Canadian Chemical Transactions

ISSN 2291-6458 (Print), ISSN 2291-6466 (Online)
Year 2013 | Volume 1 | Issue 3 | Page 157-164

Research Article

DOI:10.13179/canchemtrans.2013.01.03.0025

Isolation, Identification, Quantification and Inhibitory Activity on HCV Protease of Coumarins from *Viola yedoensis*

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Received: *May 28, 2013* ***Revised:*** *June 18, 2013* ***Accepted:*** *June 19, 2013* ***Published:*** *June 20, 2013*

Abstract: This research was aimed at investigating the possible anti-HCV activity and identifying the responsible bioactive constituents of the anti-hepatitis ethnomedicinal plant *Viola yedoensis*, using HCV protease (PR) as target. This has led to the isolation and characterization of 3 coumarins from the plant, using various chromatographic procedures and the interpretation of spectral data in comparison with already existing data reported in literatures. One of the isolated compounds, 5, 5′-bi (6, 7-dihydroxycoumarin), a dimeric coumarin, potently inhibited HCV NS3/4A protease with an IC_{50} value of 0.5 μg/ml, an uncommonly low IC_{50} value (portending an uncommonly high inhibitory activity) for a non-peptide protein binder. This dicoumarin, hereby for the first time reported for its anti-HCV PR activity, could become a molecular template for the development of novel anti-HCV drugs.

Keywords: *Viola yedoensis*; Coumarin; Bicoumarin; 5, 5′-bi (6, 7-dihydroxycoumarin); HCV Protease Inhibitor

1. INTRODUCTION

The dried whole plant of *Viola yedoensis* Makino (Violaceae) is documented as an antifebrile and detoxicant drug used in the treatment of boils, furuncles, carbuncles, acute and chronic hepatitis and other infections in Traditional Chinese Medicine (TCM) [1]. Literature shows that *V. yedoensis* contains many chemical components, such as flavonoids [2-4], coumarins [5], phytosterol, alkaloids, volatile oils, carbohydrates, organic acids [1], anthocyanins[6], sulfonated carbohydrate polymer [7], cyclotides,

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etc.[8]. Amongst these constituents, the sulfonated carbohydrate polymer and some cyclotides with macrocyclic peptide skeleton with three disulfide bonds have been reported to show potent anti-HIV activity [7-8]. However, there has been no report, hitherto, on the anti-hepatitis activity of *V. yedoensis*, despite the wide application of this plant in the management and treatment of liver diseases in TCM [1]. As a serious global problem, Hepatitis C Virus (HCV) infection is worth being worried about: Approximately 3 % of the global population is infected with HCV [9], the debilitating and lethal effects of which are manifest in its global recognition as a leading cause of cirrhosis, hepatocellular carcinoma and some other hepatic conditions requiring liver transplant. Current treatment with peginerferon-rivbavirin is effective only in half of the patients, with statistics on the incidence of resistance to treatment worrisomely remaining on a steady increase [9]. Therefore, it is particularly urgent to develop new anti-HCV drugs that are highly effective and desirably with different structural scaffolds from the currently available ones, to possibly prevent (or at least delay) the emergence of resistance. Inhibiting the NS3/4A protease, a crucial enzyme in the maturation process of HCV, has been identified as an invaluable therapeutic strategy in the development of new anti-HCV drugs [10-12]. In a program therefore aimed at discovering anti-HCV compounds, we have investigated the extracts and constituents of many traditional herbal drugs for their anti-HCV activity in a number of HCV PR inhibition assays, leading to the identification of the anti-HCV activity of some phytochemicals including triterpenes, antrodins (maleic and succinic acid derivatives) [13], and, of recent, three coumarins (**1-3**) (**Fig1)** isolated from *V. yedoensis*. One of these coumarins, (**2**), a dimeric coumarin, potently inhibited HCV PR. The relative concentrations of these coumarins in the plant were also determined by ultra-high-performance liquid chromatography–triple quadrupole mass spectrometry (UHPLC-QQQMS) method using a synthetic compound (**4**) as internal standard.

