ANTI-COVID-19 ACTIVITY COMPOUNDS FROM *Michelia crassipes*

**C. Y. Chen,[1]* C. M. Liu,[2] H. C. Yeh,[1] W. J. Li,[3]
H. T. Li,[4]* and C. H. Chuang [5,6]***

Species belonging to the *Michelia* genus are arboreous plants, growing in the temperate zones of oriental India, southern China, Malaysia, and Indonesia. Various chemical constituents isolated from different species of the genus *Michelia* include aporphines, oxoaporphines, steroids, benzenoids, and terpenoids. The important biological activities of constituents isolated from *Michelia* are anticancer, antiplasmodial and antibacterial activities [1]. Lesser-known species, such as *Michelia crassipes* Y. W. Law, are used as ornamental plants and to obtain essences. [1]. *M. crassipes* Y. W. Law, a small evergreen shrub tree in *Magnoliaceae*, is a great ornamental plant with fragrant and dark purple flowers. It became a useful cross-breeding germplasm resource. Field observation in Changsha (China) indicated that its flowers started blooming in late March and continued into early May for about 50 days [2]. In the course of screening for biologically and chemically novel agents from Formosan Magnoliaceous plants [3–7], *M. crassipes* was chosen for further phytochemical investigation. The compounds derived from the stems include one oxoaporphine, liriodenine (**1**) [8]; four benzenoids, vanillic acid (**2**) [9], vanillin (**3**) [10], *p*-hydroxybenzoic acid (**4**) [10], and *p*-hydroxybenzaldehyde (**5**) [11]; two amides, *N-trans*-feruloyltyramine (**6**) [12] and *N-cis*-feruloyltyramine (**7**) [13]; one cyclitol, (–)-liriodendritol (**8**) [14].

The newly identified novel coronavirus (SARS-CoV-2) has posed a serious threat to human health. COVID-19 is a pandemic disease worldwide and is resulted in millions of deaths and caused rampant economic damage worldwide [15, 16]. Therefore, it is very important to develop the drug against COVID-19. We chose the 3C-like protease (3CL protease) of SARS-CoV-2 which is crucial in viral replication, as our strategy to develop the compound [17, 18]. The inhibitory of efficient potential compounds were screened by protease activity assay. The 100 µM compounds **1–8** or PBS were incubated with 40 nM 3CL protease of COVID-19 for 30 min at RT, followed by the addition of the 10-µM SARS-CoV2 substrate for 60 min at 37°C. The fluorescence intensity was determined to monitor the protease activity. As shown in Table 1, the Protease activity of 3CL protease of COVID-19 could efficiently inhibit via all of our compounds. The results show the inhibitory ability of vanillin acid (**2**), *p*-hydroxybenzaldehyde (**5**), and (–)-liriodendritol (**8**), which have the potential to be developed to the anti-COVID-19 drugs. These results indicated that the **2**, **5**, and **8** of *M. crassipes* could specifically and efficiently inhibit the protease activity of 3CL protease of COVID-19.

The stems of *M. crassipes* Y. W. Law were collected from New Taipei City, Taiwan, May 2016. Plant material was identified by Prof. Fu-Yuan Lu (Department of Forestry and Natural Resources, College of Agriculture, National Chiayi University). A voucher specimen was deposited in the School of Medical and Health Sciences, Fooyin University, Kaohsiung City, Taiwan.

The air-dried stems of *M. crassipes* (1.4 kg) were extracted with MeOH (5 L × 3) at room temperature and a MeOH extract (22.8 g) was obtained upon concentration under reduced pressure. The MeOH extract was chromatographed over silica gel (800 g, 70–230 mesh) using *n*-hexane–EtOAc–MeOH mixtures as eluents to produce five fractions.

1) School of Medical and Health Sciences, Fooyin University, 83102, Kaohsiung, Taiwan, e-mail: xx377@fy.edu.tw; 2) School of Medicine, Yichun University, 576 XueFu Road, Yuanzhou District, 336000, Yichun, P. R. China; 3) School of Nursing, Fooyin University, 83102, Kaohsiung, Taiwan; 4) Department of Medical Laboratory Science and Biotechnology, Fooyin University, 83102, Kaohsiung, Taiwan, e-mail: mt085@fy.edu.tw; 5) Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan; 6) Drug Development and Value Creation Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan, e-mail: a4132600@gmail.com.

