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Antitumor chemical constituents of *Toddalia asiatica* (Linn) Lam root bark and its rational alternative medicinal parts by multivariate statistical analysis

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ABSTRACT

Toddalia asiatica (Linn) Lam (*T. asiatica*) as a traditional Miao medicine was investigated to find rational alternative medicinal parts for *T. asiatica* root bark and its antitumor chemical constituents by quantitative pharmacognostic microscopy, high performance liquid chromatography (HPLC) fingerprint and multivariate statistical analysis. A bivariate correlation analysis method based on microscopic characteristics and content of chemical constituents was established for the first time, there were some regular discoveries between powder microscopic characteristics and common chromatographic peaks of *T. asiatica* through quantitative pharmacognostic microscopy, cork cells, calcium oxalate square crystal, brown clump, starch granule and phloem fiber, as powder microscopic characteristics may be placed where the main chemical constituents were enriched. Scores plot of principal component analysis (PCA) and dendrogram of hierarchical clustering analysis (HCA) showed that 18 *T. asiatica* samples were distinguished correctly, clustered clearly into two main groups as follows: S01~S03 (root bark) and S07~S09 (stem bark) in cluster 1, S04~S06 and S10~S18 in cluster 2. Nineteen common peaks were obtained in HPLC fingerprint of *T. asiatica*, loadings plot of PCA indicated seven compounds played important roles in different part of samples (P10 > P08 > P07 > P14 > P16 > P17 > P19), peaks 04, 06, 07, 08, 10 were identified as hesperidin, 4-methoxycinnamic acid, toddalolactone, isopimpinellin and pimpinellin. MTT assay was used to determine the inhibitory activity of different medicinal parts of *T. asiatica* on human breast cancer MCF-7 cells, all parts of *T. asiatica* had different inhibitory effects on MCF-7 cell lines, root and stem barks of *T. asiatica* showed the best inhibitory activity. The relationship between chemical constituents and the inhibitions on MCF-7 cell had been established, significant antitumor constituents of *T. asiatica* were identified by correlation analysis, the order of the antitumor effect of the main compounds was P07 (toddalolactone) > P16 > P06 (4-methoxycinnamic acid), P11 > P18 > P10 (pimpinellin) > P08 (isopimpinellin) > P01 > P19 > P14 > P04 (hesperidin) > P17, which were antitumor chemical constituents of *T. asiatica* root bark. *T. asiatica* stem bark was the most rational alternative medicinal part for *T. asiatica* root bark.

KEYWORDS

alternative medicinal part, antitumor, chemometrics, natural medicine, *Toddalia asiatica* (Linn) Lam

INTRODUCTION

Toddalia asiatica (Linn) Lam (*T. asiatica*) as a traditional Miao medicine of Chinese Materia Medica (CMM), belonging to *Toddalia* Juss genus, *Rutaceae* family was first recorded in “*Zhi Wu Ming Shi Tu Kao*” of Qing Dynasty, it is widely distributed in Guizhou, Guangxi and other provinces in Southwest China, its root bark as a traditional medicinal part of *T. asiatica*, which has obvious pharmacological activities of hemostasis, anti-tumor, anti-

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inflammatory and analgesic, is widely used in the minority Miao for clinical treatment of traumatic injury, knife wound hemorrhage, tumor and rheumatic pain [1–5]. CMM as an important kind of natural medicines on the guidance of traditional Chinese medicine (TCM) theory has been used clinically for thousands of years in China, CMM have been followed up to now by their own exact pharmacological action and clinical effect. But, there is a main reason that TCM or CMM has always been questioned by western medical scholars due to complex chemical constituents and unclear pharmacological mechanisms of CMM [6], how to scientifically clarify the effective compounds of CMM has become an urgent problem to be solved with the continuous development of modern medicine.

