MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2025.20.3.16265

Phenolic Compounds Isolated from *Myristica fragrans* and Their Cytotoxic Effects on B16-F10 Melanoma Cancer Cell Lines

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Received June 12, 2025; **Accepted** July 30, 2025; **Available online** November 20, 2025

ABSTRACT. Phenolic compounds are a major type of secondary metabolite found in plants. These compounds are synthesized through shikimic and acetate-malonate pathways, resulting in the formation of numerous unique structures such as flavonoids, isoflavonoids, coumarins, lignans, lignins, as well as their bioactivities. In addition, a significant amount has been reported in nutmeg, an endemic plant of Indonesia, which has been widely used in traditional medicine. A previous study also revealed that ethyl acetate extract of the plant has notable cytotoxic effects against melanoma B16-F10. Therefore, the purpose of this study is to isolate and evaluate phenolic compounds in nutmeg for their potential to inhibit B16-F10 melanoma cancer cell growth. The seeds extract of nutmeg was separated by various chromatographic techniques to yield a total of five compounds, which were identified through spectroscopic analysis (HR-TOF-ESI-MS, IR, and NMR) as well as comparison with literature. The compounds **1**-**5** were identified as (+)-veraguensin (**1**), 3',4',5'-trimethoxycinnamyl alcohol (**2**), (+)-galbegin (**3**), (-)-polysphorin (**4**), and 7-methoxycoumarin (**5**). Cytotoxic effects were then assayed against B16-F10 melanoma cell lines using the Resazurin method. Furthermore, compound **1** displayed the highest cytotoxic activity, with an IC_{50} value of 112.71 μM.

Keywords: B16-F10 cell lines, cytotoxic activity, *Myristica fragrans*, phenolic.

INTRODUCTION

Phenolic compounds are widely distributed in the plant kingdom and represent one of the most important classes of secondary metabolites, with over 8000 types being reported (Alara *et al.*, 2021; Kauffman & Castro, 2023). These compounds are formed through shikimic and acetate-malonate pathways, leading to the production of various structural derivatives, such as lignans, coumarins, hydroxycinnamic acids, hydroxybenzoic acids, flavonoids, xanthones, chalcones, stilbenes, and polymers, including lignins and tannins (Carocho & Ferreira, 2013; Zhang *et al*, 2022; Garcia-Oliveira *et al.*, 2021). Several studies have also shown the presence of diverse biologically activities of phenolic compounds, such as anti-inflammatory, anticancer, antioxidant, and antibacterial. A promising medicinal plant with a significant number of phenolic compounds and a plethora of pharmacological effects is nutmeg (*Myristica fragrans* Houtt.) (Poornima *et al.*, 2016). Nutmeg or *M. fragrans* is a Moluccan endemic plant of Indonesia, which has also been cultivated in India, South East Asia, and the Pacific (Verma *et al.*, 2021). Traditionally, it is often used for treating gastrointestinal issues, infectious diseases, rheumatism, tumors, and skin infections (Abourashed *et al.*, 2016). *M. fragrans* has reported to contain secondary metabolites, such as terpenes, diphenyl alkanes, phenylpropanoids, lignans, and neolignan (Ha *et al.*, 2020). Secondary metabolites, particularly those derived from plants, have been widely reported to exhibit a broad range of biological activities (Riyadi *et al.*, 2023). Previous research reported that the secondary metabolites isolated from *M. fragrans* have different pharmacological effects, such as antioxidant, anti-inflammatory, and anticancer (Al-Rawi *et al.*,

2024). According to the previous research has reported that the ethyl acetate extract exhibits the highest potency against B16-F10 melanoma cells (Susianti *et al.*, 2021) and produce cytotoxic benzofuranoid neolignans (Hasbilla *et al.*, 2024). In the further study to isolate and identify structures as well as evaluate their cytotoxic activity against melanoma skin cancer B16-F10, five phenolics isolated from ethyl acetate extract of *M. fragrans* seeds. Based on the results, novelty of this research is the discovery of compounds **1**,**3-5** from *M. fragrans* for the first time.

EXPERIMENTAL SECTION

Material and Methods

UV and IR spectra were captured on Perkin Elmer Lambda 35 in methanol and Nicolet Summit FTIR spectrometer. In addition, the HR-ESI-QTOFMS-XEVO Waters mass spectrometer was applied to measure the mass spectra. The NMR spectra were used Bruker Ascend 700 MHz for ^{1}H and 175 MHz for ^{13}C with Tetra Methyl Silane (TMS) as the internal standard and $CDCl_3$ as the solvent. Optical rotation measurement was performed using an ATAGO AP-300 automatic polarimeter (Saitama, Japan). Column chromatography (CC) was conducted on normal phase SiO_2 60, (Merck, Germany) and on reversed-phase ODS, 100-200 mesh (Chromatorex® C18 DM1020T, Fuji Sylisia Chemical, LTD). Thin layer chromatography (TLC) was used to support column chromatography, applying normal phase SiO_2 60 F254s plates and reversed-phase C-18 F254s (Merck, Germany). Visualization was achieved under UV light 365 and 254 nm and spraying agent (10% H_2SO_4 in ethanol). Cytotoxic assay was tested using PrestoBlue® reagent in 96 plates of microplates and measured at 570 nm.

