Hindawi
Journal of Chemistry
Volume 2021, Article ID 4153790, 12 pages
https://doi.org/10.1155/2021/4153790

Research Article

Characterization and Antifungal Activity of Limonoid Constituents Isolated from Meliaceae Plants *Melia dubia*, *Aphanamixis polystachya*, and *Swietenia macrophylla* against Plant Pathogenic Fungi *In Vitro*

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Received 13 May 2021; Accepted 20 July 2021; Published 31 July 2021

Academic Editor: Xinyong Liu

The plants of Meliaceae are native to tropical and subtropical regions as the Americas, west India, Southeast Asia, and Southern China. Many species of the genera *Khaya*, *Swietenia*, *Aphanamixis*, and *Melia* in this family are known as medicinal plants and have biological activities such as antiviral, antimicrobial, antifeeding, insecticidal, and cytotoxic properties. The objectives of this research are to characterize and evaluate the bioactive limonoids from several plants of Meliaceae against phytopathogenic fungi. During the search of antifungal compounds from the plants of Meliaceae, the three methanol extracts of *Melia dubia*, *Aphanamixis polystachya*, and *Swietenia macrophylla* were found to suppress the mycelial growth of several phytopathogenic fungi. Nine limonoids isolated from *M. dubia* (**1**–**2**), *A. polystachya* (**3**–**5**), and *S. macrophylla* (**6**–**9**) were evaluated, for the first time, their antifungal effectiveness against nine phytopathogenic fungi *Fusarium oxysporum*, *Magnaporthe oryzae*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Alternaria* spp., and *Botrytis cinerea*, and three oomycetes *Phytophthora* species. Limonoids **2**, **3**, **6**, and **8** displayed a remarkable broad-spectrum antifungal activity against all the test fungi. *Sclerotium rolfsii* was highly sensitive to the four limonoids with IC_{50} values ranging from 79.4 to 128.0 μg/mL. Notably, chisocheton compound G (**3**) isolated from *A. polystachya* and khayanolide B (**8**) isolated from *S. macrophylla* were the most potent antifungal limonoids and exhibited a dose-dependent activity against *Phytophthora* species. Compounds **2** and **9** displayed moderate activity against *M. oryzae*. Our study results demonstrated the discovery of antifungal and lead compounds from the group of limonoids for application in the control of fungal plant diseases.

1. Introduction

The Meliaceae family has 50 genera and 1,400 species and distributed in tropical and subtropical regions as the Americas, west India, Southeast Asia, and Southern China [1, 2]. Almost all limonoids (more than 300 compounds) have been identified, and about one-third has been found in *Azadirachta indica* and *Melia azedarach* [3]. There were more than 160 limonoids isolated from four species of the genus *Swietenia* [4]. The seeds of *S. macrophylla* contain

bioactive compounds with anti-inflammatory, antimutagenicity, and antitumor activity, which has been used in traditional medicine in the world. Furthermore, the seeds of *S. macrophylla* in Malaysia are used traditionally to treat hypertension, diabetes, and relieve pain [1]. Limonoids and their derivatives are determined as the major constituents of *S. macrophylla*.

The application of limonoids in plant protection has been developed since the 1960s. To date, most of the Meliaceous limonoids have been reported to be related to antifeedant, insecticidal, cytotoxic, antimalarial, and anticancer activity. Azadirachtin is an insect growth regulator and feeding deterrent that affects more than 60 insects such as aphids, caterpillars, leafhoppers, leafminers, mealybugs, psyllids, thrips, and whiteflies by effects on feeding and reducing ecdysone hormone in insects [5]. Gedunin, nimbin, and nimbolide isolated from *Azadirachta indica* and *K. grandifoliola* have been reported to have *in vitro* antimalarial activity on *P. falciparum*. According to Roy and Saraf (2006), the C-seco limonoids with an enone system in ring "A" are potent cytotoxic and antimalarial agents. Moreover, the C-seco limonoids are two to three times more active than other limonoids and they are also highly active against herbivorous insects [3]. The well-known insecticidal limonoids (azadirachtin, salannin, and nimbin) isolated from neem have been used as an active ingredient in a number of commercial insecticide formulations and marketed in many countries [6–8]. Over the years, the well-known limonoids and neem extracts were documented as successful examples of botanical insecticides commercialized. Based on the record of known safety, mode of action, and interaction of the limonoid active ingredients, the chemical class is expected to repurpose or use widely for controlling the phytopathogens in organic agriculture [9–13]. However, to our best knowledge, there are very few studies on the activity of Meliaceous limonoids against phytopathogenic fungi. Several antifungal limonoids against fungal plant pathogens have been studied and reported to occur in the fruits of *Khaya senegalensis*, *K. ivorensis*, and *Aphanamixis polystachya*. Zhang et al. (2013) identified prieurianin-type limonoids and aphanamolide-type limonoids from the fruits of *A. polystachya* and conducted some preliminary experiments to evaluate for their fungicidal, herbicidal, and insecticidal activities [14]. Methyl angolensate and 1, 3, 7-trideacetylkhivorin isolated from the fruits of *K. ivorensis* displayed a moderate inhibition against the mycelial growth of *Botrytis cinerea*, at 1000 and 1500 μg/mL [10]. From the fruits of *K. senegalensis*, Abdelgaleil et al. (2004) also successfully isolated bioactive limonoids such as seneganolide A, 2-acetoxyseneganolide A, and methyl 6-hydroxyangolensate with antifungal effects on *B. cinerea* [9]. In our ongoing research studies on bioactive limonoids from Meliaceous plants growing in Vietnam, we successfully isolated and identified nine limonoids from *Melia dubia* (**1**–**2**), *Aphanamixis polystachya* (**3**–**5**), and *Swietenia macrophylla* (**6**–**9**). The antifungal potential of the isolated limonoids against various phytopathogenic fungi including *F. oxysporum*, *M. oryzae*, *S. rolfsii*, *R. solani*, *Alternaria* spp., *B. cinerea*, and oomycetes *Phytophthora* species was evaluated *in vitro*.

2. Materials and Methods

2.1. Plant Materials. The fruits of three species (*Melia dubia*, *Aphanamixis polystachya*, and *Swietenia macrophylla*) were collected from July to August 2017 from Pu Huong Nature Reserve, Nghe An Province; Vu Quang Nature Reserve, Ha Tinh Province; and Cat Tien National Park, Dong Nai Province, Vietnam, respectively. The plant materials were identified by Dr. Quoc Binh Nguyen, Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, Hanoi, Vietnam. The voucher samples (no. MDF-072017, APF-082017, and SMF-082017) were deposited in the same museum.

2.2. Microbial Strains and Culture Conditions. The *in vitro* antifungal activity of the botanical extracts and purified limonoids was tested based on poisoned food technique against nine phytopathogenic fungi including *Fusarium oxysporum*, *Magnaporthe oryzae*, *Sclerotium rolfsii*, *Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria* spp., *Phytophthora capsici*, *Phytophthora palmivora*, and *Phytophthora* spp. Out of oomycetes *Phytophthora* species, *Phytophthora* spp. was isolated from the infected roots of *Panax vietnamensis* Ha et Grushv., and *P. palmivora* and *P. capsici* were isolated from the infected leaves of durian (*Durio zibethinus*) and black pepper (*Piper nigrum*), respectively. Potato dextrose broth (PDB) was used to subculture, and the potato dextrose agar (PDA) medium was used to culture fungal strains for antifungal tests at 20–25°C for 2–7 days.

2.3. Isolation and Characterization of Phytochemical Constituents from Melia dubia, Aphanamixis polystachya, and Swietenia macrophylla. The dried fruit powder of *Melia dubia* (16.0 kg) was extracted with methanol at ambient temperature (20 L, 5 times), and total methanol extract was evaporated under reduced pressure to give the methanol crude extract (854 g). Then, it was suspended in water and partitioned successively with *n*-hexane, ethyl acetate, and butanol to afford *n*-hexane extract (MDH-68 g), ethyl acetate extract (MDE-272 g), butanol extract (MDB-133 g), and water-soluble fraction (80 g), respectively. The ethyl acetate extract (272 g) was applied to silica gel column chromatography and was eluted by a mixture of chloroform/methanol with gradient (100 : 0, 50 : 1, 30 : 1, 20 : 1, 10 : 1, 5 : 1, 2 : 1, and 1 : 1) to afford ten fractions (Frs. F1–F10). Fraction F2 (16.5 g) was subjected to silica gel column chromatography (150 g, 80 × 2 cm) eluting by a mixture of *n*-hexane/acetone with gradient 15 : 1 to obtain six fractions (F2.1–F2.6). Fraction F2.1 (2.5 g) was subjected to the silica gel column chromatography (300 g, 80 × 3 cm) eluting with a mixture of *n*-hexane: acetone (7 : 1) to give compound **1** (**MD1**, 21 mg). Fraction F2.2 (0.2 g) was separated by preparative HPLC (MeOH/H_2O, 80 : 40 to 90 : 10, 10 min, 16 ml min^{-1}) to afford compound **2** (**MD2**, 12 mg) (Figure S1a).

