International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2017.74.82

Research Article
Anticancer Action and Pharmacokinetics of Sesquiterpene Lactone Extracts of Yacon Leaves

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Abstract

Objective: The anticancer actions of extracts of yacon leaves mainly composed of sesquiterpene lactone were assayed with H22 tumor mice model *in vivo* and pharmacokinetics of the extracts were also examined. About 200 mg kg^{-1} of the extracts showed 35.99% tumor inhibitory rate and up-regulation of IL-2 levels and down-regulation of TNF-α. **Methodology:** High-performance liquid chromatography (HPLC) method was used for the study of pharmacokinetics of enhydrin and uvedalin of the extract with artemisinin as internal standard after oral administration at a dose of 200 and 100 mg kg^{-1}, respectively. **Results:** Their pharmacokinetics parameters were calculated as follows: The t_{max} for enhydrin and uvedalin is similar at 1.5±0 h in both doses and C_{max} for enhydrin and uvedalin were 13.416±0.210 and 8313.31±0.23 mg mL^{-1} in high dose and 6.887±0.120 and 4231.45±0.17 mg mL^{-1} in low dose, respectively. The $AUC_{0\rightarrow t}$ of enhydrin and uvedalin were 137.444±30.782 and 17345.375±613.231 mg L^{-1} h^{-1} in high dose and 43.426±19.663 and 8831.724±555.122 mg L^{-1} h^{-1} in low dose, respectively. **Conclusion:** The anti-cancer action of sesquiterpene extracts of yacon leaves was explored. Further, a simple and specific HPLC method was developed for the determination of enhydrin and uvedalin from yacon leaves extract in rat plasma. This study laid a foundation for the further utilization of yacon leaves.

Key words: Anti-cancer, high-performance liquid chromatography, pharmacokinetics, rat plasma, anti-cancer

Received: July 27, 2016 Accepted: November 06, 2016 Published: December 15, 2016

Citation: Jun Bai, Tianjiao Suo, Xu Wei, Peiyuan Dou, Xiaoku Ran, Khin Khin Win Aung, Deqiang Dou and Zheng Zeng, 2017. Anticancer action and pharmacokinetics of sesquiterpene lactone extracts of yacon leaves. Int. J. Pharmacol., 13: 74-82.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Yacon, *Smallanthus sonchifolius* (Peoepp. and Endl.) H. Robinson, which was originally cultivated in the Andean highlands of South America, was introduced into China in the 1990s. It has been reported that the tubers of yacon contain a higher concentration of oligofructans and was regarded as the fruit for diabetic patient[1]. Yacon leaves is traditionally used as anti-diabetes tea in folk Andean regions and now it has been introduced into Japan, Korea, China and Southeast Asia. There are several tea products of yacon leaves worldwide. Previously, the anti-diabetes, antioxidant and antitumor actions of yacon leaves were performed, indicating that the anti-diabetic action of ethanol extract of yacon leaves is stronger than its water extract and the polyphenols such as caffeic and chlorogenic acids and dicaffeoic quinic derivates were regarded as the active constituents for its anti-diabetes action[2,3]. Further, several smaditerpenic acids were discovered to possess the inhibitory effects on α-glucosidase similar to acarbose[4,5] and our most recent study indicated that the sesquiterpene lactone represented by enhydrin and uvedalin from yacon leaves possessed stronger anticancer activity[6]. To study the anticancer action of yacon leaves, the yacon leaves extract with over 45% total lactone was prepared with 95% EtOH and its anticancer action was evaluated by the H22 tumor mice mode. This study dealt with the component analysis of the yacon leaves extracts and its *in vivo* anticancer action as well as pharmacokinetic study of sesquiterpenoid lactones yacon leaves extracts after oral administration in rats.

MATERIALS AND METHODS

Chemicals: Enhydrin, uvedalin, chlorohydrin, chlorouvedalin and polymatin B, etc., standard samples were isolated from the yacon leaves in this laboratory and their purity were determined to be over 98% by HPLC analysis. The internal standard, artemisinin (Fig. 1) was provided by the Meilun Biological Pharmacy (Dalian, Liaoning, China). Methanol and acetonitrile (HPLC grade) were obtained from Damao Chemical Reagent Co., Ltd. (Tianjin, China) and the water used in all experiments was purified by Pincheng Pure Water System (Pincheng Technology Co., Ltd., Chengdu, China). Dichloromethane of analytical reagent grade was purchased from Damao Chemical Reagent Co., Ltd., Tianjin, China. Tween 80 from Kermel chemical reagent Co., Ltd., Tianjin, China and heparin sodium from Aladdin Biochemical Science and Technology Co., Ltd., Shanghai, China were used for anticoagulation for the plasma.