Plant Materials

Seed samples of *M. fragrans* were collected from Sarjo Village, Pasangkayu Regency, West Sulawesi Province. The samples were identified at the Celebense Herbarium at Tadulako University, Palu, Central Sulawesi with number of collection 281/UN.28.UPT-SDHS/LK/2019.

Extraction and Isolation

M. fragrans seeds (4.1 kg) were powdered and macerated in ethanol for 8 cycles (24 hours each). The filtrate was concentrated at 45 °C *via* rotary evaporation, yielding a concentrated ethanol extract (2.3 kg). This extract was fractionated with *n*-hexane, ethyl acetate, and *n*-butanol. Ethyl acetate extract (602.50 g) was subjected to vacuum liquid chromatography (VLC) on silica gel with 10% gradient of solvent *n*-hexane: ethyl acetate (100:0-0:100) and ethyl acetate: methanol (100:0-80:20) yielding 8 fractions (A-H). Fraction C (5.81 g) was subjected to silica gel column (70-230 mesh) with 5% gradient of *n*-hexane: ethyl acetate, resulting in 7 subfractions (C1-C7). Subfraction C6 (1197.6 mg) underwent further column chromatography (CC) with *n*-hexane: ethyl acetate (8:2) to produce 7 subfractions (C6A-C6G). Subfraction C6C (140.6 mg) was purified using silica gel column with *n*-hexane: ethyl acetate (6:4) yielding compound **1** (10.4 mg). Fraction D (3.20 g) was separated via CC (70-230 mesh) with a 5% gradient of *n*-hexane: ethyl acetate (100:0-60:40) resulting in 15 subfractions (D1-D15). D10 (331.6 mg) selected *via* TLC comparison with fraction C6, was subjected to CC using *n*-hexane: methylene chloride: ethyl acetate (6:2:2) yielding 7 subfractions (D10A-D10G). Subfraction of D10D (135.4 mg) underwent CC (230-400 mesh) with *n*-hexane: methylene chloride: ethyl acetate (5:3:2) producing 6 subfractions (D10D1-D10D6). Furthermore, subfraction D10D4 was separated using reversed-phase ODS column chromatography with acetonitrile: water (1:1) resulting in 5 subfractions to produce compound **2** (4.4 mg), compound **3** (8.5 mg), and **4** (10.5 mg). Subfraction of D10E (45.1 mg) underwent column chromatography with *n*-hexane: methylene chloride: ethyl acetate (6:2.5:1.5) yielding 2 subfractions (D10E1-D10E2) and further TLC analysis revealed of compound **5** (4.4 mg).

(+)-veraguensin (1), was isolated as a white gum $[\alpha]^{27,}{}_{D}$ = +15.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 230 (6.82), 280 (6.40) nm; IR ν_{max} 2960, 1515, 1456, 1215, 1115, 1030 and 812 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 700 MHz): δ_H 6.96 (2H, d, *J* = 2.0 Hz, H-2/2′), 6.93 (2H, dd, *J* = 2.0, 8.1 Hz, H-6/6'), 6.89 (2H, d, *J* = 8.1 Hz, H-5/5'), 5.12 (1H, d, *J* = 8.6 Hz, H-7′), 4.41 (1H, d, *J*= 10.4 Hz, H-7), 3.92 (6H, s, OCH_3-3′/4′), 3.88 (3H, s, OCH_3-4), 3.86 (3H, s, OCH_3-3), 2.25 (1H, *m*, H-8'), 1.79 (1H, *m*, H-8), 1.06 (3H, d, *J*= 6.5 Hz, H-9), 0.66 (3H, d, *J*= 7.7 Hz, H-9′), ^{13}C-NMR ($CDCl_3$, 175 MHz), see **Table 1**; HR-ESI-QTOFMS *m/z* 373.2001 $[M+H]^+$, (calcd. for $C_{22}H_{29}O_5$ *m/z* 373.2015) (Poornima *et al.*, 2016).

3',4',5'-trimethoxycinnamyl alcohol (2), was isolated as yellowish oily; $[\alpha]^{27,}{}_{D}$ = +1.6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 219 (7.14), 281 (6.62) nm; IR ν_{max} 3400, 2945, 1580, 1450, 1208, 1115, 1008 and 830 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 700 MHz): δ_H 6.61 (2H, s, H-2′/6′), 6.56 (1H, d, *J*= 15.2 Hz, H-3), 6.30 (1H, dt, *J*= 15.8, 5.8 Hz, H-2), 4.33 (2H, dd, *J*= 1.8, 5.8 Hz, H-1), 3.88 (3H, s, OCH_3-3′), 3.86 (3H, s, OCH_3-5′), 3.85 (3H, s, OCH_3-4′); ^{13}C-NMR ($CDCl_3$, 175 MHz), see **Table 1**; HR-ESI-QTOFMS *m/z* 225.1168 $[M+H]^+$, (calcd. for $C_{12}H_{17}O_4$ *m/z* 225.1127) (Sun *et al.*, 2016).

