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Inhibition of viral proteases by Zingiberaceae extracts and flavones isolated from *Kaempferia parviflora*

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In order to identify novel lead compounds with antiviral effect, methanol and aqueous extracts of eight medicinal plants in the Zingiberaceae family were screened for inhibition of proteases from human immunodeficiency virus type 1 (HIV-1), hepatitis C virus (HCV) and human cytomegalovirus (HCMV). In general, the methanol extracts inhibited the enzymes more effectively than the aqueous extracts. HIV-1 protease was strongly inhibited by the methanol extract of *Alpinia galanga*. This extract also inhibited HCV and HCMV proteases, but to a lower degree. HCV protease was most efficiently inhibited by the extracts from *Zingiber officinale*, with little difference between the aqueous and the methanol extracts. Many of the methanol extracts inhibited HCMV protease, but the aqueous extracts showed weak inhibition. In a first endeavor to identify the active constituents, eight flavones were isolated from the black rhizomes of *Kaempferia parviflora*. The most effective inhibitors, 5-hydroxy-7-methoxyflavone and 5,7-dimethoxyflavone, inhibited HIV-1 protease with IC_{50} values of 19 μM. Moreover, 5-hydroxy-3,7-dimethoxyflavone inhibited HCV protease and HCMV protease with IC_{50} values of 190 and 250 μM, respectively.

1. Introduction

The success in developing drugs against AIDS that target HIV-1 protease has resulted in a major interest in proteases as drug targets. Modern discovery of drugs is typically based on mechanistic and structural information about the enzyme of interest and on organic synthesis of transition state analogues. Another strategy is based on the analysis of effects of natural products on diseases or targets directly. Natural products have historically been the most important sources of drugs for mankind and still serve as important original starting points for synthesis new drugs (Newman et al. 2003).

Therefore our laboratory has been interested in searching for antiviral compounds from natural sources. In this paper, we investigated Thai herbs of the Zingiberaceae family for potential antiviral protease activity. The medicinal plants in this family have not only been used for spicy cooking but also as health-promoting herbs (Sirirugsa 1999). The rhizomes of Zingiberaceae have been shown to contain different bioactive compounds, such as curcuminoids (Lechtenberg et al. 2004), sesquiterpenes (Hong et al. 2001) and flavones (Yenjai et al. 2004). One of the plants we found to contain inhibitory compounds of all three enzymes, *Kaempferia parviflora,* has been known in Thailand as a health-promoting herb, and used for treatment of colic disorder, peptic and duodenal ulcer (Yenjai et al. 2004). It was therefore used as a starting point for identification of the active ingredients.

In the present study, proteases involved in AIDS, hepatitis C (HCV) and human cytomegalovirus (HCMV) infections were selected as targets for the antiviral compounds. These proteases are all crucial for the life cycle of the virus from which they originate, making them all attractive targets for antiviral drug therapy. They are structurally and mechanistically distinct and only distantly related to endogenous human proteases. Human immunodeficiency type 1 (HIV-1) protease is an aspartic protease consisting of two identical subunits, a type only present in retroviruses. In contrast, the HCV non-structural protein 3 (NS3) protease is a serine protease structurally related to chymotrypsin type proteases, but is in HCV linked to the viral helicase and activated by a viral peptide cofactor. Although, HCMV protease is also a serine protease, it represents a unique type of serine protease, occurring only in herpes viruses. By using different proteases it was possible to use the extracts efficiently and the specificity of inhibition could be evaluated at an early stage.

2. Investigations and results

2.1. Inhibitory effect of crude extracts

Methanol and aqueous crude extracts were prepared from eight medicinal plants in the *Zingiberaceae* family. The fresh rhizome was used, but for one of the plants (*K. galanga*) the dried rhizome was used instead. The effect of each extract on the activity of HIV-1, HCV and HCMV

Table 1: Inhibition of HIV-1, HCV and HCMV proteases by methanol extracts from *Zingiberaceae* medicinal herbs

