Journal of Chemistry, Vol. 47 (3), P. 388 - 392, 2009

A FATTY ACID, FLAVONOIDS, AND STEROIDS FROM *ZINGIBER PENISULARE* L. THEILADE (ZINGIBERACEAE)

Received 9 October 2008

TRUONG THI TO CHINH[1,2], PHAN MINH GIANG[1], PHAN TONG SON[1]

[1] *Faculty of Chemistry, College of Natural Science, Vietnam National University, Hanoi*

[2] *Vietnam Institute of Industrial Chemistry, Hanoi, Vietnam*

ABSTRACT

*The first chemical study of Zingiber penisulare I. Theilade (Zingiberaceae) resulted in the isolation of a fatty acid, eicosanoic acid (**1**), three kaempferol derivatives, 5-hydroxy-3,4',7-trimethoxyflavone (**2**), 4',5-dihydroxy-7-methoxyflavonol (**5**), and 4',5-dihydroxy-3,7-dimethoxyflavone (**6**), β-sitosterol (**3**), and 6β-hydroxystigmast-4-ene-3-one (**4**). Their chemical structures were determined by spectroscopic analyses.*

Keywords: *Zingiber penisulare*; Zingiberaceae; kaempferol; flavonol; stigmastane

I - INTRODUCTION

In the recent studies we showed the chemical profiles of volatile oils, phytosterols, and esters of glycerol and fatty acids of some *Zingiber* species (Zingiberaceae) [1, 2]. However, the analyses also indicated the complexity in separation of the minor constituents of the rhizomes of these *Zingiber* species. Therefore, the development of isolation procedures for other *Zingiber* species may be a challenging task for researchers. In the present study on *Zingiber penisulare* I. Theilade (Zingiberaceae) we developed a systematic chromatographic fractionation of the organic extracts based on polarity and the selection of suitable purification techniques resulted in the isolation of a fatty acid **1**, three kaempferol derivatives **2**, **5**, and **6**, and two sterols **3** and **4** from the *n*-hexane-soluble fraction of a MeOH extract from the rhizomes of *Z. penisulare* (Fig. 1).

II - EXPERIMENTAL

General Procedure

Melting points were determined on a Boetius melting point apparatus and are uncorrected. IR spectra were recorded on an Impact-410 Nicolet FT-IR spectrophotometer. EI-MS (70 eV) spectra were measured on a Hewlett-Packard 5989 B mass spectrometer. ^{1}H-(500 MHz) and ^{13}C-NMR (125 MHz) spectra with DEPT program were recorded on a Bruker Avance 500 NMR spectrometer. Tetramethyl silane (TMS) was used as zero internal standard. Silica gel 60 (63 - 200 μm and 63 - 100 μm, Merck, Darmstadt, Germany) was used for open column (CC) and silica gel 60 (15 - 40 and 40-63 μm, Merck, Darmstadt, Germany) for flash column (FC) chromatography. TLC was performed on precoated DC Alufolien 60 F_{254} sheets (Merck, Darmstadt, Germany) and detected by UV light (λ 254 nm) or by spraying with 1% vanillin in conc. H_2SO_4 followed by heating on a hot plate.

Plant Material

The fresh rhizomes of *Z. penisulare* were collected in Sơ Pai, Kbang, province Gia Lai, Vietnam in June 2007 and were identified by Mr. Nguyen Quoc Binh, a botanist of the Institute of Biological Resources and Ecology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

Extraction and Isolation

The fresh rhizomes (18 kg) were washed, drained, sliced, then dried in shadow, and oven-dried at 40ºC. The dried material was powdered (2 kg) and extracted with MeOH by percolation (7 times) at room temperature. The MeOH extracts were filtered; the filtrates were combined and concentrated under reduced pressure. The resultant MeOH extract was suspended in H_2O (300 ml) and partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. Removal of extraction solvents gave *n*-hexane- (**ZPH**, 35.8 g, extraction yield 1.79% on the basis of the dried material), CH_2Cl_2- (**ZPD**, 4 g, 0.2%), EtOAc- (**ZPE**, 5.9 g, 0.29%), and *n*-BuOH- (**ZPB**, 16.4 g, 0.82%) soluble fractions.

