Cytotoxic Effect of Benzofuranoid Neolignans from *Myristica fragrans* Seeds Against Melanoma B16-F10 Cancer Cells

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Abstract. *Myristica fragrans* is an indigenous plant in Indonesia, characterized by various secondary metabolites with diverse bioactivities. This plant possesses promising anti-melanoma properties capable of addressing prevalent cases of melanoma. Therefore, this study's objective was to isolate potential compounds from *Myristica fragrans* seeds and assess their cytotoxic effects on melanoma B16-F10 cells. Ethyl acetate extracts were subjected to separation through various chromatographic methods to obtain three benzofuranoid neolignans, which was conducted using spectroscopic analyses, namely UV-vis, polarimeter, HRTOF-MS, IR, and NMR. Subsequently, comparison with previously reported spectral data confirmed the identities of neolignans as (+)-licarin A (**1**), (+)-licarin B (**2**), and (+)-maceneolignan B (**3**). Cytotoxicity against melanoma B16-F10 cells was assessed using the PrestoBlue method, revealing (+)-licarin A (**1**) to display the most potent activity with an IC_{50} value of 94.15 µM.

Keywords: *B16-F10 Cancer Cells; cytotoxicity; Myristica fragrans; Myristicaceae; neolignans.*

1 Introduction

Myristica fragrans Houtt, (Myristicaceae) is an endemic plant of Indonesia, broadly spread throughout equatorial regions of the world [1]. This plant has

Received April 24th, 2024, Revised August 17th, 2024, Accepted for publication November 26th, 2024
 ISSN: 2337-5760, DOI: 10.5614/j.math.fund.sci.2024.56.2.4

various useful parts, such as the red aril (mace), the kernel (nutmeg), and essential oil, which is used as a spice and traditionally applied as a medicine for relieving flu symptoms, treating headache, diarrheic, rheumatic, anxiety, cholera, paralysis, rheumatism and aphrodisiacs [2]. *Myristica fragrans* contains various secondary metabolites such as lignans, neolignans, terpenes, diphenyl alkanes, phenylpropanoids, steroids, saponins, triterpenoids, and flavonoids with various activities for instance antimicrobial, anti-inflammatory, anticancer, radical inhibitor, and heart-protective activities [3].

Melanoma is a cancer caused by malignant cells of melanocytes [4]. Melanoma is caused mainly by UV radiation, either natural or artificial [4]. When people are exposed often to UV, the concern for melanoma increases. Melanoma occurs at an average age of 52 years, which is younger than most other tumors. In 2021, there were an estimated 106,110 diagnoses, with 7,180 deaths [4]. Melanoma is relatively common compared to other cancers [5]. Melanoma is highly metastatic and can spread massively into bone, brain, lung, liver, and lymph nodes [6]. Different therapeutic approaches have been utilized in addressing melanoma, spanning from surgical interventions to chemotherapy and radiotherapy. These treatment modalities not only result in adverse effects such as skin damage but also undermine radiation efficiency and reduce the number of cancer cells, potentially fostering resistance to chemotherapy drugs [7]. Consequently, the exploration of alternative medication emerges as a crucial endeavour, specifically in its capacity as a chemo preventive measure. These remedies, harnessed from natural sources, exhibit the potential to suppress the abnormal proliferation of melanoma cells, while offering reduced adverse effects and enhanced safety properties.

One of the researched medications is from nutmeg or *Myristica fragrans*. It has shown cytotoxic ability by inducing apoptosis of melanoma cells. Ethyl acetate extract has been shown to be the most promising extract. So far, no research has been published on nutmeg compound against melanoma cells [8]. Therefore, the objective of this research was to obtain cytotoxic compounds from *M. fragrans* seeds and investigate their activity toward B16-F10 melanoma cancer cells.