Name	Used part	% Inhibition					
		HIV-1 protease		HCV protease		HCMV protease	
		20 μg/ml	200 μg/ml	20 μg/ml	200 μg/ml	20 μg/ml	200 μg/ml
Kaempferia galanga	Dried rhizome	1.5 ± 0.8	4.9 ± 1.4	3.5 ± 2.7	20.9 ± 6.4	22.3 ± 0.8	45.6 ± 1.7
Curcuma zedoaria	Fresh rhizome	18.6 ± 2.7	97.8 ± 1.5	68.9 ± 2.4	84.7 ± 6.2	32.1 ± 1.6	94.8 ± 2.8
Curcuma longa	Fresh rhizome	67.8 ± 5.6	92.9 ± 0.9	44.6 ± 6.1	84.9 ± 2.8	68.6 ± 1.2	97.3 ± 2.4
Kaempferia parviflora	Fresh rhizome	37.1 ± 0.4	81.5 ± 0.8	17.7 ± 2.8	71.9 ± 2.3	29.6 ± 4.0	74.7 ± 3.5
Boesenbergia pandurata	Fresh rhizome	22.1 ± 2.1	64.6 ± 2.0	33.9 ± 3.5	82.0 ± 1.2	47.9 ± 3.6	96.2 ± 0.6
Zingiber zerumbet	Fresh rhizome	9.9 ± 2.8	17.8 ± 1.4	47.2 ± 3.5	85.4 ± 5.5	20.5 ± 2.4	39.0 ± 0.3
Zingiber officinale	Fresh rhizome	14.8 ± 2.7	46.0 ± 8.6	53.6 ± 0.8	96.0 ± 1.4	4.0 ± 1.5	57.2 ± 5.8
Alpinia galanga	Fresh rhizome	97.5 ± 0.4	98.2 ± 1.0	58.7 ± 1.8	82.8 ± 0.9	77.1 ± 0.3	96.6 ± 0.3

Extracts of the specified part were tested at 20 and 200 μg/ml, values are means ± SD, n = 3

Table 2: Inhibition of HIV-1, HCV and HCMV proteases by aqueous extracts from *Zingiberaceae* medicinal herbs

Name	Used part	% Inhibition					
		HIV protease		HCV protease		HCMV protease	
		20 μg/ml	200 μg/ml	20 μg/ml	200 μg/ml	20 μg/ml	200 μg/ml
Kaempferia galanga	Dried rhizome	0.7 ± 0.7	22.1 ± 1.2	8.3 ± 0.1	29.4 ± 3.9	6.7 ± 1.7	22.9 ± 0.4
Curcuma zedoaria	Fresh rhizome	26.5 ± 3.4	89.8 ± 1.2	50.6 ± 9.9	81.3 ± 1.3	13.1 ± 4.7	57.5 ± 0.2
Curcuma longa	Fresh rhizome	35.3 ± 3.5	97.0 ± 1.9	65.1 ± 5.8	84.5 ± 5.9	1.8 ± 0.9	54.1 ± 3.2
Kaempferia parviflora	Fresh rhizome	15.2 ± 0.6	48.3 ± 7.8	56.7 ± 2.3	87.2 ± 2.6	11.3 ± 6.5	33.8 ± 7.3
Boesenbergia pandurata	Fresh rhizome	35.7 ± 1.7	57.9 ± 2.0	54.0 ± 0.9	84.8 ± 2.1	13.1 ± 3.0	19.2 ± 3.9
Zingiber zerumbet	Fresh rhizome	5.0 ± 0.6	8.5 ± 0.5	7.9 ± 4.4	71.8 ± 5.5	0.9 ± 0.4	5.5 ± 4.3
Zingiber officinale	Fresh rhizome	17.6 ± 0.6	26.8 ± 0.7	43.0 ± 9.7	93.1 ± 2.2	5.8 ± 0.4	11.9 ± 8.2
Alpinia galanga	Fresh rhizome	16.5 ± 0.1	37.9 ± 1.0	12.3 ± 0.8	75.7 ± 5.2	5.2 ± 3.0	10.7 ± 3.9

Extracts from the specified parts were tested at 20 and 200 μg/ml, values are means ± SD, n = 3

proteases was tested at a concentration of 20 μg/ml and 200 μg/ml. The methanol extracts (Table 1) were overall more inhibitory than the aqueous extracts (Table 2). Only two aqueous extracts resulted in more than 90% inhibition, while nine methanol extracts exhibited at least 90% inhibition of the proteases studied. Furthermore, none of the aqueous extracts were inhibitory to this degree at the lower concentration tested, while two of the methanol extracts were. The methanol extracts that corresponded to the two aqueous extracts that showed at least 90% inhibition, were also inhibitory to this degree.

2.1.1 Inhibition of HIV-1 protease

The methanol crude extracts of *Curcuma zedoaria*, *Curcuma longa*, *Kaempferia parviflora* and *Alpinia galanga* were found to inhibit HIV-1 protease activity by more than 80% at a concentration of 200 μg/ml (Table 1). The *Alpinia galanga* extract retained more than 90% inhibition of HIV-1 protease activity at a ten-fold lower concentration. The aqueous extracts of *Curcuma zedoaria* and *Curcuma longa* showed an inhibitory effect (80% inhibition) at high concentration (Table 2).