Chromatographic equipment and conditions for HPLC analysis: The analysis were carried out on an Agilent 1100 series HPLC system (Agilent technology, Palo Alto, CA, USA) which consisted of a quaternary pump (G1311A), a vacuum degasser (G1322A), a uv-vis spectrophotometric detector (G1314A) and chemstation software (Agilent). The analytical column was a Zorbax Eclipse Plus-C18 column (150×4.6 mm i.d., 5 μm, USA) protected by a C18 guard column (35×8.0 mm, i.d., 5 μm, USA). The mobile phase for HPLC analysis for pharmacokinetic consisted of water (A) and methanol (B) (0-10 min, A 60-50%, 10-15 min, A 50-40%, v/v) was passed under vacuum through a 0.45 μm membrane filter and degassed before use. All the chromatographic measurements were performed at 30°C and a flow rate of 1 mL min^{-1} with the detection wavelength of 210 nm. The mobile phase for HPLC analysis for yacon leaves extract consisted of methanol (A) and 0.1% phosphoric acid water (B) (0-20 min, A 25-50%, 20-35 min, A 50-60%, 35-60 min, A 60-80%, 60-80 min, A 80-100%, v/v) was also passed under vacuum through a 0.45 μm membrane filter and degassed before use. All the chromatographic measurements were performed at 30°C and a flow rate of 1 mL min^{-1} with the detection wavelength of 210 nm.

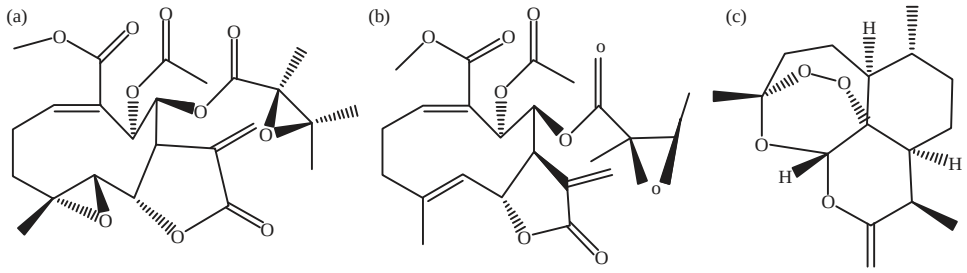

Fig. 1(a-c): Structures of (a) Enhydrin, (b) Uvedalin and (c) Artemisinin

Plant material and its extract: The yacon leaves were collected from Dalian, Liaoning Province, China in October 2012 and were identified by Professor Bing Wang, College of Pharmacy, Liaoning University of Traditional Chinese Medicine. A voucher specimen (YG-2012101) has been deposited in the College of Pharmacy, Liaoning University of Traditional Chinese Medicine.

Dried yacon leaves were extracted twice with 95% (v/v) EtOH at 35°C (0.5 h each). After precipitation in the fridge at 4°C for 12 h, the solution was filtered and the resulting solution was decolorizated with 1% activated carbon for 50 min at 70°C. The solution was evaporated in vacuo to dryness and dissolved the residue with 50% ethanol and left in the refrigerator at 4°C for a night. Following by centrifuging at 3500×g for 10 min. The resulting supernatant was concentrated to dryness to yield a pale yellow residue powder.

Animals: Male Sprague-Dawley (SD) 200-230 g were obtained from the Laboratory Animal Center of Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China, License No. 20160223). They were kept in an environmentally controlled breeding room for 1 week (Temperature: 20±2°C, relative humidity: 60±5%) before the experiments and fed with standard laboratory food and water *ad libitum* and fasted overnight before the experiment. All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Liaoning University of Traditional Chinese Medicine (131/2010).