2 Materials and Methods

2.1 General

An AP-300 polarimeter (ATAGO, Japan) was used to measure optical rotations in MeOH. The UV spectrum was obtained with an Infinite M200 plate reader (Tecan, Switzerland) in MeOH. The FT-IR spectra were obtained with a Spectrum 100 spectrometer (Perkin Elmer, USA) using KBr methods. HR-TOFMS was measured on a Xevo Q-Tof MS instrument (Waters, USA). The

NMR spectra were measured by an ECZ instrument (JEOL, Japan) in $CDCl_3$ and used tetra methyl silane (TMS) as internal standard. Silica gel 60 (Merck, 70-230 and 230-400 mesh) and Octa desylsilane (Fuji Sylisia Chemical, 100-200 mesh) was used for column chromatography (CC). Previously coated F254 plates and RP-18 F254s silica gel plates (Merck) were utilized to track the purification steps, while UV light at 254 and 365 nm was used for checking prior to spraying with 10% H_2SO_4 in ethanol and then heating.

2.2 Plant Material

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

2.3 Extraction and Isolation

The *M. fragrans* seeds were crushed into powder weighing 4.1 kg and macerated in ethanol for 4 x 24 hours. The macerate was then evaporated by a rotary evaporator at 45 °C, resulting in a concentrated ethanol extract of 2.3 kg, which was fractioned using *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc extract (602.50 g) was fractionated using a mixture of solvents *n*-hexane-EtOAc-MeOH in a 10% gradient on vacuum liquid chromatography (VLC) using silica gel (70-230 mesh), leading to eight fractions (A-H), based on TLC spots. Fraction C (5.81 g) was CC on silica gel by a mixture of solvent *n*-hexane:EtOAc in 5% gradient to obtain seven subfractions (C1-C7). Next, subfraction C2 (116.3 mg) was chromatographed on reverse phase CC on ODS with MeOH:water (3:1) to obtain three subfractions (C2A-C2C). This was followed by separation of C2B and C2C using CC silica gel by eluent of *n*-hexane:EtOAc (6:4), resulting in C2B2 and C2C1 with the same spot by TLC, which were then combined to obtain compound **1** (8.8 mg). Subfraction C3 (564.6 g) was then separated using normal phase CC with *n*-hexane:DCM:EtOAc (7:2:1) as solvent to obtain ten subfractions (C3A-C3J). Then, C3A (70 mg) was then purified to CC on silica gel with a mixture of *n*-hexane:EtOAc (50:1) to obtain three subfractions (C3A1-C3A3). Compound **2** (4.5 mg) and **3** (4.0 mg) were obtained from subfractions C3A2 and C3A3, respectively.

(+)-Licarin A (**1**), a white solid; m.p =113 – 115 °C; $[\alpha]^{25,D}$ +68.75 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 205 (6.8), 222 (7.6), 275 (6.4) nm; IR (KBr) ν_{max} 3414, 2925, 1613, 1519, 1274, 1144, 810 cm^{-1}; ^{1}H-NMR: δ_H 6.96 (1H, d, *J* = 2.0 Hz, H-2), 6.90 (1H, dd , *J* = 8.3, 2.0 Hz, H-6), 6.88 (1H, d, *J* = 8.3 Hz, H-5), 6.77 (1H, s, H-6'), 6.75 (1H, s, H-2'), 6.36 (1H, dd, *J* = 16.0, 1.5 Hz, H-7'), 6.10 (1H, dq, *J* = 16.0, 6.5 Hz, H-8'), 5.63 (1H, s, OH-4), 5.10 (1H, d, *J* = 10 Hz, H-7), 3.88

(3H, s, 3-OMe), 3.90 (3H, s, 3'-OMe), 3.44 (1H, dq, *J* = 10.0, 7.0 Hz, H-8), 1.86 (3H, dd, *J* = 7.0, 2.0 Hz, H-9'), 1.41 (3H, d, *J* = 7.1 Hz, H-9); ^{13}C-NMR: δ_C 147.0 (C-3), 147.0 (C-4'), 146.0 (C-4), 145.1 (C-3'), 133.0 (C-5'), 131.6 (C-1'), 132.1 (C-1), 130.8 (C-7'), 124.0 (C-8'), 120.1 (C-6), 113.7 (C-5), 112.9 (C-6'), 108.4 (C-2'), 108.9 (C-2), 94.0 (C-7), 56.1 (3-OMe), 56.0 (3'-OMe), 46.0 (C-8), 19.1 (C-9'), 16.9 (C-9). HRTOFMS *m*/z 327.1583 $[M+H]^+$, (calcd. for $C_{20}H_{23}O_4$ *m/z* 327.1596).