2.1.2. Inhibition of HCV protease

The methanol crude extracts of *Curcuma zedoaria*, *Curcuma longa*, *Kaempferia parviflora*, *Boesenbergia pandurata*, *Zingiber zerumbet*, *Zingiber officinale* and *Alpinia galanga* were found to inhibit HCV protease activity by more than 70% at a concentration of 200 μg/ml (Table 1). At a low concentration (20 μg/ml), the methanol crude extracts of *Curcuma zedoaria*, *Zingiber officinale* and *Alpinia galanga* elicited inhibition over 50%. For the aqueous extracts, most of the examined herbal aqueous extracts, except *Kaempferia galanga,* showed more than 70% inhibition of HCV protease at a concentration of 200 μg/ml (Table 2). At a concentration of 20 μg/ml, the aqueous extracts of *Curcuma zedoaria*, *Curcuma longa*, *Kaempferia parviflora* and *Boesenbergia pandurata* showed more than 50% inhibition.

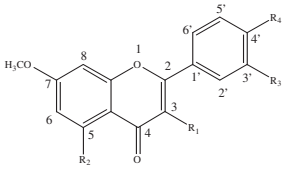
2.1.3. Inhibition of HCMV protease

The methanol crude extract of *Curcuma zedoaria*, *Curcuma longa*, *Kaempferia parviflora*, *Boesenbergia pandurata* and *Alpinia galanga* inhibited HCMV protease by more than 70% at high concentration (200 μg/ml) (Table 1). At low concentration (20 μg/ml), the methanol crude extracts of *Curcuma longa* and *Alpinia galanga* were inhibitory by more than 60%. The aqueous extracts of *Curcuma zedoaria* and *Curcuma longa* were found to inhibit HCMV protease by more than 50% at high concentration (Table 2).

2.2. Characterization of inhibitory constituents in Kaempferia parviflora

Kaempferia parviflora, a well known medicinal plant in Thailand, was used in a first effort to identify the active ingredients in one of the tested *Zingiberaceae* species. The isolated compounds were identified on the basis of their ^{1}H- and ^{13}C-NMR data, which were identical with those reported in the literature.

Although the crude methanol extract of *Kaempferia parviflora* inhibited all three proteases, the purified flavones inhibited primarily HIV protease (Table 3). HCV protease was only inhibited by 5-hydroxy-3,7-dimethoxyflavone (**1**), with an IC_{50}-value of almost 200 μM. The same com-



Compd.	R1	R2	R3	R4
1	OCH_3	OH	H	H
2	H	OH	H	H
3	OCH_3	OH	H	OCH_3
4	H	OH	H	OCH_3
5	OCH_3	OH	OCH_3	OCH_3
6	OCH_3	OCH_3	H	H
7	H	OCH_3	H	H
8	H	OCH_3	H	OCH_3

Table 3: Inhibitory activity of compounds isolated from *Kaempferia parviflora* against HIV-1, HCV and HCMV proteases, values are means ± SD, n = 3

Compd.	IC_{50} (μM)		
	HIV-1 protease	HCV protease	HCMV protease
1	66.11 ± 3.24	192.95 ± 9.87	248.56 ± 4.03
2	19.04 ± 3.11	inactive	349.11 ± 3.83
3	101.09 ± 9.92	inactive	inactive
4	77.95 ± 9.08	inactive	inactive
5	160.07 ± 5.42	inactive	inactive
6	81.28 ± 7.07	inactive	inactive
7	19.54 ± 1.25	inactive	inactive
8	109.91 ± 10.92	inactive	inactive

inactive at IC_{50} > 400 μM

pound inhibited HCMV protease with an IC_{50}-value of 250 μM. In contrast, all eight compounds inhibited HIV-1 protease. The most potent compounds, **2** and **7**, had IC_{50}-values below 20 μM, while compounds **1**, **2**, **4**, **6**, and **7** had IC_{50}-values less than 100 μM.

3. Discussion

The results show that most of the crude extracts had some protease inhibitory effect. The inhibition was specific for the different proteases as none of the extracts inhibited all the three enzymes. However, some extracts inhibited both HIV-1 protease and HCMV protease. These enzymes are not structurally related, and have completely different active site architectures and catalytic mechanisms. It is therefore unlikely that inhibition of the two proteases is caused by the same compound in the extracts. This hypothesis was confirmed by studying the inhibitory potencies of the active constituents in *Kaempferia parviflora.* Six of the isolated compounds inhibited HIV-1 protease but not the other two proteases. The most potent compounds inhibited HIV protease with IC_{50}-values of 20 μM. Under the present conditions, and assuming a K_m of 4.2 μM (Ahlsén et al. 2002), this corresponds to a K_i-value of 16 μM.

