JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# A Novel Compound with Antioxidant Activity Produced by *Serratia ureilytica* TKU013

Yao-Haur Kuo,[†] Hsiu-Ching Hsu,[‡] Yu-Chi Chen,[∥] Tzu-Wen Liang,[‡,§] and San-Lang Wang*[,‡,§,⊥]

[†]National Research Institute of Chinese Medicine, Taipei 112, Taiwan

[‡]Department of Chemistry, and [§]Life Sciences Development Center, Tamkang University, Taipei 25137, Taiwan

[∥]Department of Cosmetic Science, Vanung University, Chung-Li 32061, Taiwan

**ABSTRACT:** The secondary metabolites from the cultured supernatant of *Serratia ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source were isolated and ascertained the mechanism of biological activity. The EtOAc layer, which has high DPPH scavenging activity, was applied to silica gel column chromatography with a gradient of $CH_2Cl_2$/MeOH solvent system, to yield A–H and MeOH fractions. The DPPH scavenging activity and cytotoxic activities against Doay and HEp-2 cell lines of these fractions were examined. The active fractions were further applied to purification by RP-HPLC, to obtain seven compounds, including a novel compound, serlyticin-A (**1**), together with six known compounds, serranticin (**2**), serratamolide A (**3**), thymine (**4**), (4-hydroxyphenyl)acetic acid (**5**), methyl *p*-hydroxybenzoate (**6**), and uracil (**7**). Their structures were determined by physical and extensive spectral analyses such as 1D and 2D NMR data, as well as comparison with literature values. Furthermore, the major secondary metabolites of EtOAc extract of the cultured supernatant were examined by the fingerprinting data of the HPLC system.

**KEYWORDS:** *Serratia ureilytica, serlyticin-A, squid pen, antioxidant activity, cytotoxicity, DPPH*

## INTRODUCTION

Seafood processing and consumption generate a thousand tons of shellfish wastes in Taiwan each year. A practice in resource recovery is to grind and dry the shrimp shells and use the powder for animal or fish feed. The shrimp shell powder is of low economic value but readily available in Taiwan. The management of waste products from industries involved in the processing of marine resources, such as the canning industry, is a serious challenge. The major components (on a dry weight basis) of squid pen waste are protein (61%), chitin (38%), and minerals (1%);[1] as such, it is an important source of bioactive molecules. The bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes.[2−4] However, to date, there have been very few reports published on the bioactive compounds from the fermentation of squid pen waste. Squid pen waste undergoes rapid putrefaction. Consequently, it is necessary to preserve the material by adopting environmentally safe techniques prior to the production of bioactive components for biotechnological and pharmaceutical applications. The fermentation technique can be used to process squid pen waste.[1,5] Liquor rich in bioactive material is formed during fermentation due to the liquefaction of protein and chitin; this liquor contains peptides, amino acids, and chitooligosaccharides, among others.[5] We have recently reported our evaluation of the $\alpha,\alpha$-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activities of squid pen powder (SPP) fermented supernatants.[5] These results encouraged us to focus on the fermentation of squid pen. A literature survey revealed that shellfish wastes, such as shrimp shell waste, are rich sources of phenolic compounds.[6] Phenolic compounds have important antioxidative properties, and various phenolic substances have been reported to also possess a wide range of biological properties, including antioxidant, antimicrobial, antiinflammatory, and vasodilatory actions.[7−9] Antioxidants are considered to be key compounds in the fight against various diseases (e.g., cancer, chronic inflammation, atherosclerosis, and cardiovascular disorder) and aging processes. Moreover, the relevance of using antioxidants from natural sources has been considerably enhanced by the consumer's preference for natural products and concerns about the toxic effects of synthetic antioxidants.[5,10] The SPP fermented supernatant may be also rich in some compounds possessing amino groups to enhance its antioxidant properties. It is expected that this bioactive rich liquor will have beneficial and biological functions due to its inherent protein and chitin hydrolysis activity as well as the production of other bioactive materials during fermentation.