The dried fruit powder of *Aphanamixis polystachya* (9.0 kg) was extracted with methanol at ambient temperature (10 L, 3 times), and total methanol extract was evaporated under reduced pressure to give the methanol crude extract

(480 g). Then, it was suspended in water and partitioned successively with *n*-hexane, ethyl acetate, and butanol to afford *n*-hexane extract (APH, 68 g), ethyl acetate extract (APE, 172 g), butanol extract (APB, 87 g), and water-soluble fraction (APW, 100 g), respectively. The ethyl acetate extract (172 g) was applied to silica gel column chromatography and was eluted by a mixture of chloroform/methanol with gradient (100 : 0, 50 : 1, 30 : 1, 20 : 1, 10 : 1, 5 : 1, 2 : 1, and 1 : 1) to afford seven fractions (F1–F7). Fraction F3 (5.9 g) was subjected to silica gel column chromatography (120 g, 80 × 1.5 cm) eluting by a mixture of *n*-hexane/ethyl acetate with gradient 9 : 1 to obtain five fractions (Frs. F2.1–F2.5). Fraction F3.2 (1.68 g) was subjected to the silica gel column chromatography (150 g, 80 × 2 cm) eluting with a mixture of *n*-hexane: ethyl acetate (5 : 1) to give compound **3** (**AP1**, 11 mg). Fraction F3.3 (0.2 g) was separated by the silica gel column chromatography (*n*-hexane: ethyl acetate, 20 : 10 to 20 : 5) to afford compound **4** (**AP2**, 21 mg) and compound **5** (**AP3**, 14 mg) (Figure S1b).

The dried powdered fruits of *Swietenia macrophylla* (6.0 kg) were extracted with methanol (3 times × 10 L) at room temperature to yield 318.0 g of the crude methanol extract. The crude extract was then suspended in distillate water and introduced to liquid-liquid extraction partitioning in turn with *n*-hexane and ethyl acetate to obtain crude *n*-hexane and ethyl acetate, respectively. The ethyl acetate extract (105.0 g) was chromatographed by a silica gel column and eluted with a *n*-hexane: ethyl acetate (gradient 100 : 1 to 1 : 1) to yield 5 fractions. Fraction 4 was separated by a silica gel column, eluting with *n*-hexane-ethyl acetate (25 : 1 to 2 : 1) to give 7 subfractions from 4.1 to 4.7. Fraction 4.3 was rechromatographed by a silica column eluted with *n*-hexane: ethyl acetate (15 : 1) to afford compound **7** (**SM2,** 13 mg). Then, fraction 4.4 was rechromatographed by a silica column eluted with *n*-hexane: ethyl acetate (9 : 1) to yield compounds **6** (**SM1**, 18 mg) and **8** (**SM3**, 34 mg) were isolated from fraction 4.5 by using a silica gel column and eluted *n*-hexane: ethyl acetate (9 : 1; 4 : 1). Compound **9** (**SM4**, 12.5 mg) (Figure S1c) was obtained from the purification of fraction 5 by silica gel column chromatography eluting with CH_2Cl_2 : CH_3OH (9 : 1).

2.4. Structural Characterization of the Isolated Compounds

*2.4.1. Compound **1** (**MD1**): Dysobinin [15, 16].* Colorless crystals, m.p. 209–210°C; HR-ESI-MS *m/z* 517.2629 $[M + Na]^+$; ^{1}H NMR (DMSO-d_6, 500 MHz): *d* (ppm): 7.38 (*t*, *J* = 3.5 Hz, C_1-H), 7.24 (*s*, C_{21}-H), 7.14 (*dd*, *J* = 2.5, 10.5 Hz, C_{23}-H), 6.28 (*s*, C_{22}-H), 5.92 (*dd*, *J* = 3.5, 10.0 Hz, C_2-H), 5.46 (*d*, *J* = 3.0 Hz, C_7-H), 5.43 (*m*, C_6-H), 5.39 (*s*, C_{15}-H), 2.82 (*m*, C_{17}-H), 2.51 (*dd*, *J* = 3.0, 11.5 Hz, C_5-H), 2.04 (*s*, C_7-$OCOCH_3$), 2.02 (*s*, C_6-$OCOCH_3$), 1.33 (*s*, C_{19}-H), 1.26 (*s*, C_{30}-H), 1.19 (*s*, $C_{28,29}$-H), and 0.81 (*m*, C_{18}-H); ^{13}C NMR (DMSO-d_6, 125 MHz): *d* (ppm): C_3 (204.6), 6-OCO (170.2), 7-OCO (170.0), C_{14} (158.2), C_1 (157.2), C_{21} (142.5), C_{23} (139.6), C_2 (126.1), C_{20} (124.3), C_{15} (119.7), C_{22} (110.9), C_7 (74.5), C_6 (69.9), C_{17} (51.6), C_5 (47.9), C_{13} (47.06), C_8 (44.8), C_{10} (43.0), C_4 (40.7), C_9 (37.3), C_{16} (34.3), C_{12} (32.7), C_{18} (31.5), C_{28} (26.7), 7-$OCOCH_3$ (21.3), 6-$OCOCH_3$ (21.0), C_{29} (20.8), C_{30} (20.7), C_{19} (20.3), and C_{11} (16.3).

*2.4.2. Compound **2** (**MD2**): (3α, 8R, 9S, 20R, 24S)-20, 24-Epoxytirucalla-3, 25-Diol [17].* White crystals, m.p. 236–237°C; HR-ESI-MS *m/z* 461.3986 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 500 MHz): *δ* (ppm): 3.73 (*t*, *J* = 7.0 Hz, C_{24}-H), 3.39 (*t*, *J* = 2.5 Hz, C_3-H), 1.94 (*m*, C_2-H), 1.86 (*m*, C_{22}-H), 1.71 (*m*, C_7-H), 1.62 (*m*, C_7-H), 1.58 (*m*, C_{16}-H), 1.56 (*m*, C_{11}-H), 1.55 (*m*, C_8-H), 1.52 (*m*, C_2-H), 1.45 (*m*, $C_{6,9}$-H), 1.44 (*m*, C_{22}-H), 1.42 (*m*, C_{15}-H), 1.41 (*m*, $C_{1,23}$-H), 1.27 (*m*, C_1-H), 1.26 (*m*, C_6-H), 1.25 (*m*, $C_{5,21}$-H), 1.24 (*m*, C_{23}-H), 1.20 (*s*, C_{27}-H), 1.17 (*m*, C_{11}-H), 1.12 (*d*, *J* = 5.5 Hz, $C_{12,26}$-H), 1.07 (*t*, *J* = 17.5 Hz, C_{15}-H), 0.96 (*s*, C_{30}-H), 0.94 (*s*, C_{28}-H), 0.88 (*s*, C_{18}-H), 0.85 (*s*, C_{19}-H), and 0.84 (*s*, C_{29}-H); ^{13}C NMR ($CDCl_3$, 125 MHz): *δ* (ppm): C_{20} (86.4), C_{24} (83.3), C_3 (76.3), C_{25} (71.5), C_9 (50.6), C_{14} (50.2), $C_{5,17}$ (49.6), C_8 (42.9), C_{13} (40.6), C_4 (37.6), C_{10} (37.3), C_7 (35.7), C_{23} (35.2), C_1 (33.7), C_{15} (31.4), C_{28} (28.3), $C_{21,\ 27}$ (27.4), $C_{12,22}$ (26.2), C_2 (25.7), C_{16} (25.4), C_{26} (24.3), C_{29} (22.1), C_{11} (21.4), C_6 (18.2), C_{18} (16.6), C_{19} (16.0), and C_{30} (15.5).

*2.4.3. Compound **3** (**AP1**): Chisocheton Compound G [18].* White powder, m.p. 182–183°C; HR-ESI-MS *m/z* 549.2566 $[M + Na]^+$; ^{1}H NMR (DMSO-d_6, 500 MHz): *δ* (ppm): 7.17 (*m*, C_1-H), 5.98 (*s*, C_{21}-H), 5.93 (*d*, *J* = 10.5 Hz, C_2-H), 5.90 (*s*, C_{22}-H), 5.45 (*d*, *J* = 3.0 Hz, C_7-H), 5.42 (*d*, *J* = 3.5 Hz, C_6-H), 5.40 (*d*, *J* = 2.5 Hz, C_{15}-H), 2.52 (*s*, C_5-H), 2.05 (*s*, C_7-$OCOCH_3$), 2.02 (*s*, C_6-$OCOCH_3$), 1.33 (*s*, C_{19}-H), 1.26 (*s*, C_{30}-H), 1.19 (*s*, $C_{28,29}$-H), and 0.93 (*m*, C_{18}-H). ^{13}C NMR (DMSO-d_6, 125 MHz): *δ* (ppm): C_3 (204.9), C_{23} (171.6), C_{20} (170.4), 6-O$\underline{C}$O (170.2), 7-O$\underline{C}$O (169.5), C_{14} (157.5), C_1 (157.2), C_2 (126.1), C_{15} (119.6), C_{22} (118.6), C_{21} (99.1), C_7 (74.5), C_6 (69.8), C_{17} (52.7), C_5 (47.8), C_{13} (47.2), C_{10} (44.9), C_8 (43.1), C_4 (40.7), C_9 (37.1), C_{12} (33.2), C_{16} (33.0), C_{18} (31.5), C_{28} (26.6), 7-OCO$\underline{C}H_3$ (21.4), 6-OCO$\underline{C}H_3$ (21.1), C_{29} (20.8), C_{30} (20.6), C_{19} (20.2), and C_{11} (16.3).