The inhibition data for the compounds isolated from *Kaempferia parviflora* revealed that the degree of substitution at positions 3, 3′, 4′ and 5 of the flavone was critical for the HIV-1 protease inhibition; increased substitution correlated with decreased inhibitory effect. For example, the presence of a methoxy group at the 5-position of the flavone nucleus reduced the HIV-1 protease inhibitory potency. In contrast, the presence of a methoxy group at the 3-position of the flavone moiety had a minor effect on the inhibition (compare compounds **1** and **6**). The methoxy group at the 4′-position reduced the inhibitory effect slightly (compare compounds **4** and **8**). Compounds with two or three methoxy groups showed the lowest inhibitory effect, and the group was most acceptable when in the 3-position (compound **6**).

The use of plants as sources for antiviral agents has been explored previously (Jassim and Naji 2003). HIV protease has been shown to be inhibited by aqueous extracts of Chinese herbs (Collins et al. 1997), flavonoids (Xu et al. 2000), flavones (Brinkworth et al. 1992) and phenolics (Hatano et al. 1988). Also truncated variants of HCV protease have been shown to be inhibited by plant constituents (Hegde et al. 2003; Hussein et al. 2000), but we have not found any examples in the literature of plant extracts that inhibit HCMV protease.

HIV protease and other aspartic proteases are typically inhibited by pepstatin, a transition state analogue inhibitor that was originally isolated from Actinomycetes (Umezawa et al. 1970). Pepstatin has a K_i-value of 17 nM for HIV-1 protease (Matayoshi et al. 1990) and it has been important as an early lead compound for the development of more potent inhibitors against HIV protease. However, resistance rapidly develops towards all anti-AIDS drugs based on this type of peptidic structure. The observation that flavonoids inhibit HIV protease is significant from the perspective that they represent a different structural class of compound, essential for a new strategy for development of anti-AIDS drugs against resistant virus.

Although the flavones identified in this study have previously been described (Jaipetch et al. 1983; Vidari et al. 1971; Silva and Mundaca 1971; Franca et al. 1976; Yenjai et al. 2004), they have not been shown to be inhibitors of viral proteases. Furthermore, the flavonoids that have been described as inhibitors of HIV-1 protease (Xu et al. 2000) were different from those identified in this study. Their IC_{50}-value for quercetin (60 μM) is of the same order as we found for the current flavones, indicating that this class of compounds typically results in moderate inhibitory effects. However, this effect was sufficient for warfarin (IC_{50}-value = 30 μM), the early lead used in the discovery of tipranavir, a compound developed from warfarin and other coumarins and pyrones (Thaisrivongs and Strobach 1999). The discovery of tipranavir illustrates the potential of a flavonoid-based approach.

In conclusion, as *Kaempferia parviflora* provided novel and specific inhibitors of HIV-1 protease, *Zingiberaceae* species have a great potential as sources for novel lead compounds with specific antiviral properties. Further bioassay guided separation and isolation of the active compounds from several other herbs is therefore of interest, the active constituents in *Alpinia galanga* are already in the process of being determined. Finally, it is also of interest to determine if the inhibitory effects of the extracts are due to a combined effect of several weak inhibitors or the sole effect of a highly potent compound. Such studies require more extensive kinetic studies using the pure compounds.

4. Experimetal

4.1. Plant material

Plants were purchased from the local herbal market in Bangkok, Thailand in May 2003; *Kaempferia galanga* Linn., *Curcuma zedoaria* Roscoe, *Curcuma longa* Linn., *Kaempferia parviflora* Wall, *Boesenbergia pandurata* Holtt, *Zingiber zerumbet* Smith, *Zingiber officinale* Roscoe and *Alpinia galanga* Sw.. Voucher specimens for *K. galanga* (BKF 102680), *C. zedoaria* (BKF 128776), *C. longa* (BKF 090655), *K. parviflora* (BKF

73995), *B. pandurata* (H.B. 006041), *Z. zerumbet* (BKF 128776), *Z. officinale* (BKF 118527) and *A. galanga* (BKF 47101) have been deposited at the Forest Herbarium (BKF), Royal Forest Department, Ministry of Agriculture and the Botanical Herbarium (H.B.), Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The specimens were botanically identified by Associate Prof. Chaiyo Chaichantipyuth, Department of Pharmacognosy, Faculty of Pharmacy, Chulalongkorn University, Bangkok, Thailand.

4.2. Preparation of extracts

The methanol and aqueous extracts were prepared by cutting the rhizome (2 g) into small pieces and extraction of these either overnight with 10 ml of methanol or by boiling with 10 ml distilled water under reflux for 2 h (Lam et al. 2000). Insoluble debris was removed by filtration. The water extract was lyophilized and the methanol extract dried by evaporation. The dried extracts were subsequently stored at −20 °C.