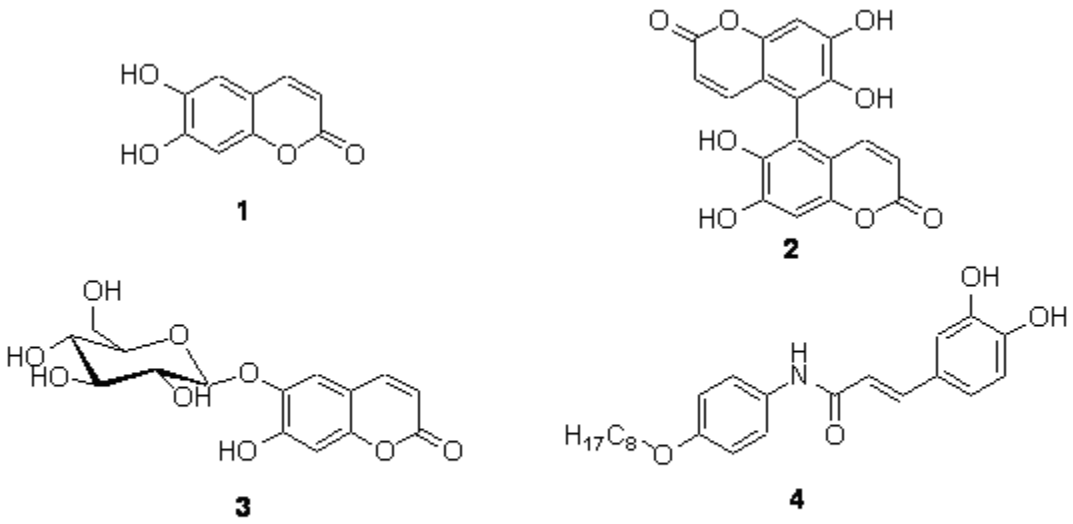

Figure 1. Structures of **1**, **2** and **3**, isolated from *Viola yedoensis* and **4**, an internal standard.

2. EXPERIMENTAL

2.1. Reagents and Apparatus

HPLC grade solvents used for UPLC-DAD-ESI-MS were purchased from Fisher scientific company (HPLC grade, New Jersey, USA). Other solvents used were of analytical grade. Silica gel (100-

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200 mesh, Qingdao Marine Chemical Inc., Qingdao, China) and octadecylsilane (ODS, 38 to 63 μm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used in column chromatography. NMR spectra were acquired with a Bruker Avance III 500 spectrometer. UHPLC-DAD-ESI-MS experiments were performed on an Agilent 1290 infinity UHPLC-DAD system (Agilent Technologies Singapore (International) Pte. Ltd., Singapore) with an autosampler and a photo-diode array detector (DAD) coupled with an Agilent 6430 triple Quad MS. Preparative HPLC (PHPLC) was carried out on a CXTH LC3000 HPLC system (Beijing Chuangxintongheng Co., Beijing, China) with a TOSOH TSKgel ODS-80Ts column. SensoLyte 520 HCV fluorimetric Protease Assay Kit (lot# 1028) and HCV NS3/4A protease (lot# 103-075) purchased from Anaspec. Com. (San Jose, CA 95131, USA), were used for the HCV protease inhibition assay.

2.2. Plant Materials

The plant material for this research was purchased from Anguo medicine market in Hebei province, China, and was identified as the whole plant of *V. yedoensis* by the authors.

2.3. Extraction and Isolation

The dried *V. yedoensis* (2 kg) was extracted with methanol under reflux 3 consecutive times lasting 2 h, 1 h and 30 min, in this order. The methanol solutions were combined and concentrated under reduced pressure. The concentrated extract was partitioned into petroleum ether, EtOAc, n-butyl alcohol and water, yielding 133 g, 81 g, 96 g and 47 g respectively after concentration to dryness. The EtOAc fraction was subjected to column chromatography over silica gel eluted with a solvent gradient of petroleum ether/EtOAc (95:5-0:100, v/v), EtOAc/EtOH (95:5, v/v) and EtOAc/EtOH/H_2O (20:0:0-10:2:1, v/v/v), successively. Similar fractions were combined according to the result of thin-layer chromatography (TLC). The EtOAc/EtOH (95:5, v/v) and EtOAc/EtOH/H_2O (22:2:1-20:2:1, v/v/v) eluted parts were combined, concentrated and subjected to an octadecylsilane (ODS) column chromatography with a solvent gradient of MeOH/H_2O (0:100-100:0, v/v). Compound **1** (200 mg) was crystallized from the MeOH/H_2O (55:45-60:40 v/v) eluted part. The MeOH/H_2O (100:0, v/v) eluted part was purified with PHPLC eluted with 20–60% MeOH/H_2O for 90 min at a flow rate of 10 ml/min to generate **2** (1.28 mg) at 35–38 min. The EtOAc/EtOH/H_2O (16:2:1-14:2:1, v/v/v) eluted part was further purified with ODS column chromatography [MeOH/H_2O (0:100-100:0, v/v)] to obtain compound **3** (18.8 mg) from MeOH/H_2O (20:80-30:70, v/v) eluted part.

