Coumarins from *Campylotropis hirtella* (Franch.) Schindl. and Their Inhibitory Activity on Prostate Specific Antigen Secreted from LNCaP Cells

Hui-Ying Han,[a] Ping Wen,[b] Hong-Wei Liu,[c] Nai-Li Wang,[d] and Xin-Sheng Yao*,[e]

[a] *Cancer Biology Group, Department of Anatomy, Faculty of Medicine, The University of Hong Kong; 21 Sassoon Road, Hong Kong, SAR, China:* [b] *College of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University; Shenyang, China:* [c] *Capital Medical University, School of Chemical Biology and Pharmaceutical Sciences; Beijing 100000, China:* [d] *Key Laboratory for Research and Development of Traditional Chinese Medicine and Natural Medicine; Shenzhen 518100, China: and* [e] *Institute of Traditional Chinese Medicine and Natural Products, Jinan University; 601 Huangpu Avenue West, Guangzhou 510632, China.* Received April 28, 2008; accepted June 17, 2008; published online June 23, 2008

***Campylotropis hirtella* (Franch.) Schindl. was used as a folk medicine for the treatment of benign prostate hyperplasia (BPH) in China. In this study, two new coumarins, 7,2′,4′-trihydroxy-5-methoxy-3-arylcoumarin (1), 6-[(1*S*,2*S**)-2-angeloyloxy-1,3-dihydroxy-3-methylbutyl]-7-methoxycoumarin, named angelol M (2), together with eleven known related compounds (3—13) were isolated from this plant under the bioassay guided fractionation. All the compounds showed activity with different levels in inhibiting prostate specific antigen (PSA) secreted from androgen dependent prostate cancer cell line, LNCaP cells, using ELISA method.**

Key words *Campylotropis hirtella*; coumarin; benign prostate hyperplasia; prostate specific antigen

Campylotropis hirtella (Franch.) Schindl., belonging to the family of Leguminosaea, has been used in traditional medicine for the treatment of benign prostate hyperplasia (BPH) in China. Previous studies on this plant revealed that it contains tannins, steroids and triterpenes.[1,2] However, there is no information available on chemical substances contributing to its clinical anti-BPH effect. Prostate specific antigen (PSA) is a well-accepted clinically marker for the diagnosis and prognosis of prostate diseases. The serum PSA level and prostate volume have been described as significant prognostic factors that predict treatment outcome in patients with BPH under medical treatment.[3,4] Therefore, to measure secreted PSA in androgen dependent prostate cancer cell line, LNCaP cells, could be used as an *in vitro* model for screening activity of anti-BPH. Our previous study has obtained a series of active flavonoids from *Brassica napus* L. pollen under this bioassay guided fractionation, which also has been used in the treatment of BPH in China.[5] In this study, we further investigated the constituents which can inhibit PSA secretion from *C. hirtella*, aiming to clarify the substance responsible for its treatment of BPH. From the EtOAc-soluble fraction of this plant, which showed activity in decreasing PSA secretion, we isolated two new coumarins (**1**, **2**), and eleven known related compounds (**3**—**13**). All the known compounds were first reported to be isolated from *C. hirtella*. And all isolated compounds showed activity at different levels against prostate specific antigen (PSA) secreted from LNCaP cells by ELISA method.

Results and Discussion

The 60% (v/v) EtOH extract of dried roots of *C. hirtella* were suspended in water, and partitioned with EtOAc and *n*-BuOH successively. The EtOAc-soluble extract showed stronger activity in decreasing the secretion of PSA in LNCaP cells compared to the other two extracts (data not shown). Thus, the EtOAc-soluble extract further subjected to column chromatography on silica gel, which resulted in 11 fractions. Among these fractions, fractions 2—7 showed inhibitory ability on PSA secretion (Fig. 1). Further purification on these active fractions by column chromatography and HPLC gave compounds **1**—**13**.

Compound **1** was obtained as yellow amorphous powder.