Our research group is interested in chitosanase, protease, biosurfactant, and bioactive compounds from the fermentation of squid pen waste, and so far has isolated several bioactivity compounds that showed antioxidative and antiproliferative activities. In our continuing study of the isolation and identification of a novel antioxidant with antitumor activity from *S. ureilytica* using squid pen as fermentation substrate, we isolated additional secondary metabolites from the cultured supernatant of *S. ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source. In this study, we investigated and evaluated the antioxidant activities of the isolated fractions and compounds of the fermented supernatant compared with synthetic antioxidants, $\alpha$-tocopherol. This led us to further select the crude extract for fractionation by testing the

Received: February 6, 2012
Revised: August 15, 2012
Accepted: August 16, 2012
Published: August 16, 2012





dx.doi.org/10.1021/jf302481n | J. Agric. Food Chem. 2012, 60, 9043−9047

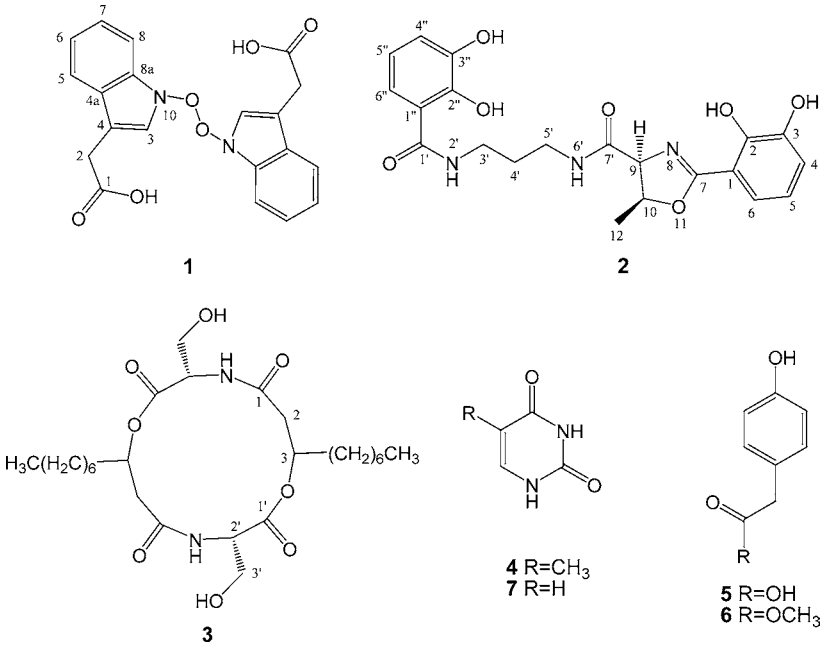


**Figure 1.** Chemical structures from the cultured supernatant of *S. ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source.

antioxidant activity of each fraction to analyze relationships between both parameters. A cytotoxic assay was also conducted with two different tumoral cells lines (Doay and HEp-2) to determine the antitumoral activities of the antioxidative fraction. We also described the isolation, structural elucidation, and DPPH radical-scavenging activity of secondary metabolites from the cultured supernatant of *S. ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source.

## MATERIALS AND METHODS

**Materials.** The SPP used in these experiments was prepared as described by Wang et al.[11] Squid pens of *Loliolus* sp. were purchased from the Shin-Ma Frozen Food Co. (I-Lan, Taiwan), washed thoroughly with tap water, and then dried. The dried materials were ground to powder for use as the carbon source in the production of antioxidant and antitumor materials. The *S. ureilytica* TKU 013 used in our experiments had been previously isolated from soil and was maintained on nutrient agar. DPPH was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were used of the highest grade available. The squid pen waste was fermented under optimized conditions as reported previously.[5] The culture conditions suitable for the production of antioxidant were found to be a shaking culture of squid pen waste in 50 mL of medium containing 1.5% SPP, 0.1% $K_2HPO_4$, and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7), at 25 °C for 4 days. The fermented broth was centrifuged (4 °C and 8200*g* for 20 min), and then the supernatant was lyophilized. The lyophilized powder was collected and stored in a polythene pouch in a freezer (−20 °C).