4.3. Isolation of pure compounds from Kaempferia parviflora

For the isolation of pure compounds from *Kaempferia parviflora,* extraction was performed with a larger amount of starting material. Consequently, 5 kg of the fresh black rhizomes of *Kaempferia parviflora* were cut and crushed. After extraction with methanol (5 l, 2 times) at room temperature, the filtrate was dried by evaporation under reduced pressure. The filtrate was extracted with hexane and subsequently evaporated, giving the hexane extract as a yellow viscous oil (0.50 g). The residue of the previous extraction was repeatedly extracted with ethyl acetate. The ethyl acetate extracts were combined and concentrated under reduced pressure to give the ethyl acetate extract as a mixture of a white solid and a yellow oil (5.15 g). The final residue (insoluble in both hexane and ethyl acetate) was evaporated, giving a brown-violet liquid (49.2 g). TLC analysis indicated that the hexane and ethyl acetate crude extracts had similar chemical compositions, the two extracts were therefore combined and subjected to silica gel column chromatography (Merck 70–230 mesh, 230–400 mesh, ASTM) using a hexane/ethyl acetate gradient to yield the pure compounds.

4.4. Structure determination

Melting points were determined with a Fisher-Johns melting point apparatus. The FT-IR spectra were recorded on a Nicolet Impact 410 spectrophotometer. The ^{1}H and ^{13}C NMR Spectra were recorded on a Varian Model Mercury 400 MHz at 400 and 100 MHz, respectively, in deuterated chloroform ($CDCl_3$). The mass spectra were acquired by a Bruker Instruments Mass Spectrometer Model Trio 2000 in MALDI-TOF mode.

(**1**) 5-Hydroxy-3,7-dimethoxyflavone (76 mg, 1.4%) (Yenjai et al. 2004): Recrystallization of compound **1** from $CHCl_3$ gave yellow needle crystals, m.p. 129–130 °C IR ν_{max} 3000, 1661, 1594. UV ETOH λ_{max} (log ε): 268 (4.38), 330 (4.10). ^{1}H NMR ($CDCl_3$): δ 3.91 (3 H, s, $OC\underline{H}_3$), 3.92 (3 H, s, $OC\underline{H}_3$), 6.41 (1 H, s, Ar$\underline{H}$), 6.50 (1 H, s, Ar$\underline{H}$), 7.56 (3 H, s, 3 × Ar$\underline{H}$), 8.11 (2 H, s, 2 × Ar$\underline{H}$), 12.56 (1 H, s, O$\underline{H}$). MALDI-TOF MS m/z: 299.21 $[M + H]^+$.

(**2**) 5-Hydroxy-7-methoxyflavone (37 mg, 0.7%) (Jaipetch et al. 1983): Compound **2** was purified by recrystallization from $CHCl_3$ to give yellow needle crystal, m.p. 170–171 °C (lit. 169–1670 °C). IR ν_{max} 3000, 1665, 1606. UV λ_{max}^{EtOH} nm (log ε): 270 (4.15), 336 (3.96). ^{1}H NMR ($CDCl_3$): δ 3.92 (3 H, s, $OC\underline{H}_3$), 6.42 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 6.54 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 6.71 (1 H, s, C=C$\underline{H}$), 7.57 (3 H, m, 3 × Ar$\underline{H}$), 7.92 (2 H, m, 2 × Ar$\underline{H}$), 12.77 (1 H, s, O$\underline{H}$). MALDI-TOF MS m/z: 269.45 $[M + H]^+$.

(**3**) 5-Hydroxy-3,7,4′-trimethoxyflavone (184 mg, 3.5%) (Jaipetch et al. 1983): Purification of compound **3** by recrystallization in $CHCl_3$ to give a yellow needle crystal, m.p. 146–148 °C. IR ν_{max} 3000, 1661, 1587. UV λ_{max}^{EtOH} nm (log ε): 270 (4.01), 346 (3.82). ^{1}H NMR ($CDCl_3$): δ 3.89 (3 H, s, $OC\underline{H}_3$), 3.91 (3 H, s, $OC\underline{H}_3$), 3.94 (3 H, s, $OC\underline{H}_3$), 6.40 (1 H, s, Ar$\underline{H}$), 6.49 (1 H, s, Ar$\underline{H}$), 7.06 (2 H, d, J = 9 Hz, 2 × Ar$\underline{H}$), 8.12 (2 H, d, J = 9 Hz, 2 × Ar$\underline{H}$), 12.70 (1 H, s, O$\underline{H}$). MALDI-TOF MS m/z: 329.48 $[M + H]^+$.