Drug administration and blood collection: In the pharmacokinetic study, 10 rats were randomly assigned to 2 groups. The samples of yacon leaves extract was prepared with 5% tween-water (v/v) ultrasonically until dissolved completely to the concentration of 20 and 10 mg mL^{-1} for oral administration with 1 mL for every 100 g b.wt. Then, the rats were anesthetized and blood samples (0.4 mL) were collected into heparinized tubes from the vena orbitalis at times of 0.083, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 16, 20, 24, 28, 36 and 40 h for oral administration and then centrifuged at 1800×g for 10 min. The obtained plasma was stored at -20°C until analysis.

Plasma sample preparation: About 50 μL of I.S. (artemisinin, 285 μg mL^{-1}), 800 μL of acetonitrile were successively pipetted into the 200 μL plasma samples followed by vortex mixing for 1 min then centrifuged at 4500×g for 10 min at 4°C and 400 μL of acetonitrile for the second time in vortex mixing for 1 min then centrifuged at 4500×g for 8 min at 4°C. The supernatant was separated and evaporated to dryness under a stream of N_2 at room temperature. The supernatant were combined and constituted the residue in 30 μL HPLC grade methonal. About 20 μL aliquot of each supernatant was analyzed by HPLC.

Preparation of calibration standards and quality control samples: Stock standard solutions of enhydrin, uvedalin and artemisinin were prepared with methanol respectively. All solutions were stored at 4°C and found to be stable for at least 1 month. Seven calibrators (2.913, 7.825, 11.74, 23.48, 46.96 and 93.92 μg mL^{-1}) of enhydrin, (0.0383, 0.0965, 0.386, 0.772, 1.544, 3.088 and 3.65 mg mL^{-1}) of uvedalin and artemisinin (285 μg mL^{-1}) were prepared by dilution of stock solutions followed by spiking with drug free plasma. Quality Control (QC) samples were prepared at three concentrations (low, middle and high) representing the whole range of the calibration curve. The low concentration of QC (7.825 μg mL^{-1} for enhydrin and 96.50μg mL^{-1} for uvedalin) was 3 times of lower limit of quantitation, high concentration (78.25 μg mL^{-1} for enhydrin and 3088 μg mL^{-1} for uvedalin) was almost at 85% of the upper limit of quantitation and middle concentration (41.73 μg mL^{-1} for enhydrin and 545 μg mL^{-1} for uvedalin) was near the average mean and the geometric mean of low and high concentration. All of the QC samples were stored at -20°C until analysis.

RESULTS

***In vivo* anticancer action of yacon leaves extracts:** At least 10 mice were used for each sample and each dose. Mean values and SD were determined by standard methods[7]. The significant difference was estimated by the one-way ANOVA. Anticancer action was observed on H22 male mice. The test was performed by observing the effect on the growth of the tumor as described previously[8]. A dose of 0.2 mL of aseptic H22 cells (about 1.0×10^7 mL^{-1}) was implanted subcutaneously at the right groin of mice. About 24 h after the tumor implantation, the test sample was administrated once a day by oral administration for 10 days with cytoxan (CTX) as a positive control. After treatments, all rats were sacrificed and the tumor growth inhibition rate was calculated. Inhibition rate:

$$\text{Percentage} = \frac{\text{C-T}}{\text{C}}\times100$$

where, C is the average tumor weight of the control group and T is the tumor weight of the treated sample group (Table 1).

