# SUPPLEMENTARY MATERIAL

# Identification and Quantitative Analysis of Bioactive Components from *Potentilla kleiniana* Wight et Arn with Anti HIV-1 Proteases Activity

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**Abstract:** *Potentilla kleiniana* Wight et Arn（**PK**, "Wu Pi Feng" in Chinese）was recorded as Miao ethnic medicine for treatment of fever, cough, ulcer, and erysipelas for thousands years. This study aimed to evaluate the antiviral activity of four PK extracts and seven compounds by using HIV-1 protease (HIV-1 PR). In addition, UPLC-HRMS was employed to identify the bioactive components. The toxicity assessment of the extracts was done before antiviral screening using a highly specific human aspartyl protease, renin protease by fluorimetric method. As a result, seven compounds and four extracts of **PK** inhibited HIV-1 PR with $IC_{50}$ range from 0.009 to 0.36 mg/mL, and did not appreciably inhibit the general human protease renin. This study first demonstrated that four **PK** extracts, ellagic acid and ursolic acid potent inhibit HIV-1 protease, could be used as an efficacious drug candidate to treat SARS-CoV-2 infection.

**Keywords:** *Potentilla kleiniana*; Bioactive components; HIV-1 protease

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# *List of Supplementary Material*

## 1. Experiments

### 1.1 Instruments and chemicals

Acetonitrile and formic acid of LC-MS grade, and isopropanol and methanol of HPLC grade, were purchased from Rhawn Reagent Co. Ltd (Shanghai, China, https://www.rhawn.cn/). DMSO was purchased from Solarbio Co. Ltd (Beijing. China). Ultra-pure water was prepared using a Milli-Q Plus water purification system (Millipore, Billerica, MA, USA). The reference compounds were purchased from Chendu De KeqiRuiKe Biological Technology Co., Ltd. (Sichuan, China), the purity of each compound was higher than 98.0% determined by HPLC analysis. Instruments of Liquid chromatography coupled with Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), Microplate Reader Synergy II ( Biotec Co. USA ), and Analytical Balance MS205DU (Mettler-Toledo Co. Switzerland ) were applied in this study. Other consumers 384 well plate (Lot #: 3573, Corning Incorporated, USA) was purchased from Corning Incorporated .

### 1.2 Materials and Methods

*1.2.1 Plant Material and Extraction*

The PK crude plant was purchased in September 2020 at Guiyang Wan Dong Medicine Market, Guizhou Province of China and identified by professor De-yuan Chen, a botanist. The specimen of the plant (voucher no. 202009/WPF) is deposited in Guiyang University of Traditional Chinese Medicine. Four dried and finely powdered samples of the nodule of Wu Pi Feng (100 g) were refluxed with $H_2O$ , 30% MeOH, 60% MeOH and 85% MeOH(1:8, v/v), respectively for 1 h, and the process was repeated twice. Then samples were filtated and concentrated under vacuum drying to obtain the water extract (PKW), 30% MeOH extract (PK30), 60% MeOH extract (PK60) and 85% MeOHextract (PK85).

*1.2.2. UPLC-MS Analysis Samples Preparation*

For each extract of *Wu Pi Feng* (water, 30% MeOH, 60% MeOH and 85% MeOH), which were reconstituted in proper amount of water, 50% methanol, methanol and isopropanol respectively to reach the concentration of 10 μg/μL for chemical profiling analysis and 100 μg/μL for quantitative data aquisition. All test solutions were filtered using a 0.45 mm filter (Sartorius Stedium Australia No: 16533K) and centrifuged (10 min, 14,000 x g) before testing.

*1.2.3. FluorimetricRenin Protease inhibition assay*

The toxicity assay was done using theSensoLyte™ 520 Renin Assay Kit Fluorimetric (Lot#: AS72040) following the previously published protocols (Wei et al. 2009).