(+)-Licarin B (**2**), a colorless oil; $[\alpha]^{25,D}$ +51.20 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 205 (6.9), 222 (6.8), 275 (6.4) nm; IR (KBr) ν_{max} 2925, 1613, 1519, 1270, 1144, 811 cm^{-1}; ^{1}H NMR: δ_H 6.91 (1H, d, *J* = 2.0 Hz, H-2), 6.87 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.77 (1H, s, H-6'), 6.76 (1H, s, H-2'), 6.73 (1H, d, *J* = 8.0 Hz, H-5), 6.36 (1H, dd, *J* = 16.0, 1.7 Hz, H-7'), 6.10 (1H, dq, *J* = 16.0, 7.0 Hz, H-8'), 5.94 (2H, s, 3-OCH_2O-4), 5.09 (1H, d *J* = 9.0 Hz, H-7), 3.88 (3H, s, OMe-3') 3.39 (1H, dq, *J* = 9.0, 7.0 Hz, H-8), 1.86 (3H, dd, *J* = 7.0, 2.0 Hz, H-9'), 1.40 (3H, d, *J* = 7.0 Hz, H-9); ^{13}C-NMR: δ_C 148.0 (C-3), 147.7 (C-4), 146.6 (C-4'), 145.2 (C-3'), 134.4 (C-1), 133.1 (C-5'), 133.0 (C-1'), 131.0 (C-7'), 124.0 (C-8'), 119.3 (C-6), 113.4 (C-6'), 109.3 (C-2'), 108.1 (C-5), 106.9 (C-2), 101.2 (4-OCH_2O-3), 93.5 (C-7), 56.0 (3'-OMe), 46.0 (C-8), 18.5 (C-9'), 16.8 (C-9). HRTOFMS *m*/z 325.1427 $[M+H]^+$, (calcd for $C_{20}H_{21}O_4$ *m/z* 325.1440).

(+)-Maceneolignan B (**3**), a yellowish oil; $[\alpha]^{25,D}$ +53.50 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 205 (7.0), 222 (6.9), 275 (6.6) nm; IR (KBr) ν_{max} 2925, 1613, 1520, 1271, 1144, 810 cm^{-1}; ^{1}H NMR: δ_H 6.77 (1H, s, H-2'), 6.74 (1H, s, H-6'), 6.60 (1H, d, *J* = 1.5 Hz, H-6), 6.59 (1H, d, *J* = 1.5 Hz, H-2), 6.35 (1H, dd, *J* = 16.0, 2.0 Hz, H-7'), 6.11 (1H, dq, *J* = 16.0, 7.0 Hz, H-8'), 5.95 (2H, s, 3-OCH_2O-4), 5.10 (1H, d, *J* = 8.0 Hz, H-7), 3.88 (3H, s, MeO-3'), 3.88 (3H, s, OMe-5), 3.40 (1H, dq, *J* = 8.0, 7.0 Hz, H-8), 1.86 (3H, dd, *J* = 7.0, 2.0 Hz, H-9'), 1.38 (3H, d, *J* = 7.0 Hz, H-9); ^{13}C-NMR: δ_C 148.3 (C-3), 147.0 (C-4'), 145.1 (C-3'), 142.8 (C-5), 134.9 (C-4), 135.0 (C-1), 132.8 (C-5'), 131.9 (C-1'), 130.8 (C-7'), 123.6 (C-8'), 114.0 (C-6'), 109.3 (C-2'), 107.2 (C-6), 101.6 (4-OCH_2O-3), 100.8 (C-2), 93.6 (C-7), 57.0 (OMe-5), 55.8 (OMe-3'), 46.1 (C-8), 18.4 (C-9'), 18.0 (C-9). HRTOFMS *m*/z 355.1532 $[M+H]^+$, (calcd. for $C_{21}H_{23}O_5$ *m/z* 355.1545).