**Instruments.** Spectroscopic measurements were taken with the following instruments: NMR, Bruker AV-400 (400 MHz) FT-NMR, Varian VNMRS (600 MHz) FT-NMR (tetramethylsilane as an internal reference at 0 ppm for $^1H$ and $^{13}C$ NMR); Joel JMS-HX 110 mass spectrophotometer, Finnigan MAT LCQ; UV spectra, Shimadzu UV-160A spectrometer; HPLC for analysis, Hitachi L-2130 pump, Shimadzu LC-6AD liquid chromatography, Hitachi diode array detector L2450, Dynamax model UV-1 absorbance, GL sciences intertsil ODS-3, 5 μm, 4.6 × 250 mm, waters RF-18, 5 μm column. Column for preparation, GL sciences intertsil ODS-3, 5 μm, 10 × 250 mm.

**Methods.** *Isolation of the Secondary Metabolites from the Cultured Supernatant.* The cultured supernatant (18 L) was extracted three times, 12 h each time, at room temperature with an equivalent volume of ethyl acetate (EtOAc). Preliminary experiments had revealed that the EtOAc layers exhibited a higher antioxidant activity than the other organic solvents tested. The EtOAc layers were combined, collected, and concentrated under reduced pressure to dryness, yielding the crude extract, which was then suspended in $CH_2Cl_2$. Compound **2** (91.0 mg) was insoluble in $CH_2Cl_2$ and isolated from EtOAc layer and then purified by recrystallization from *n*-hexane and $CH_2/Cl_2$. The EtOAc layer (2.5 g) was applied to silica gel column chromatography (CC, 230−400 mesh) with a $CH_2Cl_2$/MeOH solvent system (from 100:0 to 0:100) and then yielded nine subfractions [fraction A (103 mg), fraction B (208 mg), fraction C (55 mg), fraction D (335 mg), fraction E (159 mg), fraction F (168 mg), fraction G (182 mg), fraction H (137 mg), and fraction MeOH (26.3 mg)]. The DPPH radical-scavenging activities of these fractions were measured. The active fractions D, F, and G ($ED_{50}$ 28.51, 41.24, and 12.41 μg/mL) were further applied to Sephadex LH-20 (MeOH/$CH_2Cl_2$, 1:1) and reversed phase (RP) HPLC, eluting with MeOH/$H_2O$ solvent system (phase A, MeOH:acetic acid = 99.5:0.5 (v/v); and phase B, $H_2O$:acetic acid = 99:0.5 (v/v)). Prior to each injection, the system was equilibrated to 60/40 (A/B). Following sample injection, the phase composition changed according to the following gradient: 70/30 at 0 min, 85/15 at 30 min (convex), compound **1** (17.5 mg) was obtained from fraction D. Compound **3** (11.0 mg) was obtained from fraction F, using Sephadex LH-20, eluting with MeOH. Compounds **4** (12.0 mg), **5** (40.0 mg), **6** (4.0 mg), and **7** (35.6 mg) were obtained from fraction G with Sephadex LH-20 ($CH_2Cl_2$:MeOH = 1:1) and applied to RP-HPLC, eluting with MeOH/$H_2O$/acetic acid (35:65:0.03 (v/v)) (Figure 1).

*Fingerprinting Examined.* The fingerprinting of EtOAc extract from the cultures supernatant was assayed by a high-performance liquid chromatography (HPLC, Shimadzu SCL-10A system controller, LC-20AT pump, SPD-10A UV detector) system, and GL sciences intertsil ODS column (ODS-3, 5 μm, 4.6 × 250 mm) with a guard column packed with the same stationary phase were utilized. Phase A, $CH_3CN$, phase B, $H_2O$ (0.05% TFA (v/v)). Prior to each injection, the system was equilibrated to 15/85 (A/B), flow rate: 1 mL/min. Following sample injection (10 μL/MeOH), the phase composition changed according to the following gradient: 15/85 at 0 min, 30/70 at 20 min (convex), 60/40 at 30 min (convex), and 85/15 from 30 to 35 min (convex) for a total chromatographic run time of 35 min.

