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Isolation of Biflavonoids from Indonesian *Araucaria Cunninghamii* Mudie Leaves and Their Activity Against MCF7 Cells and 20S Proteasome

Luthfan Irfana[1], Dhea Demetri Agusta[2], Budi Arifin[1], Setyanto Tri Wahyudi[3], Suminar Setiati Achmadi[1] and Purwantiningsih Sugita[1,*]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia
[2]Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kyoto 6068502, Japan
[3]Department of Physics, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia

([*]Corresponding author's e-mail: purwantiningsih@apps.ipb.ac.id)

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Abstract

To enhance our survey of *Araucaria* biflavonoid's biological activity, *Araucaria cunninghamii* Mudie dried leaf samples from West Java, Indonesia were extracted using acetone, then purified by Sephadex column and silica gel radial chromatography to give 3 known pure compounds: 7,4′,7″,4‴-tetra-*O*-methyl cupressuflavone, 7,4′,4‴-tri-*O*-methylrobustaflavone, and 7,7″-di-*O*-methylamentoflavone. This is the first report of 7,4′,4‴-tri-*O*-methylrobustaflavone and 7,7″-di-*O*-methylamentoflavone isolation from *A. cunninghamii* Mudie species. Among the isolated compounds, 7,7″-di-*O*-methylamentoflavone showed the best activity towards the MCF7 cancer cell line (IC_{50} = 150 μM). The ELISA assay exhibited a 32 % decrease in the detected 20S proteasome concentration level in the MCF7 lysate samples treated with 25 ppm 7,7″-di-*O*-methylamentoflavone.

Keywords: Amentoflavone, Biflavonoid, Cupressuflavone, MCF7, Proteasome, Robustaflavone

Introduction

The *Araucaria* is a member of the *Araucariaceae*, which consists of 3 genera with 20 species of evergreen trees. These plants have been known to contain bioactive compounds and are traditionally utilized for ethnopharmacological purposes [1]. In a survey of bioactive compounds' local sources, our group has isolated and characterized biflavonoid compounds from 2 *Araucaria* species that grow in Indonesian soil. From the leaves of *Araucaria hunsteinii* K. Schuum, we have isolated 7,4′,7″-tri-*O*-methylcupressuflavone, 7,7″,4‴-tri-*O*-methylagathisflavone, 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone, 7,4‴-di-*O*-methylcupressuflavone, 7-*O*-methylcupressuflavone, 4′,4‴-di-*O*-methylamentoflavone (isoginkgetin), and 7,7″-di-*O*-methylagathisflavone [2,3]. The amentoflavones and cupressuflavones from *A. hunsteinii* have better *in vitro* activity against MCF7 and HeLa cells than the agathisflavones. We also have isolated 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone, 7,4′,7″-tri-*O*-methylcupressuflavone, and 7,4‴-di-*O*-methylcupressuflavone from the leaves of *Araucaria columnaris* [4]. The tri- and dimethyl ethers of cupressuflavone from *A. columnaris* demonstrated higher activity in inhibiting the proliferation of CPAE cells than the tetramethyl ethers. In this present study, we report the biflavonoids isolated from the dried leaves of *Araucaria cunninghamii* Mudie from Taman Bunga Nusantara, Indonesia, together with their associated biological activity, to improve our survey.

The polar fraction of *A. cunninghamii* leaves have been known to contain several types of biflavonoids such as 7,7″-di-*O*-methylcupressuflavone, 7,4′,7″-tri-*O*-methylcupressuflavone, 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone, 4′,4″,7,7″-tetra-*O*-methylamentoflavone, 4′,4‴,7″-tri-*O*-methylamentoflavone, and -4′,7″-di-*O*-methylamentoflavone [5-8]. Thin-layer chromatographic detection of several methyl ethers of agathisflavones and hinokiflavones has also been

reported [9]. In these classic literatures, the dried leaves were generally extracted using acetone, followed several separation techniques such as liquid-liquid extraction and chromatography, such as preparative thin layer chromatography (TLC) or magnesium silicate column chromatography and silica gel preparative TLC. The most recent study employing LC-HRMS identification of the leaves methanol extract also indicates the presences of agathisflavone derivatives, cupressuflavone methyl ethers, such as 4′,4‴- and 7′,7″-di-*O*-methylcupressuflavone, abietic acid, and β-sitosterol [10]. Another recent study on *A. cunninghamii* reported the isolation of 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone and other types of natural products, namely pheophytin a, *iso*-cupressic acid, methyl-(*E*)-communate, ladanein, and shikimic acid, utilizing ethanol extraction and silica gel chromatography with *n*-butanol, methanol, and distilled water gradient elution system [11]. Thus, the cupressuflavone and amentoflavone series are the most common biflavonoid types isolated from the leaves of *A. cunninghamii* species.