Meanwhile, under the huge clinical demand for *T. asiatica* root bark and current drug market driven by actual economic interests, whole root slice and whole stem slice of *T. asiatica* are the main forms of *T. asiatica* instead of its traditional medicinal part – root bark. This chaotic use of CMM made it necessary for us to carry out research on rational medicinal parts of *T. asiatica*, and find out more suitable alternative parts of *T. asiatica* for *T. asiatica* root bark. In recent years, some researchers had tried to address these practical challenges through studies on phytochemistry, LC fingerprinting and LC-MS/MS analysis of *T. asiatica* [7–10], our group also had carried out a series of pharmacodynamic material basis studies on *T. asiatica* root bark in the previous research work, its hemostatic activity was systematically verified through animal model experiments, a lot of natural furocoumarins from *T. asiatica* root bark had been detected, isolated and finally identified [11–15]. However, the findings of these studies still did not pay attention to the rationality of medicinal parts of *T. asiatica*, as there were no comparative studies of all possible medicinal parts of *T. asiatica*, some of them such as root core of *T. asiatica* even had been used in drug market by default until now without any relevant drug quality standards and scientific research data support.

High performance liquid chromatography (HPLC) fingerprint technology as an important analytical method had got quick development in many areas such as TCMs, food and biological samples. It can be a relatively effective method to evaluate the quality of complex TCMs as a whole [16–18]. In order to further explore the differences of all medicinal parts of *T. asiatica*, the obtained data were statistically processed by multivariate statistical analysis including bivariate analysis, hierarchical clustering analysis (HCA) and principal component analysis (PCA). Multivariate statistical analysis was used to evaluate the intrinsic quality of *T. asiatica* and to identify the chemical constituents that are most responsible for quality control of different medicinal parts of *T. asiatica* [19, 20]. HPLC fingerprint profiling combined with quantitative pharmacognostic microscopy, pharmacological activity and multivariate statistical analysis was a novel strategy for assessing different medicinal parts of *T. asiatica* in this study. Our research group had conducted deep and systematic research on different parts of *T. asiatica* and their antitumor effects for

the first time, so as to solve current chaos of *T. asiatica*, clarify on its antitumor material basis and to scientifically confirm the rational alternative medicinal part of *T. asiatica*.

MATERIALS AND METHODS

Plant materials

Eighteen batches of different medicinal parts of *T. asiatica* including root bark, root core, stem bark, stem core, near-leaf stem and leaf collected from Qixing mountain area, Duyun, Guizhou in the southwest of China were investigated (Table 1). *T. asiatica* samples were ground into powder of the homogenous 24 mesh before the experiment. All samples had been identified by Professor Zhiyou Guo, Qiannan Normal University for Nationalities, and the specimens were

Table 1. The origins of different medicinal parts of *Toddalia asiatica*

Sample no.	Medicinal parts of <i>T. asiatica</i>	Origins	Collecting time
01	Root bark	Duyun, Guizhou	July, 2018
02	Root bark	Duyun, Guizhou	July, 2018
03	Root bark	Duyun, Guizhou	July, 2018
04	Root core	Duyun, Guizhou	July, 2018
05	Root core	Duyun, Guizhou	July, 2018
06	Root core	Duyun, Guizhou	July, 2018
07	Stem bark	Duyun, Guizhou	Sept, 2018
08	Stem bark	Duyun, Guizhou	Sept, 2018
09	Stem bark	Duyun, Guizhou	Sept, 2018
10	Stem core	Duyun, Guizhou	Sept, 2018
11	Stem core	Duyun, Guizhou	Sept, 2018
12	Stem core	Duyun, Guizhou	Sept, 2018
13	Near-leaf stem	Duyun, Guizhou	August, 2018
14	Near-leaf stem	Duyun, Guizhou	August, 2018
15	Near-leaf stem	Duyun, Guizhou	August, 2018
16	Leaf	Duyun, Guizhou	August, 2018
17	Leaf	Duyun, Guizhou	August, 2018
18	Leaf	Duyun, Guizhou	August, 2018

deposited in Herbarium of Chinese Materia Medica and Ethnomedicines, School of Pharmacy, Guizhou Medical University.