*2.4.4. Compound **4** (**AP2**): Chisocheton Compound E [18].* Colorless crystals, m.p. 236–237°C; ESI-MS *m/z* 513.2 $[M + H]^+$; ^{1}H NMR (DMSO-d_6, 500 MHz): *δ* (ppm): 7.13 (*d*, *J* = 10.0 Hz, C_1-H), 5.95 (*d*, *J* = 10.0 Hz, C_2-H), 5.45 (*d*, *J* = 3.5 Hz, C_7-H), 5.42 (*d*, *J* = 6.0 Hz, C_6-H), 5.40 (*d*, *J* = 2.5 Hz, C_{15}-H), 4.49 (*t*, *J* = 3.0 Hz, C_{21}-H_a), 3.94 (*t*, *J* = 4.5 Hz, C_{21}-H_b), 2.75 (*m*, C_{20}-H), 2.52 (*s*, C_5-H), 2.04 (*s*, C_7-$OCOCH_3$), 1.99 (*s*, C_6-$OCOCH_3$), 1.29 (*s*, C_{19}-H), 1.26 (*s*, C_{30}-H), 1.18 (*s*, $C_{28,29}$-H), and 1.04 (*m*, C_{18}-H). ^{13}C NMR (DMSO-d_6, 125 MHz): *d* (ppm): C_3 (204.4), C_{23} (176.4), 6-O$\underline{C}$O (170.2), 7-O$\underline{C}$O (169.9), C_{14} (158.0), C_1 (156.8), C_2 (126.3), C_{15} (119.6), C_7 (74.4), C_{21} (72.3), C_6 (69.8), C_{17} (58.2), C_5 (48.0), C_{13} (46.6), C_{10} (44.9), C_8 (42.9), C_4 (40.8), $C_{9,\ 20}$ (37.0), C_{22} (34.8), $C_{12,\ 16}$ (33.5), C_{18} (31.7), C_{28} (26.9), 7-OCO$\underline{C}H_3$ (21.3), 6-OCO$\underline{C}H_3$ (20.9), C_{29} (20.7), C_{30} (20.4), C_{19} (20.1), and C_{11} (16.4).

*2.4.5. Compound **5** (**AP3**): 6α-Acetoxyepoxyazadiradione VI [19].* Colorless crystals, m.p. 167–168°C; ESI-MS *m/z* 525.1 $[M + H]^+$; ^{1}H NMR (DMSO-d_6, 500 MHz): *δ* (ppm): 7.55

(C_{21}-H), 7.4 (C_{23}-H), 7,12 (*d*, $J = 10$ Hz, C_1-H), 6.23 (C_{22}-H), 5.94 (*d*, $J = 10.5$ Hz, C_2-H), 5.37 (*dd*, $J = 12.5$, 2.5 Hz, C_6-H), 5.03 (*d*, $J = 2.5$ Hz, C_7-H), 3.89 (*s*, C_{17}-H), 3.43 (*s*, C_{15}-H), 2.53 (*m*, C_9-H), 2.52 (*d*, $J = 12.5$ Hz, C_5-H), 2.08 (*s*, C_7-$OCOCH_3$), 2.03 (*s*, C_6-$OCOCH_3$), 1.93 (*m*, C_{11}-H_a), 1.84 (*m*, C_{11}-H_b), 1.32 (C_{26}-H), 1.25 (C_{24}-H), 1.21 (*s*, $C_{18,19}$-H), and 1.04 (C_{25}-H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm): C_3 (204.1), 6-O$\underline{C}$OCH_3 (169.8), 7-O$\underline{C}$OCH_3 (169.7), $C_{1,16}$ (156.6), C_{23} (142.4), C_{21} (141.5), $C_{2,20}$ (126.4), C_{22} (110.8), C_7 (72.1), $C_{6,14}$ (69.7), C_{15} (57.0), $C_{13,17}$ (50.7), C_9 (48.5), C_5 (48.4), C_4 (45.0), C_8 (43.2), C_{10} (40.4), C_{24} (31.5), C_{12} (24.5), C_{19} (21.4), 6,7-OCO$\underline{C}H_3$ (21.1), $C_{25,26}$ (20.1), C_{18} (18.9), and C_{11} (16.0).

2.4.6. Compound 6 (SM1): Seneganolide [20]. White powder, m.p. 276–277°C; ESI-MS m/z 471.1 $[M + H]^+$ ($C_{26}H_{31}O_8$); ^{1}H NMR ($CDCl_3$ and CD_3OD, 500 MHz): δ (ppm): 7.40 (*d*, $J = 2.0$ Hz, C_{23}-H), 7.40 (*brs*, C_{21}-H), 6.33 (*t*, $J = 1.0$ Hz, C_{22}-H), 5.28 (*s*, C_{17}-H), 4.45 (*d*, $J = 11.5$ Hz, C_{19}-H_α), 4.21 (*d*, $J = 12.0$ Hz, C_{19}-H_β), 2.84 (*dd*, $J = 7.0$, 13.5 Hz, C_2-H), 2.83 (*dd*, $J = 7.0$, 19.5 Hz, C_{15}-H_β), 2.78 (*dd*, $J = 2.0$, 19.5 Hz, C_{15}-H_α), 2.70 (*dd*, $J = 11.0$, 15.0 Hz, C_6-H_α), 2.56 (*dd*, $J = 7.0$, 15.0 Hz, C_6-H_β), 2.32 (*dd*, $J = 10.5$, 7.0 Hz, C_5-H), 2.27 (*dd*, $J = 2.0$, 7.0 Hz, C_{14}-H), 2.20 (*td*, $J = 2.0$, 13.5 Hz, C_{30}-H_α), 2.01 (*br d*, $J = 11.5$ Hz, C_9-H), 1.87 (*dd*, $J = 7.0$, 13.5 Hz, C_{30}-H_β), 1.76 (*dd*, $J = 2.0$, 11.0 Hz, C_{12}-H_β), 1.61 (*m*, C_{11}-H_α), 1.41 (*m*, C_{11}-H_β), 1.41 (*br t*, $J = 12.5$ Hz, C_{12}-H_α), 1.32 (*s*, C_{29}-H), 1.19 (*s*, C_{28}-H), and 1.03 (*s*, C_{18}-H). ^{13}C NMR ($CDCl_3$ & CD_3OD, 125 MHz): *d* (ppm): C_3 (213.5), C_7 (174.1), C_{16} (170.6), C_{23} (143.1), C_{21} (140.9), C_{20} (120.7), C_{22} (109.6), C_1 (107.5), C_8 (80.1), C_{17} (78.1), C_{19} (74.1), C_9 (61.2), C_2 (53.0), C_{10} (46.8), C_{14} (44.7), C_4 (44.7), C_5 (38.5), C_{13} (35.3), C_{12} (34.9), C_{30} (31.4), C_6 (29.5), C_{15} (27.5), C_{28} (23.8), C_{18} (22.4), C_{11} (20.8), and C_{29} (19.6).

2.4.7. Compound 7 (SM2): Khayanone [21]. Colorless crystals, m.p. 170–171°C; ESI-MS m/z 503.1 $[M + H]^+$ ($C_{27}H_{35}O_9$); ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm): 7.45 (*brs*, C_{21}-H), 7.43 (*t*, $J = 2.0$ Hz, C_{23}-H), 6.37 (*m*, C_{22}-H), 5.60 (*s*, C_{17}-H), 4.42 (*m*, C_6-H), 3.83 (*s*, 7-$COOCH_3$), 3.14 (*d*, $J = 9.0$ Hz, C_2-H), 3.13 (*d*, $J = 15.0$ Hz, C_{30}-H_β), 2.89 (*brs*, C_6-OH), 2.82 (*dd*, $J = 19.0$, 2.0 Hz, C_{15}-H_α), 2.78 (*brs*, C_8-OH), 2.78 (*m*, C_5-H), 2.75 (*dd*, $J = 19.0$, 7.5 Hz, C_{15}-H_β), 2.36 (*ddd*, $J = 15.0$, 9.5, 2.0 Hz, C_{30}-H_α), 1.87 (*dd*, $J = 13.0$, 5.0 Hz, C_9-H), 1.82 (*m*, C_{11}-H_α), 1.75 (*dd*, $J = 7.5$, 2.0 Hz, C_{14}-H), 1.72 (*m*, C_{12}-H_α), 1.36 (*s*, C_{19}-H), 1.28 (*s*, C_{29}-H), 1.27 (*s*, C_{28}-H), 1.26 (*m*, C_{12}-H_β), 1.19 (*m*, C_{11}-H_β), and 0.99 (*s*, C_{18}-H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm): C_3 (214.2), C_1 (213.1), C_7 (175.5), C_{16} (171.2), C_{23} (143.1), C_{21} (141.1), C_{20} (120.9), C_{22} (109.8), C_{17} (76.8), C_8 (72.9), C_6 (70.7), C_9 (61.2), C_2 (54.3), 7-COO$\underline{C}$ (53.0), C_{14} (51.1), C_{10} (50.2), C_4 (50.2), C_5 (46.0), C_{30} (39.0), C_{13} (35.4), C_{12} (35.0), C_{15} (27.1), C_{29} (26.7), C_{19} (25.5), C_{28} (23.8), C_{18} (23.8), and C_{11} (22.6).

2.4.8. Compound 8 (SM3): Khayanolide B [22]. Colorless crystals, m.p. 303–304°C; ESI-MS m/z 519.2 $[M + H]^+$ ($C_{27}H_{35}O_{10}$); ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm): 7.47 (*brs*, C_{21}-H), 7.41 (*t*, $J = 2.0$ Hz, C_{23}-H), 6.42 (*m*, C_{22}-H), 5.64 (*s*, C_{17}-H), 4.50 (*dd*, $J = 9.0$, 7.0 Hz, C_2-H), 4.20 (*d*, $J = 7.0$ Hz, C_6-H_α), 3.71 (*s*, 7-$COOCH_3$), 3.40 (*d*, $J = 7.0$ Hz, C_3-H), 3.16 (*d*, $J = 19.0$ Hz, C_{15}-H_α), 3.06 (*d*, $J = 7.0$ Hz, C_5-H), 2.77 (*d*, $J = 19.0$ Hz, C_{15}-H_β), 2.60 (*d*, $J = 9.5$ Hz, C_{30}-H), 2.09 (*d*, $J = 8.0$ Hz, C_9-H), 1.89 (*d*, $J = 11.5$ Hz, C_{29}-H_α), 1.86 (*m*, C_{11}-H_α), 1.85 (*m*, C_{12}-H_β), 1.77 (*m*, C_{11}-H_α), 1.38 (*d*, $J = 11.5$ Hz, C_{29}-H_β), 1.20 (*s*, C_{19}-H), 1.10 (*s*, C_{18}-H), 1.07 (*q*, C_{28}-H), and 0.96 (*m*, C_{12}-H_α). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm): C_7 (175.4), C_{16} (171.7), C_{21} (140.9), C_{23} (142.6), C_{20} (120.6), C_{22} (110.0), C_8 (86.9), C_1 (84.2), C_{14} (81.4), C_{17} (81.2), C_3 (78.5), C_2 (72.2), C_6 (71.4), C_{30} (63.2), C_{10} (59.3), C_9 (56.0), 7-COO$\underline{C}$ (52.1), C_{29} (44.6), C_4 (42.6), C_5 (40.7), C_{13} (37.6), C_{15} (32.0), C_{12} (26.0), C_{28} (19.2), C_{19} (17.6), C_{11} (16.4), and C_{18} (14.4).

