Extraction of Yacon Leaves Enhances Enhydrin Degradation

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Summary: Sesquiterpene lactones (SLs) with various activities, which are primarily composed of enhydrin, are the primary constituents in the leaves of the yacon, *Smallanthus sonchifolius* (Peoepp. & Endl.) H. Robinson. To study the effect of heat extract on the degradation of enhydrin，the hydrolyzed products were isolated and their anticancer activity was assayed. The decoction extraction of yacon leaves under heat could enhance enhydrin degradation. However, the degradation was not observed when the pure compound (i.e., enhydrin) was refluxed. The major degradation products of enhydrin were isolated and identified to be 6-deacetyldeepoxydihydroxyenhydrin (1), deepoxydihydroxyenhydrin (2) and enhydrin chlorohydrin (3). In addition, the cytotoxic activity of the SLs obtained against human gastric cancer cells (MGC80-3) indicated that enhydrin is stronger than its degradation products. Our results further confirmed that the traditional tea using way of yacon leaves in the folks is reasonable.

Keywords: Yacon leaves, *Smallanthus sonchifolius*, Enhydrin, degradation, Anticancer activity.

Introduction

Yacon (*Smallanthus sonchifolius* (Peoepp. & Endl.) H. Robinson, family Asteraceae) is an indigenous edible plant in the Andes, South America. The yacon tubers are used in traditional Indian foods for the treatment of diabetes and intestinal disorders by the local people. Traditionally, the folks in Andean regions always processed yacon leaves into tea drinks for healthcare, especially used for diabetic patients. Pharmacological studies of the leaves have revealed a series of biological effects, such as antioxidant, anti-inflammatory, anti-diabetic, antibacterial, and antitumor actions [1-5]. Phytochemical studies have reported that the major chemical constituents in the leaves are sesquiterpene lactones (SLs), lignans and phenolic acids [2, 6-7]. Enhydrin is the major sesquiterpene lactone from the yacon leaves and exhibits NF-κB inhibition as well as antibacterial, anti-inflammatory and anti-diabetic activities [8]. Previously, the bioactive constituents of yacon leaves were engaged [9-12], further, the HPLC analysis of chemical constituents of the decoction of yacon leaves revealed that the contents of enhydrin was decreased as refluxing time went on, so the hydrolysates of enhydrin was identified by the spectroscopic analysis and their cytotoxic activity against human gastric cancer cells (MGC80-3) was also investigated compared to enhydrin. Our results further confirmed that the traditional tea using way in the folks is reasonable to benefit the medical value of yacon leaves.

Experimental

Reagents and Materials

HPLC-grade methanol was obtained from Tedia (Fairfield, USA). 5-Fluorouracil (5-FU), which was used as the positive control for the anticancer experiment, was obtained from Sigma, USA. Hydrochloric acid (analytical grade) was purchased from Kermel (Tianjin, China). The leaves of *S. sonchifolius* were collected from Dalian (Sept 10, 2013), China and identified by Professor Kang Tingguo in our university. A voucher specimen (Batch No. 20131001) has been deposited at the Pharmacognosy Laboratory, Liaoning University of TCM. Enhydrin and uvedalin with a purity of more than 98.5% were prepared in our laboratory and identified by NMR comparison with reference data [8].

Preparation of Tea Infusion

An aliquot consisting of 4 g of dried yacon leaves was weighed in an Erlenmeyer flask with a cap, and 100 mL of boiling water was added and allowed to stand for 1 h. The solution was filtered

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with a 0.45 μm Millipore film prior to analysis.

Preparation of the Decoction of Yacon Leaves

4 g of yacon leaves were accurately weighed in an Erlenmeyer flask with a cap and refluxed with 100 ml of distilled water for 1 h followed by cooling to room temperature. The solution was filtered with a 0.45μm Millipore film prior to analysis.

Refluxing of Enhydrin Solution

1.0 mg of enhydrin was dissolved in 10 ml 50% ethanol and refluxed for 1h. At 0, 30 and 60 min of refluxing time, the solution was analyzed by the HPLC as follows.