2.4. Sample Preparation

A 10 mg/ml stock solution of each of the compounds, extract and fractions was prepared in DMSO. A serial dilution of 1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, and 0.001 mg/ml was made from each stock solution using DMSO as solvent. Each concentration was evaluated in the HCV PR inhibition assay. In the quantification experiments, a caffeic acid derivative (compound **4**) synthesized in one of our earlier works [14] was used as internal standard (**I.S.**) as it contained a catechol moiety as most of the coumarins did.

2.4. HCV Protease Assay

The experiment was carried out in a 384-well black plate (BD Falcon) following the instructions provided by the supplier as follows: 2 μl of each sample solution (in DMSO) and 10 μl of a freshly prepared 50 dilution (made in assay buffers) of the enzyme substrate were put in a well. Similarly, DMSO and embelin [15] were used in place of test sample to set up negative and positive control wells respectively. This was followed by the addition of 8 μl of freshly prepared enzyme (0.5 μg/ml) into each well and subsequent incubation at 37˚C for 30 min. The fluorescence of the contents of each well was then measured at excitation /emission wavelengths of 490 nm/ 520 nm respectively by a Thermo

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Scientific Varioskan Flash (Thermo Fisher Scientific Oy D.O. Box100, FI-01621 Vantaa, Finland). Percentage inhibition was calculated as follows: **%** Inhibition = 100 × ($F_{vehicle}$ -F_{sample})/$F_{vehicle}$. The experiment was carried out in triplicates for each sample and the results were analyzed by a plot of average % inhibition versus sample concentrations. IC_{50}, i.e., concentration producing 50% inhibition was determined from each curve.

2.5. Quantification of the Chemical Components with UPLC-DAD-ESI-MS

Samples were analyzed by UPLC-DAD-QQQMS with an Agilent ZORBAX SB-C18 RRHT column (50 x 2.1 mm i.d.; particle size 1.8 μm) at 30˚C. The mobile phase comprised of 0.1% formic acid in H_2O (solvent A) and methanol (solvent B). The flow pattern was programmed as 70/30 A/B from 0.00min to 3.00min and 0/100 A/B from 3.00min to 5.00min. The injection volume was 2 μL and the rate of flow was 0.4 ml/min. MS analysis was performed in the negative ion mode, with a capillary, 3.5 kV; mass range, *m/z* 100 to 1500; Gas temperature, 350˚C; Gas flow, 11 L/min; Nebulizer, 45 psi. MS2 scan mode (negative) was chosen for qualitative analysis while multiple reaction monitoring (MRM) with negative polarity (**Figure 2**) was used for quantification of compounds **1**-**3**. The MS/MS transitions were set at *m/z* 177→133.1 for compound **1**, *m/z* 353→177.1 for compound **2**, *m/z* 339→177.0 for compound **3**, and *m/z* 382→161.1 for the **I.S.** The collision energies for compound **1**, **2**, **3** and **I.S.** were 15, 30, 15 and 30 eV, respectively.