Biflavonoid compounds have been reported to be active as antioxidants, anti-inflammatory, antiviral, anti-diabetic, anti-atherosclerosis, Alzheimer's and Parkinson's disease drugs, cytotoxic agents, antitumor and anti-angiogenesis [12]. Isoginkgetin has been reported to trigger the apoptosis of HeLa, MDA-MB-468, and MM cancer cells caused by direct inhibition of 20S proteasome activity which results in paralyzing the NF-κB signalling pathway [13]. In addition, several flavonoid compounds, such as apigenin, quercetin, and luteolin, as well as the biflavonoid compound, moreloflavone, have been reported to actively inhibit 20S proteasome activity [14]. The 20S proteasome is the core part of the larger quaternary protein unit, the 26S proteasome. This enzyme, together with ubiquitin, is a major player in protein degradation, both in the cytosol and cell nucleus, thereby influencing cell protein homeostasis. Proteasomes are known to influence cell cycle control, apoptosis, DNA repair, transcription, immune responses, and various signalling through the degradation of important proteins such as p53. Cancer cells show a higher susceptibility to proteasome inhibition than normal cells due to their excessive proteasome activity [15]. The proteasome inhibitor MG-132 has also been reported to cause apoptosis of HeLa cell lines by inhibiting P20S and increasing the production of MCPIP1, a natural inhibitor of the transcription factor NF-κB [16]. Meanwhile, resistance of MCF7 breast cancer cells to the drug Docetaxel can be overcome by administering a proteasome inhibitor which can again trigger apoptosis [17]. Therefore, the possibility of 20S proteasome inhibition by biflavonoid as the cause of their anticancer activity gaining our interest in this work.

This study aims to isolate as well as determine the structure and bioactivity of biflavonoid compounds from *A. cunninghamii* Mudie from Taman Bunga Nusantara, Indonesia. Three known compounds have been successfully isolated and determined in this work (**Figure 1**) namely 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone (1), 7,4′,4‴-tri-*O*-methylrobustaflavone (2), and 7,7″-di-*O*-methylamentoflavone (3). To explore the bioactivity of the isolated compounds we employed an *in vitro* MTT assay against the MCF7 cell line and quantified the 20S proteasome concentration of the lysate with ELISA assay. To the best of our knowledge, this is the first work that reports the isolation of 7,4′,4‴-tri-*O*-methylrobustaflavone and 7,7″-di-*O*-methylamentoflavone from *A. cunninghamii* Mudie.

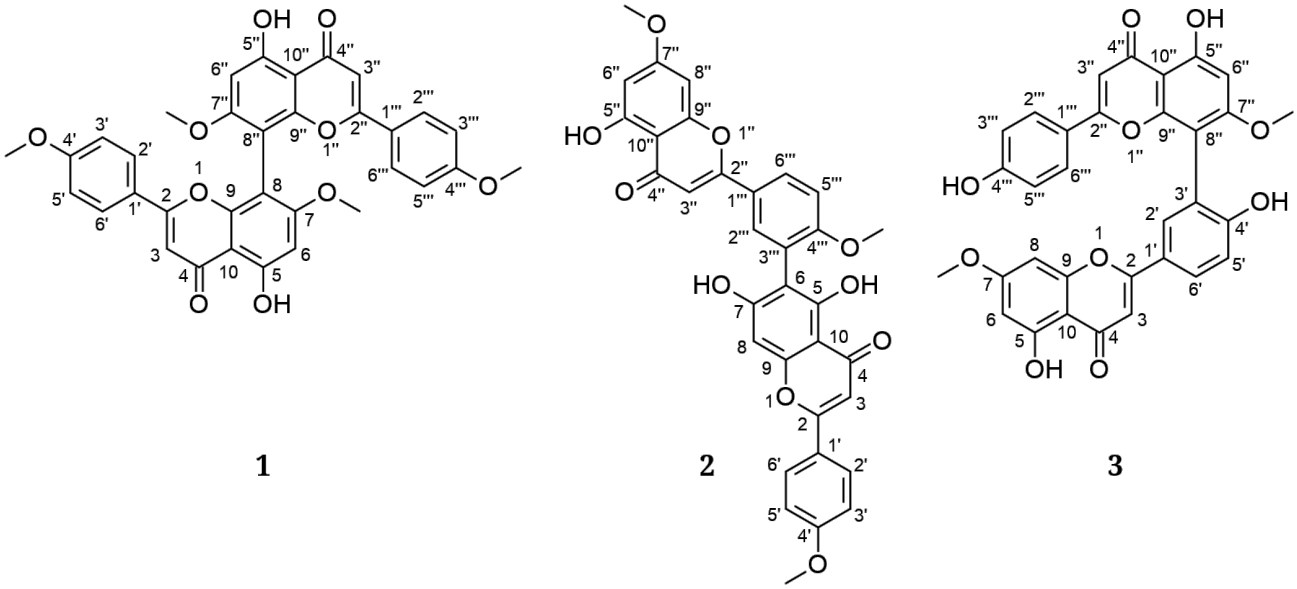

Figure 1 Biflavonoid structures isolated from *Araucaria cunninghamii* Mudie in this work: 7,4′,7″,4‴-tetra-*O*-methylcupressuflavone (1), 7,4′,4‴-tri-*O*-methylrobustaflavone (2), and 7,7″-di-*O*-methylamentoflavone (3).