HPLC Analysis

The fingerprint of the water decoction of yacon leaves was achieved using analytical HPLC with an Agilent 1260 series over an Agilent reverse-phase ZORBAXSB-C18 column (4.6×150 mm,5μm particle size) protected by a pre-column from the same company eluting with MeCN(A)-H_2O containing 0.4% H_3PO_4 (B) in a gradient as follows: initial 10:90 (A:B) increased to 25:75 for 10 min, isocratic at 25:75 for 5 min, then run to 50:50 at the 30th min, to 65:35min at the 45th min, to 80:20 at the 55th min and changed to 0:100 after 5 min followed by an isocratic hold for 10 min, and finally reconditioning for 20 min. The flow rate was 1.0 mL/min, the temperature for column oven was set at 30ºC and a DAD detector was used at a wavelength of 210 nm

Hydrolysis of Enhydrin

A solution consisting of 100 mg of enhydrin in 70% MeOH (100 mL) adjusted with 1 N HCl to a pH of 4 was refluxed for 24 h. The solution was analyzed at 1 h, 2 h, 4 h, 12 h and 24 h by HPLC using an ODS column eluting with 60% MeOH-H_2O with the detection wavelength set to 210 nm. After 80% of the enhydrin was hydrolyzed, the solution was evaporated in vacuo to dryness and then separated by semi-preparative HPLC over YMC-Pack ODS-A (250×10 mm, 5μm) eluting with 60% MeOH to yield hydrolysates 1 (8 mg), 2 (15 mg), and 3 (17 mg).

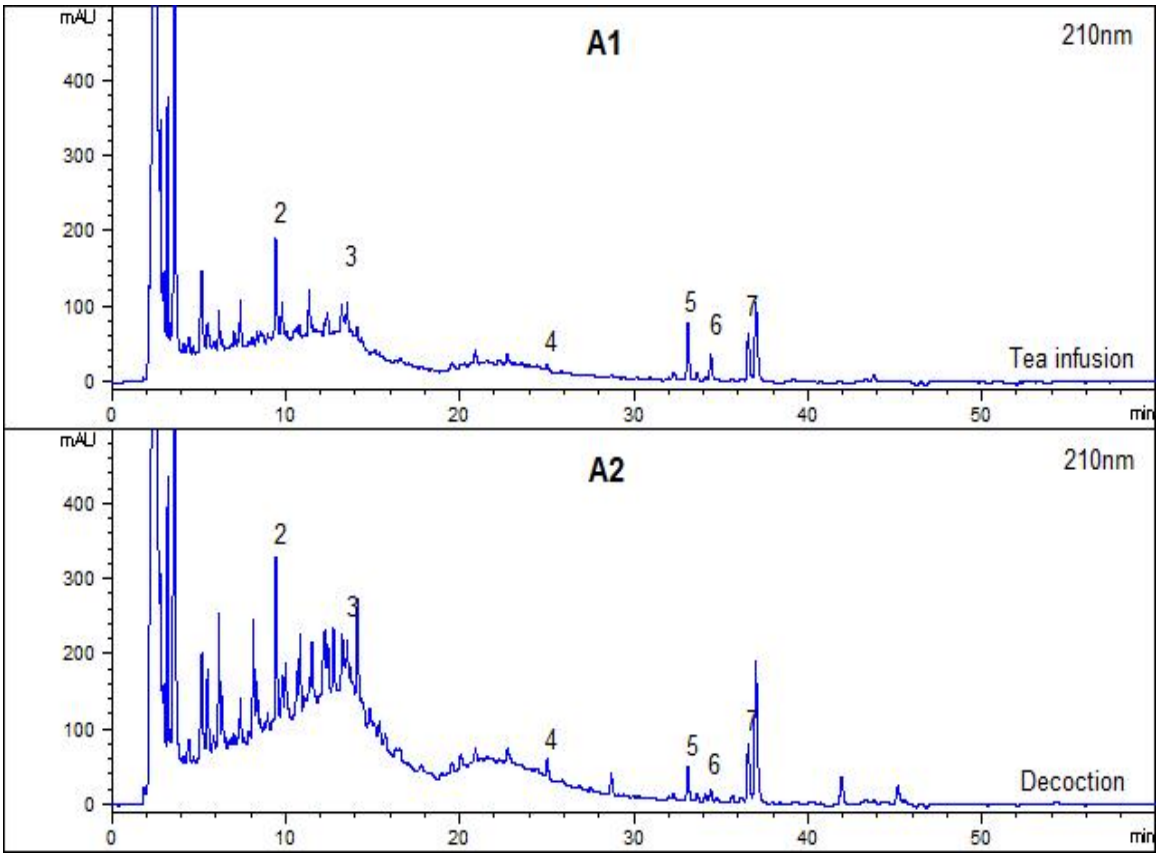
Compounds 2 and 3 were identified as the hydrolysates of enhydrin in previously reported in the literature [8] based on ^{1}H and ^{13}C NMR spectra. Compound 1 was identified as a new compound (TOF-MS (Agilent 6540Q): m/z: 458.2028 $[M+NH_4]^+$). The ^{1}H and ^{13}C NMR data are shown in Table-1.

