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Original Article

Isolation, Simultaneous Quantification of Taxifolin and Taxifolin-3-O-rhamnoside and Validation by RPHPLC

Subashini Subramanian, Shakila Ramachandran*

ABSTRACT

Background: Taxifolin (TA) is a flavonoid that has antioxidant, hepatoprotective, anti-alzheimer, anti-hyperglycemic, cardiovascular, anti-inflammatory, anti-psoriatic and anti-alzheimer properties. Taxifolin 3-*O*-rhamnoside (TAR), a glycoside of taxifolin, has antioxidant, anticonvulsant, anticancer, anti-inflammatory, and immunosuppressive effects. **Objectives:** The goal of this study is to separate TA and TAR from *Smilax china* Linn. rhizomes, as well as to develop and validate a technique for simultaneous measurement of TA and TAR. **Materials and Methods:** The hydro alcoholic extract of the rhizome of *S. china* yielded TA and TAR. HPLC system was fitted with a C_{18} column (shim-pack) of size 150 mm x 4.6 mm; 5µ, with suitable eluting mixture of methanol: water (90:10 v/v) at a flowing rate of 1 ml/min and at 254 nm wavelength for identification of peaks. Lab solution software was used to establish the quantification method for above-mentioned chemical compounds. **Results:** TAR and TA were separated using the proposed technique at R_t 2.917 and 3.924 min respectively. Over the range of 0.1-0.8 µg/ml, calibration curves were produced with a linear relationship $R^2 > 0.9941$ and 0.9963, respectively. The relative standard deviation was less than 2%. The percentage recoveries were shown to be between 97 and 102.1. TA and TAR had detection limits of 0.156 and 0.077 µg/ml; quantification limits were 0.473 and 0.234 µg/ml respectively. The technique that was devised was simple, sensitive and specific. **Conclusion:** The newly designed RP-HPLC technique has improved specificity, precision, and accuracy. The quality of *S. china* and other dietary supplements containing these two flavonoids may be successfully assessed by quantifying these two flavonoids and their glycosides.

Key words: Astilbin, Dihydroqucertin, 3,5,7,3',4'-pentahydroxy flavanone, Tuberculosis, Validation.

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INTRODUCTION

Smilax china Linn, a member of the Smilacaceae family, grows widely in tropical and temperate locations around the world, particularly in East Asia.[1,2] This plant is a perennial climber with aculeate pore skin and paired tendrils that help with climbing. *Smilax china* L. tubers have been practiced for treating TB, gout, tumour and inflammation according to several studies.[3-5] The study about the Smilax genus has grown in popularity in recent years, particularly in Asia and Europe, as the existence of phenol compounds in few specie were found which can prevent and treat a variety of malignancies. Furthermore, plant extracts of the *Smilax* genus have antioxidant and pro-apoptotic properties.[6] (2R,3R) Hayashi and Ouchi was the first one to isolate taxifolin-3-β-O-rhamnoside from the rhizome of *Astilbe thunbergii*.[7] Astilbin is another name of (2R,3R)-taxifolin-*3-β*-O-rhamnoside. It is also present in other plants consisting of *Dimorphandra mollis*, *Psychotria prumfolia, Senna obtusifolia*, *Tithonia diversifolia*,[8] *Heritiera littoralis*,[9] *Engelhardtia roxburghiana*,[10,11] *Smilacis glabrae*,[12] *Smilacis chinae*,[13] *Drimys brasiliensis*,[14] *Hymenaea courbaril*,[15] *Hymenaea stigonocarpa*,[16] *Pieris japonica*,[17] previous research has suggested that astilbin has the potency to be used in both healthy supplements and medicine because of its various bioactivities, increasing liver injury immunological activity.[7] Taxifolin (also known as 3,5,7,3,4'-pentahydroxy flavanone or dihydroquercetin) is a flavonoid and an integral ingredient of nutritional dietary supplements. It is also utilized as a nutritious meal that is high in antioxidants. *Pseudotsuga taxifolia* (Lindl.) Britton was the first plant to isolate it, followed by *Larix gmelinii* (Rupr.) Kuzen. syn *Larix dahurica* Turcz. ex Trautv. and *Larix sibirica* Ledeb,[18] Milk thistle,[19] onions,[20] Douglas fir bark,[21] and French maritime pine bark,[22] are all sources of taxifolin. It can also be present in a variety of plants. It has hardly been utilised as a single component, although it may be found in several preparations such as silymarin (Legalon TM), Pycnogenol® and Venoruton®.[23]

In previous research, taxifolin in the plasma of rabbit was determined and studied for pharmacokinetic

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using Shimadzu's high pressure liquid chromatography with a C_{18} column (Luna) with 150 mm length, 4.6 mm diameter and 5 mm particle size, pre-column (2.0 mm, the same adsorbent) and UV detector in two step linear gradient elution mode with acetonitrile and 0.3 percent trifloroacetic acid in water mobile phase and 0.1 ml flow rate has been reported.[24] In another study, method by UPLC-MS with sunfire TM C_{18} column (2.1mm x 50mm, 3.5 µm) and electrospray ionization technique using the mobile phase of acetonitrile and 0.3 percent acetic acid by eluting in gradient mode from 10 percent acetonitrile followed by 35 percent acetonitrile and then 10 percent acetonitrile for quantification in rat plasma has been reported.[25]

MATERIALS AND METHODS

Solvents

HPLC grade solvents viz., acetonitrile, methanol and deionized water were of are purchased from Merck. Hexane and ethyl acetate were of LR grade from Reachem Chemicals.

Plant Material

The proposed study's dried plant material was acquired from an industrial source in Chennai, Tamil Nadu. The Pharmacognosy department of our Institute authenticated and stored the specimen samples with them.