TABLE 1. Inhibitory Activity of Compounds against 3CL Protease of COVID-19, %

Compound	Protease activity	Inhibitory activity	Compound	Protease activity	Inhibitory activity
PBS (noninhibitory)	100.0 ± 1.3		**5**	69.3 ± 1.5	30.7 ± 1.5
1	75.2 ± 1.8	24.8 ± 1.8	**6**	77.7 ± 1.9	22.3 ± 1.9
2	60.3 ± 1.7	39.7 ± 1.7	**7**	79.1 ± 2.8	20.9 ± 2.8
3	74.9 ± 3.4	25.1 ± 3.4	**8**	63.5 ± 3.8	36.5 ± 3.8
4	89.9 ± 4.3	10.1 ± 4.3			

Part of fraction 1 (5.2 g) was subjected to silica gel chromatography by eluting with CH_2Cl_2–MeOH (70:1), enriched gradually with MeOH, to furnish five fractions (1-1–1-5). Fraction 1-2 (1.1 g) was further purified on a silica gel column using CH_2Cl_2–MeOH mixtures to obtain **6** (11 mg) and **7** (6 mg). Part of fraction 2 (6.6 g) was subjected to silica gel chromatography, by eluting with CH_2Cl_2–MeOH (50:1), enriched gradually with MeOH, to furnish five fractions (2-1–2-5). Fraction 2-2 (1.6 g) was further purified on a silica gel column using CH_2Cl_2–MeOH mixtures to obtain **4** (13 mg) and **5** (9 mg). Part of fraction 3 (4.8 g) was subjected to silica gel chromatography by eluting with CH_2Cl_2–MeOH (40:1), enriched with MeOH to furnish four further fractions (3-1–3-4). Fraction 3-2 (1.2 g) was further purified on a silica gel column using CH_2Cl_2–MeOH mixtures to obtain **2** (22 mg) and **3** (16 mg). Part of fraction 4 (6.8 g) was subjected to silica gel chromatography by eluting with CH_2Cl_2–MeOH (30:1), enriched with MeOH to furnish four further fractions (4-1–4-4). Fraction 4-2 (1.8 g) was further purified on a silica gel column using CH_2Cl_2–MeOH mixtures to obtain **1** (28 mg). Part of fraction 5 (5.1 g) was subjected to silica gel chromatography by eluting with CH_2Cl_2–MeOH (25:1), enriched with MeOH to furnish five further fractions (5-1–5-5). Fraction 5-3 (2.4 g) was further purified on a silica gel column using CH_2Cl_2–MeOH mixtures to obtain **8** (36 mg).

All of these known compounds were obtained for the first time from stems of this plant and were identified by direct comparison with authentic samples (TLC, UV, IR, ESI-MS, and NMR) and literature [8–14].

Liriodenine (1) as in [8], yellow needles (CH_2Cl_2), mp 281–283°C. UV (MeCN, λ_{max}, nm): 256, 280, 335. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.35 (2H, s, $-OCH_2O-$), 7.15 (1H, s, H-3), 7.56 (1H, td, J = 8.0, 1.0, H-9), 7.71 (1H, td, J = 8.0, 1.5, H-10), 7.75 (1H, d, J = 5.2, H-4), 8.57 (1H, dd, J = 8.0, 1.5, H-8), 8.61 (1H, dd, J = 8.0, 1.0, H-11), 8.87 (1H, d, J = 5.2, H-5).

Vanillic acid (2) as in [9]. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 3.89 (3H, s, 3-OCH_3), 6.81 (1H, d, J = 8.0, H-5), 7.51 (1H, dd, J = 8.0, 2.0, H-6), 7.58 (1H, d, J = 2.0, H-2).