2.4.9. Compound 9 (SM4): 6-Acetoxy-Methyl Angolensate [22]. White powder, m.p. 208–209°C; ESI-MS m/z 529.2 $[M + H]^+$ ($C_{29}H_{37}O_9$); ^{1}H NMR (DMSO-d_6, 500 MHz): δ (ppm): 7.58 (*m*, C_{21}-H), 7.55 (*m*, C_{23}-H), 6.47 (*m*, C_{22}-H), 5.57 (*s*, C_{17}-H), 5.29 (*s*, C_{30}-H_α), 5.10 (*s*, $C_{6,30\beta}$-H), 3.77 (*s*, 7-$COOCH_3$), 3.67 (*dd*, $J = 5.5$, 3.0 Hz, C_1-H), 3.02 (*s*, C_5-H), 2.28 (*dd*, $J = 14.0$, 3.0 Hz, C_9-H), 2.20 (*s*, 6-$OCOCH_3$), 1.47 (*s*, C_{28}-H), 1.16 (*s*, C_{19}-H), 1.06 (*s*, C_{29}-H), and 0.92 (*s*, C_{18}-H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ (ppm): C_3 (211.1), C_7 (171.7), 6-O$\underline{C}$OCH_3 (170.4), C_{16} (169.6), C_8 (146.9), C_{23} (143.9), C_{21} (141.7), C_{20} (122.3), C_{30} (112.4), C_{22} (110.8), C_{14} (81.7), C_{17} (79.9), C_1 (78.9), C_6 (73.0), 7-COO$\underline{C}H_3$ (53.1), C_9 (51.7), C_4 (49.4), C_5 (47.4), C_{10} (45.2), C_{13} (41.9), C_2 (39.9), C_{15} (34.3), C_{12} (29.3), C_{28} (25.2), C_{11} (24.8), C_{29} (24.4), C_{19} (23.0), 6-OCO$\underline{C}H_3$ (20.9), and C_{18} (14.3).

2.5. Antifungal Activity Bioassays. The organic soluble extracts and limonoids were tested for their in vitro antifungal activity by using the poisoned food technique. The tested materials were dissolved in DMSO or MeOH and then amended with a sterilized PDA medium in Petri dishes (4 cm and 6 cm diameters) to reach to final concentrations. The solvent content was not higher than 2% in the medium, and the amended dishes were let cooling at room temperature. All of the limonoids were treated in a range concentration of 37.5–500 μg/mL. Each Petri dish was inoculated with a mycelial plug in the center and incubated at 20–25°C for 2–7 days. The Petri dishes treated with 2% DMSO were used as negative controls. The positive controls treated with Score 250EC (a commercial fungicide containing 250 g/L of difenoconazole, Syngenta Vietnam Co. Ltd.) were tested against *M. oryzae*, *S. rolfsii*, and oomycetes at a dose of 1000-fold dilution (equivalent to 250 μg of active ingredient/mL). All experiments for each fungal species were performed at least with two replicates.

The diameter of mycelial growth was measured and recorded when fungal colonies nearly reached the edge of the negative control dishes. The percentage inhibition of mycelial growth (%) for the tested samples was calculated by using the formula: % inhibition $= 100 \times [(A - B)/(A - 4)]$ where A is the diameter of the mycelial growth of fungus in the negative control dishes (mm), B is the diameter of

mycelial growth of fungus in treated dishes (mm), and 4 is the diameter of PDA plug of fungal inoculum (mm).

The inhibition values were presented as means ± standard deviation and evaluated by one-way analysis of variance (ANOVA). The half-maximal inhibitory concentration (IC_{50}, μg/mL) of limonoids against the mycelial growth of the test fungi was computed from a dose-response inhibition curve by probit analysis of WINPEPI software version 11.63.

3. Results

3.1. Structural Characterization of the Isolated Compounds. Compound **1** was obtained colorless crystals. The HR-ESI-MS of compound **1** showed *m/z* 517.2629 $[M+Na]^+$, (cal. for $C_{30}H_{38}O_6Na$ 517.2566), was established to be $C_{30}H_{38}O_6$. The ^1H- and ^{13}C-NMR spectra of compound **1** showed a β-substituted furan ring at 7.24 (*s*, C_{21}-H), 7.14 (*dd*, $J = 2.5$, 10.5 Hz, C_{23}-H), 6.28 (s, C_{22}-H), and C_{21} (142.5), C_{23} (139.6), C_{20} (124.3), C_{22} (110.9), and C_{17} (51.6). The presence of two acetoxy-methyl carbons appeared at 6-OCO (170.2) and 7-OCO (170.0). Moreover, the pair of protons at 7.38 (*t*, $J = 3.5$ Hz, C_1-H) and 5.92 (*dd*, $J = 3.5$, 10.0 Hz, C_2-H) showed two olefinic protons of double bond between C_1-C_2. The five methyl groups appeared at 0.96 (*s*, C_{30}-H), 0.94 (*s*, C_{28}-H), 0.88 (*s*, C_{18}-H), 0.85 (*s*, C_{19}-H), and 0.84 (*s*, C_{29}-H) in its ^1H NMR spectrum. To the best of our knowledge, the spectrum of compound **1** suggested the skeleton of tetracyclic triterpene (tirucallane-type triterpenoid). Thus, compound **1** (**MD1**) was assigned the structure of dysobinin, when its spectrums were compared to the literature [15, 16].

Compound **2** was obtained as white crystals. The HR-ESI-MS of compound **2** showed *m/z* 461.3986 $[M+H]^+$, (cal. for $C_{30}H_{53}O_3$ 461.3995), was established to be $C_{30}H_{52}O_3$. The ^1H NMR spectrum of compound **2** appeared the five methyl groups at 0.96 (*s*, C_{30}-H), 0.94 (*s*, C_{28}-H), 0.88 (*s*, C_{18}-H), 0.85 (*s*, C_{19}-H), and 0.84 (*s*, C_{29}-H). However, compound **2** was oxygenated at position 3 [3.39 (*t*, $J = 2.5$ Hz, C_3-H) and C_3 (76.3)]. The oxygenated methine at δ_C 71.5 showed HMBC correlations to C_{26}-H and C_{27}-H and to the oxygenated methylene at δ_C 83.3. The oxygenated methine at δ_C 86.4 showed HMBC correlations to C_{21}-H and C_{17}-H. It could thus be concluded that the side chain contains an ether bridge between C_{20} and C_{24} while the second alcohol function (besides the one at C_3) is at C_{25}. Because C_{24}-H shows NOEs both to C_{26}-H and to C_{27}-H, the stereochemistry of C_{24} must be rel S. The NOE between C_{16}-Hα and C_{23}-H leads to the conclusion that the configuration at C_{20} is rel R. Again, this is in agreement with a C_{20}-oxygenated tirucallane. The hydroxy group at C_3 was determined as α-oriented. The structure of compound **2** determined by 1D- and 2D-NMR experiments was in agreement as tirucallane-type triterpenoid. The database spectra of compound **2** (**MD2**) were identical with literature values for (3α, 8R, 9S, 20R, 24S)-20, 24-epoxytirucalla-3, 25-diol [17].

Compound **3** was obtained as white powder. The HR-ESI-MS of compound **3** showed *m/z* 549.2566 $[M + Na]^+$, (cal. for $C_{30}H_{38}O_8Na$ 549.2464), was established to be $C_{30}H_{38}O_8$. The NMR spectrum of compound **3** showed five tertiary methyl groups and four methyl protons. The ^{13}C-NMR spectrum of compound **3** showed several similarities with that of compound **1**. This indicated the presence of another tirucallane triterpene with differences in the side chain, which was evidenced only a change in the structural part of the lactone ring substitutes for the furan ring. The structure of compound **3** determined by 1D- and 2D-NMR experiments agreed as tirucallane-type triterpenoid. The spectral data of compound **3** (**AP1**) were identical with literature values for chisocheton compound G [18].

Compound **4** was obtained as colorless crystals. The ESI-MS of compound **4** showed *m/z* 513.2 $[M + H]^+$ was established to be $C_{30}H_{40}O_7$. The DEPT and ^{13}C-NMR spectra of compound **4** revealed 30 carbon signals, including five methylenes, seven methyl groups (δ 31.7, 26.9, 20.7, 20, 4, 20, 1), six methines, four quaternary carbons (δ 40.8; 42.9; 44.9; 46.6), 4 olefinic carbons (δ 119.6; 126.3; 156.8; 158.0), and four carbonyl groups (δ 204.4; 176.4; 170.2; 169.9). The database spectrums of compound **4** were identical with literature values for chisocheton compound E [18]. Compound **4** (**AP2**) was previously isolated from *Chisocheton paniculatus* Hiern (Meliaceae).