*Antioxidant Assays: Measurement of the Scavenging Activity of DPPH Radicals.* The test sample (150 μL) dissolved in methanol at

various concentrations was mixed with 37.5 μL of methanolic solution containing 0.75 mM DPPH (Sigma) radicals. The mixture was shaken vigorously and left to stand for 30 min in the dark, following which the absorbance was measured at 517 nm against a blank.[12] The scavenging ability was calculated as follows: scavenging activity (%) = [($\Delta A_{517}$ of control − $\Delta A_{517}$ of sample)/$\Delta A_{517}$ of control] × 100. A curve of sample concentration against % DPPH was generated to estimate the concentration of sample needed to cause a 50% reduction of the initial DPPH concentration. This value is known as the $ED_{50}$ (dose effective in 50% of test samples/subjects, also called oxidation index) and was expressed in terms of milligrams per milliliter. This assay was performed in triplicate for each sample, and the mean values were used to calculate the $ED_{50}$. α-Tocopherol was used as the positive control ($ED_{50}$ = 13.14 ± 0.24 μg/mL).

*Antitumor Assay by MTT Testing.* The assay using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) against the Doay (Human medulloblastoma) and HEp-2 (Human laryngeal carcinoma) tumor cells was based on the methods of Huang et al.[13] Mitomycin was used as a positive control (Doay: $EC_{50}$ = 0.17 μg/mL; HEp-2: $EC_{50}$ = 0.17 μg/mL).

## RESULTS AND DISCUSSION

In this study, the EtOAc extracted (2.5 g) of cultured supernatant (18 L) was applied to silica gel column chromatography with a $CH_2Cl_2$/MeOH solvent system, to yield nine subfractions (fractions A−G and MeOH). The DPPH radical-scavenging activities of these fractions were measured. Fractions G, E, D, and F demonstrated high activity, and $ED_{50}$ values of 12.41, 20.75, 28.51, and 41.24 μg/mL, respectively. These fractions that contained several DPPH radical-scavengers were further applied to the RP-HPLC system to isolate the secondary metabolites from the cultured supernatant of *S. ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source. Finally, seven compounds were isolated and their chemical structures were characterized using the $^1$H NMR, $^{13}$C NMR, HMQC, HMBC, and MS spectral data and comparison of $^1$H and $^{13}$C NMR data with those published in the literature.

**Table 1. NMR Spectroscopic Data of Compound 1 ($CD_3OD$)[a]**

| position | $^1$H NMR[b] | $^{13}$C NMR[c] | HMBC |
|---|---|---|---|
| 1 | | 177.5 (s) | |
| 2 | 3.68 (2H, s) | 32.8 (t) | 1, 3, 4, 4a |
| 3 | 7.14 (1H, s) | 124.5 (d) | 4a, 8a |
| 4 | | 109.6 (s) | |
| 4a | | 128.8 (s) | |
| 5 | 7.54 (1H, d, *J* = 8.0 Hz) | 119.5 (d) | 4, 7, 8a |
| 6 | 6.99 (1H, dd, *J* = 8.0, 8.0 Hz) | 119.7 (d) | 4a, 8 |
| 7 | 7.07 (1H, dd, J = 8.0, 8.0 Hz) | 122.3 (d) | 5, 8a |
| 8 | 7.33 (1H, d, *J* = 8.0 Hz) | 112.1 (d) | 4a, 6, 8a |
| 8a | | 138.0 (s) | |

[a]Assignments were confirmed by $^1$H−$^1$H COSY (correlation spectroscopy), HMQC (heteronuclear multiple quantum coherence), and HMBC (heteronuclear multiple bond coherence). [b]$^1$H NMR spectrum was measured at 400 MHz. [c]The $^{13}$C NMR spectrum was measured at 100 MHz.