Cells, chemicals and reagents

MCF-7 cells were purchased from China Center for Type Culture Collection (CCTCC) of Wuhan University, less than 10 generations of cell passage. Eleven authentic compounds were used in the present study, namely, hesperidin (P04), 4-methoxycinnamic acid (P06), toddalolactone (P07), isopimpinellin (P08), pimpinellin (P10), methyl trans-4-hydroxycinnamate (P20), avenalunic acid methyl ester (P21), ferulic acid methyl ester (P22), bergapten (P23), moellendorffiline (P24) and phellopterin (P25). These were isolated and prepared from *T. asiatica*, identified by our research group in our previous research, and their purities in HPLC are more than 98%. DMEM culture medium (Lot No 8118403, GIBCO), Fetal bovine serum (Lot No 1P1701, SeraPro S601-500), Trypsin digestive fluid (Lot No 1951208, GIBCO), Phosphate buffer saline (Lot No 1015M022, Solarbio), MTT (Lot No 829Z0513, Solarbio), DMSO (Lot No 1213C0222, Solarbio), 75% medical alcohol (Lot No 190414, Guizhou Kangtai Lijian), Methanol (HPLC pure, Shanghai Sino-pharm Chemical), Glycerol (Shanghai Sinopharm Chemical), Chloral hydrate (Shanghai Macklin Biochemical), Pure water (Watsons), 96-well Cell culture plate (Lot No 180809-078, Guangzhou Jet Bio-Filtration) were purchased for the analysis.

Instruments

Agilent 1260 infinity HPLC-DAD system (Agilent), R-1001VN rotary evaporator (Zhengzhou Greatwall Scientific), SHB-III water circulating multi-purpose vacuum pump (Zhengzhou Greatwall Scientific), SB-5200D ultrasonic cleaning machine (Ningbo Scientz), TS100 Inverted Microscope (Nikon), Epoch microplate reader (Biotek), Thermo Forma 3131 carbon dioxide cell incubator (Thermo), TDL-40B centrifuge (Changzhou Langyue), HH-501 thermostatic water bath (Changzhou Langyue) and HK-UP-20 Ultra-pure water preparation system (Hefei Hongke) were used for the analysis.

Sample preparation

About 6.0 g of root bark, root core, stem bark, stem core, near-leaf stem or leaf of *T. asiatica* was soaked separately in methanol for 24 h at a solid–liquid ratio of 1:50 (g/mL), extracted three times by reflux method for 2 h each time in a 65 °C-constant temperature water bath [9, 11, 21]. Six extracts from different parts of *T. asiatica* were finally prepared. Each accurately-weighed extract of *T. asiatica* was dissolved in methanol and fixed in their respective 25-mL volumetric flasks. All sample solutions were filtered through 0.22- μ m lipophilic microporous filters before LC analysis.

The standard reference solutions were prepared by adding an accurately-weighed amount of above-mentioned eleven natural compounds to a 10-mL volumetric flask, respectively, dissolved with methanol to make 100.0 μ g/mL of the final concentrations as the stock solutions prepared for analysis.

Fig. 1. The appearance of differential parts of *Toddalia asiatica* and their powders. 1: Root bark of *T. asiatica* and its powder. 2: Root core of *T. asiatica* and its powder. 3: Stem bark of *T. asiatica* and its powder. 4: Stem core of *T. asiatica* and its powder. 5: Near-leaf stem of *T. asiatica* and its powder. 6: Leaf of *T. asiatica* and its powder

Microscopical character analysis of pharmacognostical powder

The root bark, root core, stem bark, stem core, near-leaf stem and leaf of *T. asiatica* were pulverized by a pulverizer, sieved by a 24-mesh sieve to obtain the medicinal material powders to be tested (Fig. 1). The right amount of the powder from different parts of *T. asiatica* was taken out separately, placed on a glass slide of 7.5 cm × 2.5 cm and dripped with 2~3 drops of chloral hydrate-glycerol test solution for further 2–3 s of heat treatment under the flame of an alcoholic lamp, then each sample on the glass slide was covered with a cover glass without generating bubbles. All these glass slides with sample powders were observed under an inverted microscope to do microscopical character analysis of pharmacognostical powder.