The *n*-hexane-soluble fraction (35 g) was adsorbed on silica gel of the particle size 63-200 μm (50 g) then was applied to silica gel CC (350 g) using *n*-hexane and gradient *n*-hexane-EtOAc (29:1, 9:1, 7:1, 4:1, 2:1, 1:1, 1:2, and EtOAc) solvent systems. Fourteen pooled fractions were collected on the basis of the TLC analysis (**ZPH0→ZPHXIII**). The polarities of the pooled fractions were as follows: *n*-hexane (**ZPH0**), gradient *n*-hexane-EtOAc 29:1 (**ZPHI**, **II**), 9:1 (**ZPHII**, **III**, **IV**, **V**), 7:1 (**ZPHVI**, **VII**), 4:1 (**ZPHVII**, **VIII**), 2:1 (**ZPHIX**, **X**), 1:1 (**ZPHX**, **XI**, **XII**), 1:2 (**ZPHXII**), and EtOAc (**ZPHXIII**). Pooled fraction **ZPHII** (2.49 g) was chromatographed on silica gel (CC), eluting with gradient *n*-hexane-EtOAc solvent systems (70:1, 49:1, 29:1, 19:1, and 9:1) to afford five fractions; fraction 2 (**ZPHII.2**) was washed with *n*-hexane to afford **1** (3 mg). Pooled fraction **ZPHIII** (1.41 g) was separated by silica gel CC (gradient *n*-hexane-EtOAc 15:1, 9:1, 7:1, 4:1, and 2:1) to give four fractions. Fraction 2 (**ZPHIII.2**, 0.5 g) was purified by silica gel FC (*n*-hexane-EtOAc 70:1), and the crystalls obtained were washed with *n*-hexane to afford **1** (25 mg). Fraction 3 (**ZPHIII.3**) was washed with *n*-hexane to yield **2** (97.3 mg). Pooled fraction **ZPHIV** (2.08 g) was separated on silica gel CC (gradient *n*-hexane-EtOAc 15:1, 9:1, 7:1, 4:1, and 2:1) to afford five fractions. From fractions **ZPHIV.2** (0.24 g), **ZPHIV.3** (0.84 g), **ZPHIV.4** (0.8 g) **2** was isolated as the main compound. Further purification of fraction **ZPHIV.5** (0.31 g) by washing with *n*-hexane afforded **3** (283.5 mg). Separation of the pooled fraction **ZPHVIII** (1.38 g) on silica gel CC (63-100 □m), eluted with gradient *n*-hexane-EtOAc solvent system (9:1, 4:1, and 2:1) gave three fractions. Fraction **ZPHVIII.2** was washed with *n*-hexane then purified by silica gel FC (*n*-hexane-EtOAc 9:1) to yield **4** (7 mg). Fraction **ZPHVIII.3** was purified by similar methods as those of **ZPHVIII.2** (FC, *n*-hexane-EtOAc 4:1) to give a mixture of **5** and **6** (6.5 mg). Pooled fraction **ZPHIX** (1.63 g) was chromatographed on silica gel (CC) using gradient *n*-hexane-EtOAc solvent system (2:1, 1:1, and 1:2) to give four fractions. Fraction **ZPHIX.3** was purified by FC (*n*-hexane-EtOAc 7:1 and 4:1) to yield a mixture of **5** and **6** (3 mg).

Eicosanoic acid (1): White amorphous powder. R_f 0.36 (*n*-hexane-EtOAc 7:1). EI-MS: *m/z* 312 ($C_{20}H_{40}O_2$, $M^{+\cdot}$). ^{1}H-NMR ($CDCl_3$): δ 0.88 (3H, t, *J* = 6.5 Hz, H_3-20), 1.26 (32H, br s, H, 2H-4→2H-19), 1.64 (2H, quintet, *J* = 7.5 Hz, 2H-3), 2.34 (2H, t, *J* = 7.5 Hz, 2H-2). ^{13}C-NMR ($CDCl_3$): δ 14.5 (q, C-20), 23.1, 25.1, 29.5, 29.6, 29.7, 29.8, 29.9, 30.1 (all t, C-3→C-19), 32.3 (t, C-2), 179.9 (s, C-1).