*1.2.4. Fluorimetric HIV-1 Protease inhibition assay*

SensoLyte™ 520 HIV PR Assay Kit Fluorimetric (Lot#: AK71147-1021) and HIV protease, recombinant (AS-72028, 80 ng/well) were used to evaluate the inhibitory activity following the previously published protocols (Wang et al. 2019).

*1.2.5. FluorimetricCathepsin L Protease inhibition assay*

A commercially available fluorimetricSensoLyte™ 520 Cathepsin L protease assay kit (Lot#: AS72218-1010, AnaSpec Inc. San Jose, CA, USA), was used to investigate the in vitro inhibition properties of test samples. According the manufacturer's protocol, ten microliters of Cathepsin L substrate (0.01 mM) was mixed with 2 μL of a compound solution, then 8 μL of Cathepsin L protease (0.5 μg/mL) was added in 384 well blackplates. The reaction mixture was incubated for 30 min at 37℃ and detected fluorescence signal emission at 520 nm with excitation at 490 nm. A Cathepsin L inhibitor (1 μM) served as a positive control in the same assay. Doubling dilutions to achieve final concentrations in the range of 0.1-0.001 mg/mL of tested compounds were prepared in assay buffer. The test inhibitors were assayed in triplicate; four determinations were made for the background, Cathepsin L protease activity, and the positive control. Absolute fluorescence intensity values at 490 nm were measured, and % protease inhibition and $IC_{50}$ values were calculated.

*1.2.6 Data acquisition with UPLC-MS*

The selective compounds of the extracts of *Wu Pi Feng* were chemically characterized by ultra-high performance liquid chromatography coupled with Q-ExactivePlus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was performed on a PhenomenexKinetex® C18 Column (2.1×100 mm, 1.7 μm) with an on-line filter in front of the column. The mobile phases were consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), with a gradient elution as follows: 0-2 min, 2-20% B; 2-10 min, 20-31% B; 10-13 min, 31-100% B, 13-18 min, 100% B, 18-19 min, 100-2%, 19-22 min, 2% B. Sample injection volume was 2 μL. The flow rate was set at 0.3 mL/min and the column temperature was 30 °C. The LC was coupled to the MS via a heated ESI source associated with a Q-Exactive Plus operating in full-scan dd-MS2 positive and negative polarities separately. For full MS scan, the resolution is 35,000, MS scan range was 100-1500 m/z, the ionization spray voltage was set to 4 kV, sheath gas flow rate was set to 40 L/min and auxiliary gas flow rate to 10 L/min (both in arbitrary unit). For dd-MS2, the resolution was set to 17,500 with the isolation window of 3.0 *m/z*, and NCE of 10, 20, 30. PRM mode was applied for quantitative analysis of the 10 components，with injection volume of 10 μL, and NCE of 20, 50, 80.

Data analysis was performed by using Compound Discoverer (Thermo Scientific) and relevant databases. The work flow includes retention time (RT) alignment, unknown compound detection, and compound grouping across all samples. Elemental compositions for all compounds were predicted, and chemical backgrounds were hidden by using Blank samples. mzCloud (ddMS2), ChemSpider (exact mass or formula) and local database searches against Mass Lists (exact mass and RT) and mzVault spectral libraries were included in compounds identification. Similarity search for all compounds with ddMS2 data using mzCloud was performed. MzLogic was applied to rank structures from ChemSpider and mass list search results. Xcalibur 4.2 was used for quantitative data processing.

## References

Wang X,WeiY, TianWY, SakharkarMK, LiuQ, YangX, ZhouYZ, MouCL, CaiGL, YangJ. 2019. Characterization of nine compounds isolated from the acid hydrolysate of *Lonicera fulvotomentosa*Hsu et S. C. Cheng and evaluation of their In vitro activity towardsHIV protease. Molecules, 24(24): 4526-4537.

Wei Y, Ma CM, Hattori M. 2009. Synthesis of dammarane-type triterpene derivatives and their ability to inhibit HIV and HCV proteases.Bioorg. Med.Chem, 17: 3003-3010.