(+)-galbegin (3), was gained as colorless gum; $[\alpha]^{27,D}$ = +52.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 230 (6.82), 280 (6.40) nm; IR ν_{max} 2957, 1513, 1456, 1207, 1028, 1006 and 812 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 700 MHz): δ_H 6.96 (2H, d, *J* = 2.1 Hz, H-2/2'), 6.90 (2H, dd, *J*= 2.1, 8.0 Hz, H-6/6'), 6.86 (2H, d, *J* = 8.0 Hz, H-5/5'), 4.66 (2H, d, *J* = 9.0 Hz, H-7), 3.91 (6H, s, OCH_3-3'/4'), 3.88 (3H, s, OCH_3-4), 3.86 (3H, s, OCH_3-3), 1.79 (2H, m, H-8/8'), 1.06 (6H, d,

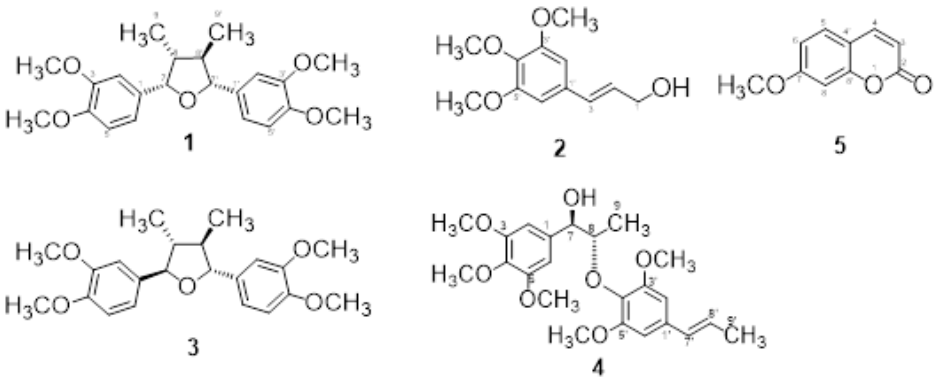

Figure 1. Isolated compounds **1**-**5** from *M. fragrans* seeds.

J = 10.8 Hz, H-9/9'),^{13}C-NMR ($CDCl_3$, 175 MHz), see **Table 1**; HR-ESI-QTOFMS *m/z* 373.2002 $[M+H]^+$, (calcd. $C_{22}H_{29}O_5$ m/z 373.2015) (Li *et al.*, 2020).

(-)-polysphorin (4), was isolated as a yellowish gum; $[\alpha]^{27,}{}_D$ = –54.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 218 (7.11), 260 (6.50) nm; IR ν_{max} 3415, 2957, 1608, 1515, 1456, 1210, 1106, 1026, and 812 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 700 MHz): δ_H 6.58 (2H, s, H-2/6), 6.44 (2H, s, H-2'/6'), 6.35 (1H, dd, *J*=1.7, 15.7 Hz, H-7'), 6.16 (1H, dq, *J*= 6.6, 15.7 Hz, H-8'), 4.81 (1H, d, *J*= 3.0 Hz, H-7), 4.34 (1H, m, H-8), 3.89 (3H, s, OCH_3-5'), 3.88 (9H, s, OCH_3-4/5/3'), 3.87 (3H, s, OCH_3-3), 1.89 (3H, dd, *J*= 1.6, 7.7 Hz, H-9'), 1.18 (3H, d, *J*= 6.4 Hz, H-9); ^{13}C-NMR (CDCl3, 175 MHz), see **Table 2**; HR-ESI-QTOFMS *m/z* 419.2078 $[M+H]^+$, (calcd. for $C_{23}H_{31}O_7$ *m/z* 419.2070) (Nagaraju *et al.*, 2012).

7-methoxycoumarin (5), was gained as yellowish solid; mp =113 – 115 °C; UV (MeOH) λ_{max} (log ε) 218 (6.36), 317 (6.01) nm; IR ν_{max} 2926, 1707, 1612, 1515, 1456, 1205, 1124 and 829 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 700 MHz): δ_H 7.63 (1H, d, *J*= 9.7 Hz, H-4), 7.37 (1H, d, *J*=8.5 Hz, H-5), 6.84 (1H, dd, *J*=8.5, 2.3 Hz, H-6), 6.82 (1H, d, *J* = 2.4 Hz, H-8), 6.33 (1H, d, *J* = 9.7 Hz, H-3), 3.93 (OCH_3-7); ^{13}C-NMR (CDCl3, 175 MHz), see **Table 2**; HR-ESI-QTOFMS *m/z* 177.0487 $[M+H]^+$, (calcd. for $C_{10}H_9O_3$ *m/z* 177.0552) (Adenekov *et al.*, 2017).