(**4**) 5-Hydroxy-7,4′-dimethoxyflavone (56 mg, 1.1%) (Vidari et al. 1971): Compound **4** was purified by recrystallization from $CHCl_3$ to give yellow needle crystal, m.p. 175–176 °C (lit. 177–179 °C). IR ν_{max} 3000, 1665, 1602. UV λ_{max}^{EtOH} nm (log ε): 250 (3.96), 322 (3.69). ^{1}H NMR ($CDCl_3$): δ 3.92 (3 H, s, $OC\underline{H}_3$), 3.93 (3 H, s, $OC\underline{H}_3$), 6.40 (1 H, s, Ar$\underline{H}$), 6.52 (1 H, s, Ar$\underline{H}$), 6.62 (1 H, s, C=C$\underline{H}$), 7.05 (2 H, d, J = 8 Hz, 2 × Ar$\underline{H}$), 7.88 (2 H, d, J = 8 Hz, 2 × Ar$\underline{H}$), 12.86 (1 H, s, O$\underline{H}$). MALDI-TOF MS m/z: 299.61 $[M + H]^+$.

(**5**) 5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (4 mg, 0.1%) (Silva and Mundaca 1971): Purification of compound **5** by recrystallization in $CHCl_3$ to give a yellow needle crystal, m.p. 158–160 °C (lit. 160–162 °C). IR ν_{max} 3000, 1649, 1587. UV λ_{max}^{EtOH} nm (log ε): 260 (4.11), 322 (3.72). ^{1}H NMR ($CDCl_3$): δ 3.89 (3 H, s, $OC\underline{H}_3$), 3.92 (3 H, s, $OC\underline{H}_3$), 4.00 (3 H, s, $OC\underline{H}_3$), 4.01 (3 H, s, $OC\underline{H}_3$), 6.39 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 6.49 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 7.03 (1H, d, J = 2 Hz, Ar$\underline{H}$), 7.73 (1H, d, J = 2 Hz, Ar$\underline{H}$), 7.77 (1 H, dd, J = 2,8 Hz, Ar$\underline{H}$), 12.68 (1 H, s, O$\underline{H}$). MALDI-TOF MS m/z: 359.59 $[M + H]^+$.

(**6**) 3,5,7-Trimethoxyflavone (46 mg, 0.9%) (Franca et al. 1976): Recrystallization of compound **6** from $CHCl_3$ gave colorless needle crystal, m.p. 200–202 °C (lit. 204–206 °C). IR ν_{max} 3000, 1661, 1594. UV λ_{max}^{EtOH} nm (log ε): 262 (4.45), 322 (4.06). ^{1}H NMR ($CDCl_3$): δ 3.92 (3 H, s, $OC\underline{H}_3$), 3.93 (3 H, s, $OC\underline{H}_3$), 4.00 (3 H, s, $OC\underline{H}_3$),6.38 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 6.55 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 7.52 (3 H, m, 3 × Ar$\underline{H}$), 8.10 (2 H, m, 2 × Ar$\underline{H}$). MALDI-TOF MS m/z: 313.23 $[M + H]^+$.

(**7**) 5,7-Dimethoxyflavone (208 mg, 4.0%) (Yenjai et al. 2004): Compound **7** was purified by recrystallization from $CHCl_3$ to give colorless needle crystal, m.p. 149–151 °C (lit. 149–150 °C). IR ν_{max} 3000, 1665, 1602. UV λ_{max}^{EtOH} nm (log ε): 262 (4.35), 302 (3.76). ^{1}H NMR ($CDCl_3$): δ 3.95 (3 H, s, $OC\underline{H}_3$), 3.99 (3 H, s, $OC\underline{H}_3$), 6.41 (1 H, s, Ar$\underline{H}$), 6.60 (1 H, s, Ar$\underline{H}$), 6.72 (1 H, s, C=C$\underline{H}$), 7.53 (3 H, m, 3 × Ar$\underline{H}$), 7.91 (2 H, m, 2 × Ar$\underline{H}$). MALDI-TOF MS m/z: 283.06 $[M + H]^+$.

(**8**) 5,7,4′-Trimethoxyflavone (306 mg, 5.88%) (Yenjai et al. 2004): Purification of compound **8** by recrystallization in $CHCl_3$ to give white powder, m.p. 160–162 °C (lit. 159–161 °C). IR ν_{max} 1658, 1596, 1504, 1435. UV λ_{max}^{EtOH} nm (log ε): 266 (4.29), 318 (3.86). ^{1}H NMR ($CDCl_3$): δ 3.92 (3 H, s, $OC\underline{H}_3$), 3.94 (3 H, s, $OC\underline{H}_3$), 3.99 (3 H, s, $OC\underline{H}_3$), 6.40 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 6.59 (1 H, d, J = 2 Hz, Ar$\underline{H}$), 6.63 (1 H, s, C=C$\underline{H}$), 7.03 (2 H, d, J = 9 Hz, 2 × Ar$\underline{H}$), 7.85 (2 H, d, J = 9 Hz, 2 × Ar$\underline{H}$). MALDI-TOF MS m/z: 313.21 $[M + H]^+$.