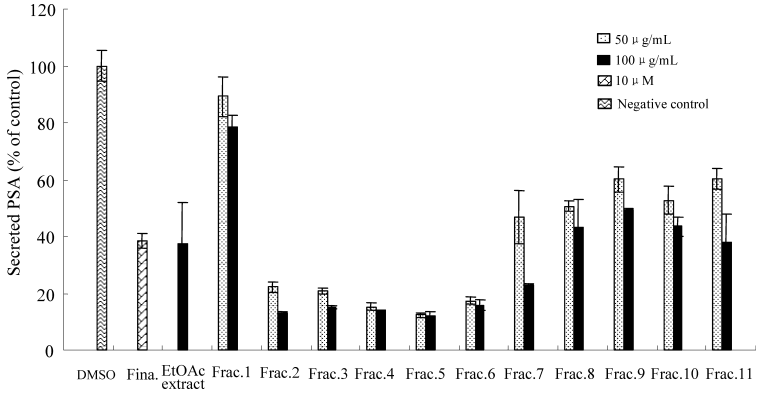

Fig. 1. Fractions from EtOAc Extracts of *C. hirtella* Inhibited the Secretion of PSA in LNCaP Cells

The results were showed as a percentage of solvent control and represented the mean and standard deviation. The finasteride (Fina.) serves as positive control.

* To whom correspondence should be addressed. e-mail: tyaoxs@jnu.edu.cn

Its molecular formula was established as $C_{16}H_{12}O_6$ by HR-TOF-MS giving a quasimolecular-ion peak $[M+Na]^+$ at *m/z* 323.0511. The UV spectroscopic data (λ_{max} 259, 352 nm) of this compound were similar to those of licofuranocoumarin and licopyranocoumarin,[6,7] suggesting a 3-arylcoumarin structure for **1**. The ^{1}H-NMR spectrum of **1** showed one singlet signal at δ 8.03 (1H) that indicated a characteristic proton located at C-4 in coumrin skeleton. The remaining signals in the ^{1}H-NMR spectrum showed the presence of a 1,2,4-trisubstituted benzene ring [δ 7.19 (1H, d, J=8.3 Hz), 6.47 (1H, d, J=2.4 Hz), 6.44 (1H, dd, J=2.4, 8.3 Hz)], a 1,2,3,5-tetrasubstituted benzene ring [δ 6.45 (1H, d, J=1.6 Hz), 6.42 (1H, d, J=1.6 Hz)], and a methoxyl group (δ 3.94, 3H, s). The aromatic protons at δ 6.47, 6.44 and 7.19 were assigned as H-3′, H-5′ and H-6′ on the basis of the coupling constants and HSQC spectrum, and further confirmed by HMBC correlations of H-3′/C-1′ (δ 115.8) and C-5′ (δ 104.5) and those of H-6′/C-2′ (δ 157.1) and C-4′ (δ 159.7). A long range correlation between the methoxy protons (δ 3.94) and carbon signal δ 158.4 was observed. Additionally, the methoxy protons (δ 3.94) correlated with H-4 (δ 8.03) in the NOESY spectrum, and so the carbon signal at δ 158.4 was assigned as C-5. One of the remaining two *meta*-coupled aromatic protons, at δ 6.45, was attributed to H-6 according to the correlation of this proton and the methoxy protons (δ 3.94) in the NOESY spectrum. The HMBC cross-peak between H-4 and C-1′ confirmed that the trisubstituted benzene ring was linked at C-3. The quaternary carbon signal at δ 160.0 was deduced as C-2, for it correlated with H-4 in the HMBC spectrum. Therefore, **1** was determined as 7,2′,4′-trihydroxy-5-methoxy-3-arylcoumarin.