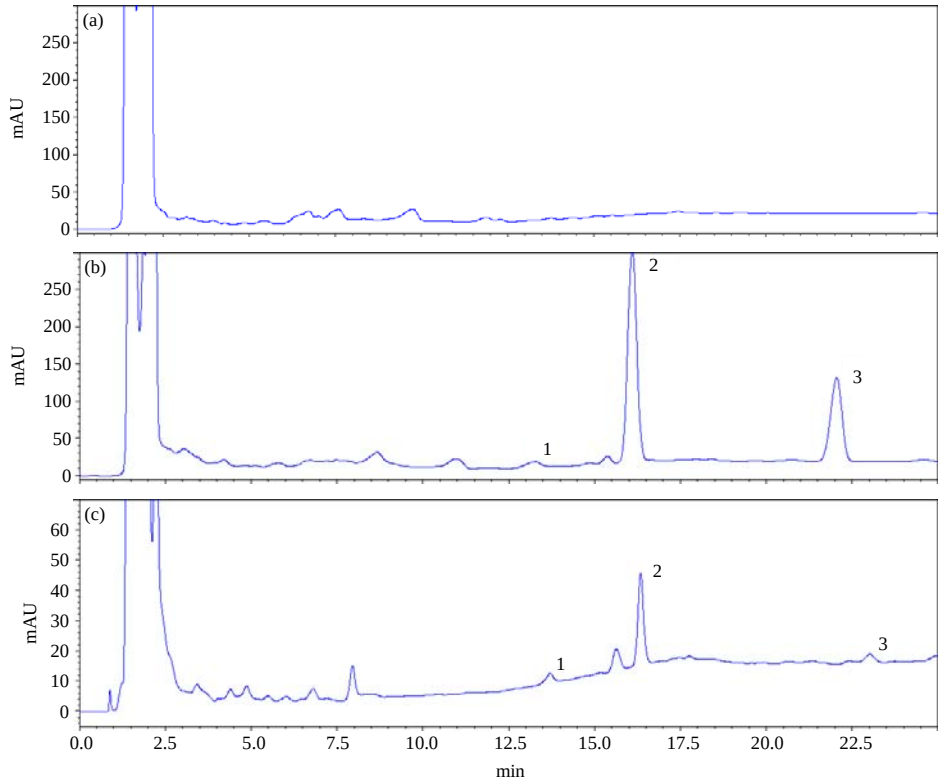

Fig. 2(a-c): Representative chromatograms of (a) Blank plasma, (b) Plasma spiked with corresponding standard samples and I.S. and (c) Plasma sample 20 min after the oral administration of yacon leaves extract, peak 1: Enhydrin, peak 2: Uvedalin, peak 3: I.S.: Artemisinin

Table 1: Effect of yacon extract on the inhibitory rate of H22 mice bearing H22 tumor ($\bar{x}\pm s$)

		No. of mice					
Groups	Doses (mg kg^{-1})	Begin	Over	Weigh (g)	Inhibitory rate (%)	IL-2 (ng L^{-1})	INF-α (ng L^{-1})
Normal	-	15	15	-	-	30.81±4.39**	70.46±10.18*
Model	-	15	15	1.13±0.29	-	20.79±2.18	78.88±5.110
Positive	40	15	11	0.04±0.02**	95.81	43.78±7.02**	86.62±14.73
Extract I	200	15	15	0.70±0.28**	35.99	26.29±3.01**	57.49±5.99**
Extract II	100	15	13	0.95±0.32	17.11	25.29±3.81**	66.22±13.40*

**p<0.01as compared with model

Method validation for the pharmacokinetics study

Selectivity: Selectivity was shown by comparing chromatograms of blank plasma obtained from rats prior to dosing with those of corresponding standard plasma samples spiked with and plasma samples from rats after the oral administration of yacon leaves extract (Fig. 2a-c).

To determine the selectivity of this method, blank rat plasma, plasma spiked with known amounts of enhydrin, uvedalin and I.S. and plasma samples from rats after oral doses of yacon leaves extract were respectively analysed. The chromatograms showed that there were no interfering peaks in the region of the peaks of the analyte and I.S. The retention times of enhydrin, uvedalin and IS were approximately 13.4, 15.5 and 22.8 min, respectively. The total run time was 30.0 min.

Linearity, LOD and LOQ: The evaluation of the linearity was performed with a seven-point calibration curve over the concentration range of 2.913-93.92 μg mL^{-1} for enhydrin and 38.25-3633 μg mL^{-1} for uvedalin.

The slope and intercept of the calibration graphs were calculated by weighted (1/cc) for both enhydrin and uvedalin least squares linear regression. The regression equation of the calibration curves was typically: $Y = 0.06752x+0.36643$ and R^2 was 0.99905, where Y is the peak area ratio of enhydrin to I.S. and x the plasma concentration of enhydrin while

Table 2: Accuracy and precision of enhydrin and uvedalin in rat's plasma (n = 5)

		RSD (%)			
	Concentration (μg mL^{-1})	Intra-day	Inter-day	Accuracy (%)	Extract recovery (%)
Enhydrin	7.825	5.44	6.00	97.8	87.17±3.26
	41.730	5.72	6.33	96.2	89.54±3.60
	78.250	4.30	5.56	95.9	90.13±1.72
Uvedalin	96.500	2.73	3.05	96.7	91.20±3.50
	545.000	4.00	11.83	99.1	87.15±1.10
	3088.000	2.84	2.78	95.4	87.00±2.00