2.4 Bioassay for Cytotoxic Activity

Resazurin reagent contained in Prestoblue was used to evaluate the activity of compounds **1** to **3** towards B16-F10 melanoma skin cancer cells [8,9]. Cells were initially plated into 96 wells containing Rosewell Park Memorial Institute (RPMI) medium, then hatched for 24 hours at 37 °C with 5% CO_2 until a density of 1.7 x 10^4 cells/well was reached. This was followed by treating the cells with samples (compounds **1** to **3**), positive control (Cisplatin), and negative control (solvent

only). During this phase, the RPMI medium was removed and a medium containing the sample (with DMSO as the solvent) at different concentrations (1,000.00, 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91, and 1.95 µg/mL) was added and then hatched for 48 hours. The Prestoblue reagent was introduced and the mixture was incubated for 2 hours to achieve a significant color change. Following that, the absorbance of each sample was measured at 570 nm using a multimode reader. The absorbance readings were then converted to determine the percent cell viability, enabling the calculation of the IC_{50} value.

3 Results and Discussion

The structures of compounds **1** to **3** were determined by comparing their ^{1}H, ^{13}C-NMR, MS spectra and optical rotation with the existing literature, corresponding to the benzofuranoid neolignans licarin A (**1**) [10], licarin B (**2**) [11], maceneolignan B (3) [11].

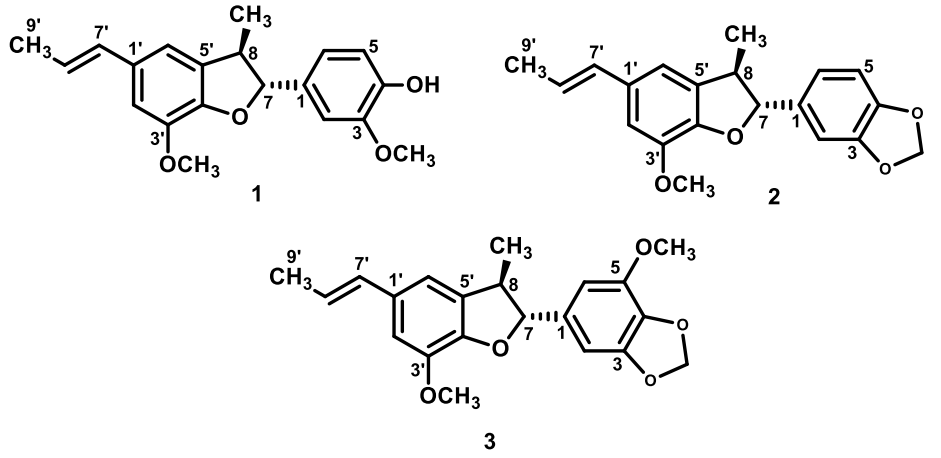

Figure 1 Structures of compounds **1-3**.

Compound **1** appeared as a white solid, with a melting point range of 113 to 115 °C. The composition was identified as $C_{20}H_{22}O_4$ by HRTOFMS showing a molecular ion peak at *m/z* 327.1583 $[M+H]^+$ (calcd. 327.1596), resulting in ten saturation degrees. Compound **1** had conjugated double bonds, i.e., the benzenoid moiety was indicated by a UV absorbance peak at 222 and 275 nm [12,13]. The IR spectrum of **1** at 3,414 cm^{-1} suggested the existence of hydroxy groups, i.e., 1,613 cm^{-1} indicating an olefinic double bond, and 1,519 cm^{-1} indicating aromatic double bond carbon. Furthermore, the ^{1}H-NMR spectrum of **1** exhibited a total of twenty-two protons, comprised of two methyls at δ_H 1.41 (3H, d, J = 7.1 Hz, H-9) and 1.86 (3H, dd, J = 7.0, 2.0 Hz, H-9') ppm; two methyls bearing oxygen at