Compound **2** was isolated as a pale yellow gum. The molecular formula was determined to be $C_{20}H_{24}O_7$ by HR-TOF-MS (*m/z* 399.1438, $[M+Na]^+$) and NMR analysis. The ^{1}H-NMR spectrum of **2** showed characteristic signals of 6,7-disubstituted coumarin skeleton [δ 6.23 (1H, d, J=9.5 Hz), 7.88 (1H, d, J=9.5 Hz), 7.68 (1H, s) and 6.94 (1H, s)], an –CH–CH– (both oxygenated) fragment [δ 5.38 (1H, d, J=8.7 Hz), 5.12 (1H, d, J=8.7 Hz)], and one methoxyl group (δ 3.88, 3H). The remaining signals [δ 1.62 (6H, s), 1.39 (3H, s), 1.23 (3H, s) and 5.88 (1H, m)] in the ^{1}H-NMR spectrum were deduced to four methyl groups and one olefinic proton. The above ^{1}H-NMR spectrum analysis suggested an angelol-type coumarin for **2**.[8] An angelol group was deduced based on the HMBC correlationship between the two methyl protons (δ 1.39, 1.23) and C-12 (δ 80.1) and C-13 (δ 73.8), and the protons at δ 5.38 and 5.12 were attributed to C-11 (δ 68.1) and C-12 according to their coupling and HSQC correlation. In the HMBC spectrum, two remaining methyl protons (δ 1.62, 6H) correlated with C-2′ (δ 129.4), C-3′ (δ 138.9) and C-1′ (δ 167.5), indicating an angeloyl group. The long-range correlations of H-11/C-5 (δ 129.4) and C-6 (δ 129.4) established the linkage between C-6 and C-11. The angeloyl group was located at C-12, confirmed by HMBC correlation of H-12/C-1′. All the above evidence suggested this compound was very similar to angelol A,[8] which also was obtained in this study. Their ^{1}H- and ^{13}C-NMR data in MeOH were showed in Table 1. The major difference was that the coupling constant between H-11 and H-12 was 8.7 Hz for **2**, not 1.7 Hz for angelol A, which suggested compound **2** was the stereoisomer of angelol A.[8] So its relative configuration between C-11 and C-12 was deduced to be same to angelol E, and different with angelol A.[8] According to the previous study report, angelol-type coumarins showed plane optical rotatory dispersion (ORD) curve.[8—10] The absolute configuration of C-11 in the angelol-type coumarins was determined from their ORD spectra. If the configuration of C-11 was *R*, the ORD spectrum of the coumarin should have a negative plane curve. Conversely, if it was *S*, the ORD should present a positive curve.[8] Compound **2** showed the data $[\alpha]_D^{26}$ +69.2° (c=1.0, MeOH, 589 nm), implied it should have a positive ORD curve, so its C-11 should be *S*-configuration. Because of the configuration of C-11 in 2 opposite to angelol A, we still have not enough

Table 1. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Data for Compounds **2** and **3**

Position	Compound **2** (in CD_3OD)		Compound **3** (in CD_3OD)	
	δ_H	δ_C	δ_H	δ_C
1				
2		163.3		163.4
3	6.23 (1H, d, J=9.5 Hz)	113.4	6.22 (1H, d, J=9.4 Hz)	113.4
4	7.88 (1H, d, J=9.5 Hz)	145.9	7.83 (1H, d, J=9.4 Hz)	145.9
5	7.68 (1H, s)	129.4	7.62 (1H, s)	128.0
6		129.4		129.1
7		162.0		160.9
8	6.94 (1H, s)	99.4	6.90 (1H, s)	99.5
9		156.6		156.5
10		113.4		113.2
11	5.38 (1H, d, J=8.7 Hz)	68.1	5.60 (1H, d, J=1.7 Hz)	68.1
12	5.12 (1H, d, J=8.7 Hz)	80.1	5.14 (1H, d, J=1.7 Hz)	78.0
13		73.8		74.3
14	1.23 (3H, s)	27.3	1.25 (3H, s)	27.3
15	1.39 (3H, s)	25.4	1.26 (3H, s)	27.4
1′		167.5		168.5
2′		128.8		129.1
3′	5.88 (1H, m)	138.9	5.89 (1H, m)	138.4
4′	1.62 (3H, s)	15.6	1.76 (3H, m)	15.6
5′	1.62 (3H, s)	20.3	1.62 (3H, m)	20.6
7-OCH_3	3.88 (3H, s)	56.6	3.98 (3H, s)	56.8

Table 2. Inhibition (IC_{50} Values) of PSA Secretion in LNCaP Cells by Thirteen Coumarins Isolated from *C. hirtella*

IC_{50}	Compounds												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
μg/ml	24.2	102.0	63.4	97.1	57.2	42.0	38.8	33.0	57.3	43.9	34.7	66.1	74.4
μM	80.7	271.2	168.6	256.2	152.1	194.3	157.7	152.8	268.9	178.1	120.5	344.3	277.4

information to establish the absolute configuration of C-12 in 2 basing on present data and literature reference. Therefore, presently, **2** was deduced as 6-[(1*S*,2*S**)-2-angeloyloxy-1,3-dihydroxy-3-methylbutyl]-7-methoxycoumarin, named angelol M.

The structures of eleven known coumarins were elucidated by means of spectral data analyses and comparison with literature data. They were identified as angelol A (**3**), angelol B (**4**), angelol G (**5**),[8] xathotoxin (**6**), isopimpinellin (**7**), bergapten (**8**),[11] alloimperatorin (**9**),[12] columbianetin (**10**),[13] lomatin acetate (**11**),[14] 8-*O*-methylmellein (**12**),[15] and 3,9-dihydroxy coumestan (**13**).[16] All these known compounds were reported from this plant for the first time.

