DOI: 10.19600/j.cnki.issn2152 - 3924.2023.04.008

Determination of Total Flavonoids in Leaves of *Tetracera asiatica*

Fangfang DA[1,2*], Caoju MO[1], Wenxiu HUANG[3], Yulin LU[4], Qiying YANG[1], Qing ZHANG[1]

1. School of Chinese Ethnic Medicine, Guizhou Minzu University, Guiyang 550025, China; 2. Key Laboratory of the Development and Utilization of Guizhou Minority Medical Resources (Guizhou Minzu University), State Ethnic Affairs Commission, Guiyang 550025, China; 3. School of Pharmacy, Guangxi University of Chinese Medicine, Nanning 530001, China; 4. Bio-Thera Solutions, Ltd., Guangzhou 510700, China

Abstract [**Objectives**] This study was conducted to determine and compare the contents of flavonoids in the leaves of *Tetracera asiatica*, an ethnic medicine from different habitats, in order to provide a basis for the quality control of its medicinal materials. [**Methods**] The flavonoid compounds in the leaves of *T. asiatica* were reflux-extracted in a water bath, and the total flavonoid content in *T. asiatica* was determined by ultraviolet-visible spectrophotometry. [**Results**] The total flavonoid content in *T. asiatica* leaves varied according to different habitats. The highest content was found in Gaofeng (19.41%) of Guangxi, followed by Huizhou (16.88%) of Guangdong and Hong Kong (16.76%). The lowest total flavonoid content was found in Shangcuntang (3.91%) of Bobai County, Guangxi, followed by Beiliu (4.15%), Guangxi. The total flavonoid contents in the samples ranged from 3.91% to 19.41%, with an average content of about 12.36%. [**Conclusions**] The UV spectrophotometry method is easy to operate, and has good repeatability, accurate detection results, and high reliability, and can be used for the determination of flavonoids in *T. asiatica*.

Key words Leaves of *Tetracera asiatica*, Total flavonoids, Content determination, Habitat

1 Introduction

Tetracera asiatica (Lour.) Hoogland, also known as Seteng, Xueteng, Caomiteng, Gouya and Gousheteng, belonging to Dilleniaceae, can be medicated with its whole plant[1]. *T. asiatica* is an evergreen plant that grows all year round, usually up to 20 m or longer in length. The plants mostly have branches that intertwine with each other, and their branches are rough. Their leaves are often covered with hair, and are extremely rough. The medicinal parts of *T. asiatica* are often roots, stems, and leaves[2-4]. It is commonly used in the Zhuang and Li ethnic groups to treat long-term diarrhea, anal prolapse, bleeding with defecation, male ejaculation, female vaginal discharge, uterine prolapse, bruises, liver and spleen enlargement, constipation, *etc.*[5-6]. *T. asiatica* mainly grows in low-altitude mountains, jungles, shrubs, *etc.*, and it is concentrated in tropical and subtropical regions such as Guangxi, Guangdong, Yunnan, Hong Kong, Hainan, *etc.*, with abundant plant resources and great development potential. The medicinal herb is rich in various bioactive components such as tannins, flavonoids, and terpenoids. At present, there is not much research on the leaves of *T. asiatica* at home and abroad, and most literature research mainly focuses on the types of its chemical composition and pharmacological effects of its roots and stems. No specific research has been conducted on the leaves of *T. asiatica*, while the leaves are easy to collect, and effectively utilizing them can effectively protect medicinal resources. The previous studies of our research team found in the early stage showed that flavonoids from the roots and stems of *T. asiatica* have significant hepatoprotective effects[7-9], and flavonoids from *T. asiatica* leaves have potential pharmacological effects. Therefore, with the leaves of *T. asiatica* as the research object, ultraviolet-visible spectrophotometry was applied to determine the contents of flavonoids in *T. asiatica* leaves from different habitats in China, providing a scientific basis and data support for the sustainable utilization of *T. asiatica* medicinal resources in the future.