5-Hydroxy-3,4',7-trimethoxyflavone (2): Yellow needles, m.p 134-136ºC (Lit. [3]: 145 - 147ºC). R_f 0.37 (*n*-hexane-EtOAc 4:1). EI-MS: *m/z* 328 ($C_{18}H_{16}O_6$, $M^{+\cdot}$, 100), 327 (75.8), 285 (58.6), 167 (13.2), 166 (5.9), 138 (9.9), 135 (57.3), 107 (17.9). ^{1}H-NMR ($CDCl_3$): δ 3.86 (3H, s, 4'-OCH_3), 3.87 (3H, s, 7-OCH_3), 3.89 (3H, s, 3-OCH_3), 6.35 (1H, d, *J* = 2.0 Hz, H-8), 6.44 (1H, d, *J* = 2.0 Hz, H-6), 7.02 (2H, d, *J* = 9.0 Hz, H-3′, H-5′), 8.07 (2H, dd, *J* = 9.0 Hz, H-

2′, H-6′), 12.6 (1H, s, 5-OH). ^{13}C-NMR/DEPT ($CDCl_3$): δ55.4 (q, 4'-OCH_3), 55.8 (q, 7-OCH_3), 60.2 (q, 3-OCH_3), 92.2 (d, C-8), 97.8 (d, C-6), 106.1 (s, C-10), 114.1 (d, C-3′, C-5′), 122.9 (s, C-1′), 130.2 (d, C-2′, C-6′), 138.9 (s, C-3), 155.9 (s, C-2), 156.8 (s, C-9), 161.7 (s, C-4′), 162.1 (s, C-5), 165.4 (s, C-7), 178.8 (s, C-4).

β-Sitosterol (3): White rods, m.p. 134-135ºC. R_f 0.34 (*n*-hexane-EtOAc 4:1). IR (KBr): ν_{max} cm^{-1} 3426, 2945, 2873, 1639, 1461, 1378, 1237, 1061, 960, 803.

6β-Hydroxystigmast-4-ene-3-one (4): Yellow needles, m.p. 210 - 211ºC (Lit. [3]: 213-215ºC). R_f 0.37 (*n*-hexane-EtOAc 4:1). EI-MS: *m/z* 428 ($C_{29}H_{48}O_2$, $M^{+\cdot}$, 100), 413 (54), 410 (30.7), 287 (29.3), 269 (36.3), 245 (32.7), 227 (45.3), 152 (99). ^{1}H-NMR ($CDCl_3$): δ 0.74 (3H, s, H_3-18), 0.81 (3H, d, *J* = 7.0 Hz, H_3-26), 0.84 (3H, d, *J* = 6.5 Hz, H_3-27), 0.85 (3H, t, *J* = 7.5 Hz, H_3-29), 0.92 (3H, d, *J* = 6.5 Hz, H_3-21), 1.38 (3H, s, H_3-19), 2.38 (1H, dt, *J* = 17.0 Hz, 7.0 Hz, H-2a), 2.51 (1H, ddd, *J* = 15.0 Hz, 12.5 Hz, 5.0 Hz, H-2b), 4.35 (1H, br s, H-6), 5.81 (1H, br s, H-4). ^{13}C-NMR/DEPT ($CDCl_3$): δ 11.9 (q, C-18), 12.0 (q, C-29), 18.7 (q, C-21), 19.0 (q, C-26), 19.5 (q, C-27), 19.8 (q, C-19, 20.9 (t, C-11), 23.1 (t, C-28), 24.2 (t, C-15), 26.1 (t, C-23), 28.2 (t, C-16), 29.2 (d, C-25), 29.7 (d, C-8), 33.9 (t, C-22), 34.3 (t, C-2), 36.1 (d, C-20), 37.1 (t, C-1), 38.0 (s, C-10), 38.6 (t, C-7), 39.6 (t, C-12), 42.5 (s, C-13), 45.9 (d, C-24), 53.7 (d, C-9), 55.9 (d, C-17), 56.1 (d, C-14), 73.3 (d, C-6), 126.3 (d, C-4), 168.6 (s, C-5), 200.2 (s, C-3).

Mixture of 4',5-dihydroxy-7-methoxyflavonol (5) and 4′,5-dihydroxy-3,7-dimethoxyflavone (6): Yellow amorphous powder. R_f 0.35 (*n*-hexane-EtOAc 3:1).

4',5-Dihydroxy-7-methoxyflavonol (5): EI-MS: *m/z* 300 ($C_{16}H_{12}O_6$, $M^{+\cdot}$). ^{1}H-NMR ($CDCl_3$+CD_3OD): δ 3.80 (3H, s, 7-OCH_3), 6.26 (1H, d, *J* = 2.0 Hz, H-8), 6.40 (1H, d, *J* = 2.0 Hz, H-6), 6.86 (2H, d, *J* = 9.0 Hz, H-3′, H-5′), 8.01 (2H, dd, *J* = 9.0 Hz, H-2′, H-6').

