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# CHEMICAL CONSTITUENTS AND CYTOTOXICITY FROM THE STEM BARK OF *CITRUS MEDICA*

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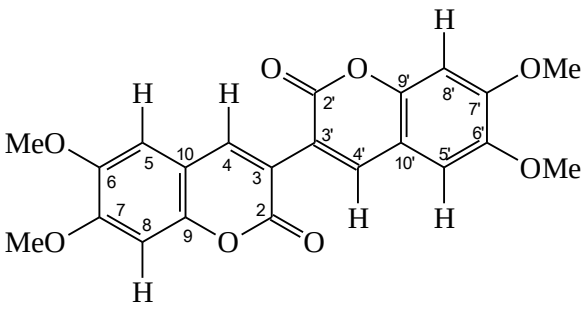
**Abstract** – A new biscoumarin, namely citrumedin-A [6,6',7,7'-tetramethoxy-3,3'-biscoumarin] (**1**), together with twenty two known compounds were isolated from the stem bark of *Citrus medica* L. var. *sarcodactylis* Swingle. The structure of this new compound was completely elucidated using a combination of 2D NMR techniques (COSY, NOESY, HMQC and HMBC) and HR-EI-MS analyses. The known compounds were identified by comparison of their spectroscopic and physical data with those reported in the literatures. The isolates were also screened their cytotoxicity against Daoy, Hep2, MCF-7 and Hela cell lines. Lonchocarpol A (**12**) showed marginally cytotoxic activity against those four cancer cell lines.

*Citrus medica* L. var. *sarcodactylis* Swingle belongs to genus of *Citrus* (Rutaceae) and mainly distributed in the south of China, India, Vietnam and Malaysia. It was introduced from Fujian, China to Taiwan around 1700. The fruit of the genus of *Citrus* can be used as food, and its peel, leaves and root can be also used as folk medicine or spice in Taiwan.[1] Several active constituents, such as coumarins,[2] flavonoids,[3] tetranortriterpenoids[4] and monoterpenoids,[5] were isolated and reported from the *Citrus* species. Recently, Wu *et al.* [6~23] reported the isolation of several acridone alkoids from *Citrus* genus.

*C. medica* L. var. *sarcodactylis* Swingle is used as a folk medicine for the treatment of stomach ache, edema, headache, rheumatism, infectious hepatitis and arthritis in Taiwan.[24] In continuation of our interest on the bioactive compounds from the natural resource led us to investigate the constituents and bioactivity of the

stem bark of *C. medica* L. var. *sarcodactylis* Swingle. In this paper, we reported the isolation and structural elucidation a new biscoumarin, citrumedin-A (**1**) together with twenty two known compounds including seven coumarins, seven flavonoids, four tetranortriterpenoids, two steroids, one triterpenoid and one benzenoid from the stem bark of this plant and their cytotoxic activity of the isolates.

Compound **1** was isolated as yellow needles, mp 251-253$^{o}$C. The high resolution electron impact mass spectrum (HR-EIMS) of **1** showed a molecular ion peak ($[M]^{+}$) at *m/z* 410.1004 which is in agreement with the molecular formula $C_{22}H_{18}O_8$. The IR spectrum revealed the presence of carbonyl group (1717cm$^{-1}$) and aromatic ring (1614, 1548 and 1511 cm$^{-1}$). The UV spectrum appeared the maximum absorption at 233, 257, 266sh, 301 and 351 nm to indicate **1** having a 6, 7-dioxygented coumarin skeleton.[25] The $^{13}$C-NMR spectroscopic data (Table 1) showed 11 carbon signals, which were only half of the number of carbon atoms in the molecular formula of $C_{22}H_{18}O_8$. The $^{1}$H-NMR spectrum of **1** was very simple and suggested a symmetric biscoumarin. The lack of the characteristic signal of H-3 at δ 6.2~6.3 as in a simple coumarin suggested a C3-C3' linkage between two monomer coumarin units. Three singlet aromatic signals at δ 7.79 (2H), 6.86 (2H) and 6.81 (2H) were assigned to H-4 and H-4', H-8 and H-8', H-5 and H-5', respectively. They were confirmed by the long-range correlations between δ 7.79 (H-4 and H-4') with δ 158.0 (C-2 and C-2'), δ 6.81 (H-5 and H-5') with δ 140.3 (C-4 and C-4') and δ 6.86 (H-8 and H-8') with δ 111.6 (C-10 and C-10') in the heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum (Figure 1). Other proton signals were characteristic of four methoxy groups at δ 3.96 (6H, s) and 3.92 (6H, s) attached to C-7 and C-7', C-6 and C-6', respectively. These assignments were further supported by the presence of the NOE correlations between the signal at δ 3.96 with δ 6.86 (H-8 and H-8') and δ 3.92 with δ 6.81 (H-5 and H-5') in the nuclear overhauser and exchange spectroscopy (NOESY) experiment (Figure 2). On the basis of the above results, the structure of compound **1** was assigned as citrumedin-A (**1**) [6,6',7,7'-tetramethoxy-3,3'-biscoumarin]. It is rarely example that the presence of the C3-C3' linkage between two monomer coumarin units in natural products.