Table 3: Pharmacokinetic parameters of enhydrin and uvedalin in rats (Mean±SD, n = 5) after oral administration at a dose of 200 and 100 mg kg^{-1}, respectively

	Oral administration			
	Enhydrin oral (mg kg^{-1})		Uvedalin oral (mg kg^{-1})	
Parameters	200	100	200	100
Cmax (μg mL^{-1})	13.416±0.210	6.887±0.120	8313.31±0.23	4231.450±0.17
Tmax (h)	1.5±0	1.500±0.00	1.5±0	1.500±0
Vz (L kg^{-1})	23.956±3.98	16.584±3.67	0.013±0.213	0.022±0.015
$t_{1/2\alpha}$ (h)	7.554±1.003	6.034±0.998	7.699±1.012	6.566±1.190
$t_{1/2z}$ (h)	7.054±0.43	6.198±0.676	7.628±1.739	6.796±2.178
AUC_{0-t} (mg L^{-1} h^{-1})	137.444±30.782	43.426±19.663	17345.375±613.231	8831.724±555.122
$AUC_{0-\infty}$ (mg L^{-1} h^{-1})	139.094±33.454	45.225±21.778	17359.473±663.122	8832.021±523.740
MRT_{0-t} (h)	2234.307±191.10	274.014±63.28	6.76±0.914	66979.658±783.169
$MRT_{0-\infty}$ (h)	2380.63±179.63	330.668±95.459	6.822±1.213	67011.072±992.781

$Y = 4.1434x+0.14435$, R^2 was 0.99785, where Y is the peak area ratio of uvedalin to I.S. and x the plasma concentration of uvedalin. The limit of detection (LOD) for enhydrin and uvedalin were 2.913 and 38.25 μg mL^{-1} (S/N = 3) while the limit of quantification (LOQ) were 7.825 and 96.5 μg mL^{-1} (S/N = 10).

Precision and accuracy: The RSD of three concentrations for enhydrin and uvedalin were ranged from 4.3-5.72 and 2.73-4.0% for intra-day assay, 5.56-6.33 and 2.78-11.83% for inter-day assay, respectively. The REs of intra and inter-day accuracy were within 2.2-4.1 and 0.9-4.6%. It is required that the precision (RSD) determined at each concentration level should not exceed 15% and accuracy (RE) be within ±15% of the actual value which conform to the criteria for the analysis of biological sample according to the guidance of US Food Drug and Administration (USFDA, 2001). These results suggested that the procedures described were satisfactory with respect to both precision and accuracy (Table 2).

Extract recovery: The average extract recoveries of enhydrin at three concentrations were 87.17±3.26, 89.54±3.60 and 90.13±1.72 and for uvedalin 91.2±3.50, 87.15±1.10 and 87.0±2.00%. The recoveries of I.S. was 90.41±1.97% suggesting that there was negligible loss during extract. The high recovery could be attributed to the high solubility of enhydrin and uvedalin in acetonitrile and the two-step protein precipitation used in the sample preparation.

Stability: The mean recoveries of the short-term stability, long-term stability and freeze-thaw stability of enhydrin and uvedalin in rat plasma were more than 95.66±0.43, 95.06±1.21 and 94.42±0.97%, respectively. The results showed that the drug in rat plasma was stable during chromatography, extract and sample storage processes and could not be degraded under these conditions.

Pharmacokinetic studies: Pharmacokinetic data were processed by DAS 2.0 software (China Mathematical Pharmacology Professional Committee of China, Shanghai, China). The plasma concentration-time curves of enhydrin and uvedalin in rats following oral administration of 200 and 100 mg kg^{-1} b.wt., are shown in Fig. 3, demonstrating that these two lactones were eliminated rapidly from the plasma. The plasma concentrations of enhydrin was detectable for only up to 8 h in rats by low dosage and 24 h in rats by high dosage, indicating that enhydrin was absorbed and eliminated in a day. While, uvedalin was detectable for only up to 36 h in rats by low dosage and 40 h in rats by high dosage, indicating that uvedalin was absorbed and eliminated slower than enhydrin. A two-compartment open model (Weight = 1/cc) gave the best fit to the plasma concentration-time curves obtained in rats. After administered with 100 and 200 mg kg^{-1} of yacon leaves extract, there was significant dose dependent increase in $t_{1/2}$. All pharmacokinetic parameter values are summarised in Table 3.