Cytotoxic Activity

Evaluation of cytotoxic activity compounds **1**-**5** on B16-F10 melanoma skin cancer cells was determined using the resazurin method with Prestoblue® reagent (Susianti *et al.*, 2021; Safriansyah *et al.*, 2024; Riyadi *et al.*, 2024). This procedure started by adding the cells to 96-well plates that were filled with Roswell Park Memorial Institute (RPMI) medium. Subsequently, the cells were cultured for a full day at 37 °C and 5% CO_2 until it was 1.7 x 10^4 cells/well. After this procedure, samples, a positive control, and a negative control were used to treat the cells. During this phase, RPMI medium in the plates was removed, and a medium containing the sample (with DMSO as the solvent) at different concentrations (500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, and 3.91 μg/mL) was introduced, with positive control (cisplatin). Cells treated with the samples were then incubated for 48 hours. Finally, Prestoblue® reagent was introduced, and the incubated mixture (2 hours) will produced the noticeable color change. Following this, the absorbance of each sample was measured using a multimode reader at a wavelength of 570 nm. Subsequently, the absorbance readings were converted to determine the percent cell viability, enabling the calculation of IC_{50} value.

RESULTS AND DISCUSSION

Five known phenolics derivatives (**1**-**5**) have been isolated, except for compound **2**, the rest were the first time isolated from *M. fragrans* seeds. Those compounds, namely (+)-veraguensin (**1**), 3',4',5'-trimethoxycinnamyl alcohol (**2**), (+)-galbegin (**3**), (-)-polysphorin (**4**), and 7-methoxycoumarin (**5**). Their chemical structure is shown in **Figure 1.**

Compound **1** was assigned a molecular formula of $C_{22}H_{28}O_5$ based on HR-ESI-QTOFMS. Its $[M+H]^+$ molecular ion peak appeared at *m/z* 373.2001, similarly with the calculated value for $C_{22}H_{29}O_5$, *m/z* 373.2015, indicating of nine degree of saturation. In addition, UV spectrum revealed the presence of conjugated double bonds by the peak at 230 and 280 nm (Supratman *et al.*, 2020). IR spectrum further exhibited a spectrum of aliphatic carbon at 2960 cm^{-1}, conjugated aromatic double bond at 1515 and 1456 cm^{-1}, and ether bond at 1215, 1115, and 1030 cm^{-1}. ^{1}H-NMR spectrum showed two methyl protons at δ_H 0.66 (3H, d, *J* = 7.7 Hz, CH_3-9') and 1.06 (3H, d, *J*=6.5 Hz, CH_3-9), two aliphatic methines at δ_H 1.79 (1H, m, H-8) and 2.25 (1H, m, H-8'), two oxygenated methines at δ_H 4.41 (1H, d, *J* = 10.4 Hz, H-7) and 5.12 (1H, d, *J* = 8.6 Hz, H-7'). In addition, four oxygenated methyls were observed at δ_H 3.86 (3H, s, OCH_3-3), 3.88 (3H, s, OCH_3-4), 3.92 (6H, s, OCH_3-3' and 4'). A total of six aromatic protons appeared at δ_H 6.89 (2H, d, *J* = 8.1 Hz, H-5/5'), 6.93 (2H, dd, *J* = 2.0, 8.1 Hz, H-6/6'), and 6.96 (2H, d, *J* = 2.0 Hz, H-2 and 2'). This coupling constant indicated the presence of 1,2,4 tri-substituted aromatic ring. ^{13}C and DEPT spectra followed by HSQC displayed two methyls, two methines, two oxygenated methines, four oxygenated methyls, six aromatic methines, two

quartenary carbons, and four oxygenated quartenary carbons, yielding a total of 22 carbons. The molecular formula $C_{22}H_{28}O_5$ indicated nine degrees of saturation, eight were attributed to two benzene moieties, while the remaining was associated with a cyclic moiety. The presence of two oxygenated methines at δ_H 4.41 and 5.12, each exhibiting a large coupling constant (>5 Hz) implied an oxygenated cyclic structure.

The placement of functional groups was identified using ^{1}H-^{1}H COSY and HMBC spectra, and the results were displayed in **Figure 2**. ^{1}H-^{1}H COSY spectrum displayed the correlations of H-7/H-8/H-9, H-7'/H-8'/H-9' and H-8/H-8', indicating tetrahydrofuran fragment. The HMBC spectrum of **1** implied the occurrence of tetrahydrofuran fragment bonded to benzene moiety, deducing that compound **1** possessed a tetrahydrofuran lignan skeleton. In addition, four oxygenated methyls were also confirmed by HMBC correlations (**Figure 2**), verifying that C-3/4/3'/4' were bonded to oxygenated methyls. The tetrahydrofuran lignan had four chiral carbons and a ^{1}H-^{1}H NOESY experiment was conducted to determine the correlation between H-9/9', H-8/8', and H-7/7'. From NOESY correlations (**Figure 3**) it was confirmed the α orientation with correlation between H-9' to H-8, H-7 and H-7'. The β orientation of H-9 to H-8' were then elucidated as the same as its analogs of those reported veraguensin (Poornima *et al.*, 2016). Therefore, compound **1** was identified to be veraguensin, and to separate enantiomeric possibilities, the optical rotary experiment was performed and resulted in (+) value, (+)-veraguensin had 7*S*, 7'*R*, 8*R*, 8'*R* conformation (Poornima *et al.*, 2016). Compound **1** concluded to be (+)-veraguensin, was found in the *Schisandra* genus (Poornima *et al.*, 2016) as seen in **Table 1**, and was reported for the first time from *M. fragrans*.