Received: December 6, 2022 Accepted: July 7, 2023
Supported by Regional Fund of the National Natural Science Foundation of China (82160820); General Project of Natural Science Foundation of Guizhou Province (QKHJC-ZK [2023] YB 153); Startup Foundation for Introduced Doctors in 2018 (2018BS047).
* Corresponding author. E-mail: 604178071@qq.com

2 Experimental materials

2.1 Medicinal materials The medicinal materials of *T. asiatica* were collected from different regions in China and identified by Associate Professor Dai Zhonghua of Guangxi University of Chinese Medicine as the leaves of *T. asiatica* (Lour.) Hoogl. After low-temperature drying, the samples were crushed into coarse powder for later use.

2.2 Instruments UV2600 ultraviolet-visible spectrophotometer (Agilent); B35005-MT ultrasonic cleaning instrument (Branson Ultrasonics (Shanghai) Co., Ltd); electronic analytical balance (Sartorius Instrument (Beijing) Co., Ltd.); HH-S2 digital display constant-temperature water bath pot (Jintan Medical Apparatus Factory); crusher.

2.3 Reagets Rutin reference substance (National Institute for the Control of Pharmaceutical and Biological Products, batch number ZX63-UTXV, for content testing); sodium nitrite, aluminum nitrate, sodium hydroxide, methanol, and ethanol, all analytically pure; pure water.

3 Methods and results

3.1 Preparation of reference solution First, 5 mg of the reference substance rutin was accurately weighed, and added into a

25 mL dry conical flask. Next, 10 mL of 60% ethanol was added to perform ultrasonic dissolution. After cooling, 60% ethanol was added to the mark, and the rutin reference solution was obtained after shaking well.

3.2 Preparation of test solution First, 1 g of the coarse powder of *T. asiatica* sample (Rongxian County, Dijin Village) was accurately weighed and added into a 150 mL conical flask. Next, 25 mL of 60% ethanol was added, and the bottle containing the sample was weighed to record the total weight. The sample was reflux-extracted once in a 90 ℃ water bath, with an extraction time of 1 h. After cooling down, 60% ethanol was added to the conical flask using a dropper to replenish the weight loss to the previously recorded weight. Finally, 3 mL of filtrate was added into a 25 mL dry volumetric flask, and added with 60% ethanol to constant volume, and the sample solution of *T. asiatica* was obtained after shaking well.

3.3 Selection of measurement wavelength First, 1 mL of the rutin reference solution and 1 mL of the *T. asiatica* test solution were accurately measured, and transferred to a 10 mL dry volumetric flask, respectively. Each solution was added with 0.5 mL of 5% $NaNO_2$ solution, shaken well, and stood for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and after standing for 5 min, 5 mL of 4% NaOH solution was added. Next, each solution was diluted with 60% ethanol solution to 10 mL, shaken well, and stood for 10 min. Finally, with 60% ethanol as the reference solution, scanning was performed within a wavelength range of 200 – 800 nm according to the ultraviolet-visible spectrophotometry (General Rule 0401 of the 2020 edition of the *Chinese Pharmacopoeia*), and the results showed that there was a maximum absorption wavelength at 501 nm. Therefore, 501 nm was chosen as the measurement wavelength for this experiment.

3.4 Methodology investigation

3.4.1 Investigation of linear relationship. A pipette was employed to precisely transfer 0, 0.1, 0.5, 1, 2 and 3 mL of the rutin reference solution prepared above into 10 mL volumetric flasks, respectively, and 0.5 mL of 5% $NaNO_2$ solution was added into each flask, followed by shaking and standing still for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and each solution was shaken well and stood for 5 min. Next, a pipette was used to transfer 5 mL of 4% NaOH solution into each solution, and various solutions were diluted with 60% ethanol solution to constant volume, and shaken well and stood for 10 min. With 60% ethanol as the blank reference solution, the absorbance values of different concentrations of rutin were measured at a wavelength of 501 nm for 3 times in parallel to obtain the calibration curve of rutin reference solutions. Regression calculation was performed by the least squares method, and the relationship curve between the absorbance value (Y) and the concentration (X) of rutin standard solution was obtained: $Y = 11.813x - 0.001$, $R^2 = 0.9999$. The standard solutions of rutin had a good linear relationship with absorbance within the concentration range of (0.004 – 0.120) mg/mL.