Table-1: ^{1}H-NMR and ^{13}C-NMR data of 1 in $CDCl_3$.

No.	^{13}C-NMR	^{1}H-NMR	HMBC
1a	59.09		
2	35.44	2.32 m, 1.21 m	C-1a; C-3; C-12
3	24.71	2.58 m, 2.51 m	C-2; C-4
4	145.83	7.02(1H, t, J=7.95Hz)	C-3; C-5
5	132.79		
6	70.82	4.42(1H, d, J=8.35Hz)	C-5; C-7
7	74.08	6.34(1H, dd, J=8.60Hz)	C-6; C-7a; C-1'
7a	45.72	2.87(1H, dd, J=9.25Hz)	C-7; C-8; C-10a
8	133.09		
9	168.3		
10a	75.89	4.27(1H, t , J=9.65Hz)	C-7a; C-10b
10b	62.81	2.66 (1H, dd, J=9.65Hz)	C-1a; C-10a
11	123.04	Ha:5.79d,J=2.45Hz; Hb:6.32 d,J=3.10Hz	C-8; C-9
12	17.62	1.59 (3H,s)	C-1a; C-2
13	166.53		
14	52.54	3.83(3H, s)	C-13
1'	175.95		
2'	77.68		
3'	71.99	3.87 (q, J=6.15Hz)	C-4'
4'	16.27	1.19(3H, d, J=6.25Hz)	C-3'
5'	21.51	1.32(3H, s)	C-2'

Cytotoxicity Assay

The cytotoxicity bioassay was performed against human gastric cancer cells (MGC80-3, Cell bank, Shanghai Institute for Biological Sciences, Shanghai, China). The cells were maintained in RPMI 1640 (Gibco) containing 10% FBS (Gibco), 100 UmL^{-1}penicillin sodium salt and 100 U mL^{-1} streptomycin sulfate. The cells were grown to 80% confluence, trypsinized with 0.05% trypsin-2 mM EDTA and plated for experimental use. In all of the experiments, the cells were grown in RPMI-1640 medium with 10% FBS for 24 h prior to treatment. All of the compounds were dissolved in DMSO at a concentration of 100 mM followed by dilution in a tissue culture medium and filtered prior to use. 100 μl/well of 5×10^4 /ml cells were seeded in 96-well tissue culture plates and treated with the tested compounds or vehicle (0.1% DMSO) at various concentrations in triplicated and incubated for 48 h followed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay at 492 nm. Briefly, the IC_{50} values of the tested compounds on the MGC80-3 cell lines were obtained from the concentration effect curves.



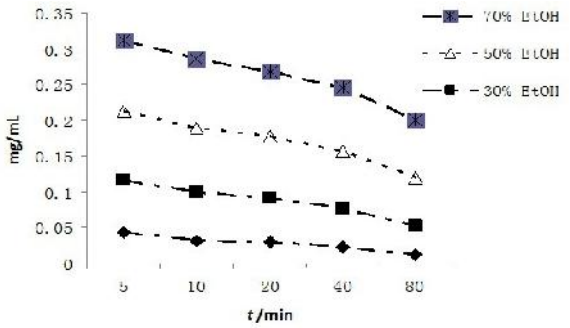
Note: During the course of chemical comparison of tea infusion and decoction of yacon leaves, enhydrin was discovered to be decreased as refluxing time went on. Several peaks were identified with retention times of authentic standards as:1: chlorogenc acid; 2: caffeic acid; 5：enhydrin; 6: smallanthaditerpenic acid A; 7: uvedalin

Fig. 1: HPLC chromatograms of the tea infusion (A1) and decoction (A2) of yacon leaves.