Compound **2** appeared as yellowish oily, with the molecular formula of $C_{12}H_{17}O_4$ in the $[M+H]^+$ peak at *m/z* 225.1168 calculated for $C_{12}H_{17}O_4$ *m/z* 225.1127, showing the presence five degree of saturation. UV spectrum revealed the presence of conjugated double bonds by the peaks at 219 and 280 nm (Salam *et al.*, 2018), and IR spectrum presented the OH group (3400 cm^{-1}), C-H bond (2945 cm^{-1}), olefinic bond (1580 cm^{-1}), conjugated aromatic double bond (1450 cm^{-1}), and ether bond (1208 and 1115 cm^{-1}). In addition, ^{1}H-NMR spectrum indicated the presence of three methoxy groups at δ_H 3.85 (3H, s, OCH_3-4'), 3.86 (3H, s, OCH_3-5') and 3.88 (3H, s, OCH_3-3') ppm, one oxygenated methylene at δ_H 4.33 (2H, dd, *J*= 1.8, 5.8 Hz, H-1), two olefinic methines at δ_H 6.56 (1H, d, *J*= 15.9 Hz, H-3), 6.30 (1H, dt, *J* = 15.8, 5.8 Hz, H-2) by the large *J* coupling (16 Hz), which implied the presence of *trans*-olefinic methine, and two aromatic methine at δ_H 6.62 (2H, s, H-2'/6') ppm. The ^{13}C and DEPT spectra showed the presence of twelve carbons, consisted of three oxygenated methyls, one oxygenated methylene, two aromatic methines, two olefinic methines, one quartenary aromatic carbon, and three oxygenated quartenary carbons. From the ^{1}H and ^{13}C spectrum, compound **2** was implied to be a phenylpropanoid compound and by the comparison according to the literature (Sun *et al.*, 2016), this compound was defined as 3',4',5'- trimethoxy cinnamyl alcohol and had been isolated from *M. fragrans* (Hattori *et al.*, 1988).

Compound **3** appeared as a white gum, and its molecular formula $C_{22}H_{28}O_5$ by HR-ESI-QTOFMS. This showed $[M+H]^+$ molecular ion peak at *m/z* 373.2002, calculated for $C_{22}H_{29}O_5$, *m/z* 373.2015, which resulted in nine degrees of saturation. UV spectrum revealed the presence of conjugated double bonds by the peak at 230 and 280 nm (Salam *et al.*, 2018). In addition, IR spectrum presented an aliphatic carbon at 2957 cm^{-1}, conjugated aromatic double bond at 1513 and 1456 cm^{-1}, and ether bond at 1207, and 1028 cm^{-1}. ^{1}H-NMR and ^{13}C-NMR signals were similar to compound **1,** although it exhibited different signals on carbon and proton for numbers 7/7', 8/8', and 9/9. This differentiated their dimensional conformation, based on NOESY signals, H-9' was on the same plane as H-7' and H-8, while H-9 was on the same plane as H-8' and H-7. According to this analysis, compound **3** was confirmed to be galbegin (Rye & Barker, 2011). In addition, optical rotation was performed to separate their enantiomeric possibilities then resulting (+) value, according to (Rye & Barker, 2011), (+)-galbegin had 7*R*, 8*R*, 7'*R,* and 8'*R* conformation. **Figure 3**, showed the epimer relationship among compound **1** and **3**, this data also strengthened by the difference of optical rotation value (+)-galbegin (compound **3**) had +52.3 and (+)-veraguensin had +15.0. The (+)-galbegin was isolated in *Machilus* genus (Li *et al.*, 2020), this compound was reported for the first time from *M. fragrans*.

Compound **4** was gained as yellowish gum, its molecular formula of $C_{23}H_{30}O_7$ appeared from $[M+H]^+$ peak at 419.2078 (calcd. for $C_{23}H_{31}O_7$, *m/z* 419.2070), which was the set value for 9 degrees of saturation. UV spectrum at 218 and 260 nm showed the presence of conjugated double bonds (Salam *et al.*, 2018). In this study, IR spectrum showed the OH spectra at 3415 cm^{-1}, aliphatic carbon at 2957 cm^{-1}, olefinic bond at 1608 cm^{-1}, conjugated aromatic double bond at 1515 and 1456 cm^{-1}, and ether bond at 1210, 1106, and 1026 cm^{-1}. ^{1}H-NMR spectrum indicated two methyls, which consisted of aliphatic methyl at δ_H 1.18 (3H, d, *J*= 6.4 Hz, H-9) and 1 olefinic methyl at δ_H 1.89 (3H, dd, *J*= 1.6, 7.7 Hz, H-9') ppm, five oxygenated methyls at δ_H 3.87 (3H, s, OCH_3-3), 3.88 (9H, s, OCH_3-4/5/3'), 3.89 (3H, s, OCH_3-5') ppm, 2 oxygenated methines