δ_H 3.88 (3H, s, OMe-3), 3.90 (3H, s, OMe-3') ppm; one aliphatic methine at δ_H 3.44 (1H, dq, J = 10.0, 7.0 Hz, H-8) ppm; a methine bearing oxygen at δ_H 5.12 (1H, d, J = 10 Hz, H-7) ppm; which indicated the existence of furanoid methines; two olefinic methines at δ_H 6.36 (1H, dd, J = 16.0, 1.5 Hz, H-7') and 6.10 (1H, dq, J = 16.0, 6.5 Hz, H-8') ppm, which indicated a *trans* olefinic proton; five aromatic methines at δ_H 6.77 (1H, s, H-6'), 6.75 (1H, s, H-2'), 6.96 (1H, d, J = 2.0 Hz, H-2), 6.90 (1H, dd, J = 8.0, 2.0 Hz, H-6), and 6.88 (1H, d, J = 8.0 Hz, H-5) ppm, which indicated the presence of 1,2,4 substituted protons (δ_H 6.90 (H-6) that is *meta* to δ_H 6.96 (H-2) and *ortho* to δ_H 6.88 (H-5) ppm); and one aromatic hydroxy at δ_H 5.63 (1H, s, OH-4) ppm. ^{13}C-NMR data of **1** displayed twenty carbons consisting of two methyls at δ_C 17.6 (C-9) and 19.1 (C-9'); two oxygenated methyls at δ_C 56.1 (OMe-3) and 56.0 (OMe-3'); an aliphatic methine at δ_C 46.0 (C-8); a methine bearing oxygen at δ_C 94.0 (C-7); and two olefinic methines at δ_C 131.0 (C-7'), and 123.6 (C-8'). In addition, five aromatic methines were observed at δ_C 120.1 (C-6), 113.7 (C-5), 112.9 (C-6'), 109.2 (C-2'), and 108.4 (C-2); three aromatic carbons at δ_C 133.0 (C-5'), 132,3 (C-1'), and 132.1 (C-1). Four quaternary carbons bearing oxygen δ_C at 146.8 (C-3), 147.0 (C-4'), 146.0 (C-4), and 144.2 (C-3') were also identified in the ^{13}C-NMR spectrum. Compound **1** was composed of $C_{20}H_{22}O_4$, which corresponds to ten saturation degrees, where eight degrees come from two benzenoid moiety, one comes from sp^2 or olefinic bonds, and the remaining one comes from cyclical furanoid. This leads to the identification of compound **1** as benzofuranoid neolignan [3].

Based on the ^{1}H-^{1}H COSY spectra of compound **1** (Figure 2), there were fragments of olefinic (H-7' – H-8' – H-9'), aliphatic, (H-7 – H-8 – H-9), and *ortho* aromatic protons (H-5 – H-6). Based on the HMBC spectra of compound **1** (Figure 2), there is a connectivity of δ_H 6.36 (H-7') to δ_C 132.3 (C-1'), which confirmed that C-1' bonded to olefinic fragments (C-7'– C-8'– C-9'). There was also a correlation of δ_H 5.20 (H-7) to δ_C 146.6 (C-4') and 132.1 (C-1), δ_H 3.44 (H-8) to δ_C 114.1 (C-5), which confirmed the presence of aliphatic fragments (C7 – C8 – C9), forming a cyclical furanoid bond to the benzene. The correlation of side groups consisted of two methoxy δ_H 3.87 (3'-OCH_3) to δ_C 144.2 (C-3') and δ_H 3.88 (3-OCH_3) to δ_C 146.8 (C-3) and one hydroxy group at δ_H 5.63 (4-OH) to δ_C 145.8 (C-4), confirming their position in the structure.