Compound **5** was obtained as colorless crystals. The ESI-MS spectrum of compound **5** showed an ion peak at *m/z* 525.1 $[M + H]^+$ ($C_{30}H_{37}O_8$). It was also clear from its NMR spectral data that **5** contained five methyl groups and two methyl esters. The presence of a β-furyl moiety and one methyl ester group was also identified from the spectra. The ^{13}C-NMR and DEPT spectra of **5** showed signals of 29 carbons, including the peaks of 6 methyls, 5 methylenes, 8 methines, and 10 tertiary carbons. Based on these data and the comparison with the literature [19], compound **5** (**AP3**) is confirmed to be methyl 6-acetoxy angolensate (6α-acetoxyepoxyazadiradione VI).

The ESI-MS spectrum of compound **6** showed a *quasi*-molecular ion peak at *m/z* 471.1 $[M + H]^+$ ($C_{26}H_{31}O_8$), which gave a molecular formula of $C_{26}H_{30}O_8$. The 1D-NMR (^1H-, ^{13}C-) spectra of compound **6** revealed the presence of two double bonds and three carbonyl groups (two esters and a ketone). Moreover, 3 methyls, 6 methylenes, 8 methines, and 9 quaternary carbons were also observed. In addition, compound **6** possessed a β-furyl moiety which was characterized by its NMR spectra. Furthermore, the 2D-NMR (^1H-^1H COSY and HMBC) spectra of **6** suggested that its structure was mexicanolide skeleton. The chemical shifts of C_1 (107.5) and C_8 (80.1) were confirmed the presence of the hemiacetal linkage, which has been observed in many mexicanolides. The methine proton H_2 (2.84) had long-range correlations with a carbonyl group C_3 (213.5), C_1 (107.5), C_{30} (31.4), C_{10} (44.7), C_4 (46.8), and C_8 (80.1). Meanwhile, another methine proton H_5 (2.32) had a cross peak with C_6 (29.5), C_{28} (23.8), C_{29} (19.6), C_4 (44.7), C_3 (213.5), C_7 (174.1), C_{10} (46.8), C_{19} (74.1), and C_9 (61.2) in its HMBC spectrum. In addition, the studies using the spin-decoupling ^1H-^1H COSY spectrum was identified that H_9 (2.01) was coupled to $H_{11\beta}$ (1.41), $H_{12\alpha}$ (1.41), and $H_{12\beta}$ (1.76). Consequently, confirmed **6** (**SM1**) was found to be seneganolide [20] as shown in Figure 1.

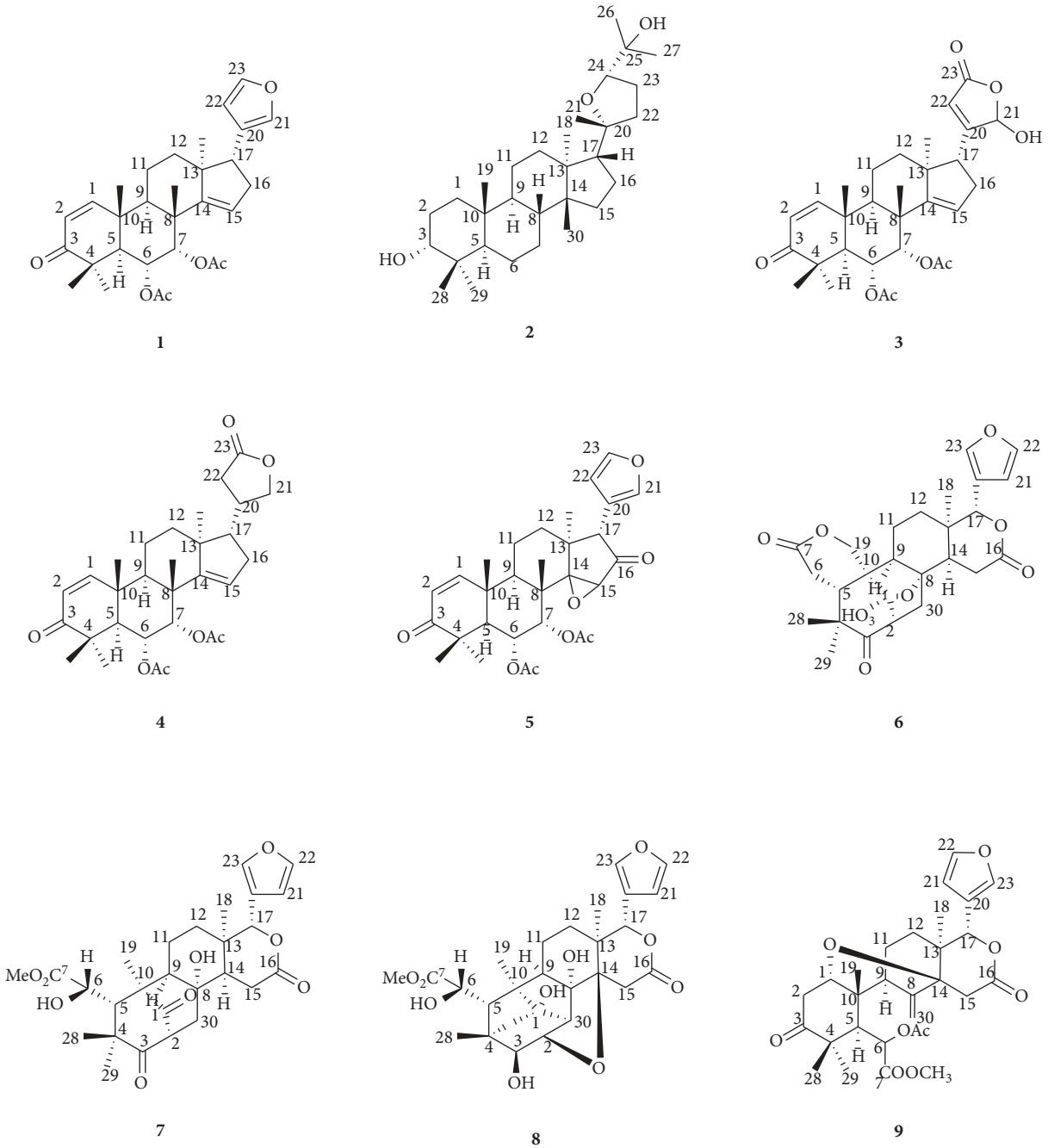

Figure 1: Chemical structures of the limonoids isolated from *Melia dubia* (**1**–**2**), *Aphanamixis polystachya* (**3**–**5**), and *Swietenia macrophylla* (**6**–**9**). (**1**) Dysobinin. (**2**) (3α, 8R, 9S, 20R, 24S)-20,24-Epoxytirucalla-3, 25-diol. (**3**) Chisocheton compound G. (**4**) Chisocheton compound E. (**5**) 6α-Acetoxyepoxyazadiradione VI. (**6**) Seneganolide. (**7**) Khayanone. (**8**) Khayanolide B. (**9**) 6-Acetoxy-methyl angolensate.

Compound **7** was isolated as colorless crystals. Its molecular formula was established as $C_{27}H_{34}O_9$ by the ESI-MS spectrum with *m/z* 503.1 $[M+H]^+$ and NMR spectral data. The structure of compound **7** could be deduced by comparing its NMR spectral data with those of compound **6**. The ^1H and ^{13}C NMR spectral data of **7** suggested the presence of two double bonds (furan ring) and 4 carbonyl groups (3 ketones and 1 ester). These NMR spectral data of compound **7** were identical with those of khayanone. Thus, we can conclude that **7** (**SM2**) is khayanone [21].

Compound **8** was isolated as colorless crystals. Its ESI-MS indicated the molecular formula of $C_{27}H_{35}O$ (*m/z* 519.2 $[M+H]^+$). The NMR spectra of compound **8** are mostly similar to those of compounds **6** and **7** except for some differences in the chemical shifts of C_1, C_{19}, and C_3, suggesting **8** to have the type of phragmalin structure. The comparison of NMR data of compound **8** with the literature data [22] confirmed **8** (**SM3**) to be khayanolide B.

Compound **9** was purified as white powder. Its molecular formula was determined as $C_{29}H_{36}O_9$ by ESI-MS. The

structure of **9** revealed the presence of five methyl groups at δ_H 0.92; 1.16; 1.47; 1.06; 2.20; and 3.77 by the ^{1}H NMR spectrum. Its ^{13}C NMR spectrum indicated the presence of 29 carbons, including 6 methyls, 5 methylenes, 8 methine groups, and 10 tertiary carbons. The β-furyl moiety and a methyl ester were also detected from its ^{13}C-NMR and DEPT spectra. Based on these data and the comparison with literature data [22], compound **9** (**SM4**) is indicated to be 6-acetoxy-methyl angolensate.