4',5-Dihydroxy-3,7-dimethoxyflavone (6): EI-MS: *m/z* 314 ($C_{17}H_{14}O_6$, $M^{+\cdot}$). ^{1}H-NMR ($CDCl_3$+CD_3OD): δ 3.72 (3H, s, 3-OCH_3), 3.79 (3H, s, 7-OCH_3), 6.26 (1H, br s, H-8), 6.38 (1H, d, *J* = 2.0 Hz, H-6), 6.86 (2H, d, *J* = 9.0 Hz, H-3′, H-5′), 7.91 (2H, dd, *J* = 9.0 Hz, H-2′, H-6′). ^{13}C-NMR/DEPT ($CDCl_3$+CD_3OD): δ 55.8 (q, 7-OCH_3), 60.0 (q, 3-OCH_3), 92.2 (d, C-8), 97.9 (d, C-6), 105.8 (s, C-10), 115.6 (d, C-3′, C-5′), 121.5 (s, C-1′), 130.3 (d, C-2′, C-6′), 138.6 (s, C-3), 155.0 (s, C-2), 156.8 (s, C-9), 160.0 (s, C-4′), 161.5 (s, C-5), 165.5 (s, C-7), 178.8 (s, C-4).

III - RESULTS AND DISCUSSION

The MeOH extract of the dried rhizomes of *Z. penisulare* was prepared by percolation at room temperature and subjected to successive partition between H_2O and *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. The *n*-hexane-soluble fraction (**ZPH**, extraction yield 1.79% of the dried plant material) was polarity-fractionated by gradient *n*-hexane-EtOAc solvent systems. Fourteen pooled fractions were collected on the basis of TLC analyses and polarities of the solvent systems. Further chromatography on silica gel (CC and FC) led to the isolation of **1** and **2** from the pooled fractions eluted with *n*-hexane-EtOAc 29:1 and 9:1, **2** and **3** from the pooled fraction eluted with *n*-hexane-EtOAc 9:1, **4** from the pooled fraction eluted with *n*-hexane-EtOAc 4:1, and **5** and **6** from the pooled fraction eluted with *n*-hexane-EtOAc 2:1.

Compound **1** was isolated as a white amorphous powder. The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited the presence of a fatty acid [δ_H 0.88 (3H, t, *J* = 6.5 Hz), 1.26 (32H, br s), 1.64 (2H, quintet, *J* = 7.5 Hz) and 2.34 (2H, t, *J* = 7.5 Hz); δ_C 14.5 (q), 23.1, 25.1, 29.5, 29.6, 29.7, 29.8, 29.9, 30.1, 32.3 (all t), and 179.9 (s)]. The molecular formula of **1** was deduced from the EI-MS spectrum and ^{1}H-NMR data of **1** to be $C_{20}H_{40}O_2$ (*m/z* 312, $M^{+\cdot}$). Therefore, compound **1** was determined to be eicosanoic acid (arachic acid). **1** is a constituent of *Arachis hypogaea* and widely distributed in other seed oils [3].

Compound **2** was isolated as yellow needles, m.p. 134 - 136ºC. The EI-MS spectrum of **2** (*m/z*

328, $M^{+\cdot}$) and NMR data determined the molecular formula $C_{18}H_{16}O_6$. In the ^{1}H- and ^{13}C-NMR spectra of **2** the presence of three methoxyl groups were clearly indicated [δ_H 3.86 (3H, s), 3.87 (3H, s), and 3.89 (3H, s); δ_C 55.4 (q), 55.8 (q), and 60.2 (q)]. The other aromatic proton signals at δ_H 6.35 (1H, d, J = 2.0 Hz), 6.44 (1H, d, J = 2.0 Hz), 7.02 (2H, d, J = 9.0 Hz), and 8.07 (2H, dd, J = 9.0 Hz) were characteristic of a 4',5,7-oxysubstituted flavonol skeleton. The downfield signal of at δ_H 12.6 (1H, s) determined the only hydroxyl group at C-5 position, therefore, the three methoxyl groups were placed at C-3, C-4', and C-7. Furthermore, the comparison of the ^{13}C-NMR spectroscopic data of **2** with those of kaempferol [4] confirmed the suggestion. Key EI-MS fragmentations of **2** were shown in the Fig. 2 and the fragments of *m/z* 167, 166 and 135, 107 were useful in the assignment of the methoxyl groups. Therefore, **2** was determined to be 5-hydroxy-3,4',7-trimethoxyflavone.