4.5. HIV-1 protease activity assay

HIV-1 protease was expressed, purified and the inhibition measured with a fluorometric method as previously published (Danielson et al. 1998; Nillroth et al. 1997). Inhibitors and the fluorogenic substrate Dabcyl-γ-Abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Edans (Bachem, Bubendorf, Switzerland) were dissolved in DMSO before being used. Measurements were performed in 100 mM sodium acetate, 1 mM EDTA, 1 M NaCl, pH 4.7 and 3% dimethyl sulfoxide (DMSO) (v/v) (accounting for DMSO added with inhibitors and substrate) in a final volume of 300 µl. The enzyme was prediluted in buffer containing 1 mg/ml bovine serum albumin, and the reaction was initiated by addition of enzyme. The final concentrations of substrate and enzyme were 5.2 µM and 7.5 nM, respectively. The increase in fluorescence was monitored over a period of 5 min at 30 °C using λ_{exc} = 355 nm, and λ_{em} = 500 nm.

The IC_{50} values of isolated compounds targeting the HIV-1 protease were determined by varying the tested compounds concentrations between 10–100 µM for compounds **1**–**2**, **4**–**7** and 80–150 µM for compounds **3**, **8**.

4.6. HCV protease assay

Full-length HCV NS3 protease was expressed, purified and the inhibition measured as previously published (Poliakov et al. 2002). Briefly, the hydrolysis of a depsipeptide substrate, Ac-DED(Edans)EEAbuψ(COO)ASK(Dabcyl)-NH_2 (AnaSpec, San Jose, CA, USA) was measured continuously over time in a fluorescence plate reader (Fluoroskan, Ascent Labsystems, Stockholm, Sweden) using λ_{exc} = 355 nm, and λ_{em} = 500 nm. The enzyme was incubated in 50 mM HEPES pH 7.5, 10 mM DTT, 40% glycerol, 0.1% n-octyl-β-D-glucoside, 3.3% DMSO with 25 µM cofactor (KKGSVVIVGRIVLSGK) and inhibitor at 30 °C for 10 min, the reaction was initiated by addition of substrate. Inhibition measurements were performed with 1 nM enzyme, 0.5 µM substrate, and a final concentration of 3.3% DMSO (final volume 300 µl). Inhibitors were dissolved in DMSO, a mixture of DMSO and assay buffer, or assay buffer alone, sonicated for 30 s and vortexed. The solutions were stored at −20 °C between measurements.

The IC_{50} value of compound **1** was determined by varying the concentration from 170–220 µM.

4.7 HCMV protease assay

HCMV protease was expressed, purified and the activity measured essentially as previously published (Geitmann and Danielson 2004), (Holskin et al. 1995). Enzymatic assays were performed in 50 mM TES, pH 7.6, 0.1 mM EDTA, 25% glycerol and 3.3% DMSO (v/v) in a final volume of 300 µl. Inhibitors and the fluorogenic substrate Dabcyl-Arg-Gly-Val-Val-Asn-Ala-Ser-Ser-Arg-Leu-Ala-Edans (Bachem, Bubendorf, Switzerland) were dissolved in DMSO before use. The reaction was initiated by addition of enzyme (pre-diluted in buffer containing 1 mg/ml bovine serum albumin). The final concentration of substrate and enzyme were 3 µM and 120 nM monomer, respectively. The increase in fluorescence (λ_{exc} = 355 nm, and λ_{em} = 500 nm) was monitored over a period of 5 min at 30 °C in assay buffer.

The IC_{50} value of compound **1** was determined by varying the concentration from 220–270 µM, and for compound **2** for a corresponding range spanning the IC_{50}-value was used.

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References

Ahlsén G, Hultén J, Shuman CF, Poliakov A, Lindgren MT, Alterman M, Samuelsson B, Hallberg A, Danielson UH (2002) Resistance profiles of cyclic sulfamide inhibitors of HIV-1 protease. Antivir Chem Chemother 13: 27–37.

Brinkworth RI, Stoermer MJ, Fairlie DP (1992) Flavones are inhibitors of HIV-1 proteinase. Biochem Biophys Res Commun 188: 631–637.

Collins RA, Ng TB, Fong WP, Wan CC, Yeung HW (1997) A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs. Life Sci 60: PL345–351.