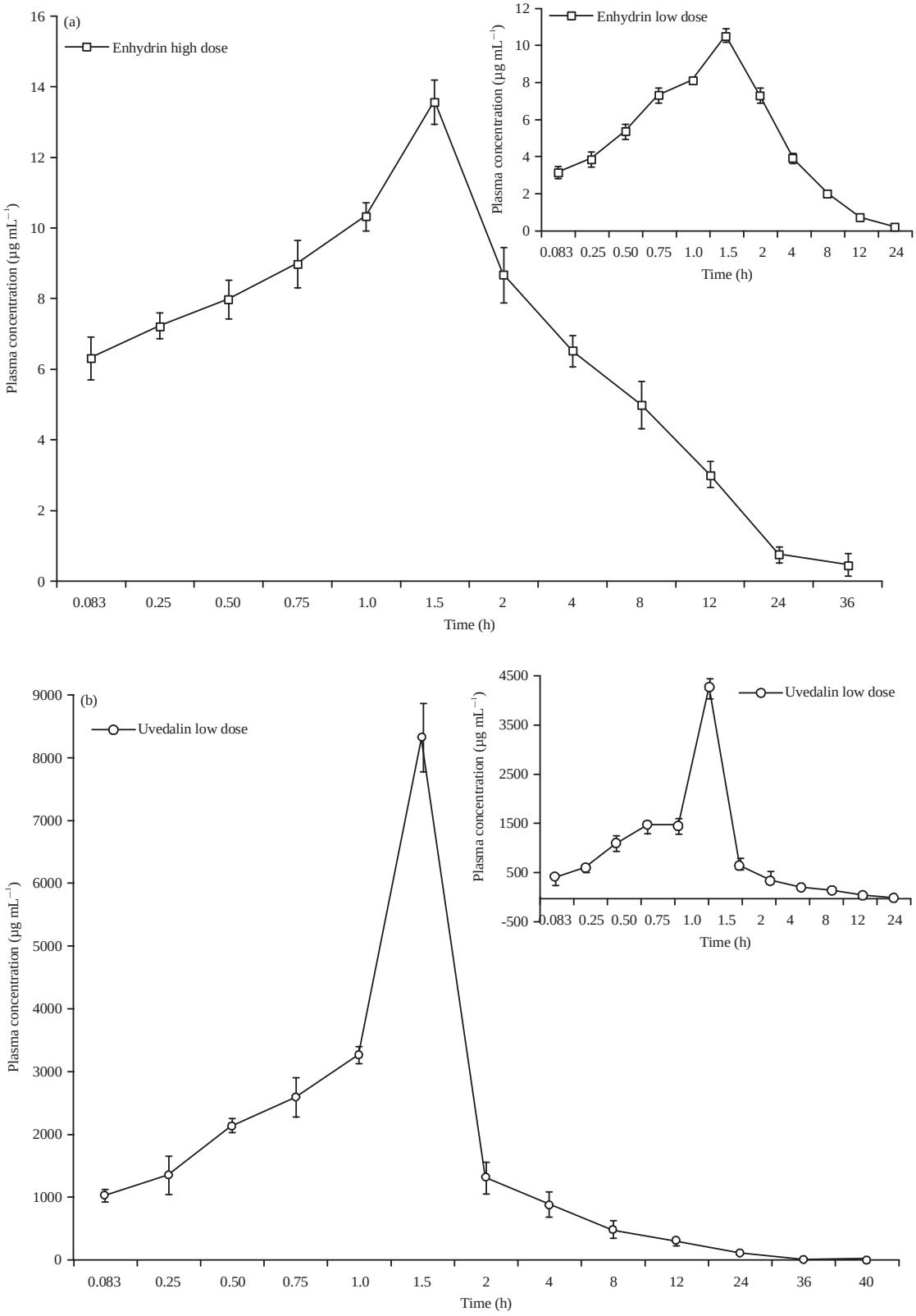

Fig. 3(a-b): Plasma concentration-time profile of yacon leaves extract after oral administration (200 mg kg^{-1} for high dose and 100 mg kg^{-1} for low dose)