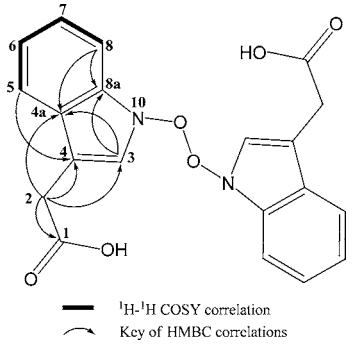


**Figure 2.** $^1$H−$^1$H COSY correlations and key of HMBC correlations for compound **1**.

Compound **1** was isolated as a yellow powder. The IR (KBr) spectrum showed absorption bands due to a hydroxyl group (3366 $cm^{-1}$) and carbonyl (1713 $cm^{-1}$). The $^{13}$C NMR spectrum (100 MHz, $CD_3OD$; Table 1) showed resonances for 10 carbons, which can be differentiated by DEPT experiments into one methylene (δ 32.8), five methine (δ 112.1, 119.5, 119.7, 122.3, 124.5, 128.8), and four quaternary carbons (δ 109.6, 128.8, 138.0, 177.5). Furthermore, the UV absorption bands were observed at 244, 261, and 299 nm, which implied that compound **1** could be an indole-type compound. The $^1$H NMR spectrum of **1** showed one singlet signal at δ 3.68 (s, 2H), which had HMBC (heteronuclear multiple-bond connectivity, Figure 3) correlations with three quaternary carbons (δ 109.6, C-4; 124.5, C-3; 128.8, C-4a) and a carbonyl carbon (δ 177.5, C-1). The planar skeleton structure of compound **1** is established by $^1$H−$^1$H COSY and HMBC spectral data. Through the literature search, we found that compound **1** possessed an indole skeleton as 3-indoleacetic acid, due to the similar signals of $^1$H and $^{13}$C NMR spectral data: $\delta_H$ 3.65 (2H, s), 7.02 (t, *J* = 7.5 Hz), 7.11 (t, *J* = 7.5 Hz), 7.17 (1H, s), 7.35 (t, *J* = 8.0, 8.0 Hz), 7.53 (d, *J* = 7.9 Hz); $\delta_C$ 177.7, 138.1, 128.3, 124.7, 122.6, 121.1, 119.4, 112.3, 108.9, and 33.9.[14] Moreover, the molecular formula of **1** was determined to be $C_{20}H_{16}N_2O_6$ (calcd 380.1008) from its ESI-MS spectral data $[M + H]^+$ *m*/*z* 381.2629. These agreed that compound **1** contained two *O*-10*N*-indole skeletons connected by *N*−*O*−*O*−*N* structures. Together with the above findings, compound **1** was established as [1,1-peroxybis(1*H*-indol-3,1-diyl)]diacetic acid and has been named serlyticin-A.

The molecular formula of compound **2** was determined to be $C_{21}H_{23}N_3O_7$ from its ESI-MS *m*/*z* 452 $[M + Na]^+$, 428 $[M - H]^-$; HRESI-MS *m*/*z* 430.1648 $[M + H]^+$, 428.1471 $[M - H]^-$ (calcd 429.1536, $C_{21}H_{23}N_3O_7$). The $^1$H NMR (400 MHz, pyridine-$d_5$) spectrum showed the following signals: δ 1.48 (3H, d, *J* = 6.4 Hz, H-12), 1.87 (2H, m, H-4′), 3.50 (2H, m, H-5′), 3.60 (2H, m, H-3′), 4.67 (d, *J* = 8.0 Hz, H-9), 5.19 (m, H-10), 6.82 (t, *J* = 8.0 Hz, H-5″), 6.88 (t, *J* = 8.0 Hz, H-5), 7.33 (dd, *J* = 8, 1.2 Hz, H-4), 7.34 (dd, *J* = 8.0, 1.2 Hz, H-4″), 7.35 (dd, *J* = 8.0, 1.2 Hz, H-6), 7.71 (dd, *J* = 8.0, 1.2 Hz, H-6″), 8.88 (t, *J* = 6.0, NH-2′) and 9.48 (t, *J* = 6.0 Hz, NH-6′). The $^{13}$C NMR (100 MHz, pyridine-$d_5$) spectrum showed 21 signals: δ 21.0 (q, C-12), 29.9 (t, C-4′), 36.6 (t, C-5′), 36.7 (t, C-3′), 75.0 (d, C-9), 79.6 (d, C-10), 111.1 (s, C-1), 116.3 (s, C-1″), 117.6 (d, C-6″), 118.7 (d, C-4), 118.8 (d, C-5″), 119.2 (d, C-5), 119.7 (d, C-4″), 120.7 (d, C-6), 147.4 (s, C-3), 148.1 (s, C-3″), 149.5 (s, C-2), 151.0 (s, C-2″), 167.2 (s, C-7), 171.1 (s, C-1′), and 170.9 (s, C-7′). On the basis of the above observation, compound **2** was a novel compound, which was isolated and published in 2010 by our laboratory[15] (Figure 2).