Chromatographic condition

Chromatographic separation was performed on a Diamonsil C18 column (250 mm × 4.6 mm, 5 μm) in a HPLC-DAD system (Agilent), the mobile phase was methanol (A)–water (B) with gradient elution of 0–10 min A:B (5:95) → (25:75), 10–50 min A:B (25:75) → (50:50), 50–80 min A:B (50:50) → (80:20), 80–95 min A:B (80:20) → (90:10) and 95–100 min A:B (90:10) → (90:10) [9, 10]. The flow rate was 1.0 mL/min, detection wavelength was 230 nm, column temperature was maintained at 25 °C and the injection volume was 20.0 μL by automatic sampling system at 4 °C.

Evaluation of anti-tumor activities of different parts of *T. asiatica*

MCF-7 cells in logarithmic growth stage were selected and inoculated into 96-well plates at a cell density of 1×10^4 /well with 100.0 μL DMEM culture medium, six duplicates in each group 12.5~200.0 μg/mL extracts of six medicinal parts of *T. asiatica* (root bark, root core, stem bark, stem core, near-leaf stem and leaf) in 0.1%-DMSO DMEM culture medium were added respectively and incubated at 37 °C, 5% CO₂ for 24 h in the dark, while 0.1%-DMSO DMEM culture medium without drug treatment was set up as Blank control group. Then, 15 μL of 5% MTT solution was added to each well in the dark. These cells were continuously incubated at 37 °C, 5% CO₂ for 4 h, the supernatant was discarded, 150 μL DMSO was added to each well and shaken for 10 min. Subsequently, the optical density (OD) of each well at 490 nm was determined by a microplate reader (Bio-Rad Laboratories, Inc.), the inhibitory rate (%) was finally calculated by the following formula:

$$\text{Inhibitory rate(\%)} = \frac{\text{OD}_{\text{Blank}} - \text{OD}_{\text{Sample}}}{\text{OD}_{\text{Blank}}} \times 100\%$$

Correlation analysis

Quantitative pharmacognostic microscopy and multivariate statistical analysis of antitumor chemical constituents of *T. asiatica* need to be analyzed by means of correlation analysis

method, which contained bivariate analysis, HCA and PCA. Bivariate analysis and HCA were performed by SPSS 22.0 (SPSS Inc), PCA was carried out by SIMCA 14.1 Software (Umetrics, Sweden).

RESULTS AND DISCUSSION

Pharmacognostical powder analysis

Microscopic characteristics of powders of different medicinal parts of *T. asiatica* are: 1) powder of the root bark was brown, the powder microscopic characteristics were the most abundant, there were much more brown cork cells, calcium oxalate square crystals, brown clumps and reticulate vessels. Xylem fibers, non-glandular hairs, oil cells, starch granules and phloem fibers could also be characterized in the root bark; 2) powder of the root core was yellowish green, its microscopic characteristics contained yellowish brown cork cells and reticulate vessels, no calcium oxalate square crystals and stone cells were found here; 3) *T. asiatica* stem bark was brown, more cork cells, calcium oxalate square crystals, brown clumps and starch granules were its main microscopic powder characteristics, which were similar to *T. asiatica* root bark; 4) *T. asiatica* stem core was brown yellow, there were many wood fibers containing calcium oxalate square crystals in the powder, cork cells and reticulate vessels were also common herein; 5) the near-leaf stem was brown, it had wood fibers, cork cells, non-glandular hairs and reticulate vessels; 6) the leaf was light green in color, brown oil cells, non-glandular hairs, vessels, parenchyma cells and wood fibers were its main powder microscopic characteristics (Fig. 2).

In our opinion, the bioactive chemical constituents of *T. asiatica* were definitely from this TCM, which showed us its own powder microscopic characteristics in the field of view of a microscope, and this prompted us to try to build correlation between powder microscopic characteristics of *T. asiatica* and 19 common chromatographic peaks through quantitative pharmacognostic microscopy. Correlation analysis method helped us to calculate their correlation coefficients, and the final data was listed in Table 2. Cork cells, calcium oxalate square crystal, brown clump, starch granule and phloem fiber obtained relatively high frequency of occurrence with common peaks (01~19) from these results, these microscopic characteristics may be placed where the main chemical constituents were concentrated and enriched, it can guide us to more purposefully extract and purify the target natural compounds. For example, phloem fiber had rich compounds of P06 (0.805, $P < 0.01$), P11 (0.539, $P < 0.05$) and P18 (0.787, $P < 0.01$).