3.4.2 Precision test. First, 1.0 mL of the rutin reference solution prepared above was transferred into a 10 mL volumetric flask, and added with 0.5 mL of 5% $NaNO_2$ solution to obtain a solution, which was shaken well and stood still for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and the solution was shaken well and stood for 5 min. Next, 5 mL of 4% NaOH solution was added into the solution, which was then diluted with 60% ethanol solution to constant volume, and shaken well and stood for 10 min. The absorbance of the resultant solution was continuously measured 6 times, and the results showed that the precision of the test method was good, and the reliability was high, so it could meet the standard for content determination. The results showed that the average absorbance was 0.452 43, and the *RSD* was 0.03%.

3.4.3 Stability test. First, 1.0 mL of the *T. asiatica* test solution (Dijin Village, Rongxian County) was transferred into a 10 mL volumetric flask with a pipette, and added with 0.5 mL of 5% $NaNO_2$ solution to obtain a solution, which was shaken well and stood still for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and the solution was shaken well and stood for 5 min. Next, 5 mL of 4% NaOH solution was added, and the obtained solution was diluted with 60% ethanol solution to constant volume, and shaken well and stood for 10 min. Finally, the absorbance was measured at 0, 10, 20, 30, 40, 50, 60, 70, and 80 min after standing at room temperature. The experimental results showed that the stability was good within 70 min after color development, which could meet the experimental detection requirements.

3.4.4 Repeatability experiments. First, six portions of the powder of *T. asiatica* leaves (Rongxian County, Dijin Village) were accurately weighed, 1 g each. Each portion was added into a 150 mL conical flask, and added with 25 mL of 60% ethanol, and the bottle containing the sample was weighed to record the total weight. Each sample was reflux-extracted once in a 90 ℃ water bath, with an extraction time of 1 h. After cooling down, 60% ethanol was added to each conical flask dropwise to replenish the weight loss to the previously recorded weight. Next, 3 mL of each filtrate was added into a 25 mL dry volumetric flask, and added with 60% ethanol to constant volume, and six parallel sample solutions of *T. asiatica* were obtained after shaking well. Next, each solution was added with 0.5 mL of 5% $NaNO_2$ solution, and shaken well and stood still for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and the solution was shaken well and stood for 5 min. Next, 5 mL of 4% NaOH solution was added, and the obtained solution was diluted with 60% ethanol solution to constant volume, and shaken well and stood for 10 min. Finally, the absorbance values of various sample solutions were determined using a UV spectrophotometer to calculate the total flavonoid contents in them. The results showed that the average total flavonoid content was 10.21%, and the *RSD* was 1.99%.

3.4.5 Recovery test. First, six portions of the powder of *T. asiatica* leaves (Rongxian County, Dijin Village) were accurately weighed, 1 g each. Next, a certain amount of rutin reference substance was added to each portion of sample. Each mixture was

added into a 150 mL conical flask, and added with 25 mL of 60% ethanol, and the bottle containing the sample was weighed to record the total weight. Each sample was reflux-extracted once in a 90 ℃ water bath, with an extraction time of 1 h. After cooling down, 60% ethanol was added to each conical flask dropwise to replenish the weight loss to the previously recorded weight. Next, 3 mL of each filtrate was added into a 25 mL dry volumetric flask, and added with 60% ethanol to constant volume, and six parallel sample solutions of *T. asiatica* were obtained after shaking well. Next, each solution was added with 0.5 mL of 5% $NaNO_2$ solution, and shaken well and stood still for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and the solution was shaken well and stood for 5 min. Next, 5 mL of 4% NaOH solution was added, and the obtained solution was diluted with 60% ethanol solution to constant volume, and shaken well and stood for 10 min. Finally, the total flavonoid contents in various sample solutions were determined, as shown in Table 1.