The effects of compounds **1**—**13** on PSA secretion in LNCaP cells were tested using ELISA bioassay. The IC_{50} values were shown in Table 2. Compound **1** was the most active one. Our previous study and other researchers have indicated that flavonoid compounds often exhibit significant activity against PSA secretion in LNCaP cells.[5,17,18] Compound **1**, belonging to 3-arylcoumarin, has a similar skeleton with the nucleus of flavonoids. It may be the basic unit C_6–C_3–C_6 in **1** that plays an essential role in its bioactivity against PSA secreted from LNCaP cells. Compound **13**, a typical phytoestrogen, had a weak inhibition on PSA secretion, suggesting estrogen-like effect should not be the mechanism of inhibition of PSA secretion by these coumarins. Other compounds including angelol-type coumarins (**2**—**5**), furano-coumarins (**6**—**10**), pyrano-coumarin (**11**) and isocoumarin (**12**), showed moderate or weak activity against PSA secretion. Compared with flavonoids from *Brassica napus* L. pollen which also has been used in traditional Chinese medicine for the treatment of BPH, the coumarins from *C. hirtella* showed relatively lower effect on PSA secretion.[5] The reason may be that these coumarins involved in other mechanism also contributing to the treatment of BPH for *C. hirtella*. Previous study demonstrated that coumarin and its major human metabolite, 7-hydroxycoumarin, inhibited proliferation of several cancer cell lines, including LNCaP cells. And PSA secretion from LNCaP was also suppressed by these two compounds.[19] Although the effective concentration of coumarin and 7-hydroxycoumarin was more than 100 μg/ml, these results suggest coumarins may have positive effect on the treatment of prostate diseases. In our study, the IC_{50} values of isolated coumarins on inhibiting secretion of PSA from LNCaP cells were ranged from 24 to 102 μg/ml, which indicated that these compounds should be more effective than coumarin and 7-hydroxycoumarin. In the Fig. 3, we also showed the proliferation inhibitory effects on LNCaP cells by new compounds **1** and **2** through MTS assay, and their IC_{50} values were 61.2 and 83.7 μg/ml, respectively.

Table 3. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) Data for Compound **1**

Position	Compound **1** [in $(CD_3)_2CO$]	
	δ_H	δ_C
1		
2		160.0
3		120.9
4	8.03 (1H, s)	137.8
5		158.4
6	6.45 (1H, d, J=1.6 Hz)	96.1
7		162.9
8	6.42 (1H, d, J=1.6 Hz)	95.6
9		156.9
10		104.4
1′		115.8
2′		157.1
3′	6.47 (1H, d, J=2.4 Hz)	108.2
4′		159.7
5′	6.44 (1H, dd, J=2.4, 8.3 Hz)	104.5
6′	7.19 (1H, d, J=8.3 Hz)	132.6
5-OCH_3	3.94 (3H, s)	56.6

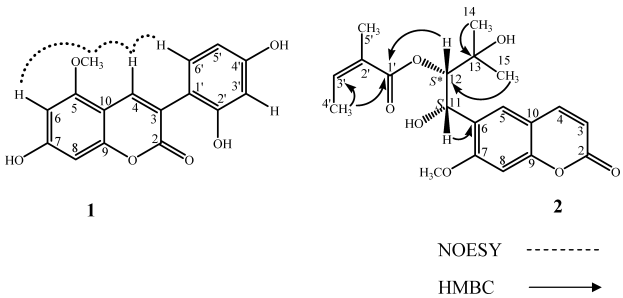

Fig. 2. Key HMBC and NOESY Correlations of Compounds **1** and **2**

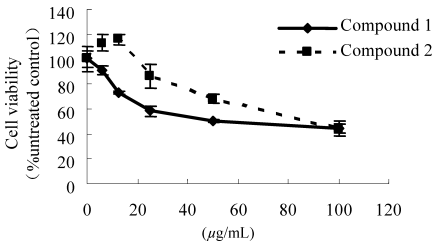

Fig. 3. Effects on Cell Viability of Compounds **1** and **2** Examined by MTS Assay

Results represented the OD ratio between the treated and untreated cells. Each data point is represented by mean±standard deviations.