Chromatographic fingerprint analysis

The chromatographic fingerprints of root bark, root core, stem bark, stem core, near-leaf stem and leaf of *T. asiatica* had been established, a total of 19 common chromatographic peaks (01~19) in different medicinal parts of *T. asiatica* were confirmed, in Fig. 3, showing large peak areas

Fig. 2. Microscopic characteristics of fine powders of different parts of *Toddalia asiatica* (10 × 40). 1: Stone cells. 2: Calcium oxalate square crystals. 3: Reticulate vessels. 4: Wood fibers. 5: Fiber containing crystals. 6: Catheters. 7: Cork cells. 8: Parenchyma cells. 9: Non-glandular hairs

and good separation from adjacent peaks. The total peak areas of 19 common peaks were more than 90% of the total peak areas. By comparing with the chromatographic peaks of our eleven reference substances, the five common chromatographic peaks 04, 06, 07, 08 and 10 of *T. asiatica* were determined to be hesperidin, 4-methoxycinnamic acid, toddalolactone, isopimpinellin and pimpinellin, respectively.

The LC fingerprints were matched automatically by use of the Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004), similarities of LC fingerprints of samples no. 1~18 of *T. asiatica* were calculated by the cosine value method of vectorial angle, and the results were detailed below: 0.9507–0.9765 of samples no. 1–3, 0.8306~0.8570 of samples no. 4~6, 0.9824~0.9881 of samples no. 7~9, 0.8207~0.8940 of samples no. 10~12, 0.5571~0.5667 of samples no. 13~15 and 0.1328~0.1366 of samples no. 16–18. The degree of chromatographic fingerprint similarity between *T. asiatica* root bark and *T. asiatica* stem bark was much higher than that of other parts. HCA was used in our study to find relatively homogeneous clusters of all six different medicinal parts of *T. asiatica* based on the peak areas of the 19 common peaks as the measured characteristics, and the results were shown in Fig. 4D, it was clear that the six parts of *T. asiatica* were able to be classified into two broad categories. Samples no. 1~3 of *T. asiatica* root bark and samples no. 7~9 of *T. asiatica* stem bark were in the first category, and the other samples of *T. asiatica* were in the second category, which was further classified into two subclusters: samples no. 4~6 of *T. asiatica* root

Table 2. Correlation coefficients between nineteen common chromatographic peaks and powder microscopic characteristics of different parts of *Toddalia asiatica*