Materials and methods

Plant sample

Samples of *A. cunninghamii* leaves were obtained from the Taman Bunga Nusantara, Cianjur, West Java, Indonesia (6.7276° S, 107.0794° E), on December 1, 2023. The factors underlying the selection of sample locations were the relatively close distance to the IPB University campus, the large number of *Araucaria* plant collections, and the phytochemical content of the samples that had never been studied before. The sample has been determined by *Herbarium Bandungense SITH ITB (FIPIA)*, Indonesia, as *Araucaria cunninghamii* Mudie with specimen voucher number FIPIA-DEP64.

Chemicals

Sephadex LH-20 (GE Healthcare), silica gel 60 PF254 (Merck), silica gel F254 TLC plate (Merck), Merck pro analysis solvents such as acetone, hexane, methanol, chloroform, and dichloromethane; MCF7 cells (ATCC HTB 22), 3-(4,5-dimethyliazol-2-il)-2,5-diphenyltetrazolium bromide (MTT, Merck), D-MEM, RPMI 1640, FBS 5 %, *Penicillin* 100 U/mL, and streptomycin 100 µg/mL, and Proteasome Elisa Kit (BML-PW0575, Enzo).

Instruments

Ultraviolet–visible (UV-Vis) spectrophotometer (Thermo Scientific Genesys 10), liquid chromatography tandem mass spectrometer (LC-MS/MS, Waters), nuclear magnetic resonance (NMR) DD2 console system (Agilent), and microplate reader (Bio-Rad).

Extraction and isolation

The extraction and isolation of biflavonoid compounds in this work are summarized in **Figure 2**. The dried leaves sample (711.4 g) were extracted 3 times using acetone at room temperature for 24 h each. The combined crude extract (68.9 g) was subsequently concentrated under vacuum. The extract was partitioned between hexane and methanol (1:2) to give a methanol-soluble fraction (44.2 g). The concentrated methanol fraction was then redissolved in acetone and filtered to separate the acetone-soluble fraction (38.8 g) and tannins precipitate (5.4 g). Every 5 g portion of the concentrated acetone-soluble fraction was separated further by vacuum column chromatography (3.0×85.0 cm^2) using Sephadex LH-20 (150 g) and methanol as the eluent. TLC with chloroform-methanol (19:1) eluent and cerium sulfate colouring agent was used to group the 14 fractions as flavonoids (F6, F7-8, and F10-13) and non-flavonoids. The flavonoid fractions were subsequently purified by gradient elution silica gel radial chromatography. The typical gradient elution starts from 100 % hexane, hexane-dichloromethane (DCM) (1:1), 100 % DCM, DCM-ethanol (100:1, 75:1, 50:1, and 30:1), then purged with methanol and methanol-water.

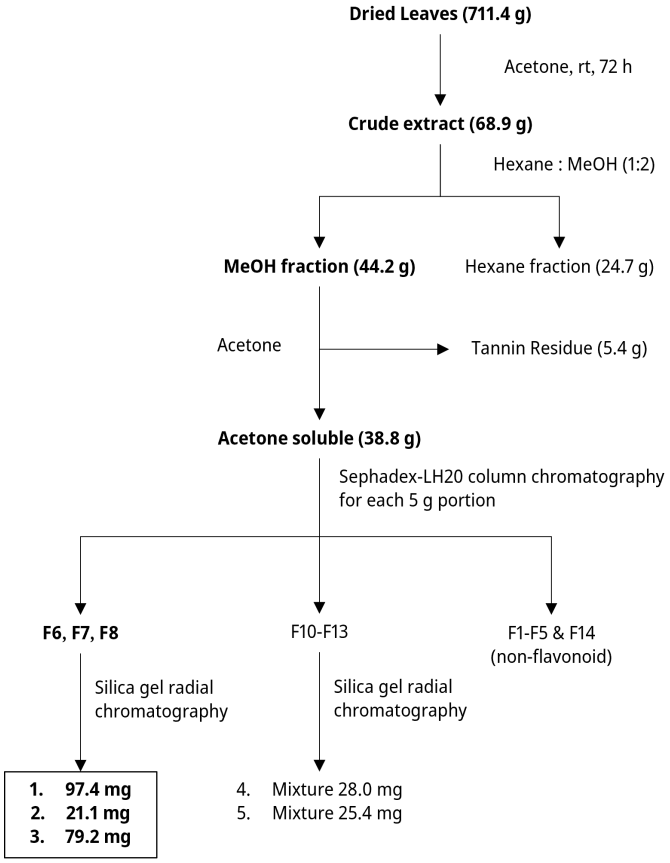

Figure 2 Isolation steps of *A. cunninghamii* biflavonoids.

Structure elucidation

The structure of the isolated compounds were determined by spectroscopic measurement that includes: UV-Vis spectra in methanol solvent (100 ppm); LC-MS/MS-C18 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), COSY, HMBC, and HSQC in acetone-d6 solvent.

Cell viability MTT assay

MCF7 cells (ATCC HTB 22) were grown at a concentration of 5000 cells in 100 µL of growth medium. DMSO solution series of 1 - 3 were added in triplicate wells after the cells reached 50 % confluency (24 h) except for the negative control wells. The selection of the treatment concentration range used in this study was based on our previous work [3]. The MTT test was carried out on the third day, by adding 10 µL MTT (5 mg/mL) in ethanol per well with 4 h incubation time at 37 °C. Optical density reading was carried out with a measurement filter at 595 nm and a reference filter at 655 nm using a microplate reader.