Table 1 Results of recovery test on *Tetracera asiatica*

Sample weight//g	Amount in sample//mg	Added amount//mg	Measured amount//mg	Recovery//%	Average recovery//%	*RSD*//%
1.000 4	0.490 28	0.392 23	0.089 26	102.08	99.96	2.26
1.000 1	0.490 13	0.391 83	0.088 05	99.72		
1.000 6	0.490 37	0.490 29	0.096 34	96.49		
1.000 5	0.490 33	0.490 29	0.098 62	101.15		
1.000 1	0.490 13	0.587 95	0.010 693	98.22		
1.000 3	0.490 23	0.588 35	0.010 887	102.08		

3.5 Determination of total flavonoid contents in medicinal materials of *T. asiatica* leaves from different habitats First, samples of *T. asiatica* leaves from different habitats were accurately weighed, 1 g each. Each sample was added into a 150 mL conical flask, and added with 25 mL of 60% ethanol, and the bottle containing the sample was weighed to record the total weight. Next, each sample was reflux-extracted once in a 90 ℃ water bath, with an extraction time of 1 h. After cooling down, 60% ethanol was added to each conical flask dropwise to replenish the weight loss to the previously recorded weight. Next, 3 mL of each filtrate was added into a 25 mL dry volumetric flask, and added with 60% ethanol to constant volume, and various solutions of *T. asiatica* were obtained after shaking well. Next, 1 mL of each test solution was added with 0.5 mL of 5% $NaNO_2$ solution, and shaken well and stood still for 5 min. Next, 0.5 mL of 10% $Al(NO_3)_3$ solution was added, and the solution was shaken well and stood for 5 min. Next, 5 mL of 4% NaOH solution was added, and the obtained solution was diluted with 60% ethanol solution to constant volume, and shaken well and stood for 10 min. Finally, the absorbance values were determined using an ultraviolet-visible spectrophotometer, and the average values of total flavonoid content in the samples were obtained. The results showed that the total flavonoids in the samples of *T. asiatica* leaves ranged from 3.91% to 19.41%, with an average content of 12.36%, as shown in Table 2.

Table 2 Total flavonoid contents in medicinal materials of *Tetracera asiatica* leaves from different habitats ($n=3$, %)

Habitat	Content	*RSD*
Dijin Village, Rongxian County, Guangxi	10.80	0.24
Xianhu in Qingxiu District, Guangxi	8.57	0.02
Beiliu City, Guangxi	4.15	2.06
Lingshan County, Guangxi	10.18	0.35
Shangsi County, Guangxi	11.00	0.15
Bailiang Town, Rongxian County, Guangxi	13.98	0.10
Gaofeng, Nanning City, Guangxi	19.41	1.80
Bobai County, Guangxi	14.94	0.08
Xiaogaofeng, Nanning City, Guangxi	12.28	1.22
Hengxian County, Guangxi	12.94	0.15
Huizhou City, Guangdong Province	16.88	0.32
Shangcuntang, Bobai County, Guangxi	3.91	0.67
Hong Kong Special Administrative Region	16.76	2.49
Qinbei District, Qinzhou City, Guangxi	14.63	0.74
Fangchenggang City, Guangxi	15.18	2.35
Hengxian County, Guangxi	15.08	1.60
Shiwan Mountain, Shangsi County, Guangxi	15.56	2.26
Yulin City, Guangxi	5.79	2.75

4 Conclusions

Through this study, it was found that there were differences in the total flavonoid contents of *T. asiatica* leaves from different habitats. Among them, the highest content was found in Gaofeng (19.41%) of Guangxi, followed by Huizhou (16.88%) of Guangdong and Hong Kong (16.76%). The lowest total flavonoid content was found in Shangcuntang (3.91%) of Bobai County, Guangxi, followed by Beiliu (4.15%). The total flavonoid contents in the samples ranged from 3.91% to 19.41%, with an average content of about 12.36%. According to the above experimental results, there were significant differences in total flavonoid content in *T. asiatica* leaves in southern China. If you want to collect *T. asiatica* leaves as raw materials, it is recommended to go to Gaofeng in Nanning, Guangxi, Huizhou in Guangdong, and Hong Kong for sampling, rather than Shangcuntang in Bobai County, Guangxi, and Beiliu in Guangxi. Overall, the soil quality, altitude, climate, environmental temperature, humidity, and light intensity of different regions may all affect the total flavonoid content in the leaves of *T. asiatica*. These various influencing factors need further investigation. In this study, we established a content detection method for the flavonoid component rutin in the ethnic medicine *T. asiatica*, and validated the method. The validation content in-

cluded linear range, *T. asiatica* precision, stability, repeatability, and spiked recovery test. The results showed that the method is easy to operate, and has good repeatability, and the detection results are accurate and reliable, so it can be used for the determination of flavonoid content in *T. asiatica* leaves.