Table 1. ^{13}C-NMR ($CDCl_3$, 125 MHz) data comparison of compounds **1-3** with literature

Position Carbon	**1**	Veraguensin	**2**	3',4',5'-Trimethoxy cinnamyl alcohol	**3**	Galbegin
	δc (mult.)	δc (mult.)	δc (mult.)	δc (mult.)	δc (mult.)	δc (mult.)
1	134.4 (C)	135.5 (C)	63.7 (CH_2)	63.6 (CH_2)	134.3 (C)	134.9 (C)
2	109.2 (CH)	109.2 (CH)	128.0 (CH)	128.1 (CH)	108.5 (CH)	109.2 (CH)
3	146.2 (C)	148.9 (C)	131.2 (CH)	131.1 (CH)	147.0 (C)	148.5 (C)
4	146.5 (C)	148.2 (C)	-	-	147.0 (C)	149.1 (C)
5	109.5 (CH)	110.7 (CH)	-	-	109.5 (CH)	110.8 (CH)
6	120.0 (CH)	118.3 (CH)	-	-	119.9 (CH)	118.6 (CH)
7	87.4 (CH)	85.4 (CH)	-	-	88.4 (CH)	88.3 (CH)
8	47.8 (CH)	47.3 (CH)	-	-	51.1 (CH)	51.0 (CH)
9	15.1 (CH_3)	15.6 (CH_3)	-	-	13.8 (CH_3)	13.9 (CH_3)
1′	132.9 (C)	133.0 (C)	132.4 (C)	132.5 (C)	134.3 (C)	134.9 (C)
2′	108.6 (CH)	108.9 (CH)	103.6 (CH)	103.4 (CH)	109.3 (CH)	109.2 (CH)
3′	146.6 (C)	148.5 (C)	153.4 (C)	153.4 (C)	147.0 (C)	148.5 (C)
4′	146.7 (C)	147.6 (C)	138.0 (C)	138.2 (C)	147.0 (C)	149.1 (C)
5′	109.8 (CH)	110.8 (CH)	153.4 (C)	153.4 (C)	109.7 (CH)	110.8 (CH)
6′	119.4 (CH)	117.9 (CH)	103.6 (C)	103.8 (C)	119.9 (CH)	118.6 (CH)
7′	83.2 (CH)	84.5 (CH)	-	-	88.6 (CH)	88.3 (CH)
8′	46.0 (CH)	43.2 (CH)	-	-	51.1 (CH)	51.0 (CH)
9′	15.0 (CH_3)	14.9 (CH_3)	-	-	14.0 (CH_3)	13.9 (CH_3)
3-OCH_3	55.89 (CH_3)	55.6 (CH_3)	-	-	55.9 (CH_3)	55.9 (CH_3)
4-OCH_3	55.92 (CH_3)	55.6 (CH_3)	-	-	55.9 (CH_3)	55.9 (CH_3)
3′-OCH_3	55.94 (CH_3)	55.7 (CH_3)	56.1 (CH_3)	56.2 (CH_3)	55.9 (CH_3)	55.9 (CH_3)
4′-OCH_3	56.00 (CH_3)	55.7 (CH_3)	61.0 (CH_3)	60.9 (CH_3)	56.0 (CH_3)	55.9 (CH_3)
5′-OCH_3	-	-	56.1 (CH_3)	56.2 (CH_3)	-	-

at δ_H 4.34 (1H, m, H-8) and 4.81 (1H, d, *J*= 3.0 Hz, H-7) ppm, 2 olefinic methine at δ_H 6.35 (1H, dd, *J*=1.7, 15.7 Hz, H-7'), 6.16 (1H, dq, *J*= 6.6, 15.7 Hz, H-8') ppm, and 4 aromatic methines at δ_H 6.58 (2H, s, H-2/6), 6.44 (2H, s, H-2'/6'). In ^{13}C-NMR, DEPT followed by HSQC spectra, there were 23 carbons including one aliphatic methyl, one olefinic methyl, five oxygenated methyls, two oxygenated methines, four aromatic methines, two olefinic methines, two quaternary methines, and six quaternary oxygenated methyls. From the molecular formula ($C_{23}H_{30}O_7$) which resulted in nine saturation degrees, eight from two aromatic groups, and one from olefinic carbons. From these characteristics, compound **4** could be a benzofuranoid or 8-*O*-4' type of neolignan.