Cell lysate 20S proteasome level ELISA assay

This assay was conducted by following the general procedure explained by the manufacturer of the 20S Proteasome ELISA Kit. The 96-well plate for ELISA analysis was prepared by dispensing 100 µL of a diluted 1:500 solution of Proteasome 20S alpha6 subunit monoclonal antibody into each well of the plate using a pipette. Afterwards, the plate was sealed with plastic wrap and incubated at 4 °C overnight. Subsequently, 400 µL of 1X Wash Buffer is introduced into each well, followed by the addition of 300 µL of blocking buffer (BML-KW0855-0030), and incubation for 2 h at ambient temperature. MCF7 cells (ATCC HTB 22) were grown and treated with compounds 1 - 3 with the same procedure described in the MTT assay section. Cell lysate samples were prepared by washing the samples and control cells with PBS, lysing with lysis buffer

(BML-KW0865-0005), briefly vortexing, incubation for 15 min at 4 °C, centrifuging at 10,000 rpm for 5 min, and transferring the supernatant to a separate tube. Next, 100 µL of ELISA Buffer (BML-KW0860-0100), 20S Proteasome (BML-PW8720-0010) dilutions (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 µg/mL), and cell lysate samples are pipetted onto the ELISA plate. Subsequently, the plate is sealed tightly and placed in an incubator at room temperature for 1 h. The contents are subsequently rinsed with 400 µL of 1X wash buffer, repeated 4 times, and the plate is then rinsed with 100 µL of TMB substrate. Ultimately, a volume of 100 µL of Stop Solution 2 (1N HCl solution in water) is introduced into every well. The measurement of optical density is taken at 450 nm with the correction factor reads between 570 and 590 nm with the same microplate reader as the MTT assay.

Statistical analysis

Plate reader raw data from each activity assay was statistically analyzed with the statistical software R. Each data was tested for normality and outlier detections before inferential statistic testing with ANOVA and *t*-test. Apart from blank corrections, data preprocessing may include median polish to account for row and column effects from the relative position of the well. The optical density (OD) ratio (treatment to control) and compound concentrations were fitted to the *n*-parameter log-logistic functions (n = 2, 4, or 5) to get the estimate of IC_{50} values with the R package nplr [18]. Each IC_{50} estimate was reported together with the standard error of estimation at a 2-tailed 95 % confidence interval.

Results and discussion

Biflavonoid isolation results

Maceration of 711.4 g of *A. cunninghamii* Mudie dried leaves yielded 9.68 % dry weight of crude acetone extract. The subsequent fractionations of the crude extract then outcomes 56.34 % acetone soluble fraction, 35.86 % hexane soluble fraction, and 7.80 % tannins by crude extract weight. Furthermore, the Sephadex column followed by silica gel radial chromatography of the acetone soluble fraction yielded flavonoid fractions F6, F7, and F8 (**Figure 2**). Compound 1 was obtained from radial chromatography eluate of F6 as well as parts of F7 and 8. Meanwhile, other radial chromatography eluates of F7 and 8 gave compounds 2 and 3. On the other hand, F10-13 yielded mixtures that were too small in quantity to purify. Thus, only 3 compounds can be analyzed in the next steps. Although not as common as the other biflavonoid series, parent and substituted robustaflavone compounds have been isolated from other *Araucaria* species, such as robustaflavone from *Araucaria rulei* F. Muell Iyas *et al*. [19], 7″-*O*-methylrobustaflavone from *Araucaria angustifolia* (Bert) O. Kuntze Freitas *et al*. [20], and 7,4′,7″,4‴-tetra-*O*-methylrobustaflavone from *Araucaria columnaris* (G. Forst) Hook [21]. Compound 3 was first isolated from *Araucaria excelsa* Lamb from India [22]. More recent works have isolated 3 from other types of plants, such as *Podocarpus imbricatus* Gu *et al*. [23] and *Ouratea castaneifolia* [24].

Structure elucidation of compound 1

The ^{1}H and ^{13}C NMR shifts of all isolated compounds are summarized in **Tables 1** and **2**, respectively. All spectral information is available in Supporting Information S1. UV measurement of 100 ppm solution of 1 in MeOH exhibits absorption maxima at 269 nm (log ε = 3.6) and 322 nm (log ε = 3.5). Shift reagents only show the presences of strongly chelated 5/5″-OH groups, 1+AlC_3: 278 nm (log ε = 3.6), 341 nm (log ε = 3.5); 1+AlC_3+HCl: 278 nm (log ε = 3.6), 341 nm (log ε = 3.5)). LC-MS detected the largest peak at *Rt* 13.49 min with ESI-MS *m*/*z* 595.1612 $[M+H]^+$ calculated for $C_{34}H_{26}O_{10}$, indicating 2 apigenin units with 4 methyl substituents. Comparison of ^{1}H and ^{13}C NMR signals of **1** with several literatures [3,4,25-27] show good agreement. Overall, the ^{1}H and ^{13}C NMR spectra of **1** shows the characteristic simplicity of a symmetrical chemical environment, together with the non-existent C8/8″ proton signals, this concludes that the 2 apigenin units were 8–8″-linked. 2D HMBC measurement verified the connectivity of proton and carbon signals of 1 (**Figure 3(a)**).

