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LC–MS/MS CHARACTERIZATION OF BIOACTIVE SECONDARY METABOLITES FROM TWIGS OF *ARAUCARIA CUNNINGHAMII*

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ABSTRACT

Araucaria cunninghamii is a member of the Araucaria genus known to contain diverse bioactive secondary metabolites. This study aimed to identify and characterize the secondary metabolites present in the twigs of *A. cunninghamii* using LC–MS/MS. Methanolic extracts of *A. cunninghamii* twigs were chromatographically fractionated and analyzed using LC–HRMS/MS. Fraction FAn14D revealed six compounds, including meranzin and osthol (coumarins), phenylpropanoic acid and benzoic acid (phenolic acids), 4′,4‴,7,7″-tetra-*O*-methylcupressuflavone (biflavonoid), and (2*E*)-3-(3,4-dihydroxyphenyl) acrylate 9,19-cyclolanost-24-en-3-yl (triterpenoid lanostanoid). Benzoic acid was the predominant compound (45% peak area. negative ionization mode). while dioctyl adipate was identified as an instrumental artifact.

KEYWORDS: *Araucaria cunninghamii*, benzoic acid, biflavonoid, LC-MS/MS, secondary metabolites.

INTRODUCTION

The genus Araucaria (family Araucariaceae) comprises 19 species of evergreen conifers distributed in tropical and subtropical regions. Members of this genus are recognized as rich sources of biflavonoids, a distinctive class of dimeric flavonoids that exhibit a wide range of biological activities, including antibacterial,[1] antimicrobial,[2,3] antifungal,[4] antioxidants,[4–12] antiviral,[2,8–11] antitumor,[13] anticancer,[14–19] antidiabetic,[20–24] and anti-inflammatory effects.[12,21,25,26,27]

Previous studies have successfully isolated nine biflavonoids classified as cupressuflavone, amentoflavone, agathisflavone, and robustaflavone types from leaves acetone extract of *A. hunsteinii*, *A. columnaris*, and *A. cunninghamii*.[7,18,19,28] Samples of *A. hunsteinii* and *A. columnaris* were collected from the Bogor Botanical Garden. Indonesia. whereas *A. cunninghamii* was obtained from Taman Bunga Nusantara. Indonesia. However, research on the twigs of *A. cunninghamii* remains scarce compared with its leaves. Chen *et al.*[1] identified several metabolites, including 5-*p*-cis-coumaroylquinic acid, quinic acid, and biflavonoids such as 5,5″-dihydroxy-7,7″,4′,4‴-tetramethoxy biflavone and 4′,7,7″-trimethoxy cupressuflavone, from twig and leaf extracts of *A. cunninghamii*, while labdane-type diterpenes were found in its resin.[29] Environmental factors such as light intensity, temperature, humidity, and geographical location can influence the biosynthesis of secondary metabolites. Considering these gaps this study aimed to characterize and identify secondary metabolites from *A. cunninghamii* twigs using LC–HRMS/MS, thereby providing a foundation for future investigations on their bioactivity and pharmacological potential.

MATERIALS AND METHODS

Twigs of *A. cunninghamii* were collected from Taman Bunga Nusantara, Cianjur, Indonesia (voucher: FIPIA-DEP61). Reagents included analytical-grade methanol, n-hexane, Merck® silica gel 60 F_{254} (0.25 mm), $CeSO_4 \cdot 4H_2O$, and LC-MS/MS-grade solvents. The instruments used included standard laboratory glassware, a Sephadex LH-20 column chromatography setup (30 × 20 cm), and a Thermo Scientific Vanquish Flex UHPLC system coupled with a Q Exactive Plus Orbitrap High-Resolution Mass Spectrometer (Thermo Scientific, Munich, Germany). Data acquisition was performed with Xcalibur 2.0.7, and metabolomic analysis was conducted using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

Fifty grams of dry twigs were macerated in methanol (1:5 w/v) for 72 hours with solvent renewal every 24 hours at room temperature. The filtrates were pooled and concentrated under reduced pressure. The crude extract was defatted with n-hexane and re-dissolved in methanol and treated to remove chlorophyll and tannins. Silica-gel column chromatography (CC) was performed after vacuum liquid chromatography (VLC) on the resultant fraction. A C18 column with a mobile phase consisting of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid was used for the LC-HRMS/MS analysis of the most simplified fraction (FAn14D) during a 33-minute gradient program at a flow rate of 0.2 mL/min.