Results and Discussion

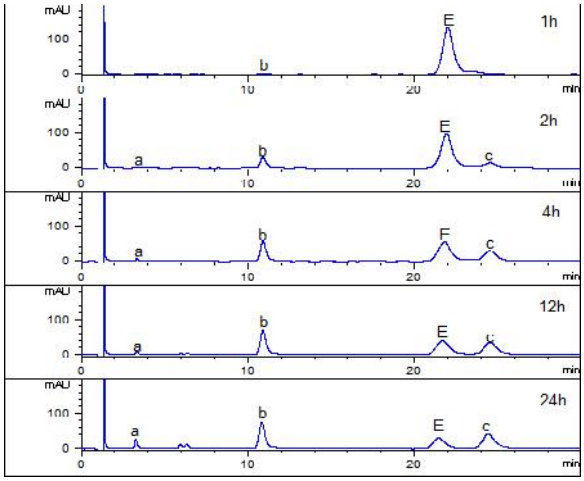
Traditionally, yacon leaves were always used as tea in the folks for the healthcare of local people. To compare the chemical constituents of yacon leaves with their tea infusion, we found that the content of enhydrin in the decoction was substantially decreased compared to that in the tea infusion (Fig. 1). Therefore, this decrease in enhydrin was further investigated using different extraction solvents and extraction times. As shown in Fig. 2, the extraction yield of enhydrin from the yacon leaves increased as the proportion of alcohol in the extraction solvents increased. In addition, the enhydrin content decreased in a time dependent fashion in all of the extraction solvents. However, when pure enhydrin was refluxed with 70% aqueous ethanol for 1h, the content of enhydrin exhibited almost no change and its peak areas were 2190, 2150 and 2150 for 0h, 0.5h and 1h refluxing, respectively.

Because phenolic acids, such as chlorogenic acid and caffeic acid, are also major components in yacon leaves and the extract solution exhibited weak acidity (PH = 4). We assumed that the degradation of enhydrin during extraction was caused by the acidic hydrolysis induced with the phenolic acidity. To validate this hypothesis, we hydrolyzed enhydrin under refluxing conditions in a hydrochloric acid solution, which adjusted the pH to 4. The production of the hydrolysates was monitored by HPLC analysis for 24 hrs. As shown in Fig. 3, three hydrolysates (1, 2, 3) were observed, and they were produced in the order of 2, 3 and 1 during hydrolysis. Then, the three hydrolysates were isolated by preparative HPLC to afford two known compounds (i.e., deepoxydihydroxyenhydrin (2) and enhydrin chlorohydrin (3)). The structures of these compounds were identified by comparison to previously reported spectroscopic data [8], and a new compound (1) was determined by extensive spectroscopic analysis.



Note: The experiments indicated that the concentration of enhydrin was decreased more quickly as the alcohol proportion in the solution become lower under refluxing condition.

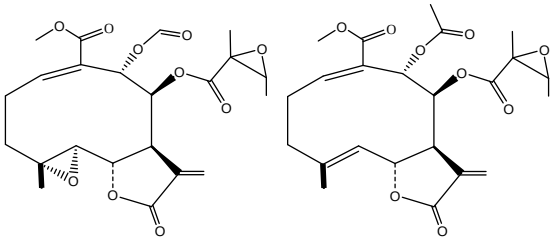
Fig. 2: Time-dependent decrease of enhydrin in solvents with different alcohol proportions.



Note: The HPLC analysis of enhydrin hydrolysates indicated that peak b was firstly transformed, then peak c and peak a. After isolation and identification of the hydrolysates, the peaks in HPLC can be marked as follows: Peak a : compound **1**; Peak b:compound **2**; Peak c: compound **3**; Peak E:enhydrin.