**1**

**Table 1** $^{1}$H NMR and $^{13}$C NMR data of **1**

| C | **1** | |
|---|---|---|
| | $\delta_H$(ppm) | $\delta_C$ |
| 2, 2' | | 158.0 |
| 3, 3' | | 119.4 |
| 4, 4' | 7.79 s | 140.3 |
| 5, 5' | 6.81 s | 102.9 |
| 6, 6' | | 147.1 |
| 7, 7' | | 153.2 |
| 8, 8' | 6.86 s | 100.1 |
| 9, 9' | | 149.0 |
| 10, 10' | | 111.6 |
| 6, 6'-$OCH_3$ | 3.92 s | 56.6 |
| 7, 7'-$OCH_3$ | 3.96 s | 56.7 |

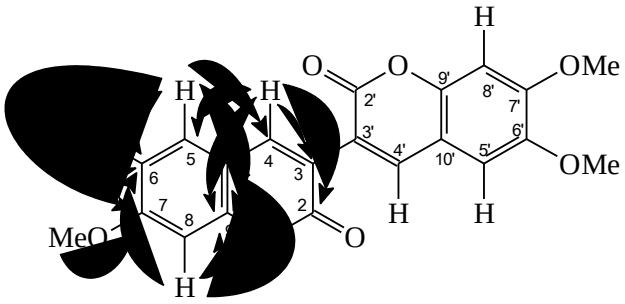



**Figure. 1** HMBC correlations of **1**

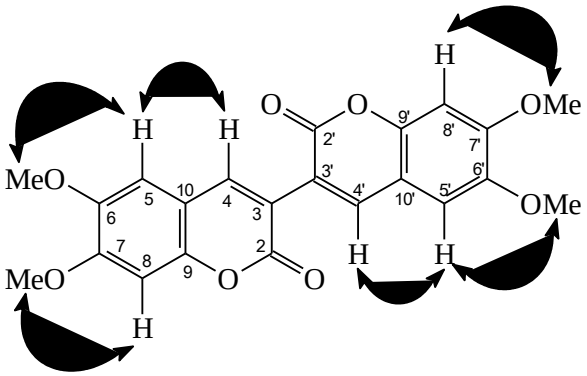
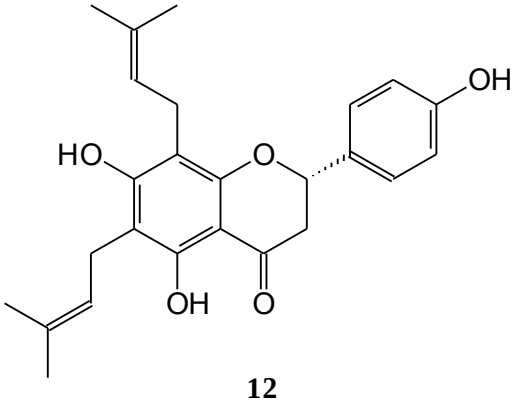



**Figure. 2** NOE correlations of **1**

The known compounds, 5,7-dimethoxycoumarin **(2)**,[26] xanthyletin **(3)**,[12] 6,7-dimethoxycoumarin **(4)**,[26] 7-methoxycoumarin **(5)**,[26] leptodactylone **(6)**,[27] 7,8-dimethoxycoumarin **(7)**,[26] nordentatin **(8)**,[12] lupinifolin **(9)**,[28] erythrisenegalone **(10)**,[28] atalantoflavon **(11)**,[29] lonchocarpol A**(12)**,[30] hiravanone **(13)**,[31] C-glycosylflavone-vitexin **(14)**,[32] citflavanone **(15)**,[33] limonin **(16)**,[34] nomilin **(17)**,[34] citrusin **(18)**,[35] limonexic acid **(19)**,[36] β-sitosterol **(20)**,[36] stigmasterol **(21)**,[36] lupeol **(22)**[37] and *cis-p*-coumaric acid **(23)**[38]

were also isolated. Their structures were identified by comparison of their spectroscopic data (UV, IR, NMR and mass spectrometry) with values in the literature.

The isolated compounds were subjected to evaluate their cytotoxic activity against tumor cell lines Daoy (human medulloblastoma), Hep-2 (human laryngeal carcinoma), MCF-7 (human breast adenocarcinoma), and Hela (human cervical epitheloid carcinoma), as described previously,[39] and the $ED_{50}$ values are summarized in Table 2. Only compound **12** displayed moderate cytotoxicity with the $ED_{50}$ values of 5.03, 6.29, 6.64, 5.09 μg/mL against the Daoy, Hep-2, MCF-7, and Hela tumor cell lines, respectively. The above results indicated this plant can be safely used in folk medicine due to its marginally cytotoxic activity.