RESULTS AND DISCUSSION

Using both ionization modes enhanced metabolite coverage and ensured the detection of compounds with varying polarity. (**Figure 1.).** Triterpenoids, biflavonoids, phenolic acids, and coumarins were the six secondary metabolites that were found (**Table 1.).** Benzoic acid was the predominant metabolite (45% area), followed by minor components such as meranzin, ostol, and 4′,4‴,7,7″-tetra-O-methylcupressuflavone. The characteristic fragmentation peaks at m/z 135 and 121 confirmed the presence of biflavonoid-type compounds.

Two phenolic acids, 3-phenylpropanoic acid (m/z 150.0681 $[M+H]^+$, *rt* 20.39 min) and benzoic acid (m/z 122.0359 $[M+H]^+$, *rt* 6.85 min), were detected. Similar derivatives have been reported in *A. cunninghamii* twigs.[1] 3-Phenylpropanoic acid, previously isolated from *Nicotiana spp*., exhibits antibacterial activity against *Staphylococcus aureus.*[30]

The biflavonoid identified at a retention time of 22.79 min (m/z 594.1526 $[M+H]^+$) corresponded to 4′,4‴,7,7″-tetra-O-methylcupressuflavone, whose MS fragmentation pattern (**Figure 2**) matched that reported by Sugita et al.,[18] Kurniawanti et al.[7] and Sugita et al.,[31] This compound is newly reported in the twigs of *A. cunninghamii*.

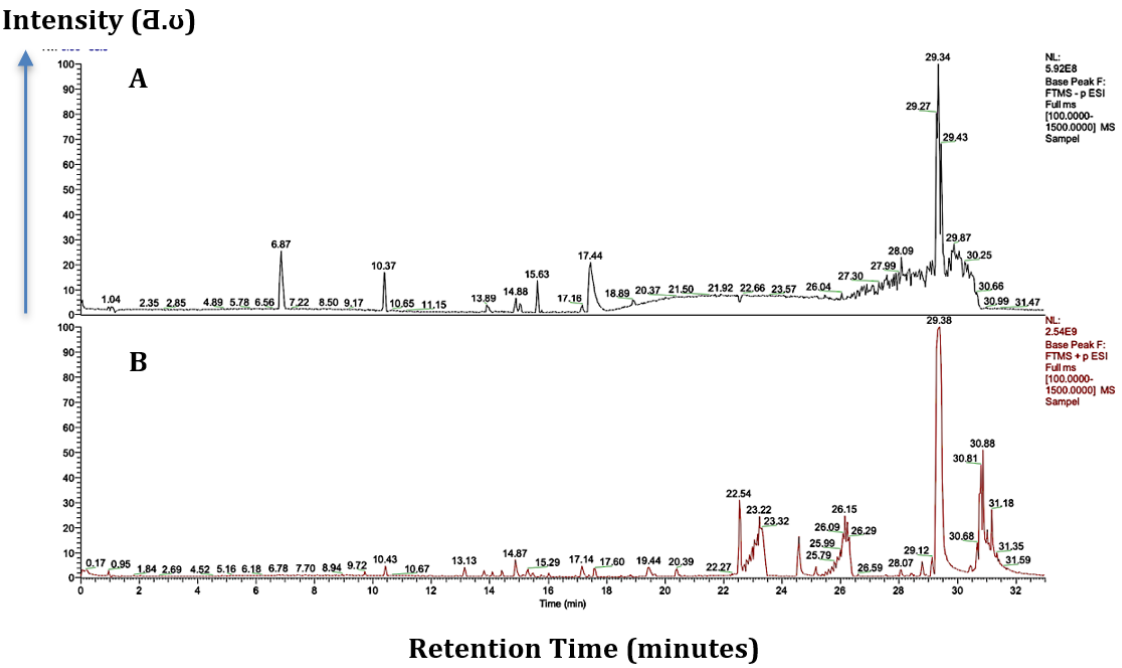


Figure 1: Base peak chromatogram of *A. cunninghamii* twig extract in (A) negative and (B) positive modes.