3.2. In Vitro Antifungal Efficacy of the Isolated Limonoids. In a preliminary study, we found that the methanol extracts of *M. dubia*, *A. polystachya*, and *S. macrophylla* suppress the mycelial growth of various phytopathogenic fungi at 1000 and 2000 μg/mL (Table S1). The pure limonoids were tested for their *in vitro* antifungal activity against nine phytopathogenic agents including *F. oxysporum*, *M. oryzae*, *S. rolfsii*, *R. solani*, *Alternaria* spp., and *B. cinerea*, and three oomycetes *Phytophthora* species. The development of mycelial growth of *F. oxysporum* and *M. oryzae* was affected by almost all the test limonoids. However, *F. oxysporum* was less sensitive to compounds **1** and **5** on the basis of the evidence of the mycelial growth inhibition at 250 and 500 μg/mL (Table 1). The mycelial growth of this fungus was slightly suppressed by compounds **2**, **3**, **4**, **6**, and **8** (Table 1 and Figure 2). Compound **3** gave the highest inhibition (IC_{50} = 480.5 μg/mL) for *F. oxysporum* (Table 2). The mycelial growth of *M. oryzae*, the causal agent of rice blast, was solidity suppressed when treated with **2**, **3**, **8**, and **9** (Tables 1 and 2). At a concentration of 500 μg/mL, compounds **2**, **8**, and **9** also significantly reduced the mycelial growth of *M. oryzae* with inhibitions of 68.9%, 97.1%, and 66.0%, respectively, while compounds **1**, **5**, and **7** caused weak inhibitions (data not shown). The isolated limonoids **2**, **3**, **6**, and **8** significantly suppressed the oomycetes *Phytophthora* spp., *P. capsici*, and *P. palmivora* at concentrations ranging from 62.5 to 500 μg/mL (Figure 3 and S2-4). Compounds **3** and **8** were the most potent limonoids against the tested oomycetes. The IC_{50} values of these four limonoids are presented in Table 2. At 250 and 500 μg/mL, compound **8** inhibited by 69.3% and 79.7% of the mycelial growth of *Phytophthora* spp., respectively (data not shown). Compound **3** displayed a dose-dependent activity against the test oomycetes *Phytophthora* spp. and *P. capsici* (Figure 2). This compound was also most active against *Phytophthora* spp and *P. capsici*; it caused 75.71% and 70.66% inhibition for the mycelial growth of the test oomycetes at 250 and 500 μg/mL, respectively (Figure S2 and 3). Similar to the antifungal activity of **3**, compound **8** also had a dose-dependent activity against *Phytophthora* spp. and *P. capsici*. However, it seems that the fungal efficacy of **8** (IC_{50} = 97.1 μg/mL) was better than that of **3** (IC_{50} = 178.5 μg/mL) when tested against *Phytophthora* spp. In contrast, the plant pathogens *F. oxysporum*, *M. oryzae*, and *P. palmivora* were strongly inhibited in the treatment with **3** compared with those with **8** (Figure 2, S2-4, and Table 2).

Besides, *S. rolfsii* was strongly inhibited by limonoids **2**, **3**, **6**, and **8** *in vitro* (Table 1 and Figure 2). All of the four compounds displayed a dose-dependent antifungal activity against *S. rolfsii*. Compounds **2** and **6** were the best inhibitor against the mycelial growth of *S. rolfsii* with IC_{50} values of 94.0 and 79.4 μg/mL, respectively. Compounds **3** and **8** also strongly inhibited this fungus with IC_{50} values of 128.0 and 124.5 μg/mL, respectively (Table 2).

Table 1: Control value (%) of limonoids **1**, **4**, **5**, 7, and **9** against *Fusarium oxysporum*, *Magnaporthe oryzae*, and *Sclerotium rolfsii*.

Fungi	Conc. (μg/mL)	Control value (%)				
		1[b]	**4**	**5**	**7**	**9**
FO[a]	250	11.73 ± 7.64[c]	28.67 ± 2.38	18.51[d]	nt	nt
	500	25.03 ± 5.93	30.63 ± 0.58	22.43	nt	nt
MG	250	13.84 ± 5.97	24.89 ± 0.69	25.67	6.60	14.15
	500	27.67 ± 3.14	33.33 ± 0.42	28.61	16.04	66.04
SR	250	Nt	nt	nt	nt	nt
	500	Nt	nt	nt	nt	nt

[a]FO : *Fusarium oxysporum*, MG : *Magnaporthe oryzae*, and SR : *Sclerotium rolfsii*. [b]Compounds: **1**, dysobinin; **4**, chisocheton compound E; **5**, 6α-acetoxyepoxyazadiradione VI; **7**, khayanone; **9**, 6-acetoxy-methyl angolensate. [c]The values are the average of two determinations ± standard error of the mean. nt: not tested. [d]Values are obtained through a single treatment.

Of the limonoids isolated from *S. macrophylla* (**6**–**9**), compounds **6**–**8** belong to mexicanolide-type limonoids. Compound **6** moderately inhibited the mycelial growth of *R. solani* (57.9%) while **8** did not when tested at a concentration of 250 μg/mL (data not shown). Compounds **6** (seneganolide) and **8** (khayanolide B) affected the mycelial growth of various oomycetes and fungi (Table 2 and Figure 2). Compounds **7** and **9** were isolated in a limited amount and were tested only with *M. oryzae*. Of those, limonoid **9** belonging to andirobin-type limonoid was highly active against *M. oryzae*.

Compounds **1** and **2** were isolated from *M. dubia* in the work, and their antifungal activity was evaluated against various phytopathogenic fungi. Dysobinin (**1**) was tested against only *F. oxysporum* and *M. oryzae* because of lacking isolated weight. At a concentration of 500 μg/mL, compound **2** exhibited discernible effects on *F. oxysporum* (32.4%), *M. oryzae* (68.8%), *Phytophthora* spp. (46.4%), and *P. capsici* (30.2%) (data not shown). Compared with **2**, compound dysobinin (**1**) was less active against *F. oxysporum* and *M. oryzae*. The mycelial growth of *B. cinerea*, *R. solani*, and *Alternaria* spp. was also weakly inhibited by compounds **2**, **3**, **6**, and **8** with an inhibition range of 12.8–34.8% at concentrations of 250 and 500 μg/mL (data not shown). All positive controls against the mycelial growth of *M. oryzae*, *S. rolfsii*, and oomycetes exhibited by 100% suppression when treated with difenoconazole at 250 μg/mL (data not shown).

4. Discussion

Limonoids are known as the main secondary metabolites produced by the plants from *Melia* and *Citrus* genera. The insecticidal limonoids have been applied to pest management in agriculture for a long time. However, the reports of the antifungal activity of limonoids from Meliaceae family have been rare until now. The notable antifungal activity of the three methanol extracts of *M. dubia*, *A. polystachya*, and

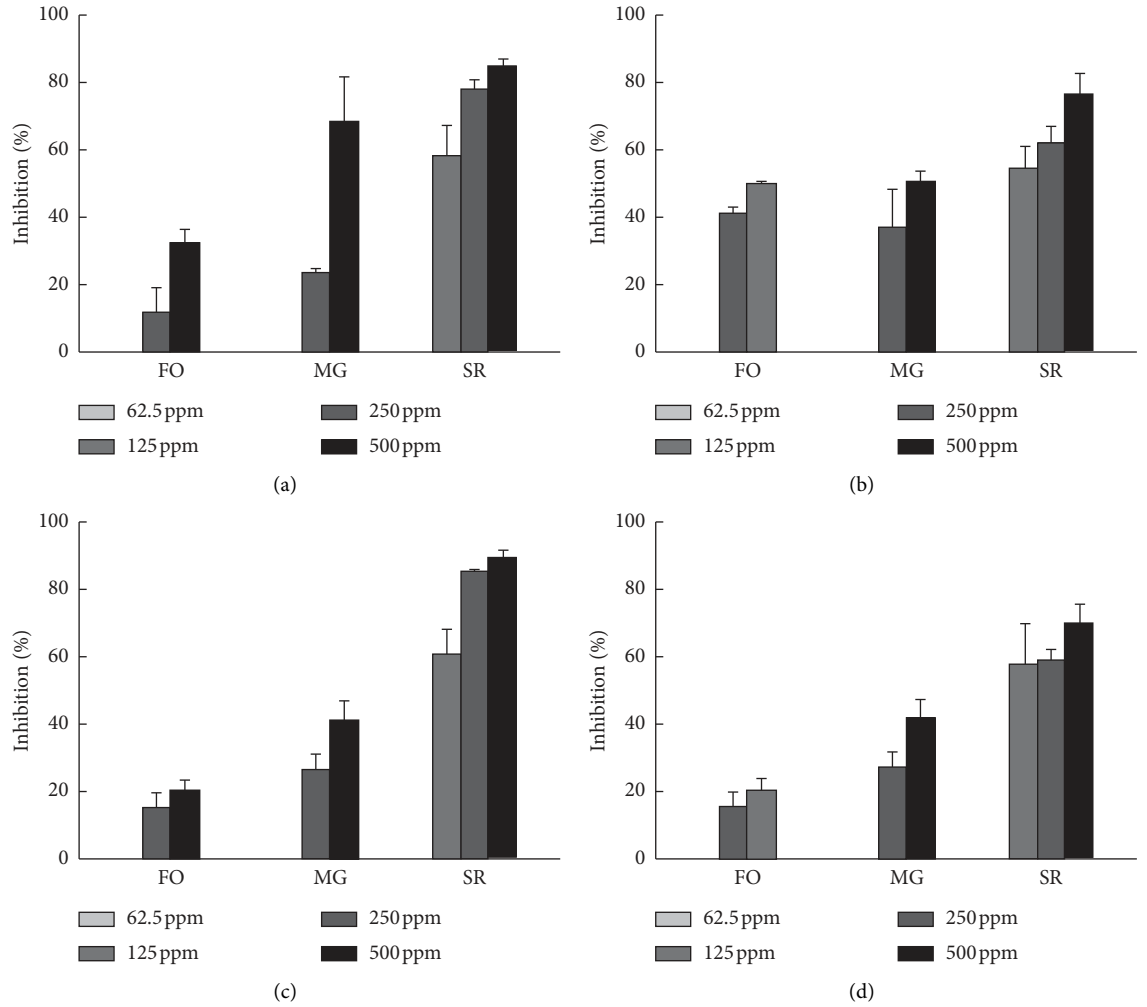

Figure 2: *In vitro* antifungal activity of the isolated limonoids **2**, **3**, **6**, and **8** against three phytopathogenic fungi. FO : *Fusarium oxysporum*; MG : *Magnaporthe oryzae*; SR : *Sclerotium rolfsii*. (a) Compound **2**. (b) Compound **3**. (c) Compound **6**. (d) Compound **8**.

