cell inhibition and α-glucosidase enzyme from the cupressuflavone group. [a]Sugita *et al.* [24] and [b]Sugita *et al*. [14]

4. CONCLUSIONS

This study successfully isolated and characterized two biflavonoid compounds from the acetone extract of *A. columnaris* leaves and *A. hunsteinii* twigs, namely 7-*O*-methylcupressuflavone and 4',4''',7,7''-tetra-*O*-methylcupressuflavone. These compounds were confirmed by 1D and 2D NMR spectroscopy as well as LC-MS/MS analysis. The results indicate that 7-*O*-methylcupressuflavone exhibits more significant pharmacological activity than 4',4''',7,7''-tetra-*O*-methylcupressuflavone particularly in inhibiting MCF-7 and HeLa cancer cells as well as the α-glucosidase enzyme. This study reinforces the potential of biflavonoids from the Araucaria genus as bioactive compounds with applications in pharmaceutical research and chemotaxonomy. Further studies are needed to explore the structure-activity relationship and pharmacological mechanisms of these compounds.

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