The evaluation of the NMR spectra of compound **1** was compared to data from the literature, suggesting compound **1** to be licarin A [10]. Licarin A(1) is a benzofuranoid neolignan with two chiral carbons, which could be determined by their H-9 and H-7 chemical shift and vicinal coupling, NOESY connectivity between H-9 and H-7, and optical rotation in comparison with the existing literature. H-7 and H-9 pointed in the same direction, i.e., *cis* had δ_H 5.7-5.8 (H-7) and δ_H 0.7-0.8 (H-9) with $J_{7,9}$ = 2-5 Hz. Meanwhile, *trans* appeared at δ_H 1.3-1.4

(H-9) and δ_H 5.10 (H-7) with $J_{7,9}$ = 8-10 Hz [3,14].

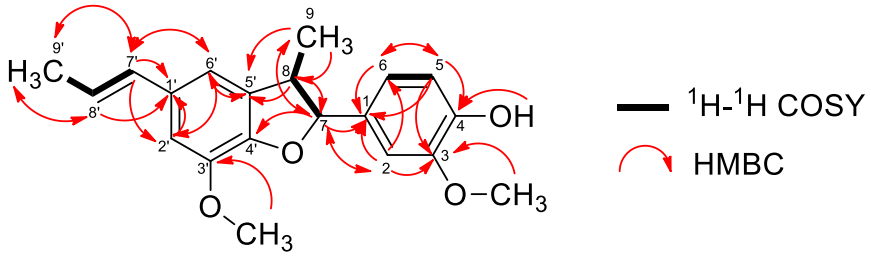

Figure 2 2D NMR correlations for compound **1**.

Compound **1** had δ_H 1.41 (H-9) and 5.10 (H-7) ppm, along with a large coupling (J = 10.0 Hz) between δ_H 5.10 and 3.44 ppm. NOESY correlation implied (Figure 3) H-7 and H-9 in the same plane, which means H-8 and H-7 had a *trans* correlation. Optical rotation was done to support the result. Based on the optical rotary value ($[\alpha]^{25,D}$ = +68.75), compound **1** had a (+) configuration, according to 8*R* and *7R* stereochemistry [10]. Therefore, it is implied that compound **1** was (+)-licarin A.

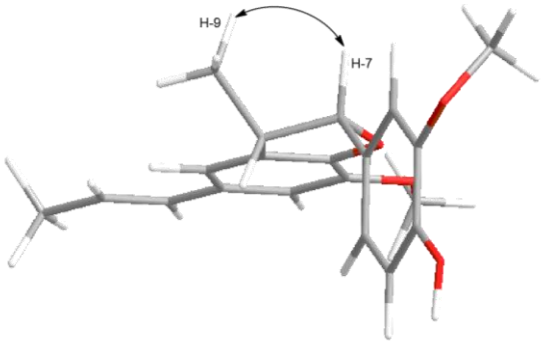

Figure 3 NOESY correlation for compound **1**.

Compound **2** appeared as a colorless oil, with a composition of $C_{20}H_{20}O_4$, as determined from the $[M+H]^+$ peak at *m/z* 325.1427 (calcd. 325.1440), resulting in eleven saturation degrees. The UV absorbance at 222 and 275 nm suggests the presence of conjugated double bonds such as benzene groups [12,13]. The IR spectrum of **2** at 1,613 cm^{-1} showed the existence of an aliphatic double bond, while the wavenumber at 1,519 cm^{-1} showed aromatic double bonds. The ^{1}H-NMR spectrum of **2** showed a total of twenty protons, comprised of two methyls at δ_H 1.40 (3H, d, J = 7.0 Hz, H-9) and 1.86 (3H, dd, J = 7.0, 2.0 Hz, H-9') ppm; one oxygenated methyl at δ_H 3.88 (3H, s, OMe-3') ppm; one methylenedioxy at