S. macrophylla has prompted us to search the antifungal ingredients in the samples and evaluate their *in vitro* antifungal efficacy against phytopathogenic fungi. In our study, nine limonoids were tested for their antifungal activity against nine phytopathogenic fungi. Five potent antifungal limonoids **2**, **3**, **6**, **8**, and **9** significantly inhibited the test fungi in a broad spectrum. *S. rolfsii* was also found to be the best sensitive to the isolated limonoids **2**, **3**, **6**, and **8**; for this fungus, all of the compounds displayed a strong antifungal activity in a dose-dependent manner and IC_{50} values in a range of 79.4–128.0 μg/mL (Figure 2 and Table 2). *F. oxysporum* seemed to be less sensitive to all test fungi, except for compound **3** when tested at a concentration of 500 μg/mL. Remarkably, compounds **3** and **8** were the most active and showed a broad-spectrum antifungal activity against various fungal plant pathogens such as *F. oxysporum*, *M. oryzae*, and *S. rolfsii*, and the three oomycete strains of *Phytophthora* species. Compounds **1**-**5** belong to the class of ring D-opened limonoid, and the class includes some antifungal limonoids such as 3-deacetylkhivorin, 1, 3, 7-trideacetylkhivorin, 7-deacetylgedunin, and 7-deacetoxy-7-oxogedunin isolated from *Khaya ivorensis* and reported to be weakly and moderately active against the plant fungus *Botrytis cinerea* at the concentrations higher than 1000 μg/mL [10].

A. polystachya grows abundantly in India, China, and Southeast Asia and has been used as medicinal materials in traditional medicine. The structural characterization and bioactivities of various limonoids from the fruits of *A. polystachya* were investigated in previous studies [3, 23, 24]. Especially, the reports of the antifungal activity of limonoids from this plant have been rare until now. Srivastava et al. (2003) reported the isolation of an andirobin-type limonoid named amoorinin-3-*O*-α-L-rhamnopyranosyl-(1–6)-β-D-glucopyranoside from *A. polystachya* with antifungal activity against *Aspergillus niger* and *Candida albicans* [25]. In a previous study by Zhang et al. (2013), A, B−seco limonoids, aphapolynin C, rohituka-15, aphanamolide A, and aphapolynin A from the fruits of *A. polystachya* were evaluated antifungal property against various phytopathogenic fungi such as *Alternaria solani*, *Botryotinia fuckeliana*, *Gibberella*

Table 2: The half-maximal inhibitory concentration (IC_{50}, μg/mL) of limonoids **2**, **3**, **6**, **8**, and **9** against the mycelial growth of phytopathogenic fungi and oomycetes *in vitro*.

Fungi	The half-maximal inhibitory concentration (IC_{50}) (μg/mL)				
	2	**3**	**6**	**8**	**9**
FO	>500	480.5 ± 20.9	>500	>500	nt
MG	386.3 ± 2.8	490.0 ± 21.1	>500	318.3 ± 1.2	422.8 ± 3.9
SR	94.0 ± 2.5	128.0 ± 3.6	79.4 ± 2.1	124.5 ± 4.9	nt
PS	>500	178.5 ± 4.2	434.3 ± 10.8	97.1 ± 3.4	nt
PC	>500	262.4 ± 10.6	>500	280 ± 11.4	nt
PP	nt	351.5 ± 9.6	>500	>500	nt

[a]FO: *Fusarium oxysporum*; MG: *Magnaporthe oryzae*; SR: *Sclerotium rolfsii* BV; PS: *Phytophthora* spp.; PC: *Phytophthora capsici*; PP: *Phytophthora palmivora*. [b]Compounds: **2**, (3α,8R,9S,20R,24S)-20,24-epoxytirucalla-3,25-diol; **3**, chisocheton compound G; **6**, seneganolide; **8**, khayanolide B. nt: not tested.

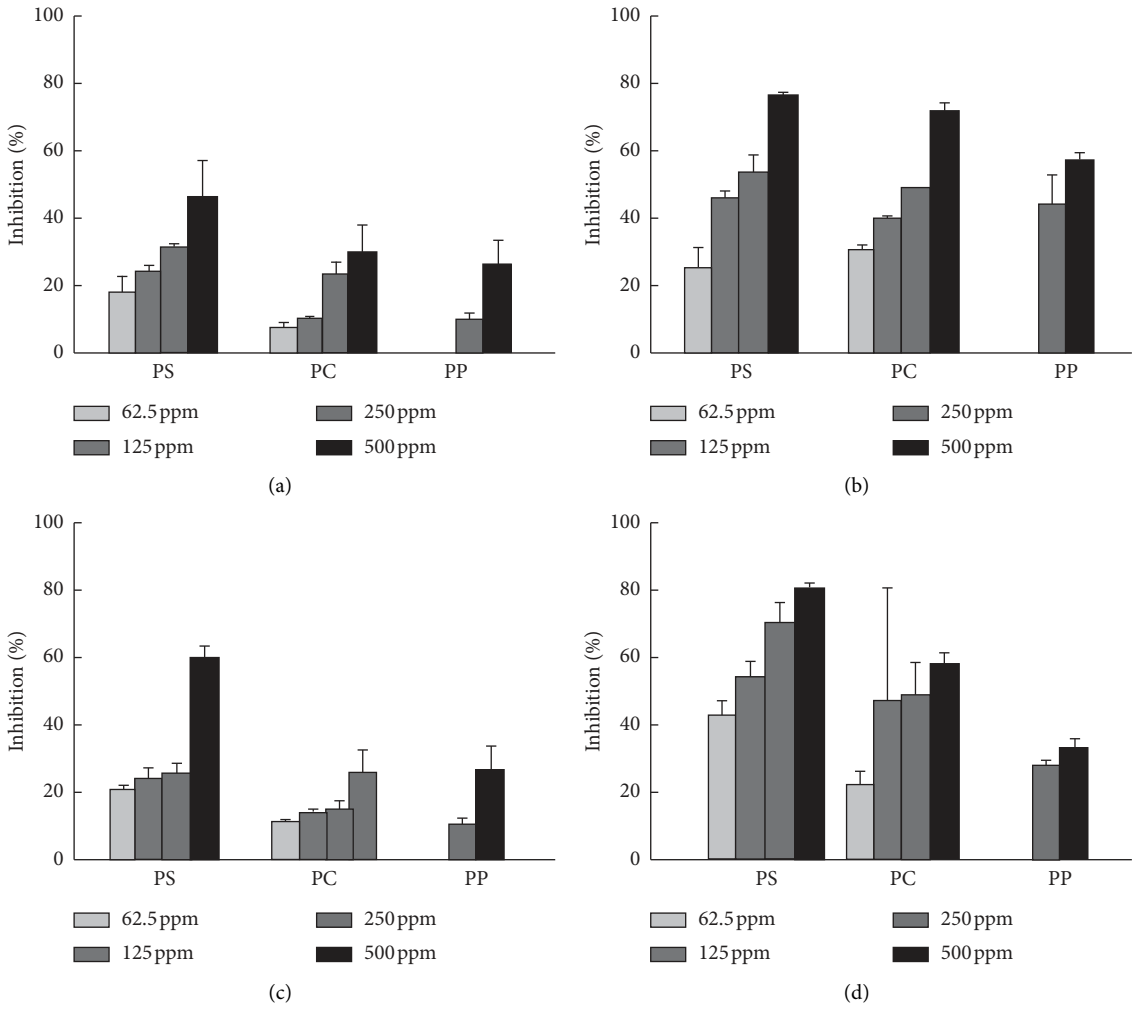

Figure 3: *In vitro* antifungal activity of the isolated limonoids **2**, **3**, **6**, and **8** against oomycete *Phytophthora* strains. PS: *Phytophthora* spp.; PC: *Phytophthora capsici*; PP: *Phytophthora palmivora*. (a) Compound **2**. (b) Compound **3**. (c) Compound **6**. (d) Compound **8**.

zeae, *Septoria tritici*, *P. infestans*, *Pythium dissimile*, and *Uromyces viciae-fabae* [14]. Compounds rohituka-15 and aphanamolide A completely inhibited *P. dissimile*, and aphapolynin A did partial inhibition at a concentration of 20 μg/mL. Aphapolynin C and aphapolynin A displayed strong inhibition against *U. viciae-fabae* at 100 μg/mL. However, all of the test compounds were inactive against oomycete *P. infestans* at concentrations of 200 and 60 μg/mL.

In contrast, we reported here that compounds **3**, **4**, and **5** are isolated from *A. polystachya* and have a similar carbon skeleton except for the substitute attached to C-17 (4-hydroxybut-2-enolide for **3**, butanolide for **4**, and furan ring for **5**). Chisocheton compound G (**3**) showed the best mycelial growth inhibition against *Phytophthora* species, while **4** and **5** were almost inactive against the oomycete. This may be due to the structure of **3** contains 4-hydroxybut-2-enolide

moiety at C-17, which is similar to the head moiety of annonaceous acetogenins with a potential of antioomycete activity and cytotoxicity [26].