	Cork cells	Xylem fiber	Non-glandular hair	Calcium oxalate square crystal	Vessels	Oil cells	Parenchyma cells	Brown clump	Stone cells	Starch granule	Phloem fiber
P01	0.685**	−0.741**	−0.628*	0.552*	−0.823**	−0.715**	−0.400	0.772**	−0.218	0.455	0.879**
P02	−0.294**	0.882**	0.906**	−0.320	0.659**	0.898**	0.834**	−0.325	0.725**	0.211	−0.514
P03	−0.266	0.595*	0.815**	0.238	0.275	0.688**	0.783**	−0.045	0.724**	0.468	−0.538
P04	0.748**	−0.661**	−0.400	0.951**	−0.727**	−0.547*	−0.336	0.504	−0.086	0.424	0.312
P05	−0.003	0.183	0.415	0.579*	−0.114	0.293	0.455	0.302	0.710**	0.772**	0.180
P06	−0.115	−0.161	−0.274	0.073	−0.065	−0.175	−0.278	0.095	0.158	0.236	0.805**
P07	0.563*	−0.691**	−0.592*	0.683**	−0.661**	−0.636*	−0.508	0.501	−0.112	0.393	0.799**
P08	0.425	−0.487	−0.341	0.726**	−0.577*	−0.411	−0.243	0.546*	0.190	0.631*	0.809**
P09	−0.814**	0.981**	0.971**	−0.417	0.766**	0.987**	0.882**	−0.397	0.783**	0.192	−0.520*
P10	0.435	−0.507	−0.367	0.718**	−0.586*	−0.433	−0.268	0.547*	0.163	0.613*	0.821**
P11	−0.296	0.140	0.087	0.076	0.125	0.153	0.054	0.006	0.444	0.360	0.539*
P12	0.486	−0.287	−0.012	0.731**	−0.536*	−0.185	0.119	0.559*	0.228	0.621*	0.229
P13	−0.796**	0.840**	0.821**	−0.206	0.654**	0.864**	0.724**	−0.297	0.918**	0.413	0.073
P14	0.634*	−0.563*	−0.313	0.882**	−0.757**	−0.463	−0.146	0.735**	0.158	0.735**	0.689**
P15	0.751**	−0.445	−0.085	0.862**	−0.759**	−0.329	0.118	0.752**	0.083	0.648**	0.132
P16	0.244	−0.402	−0.349	0.530*	−0.414	−0.354	−0.293	0.392	0.184	0.509	0.862**
P17	0.611*	−0.473	−0.193	0.874**	−0.707**	−0.364	−0.031	0.703**	0.201	0.732**	0.516*
P18	−0.120	−0.144	−0.236	0.145	−0.057	−0.144	−0.259	0.079	0.218	0.280	0.787**
P19	0.011	0.065	0.174	0.246	−0.160	0.105	0.289	0.366	0.416	0.563*	0.375

Notes: * $P < 0.05$ statistically significant correlation, ** $P < 0.01$ statistically very significant correlation.

Bold values are chromatographic peaks.

Fig. 3. HPLC-UV chromatograms of standard references (A) and different medicinal parts of *Toddalia asiatica* (B)

core, samples no. 10~12 of *T. asiatica* stem core and samples no. 13~15 of *T. asiatica* near-leaf stem as one subcluster, and samples no. 16~18 of *T. asiatica* leaf as the other subcluster.

PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a number of uncorrelated variables called principal components. It is the unsupervised multivariate data analysis approach, and appropriate when a function of many attributes is involved in differences between samples. PCA was able to provide the accurate component that played the most important role in the discrimination of the different medicinal parts of *T. asiatica*, PCA on 19 common peaks of LC fingerprints of *T. asiatica* samples was obtained to find the possible chemical markers for the discrimination of different medicinal parts of *T. asiatica*. The 3D matrix was composed of 18 observations and 19 variables, which indicated samples and the various markers measured by HPLC, respectively. Based on eigenvalues higher than 1, the first ($\lambda_1 = 10.3$), the second ($\lambda_2 = 3.32$) and the third ($\lambda_3 = 2.84$) uncorrelated principal components accounted for 57.3%, 18.5% and 15.8% contribution rate of variance, respectively, and cumulative contribution rate of these three principal components had

reached 91.5% (Fig. 4B). Eighteen batches of samples were divided into four groups in 3D score plot: Group 1 – S01~S03; Group 2 – S07~S09; Group 3 – S04~S06, S10~12 and S13~15; and Group 4 – S16~S18. Group 1 and Group 2 would be merged into one group in 2D score plot (Fig. 4A). The three principal components were assigned to evaluate the similarities and differences of the samples. The loading plot (Fig. 4C) indicated that seven compounds played important roles in the samples, $P_{10} > P_{08} > P_{07} > P_{14} > P_{16} > P_{17} > P_{19}$. In particular, P10, P8 and P7 showed more influence on the discrimination of different medicinal parts of *T. asiatica* than other peaks, which might be the chemical markers for quality control of *T. asiatica*. Peaks 10, 08 and 07 were also confirmed to natural compounds pimpinellin, isopimpinellin and toddalolactone.