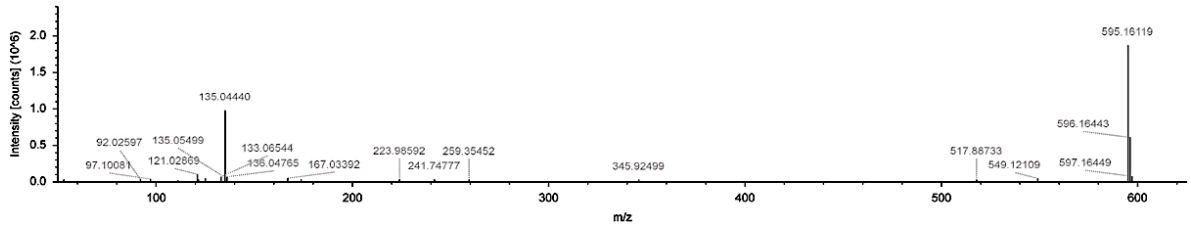
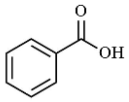
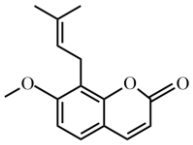
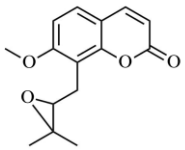
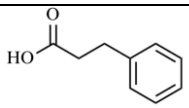
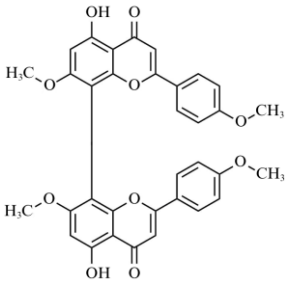
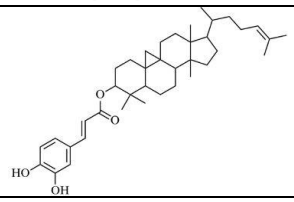
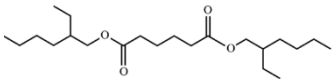


Figure 2: Mass spectrum identified at a retention time of 22.79 min (m/z 594.1526 $[M+H]^+$).

Two coumarin derivatives were detected, namely meranzin and osthol. Osthol ($C_{15}H_{16}O_3$) emerged at 13.79 minutes (m/z 244.1099 $[M+H]^+$), whereas meranzin ($C_{15}H_{16}O_4$) was found at a retention time of 14.41 minutes (m/z 260.1049 $[M+H]^+$). While osthol is frequently found in members of the Apiaceae family,[32] meranzin has previously been isolated from *Citrus aurantium*.[33] Additionally, coumarins have been found in *A. columnaris* Hook,[6] confirming that the genus Araucaria contains them. The antidepressant properties of meranzin and osthol, two of the known coumarin derivatives, have been researched the most. Interestingly, meranzin is the only coumarin derivative that is now known to be a part of the brain-gut-microbiota antidepressant route.[33]

Table 1: Putative identification of *A. cunninghamii* twig extracts by LC–MS/MS.

Struture	Retention time (minutes)	Molecular weight (*m/z*)	Molecular formula	Class	*Mass error* (ppm)
	6.85	122.0359	$C_7H_6O_2$	Phenolic acid	–7.50
	13.79	244.1099	$C_{15}H_{16}O_3$	Coumarin	+1.18
	14.41	260.1049	$C_{15}H_{16}O_4$	Coumarin	+1.00
	20.39	150.0681	$C_9H_{10}O_2$	Phenolic acid	+1.06
	22.79	594.1526	$C_{34}H_{26}O_{10}$	Biflavonoid	+0.68
	25.89	588.4179	$C_{39}H_{56}O_4$	Terpenoid	+4.52
	29.34	379.3083	$C_{22}H_{42}O_4$	Ester	+0.25

The triterpenoid (2*E*)-3-(3,4-dihydroxyphenyl) acrylate 9,19-cyclolanost-24-en-3-yl (m/z 588.4179 $[M+H]^+$, a retention time of 25.89 min) belongs to the lanostanoid group, analogous to compounds found in *Calystegia sylvatica*.[34] The major signal in the positive mode corresponded to dioctyl adipate (DOA, m/z 408.2645 $[M+H]^+$), a known plasticizer artifact, emphasizing the need for stringent quality control to prevent contamination during analysis.

CONCLUSION

This study successfully identified and characterized six secondary metabolites from *A. cunninghamii* twig extracts. Benzoic acid was the predominant compound, while coumarins, phenolic acids, biflavonoids, and triterpenoids were also detected. These findings enrich the phytochemical understanding of *A. cunninghamii* and provide a basis for exploring its pharmacological potential.

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