The other known compounds, serratamolide A (**3**), thymine (**4**), (4-hydroxy-phenyl)-acetic acid (**5**), methyl *p*-hydroxybenzoate (**6**), and uracil (**7**), were unequivocally identified by NMR spectroscopic analysis and comparisons with authentic samples and data reported in the literature.[16−20] In this study,


dx.doi.org/10.1021/jf302481n | J. Agric. Food Chem. 2012, 60, 9043−9047

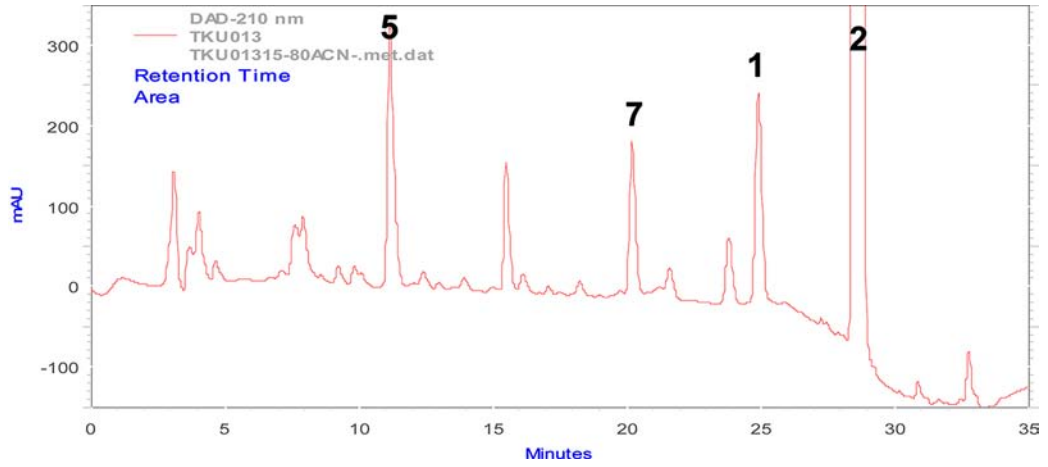


**Figure 3.** Major components of EtOAc extract of the cultured supernatant. 10 μL of EtOAc extract (dissolved in MeOH) was injected with a flow rate of 1.0 mL/min. Absorbance at 210 nm. The major secondary metabolites from the cultured supernatant of *S. ureilytica* TKU013 were compounds **1**, **2**, **5**, and **7**, which were identified by HPLC fingerprinting.

**Table 2. Inhibition Activity of Cell Proliferation of Fractions A−H and Fraction MeOH by MTT Assay**[a]

| | growth inhibition (%) | |
|---|---|---|
| sample | Doay | HEp-2 |
| fraction A | 25.36 | 17.37 |
| fraction B | 1.58 | 9.43 |
| fraction C | 1.31 | 2.40 |
| fraction D | 28.99 | 5.24 |
| fraction E | 43.54 | 44.64 |
| fraction F | 46.30 | 39.68 |
| fraction G | 17.47 | 2.50 |
| fraction H | 24.24 | 8.47 |
| fraction MeOH | 59.52 | 37.56 |

[a]Cell line: Doay (human medulloblastoma), HEp-2 (human laryngeal carcinoma). Test concentration: 80 μg/mL. Positive control is Mitomycin *c*: Doay, $ED_{50}$ = 0.17 μg/mL; HEp-2, $ED_{50}$ = 0.17 μg/mL.