Table 1 ^{1}H NMR signals of isolated compound 1 - 3 recorded in acetone-d_6 at 500 MHz.

Proton position	Chemical shift, δ/ppm (integration, multiplicity)		
	Compound 1	**Compound 2**	**Compound 3**
3	6.71 (2H, *s*)	6.79 (1H, *s*)	6.77 (1H, *s*)
6	6.68 (2H, *s*)	6.30 (1H, *d*, 2.1)	6.42 (1H, *s*)
8	-	6.66 (1H, *d*, 2.2)	7.00 (1H, *s*)
2′	7.60 (4H, *d*, 8.7)	8.14 (1H, *d*, 2.5)	7.69 (1H, *d*, 2.7)
3′	6.96 (4H, *d*, 8.9)	-	-
5′	3′	7.37 (1H, *d*, 8.7)	7.07 (1H, *d*, 8.8)
6′	2′	8.17 (1H, *dd*, 8.7, 2.5)	8.03 (1H, *d*, 8.7)
3″	3	6.70 (1H, *s*)	6.68 (1H, *s*)
6″	6	-	6.67 (1H, *s*)
8″	-	6.46 (1H, *s*)	-
2‴,3‴	2′	7.64 (2H, *d*, 8.8)	7.53 (2H, *d*, 8.4)
5‴, 6‴	3′	6.93 (2H, *d*,8.7)	6.86 (2H, *d*, 8.4)
5-OH	13.35 (2H, *s*)	12.93 (1H, *s*)	13.09 (1H, *s*)
4′-OH	-	-	9.29 (1H, *s*)
5″-OH	5-OH	13.12 (1H, *s*)	13.36 (1H, *s*)
7″-OH	-	9.55 (1H, *s*)	-
4‴-OH	-	-	9.33 (1H, *s*)
7-OCH_3	3.88 (6H, *s*)	3.87 (1H, *s*)	3.87 (1H, *s*)
7″-OCH_3	7-OCH_3	-	3.80 (1H, *s*)
4′-OCH_3	3.82 (6H, *s*)	3.86 (1H, *s*)	-
4‴-OCH_3	4′-OCH_3	3.79 (1H, *s*)	-

Structure elucidation of compound 2

LC-MS showed that 2 eluted at *Rt* 12.562 min, with ESI-MS *m*/*z* 581.1448 $[M+H]^+$ with molecular formula $C_{33}H_{25}O_{10}$. The UV spectrum of 100 ppm 2 solution in MeOH shows absorption at λ_{max} 272 nm (log ε = 3.6) and 325 nm (log ε = 3.5). Besides showing chelated 5/5″-OH groups existence (2+$AlCl_3$: 301 (log ε = 3.5), 338 (log ε = 3.5); 2+$AlCl_3$+HCl: 301 (log ε =3.5), 342 (log ε = 3.5)), shift reagents also hinted the presence of 7/7″-OH in which band II shifted from 272 nm to 316 nm (log ε = 3.5) after addition of NaOAc solution. The proton NMR assignment of 2 shows 7″-OH as a broad signal ($\Delta\nu_{½}$ = 100 Hz) at δ = 9.55 ppm which cannot be differentiated from the baseline. Several factors such as the sample's pH, measurement temperature, type of solvent, or impurities *e.g*. proton exchange with trace H_2O may lead to the broadening of free phenolic proton NMR signal [28]. Comparison between 2 and published NMR data of 7,4′,4‴-tri-*O*-methylrobustaflavone Chen *et al*. [29] shows agreement with maximum chemical shift differences of 2.4×10^{-3} Hz. Furthermore, the 3‴,6-bond between the 2 apigenin units in 2 is supported by observed HMBC correlation (**Figure 3(b)**) between the proton of C2‴ and C6 and C7.

Table 2 ^{13}C NMR signals of isolated compound 1 - 3 recorded in acetone-d_6 at 125 MHz.

Carbon position	Chemical shift, δ/ppm		
	Compound 1	Compound 2	Compound 3
2	162.94	162.65	163.37
3	102.29	102.25	102.56
4	181.73	181.53	181.40
5	161.83	160.76	160.16
6	94.24	126.95	97.83
7	162.64	160.56	162.60
8	98.55	97.83	89.41
9	153.60	153.92	157.09
10	103.83	103.48	103.61
1’	122.16	122.34	121.26
2’	126.78	126.86	126.85
3’	113.53	113.44	122.52
4’	161.86	161.72	160.31
5’	113.53	113.44	115.10
6’	126.78	126.86	127.50
2’’	162.94	163.12	163.15
3’’	102.29	102.98	101.72
4’’	181.73	181.30	181.75
5’’	161.83	161.15	161.79
6’’	94.24	96.94	94.19
7’’	162.64	164.71	161.69
8’’	98.55	91.40	103.89
9’’	153.6	156.86	154.33
10’’	103.83	104.18	103.75
1’’’	122.16	122.21	121.10
2’’’	126.78	130.26	127.01
3’’’	113.53	120.94	114.98
4’’’	161.86	160.15	163.30
5’’’	113.53	110.62	114.98
6’’’	126.78	127.26	127.01
OMe-7	55.00	-	54.98
OMe-4’	54.08	54.03	-
OMe-7’’	55.00	-	54.03
OMe-4’’’	54.08	54.96	-