**12**

**Table 2.** The cytotoxicity of compounds **2**~**4**, **9**~**12, 14, 16~19** and **22**

| Tested compounds | $ED_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | **Daoy** | **Hep2** | **MCF-7** | **Hela** |
| **2** | (-) | (-) | (-) | (-) |
| **3** | (-) | (-) | (-) | (-) |
| **4** | (-) | (-) | (-) | (-) |
| **9** | 10.44 | 12.46 | 13.09 | 13.24 |
| **10** | 15.39 | 18.41 | 23.58 | 15.32 |
| **11** | (-) | (-) | (-) | (-) |
| **12** | 5.03 | 6.29 | 6.64 | 5.09 |
| **14** | (-) | (-) | (-) | (-) |
| **16** | (-) | (-) | (-) | (-) |
| **17** | (-) | (-) | (-) | (-) |
| **18** | (-) | (-) | (-) | (-) |
| **19** | (-) | (-) | (-) | (-) |
| **22** | (-) | (-) | (-) | (-) |
| Mitomycin *C* | 0.06 | 0.10 | 0.14 | 0.15 |

(-): $ED_{50}$ ＞ 40 μg/mL

## EXPERIMENTAL

### General Procedure

Melting points were determined on a Yanagimoto MP-S3 apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-370 polarimeter. IR and UV spectra were recorded on Shimadzu FTIR-8501 and Hitachi UV-3210 spectrophotometers, respectively. EIMS was obtained on a VG-70-250S mass spectrometer. The $^{1}$H- and $^{13}$C-NMR, DEPT, COSY, HMQC, NOESY, and HMBC experiments were recorded on a Bruker AMX-400 spectrometer. Standard pulse sequences and parameters were used for the NMR experiments and all chemical shifts were reported in parts per million (ppm, δ). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, E. Merck).

### Plant Material

The stem bark of *Citrus medica* L. var. *sarcodactylis* Swingle was collected from Changhua Hsien, Taiwan, in September 2002 and verified by Prof. C.-S. Kuoh. A voucher specimen (TSWu 91023) has been deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan.

### Extraction and Isolation

The dried and powdered stem bark of *Citrus medica* L. var. *sarcodactylis* Swingle (1.34 kg) was extracted with acetone at rt and concentrated under reduced pressure to give a dark brown syrup (87g). The syrup was partitioned succesively between $H_2O$ and $CHCl_3$. The $CHCl_3$ layer (19.4g) was chromatographed directly on silica gel and eluted with a gradient of *n*-hexane and EtOAc to afford 10 fractions. Fraction 4 was rechromatographed on silica gel and eluted with *n*-hexane- EtOAc (10:1, v/v) to give **22** (37 mg). Fractions 6 was rechromatographed on silica gel and eluted with *n*-hexane-$Me_2CO$ (5:1, v/v) to give **2** (43 mg) , **3** (28 mg), **9** (3 mg), **10** (2 mg), **12** (22 mg), **13** (1 mg), **20** and **21** (312 mg). Fraction 7 underwent series of chromatographic separations on silica gel using $CHCl_3$- EtOAc (49:1, v/v) as eluent to afford **1** (1 mg), **4** (3.5 mg), **5** (1 mg), **6** (1.5 mg), **8** (1 mg), **11** (82 mg), **15** (1 mg) and **23** (1 mg), successively. Fraction 8 was underwent chromatography on silica gel using $CHCl_3$- $Me_2CO$ (20:1, v/v) as eluent to afford **3** (2 mg). Fraction 9 was also separated by chromatography on silica gel using $CHCl_3$- $Me_2CO$ (15:1, v/v) to give **16** (726 mg), **17** (834 mg), **18** (56 mg) and **19** (27 mg), respectively. The $H_2O$ layer (67.6g) subjected to column chromatography on silica gel and eluted with gradient of EtOAc-$Me_2CO$ gave 5 fractions. Fraction 2 was separated by column chromatography on silica gel using $CHCl_3$-MeOH (6:1, v/v) as eluent to give **14** (13 mg).

### Citrumedin-A (1)

Yellow needles, mp 251-253 $^{o}$C. UV λmax (MeOH) nm (log ε): 233 (4.47), 257 (1.78), 266sh (1.42), 301 (1.69), 351 (4.08); IR (KBr) cm$^{-1}$: 2916, 2849, 1717, 1614, 1549, 1511, 1250, 1149, 1012, 986; $^{1}$H- and

$^{13}$C-NMR data, see Tables 1; FAB-MS m/z: 411 ([M+H]$^+$, 1); HR-EIMS m/z:410.1004 (calcd. for $C_{22}H_{18}O_8$: 410.1002).

**In Vitro Cytotoxicity Assay**

All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 1500-7500 cells per well with compounds added from DMSO-diluted stock. After 3 days in culture, attached cells were fixed with cold 50 % trichloroacetic acid and then stained with 0.4 % sulforhodamine B (SRB). The absorbency at 562 nm was measured using a microplate reader after solubilizing the bound dye. The mean $EC_{50}$ is the concentration of agent that reduces cell growth by 50 % under the experimental conditions and is the average from at least three independent determinations that were reproducible and statistically significant. Four human cancer cell lines, Daoy, Hep2, MCF-7, and Hela were used in the assay. Mitomycin C (5 μM, final concentration) and DMSO (0.3 %, final concentration) were used as positive and vehicle controls. Results were expressed as percent of DMSO control.

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