References

[1] Food and Drug Administration of Guangxi Zhuang Autonomous Region. Quality standards of Zhuang medicine, volume 2 (2011 edition)[S]. Nanning: Guangxi Science and Technology Press, 2011. (in Chinese).

[2] ZHU H, WEI SJ. Pharmacognosy of Zhuang medicine[M]. Nanning: Guangxi Nationalities Publishing House, 2006. (in Chinese).

[3] Health Department, Logistics Department, Guangzhou Army. Handbook of commonly used Chinese herbal medicines[M]. Beijing: People's Medical Publishing House, 1969. (in Chinese).

[4] ZHU H, TIAN H, CAI Y. Zhuang pharmacy[M]. Nanning: Guangxi Science and Technology Press, 2015. (in Chinese).

[5] ZHU H, FU P. Study on the Zhuang medicinal materials of *Tetracera asiatica*, Caryopteris incana, Sphenomeris chinensis, and Hedyotis auricularia[M]. Nanning: Guangxi Science and Technology Press, 2016. (in Chinese).

[6] DAI HF, MEI WL. Ethnic medicine records of Li nationality, volume 1 [M]. Beijing: Science and technology of China Press, 2008. (in Chinese).

[7] DA FF. Application of *Tetracera asiatica* and its extract in the preparation of drugs for the treatment and/or prevention of alcoholic liver injury[P]. Guangxi Zhuang Autonomous Region: CN110787196B, 2022-03-22. (in Chinese).

[8] ZHU H, DA FF, DAI ZH, *et al.* Application of *Tetracera asiatica* and its extract in the preparation of drugs for the treatment and/or prevention of chemical liver injury [P]. Guangxi Zhuang Autonomous Region: CN110787195A, 2020-02-14. (in Chinese).

[9] ZHU H, DA FF, FU P, *et al.* Application of *Tetracera asiatica* and its extracts in the preparation of drugs for the treatment and/or prevention of liver cancer[P]. Guangxi: CN107982292A, 2018-05-04. (in Chinese).

(From page 26)

Cyt-c were significantly up-regulated, inducing apoptosis. The results of flow cytometry showed that the number of G_0/G_1 phase cells in ODA group was significantly higher than that in solvent control group, while the number of G_2/M phase cells significantly decreased, and the apoptosis rate increased with the increase of drug concentration. ELISA detection results showed that ODA up-regulated the expression of caspase-9 in glioma cells, initiated the apoptosis signal pathway, led to apoptosis of glioma cells, and then exerted the anti-tumor effect of ODA[19].

In this paper, the inhibitory mechanism of ODA on proliferation of glioma cells was explored. ODA can damage cell membrane structure, increase cell membrane permeability, induce Cyt-c release from mitochondria, induce up-regulation of P21, caspase-9 protein expression, down-regulation of P13K, PKB/Akt, P53 protein expression, decrease the activity of PI3K-Akt signal transduction pathway, block cell division cycle, and lead to apoptosis and death of glioma cells. ODA can be used as a new drug resource for P53, P21, PI3K and PKB/AKT target therapy for nervous system tumors.

References

[1] LIU H, WANG J, TAO Y, *et al.* Curumol inhibits colorectal cancer proliferation by targeting miR-21 and modulated PTEN/PI3K/Akt pathways [J]. Life Sciences, 2019(221): 354 – 361.

[2] ZOU SJ, CHEN ZF, LI M. Expression of PI3K and AKT2 in gastric cancer and its elationship with clinicopathological features and survival of patients[J]. Journal of Practical Oncology, 2011, 26(4): 346 – 350.