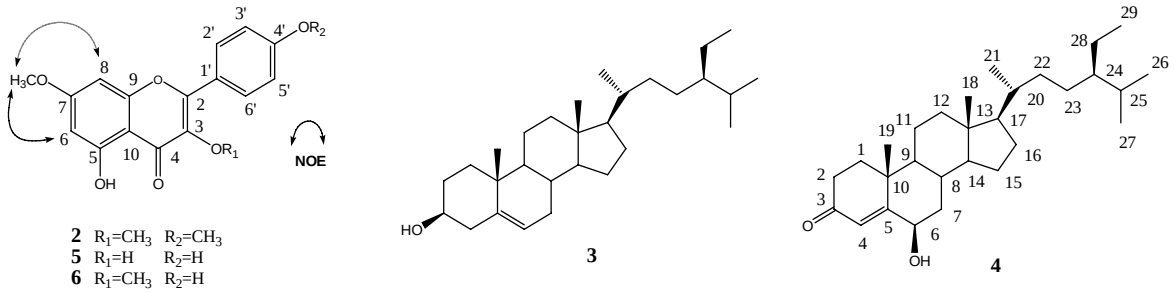

Figure 1: Chemical structures of the isolates **2**-**6** and diagnostic NOESY interactions of **5** and **6**

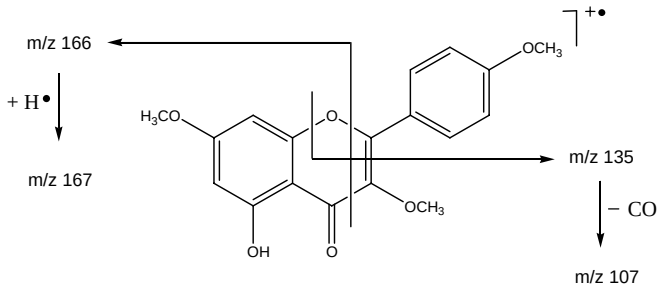

Figure 2: Diagnostic EI-MS fragmentations of **2**

Compound **3** (β-sitosterol) was isolated as white rods, m.p. 134 - 135ºC. The compound was identified on the basis of co-TLC analysis [R_f 0.34 (*n*-hexane-EtOAc 4:1)] and the superimposability of the IR spectrum of **3** with that of the authentic β-sitosterol.

Compound **4** was isolated as yellow needles, m.p. 210 - 211ºC. The molecular formula of **4** ($C_{29}H_{48}O_2$) was suggested from the EI-MS spectrum (*m/z* 428, $M^{+\cdot}$) and NMR data. The ^{1}H-NMR spectrum of **4** exhibited two tertiary methyl groups [δ_H 0.74 (3H, s) and 1.38 (3H, s)], three secondary methyl groups [δ_H 0.81 (3H, d, J = 7.0 Hz), 0.84 (3H, d, J = 6.5 Hz), and 0.92 (3H, d, J = 6.5 Hz)], and a primary methyl group [δ_H 0.85 (3H, t, J = 7.5 Hz)], an oxymethine [δ_H 4.35 (1H, br s)], and an olefinic proton of a trisubstituted double bond [δ_H 5.81 (1H, br s)]. In the ^{13}C-NMR with DEPT program the signals were assigned to six methyl groups [δ_C 11.9 (q), 12.0 (q), 18.7 (q), 19.0 (q), 19.5 (q), and 19.8 (q)], ten methylenes [δ_C 20.9 (t), 23.1 (t), 24.2 (t), 26.1 (t), 28.2 (t), 33.9 (t), 34.3 (t), 37.1 (t), 38.6 (t), and 39.6 (t)], eight methines [δ_C 29.2 (d), 29.7 (d), 36.1 (d), 45.9 (d), 53.7 (d), 55.9 (d), 56.1 (d), and δ_C 73.3 (d)], two quaternary carbons [δ_C 38.0 (s) and 42.5 (s)], and a trisubstituted double bond [δ_H 126.3 (d)