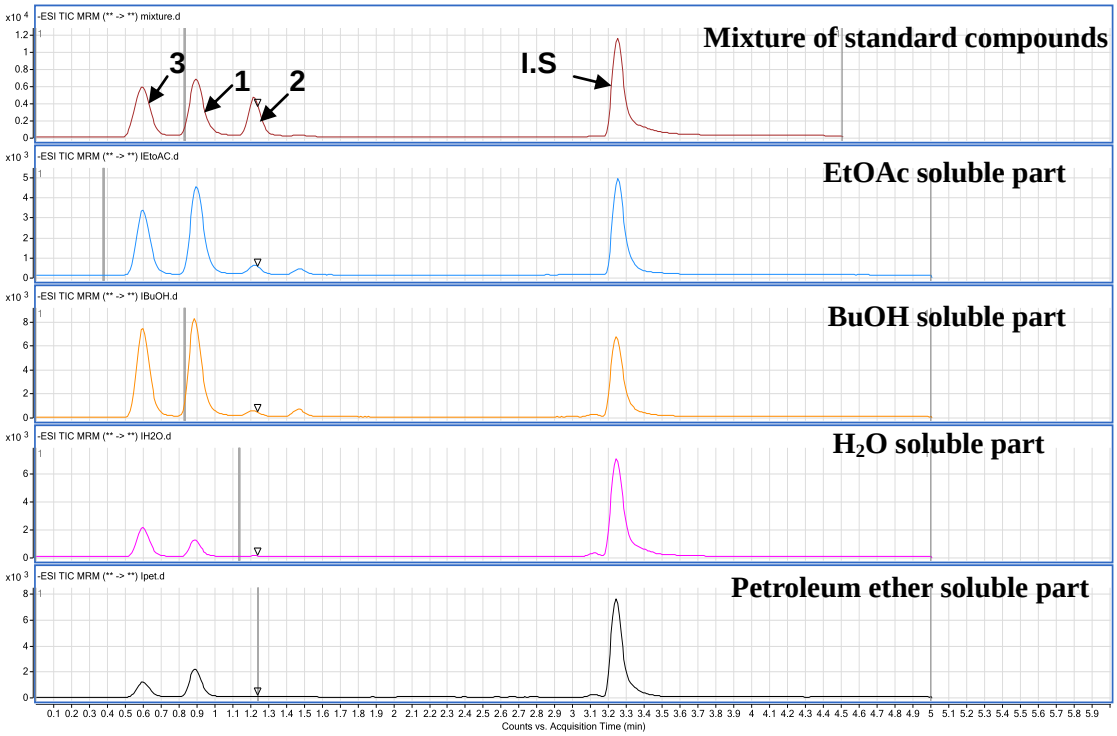

Figure 2. UHPLC-MS profiles of compounds **1**-**4** in different extracts of *Viola yedoensis*

Pure compounds **1**-**3** in DMSO at eight concentrations of 4-fold serial dilutions were used for the standard curves with the highest concentration of 16.67 μg/ml. The internal standard (**4**) concentration

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was kept at 5 µg/ml. Standard curves were plotted using the concentration of compound as X-axis and the area ratio of compound to internal standard as Y-axis. Injection volumes were 2 µl for all compounds and extract.

3. RESULTS AND DISCUSSIONS

3.1. Identification of the Isolated Components of *V. yedoensis*

The structures of compounds **1-3** were determined by the interpretation of their MS and NMR data *vis-a-vis* already existing data. Compound **2**, via its 2D NMR data, was found to be a symmetric analogue of the non-symmetric dicoumarin, dimeresculetin, earlier isolated from the same plant [16]. Compound **1** was identified as 6,7-dihydroxycoumarin, **2** as 5, 5′-bi (6, 7-dihydroxycoumarin) [17-19] and **3**, a coumarin glycoside, first isolated from *Viola* by Zhou, *et al.* (2009), as esculin [20].