[3] FU XQ, FENG JR, ZENG D, *et al.* PAK4 confers cisplatin resistance in gastric cancer cells *via* PI3K/Ak and MEK/Erk-dependent pathways[J]. Bioscience Reports, 2014, 34(2): 59 – 67.

[4] SHI J, YAO DM, LIU W, *et al.* Highly frequent PIK3CA amplification is associated with poor prognosis in gastric cancer[J]. BMC Cancer, 2012, 12(1): 50 – 61.

[5] GUO D, ZHANG B, LIU S, *et al.* Xanthohumol induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI3K/Akt/m TOR-kinase in human gastric cancer cells[J]. Biomed Pharmacoth, 2018(106): 1300 – 1306.

[6] ZHOU J, PEI Y, CHEN G, *et al.* SPC24 regulates breast cancer progression by PI3K/AKT signaling[J]. Gene, 2018(675): 272 – 277.

[7] CORTI F, NICHETTI F, RAIMONDI A, *et al.* Targeting the PI3K/AKT/mTOR pathway in biliary tract cancers: A review of current evidences and future perspectives[J]. Cancer Treatment Reviews, 2019 (72): 45 – 55.

[8] PARK S, KIM YS, KIM DY, *et al.* PI3K pathway in prostate cancer: All resistant roads lead to PI3K[J]. Biochimica Et Biophysica Acta-reviews On Cancer, 2018, 1870(2): 198 – 206.

[9] ZHONG X, WANG AJ, WANG HY. DHA-inhibited prolifera-tion through the PTEN/PI3K/Akt pathway in gastric cancer SGC 7901 cells[J]. Chinese Journal of Clinical Oncology, 2013, 40(4): 190 – 194.

[10] CAO WG, YANG WP, FAN R, *et al.* MiR-34a regulates cisplatin-induce gastric cancer cell death by modulating PI3K/AKT/survivin pathway [J]. Tumor Biology, 2014, 35(2): 1287 – 1295.

[11] ZHANG HY, CHEN JB, LU HZ. Research progress on the role of PI3K/Akt signaling pathway in tumor angiogenesis[J]. Shandong Medical Journal, 2012, 52(47): 98 – 100. (in Chinese).

[12] VELOSO M, FRITZ W, KLAUS K. P53 gene status and expression of p53, mdm2, and 21Wafl/Cipl proteins in colorectal cancer[J]. Virchous Arch, 2000, 437(3): 241 – 247.

[13] LI HX, LIU F, XIA JH. Effects of miR-670-5p on proliferation, migration and invasion of lung cancer cells[J]. Chinese Journal of Applied Physiology, 2021, 37(5): 500 – 505. (in Chinese).

[14] WANG Y, HE SY, ZHU RT, *et al.* The effects of shikonin on liver cancer cells SMMC-7721 apoptosis and its mechanism[J]. Chinese Journal of Applied Physiology, 2021, 37(4): 415 – 418. (in Chinese).

[15] DING L, SHAO SH, HE MQ, *et al.* Effects of Betulinic acid on apoptosis in human gastric cancer SGC-7901 cells[J]. Chinese Journal of Applied Physiology, 2021, 37(5): 495 – 499. (in Chinese).

[16] CHEN YN, SHAO SL, HE MQ, *et al.* Effects of Betulinic acid on proliferation in human gastric cancer SGC-7901 cells[J]. Chinese Journal of Applied Physiology, 2020, 36(6): 628 – 632. (in Chinese).

[17] LIU JP, SHAO SL, HE MQ, *et al.* Effect of betulinic acid on proliferation of human gastric cancer MGC-803 cell line[J]. Chinese Journal of Applied Physiology, 2021, 37(3): 257 – 261. (in Chinese).

[18] FAN H, SHAO SL, HE MQ, *et al.* Effects of betulinic acid on apoptosis of human gastric cancer MGC-803 cells[J]. Chinese Journal of Applied Physiology, 2021, 37(4): 419 – 422. (in Chinese).

[19] YU R, YU BX, CHEN JF, *et al.* Anti-tumor effects of at-ractylenolide I on bladder cancer cells[J]. Journal of Experimental Clinical Cancer Research, 2016, 35(40): 1 – 10.