δ_H 5.94 (2H, s, 3-OCH_2O-4) ppm; one aliphatic methine at δ_H 3.39 (1H, dq, *J* = 8.9, 7.0 Hz, H-8); a methine bearing oxygen at δ_H 5.09 (1H, d *J* = 9.1 Hz, H-7) ppm, which indicates furanoid methines; olefinic methines at δ_H 6.36 (1H, dd, *J* = 16.0, 1.7 Hz, H-7') and 6.10 (1H, dq, *J* = 16.2, 7.0 Hz, H-8') ppm, which indicates a *trans* olefinic correlation by their huge coupling value; five aromatic methines at δ_H 6.77 (1H, s, H-6'), 6.76 (1H, s, H-2') ppm, 6.91 (1H, d, *J* = 2.0 Hz, H-2), 6.87 (1H, dd, *J* = 8.4, 2.0 Hz, H-6), and 6.73 (1H, d, *J* = 8.4 Hz, H-5) ppm, which indicate 1,2,4 proton substitution (δ_H 6.87 is *meta* to δ_H 6.91 and *ortho* to δ_H 6.73 ppm). From the ^{13}C-NMR spectrum of **2**, there was a total of twenty carbons, i.e., two methyls at δ_C 17.8 (C-9) and 18.5 (C-9'); one oxygenated methyl at δ_C 55.9 (OMe-3'); one aliphatic methine at δ_C 46.0 (C-8); a methine bearing oxygen at δ_C 93.5 (C-7); two olefinic methines at δ_C 131.0 (C-7'), 123.6 (C-8'); five aromatic methines at δ_C 120.3 (C-6), 113.4 (C-6'), 109.3 (C-2'), 108.1 (C-5), and 106.9 (C-2); one methylenedioxy at δ_C 101.2 (4-OCH_2O-3); three aromatic quaternary carbons at δ_C 135.0 (C-1), 133.1 (C-5'), 132.3 (C-1'); and four oxygenated quaternary signals at δ_C 147.5 (C-3), 148.0 (C-4), 145.9 (C-4'), and 145.1 (C-3'). Compound **2** had a molecular formula of $C_{20}H_{20}O_4$, which corresponds to eleven saturation degrees, where eight values come from two benzene groups, one comes from sp^2 olefinic groups, one comes from cyclic furanoid, and the remaining is suggested to come from the side chain methylenedioxy.

Compound **2** shared the same skeleton as compound **1**, a benzofuranoid neolignan, with the alteration of methoxy and hydroxy in C-3 and C-4 into closed methylenedioxy (-OCH_2O-). Based on comparison with the literature, compound **2** was identified as licarin B [11]. As mentioned, the stereochemistry licarin B could be determined as licarin A. The vicinal coupling between H-9 and H-7 valued ($J_{7,9}$ = 9.0 Hz) exhibited a *trans* vicinal coupling between H-7 and H-8, supported by optical rotation value ($[\alpha]^{25,D}$ = +51.20) and by the existing data, which suggest that compound **2** had an *8R* and *7R* configuration, which means compound **2** was (+)-licarin B [11].

Compound **3** appeared as a yellowish gum, with a composition of $C_{21}H_{22}O_5$, as identified from the $[M+H]^+$ peak at *m/z* 355.1532 (calcd. 355.1545), indicating eleven saturation degrees. The presence of conjugated double bonds such as benzenoid moiety was indicated by UV absorbance peaks at 222 and 275 nm [12,13]. The IR wavenumber at 1613 cm^{-1} showed the presence of an aliphatic double bond and the IR wavenumber at 1520 cm^{-1} showed the existence of aromatic double bonds. The spectrum of ^{1}H-NMR of compound **3** showed a total of twenty two protons, comprising two methyls at δ_H 1.38 (3H, d, *J* = 7.0 Hz, H-9) and 1.86 (3H, dd, *J* = 7.0, 2.0 Hz, H-9') ppm; two oxygenated methyls at δ_H 3.88 (3H, s, MeO-3') and 3.88 (3H, s, OMe-5) ppm; one methylenedioxy at δ_H