## 2. Figures

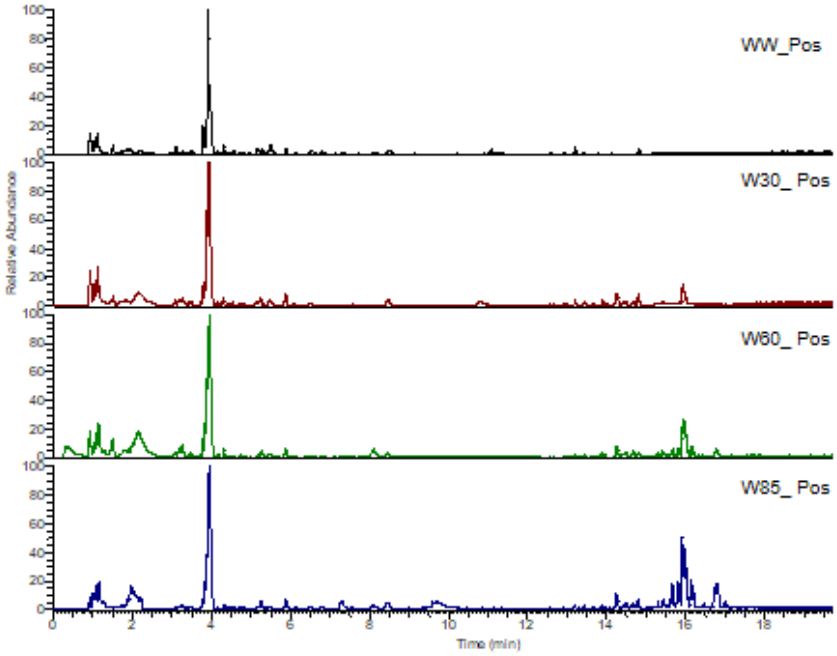



**Figure S1.** Some compounds of **PK** extracts analyzed by UPLC-MS/MS (positive)

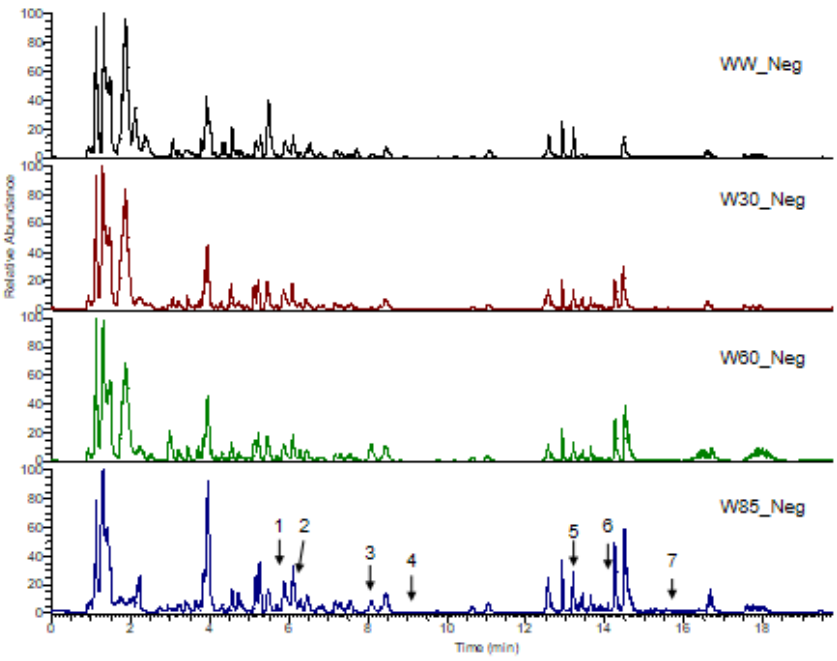



**Figure S2.** Some compounds of **PK** extracts analyzed by UPLC- HRMS (negative)

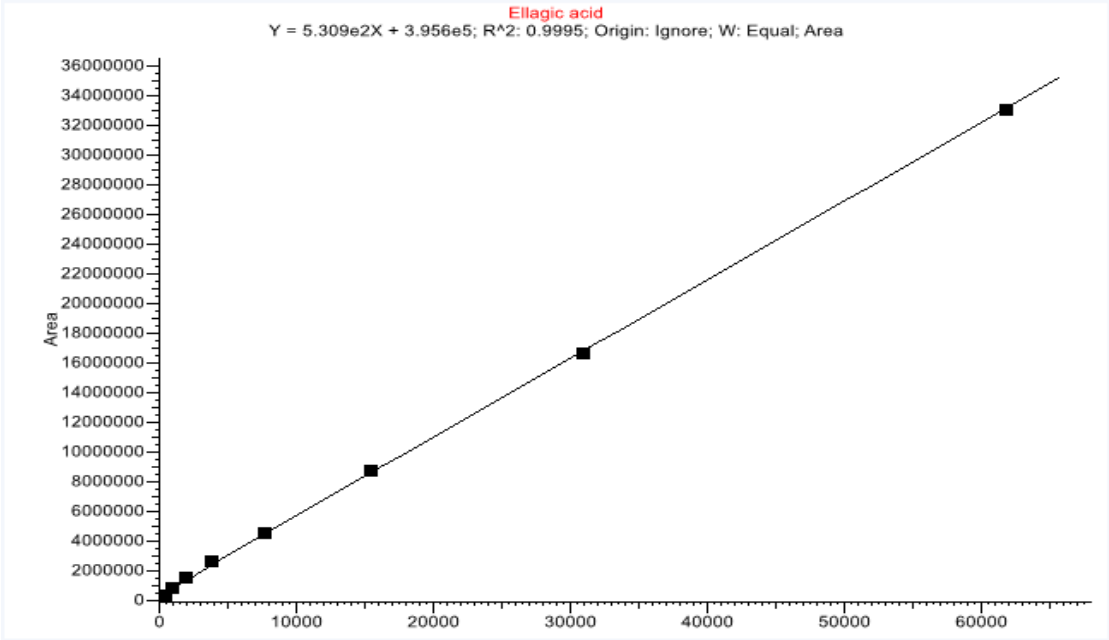