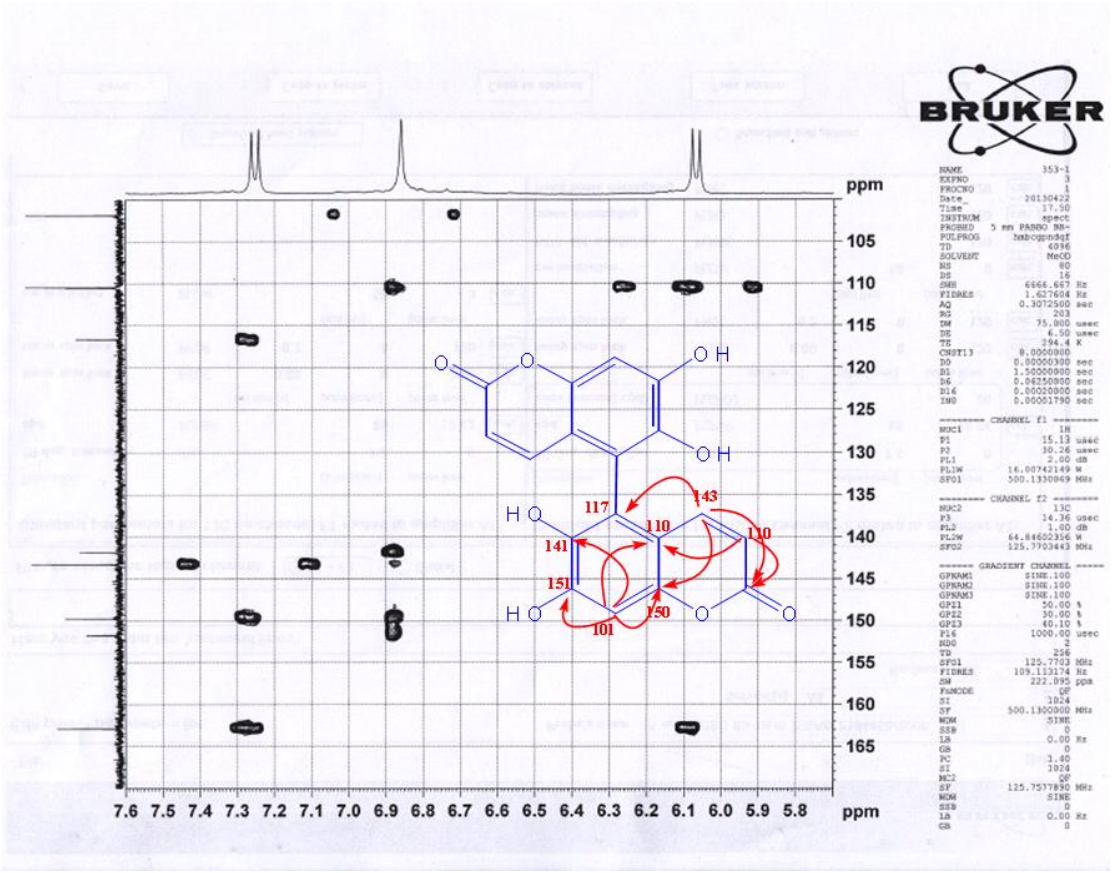

Figure 3: HMBC spectrum of compound **2,** with arrows indicating important long range *H* – *C* correlations.

3.2. HCV Protease Inhibitory Activity

Table 1 shows the inhibitory activity of the methanolic extract, various fractions and the three isolated compounds on HCV PR. Each of the methanol extract, petroleum ether, EtOAc and n-butanol fractions showed inhibitory activity on HCV PR at 100 µg/ml with percentage inhibitions of 86.53%, 67.90%, 65.01% and 52.10%, respectively, while the H_2O fraction showed very low % inhibition (32.7%). The results suggest that the active constituents of the methanol extract are concentrated in the petroleum ether and EtOAc soluble fractions. The fact that the highest percentage HCV PR inhibition was

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obtained with the crude methanolic extract suggests the possible synegystic effects of the active components on each other or at least their potentiation by the presence of some other factors that may not necessarily have anti-HCV PR activity. In the bioassay of the pure compounds, compound **2** showed the highest percentage inhibition of 94.25 % at a concentration of 100 µg/ml, while compound **1** and **3** were almost inactive. Compound **2** is therefore a major contributor to the anti-HCV PR activity of *V. yedoensis*, and is most likely a major contributor to the antihepatitis ethnotherapeutic use of the plant [1]. The plot of average % inhibition versus concentration obtained from the assay of different concentrations of compound **2** yielded an IC_{50} of 0.5 µg/ml (**Table 2**). This IC_{50} value, is much lower than the 87.7 µg/ml IC_{50} value obtained for the positive control, embelin, in the same experiment, indicating that the dimeric coumarin inhibited HCV PR much more strongly than the positive control. Moreover, an IC_{50} value as low as 0.5 µg/ml is rarely observed for non-peptides in enzyme inhibition assays, revealing the unusually high affinity of this dicoumarin for HCV PR and thereby suggesting the untapped potentials of dicoumarins in expanding the frontiers of anti-HCV drug discovery.

Table 1 HCV protease inhibitory activity of the constituents and extracts of *Viola yedoensis*.