The placement of functional groups was identified by ^{1}H-^{1}H COSY and HMBC spectra, and the results were depicted in **Figure 2**. ^{1}H-^{1}H COSY spectrum revealed the correlations in $H_7/H_8/H_9$, and $H_{7'}/H_{8'}$-/$H_{9'}$ implying aliphatic fragment and olefinic fragment. Furthermore, the HMBC spectrum revealed the fragment correlation to aromatic moiety validating that compound **4** was an 8-*O*-4' type of neolignan. This had variations of stereochemistry in their C7-C8 bond, whether it was *threo* or *erythro* as when NOESY spectrum could not determine this relative configuration due to the C7-C8 bond rotating randomly (Teponno *et al.*, 2016). One of the ways to determine their relative configuration was by $J_{7,8}$ value. According to (Yu *et al.*, 2022), *erythro* (7*R*,8*S*/7*S*,8*R*) had $J_{7,8}$ = 2-5 Hz, while *threo* (7*R*,8*R*/7*S*,8*S*) had $J_{7,8}$ = 6-8 Hz. Compound **4** had $J_{7,8}$ = 3.0 Hz, suggesting *erythro* 8-*O*-4'-type of neolignan and by comparison to the literature, it was identified as a polysphorin (Nagaraju *et al.*, 2012). A polarimeter was conducted to differentiate two enantiomer possibilities, compound **4** had a (-) value, according to (Nagaraju *et al.*, 2012), (-)-polysphorin has 7*R*,8*S* configuration. This compound was found in *Magnolia* genus (Gao *et al.*, 2012) and was isolated for the first time in *M. fragrans*.

Compound **5,** a yellowish solid, with a melting point range of 113 to 115 °C. Its molecular formula was confirmed to $C_{10}H_8O_3$ from $[M+H]^+$ calculated for $C_{10}H_9O_3$, *m/z* 177.0552, which resulted in seven degrees of saturation. UV spectrum peak at 218 and 317 nm, revealed the presence of conjugated double bonds, and IR spectrum presented the aliphatic carbon (2926 cm^{-1}), ester carbonyl (1707 cm^{-1}), olefinic carbon (1612 cm^{-1}), aromatic carbon (1515 and 1456 cm^{-1}), and ether bonds (1205 and 1124 cm^{-1}). In addition, ^{1}H-NMR spectrum displayed one oxygenated methyl at δ_H 3.93 (7-OCH_3) ppm, three aromatic protons at δ_H 6.82 (1H, d, *J* = 2.4 Hz, H-8), 6.84 (1H, dd, *J* = 8.5, 2.3 Hz, H-6), 7.37 (1H, d, *J* = 8.5 Hz, H-

5) followed by their coupling constant, this showed 1,2,4-trisubstituted aromatic proton. ^{13}C and DEPT spectra displayed ten carbons, including one oxygenated methyl, three aromatic methines, two oxygenated carbons, one quartenary carbon, two olefinic methines, and one ester carbonyl. From seven degrees of saturation, four was from aromatic, one from olefinic, one from carbonyl, and the remaining was from cyclic ester. From these results, compound **5** was confirmed to have a coumarin skeleton, and compared to the literature, compound **5** is 7-methoxy coumarin. This compound was found in *Artemisia* genus (Adenekov *et al.*, 2017) and reported for the first time from *M. fragrans*.

Table 2. ^{13}C-NMR ($CDCl_3$, 125 MHz) data comparison of compounds **4** and **5** with literature

Position Carbon	**4** δc (mult.)ppm	Polysphorin δc (mult.)ppm	**5** δc (mult.) ppm	7′-Methoxy coumarin δc (mult.) ppm
1	135.3 (C)	136.4 (C)	-	-
2	104.0 (CH)	104.3 (CH)	162.7 (C)	162.9 (C)
3	152.7 (C)	153.1 (C)	113.2 (CH)	113.3 (CH)
4	135.9 (C)	137.5 (C)	144.2 (CH)	144.5 (CH)
5	147.0 (C)	148.0 (C)	129.0 (CH)	129.0 (CH)
6	102.8 (CH)	103.0 (CH)	112.5 (CH)	112.5 (CH)
7	73.7 (CH)	73.8 (CH)	160.1 (C)	160.1 (C)
8	82.6 (CH)	83.0 (CH)	101.3 (CH)	101.3 (CH)
9	13.4 (CH_3)	13.3 (CH_3)	-	-
1′	133.8 (C)	133.9 (C)	-	-
2′	104.9 (CH)	105.0 (CH)	-	-
3′	151.4 (C)	152.8 (C)	-	-
4′	137.1 (C)	136.4 (C)	112.5 (C)	112.5 (C)
5′	151.5 (C)	152.8 (C)	-	-
6′	104.9 (CH)	102.9 (CH)	-	-
7′	130.4 (CH)	130.7 (CH)	-	-
8′	125.1 (CH)	125.7 (CH)	156.3 (C)	156.3 (C)
9′	18.4 (CH_3)	18.4 (CH_3)	-	-
3-OCH_3	55.8 (CH_3)	56.0 (CH_3)	-	-
4-OCH_3	56.0 (CH_3)	55.9 (CH_3)	-	-
5- OCH_3	56.0 (CH_3)	56.0 (CH_3)	-	-
3′-OCH_3	56.0 (CH_3)	56.1 (CH_3)	-	-
5′-OCH_3	56.0 (CH_3)	56.1 (CH_3)	-	-
7- OCH_3	-	-	56.0 (CH_3)	56.1 (CH_3)