**Figure S3.** The Regression Equation of Ellagic acid

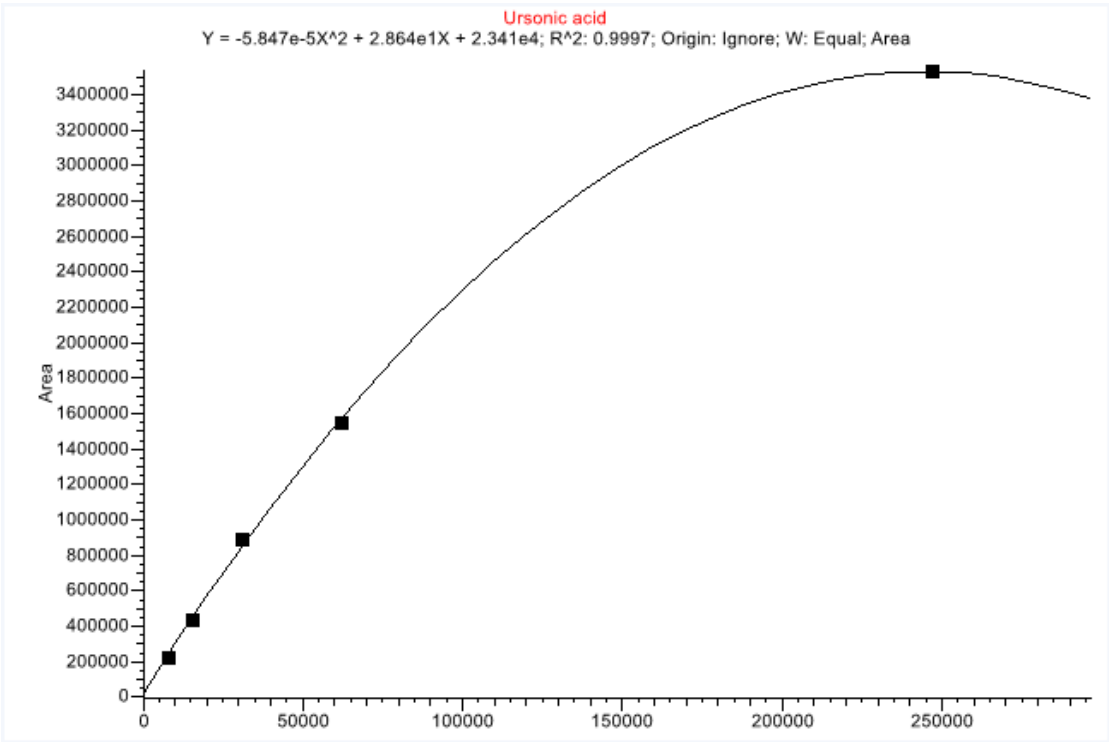



**Figure S4.** The Regression Equation of Ursolic acid

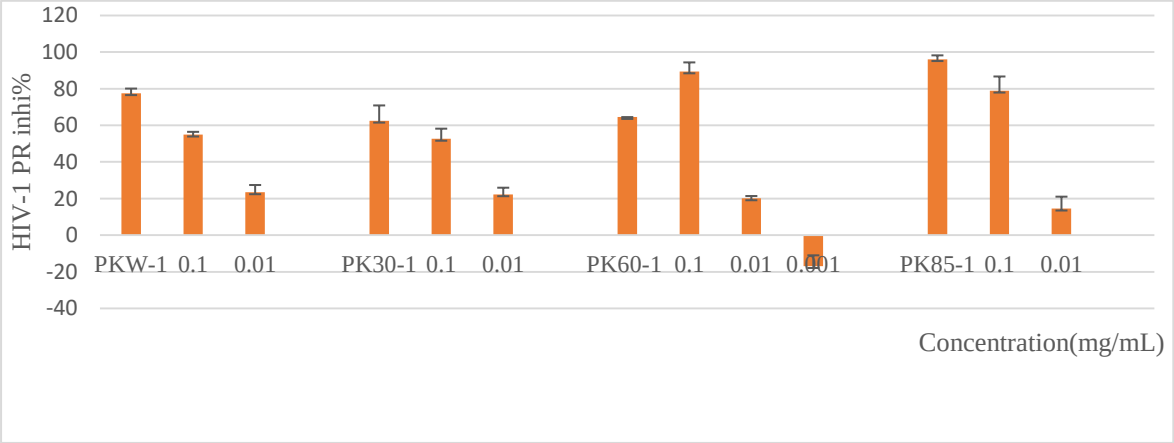



**Figure S5.** Four extracts of *Potentilla kleiniana* against HIV-1 PR (n=3)

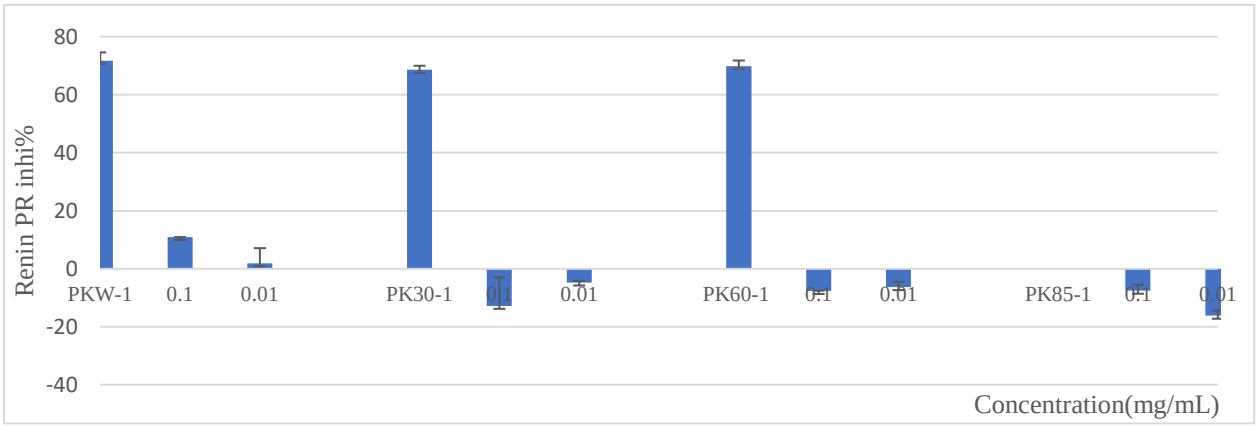