Sample	Inhibition % at 100 µg/ml ± S.D.
MeOH extract	86.5% ± 3.05
Petroleum ether soluble part	67.9% ± 1.54
EtOAC soluble part	65.0% ± 4.15
BuOH soluble part	52.1% ± 4.55
H_2O soluble part	32.7% ± 4.31
Compound **1**	4.4% ± 8.64
Compound **2**	94.2% ± 0.60
Compound **3**	23.1% ± 3.18
Positive Control	54.9% ± 10.69

Table 2. HCV protease inhibitory activity of compound **2** at different concentrations

Inhibition % ± S.D.					IC_{50}
100 µg/ml	10 µg/ml	1 µg/ml	0.5 µg/ml	0.1 µg/ml	(µg/ml)
94.2 ± 0.60	86.1 ± 0.44	72.2 ± 0.98	50.0 ± 4.46	13.4 ± 3.56	0.5

Table 3. UPLC-ESIMS data for the constituents of *Viola yedoensis*.

	Retention time(min)	Equation of Calibration curve[a]	r	LOD[b](µg/ml)
Compound **1**	1.56	y=4.065608x+0.0007972	0.9999	0.0010
Compound **2**	1.86	y=4.374285x-0.002410	0.9995	0.0041
Compound **3**	1.26	y=3.954590x-0.002690	0.9996	0.0041

[a] y is the area ratios of standard compounds to internal standard and x is the concentration of standard compounds in µg/ml. [b] LOD: limit of detection.

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3.3. Quantification of the Constituents from *Viola yedoensis* Using UHPLC-ESI-MS

As shown in **Table 3**, the calibration curves with the area ratios of standard compounds to internal standard as Y-axis and the concentrations of standard compounds as X-axis displayed good linearity. As shown in **Table 4**, the quantification results of compounds **1-3** showed that the EtOAC soluble part and BuOH soluble part contained higher levels of compound **2**, while little amount of **2** was found in petroleum ether soluble part. We deduced that compound **2** is the major contributor to the HCV protease inhibitory activity of the EtOAC and BuOH fractions, while some yet unidentified compounds played important roles in the HCV PR inhibition by the petroleum ether fraction.

Table 4. Contents of the chemical components in different extracts of *Viola yedoensis* (mg/g dry powder).

Compounds	Petroleum ether soluble part	EtOAC soluble part	BuOH soluble part	H_2O soluble part
Compound **1**	3.57	3.94	21.12	1.77
Compound **2**	0.10	1.20	0.79	0.13
Compound **3**	2.02	9.53	17.01	3.44

4. CONCLUSIONS

The MeOH extract of *V. yedoensis* was found to exhibit strong inhibitory activity against HCV NS3/4A protease. Amongst the different fractions, the petroleum ether and the EtOAc soluble parts were the most active, indicating that there were active components which are considerably lipophilic in this herbal drug. Three coumarins were isolated from the EtOAc fraction, including a dicoumarin (compound **2**) which showed a remarkable HCV protease inhibition with an IC_{50} of 0.5 μg/ml, an enzyme inhibition potency rarely encountered with non-peptide compounds. This work has, for the first time, reported the anti-HCV protease activity of the dicoumarin, 5, 5′-bi (6, 7-dihydroxycoumarin), isolated from *V. yedoensis*. Thus, it has provided a scientific evidence for the traditional use of *V. yedoensis* as a herbal medicine for liver diseases, the discovery of 5, 5′-bi (6, 7-dihydroxycoumarin) as a potential lead in the development of new anti-HCV drugs remaining of no less importance. Finally, the discovery, by this work, of the uncommonly high affinity of a dicoumarin for the viral enzyme may just have initiated an expansion of the frontiers of anti-HCV drug design.

ACKNOWLEDGEMENTS

This work was supported by a grant from The *National Natural Science Foundation* of China (#81160511) and by a grant for undergraduate innovative research from Inner Mongolia University. We thank Mr. Meng He for NMR data acquisition.

REFERENCES AND NOTES

[1] China Pharmacopoeia Committee. Pharmacopoeia of the People's Republic of China. Chemical Industry Press. Beijing, **2005**, pp237.

[2] Xie, C.; Veitch, N. C.; Houghton, P. J.; Simmonds, M. S. Flavone C-glycosides from *Viola yedoensis* Makino. *Chem. Pharm. Bull*. **2003**, *51*, 1204-1207.

[3] Xu, J. Z.; Zeng S. S.; Qu H. B. Chemical constituents from Viola yedoensis. *Zhongcaoyao* **2010**, *41*, 1423-1425.