antioxidative and antitumoral fractions and seven antioxidative compounds were yielded from the cultured supernatant of *S. ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source. Their chemical structures were elucidated as serlyticin-A (**1**), serranticin (**2**), serratamolide A (**3**), thymine (**4**), (4-hydroxy-phenyl)-acetic acid (**5**), methyl *p*-hydroxybenzoate (**6**), and uracil (**7**) by analyses of their $^{1}H$, $^{13}C$, $^{1}H-^{1}H$ COSY, HMQC, HMBC, and MS spectral data. Compound **2** was a novel compound, and it could be speculatively produced from fermentation process. Furthermore, the major secondary metabolites (**1**−**7**) from the cultured supernatant of *S. ureilytica* TKU013 were also identified by HPLC fingerprinting. Compounds **1** and **7** showed the gentle DPPH radical-scavenging activity with $ED_{50}$ values of 110.67 ± 1.75 and 142.39 ± 0.30 μg/mL, respectively. Notably, compound **2** ($ED_{50}$ = 3.28 ± 0.03 μg/mL) showed a more potent DPPH radical-scavenging activity than that of α-tocopherol ($ED_{50}$ = 13.14 ± 0.24 μg/mL), whereas **3**−**6** were not prominent for DPPH radical-scavenging activity (data not shown). The cytotoxic activities of the subfractions from the EtOAc extract of cultured supernatant of *S. ureilytica* TKU013 with squid pen as the sole carbon/nitrogen source were tested against two tumoral cell lines, Doay (human medulloblastoma) and HEp-2 (human laryngeal carcinoma). The results showed that both Doay and HEp-2 cells were not significantly inhibited by these fractions. All of these fractions (80 μg/mL) showed inhibition rates less than about 50% against Doay and HEp-2 tumoral cell lines (Table 2). Our data may contribute to a rational basis for the use of squid pen in the pharmacotherapy of diseases related to antioxidant and antitumor actions and provide a useful example of utilizing this biowaste material as a valuable ingredient.

## AUTHOR INFORMATION


### Corresponding Author

*Tel.: +886-2626-9425. Fax: +886-2-2620-9924. E-mail: sabulo@mail.tku.edu.tw.

### Present Address

⊥151 Yinchuan Road, Tamsui, New Taipei, Taiwan.



### Funding

This work was supported in part by grants from the National Science Council and the National Research Institute of Chinese Medicine, Taiwan.


### Notes

The authors declare no competing financial interest.

## REFERENCES


(1) Wang, S. L.; Kao, T. Y.; Wang, C. L.; Yen, Y. H.; Chen, M. K.; Chen, Y. H. A solvent stable metalloprotease produced by *Bacillus* sp. TKU004 and its application in the deproteinization of squid pen for β-chitin preparation. *Enzyme Microb. Technol.* **2006**, *39*, 724−731.

(2) Synowiecki, J.; Al-Khateeb, N. A. A. Q. The recovery of protein hydrolysate during enzymatic isolation of chitin from shrimp *Crangon crangon* processing discards. *Food Chem.* **2000**, *68*, 147−152.

(3) Wang, S. L.; Yeh, P. Y. Production of a surfactant- and solventstable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by *Bacillus subtilis* TKU007. *Process Biochem.* **2006**, *41*, 1545−1552.

(4) Wang, S. L.; Wang, C. Y.; Huang, T. Y. Microbial reclamation of squid pen for the production of a novel extracellular serine protease by *Lactobacillus paracasei* subsp *paracasei* TKU012. *Bioresour. Technol.* **2008**, *99*, 3411−3417.

(5) Wang, S. L.; Lin, C. L.; Liang, T. W.; Liu, K. C.; Kuo, Y. H. Conversion of squid pen by *Serratia ureilytica* for the production of enzymes and antioxidants. *Bioresour. Technol.* **2009**, *100*, 316−323.

