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# Inhibitory Components from *Glycosmis stenocarpa* on Pepper Mild Mottle Virus

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The goal of this study was to identify a source of natural plant compounds with inhibitory activity against pepper mild mottle virus (PMMoV). We showed, using a half-leaf assay, that murrayafoline-A (**1**) and isomahanine (**2**) isolated from the aerial parts of *Glycosmis stenocarpa* have inhibitory activity against PMMoV through curative, inactivation, and protection effects. Using a leaf-disk assay, we confirmed that **2** inhibited virus replication in *Nicotiana benthamiana*. Using electron microscopy, we found that a mixture of the virus with **2** resulted in damage to the rod-shaped virus.

**Keywords:** Pepper mild mottle virus, *Glycosmis stenocarpa*, isomahanine, electron miscroscopy, antiviral agent

Pepper (*Capsicum annuum* L.) is an economically important vegetable crop, with production of 2.99 million tons in 2011 [8]. Peppers are sources of spice, flavor, and capsaicin, which is used for pharmaceutical purposes [5, 8]. Pepper mild mottle virus (PMMoV), which is in the genus *Tobamovirus*, is a rigid rod-shaped virus and an important pepper pathogen. PMMoV is known to be transmitted to peppers via gloves, clothing, and hands, and plants are infected by the virus in seeds and soil [6,7]. Peppers infected with PMMoV exhibit a variety of symptoms, such as mottling and yellow/green mosaic in leaves, and small, malformed, mottled fruit, which cause significant economic losses in pepper production. Until 2005, methyl bromide was used to fumigate soil against PMMoV [1, 7].

*Glycosmis stenocarpa* (*G. stenocarpa*) belongs in the Rutaceae family and is a shrub distributed in northern Vietnam as an endemic plant [4]. This plant is known for containing carbazole alkaloids, such as murrayafoline-A, murrayanine, isomahanine, and bisisomahanine [4]. Among them, murrayafoline-A is the major component and has been reported to have inhibitory activity against colon cancer cells, cytotoxicity against MoLT-4 (leukemia) and HOP-18 (lung cancer), and anti-inflammatory activity [3, 4, 10]. The carbazole structure is similar to the structure of carboline alkaloids, except for a nitrogen in pyridine [2, 4]. Carbazole and its derivatives were previously reported to have inhibitory activity against tobacco mosaic virus (TMV) in the *Tobamovirus* genus [2]. Based on this information, we

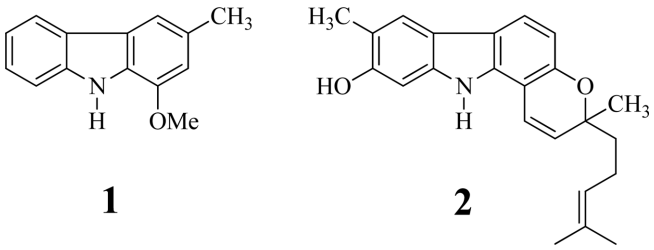


**Fig. 1.** The structure of isolated compounds from *G. stenocarpa*.

selected carbazole derivatives to develop PMMoV inhibitors. We tested two compounds, **1** and **2** isolated from methanol extracts of *G. stenocarpa*, for inhibitory activity against PMMoV.

Methanol extracts of *G. stenocarpa* leaves and stems were partitioned into *n*-hexane, chloroform, and water fractions. The chloroform fraction was separated by silica gel, and C-18 open column chromatography was used to isolate compounds **1** and **2** [4, 9]. Their chemical structures were identified by comparing their masses and $^{1}$H- and $^{13}$C-NMR spectra with previously published structures. The two compounds were identified as murrayafoline (**1**) and isomahanine (**2**) (Fig. 1) [4, 9].

To investigate the inhibitory activity of the isolated compounds on PMMoV, we used a half-leaf assay on stage 4–5 *Nicotiana glutinosa* leaves in vitro [11]. The inhibitory rates of the compounds were calculated based upon the number of local lesions on infected and control leaves, after treatment with 500 μg/ml of the inhibitor. Ribavirin (500 μg/ml) was used as a positive control (6.7 ± 8.5%). Compounds **1** and **2** had inhibition rates of 15.2 ± 5.2% and 49.5 ± 7.1%, respectively (Table 1).