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[4] Chen, H. L.; Dong, X. P.; Zhang, M.; Pei, J.; Tang, P. R; Zhang, Y. Chemical constituents in Viola yedoensis, *Zhongcaoyao* **2010**, *41*, 874-877.
[5] Yang, P. P.; Yan, F. L. Study on chemical constituents of *Viola yedoensis*. *J Xinxiang Med Coll.* **2008**, *25*, 185-187.
[6] Zhang, J.; Wang, L. S.; Gao, J. M.; Xu, Y. J.; Li, L. F.; Li, C. H. Rapid separation and identification of anthocyanins from flowers of *Viola yedoensis* and *V. prionantha* by high-performance liquid chromatography-photodiode array detection-electrospray ionisation mass spectrometry. *Phytochem analysis* **2012**, *23*, 16-22.
[7] Ngan, F.; Chang, R. S.; Tabba, H. D.; Smith, K. M. Isolation, purification and partial characterization of an active anti-HIV compound from the Chinese medicinal herb *Viola yedoensis*. *Antivir Res.* **1988**, *10*, 107-115.
[8] Wang, C. K. L.; Colgrave, M. L.; Gustafson, K. R.; Ireland, D. C.; Goransson, U.; Craik, D. J. Anti-HIV cyclotides from the Chinese medicinal herb *Viola yedoensis*. *J. Nat. Prod.* **2008**, *71*, 47-52.
[9] Stauber, R. E.; Stadlbauer, V. Novel approaches for therapy of chronic hepatitis C. *J. Clin. Virol.* **2006**, *36*, 87-94.
[10] Chu, M.; Mierzwa, R.; Truumees, I.; King, A.; Patel, M.; Berrie, R.; Hart, A.; Butkiewicz, N.; Mahapatra, B. D.; Chan, T. M.; Puar, M. S. Structure of Sch 68631: a new hepatitis C proteinase inhibitor from *Stretomyces sp*. *Tetrahedron Lett*. **1996**, *37*, 7229.
[11] Clercq, E. D. The design of drugs for HIV and HCV. *Nat Rev Drug Discov.* **2007**, *6*, 1001-1018.
[12] Kakiuchi, N.; Fukuda, K.; Nishikawa, F.; Nishikawa, S.; Shimotohno, K. Inhibition of hepatitis C virus serine protease in living cells by RNA aptamers detected using fluorescent protein substrates. *Comb Chem High Throughput Screen.* **2003**, *6*, 155-160.
[13] Hattori, M.; Ma, C. M.; Wei, Y.; El Dine, R. S.; Sato, N. Survey of Anti-HIV and Anti-HCV Compounds from Natural Sources. *Can Chem Trans.* **2013**, *1*, 116-140.
[14] Ma, C. M.; Abe, T.; Komiyama, T.; Wang, W.; Hattori, M.; Daneshtalab, M. Synthesis, anti-fungal and 1,3-ß-D-glucan synthase inhibitory activities of caffeic and quinic acid derivatives. *Bioorg Med Chem.* **2010**, *18*, 7009-7014.
[15] Hussein, G.; Nakamura N.; Hattori M.; Kakiuchi N.; Shimotohno K. Inhibitory effects of Sudanese medicinal plant extracts on hepatitis C virus (HCV) protease. *Phytother Res.* ***2000***, *14*, 510-516.
[16] Zhou, H. Y.; Hong, J. L.; Shu, P.; Ni, Y. J.; Qin, M. J. A new dicoumarin and anticoagulant activity from *Viola yedoensis* Makino. *Fitoterapia*, **2009**, *80*, 283–285
[17] Gao, G. C.; Wu, P.; Cao, H. L.; Lin, L. D.; Wei, X. Y. Phenolic compounds from *Merremia boisiana* (Convolvulaceae). *J. Trop. Subtrop. Bot*. **2006**, *14*, 234.
[18] Yu, D. Q.; Yang, J. S. Handbook of analytical chemistry. The seventh section, Chemical industry press, Beijing, **1999**, pp 449.
[19] Huang, J.; Yang, J.; Xue, Q.; Yu, L.; Zhang, D. Studies on chemical constituents from herbs of *Viola yedoensis*. Zhongguo Zhongyao Zazhi. **2009**, *4*, 1114-1116.
[20] Zhou, H. Y.; Qin, M. J.; Hong, J. L.; Ni, Y. J.; Wu, G. Chemical constituents of *Viola yedoensis*. *Chin J Nat Med*. **2009**, *7*, 290-292.

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