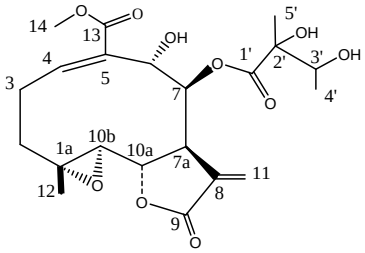
Fig. 3: HPLC profile of the hydrolysis of enhydrin at different times.

Compound 1 was obtained as a white powder. The molecular formula was determined to be $C_{21}H_{28}O_{10}$ from the pseudo-molecular ion at m/z 458.2028 $[M+NH_4]^+$ (calculated as 458.2026) in its HR-ESI-MS. In the ^{1}H NMR spectrum, two methyl singlets at δ 1.32 (H_3-5') and 1.59 (H_3-12), a methyl doublet at δ 1.19 (H_3-4') and a methoxy singlet at δ 3.83 (H_3-14) were observed (Table-1). In the ^{13}C NMR spectrum, three carboxyl resonances at δ 166.53, 168.30 and 175.95 as well as four olefinic carbons at δ 145.83 (C-4), 132.79 (C-5), 133.09 (C-8) and 123.04 (C-11) were observed. By comparing the ^{1}H and ^{13}C NMR data of 1 to those of 2, the resonances assigned to the 6-acetyl moiety in 2 disappeared in 1, and the proton resonance assigned to H-6 (δ 4.42) in 1 was shifted upfield (δ 5.88) in 2 by 1.46 ppm. These results indicated that 1 is the 6-deacetyl of 2. Further, heteronuclear multiple bond coherence (HMBC) and ^{13}C-^{1}H COSY of 1 further confirmed the structure and its ^{1}H-NMR and ^{13}C-NMR data were assigned as shown in Table-1. Thus the structure of 1 was characterized to be 14-methyl-7-((2,3-dihydroxy-2-Methyl-butanoyl)oxy)-6-hydroxy-1-methyl-8-methylene-9-oxo-cyclodeca [1,2-b] furan-5-carboxylate. According to the mother nuclei, it could also be named in short as 6-deacetyldeepoxydihydroxyenhydrin.

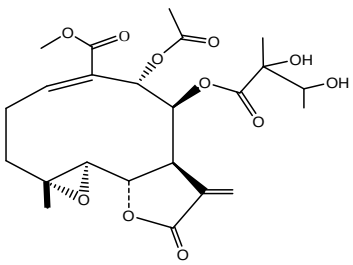


Enhydrin **Uvedalin**

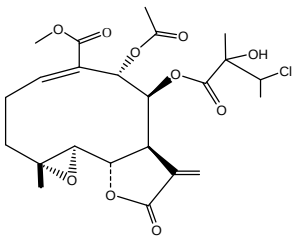
Fig. 4: Chemical structures.



(1)



(2)



(3)

Fig. 5: Chemical structures of SLs from yacon leaves.

Based on the acid hydrolysis result for enhydrin, both infusion and decoction of the yacon leaves were re-examined for the existence of degradation products from enhydrin. As expected, both compounds 1 and 2 were confirmed in the extract solution (Fig. 1), which indicated that the degradation of enhydrin was most likely due to hydrolysis of the 2',3'-epoxy moiety and 6-deacetylation.

Because sesquiterpene lactones from Yacon leaves exhibit cytotoxic activity [9], we also assessed the cytotoxic activity of enhydrin, uvedalin and the three hydrolysates (1-3) from enhydrin against human gastric cancer cells (MGC80-3). The results are shown in Table-2. Enhydrin and uvedalin, whose structures are only different by the epoxide or olefin moiety at positions 1a and 10b, exhibited nearly the same cytotoxic potency. The activity substantially decreased for hydrolysates 1 and 2, suggesting that the 2',3'-epoxy moiety is important for the activity. However, the substitution of 3'-chlroride (3) instead of a hydroxyl restored the activity.

Table-2: IC_{50} of cytotoxicity of SILs from yacon leaves.