We evaluated the antiviral activities of the compounds in vivo, according to previously published methods (Table 1) [11]. Compound **1** exhibited inhibition rates of 10.1 ± 6.1%, 4.5 ± 7.6%, and 12.7 ± 2.7% against PMMoV through curative, inactivation, and protective mechanisms, respectively. In contrast, compound **2** showed inhibition rates of 46.2 ± 5.7%, 15.2 ± 2.9%, and 42.1 ± 6.4%, also through curative, inactivation, and protective mechanisms, respectively. These results were similar to those of the half-leaf assay, except for the inactivation effect of **2** (Table 1) [11].

**Table 1.** Anti-PMMoV activities of compounds **1** and **2**.

| Inhibitor | Inhibition rate at 500 μg/ml (%) | | | |
|---|---|---|---|---|
| | Half-leaf assay | Curative effect | Inactivation effect | Protective effect |
| **1** | 15.2 ± 5.2 | 10.1 ± 6.1 | 4.5 ± 7.6 | 12.7 ± 2.7 |
| **2** | 49.5 ± 7.1 | 46.2 ± 5.7 | 15.2 ± 2.9 | 42.1 ± 6.4 |
| Ribavirin[a] | 6.7 ± 8.5 | | | |

[a]Positive control.

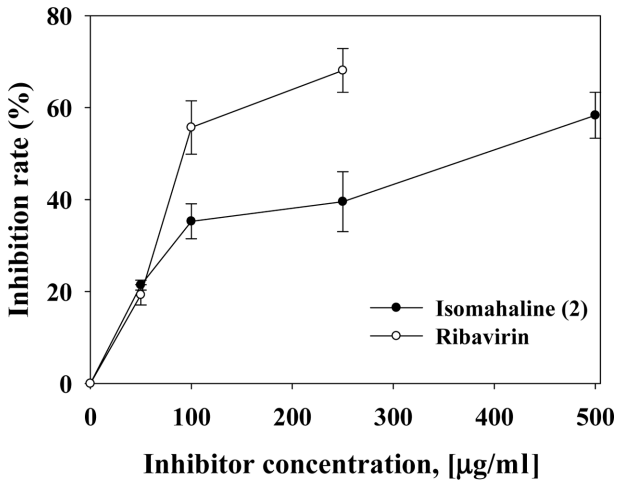


**Fig. 2.** Inhibition rate of compound **2** on the replication of PMMoV by leaf-disk assay.

To study its inhibitory activity on virus proliferation, we applied the potential antiviral inhibitor **2** to *Nicotiana benthamiana* leaves in a leaf-disk assay. The leaves were then inoculated with PMMoV at a concentration of 2 μg/ml, cultivated for 6 h at room temperature, and then cut into 1-cm-diameter disks. The virus on the leaf disks was incubated for 48 h in several concentrations of **2**, and then the amount of the replicated virus was determined using a double antibody sandwich-enzyme-linked immunosorbent assay with absorbance at 405 nm monitored with a UV-Vis spectrophotometer [2]. As shown in Fig. 2, **2** exhibited inhibition rates of 58.3 ± 5.0%, 39.5 ± 6.5%, 35.2 ± 3.8%, and 21.4 ± 1.1% of the value between the control and negative control in a dose-dependent manner against PMMoV. However, the replication inhibitor ribavirin (94.2 ± 1.5 μg/ml) had a lower $IC_{50}$ value of 392.5 ± 2.1 μg/ml against the virus than **2** (Table 2).

To determine the direct influence of inhibitors on PMMoV,

**Table 2.** $IC_{50}$ value of the potential inhibitor on the replication of PMMoV.

| Inhibitor | $IC_{50}$ (μg/ml) |
|---|---|
| **2** | 392.5 ± 2.1 |
| Ribavirin[a] | 94.2 ± 1.5 |

[a]Positive control.

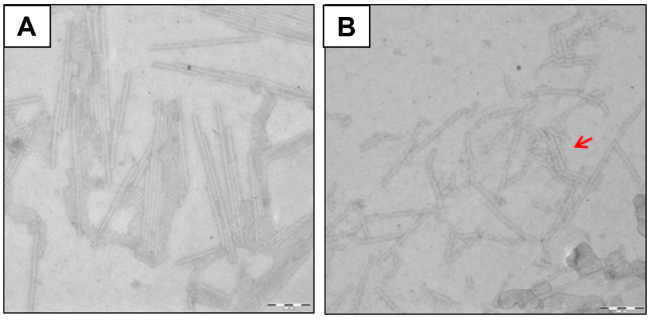


**Fig. 3.** The rod shape of PMMoV treated with methanol (**A**) and compound 2 (**B**) after 24 h, respectively (Segmented PMMoV was indicated by the red arrow).

the inhibitor mixtures and the control (methanol) were stored at room temperature for 24 h and then observed with an electron microscope. As shown in Fig. 3, the virus treated with control (5% final volume methanol) kept their clear rod shape and their adhesion and aggregation. Treatment with the inhibitor at a concentration of 100 μg/ml resulted in viruses that were separated into rod-shaped particles that exhibited no aggregation.