Vanillin (3) as in [10].

p-Hydroxybenzoic acid (4) as in [11]. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.85 (2H, d, J = 8.6, H-3, 5), 7.96 (2H, d, J = 8.6, H-2, 6).

p-Hydroxybenzaldehyde (5) as in [12].

N-trans-Feruloyltyramine (6) as in [13]. UV (MeCN, λ_{max}, nm): 220, 295, 320. IR (ν_{max}, cm^{-1}): 3300, 1650. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): feruloyl moiety: 3.87 (3H, s, 3′-OCH_3), 6.15 (1H, d, J = 15.6, H-2), 6.83 (1H, d, J = 8.0, H-5′), 7.01 (1H, dd, J = 8.0, 2.0, H-6′), 6.95 (1H, d, J = 2.0, H-2′), 7.46 (1H, d, J = 15.6, H-3); tyramine moiety: 2.75 (2H, t, J = 6.8, H-2), 3.54 (2H, t, J = 6.8, H-1), 6.75 (2H, d, J = 8.8, H-3′, 5′), 7.02 (2H, d, J = 8.8, H-2′, 6′).

N-cis-Feruloyltyramine (7) as in [14], brown powder ($CHCl_3$). UV (MeCN, λ_{max}, nm): 220, 295, 318. IR (ν_{max}, cm^{-1}): 3350, 1650. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): feruloyl moiety: 3.84 (3H, s, 3′-OCH_3), 5.81 (1H, d, J = 12.8, H-2), 6.61 (1H, d, J = 12.8, H-3), 6.73 (1H, d, J = 8.4, H-5′), 6.92 (1H, dd, J = 8.4, 2.0, H-6′), 7.35 (1H, d, J = 2.0, H-2′); tyramine moiety: 2.69 (2H, t, J = 7.6, H-2), 3.40 (2H, t, J = 7.6, H-1), 6.68 (2H, d, J = 8.4, H-3′, 5′), 6.99 (2H, d, J = 8.4, H-2′, 6′).

(–)-Liriodendritol (8) as in [14], white needles (pyridine), mp 226–228°C, $[\alpha]_D^{25}$ –26.3° (c 1.45, H_2O). IR (ν_{max}, cm^{-1}): 3300, 2900, 1370, 1100. ^{1}H NMR (400 MHz, C_5D_5N, δ, ppm, J/Hz): 3.41 (1H, dd, J = 9.6, 2.8, H-1), 3.55 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 4.02 (1H, dd, J = 9.6, 2.4, H-3), 4.04 (1H, t, J = 9.2, H-5), 4.17 (1H, t, J = 9.2, H-4), 4.69 (1H, t, J = 9.2, H-6), 4.72 (1H, br.s, H-2), 5.03 (2H, br.s, 2 × OH), 6.70 (2H, br.s, 2 × OH).

The Expression and Purification of Recombinant 3CL Protease of SARS-CoV-2. *E. coli* strain BL21 transformed with pQE60/3CL protease of SARS-CoV-2 plasmids was grown to log phase and BL21 were suspended in phosphate-buffer saline (PBS) for sonication. After sonication, the NS2B-NS3 protease was purified via Ni-NTA Agarose (Thermo, USA).

Inhibitory activity of compounds against 3CL protease of COVID-19 for 30 min at RT, followed by the addition of the 10 μM SARS-CoV2 substrate for 60 min at 37°C. The 100 μM compounds or PBS were incubated with 40 nM 3CL protease of COVID-19 in an aqueous cleavage buffer (200 mMTris [pH 9.0], 20% glycerol) for 30 min at RT, followed by the addition of the 10μM SARS-CoV2 substrate for 60 min at 37°C. Protease activity was determined by monitoring fluorescence intensity (excitation 340 nm, emission 465 nm) generated by cleavage from the peptide substrate. Compounds were assayed for *in vitro* protease inhibition along with "no inhibitor" (i.e., protease + PBS + substrate) and no protease (i.e., substrate alone) controls. Protease activities of each reaction were normalized to the "no inhibitor" control. Each value was expressed as mean ± SD from triplicate independent experiments.