Structure elucidation of compound 3

Compound 3 detected by LC-MS at *Rt* 10.681 min, with *m/z* 567.1288 $[M+H]^+$, calculated for $C_{32}H_{22}O_{10}$. Solution of 3 in MeOH shows UV absorption maxima at 272 nm (log ε = 3.6) and 316 nm (log ε = 3.5). The presence of phenolic OH groups at C4′ and 4‴ hinted by

the shifting of band II from 316 to 386 nm (log ε = 3.5) after treatment with NaOAc solution. The proton NMR confirmed the 4′- and 4‴-OH groups as weak singlet signals at 9.29 and 9.33 ppm (**Table 1**) possibly due to exchange with H_2O impurity. The characteristic 3′–8″ linkage between 2 apigenin units of 3 is verified by HMBC correlation between the proton of C2′ (7.69 ppm) and C7″ signal at 161.69 ppm. Apart from the more up-field phenolic free proton signals that may have been caused by the difference in the deuterated solvent used, NMR data of 3 obtained here agrees with available recent published references [23,24].

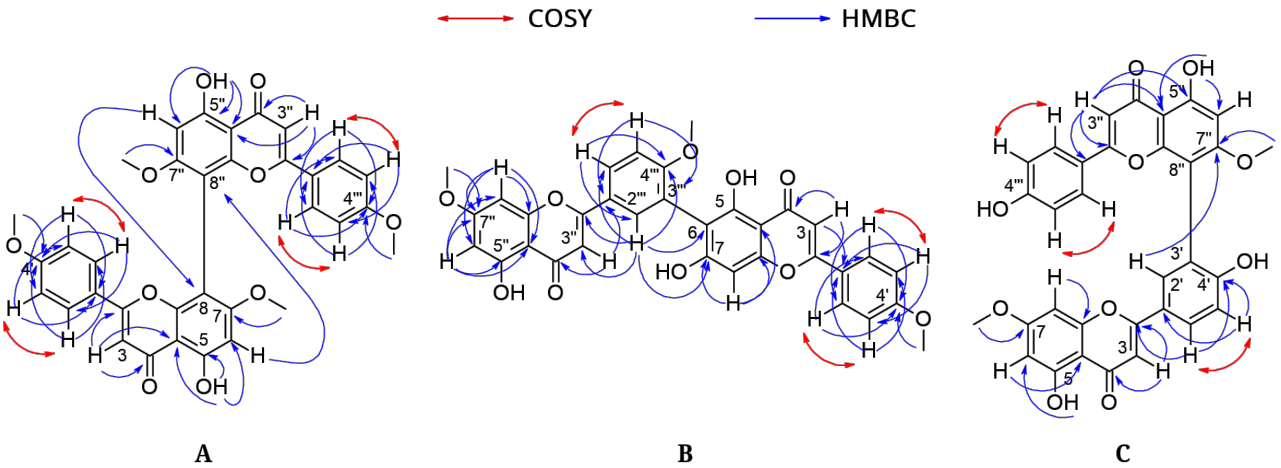

Figure 3 Observed COSY and HMBC correlations of 1 (A), 2 (B) and 3 (C).

***In-vitro* MTT assay to MCF7 cells**

The cytotoxic activity of compounds 1 - 3 on the MCF7 cell line was estimated with an MTT assay (**Figure 4**). MCF7 cell samples were treated with different biflavonoid concentrations (0, 31.25, 62.5, 125, 250 and 500 ppm) for 72 h before the MTT addition. For every compound, the ANOVA test (α = 0.05) concludes that treatment doses give significantly different OD responses. According to the 2-sided *t*-test (α = 0.05), all treatments are significantly different compared to the untreated control. To estimate the IC_{50} value, assay data of each compound was fitted to the most suitable (goodness of fit > 90 %) logistic models (Supporting Information S2). Among the isolated biflavonoids, the amentoflavone compound, 3, shows the highest *in vitro* activity estimate (IC_{50} = 150.04 ± 23.97 µM) followed by the cupressuflavone, 1 (IC_{50} = 1301.80 ± 173.86 µM), and robustaflavone, 2 (IC_{50} = 2503.91 ± 206.25 µM). The choice of concentration range is not ideal for accurate estimation, especially for 2 and 3. Compared with our previous linear regression estimates towards the cell line [3], compound 1 is estimated to have significantly lower activity with a wider confidence interval against MCF7 cells. Nevertheless, OD is a sum of various complex factors at the cellular level, including MTT uptake and formazan crystals extrusion, that could be affected by various experimental conditions [30]. The pre-assay and assay optimization hopefully will be the subjects of our future follow-up study. The MCF7 control cell morphology generally possesses an epithelial-like appearance and is assembled into a dome cluster (**Figure 5**). Anticancer drugs such as doxorubicin have been reported to change MCF7 cells morphology to be more rounded, condensed and detached from their cluster [31]. Visual comparison against untreated control showed that treatment with 62.5 ppm compound 3 was the lowest concentration that caused visible morphological disruption. At the same treatment concentration, the number of rounded detached cells is less apparent in compounds 1 and 2, consistent with the *in-vitro* IC_{50} levels trend observed in this study.