PMMoV is an important pathogen that causes reductions in crop production [1]. Antiviral agents to protect peppers from PMMoV infection are not available globally. Therefore, the goal of this study was to develop antiviral agents from natural products. β-carboline alkaloids were previously reported to have inhibitory effects on TMV, which is in the *Tobamovirus* genus [2]. This information led us to isolate murrayafoline (**1**) and isomahanine (**2**) carbazole alkaloids from *G. stenocarpa*. Compared with **1** and ribavirin (positive control), **2** had a stronger inhibitory effect on PMMoV both in vitro and in vivo. This compound (**2**) showed curative and protective effects on PMMoV but had no inactivation effect. These results suggested that leaves had soaked up the compound **2,** and then it may inhibit the replication of PMMoV. In leaf-disk assay, about a 4-fold concentration of **2** from natural plant was shown to possess the equivalent inhibitory activity as Ribavirin. Additionally, **2** interrupted virus aggregation and damaged the rod-shaped virus when preserving the mixture between PMMoV and inhibitor (**2**). These results suggested that isomahanine (**2**) could be a lead compound to develop natural antiviral agents against PMMoV to increase pepper production in the greenhouse and the field.

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## References

1. Antignus Y, Lachman O, Pearlsman M, Rosner A. 2008. A new pathotype of pepper mild mottle virus (PMMoV) overcomes the $L^4$ resistance genotype of pepper cultivars. *Plant Dis*. **92:** 1033-1037.
2. Chen J, Yan X-H, Dong J-H, Sang P, Fang X, Di Y-T, *et al*. 2009. Tobacco mosaic virus (TMV) inhibitors from *Picrasma quassioides* Been. *J. Agric. Food Chem*. **57:** 6590-6595.
3. Itoigawa M, Kashiwada Y, Ito C, Furukawa K, Tachibana Y, Bastow KF, Lee KH. 200. Antitumor agents, 203, carbazole alkaloid murrayaquinone A and related synthetic carbazole quinones as cytotoxic agents. *J. Nat. Prod*. **63:** 893-897.
4. Kim JH, Morgan AMA, Tai BH, Van DT, Cuong NM, Kim YH. 2015. Inhibition of soluble epoxide hydrolase activity by compounds isolated from the aerial parts of *Glycosmis stenocarpa*. *J. Enzyme Inhib. Med. Chem*. **31:** 640-644.
5. Kim M-K, Seo J-K, Kwak H-R, Kim J-S, Kim K-H, Cha B-J, Choi H-S. 2014. Molecular genetic analysis of cucumber mosaic virus population infecting pepper suggests unique patterns of evolution in Korea. *Virology* **104:** 993-1000.
6. Oka N, Ohki T, Honda Y, Nagaoka K, Takenaka M. 2007. Inhibition of pepper mild mottle virus with commercial cellulases. *J. Phytopathol.* **156:** 65-67.
7. Petrov N. 2014. Effect of pepper mild mottle virus infection on pepper and tomato plants. *Sci. Technol.* **4:** 61-64.
8. Reddy UK, Almeida A, Abburi VL, Alaparthi SB, Unselt D, Hankins G, *et al*. 2014. Identification of gene-specific polymorphisms and association with capsaicin pathway metabolites in *Capsicum annuum* L. collections. *PLoS One* **9:** e86393.
9. Reisch J, Goj O, Wickramasinghe A, Brandara Herath HMT, Henkel G. 1992. Carbazole alkaloids from seeds of *Murray koenigii*. *Phytochemistry* **31:** 2877-2879.
10. Son M-J, Chidipi B, Kim J-C, Huong TT, Tai BH, Kim YH, *et al*. 2014. Alterations of contractions and L-type $Ca^{2+}$ currents by murrayafoline-A rat ventricular myocytes. *Eur. J. Pharmacol*. **740:** 81-87.
11. Xiao J-J, Liao M, Chu M-J, Ren Z-L, Zhang X, Lv X-H, Cao H-Q. 2015. Design, synthesis and anti-tobacco mosaic virus (TMV) activity of 5-chloro-*N*-(4-cyano-1-aryl-1*H*-pyrazol-5-ly)-1-aryl-3-methyl-1*H*-pyrazole-4-carboxamide derivatives. *Molecules* **20:** 807-821.