Experimental

General Procedures Optical rotations were measured using a Jasco P-1020 polarimeter. UV spectra were obtained with Shimadzu UV2401PC UV–Vis recording spectrophotometer in MeOH. IR spectrum was measured using a Shimadzu FTIR 8400 spectrophotometer as KBr discs. NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer (400 MHz for

^{1}H, 100 MHz for ^{13}C). ESI-MS spectra were obtained on a Bruker Esquire 2000 mass spectrometer. HR-TOF-MS spectra were performed on a Micromass mass spectrometer. The analytical and preparative HPLC were performed on a Shimadzu Pak with RI detector using a Shim-pack VP-ODS column (4.6×250 mm) and a Shim-pack PREP-ODS column (20×250 mm), respectively. Column chromatography was carried out on silica gel (200—300 mesh, Qingdao Haiyang Chemical Group Corp., Qingdao, China), Sephadex LH-20 (Amersham Biosciences AB) and ODS (60—80 μm, YMC) as packing materials. Silica gel G was used for analytical TLC.

Plant Material Fresh roots of *C. hirtella* were collected from Kunming, Yunnan province, China in October, 2004 and authenticated by associate professor Junkui Cai, Chinese Academy of Sciences in Kunming. A voucher specimen was kept in the Key laboratory for Research and Development of New Drugs, Shenzhen 518057, China.

Extraction and Isolation Dried roots of the plant (15 kg) were ground and extracted with 60% EtOH for three times. After evaporation of the solvent *in vacuo*, the residues (900 g) were resuspended in water, and partitioned with EtOAc and *n*-BuOH successively. The EtOAc-soluble extract (94 g) with the activity of inhibition of PSA secretion subjected to column chromatography on silica gel with elution of $CHCl_3$–MeOH gradient (100 : 0→0 : 100), which resulted in 11 fractions, and active components of the extract mainly distributed in fractions 2—7. Fraction 2 was further chromatographed on silica by gradient elution with cyclohexane/acetoacetate (9 : 1→0 : 1) to give 6 subfractions. The subfraction 3 was passed through a Sephadex LH-20 column with $CHCl_3$–MeOH (1 : 1) as an eluent, then applied to an ODS column eluting with MeOH/H_2O (5 : 5→7 : 3), finally compounds **6** (6 mg), **7** (11 mg), **8** (5 mg), **9** (5 mg), **10** (6 mg), **11** (11 mg), and **12** (6 mg) crystallized. Fraction 3 was first passed through Sephadex LH-20 column with $CHCl_3$–MeOH (1 : 1) as an eluent, and then chromatographed on ODS column eluting with MeOH–H_2O (3 : 7→9 : 1). The eluents of 50% and 70% MeOH were purified by ODS preparative HPLC (45% MeOH), which yielded compounds **2** (20 mg), **3** (16 mg), **4** (22 mg) and **5** (19 mg). After passed over a Sephadex LH-20 column ($CHCl_3$–MeOH 1 : 1), faction 5 was carried on preparative TLC, then compound **13** (7 mg) was obtained. Fraction 6 was passed over Sephadex LH-20 column with $CHCl_3$–MeOH (1 : 1), then chromatographed on ODS column with MeOH–H_2O as an eluent(3 : 7→9 : 1). The eluent of 50% MeOH was purified by ODS preparative HPLC (45% MeOH), and yielded compound **1** (12 mg).

7,2′,4′-Trihydroxy-5-methoxy-3-arylcoumarin (**1**), ESI-MS *m/z*: 323 $[M+Na]^+$, 623 $[2M+Na]^+$, 299 $[M-H]^-$, 599 $[2M-H]^-$. HR-TOF-MS *m/z*: 323.0511 (Calcd for $C_{16}H_{12}O_6$, 323.0532). UV (CH_3OH) λ_{max} nm, 259, 352. IR ν_{max}^{KBr}, 3278, 1685, 1276, 1107, 1593, 1481 cm^{-1}. ^{1}H- and ^{13}C-NMR, see Table 3.

6-[(1*S*,2*S*)-2-Angeloyloxy-1,3-dihydroxy-3-methylbutyl]-7-methoxycoumarin, angelol M (**2**), $[\alpha]_D^{26}$ +69.2° (*c*=0.1, MeOH, 589 nm). ESI-MS *m/z*: 399 $[M+Na]^+$. HR-TOF-MS *m/z*: 399.1438 (Calcd for $C_{20}H_{24}O_7$, 399.1420). UV (CH_3OH) λ_{max} nm, 220, 326. ^{1}H- and ^{13}C-NMR, see Table 1.

Bioactivity Assay. Cell Culture and Test Compounds Preparation LNCaP cells were maintained at 37 °C in RPMI 1640 (Gibco, U.S.A.) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin–streptomycin (Hyclone, U.S.A.). The fractions and isolated compounds (**1**—**13**) were dissolved in DMSO to obtain the desired concentrations.