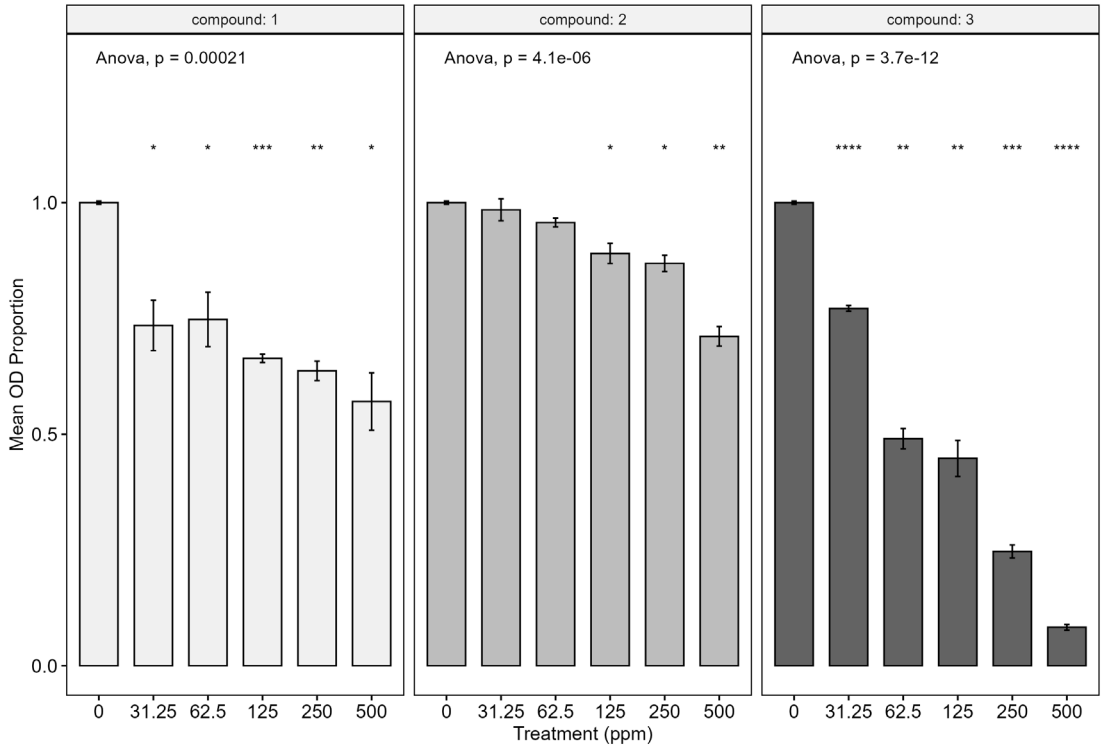

Figure 4 MTT assay results measured by optical density (OD) at 595 nm.

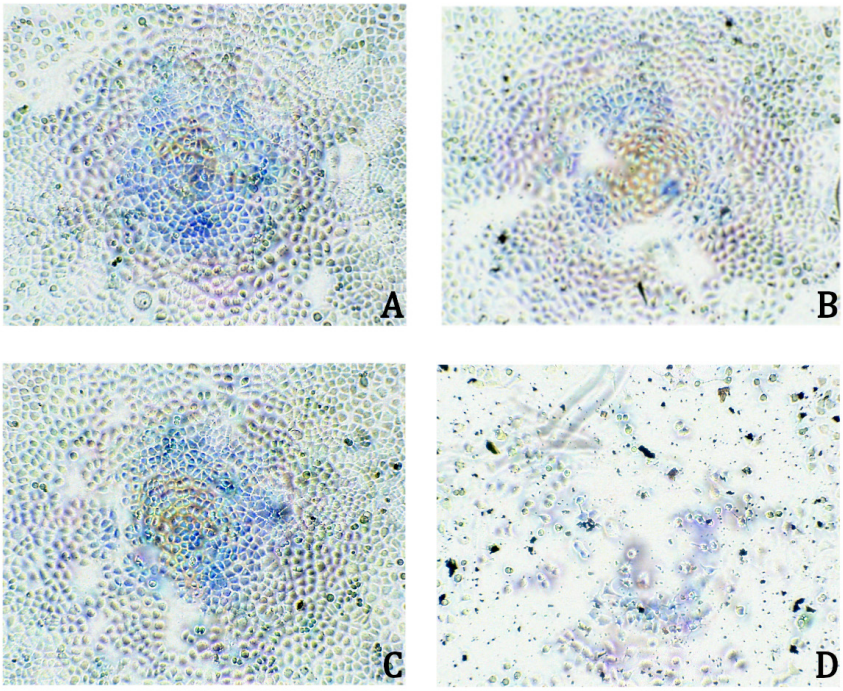

Figure 5 Light microscope observation at 400× magnification of untreated (control, A) and treated (6.25 ppm of compound 1 (B), 2 (C) and 3 (D)) MCF7 cell samples.