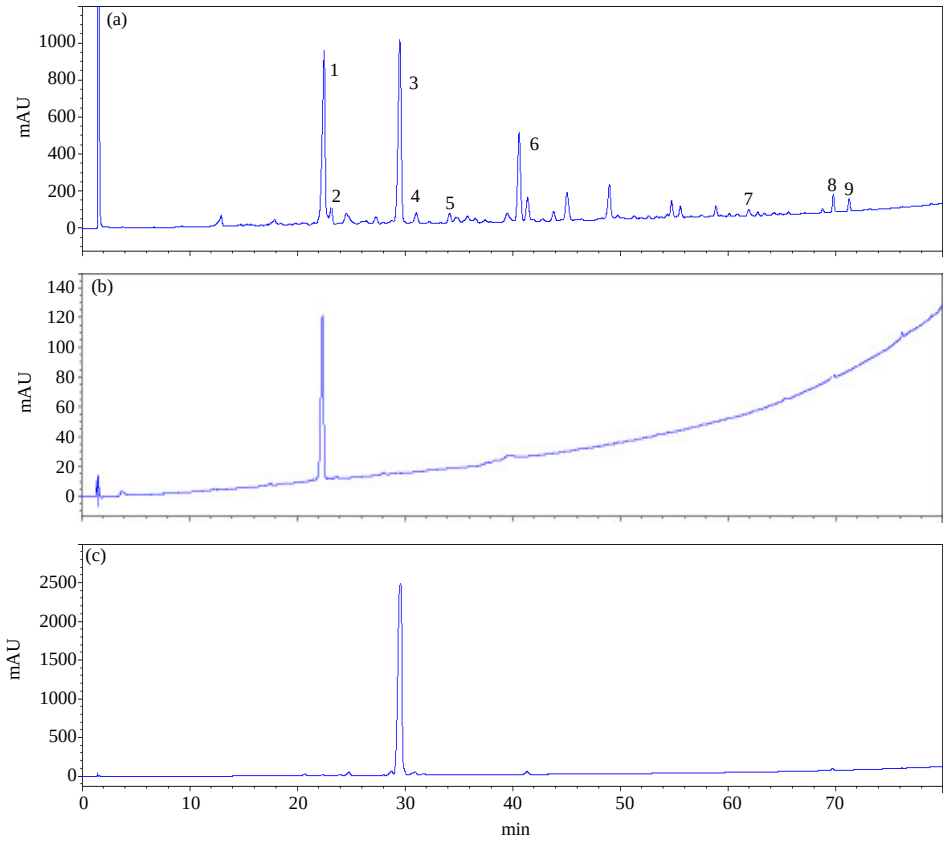

Fig. 4(a-c): HPLC chromatograms of (a) Yacon leaves extract (b) Standard sample of enhydrin and (c) Uvedalin, 1: Enhydrin, 2: Chlorohydrin, 3: Uvedalin, 4: Chlorouvedalin, 5: Fluctuanin, 6: Polymatin B, 7: 15α-angeloyloxy kauren-19-oic acid 16-epoxide, 8: 8β-tigloyloxymelapolide-14-oicacid methyl ester, 9: Kaurenoic acid

HPLC analysis of yacon leaves extract: The HPLC peak in the yacon leaves extract was analyzed according to the standard samples and exhibited as Fig. 4 indicating that the sesquiterpene lactones in the HPLC chromatogram accounted for over 45.01% total peak area.

DISCUSSION

To obtain suitable retention time and good separation for the analysis, the mobile phase was chosen after several trials in various proportions with methanol-water (15:85-75:25), then the mobile phase for HPLC analysis for pharmacokinetic consisted of water (A) and methanol (B) (0-10 min, A 60-50%, 10-15 min, A 50-40%, v/v) has been chosen. To simultaneously acquire high extraction recovery and precision of enhydrin and uvedalin, acetonitrile was selected as the precipitant after several extraction solvents including methanol, acetonitrile and dichloromethane, in different ratios being tried to precipitate the protein. Eventually, acetonitrile was added in the plasma. In addition, different amount of acetonitrile was respectively tried and added in the plasma. The pharmacokinetics relative parameters were calculated by DAS 2.0 software and comparing the parameters of AIC and R^2, a two-compartment open model (weight = 1/cc) is best fit to oral administration, respectively.