Antitumor activities of different medicinal parts of *T. asiatica*

Antitumor activities of different medicinal parts (root bark, root core, stem bark, stem core, near-leaf stem and leaf) of *T. asiatica* were considered to deeply evaluate their differences

Fig. 4. Scores plot of PCA (A, B), loadings plot of PCA (C), dendrogram and heatmap of HCA (D) of different parts of *Toddalia asiatica*. Red color represents to higher concentration, while blue color corresponds to lower concentration. S1, S2, S3: Root bark of *T. asiatica*. S4, S5, S6: Root core of *T. asiatica*. S7, S8, S9: Stem bark of *T. asiatica*. S10, S11, S12: Stem core of *T. asiatica*. S13, S14, S15: Near-leaf stem of *T. asiatica*. S16, S17, S18: Leaf of *T. asiatica*. P: Liquid chromatography peak

through classical MTT experimental method. Anti-tumor mean inhibitory rate (%) of 100.0 $\mu\text{g/mL}$ different medicinal parts of *T. asiatica* on human breast cancer MCF-7 cells showed clear pharmacodynamic action law: root bark (58.23%), stem bark (48.03%) > root core (27.01%), stem core (33.01%) > near-leaf stem (7.69%), leaf (6.64%), and the inhibition rate showed a significant upward trend with the increase of test sample concentration of treated MCF-7 cells. *T. asiatica* root bark and root core can significantly inhibit the proliferation of MCF-7 breast cancer cells ($P < 0.01$) than other parts (Fig. 5). Through bivariate analysis, correlation between 19 characteristic peaks and antitumor inhibition rate of different medicinal parts of *T. asiatica*

had been established, and reflected in the form of correlation coefficients of peaks 01~19 ($*P < 0.05$, $**P < 0.01$): 0.739** (P01), -0.719^{**} (P02), -0.673^{**} (P03), 0.555* (P04), 0.166 (P05), 0.783** (P06), 0.854** (P07), 0.751** (P08), -0.537^{**} (P09), 0.769** (P10), 0.783** (P11), 0.337 (P12), -0.096 (P13), 0.600* (P14), 0.054 (P15), 0.813** (P16), 0.550* (P17), 0.775** (P18), 0.717** (P19). The above results of statistical analysis suggested that some compounds in *T. asiatica* showed strong antitumor activities, their orders were P07 (toddalolactone) > P16 > P06 (4-methoxycinnamic acid), P11 > P18 > P10 (pimpinellin) > P08 (isopimpinellin) > P01 > P19 > P14 > P04 (hesperidin) > P17 in turn.

Fig. 5. The inhibitory effect of extracts from different parts of the *Toddalia asiatica* on MCF-7 cells

CONCLUSION

A novel strategy integrating quantitative pharmacognostic microscopy, pharmacological activity and multivariate statistical analysis was successfully set up herein for assessing different medicinal parts of *T. asiatica*, an HPLC-UV method was established to evaluate the quality of different medicinal parts of *T. asiatica*, antitumor chemical constituents of *T. asiatica* and rational alternative parts for the root bark by quantitative

pharmacognostic microscopy and multivariate statistical analysis. The powder microscopic characteristics of different medicinal parts of *T. asiatica* had gone through systematic analysis and comparison. Cork cells, calcium oxalate square crystal, brown clump, reticulate vessels, oil cells, stone cells and xylem fibers were the main powder microscopic characteristics of *T. asiatica*. The main natural compounds of *T. asiatica* were concentrated in cork cells, calcium oxalate square crystals, brown clumps, starch granules and phloem fibers. The results of multivariate statistical analysis illustrated that different medicinal parts of *T. asiatica* could be classified, and pimpinellin, isopimpinellin and toddalolactone were highlighted as potential chemical markers for discrimination of different medicinal parts of *T. asiatica* and quality control of *T. asiatica*. This study fills the blank space for the research work of rational medicinal parts of *T. asiatica*, and is helpful in establishing a scientific and rational method for explaining *T. asiatica* stem bark as a rational alternative medicinal part for *T. asiatica* root bark and solving current chaos of *T. asiatica* in the drug market.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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ABBREVIATIONS

CMM	Chinese materia medica
HCA	hierarchical clustering analysis
HPLC	high performance liquid chromatography
MTT	methyl thiazolyl tetrazolium
PCA	principal component analysis
TCM	traditional Chinese medicine

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