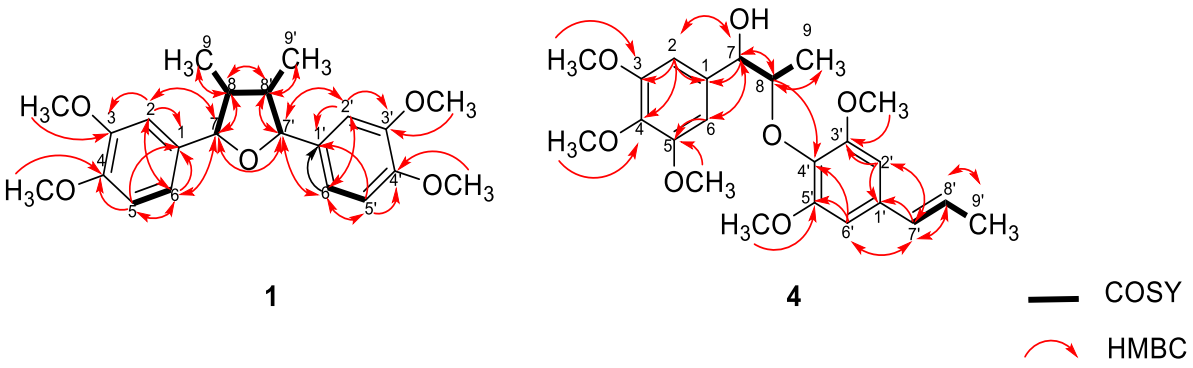

Figure 2. Key HMBC and ^1H-^1H COSY correlations of compounds **1** and **4**

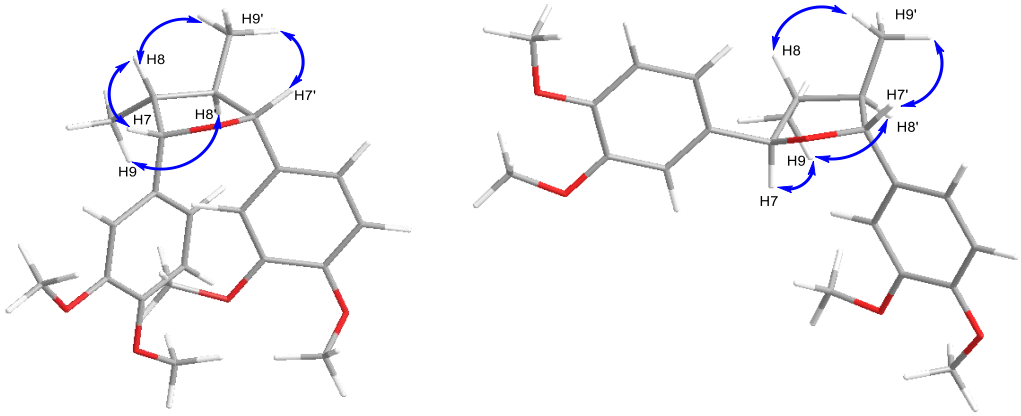

Figure 3. Key ^{1}H-^{1}H NOESY correlations of compounds **1** and **3**

Table 3. Cytotoxic activity of compounds **1**-**5** and ethyl acetate extract against B16-F10 melanoma cancer cell lines

Compounds	IC_{50} (μM)
(+)-veraguensin (**1**)	112.71
3',4',5'-trimethoxycinnamyl alcohol (**2**)	739.38
(+)-galbegin (**3**)	147.15
(-)-polysphorin (**4**)	236.51
7-methoxycoumarin (**5**)	265.68
Ethyl acetate extract	21.66
Cisplatin	16.24

Cytotoxic Activity

Compounds **1**-**5** were assayed for cytotoxic activity against the B16-F10 melanoma skin cancer cell lines using Presto Blue reagent according to a method described previously (Safriansyah *et al.*, 2024; Supratman *et al.*, 2020), as shown in **Table 3**. Among these compounds, compound **1** exhibited the highest cytotoxic value with IC_{50} 112.71 μM. The structure activity relationship showed that the differences of configuration in C-7 in compound **1** and **3** might decrease the IC_{50} value. Meanwhile the *erythro* 8-*O*-4'-type of neolignan structure (compound **4**) and the coumarin derivate showed moderate cytotoxic activity. Among all the characterized compounds, the phenylpropanoid (compound **2**) showed the lowest IC_{50} value of 739.38 μM.

CONCLUSIONS

Five isolated phenolic compounds consisted of two tetrahydrofuran lignans, namely (+)-veraguensin (**1**) and (+)-galbegin (**3**), one phenylpropanoid named 3',4',5'-trimethoxycinnamyl alcohol (**2**), one 8-*O*-4' neolignan named (-)-polysphorin (**4**), and one coumarin named 7-methoxycoumarin (**5**). Compounds **1**, **3**, **4**, and **5** were isolated for the first time from *Myristica* genus. In addition, compound **1** had the strongest activity, with IC_{50} value of 112.71 μM, suggesting the configuration of C-7 influenced the cytotoxic activity.

ACKNOWLEDGEMENTS

This research was funded by Universitas Padjadjaran from Academic Leadership Grant, No: 1959/UN6.3.1/PT.00/2024 by Unang Supratman and Post-Doctoral Grant, No: 1195/UN6.3.1/PT.00/2025 provided by Unang Supratman.

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