and 168.6 (s)], which was conjugated with a ketone group [δ_C 200.2 (s)]. When compared to the ^{13}C-NMR data of *β*-sitosterol (our authentic sample), stigmast-4-ene-3,6-dione [5], and stigmastane-3,6-dione [5] the differences observed for the carbons of rings A and B of the stigmastane skeleton suggested the 6-hydroxysigmast-4-ene-3-one structure of **4**. The β-orientation of the hydroxyl group at C-6 was assigned on the basis of the small coupling constant (br s) of H-6α (equatorial) with 2H-7. This orientation was in full agreement with a γ-gauche effect ($\Delta\delta_C$ −5.8 ppm) observed for C-8 when the ^{13}C-NMR spectroscopic data of **4** and cholestanol [6] were compared. The absolute configuration of C-24 was deduced to be 24*R* since the carbon-13 chemical shifts of C-24 (δ_C 45.9) and the side chain at C-17 were in full agreement with the corresponding values of β-sitosterol (δ_C 45.9, C-24), stigmast-4-ene-3,6-dione (δ_C 45.8, C-24), and stigmastane-3,6-dione (δ_C 45.8, C-24) [5]. Therefore, the structure of **4** was determined to be 6*β*-hydroxystigmast-4-ene-3-one; this compound was found previously in aquatic fern *Azolla nilotica* (Azollaceae) [7] and had the same ^{1}H- and ^{13}C-NMR spectroscopic data as those of **4**.

A mixture of compound **5** and **6** were isolated as a yellow amorphous powder. The ^{1}H-NMR spectrum of the mixture showed two signal groups of two methylated kaempferol in a ratio of ca. 1:2 (**5**:**6**). The compounds were shown to possess methoxyl groups at δ_H 3.80 (3H, s) (**5**) and 3.72 (3H, s) and 3.79 (3H, s) (**6**). **5** and **6** were suggested to be kaempferol derivatives since the aromatic proton signals [δ_H 6.26 (1H, d, *J* = 2.0 Hz), 6.40 (1H, d, *J* = 2.0 Hz), 6.86 (2H, d, *J* = 9.0 Hz), and 8.01 (2H, dd, *J* = 9.0 Hz) (**5**) and 6.26 (1H, br s), 6.38 (1H, d, *J* = 2.0 Hz), 6.86 (2H, d, *J* = 9.0 Hz), and 7.91 (2H, dd, *J* = 9.0 Hz) (**6**)] were in a similar pattern to that of kaempferol [4]. In **6** the placement of the methoxyl groups was determined to be at C-3 and C-7 on comparison of the ^{13}C-NMR spectroscopic data of **3** and **6**. The carbon-13 chemical shift of C-4 is known to be sensitive to the substitution at C-5 [8] and the value of δ_C 178.8 (s) was indicative of a 5-hydroxylated flavonol. The similarity in the proton chemical shifts of **5** and **6** suggested the placement of the only methoxyl group of **5** at C-7. The EI-MS spectrum of the mixture showed the molecular ion peaks at *m/z* 300 ($C_{16}H_{12}O_6$) (**5**) and 314 ($C_{17}H_{14}O_6$) (**6**), which were in good agreement with the proposed structures. The NOESY spectrum of the mixture (Fig. 1) confirmed the assignments of the methoxyl groups of **5** and **6**. Therefore, **5** was determined to be 4',5-dihydroxy-7-methoxyflavonol (rhamnocitrin) and **6** to be 4',5-dihydroxy-3,7-dimethoxyflavone.

Acknowledgments: *This work was supported by the International Foundation for Science (IFS, Stockholm, Sweden) through a Research Grant to P. M. G. and the Basic Research Program in Natural Science of Vietnam.*

REFERENCES

1. Le Huyen Tram, Phan Minh Giang, Phan Tong Son. Pharmaceutical Journal (Vietnam), **47** (9), 8-11 (2007).
2. Phan Minh Giang, Le Huyen Tram, Phan Tong Son. Pharmaceutical Journal (Vietnam), **47** (11), 17-20 (2007).
3. Dictionary of Natural Products. Chapmann and Hall/CRC 1985-2005 (2005).
4. The Flavonoids: Advances in Research, ed. J. B. Harborne, T. J. Mabry, Chapman and Hall, London (1982).
5. J. C. Lim, J. H. Park, M. Budesinsky, A. Kasal, Y. H. Han, B. S. Koo, S. I. Lee, D. U. Lee. Chem. Pharm. Bull., **53** (5), 561 - 564 (2005).
6. L. J. Goad, T. Akihisha. Analysis of Sterols, Chapmann & Hall, London (1997).
7. Y. Arai, T. Nakagawa, M. Hitosugi, K. Shiojima, H. Ageta, O. Basher. Phytochemistry, **48** (3), 471 - 474 (1998).
8. Carbon-13 NMR of Flavonoids, ed. P. K. Agrawal, Elservier, Amsterdam (1989).