REFERENCES

1. D. Kumar, S. Kumar, S. Taprial, D. Kashyap, A. Kumar, and O. Prakash, *J. Chin. Integr. Med.*, **10**, 1336 (2012).
2. Y. Chai, X. Jin, and M. Cai, *Acta Hortic.*, **1185**, 297 (2017).
3. M. J. Cheng, W. L. Lo, J. C. Huang, Y. T. Yeh, Z. L. Hong, Y. C. Lu, M. S. Chang, and C. Y. Chen, *Nat. Prod. Res.*, **24**, 682 (2010).
4. C. Y. Chen, *Nat. Prod. Res.*, **24**, 1830 (2010).
5. W. L. Lo, Y. C. Wu, T. J. Hsieh, S. H. Kuo, H. C. Lin, and C. Y. Chen, *Chin. Pharm. J.*, **56**, 69 (2004).
6. C. Y. Chen, L. Y. Huang, L. J. Chen, W. L. Lo, S. Y. Kuo, Y. D. Wang, S. H. Kuo, and T. J. Hsieh, *Chem. Nat. Compd.*, **44**, 137 (2008).
7. C. Y. Chen, T. Z. Liu, W. C. Tseng, F. J. Lu, R. P. Hung, C. H. Chen, and C. H. Chen, *Food Chem. Toxicol.*, **46**, 2694 (2008).
8. C. Y. Chen, F. R. Chang, and Y. C. Wu, *J. Chin. Chem. Soc.*, **44**, 313 (1997).
9. C. Y. Chen, F. R. Chang, and Y. C. Wu, *J. Chin. Chem. Soc.*, **46**, 77 (1999).
10. C. Y. Chen, C. L. Lin, C. L. Kao, H. C. Yeh, H. T. Li, and C. T. Chang, *Chem. Nat. Compd.*, **55**, 1176 (2019).
11. C. T. Chen, C. L. Kao, W. J. Li, H. C. Yeh, H. T. Li, and C. Y. Chen, *Chem. Nat. Compd.*, **55**, 1185 (2019).
12. C. Y. Wu, C. L. Kao, H. T. Li, and C. Y. Chen, *Chem. Nat. Compd.*, **55**, 175 (2019).
13. T. Okuyama, S. Shibata, M. Hoson, T. Kawada, H. Osada, and T. Noguchi, *Planta Med.*, **23**, 171 (1986).
14. A. Stadelmaier and R. R. Schmidt, *Carbohydr. Res.*, **338**, 2557 (2003).
15. F. Wu, S. Zhao, B. Yu, Y. M. Chen, W. Wang, Z. G. Song, Y. Hu, Z. W. Tao, J. H. Tian, Y. Y. Pei, M. L. Yuan, Y. L. Zhang, F. H. Dai, Y. Liu, Q. M. Wang, J. J. Zheng, L. Xu, E. C. Holmes, and Y. Z. Zhang, *Nature*, **579**, 265 (2020).
16. P. Zhou, X. L. Yang, X. G. Wang, B. Hu, L. Zhang, W. Zhang, H. R. Si, Y. Zhu, B. Li, C. L. Huang, H. D. Chen, J. Chen, Y. Luo, H. Guo, R. D. Jiang, M. Q. Liu, Y. Chen, X. R. Shen, X. Wang, X. S. Zheng, K. Zhao, Q. J. Chen, F. Deng, L. L. Liu, B. Yan, F. X. Zhan, Y. Y. Wang, G. F. Xiao, and Z. L. Shi, *Nature*, **579**, 270 (2020).
17. L. Zhang, D. Lin, X. Sun, U. Curth, C. Drosten, L. Sauerhering, S. Becker, K. Rox, and R. Hilgenfeld, *Science*, **368**, 409 (2020).
18. S. Iketani, F. Forouhar, H. Liu, S. J. Hong, F. Y. Lin, M. S. Nair, A. Zask, Y. Huang, L. Xing, B. R. Stockwell, A. Chavez, and D. D. Ho, *Nat. Commun.*, **12**, 2016 (2021).