5.95 (2H, s, 3-OCH_2O-4) ppm; one aliphatic methine at δ_H 3.40 (1H, dq, *J* = 8.0, 7.0 Hz, H-8); a methine bearing oxygen at δ_H 5.10 (1H, d, *J* = 8.0 Hz, H-7) ppm, which indicates furanoid methines; two methines sp^2 at δ_H 6.35 (1H, dd, *J* = 16.0, 1.5 Hz, H-7') and 6.11 (1H, dq, *J* = 16.0, 7.0 Hz, H-8') ppm, which indicate a *trans* olefinic proton; four aromatic methines at δ_H 6.77 (1H, s, H-2'), 6.74 (1H, s, H-6') ppm, and 6.60 (1H, d, *J* = 1.5 Hz, H-6), 6.59 (1H, d, *J* = 1.5 Hz, H-2) ppm, which indicate a *meta* correlation between H-6 and H-2. From the ^{13}C-NMR spectrum of **3**, there was a total of twenty-one carbons, comprising two methyls 18.4 (C-9'), 17.9 (C-9); two oxygenated methyls at δ_C 56.7 (OMe-5), 56.0 (OMe-3'); one aliphatic methine at δ_C 45.9 (C-8); an oxygenated methine at δ_C 93.6 (C-7); four aromatic methines at δ_C 100.8 (C-2), 114.0 (C-6'), 109.3 (C-2'), 107.0 (C-6); two olefinic methines at δ_C 130.8 (C-7'), 123.6 (C-8'); one methylenedioxy at δ_C 101.6 (4-OCH_2O-3); three aromatic quaternary carbons 135.0 (C-1), 132.8 (C-5'), 131.9 (C-1'); and five quaternary carbons bearing oxygen at δ_C 148.3 (C-3), 147.0 (C-4'), 143.7 (C-3'), 142.8 (C-5). Compound **3** had a molecular formula of $C_{21}H_{22}O5$, which corresponds to eleven saturation degrees, where eight values come from two benzene groups, one comes from sp^2 olefinic groups, one comes from cyclic furanoid, and the remaining is suggested to come from the side chain methylenedioxy.

Compound **3** had the same structure as compound **2** with the addition of methoxy at C-5. By comparing with NMR, compound **3** was identified as maceneolignan B [11]. The stereochemistry, as mentioned before, was characterized by a large *J* value between H-7 and H-9 ($J_{7,9}$ = 8.0 Hz) and exhibited a *trans* vicinal coupling between H-7 and H-8, as was supported by optical rotation ($[\alpha]^{25,D}$ = +53.50), yielding (+) and comparison with the literature suggested that compound **3** had *8R* and *7R*, which means compound **3** was (+)-maceneolignan B [11].

Table 1 Compounds **1**-**3** cytotoxic activity against melanoma B16-F10 (*in vitro*).

Compounds	IC_{50} (μM)
(+)-licarin A (**1**)	94.15
(+)-licarin B (**2**)	1449.75
(+)-maceneolignan B (**3**)	116.14
Cisplatin	43.00

The cytotoxicity of benzofuranoid neolignans **1** to **3** was evaluated against skin cancer melanoma B16-F10 cell lines according to the explained method utilizing Cisplatin as positive control. Of all the compounds, (+)-licarin A (**1**) had the most potent activity against melanoma B16-F10 with an IC_{50} of 94.15 μM. Comparison among compounds **1** to **3** showed that the OH groups had C-4 high cytotoxicity, while closing the groups resulted in weaker activity. Furthermore, comparison between (+)-licarin B (**2**) and (+)-maceneolignan B (**3**) activity showed that the OCH_3 groups at C-5 had a significant increase in cytotoxic effect.

4 Conclusions

In conclusion, this study successfully isolated three benzofuranoid neolignans from *Myristica fragrans* seeds, namely, (+)-licarin A (**1**), (+)-licarin B (**2**), and (+)-maceneolignan B (**3**). Based on the results, (+)-licarin A (**1**) had the most cytotoxic effect against melanoma B16-F10 cells with an IC_{50} value of 94.15 μM, indicating the significant influence of hydroxy groups.

Acknowledgement

This study was partially supported by Academic Leadership Grant No. 1630/UN6.3.1/PT.00/2024 from Universitas Padjadjaran, Indonesia, awarded to US.

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