Swietenia species biosynthesize more than 160 limonoids including 77 mexicanolide-type limonoids (most from *S. mahagoni* and *S. macrophylla*). The Meliaceous plant *S. macrophylla* grows widely in Central and South American countries, India, Malaysia, and China [1, 4]. It is rare to see in Vietnam, and the phytochemical investigation, as well as antifungal properties of the local species, has not been reported much. From the dried fruits of *S. macrophylla* growing in Vietnam, four limonoids **6**–**9** were isolated by various chromatographic methods. Out of them, mexicanolide-type limonoids seneganolide (**6**) and khayanolide B (**8**) showed the best antioomycete activity against *Phytophthora* species. Especially, khayanolide B (**8**) was the most active against *M. oryzae*, *Phytophthora* spp., and *P. capsici*. In the same group of mexicanolide carbon skeleton, three limonoids seneganolide A, 2-acetoxyseneganolide A, and methyl 6-hydroxyangolensate were reported to isolate from the fruits of *Khaya senegalensis* and tested against phytopathogenic fungus *Botrytis cinerea* at relatively high concentrations of 1000 and 1500 μg/mL. Limonoid 2-acetoxyseneganolide A suppressed the mycelial growth of *B. cinerea* at 1000 μg/mL (61.50%) and 1500 μg/mL (68.33%), while seneganolide A and methyl 6-hydroxyangolensate showed a weaker inhibition [9]. Khayanolide B (**8**) was also reported as an antifeedant substance against *Spodoptera littoralis* with an EC_{50} of 2.19 mg/kg [2]. Limonoid swietenolide isolated from *S. macrophylla* was documented to have antifeedant activity with antifeedant index of 94.1 against fall armyworm (*Spodoptera frugiperda*). 6-*O*-acetylswietenolide, 3, 6-*O*, *O*-diacetylswietenolide, and swietemahonin F exhibited antifeedant indices of 72.2, 72.0, and 70.2, respectively [1]. According to Sun et al. (2018), swietenine from this plant also showed the greatest potency with a 50% antifeedant index concentration of 2.49 against *S. frugiperda* [4].

As for the source rich in limonoids in Meliaceae species, *A. indica* and *M. dubia* have been described as potential plant systems that contain a wide range of bioactive limonoids that are both chemically and structurally complex [27]. Salannin, a well-known insecticidal limonoid, was found to be produced by *A. indica* and *M. dubia*. The bark of *M. dubia* was also reported to contain cytotoxic euphane-type triterpenes against P388 cancer cells [7]. However, there are no reports on antifungal activity of *M. dubia* against phytopathogenic fungi up to now. In a preliminary study by Mahmoud et al. (2011), the extracts derived from the leaves and seeds of *A. indica* were assessed for antifungal activity against six human fungal pathogens *Aspergillus flavus*, *A. fumigatus*, *A. Niger*, *A. terreus*, *C. albicans*, and *M. gypseum* [28]. Nimonol with a molecular formula of $C_{28}H_{36}O_5$ was separated and identified as the main compound of ethyl acetate extract of *A. indica*, but it displayed no inhibitory effect on all the six fungal pathogens tested [28]. In contrast, Alvarez-Caballero and Coy-Barrera (2019) described that azadirone-type limonoids such as nimonol, 14, 15-epoxynimonol, isomeldenin, zafaral, and *O*-acetyl-7-deacetylnimocinol isolated from *A. indica* possess a good inhibition against conidia germination of *F. oxysporum*. Nimonol was the best inhibitor; of which inhibition against *F. oxysporum* conidia germination was observed at IC_{50} of 1.48 μM. Alvarez-Caballero and Coy-Barrera (2019) also demonstrated that the presence of a 14, 15-epoxy group and the saturation at 1, 2-olefilic positions in the structure of azadirone-type limonoids led to a diminished effect on fungal conidia germination [11]. In our study, **1**, **3**, **4**, and **5** were isolated and identified as azadirone-type limonoids. Among them, compound **3** showed a broad-spectrum activity with potent suppression against almost all of the test fungi in the concentration ranging from 37.5 to 500 μg/mL (Table 2 and Figure 2), while compounds **1**, **4** and **5** showed a little effect on the growth of *F. oxysporum* and *M. grisea*. This suggested an implication of structure-antifungal activity relationship of the studied azadirone-type limonoids. The 2-hydroxybutanolide groups at C-17 in the structure of **3** may make an enhancing effect on inhibition against fungi. Concerned to the antifungal activity of azadirone-type limonoids, 6α-acetoxyazadirone and 1, 2-dihydro-6α-acetoxyazadirone were also briefly reported to possess strong antifungal activity against pathogenic fungi *Curvularia verruciformis*, *Dreschleva oryzae*, and *A. solani*, but no information of inhibition effectiveness, biological testing methods, and test concentration was provided in the article [8, 29]. According to Govindachari et al. (1998), the neem oil derived from the seeds of *A. indica* showed antifungal activity against phytopathogenic fungi and contains an abundance of limonoids such as azadiradione, nimbin, 6-deacetylnimbin, salannin, and epoxyazadiradione [12]. However, these compounds in pure form have not much affected the mycelial growth of *D. oryzae*, *F. oxysporum* f.sp. *vasinfectum*, and *Alternaria tenuis* at a high concentration of 1000 μg/mL. Only 6-deacetylnimbin and nimbin inhibited *D. oryzae*, the causal agent of rice brown leaf spot disease, by 63.3 and 64.8%, respectively. Besides, either additive or synergistic influence of the mixture containing those terpenoids was observed with an inhibition of 70% for *D. oryzae* at 1000 μg/mL [12].

As for the antifungal effects on soilborne plant pathogenic fungi, Sharma et al. (2003) reported that azadirachtins A, B, and H possessing antifungal potential against *R. solani* and *S. rolfsii* were efficiently isolated from the seed kernels of *A. indica*. Azadirachtin A inhibited the mycelial growth of *R. solani* and *S. rolfsii* at ED_{50} values of 104.8 μg/mL (purity of 90% for azadirachtin A) and 93.6 μg/mL, respectively. Azadirachtins B and H exhibited a better inhibition with ED_{50} values varying from 43.9 to 85.9 μg/mL for the two fungi [30]. Therefore, in addition to azadirachtins A, B, and H belonging to the ring C-seco limonoid group, the isolated compounds **2**, **3**, **6**, and **8** in our study are different structure type limonoids with potent inhibition against *S. rolfsii* were consequently reported.

Our study has described the antifungal property of the limonoids isolated from *A. polystachya*, *M. dubia*, and *S. macrophylla* against various phytopathogenic fungi for the first time. To discover more potent antifungal limonoids, experiments in the mode of action and the relationship

between structures and antifungal activity of limonoids need to conduct. In further studies, the *in vivo* antifungal efficacy and phytotoxicity of the bioactive limonoids, which could be formulated evenly into a ready-to-use formulation, should be evaluated to determine their potential disease control efficacy on various crops.

5. Conclusions

Considering the application of limonoids in crop protection, in our work, the chemical structural characterization and antifungal activity evaluation of nine limonoids isolated from *M. dubia* (**1**–**2**), *A. polystachya* (**3**–**5**), and *S. macrophylla* (**6**–**9**) were described and pointed our promising active compounds against various phytopathogenic fungi. Limonoids **2**, **3**, **6**, **8**, and **9** were found to be promising candidates with a broad antifungal spectrum. Compounds **2**, **8**, and **9** displayed moderate activity against *M. oryzae*, while *S. rolfsii* and *Phytophthora* species were the most susceptible species to the test limonoids. It is noteworthy that chisocheton compound G (**3**) isolated from *A. polystachya* and khayanolide B (**8**) isolated from *S. macrophylla* were the most potent antifungal limonoids against *M. oryzae* and *Phytophthora* species. Our findings may suggest and encourage more work on the discovery of antifungal limonoids and lead compounds from the group of limonoids.

Data Availability

The data used to support the findings of this study are included in the supplementary materials and available from the corresponding authors upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Thang Tran Dinh, Hoang Dinh Vu, Dang Ngoc Quang, and Quang Le Dang were responsible for conceptualization, methodology, and supervision. Thanh Nguyen Tan, Hieu Tran Trung, Tuan Nguyen Ngoc, Hien Vu Thi, Dang Ngoc Quang, and Thang Tran Dinh were responsible for extraction, purification, and structure identification for isolated compounds. Thanh Huong Nguyen, Hoai Thu Thi Do, and Quang Le Dang were responsible for antifungal bioassay and biological data analysis. Thanh Nguyen Tan, Hoai Thu Thi Do, Quang Le Dang, and Thang Tran Dinh were responsible for writing – original draft preparation, and editing. All authors read and approved the submitted manuscript.

Acknowledgments

The authors thank Thanh Huong Nguyen for the bioassay's assistance. This research was funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant no. 104.01-2018.315.

Supplementary Materials

Figure S1a: isolation scheme of isolated compounds **1-2** from the fruits of *Melia dubia*. Figure S1b: isolation scheme of isolated compounds **3-5** from the fruits of *Aphanamixis polystachya*. Figure S1c: isolation scheme of isolated compounds **6-9** from the fruits of *Swietenia macrophylla*. Figure S2: *in vitro* antifungal activity of the most active compounds **3** and **8** against the mycelial growth of *Phytophthora* spp. Figure S3: *in vitro* antifungal activity of the most active compounds **3** and **8** against the mycelial growth of *Phytophthora capsici*. Figure S4: *in vitro* antifungal activity of the most active compounds **3, 6**, and **8** against the mycelial growth of *Phytophthora palmivora*. Figure S5: *in vitro* antifungal activity of compound **2** against the mycelial growth of *Magnaporthe oryzae*. Figure S6: *in vitro* antifungal activity of the most active compounds **2, 3, 6**, and **8** against the mycelial growth of *Sclerotium rolfsii*. Table S1: *in vitro* inhibitory activity of MeOH extracts from *Melia dubia*, *Aphanamixis polystachya*, and *Swietenia macrophylla* against *Magnaporthe oryzae*, *Phytophthora capsici*, and *Sclerotium rolfsii*. (*Supplementary Materials*)

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