Japanese scholars study ever found that yacon can effectively reduce the effect of hypertension on the digestive, circulatory system diseases and colon cancer and so on that it all have a certain effect which can support for this study in anticancer effect[9]. In the further study of this, they also changed the screening method and tried to find novel compounds. As a result, six melampolide-type sesquiterpene lactones were identified as antibacterial compounds against *Bacillus subtilis* that enhydrin and uvedalin were also included. Research on protective effects of yacon intake on

experimental colon carcinogenesis also had investigated the potential beneficial of the treatment on yacon inhibits the development of aberrant crypt foci and colon tumor. Besides, cell proliferation was also reduced in the groups orally treated with dietary yacon. They also confirmed that yacon is a potential chemopreventive agent against colon carcinogenesis[10]. The cytotoxic activity of the sesquiterpene lactones in the leaves of yacon obtained against human gastric cancer cells (MGC80-3) indicated that enhydrin is stronger than its degradation products in the study of extraction of yacon leaves enhances enhydrin degradation[11]. And study on the separation of the chemical constituents of 60% EtOH extract of yacon leaves were also yield that the sesquiterpene lactone from yacon leaves showed cytotoxic activity evaluated on two human tumour cell lines[6]. In a new study conveyed that new sesquiterpene lactone extracted from eight yacon leaf varieties were essential for the high cytotoxicity and some sesquiterpene lactone would have potential as anticancer agents[12]. Besides, the extraction of yacon leaves and chromatographic separation yielded two new antibacterial melampolide-type sesquiterpene lactones, together with uvedalin and enhydrin exhibited potent antimicrobial activity against *Bacillus subtilis* and *Pyricularia oryzae* in the finding of De Moura *et al*.[9] study. Many results suggested that yacon can also propose a novel biological function of traditional food yacon for suppressing melanin synthesis[13]. Exploitation of yacon in breast cancer prevention using preclinical rat model was carried out by the study from evaluating the circulating factors and their association with the carcinogenesis. They had successfully determined the cellular signaling pathways which had improved the rarely evaluated preventive activity for breast cancer and laid a foundation for the anticancer activity research on yacon leaves extraction[14]. As results showed in the study on yacon decoction that significantly decreased high blood glucose level in diabetic rats and improved insulin production[2] together with the bioavailability of isoflavone in plasma and its role as lowering blood glucose levels in hyperglycemia rats, biological effects in improving health with sesquiterpene lactone should be considered not only for the diabetic function but also in the pharmacokinetics study in the future[15]. These study better confirmed that yacon had become a popular study in recent years for its wildly function like anti-diabetes and anti-cancer activity. But there were few research on the instruction for pharmacokinetics. In this study the anticancer actions of extracts of yacon leaves mainly composed of enhydrin and uvedalin were assayed simultaneously which is a breakthrough in the study. This finding and method of analysing with two components was also the first time to carry out in contradiction with the latest previous studies. The anti-cancer action of sesquiterpene extracts of yacon leaves was explored both *in vivo* with H22 tumor mice model and pharmacokinetics of the extracts which was also the innovation in yacon leaves extraction's study.

CONCLUSION

The anti-cancer action of sesquiterpene extracts of yacon leaves was explored. Furthermore, a simple and specific HPLC method was developed for the determination of enhydrin and uvedalin from yacon leaves extract in rat plasma, which was successfully applied to an *in vivo* study in rats. Pharmacokinetics data processed by DAS 2.0 software demonstrate that these 2 lactones were eliminated rapidly from the plasma. A two-compartment open model (Weight = 1/cc) gave the best fit to the plasma concentration-time curves obtained in rats. To study the anticancer action of yacon leaves, the yacon leaves extract with over 45% total lactone was also prepared with 95% EtOH and its anticancer action was evaluated by the H22 tumor mice mode. This study dealt with the component analysis of the yacon leaves extracts and its *in vivo* anticancer action. This study can also laid a foundation for the further utilization of yacon leaves.

ACKNOWLEDGMENT

This study was financed by Program for Liaoning Innovative Research Team in University (No. LT2013020).

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