(6) Seymour, T. A.; Li, S. J.; Morrissey, M. T. Characterisation of a natural antioxidant from shrimp shell waste. *J. Agric. Food Chem.* **1996**, *44*, 682−685.

(7) Farombi, E. O.; Britton, G.; Emerole, G. O. Evaluation of the antioxidant activity and partial characterisation of extracts from browned yam flour diet. *Food Res. Int.* **2000**, *33*, 493−499.
(8) Canadanovic-Brunet, J. M.; Djilas, S. M.; Cetkovic, G. S.; Tumbas, V. T.; Mandic, A. I.; Canadanovic, V. M. Antioxidant activities of different *Teucrium montanum L.* extracts. *Int. J. Food Sci. Technol.* **2006**, *41*, 667−673.
(9) Diouf, P. N.; Stevanovic, T.; Cloutier, A. Study on chemical composition, antioxidant and anti-inflammatory activities of hot water extract from *Picea mariana* bark and its proanthocyanidin-rich fractions. *Food Chem.* **2009**, *113*, 897−902.
(10) Ito, N.; Hirose, M.; Fukushima, S.; Tsuda, H.; Shirai, T.; Tatematsu, M. Studies on antioxidants: Their carcinogenic and modifying effects on chemical carcinogenesis. *Food Chem. Toxicol.* **1986**, *24*, 1071−1082.
(11) Wang, S. L.; Shin, I. L.; Liang, T. W.; Wang, C. H. Purification and characterization of two antifungal chitinases extracellularly produced by *Bacillus amyloliquefaciens* V656 in a shrimp and crab shell powder medium. *J. Agric. Food Chem.* **2002**, *50*, 2241−2248.
(12) Shimada, K.; Fujikawa, K.; Yahara, K.; Nakamura, T. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food Chem.* **1992**, *40*, 945−948.
(13) Huang, H. C.; Liaw, C. C.; Zhang, L. J.; Ho, H. U.; Yang, L. M.; Shen, Y. C.; Kuo, Y. H. Triterpenoidal saponins from hydrocotyle sibthorpioides. *Phytochemistry* **2008**, *69*, 1597−1603.
(14) Sun, D. D.; Dong, W. W.; Li, X.; Zhang, H. Q. Indole alkaloids from the roots of *Isatis ingigotica* and their antiherpes simplex virus type 2 (HSV-2) activity in vitro. *Chem. Nat. Compd.* **2010**, *46*, 763−766.
(15) Kuo, Y. H.; Liang, T. W.; Liu, K. C.; Hsu, Y. W.; Hsu, H. C.; Wang, S. L. Isolation and identification of a novel antioxidant with antitumour activity from *Serratia ureilytica* using squid pen as fermentation substrate. *Mar. Biotechnol.* **2010**, *13*, 451−461.
(16) Deepti, D.; Rolf, J.; Gabriella, M.; Manfred, N.; Bhavdish, N. J.; Victor, W. Antimycobacterial serratamolides and diacyl peptoglucosamine derivatives from *Serratia* sp. *J. Nat. Prod.* **2008**, *71*, 637−641.
(17) Kokko, J. P.; Goldstein, J. H.; Mandell, L. L. A nuclear magnetic resonance investigation of tautomerism and substituent effects in some pyrimidines and related nucleosides. *J. Am. Chem. Soc.* **1961**, *83*, 2909−2911.
(18) Scott, K. N. NMR parameters of biologically important aromatic acids. *J. Magn. Reson.* **1969**, *6*, 55−73.
(19) Pietersl Luc, A. C.; Agnes, M. V. Z.; Vrieling, K. Determination of the pyrrolizidine alkaloids from *Senecio jacobaea* by $^{1}H$ and $^{13}C$ NMR spectroscopy. *Magn. Reson. Chem.* **1989**, *27*, 754−759.
(20) Ganapathy, S.; Naito, A.; McDowell, C. A. Paramagnetic doping as an aid in obtaining high-resolution carbon- 13 NMR spectra of biomolecules in the solid state. *J. Am. Chem. Soc.* **1981**, *103*, 6011−6015.