**Figure S6.** Four extracts of *Potentilla kleiniana* against Renin PR (n=3)

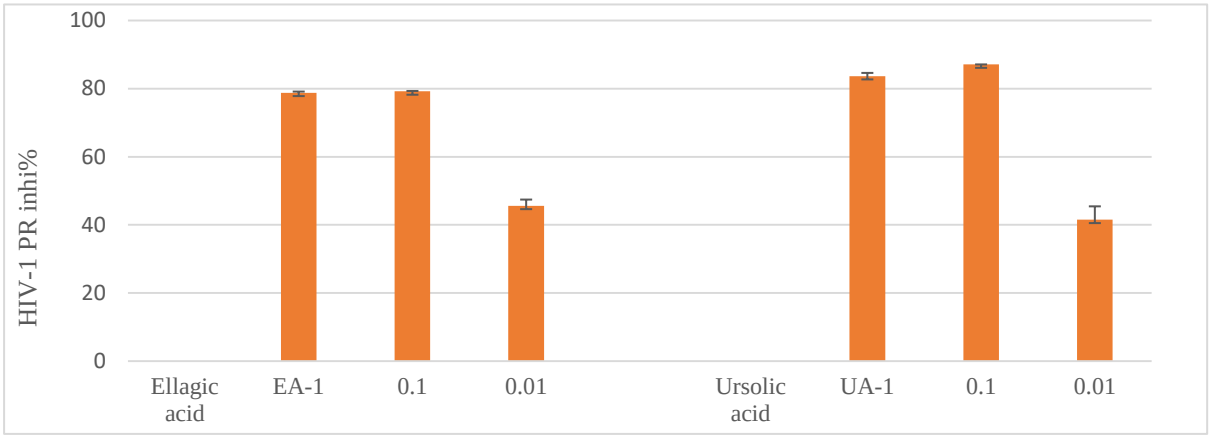



**Figure S7.** Ellagic acid and Ursolic acid inhibit HIV-1 PR (n=3)

**Table S1 Inhibition of *PK* extracts against HIV-1, Cathepsin L and Renin proteases (n=3)**

| Name | $IC_{50}$ (mg/mL) ±RSD % | |
|---|---|---|
| | HIV-1 PR | Renin PR |
| **PKW** | 0.03 ± 0.184% | 0.4± 5.60% |
| **PK60** | 0.05 ± 1.10% | 0.7 ± 0.28% |
| **PK30** | 0.06± 0.123% | 0.8 ± 2.91% |
| **PK85** | 0.048 ± 1.07% | >100 |
| **PC1** | 0.25 ± 0.03% (μM) | - |
| **PC2** | - | 0.95 ± 0.021% (μM) |

PC1: Pepstatin A, positive control for HIV-1 protease.
PC2: Positive control for renin protease.