MTS Assay Cell viability was measured using MTS(3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium) proliferation assay kit (Promega) as described by the manufacturer and previous study.[5)]

Detection of Secreted PSA by ELISA Ten thousand cells were seeded in 96-well plates and cultured for 2 d. The cells were subsequently incubated with test compounds or fractions for another 48 h. The secreted PSA in the medium was detected by ELISA as previously described.[5)] The mean absorbance value (OD490) for each set of reference standards, controls, and samples were calculated. A standard curve was constructed by plotting the mean absorbance of each reference standard against its concentration in ng/ml. The calculated concentrations were further normalized to MTS OD values, which offset the possible influence on PSA resulted from cytotoxicity of the tested compounds. The final results were represented as the ratio of PSA released from the compound-treated cells over that of vehicle-treated cells. Each data point represented the mean and standard deviation of triplicate experiments.

Acknowledgements This work was supported by grants from Traditional Chinese Medicine and Nature Drug Research Institute in Zhejiang province of China. We thank Shanghai Institute of Materia Medica of Chinese Academy of Sciences for measuring the HR-TOF-MS. We also thank Huang Jinghui and Jin Sanlin for NMR spectra, and Shao Peng for IR spectroscopy measurements.

References

1) Lu X. Z., Xu W. B., Shen J. X., *Zhongguo Zhongyao Zazhi*, **22**, 680—682 (1997).
2) Qin L., Chen X. M., Chen W. X., Yang L., Zhang C. K., *Tianran Chanwu Yanjiu Yu Kaifa*, **3**, 31—34 (1991).
3) Roehrborn C. G., Boyle P., Bergner D., Gray T., Gittelman M., Shown T., Melman A., Bracken R. B., deVere White R., Taylor A., Wang D., Waldstreicher J., *Urology*, **54**, 662—669 (1999a).
4) Roehrborn C. G., McConnell J. D., Lieber M., Kaplan S., Geller J., Malek G. H., Castellanos R., Coffield S., Saltzman B., Resnick M., Cook T. J., Waldstreicher J., *Urology*, **53**, 473—480 (1999b).
5) Han H. Y., Shan S., Zhang X., Wang N. L., Lu X. P., Yao X. S., *Phytomedicine*, **14**, 338—343 (2007).
6) Hatano T., Aga Y., Shintani Y., Ito H., Okuda T., Yoshida T., *Phytochemistry*, **55**, 959—963 (2000).
7) Hatano T., Yasuhara T., Miyamoto K., Okuda T., *Chem. Pharm. Bull.*, **36**, 2286—2288 (1988).
8) Baba K., Matsuyama Y., Koazwa M., *Chem. Pharm. Bull.*, **30**, 2025—2035 (1982).
9) Liu J. H., Xu S. X., Yao X. S., Kobayashi H. *Phytochemistry*, **39**, 1099—1101 (1995).
10) Baba K., Matsuyama Y., Ishida T., Inoue M., Koazwa M., *Chem. Pharm. Bull.*, **30**, 2036—2044 (1982).
11) Kutney J. P., Verma A. K., Young R. N., *Tetrahedron*, **28**, 5091—5104 (1972).
12) Liu Y. Y., Zhang C., Li L., Xiao Y. Q., *China J. Chin. Mater. Med.*, **131**, 309—311 (2006).
13) Ishi H., Hosoya K., Ishikawa T., Haginiwa J., *Yakugaku Zasshi*, **94**, 309—321 (1974).
14) Matsuda H., Murakami T., Nishida N., Kageura T., Yoshikawa M., *Chem. Pharm. Bull.*, **48**, 1429—1435 (2000).
15) Thomas S., Kula M. Regina., Müller Michael., *Synthesis*, **12**, 2045—2048 (1999).
16) Liu Y., Ma X. X., Chen H. B., *J. Chin. Pharm. Sci.*, **14**, 75—78 (2005).
17) Rachel S. R. Z., David J. A. J., Theodore J. B., Eleftherios P. D., *Clin. Chim. Acta*, **317**, 17—26 (2002).
18) Davis J., Muqim N., Bhuiyan M., Kucuk P., Sarkar F., *Int. J. Oncol.*, **16**, 1091—1097 (2000).
19) Marshall M. E., Kervin K., Benefield C., Umerani A., Albainy-Jenei A., Zhao Q., Khazaeli M. B., *J. Cancer Res. Clin. Oncol.*, **120** (Suppl.), S3—S10 (1994).