MCF7 cell lysates' 20S proteasome level

Based on the *in-vitro* IC_{50} trend obtained, isolated biflavonoid effects on proteasome levels in MCF7 lysate samples were measured by ELISA assay in different concentration ranges (**Figure 6**). Compared to untreated control (*t*-test; $\alpha = 0.05$), 3 is estimated to lower 32 % (*p*

= 0.0172) proteasome level at 25 ppm (44.13 μM), while 1 and 2 at 1000 ppm can only reduce 28 (p = 0.0203) and 16 % (p = 0.0289), respectively. However, the test that we have conducted is not suitable for estimating the IC_{50} of the isolated compound to 20S proteasomes, since the ELISA kit cannot distinguish between the active and inactive form of the protein target. Proteasome inhibitor (MG132) induced G2/M arrest in MCF7 cells which resulted in apoptosis; However, an autophagy inhibitor (3-methyladenine) was needed to keep the cancer cells from recovering proliferative capacity after treatment removal [32]. Unfortunately, there is no anti-proteasome activity assay reported yet for compounds 1 - 3. However, the neuroprotective activity of compounds 1 and 3 against amyloid-β42-induced toxicity in rat PC-12 cells have been reported to be 46.7 and 52.6%,respectively [33]. In addition, compound 3 is also classified as active against β-secretase 1 (BACE1, IC_{50} = 6.25 μM) [34] and compound 1 has IC_{50} above 50 μM against amyloid-β precursor protein [35]. Meanwhile, the ubiquitin-proteasome system has been confirmed to act as a modulator of neurodegeneration in several neurodegenerative diseases [36].

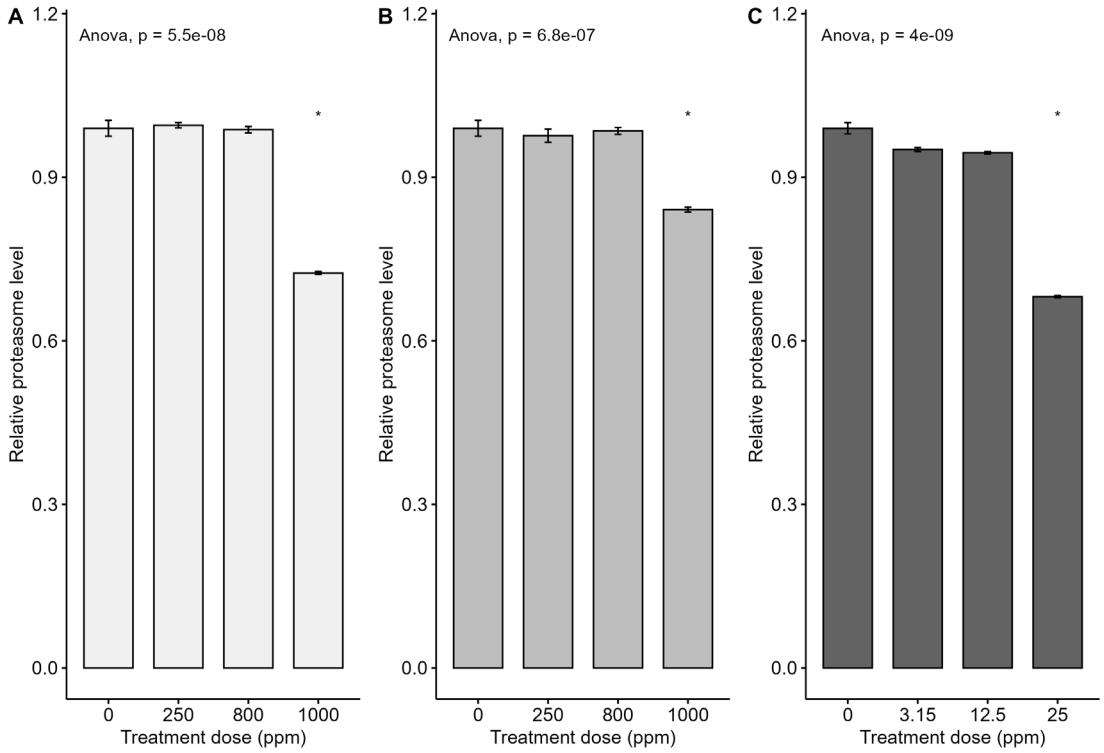

Figure 6 ELISA assay results measuring 20S proteasome level at MCF7 cell lysates treated with 1 (A), 2 (B) and 3 (C).

Conclusions

Three known biflavonoids have been isolated from *Araucaria cunninghamii* leaves samples from Indonesia. This is the first report of 7,4′,4″-tri-*O*-methylrobustaflavone (2) and 7,7″-di-*O*-methylamentoflavone (3) isolation from *A. cunninghamii* Mudie species. The 3 compounds showed moderate (3) to weak (2 & 1) activity towards the MCF7 breast cancer cell line as MTT results suggest. ELISA assays reveal that treatment with the isolated biflavonoids decreases measured 20S proteasome concentrations on MCF7 cells lysates in the same order as their cytotoxicity. However, further studies are needed to reveal the underlying connection between the isolated biflavonoids' cytotoxicity and proteasome level-lowering activity.

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