**Table S2** Some compounds information of **PK** extracts

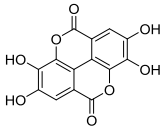
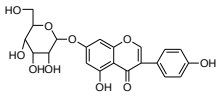
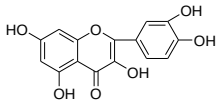
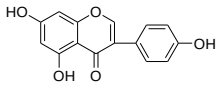
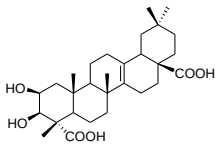
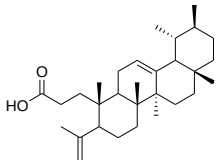
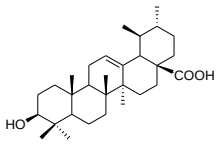
| NO. | $R_t$ [min] | Name | Structures | Formula |
|---|---|---|---|---|
| **1** | 5.83 | Ellagic acid |  | $C_{14}H_6O_8$ |
| **2** | 6.19 | Genistin |  | $C_{15}H_{10}O_7$ |
| **3** | 8.23 | Quercetin |  | $C_{15}H_{10}O_5$ |
| **4** | 9.36 | Genistein |  | $C_{15}H_{10}O_5$ |
| **5** | 13.25 | Polygalic acid |  | $C_{29}H_{44}O_6$ |
| **6** | 14.34 | Roburic acid |  | $C_{30}H_{48}O_2$ |
| **7** | 15.54 | Ursolic acid |  | $C_{30}H_{48}O_3$ |

**Table S3** The inhibition of selective compounds from ***PK*** extracts against HIV-1 protease (n=3)

| No. | Name | HIV-1 Protease | |
|---|---|---|---|
| | | **Inhi % ± SD at 1.0 (mg/mL)** | **$IC_{50}$±RSD %（mg/mL）** |
| **1** | Ellagic acid | 78.0 ± 0.57 | 0.009 ± 0.03% |
| **7** | Ursolic acid | 83.7 ± 0.91 | 0.014 ± 0.10% |
| **5** | Polygalic acid | 85.8 ± 1.45 | 0.089 ± 2.50% |
| **3** | Quercetin | 94.4 ± 7.86 | 0.19 ± 2.76% |
| **2** | Genistin | 75.0±2.11 | 0.36 ± 3.10% |
| **4** | Genistein | 75.0 ± 2.11 | 0.36 ± 3.10% |
| **6** | Roburic acid | 74.5 ± 1.74 | 0.36 ± 2.32% |
| **PC** | Pepstatin A | | 0.45 ±0.088%（μM） |

PC: HIV-1 PR inhibitor.

**Table S4** The content of seven selective compounds in different extracts of PK extracts (ng/mg)

| No. | Rt (min) | Name | PKW | PK30 | PK60 | PK85 | Regression Equation |
|---|---|---|---|---|---|---|---|
| | | | | Content（ng/mg） | | | |
| **1** | 5.83 | Ellagic acid | 8228.45 | 11112.06 | 15774.37 | 11754.80 | Y=5.309e2X+3.956e5;R^2:0.9995 |
| **2** | 6.19 | Genistin | 43.08 | 186.31 | 210.60 | 88.31 | Y=1.55e4X+1.78e6; R^2:0.9983 |
| **3** | 8.23 | Quercetin | 0.69 | 0.52 | 0.75 | 0.85 | Y=5.795e5X-1.571e5;R^2:0.9995 |
| **4** | 9.36 | Genistein | 1.57 | 6.83 | 10.39 | 13.87 | Y=6.924e4X+5.14e5; R^2:0.9962 |
| **5** | 13.25 | Polygalic acid | 161.70 | 487.75 | 500.91 | 1203.20 | Y=-2.88e-4X^2+3.482e1X-7.78e4; R^2:0.999 |
| **6** | 14.34 | Roburic acid | 7.26 | 43.36 | 341.64 | 342.70 | Y=7.601e2X+9.469e3; R^2:0.9924 |
| **7** | 15.54 | Ursolic acid | 483.05 | 7468.77 | 10810.74 | 16114.86 | Y=-5.847e-5X^2+2.864e1X+2.341e4; R^2=0.9997 |