Danielson UH, Lindgren MT, Markgren P-O, Nillroth U (1998) Investigation of an allosteric site of HIV-1 proteinase involved in inhibition by Cu^{2+}. Adv Exp Med Biol 436: 99–103.

Franca NC, Gottlieb OR, Magalhaes MT, Mendes PH, Maia JGS, Da Silva ML et al. (1976) Tri-*O*-methylgalangin from *Aniba riparia*. Phytochemistry 15: 572–573.

Geitmann M, Danielson UH (2004) Detection of conformational change in the human cytomegalovirus protease upon substrate binding using optical biosensor technology. Anal Biochem 332: 203–214.

Hatano T, Yasuhara T, Miyamoto K, Okuda T (1988) Anti-human immunodeficiency virus phenolics from licorice. Chem Pharm Bull (Tokyo) 36: 2286–2288.

Hegde VR, Pu H, Patel M, Das PR, Butkiewicz N, Arreaza G, Gullo VP, Chan TM (2003) Two antiviral compounds from the plant *Stylogne cauliflora* as inhibitors of HCV NS3 protease. Bioorg Med Chem Lett 13: 2925–2928.

Holskin BP, Bukhtiyarova M, Dunn BM, Baur P, De Chastonay J, Pennington MW (1995) A continuous fluorescence-based assay of human cytomegalovirus protease using a peptide substrate. Anal Biochem 227: 148–155.

Hong CH, Kim Y, Lee SK (2001) Sesquiterpenoids from the rhizome of *Curcuma zedoaria*. Arch Pharm Res 24: 424–426.

Hussein G, Miyashiro H, Nakamura N, Hattori M, Kakiuchi N, Shimotohno K (2000) Inhibitory effects of sudanese medicinal plant extracts on hepatitis C virus (HCV) protease. Phytother Res 14: 510–516.

Lam TL, Lam ML, Au TK, Ip DT, Ng TB, Fong WP, Wan DC (2000) A comparison of human immunodeficiency virus type-1 protease inhibition activities by the aqueous and methanol extracts of Chinese medicinal herbs. Life Sci 67: 2889–2896.

Jaipetch T, Reutrakul V, Tuntiwachwuttikul P, Santisuk T (1983) Flavonoids in the black rhizomes of *Boesenbergia panduta*. Phytochemistry 22: 625–626.

Jassim SA, Naji MA (2003) Novel antiviral agents: a medicinal plant perspective. J Appl Microbiol 95: 412–427.

Lechtenberg M, Quandt B, Nahrstedt A (2004) Quantitative determination of curcuminoids in Curcuma rhizomes and rapid differentiation of *Curcuma domestica* Val. and *Curcuma xanthorrhiza* Roxb. by capillary electrophoresis. Phytochem Anal 15: 152–158.

Matayoshi ED, Wang GT, Krafft GA, Erickson J (1990) Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. Science 247: 954–958.

Newman DJ, Cragg GM, Snader KM (2003) Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 66: 1022–1037.

Nillroth U, Vrang L, Markgren P-O, Hultén J, Hallberg A, Danielson UH (1997) Human immunodeficiency virus type 1 proteinase resistance to symmetric cyclic urea inhibitor analogs. Antivir Chem Chemother 41: 2383–2388.

Poliakov A, Hubatsch I, Shuman CF, Stenberg G, Danielson UH (2002) Expression and purification of recombinant full-length NS3 protease-helicase from a new variant of hepatitis C virus. Protein Expr Purif 25: 363–371.

Silva M, Mundaca JM (1971) Flavonoid and triterpene constituents of *Baccharis rhomboidalis*. Phytochemistry 10: 1942–1943.

Sirirugsa P (1998) Thai Zingiberaceae: Species diversity and their uses. Pure Appl Chem 70: 2111–2118. (http://www.iupac.org/symposia/proceedings/phuket97/sirirugsa.html)

Thaisrivongs S, Strohbach JW (1999) Structure-based discovery of Tipranavir disodium (PNU-140690E): a potent, orally bioavailable, nonpeptidic HIV protease inhibitor. Biopolymers 51: 51–58.

Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M (1970) Pepstatin, a new pepsin inhibitor produced by Actinomycetes. J Antibiot (Tokyo) 23: 259–262.

Vidari G, Finza PV, Bernardi MD (1971) Flavonols and quinones in stems of *Aframomum giganteum.* Phytochemistry 10: 3335–3339.

Yenjai C, Prasanphen K, Daodee S, Wongpanich V, Kittakoop P (2004) Bioactive flavonoids from *Kaempferia parviflora*. Fitoterapia 75: 89–92.

Xu HX, Wan M, Dong H, But PP, Foo LY (2000) Inhibitory activity of flavonoids and tannins against HIV-1 protease. Biol Pharm Bull 23: 1072–1076.