Samples	IC_{50} («M)
5-Fu	56.37
Enhydrin	9.12
Uvedalin	8.14
6-deacetyldeepoxydihydroxyenhydrin (1)	115.14
Deepoxydihydroxyenhydrin (2)	37.64
Ehhydrin chlorohydrin (3)	9.42

Conclusion

In conclusion, for the first time, we discovered that enhydrin is easily hydrolyzed during the heat extraction of yacon leaves, and the chemical structures of the hydrolysates were identified. In addition, the cytotoxic activity of hydrolysates 1 and 2 is lower than enhydrin and enhydrin is labile in the process of heat extraction, enlightening that the tea is a better way for taking yacon leaves than decoction in prevention and treatment for cancer. These results will benefit the future utilization of yacon leaves.

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References

1. V. Katerina，C. Ladislav，M. Alexandr，U. Jitka，S. Vilim, Antioxidant Activity of Extracts from the Leaves of Smallanthus sonchifolius, *Eur. J. Nutrition*, **42**, 61 (2003).
2. R. B. Oliveira, C. A. Daniela, S. Adriana, G. H. Thais, F. H. Lucia, C. P. Anna, D. Costa, B. Fernando, Topical Anti-Inflammatory Activity of Yacon Leaf Extracts, *Rev. Brasil. de Farmacog.*, **23**, 497 (2013).
3. S. B. Genta, W. M. Cabrera, M. I. Mercadob, A. Grauc, C. A. Catalánb, S. S. Sáncheza, Hypoglycemic Activity of Leaf Organic Extracts from Smallanthus sonchifolius: Constituents of the Most Active Fractions, *Chem. Bio. Interactions* **185**, 143 (2010).
4. F. Q. Lin, M. Hasegawa, O. Kodama, Purification and Identification of Antimicrobial Sesquiterpene Lactones from Yacon (Smallanthus sonchifolius) Leaves, *Biosci. Biotech. and Biochem.* **67**, 2154 (2003).
5. D. Moura, C. A. Nelci, F. R. Brunno, K. Sivieri, L. H. Urbano, C. Cabello, M. Rodrigues, Protective Effects of Yacon (Smallanthus sonchifolius) Intake on Experimental Colon Carcinogenesis, *Food Chem. Toxicol.*, **50**, 2902 (2012).
6. J. L. Lv, Z. Liao, L. Wang, G. Y. Yang, Y. L. Yang, Extraction of Lignan from the Leaves of Smallanthus sonchifolius and its Antioxidant Activity, *Asian J. Chem.*, **24**, 312 (2012).
7. D. Jirovsky, D. Horakova, M. Kotoucek, K. Valentova and J. Ulrichova. Analysis of Phenolic Acids in Plant Materials using HPLC with Amperometric Detection at a Platinum Tubular Electrode, *J. Sep. Sci.* **26**, 739 (2003).
8. G. Y. Ma, S. I. Khan, G. Benavides, W. Schuehly, N. H. Fischer, I. A. Khan, D. S. Pasco, Inhibition of NF-κB-Mediated Transcription and Induction of Apoptosis by Melampolides and Repandolides, *Cancer Chemother. Pharmacol.* **60**, 35 (2007).
9. D. Q. Dou，F. Tian, Y. K. Qiu, Z. Xiang, B. X. Xu, T. G. Kang and F. Dong, Studies on Chemical Constituents of the Leaves of Smallantus sonchifolius (yacon): Structures of Two New Diterpenes, *Nat. Prod. Res.* **24**, 40 (2010).
10. Z. Xiang, K. Gai, D. Q. Dou, T. G. Kang, Y. Y. Shi and F. Dong. New Compounds from Leaves of Smallanthus sonchifolius, *Nat. Prod. Comm.*, **4**, 1201 (2009).
11. D. Q. Dou, F. Tian, Y. K. Qiu, T. G. Kang, F. Dong. Structure Elucidation and Complete NMR Spectral Assignments of Four New Diterpenoids from Smallantus sonchifolius, *Mag. Reson. Chem.*, **46**:775 (2008).
12. Z. Xiang, F. He, T. G. Kang, D. Q. Dou, K. Gai, Y. Y. Shi, Y. H. Kim, F. Dong. Anti-Diabetes Constituents in Leaves of Smallanthus sonchifolius, *Nat. Prod. Commun.*, **5**,95 (2010).