Isolation

Over silica gel, the hydroalcoholic extract (50 g) was column chromatographed (60-120 mesh). Eluted using various eutectic mixture like hexane: ethyl acetate combination ratios. Using a rota-vacuum, the comparable fractions were mixed and evaporated. Compound 1 (yield 56 mg) was obtained from the fractions collected while eluting with hexane: ethyl acetate (80:20), whereas compound 2 (yield 65 mg) was obtained from the fractions collected while eluting with hexane: ethyl acetate (65:35). (Yield 135 mg). With a mobile phase of hexane: ethyl acetate (6:4, v/v), these compounds yielded a single spot at Rf 0.41 and 0.83 on TLC. NMR was used to analyze the spectral data, which was compared to taxifolin,[26] and taxifolin 3-*O*-rhamnoside.[27]

Instrumentation

HPLC experiments utilized a Shimadzu LC-20AP (Shimadzu Corporation, Kyoto, Japan). Column oven, degassing unit, low pressure gradient unit and isocratic unit were all parts of the LC-20AP system. In this experiment, a quaternary mixing pump with low pressure, a Shimadzu SPD-M20A Photodiode-Array detector with isothermal flow cell, and an RP-C_{18} column with 20-aliquot sample loop capacity, as well as a 20 microliter Hamilton injector were employed. The HPLC equipment collected and analysed data using lab solution. In order to get the UV spectra, a Shimadzu spectrometer was employed.

Selection of Wavelength

A standard 1 mg per ml solution was made in methanol. The RP-C_{18} column was injected with 20 µg/ml solutions using the Hamilton injector and the UV spectra was acquired by scanning the standard solution between 190 and 800 nm. Because of the maximum absorbance by TA and TAR were monitored in the eluents using a PDA detector at 254 nm.

Preparation of standard and sample solutions

When making samples for the study, 10 mg of the *S. china* sample was dissolved between three different solvents: ethanol, methanol, and an alcohol-based hydro-alcoholic solution. To make up the standards TA and TAR, 7 mg of compounds was dissolved in 7 ml of methanol in volumetric flasks (1 mg/ml). The mixture was then thoroughly mixed and filtered with Whatman filter paper before being filtered again through a 0.2 m membrane filter. To achieve the final concentration, the volume included up to the mark of methanol. The dilution series of 0.1, 0.2, 0.4, 0.6, and 0.8 µg/ml were produced in methanol and utilized as destination concentrations at 100 % from the standard stock solution described above.

Assay of the compounds

The ICH Protocol was used to design and validate a method for simultaneous quantification. The sample was tested three times with the optimum chromatographic settings. For each sample, chromatograms were made and the quantity of each was recorded, as well as the standard deviation and % RSD. The RP-HPLC-developed approach for measuring compounds (TA and TAR) was validated in accordance with the standard criteria in terms of its specificity, linearity, sensitivity, reliability, limit of detection, and limit of quantification.

RESULTS

Specificity/Selectivity

Specificity refers to a method's ability to analyze analyte responses when other receivers and contaminants are present. To prove their sensitivity, *S. china* sample extracts were compared to a chromatogram of a reference solution for the presence of receivers, contaminants, and other degradation. One may observe what type of reaction one receive by varying the concentration of the analytes one is measuring using the linearity and range of an analytical technique. It was decided to evaluate the linearity of the proposed technique using 0.1-0.8 µg/ml. In the mobile phase, these standards were prepared using a standard mother solution containing 1 mg/ml using optimum chromatographic conditions, the linearity standards were injected three times, and the chromatograms were recorded as evidence. To determine linearity, a graph was made with concentration (µg/ml) on the X-axis and compound area (TA and TAR) on the Y-axis, and then the correlation coefficient was calculated.

Precision and accuracy

The precision of the proposed approach was tested using interday and intraday precision experiments. Five indiscriminate injections of five distinct concentrations, namely 0.1, 0.2, 0.4, 0.6, and 0.8 µg/ml. They were employed to check the reliability of the approach once it had been done, and were injected the above mentioned samples on the same days. Internal accuracy was also examined by injecting the same samples on several days to see whether there was any variation. The average and relative percentage of the mean standard deviation were computed. In the recovery experiments, the accuracy of the methods was reported. It was accomplished using the conventional addition approach, i.e., by adding a known concentration standards to a known sample and analysing the results using the optimum chromatographic conditions The recovery tests were conducted at four different concentrations (0.1-0.8 µg/ml) and the percentage of standard deviation, relative standard deviation and specific recovery were determined.

Limit of detection and quantification

Sensitive techniques are those that can detect analytes at very low concentrations. By testing the lowest concentration of standard solution by the newly established RP-HPLC techniques, the limit of detection and limit of quantitation were calculated. The LOD is the lowest detectable concentration of analyte that can produce a detectable response (s/n ratio 3). The LOQ is the lowest concentration of an analyte at which a measurable response can be seen (s/n ratio 10). The formula used to determine LOD and LOQ values are:

$$LOD = 3.3\sigma \div S \text{ and } LOQ = 10\sigma \div S$$

Where S = slope of the deviation curve and σ = standard deviation.

Robustness and Ruggedness

Ruggedness and robustness of the method were assessed by altering the investigation parameters (analyst, reagents and columns) and enhancing the chromatographic conditions (pH of solvents, composition of mobile phase, changing the ratios of mobile phase and rate of flow of mobile phase).

System suitability

The analytical development and validation include consideration of system adaption features to ensure the functionality of system. HPLC characteristics such as peak retention times (R*t*) and asymmetry factors (A) were assessed after four injections of the compounds (TA and TAR) at a concentration of 10 mg/ml each.

DISCUSSION

Shao *et al.* has isolated six phenolic compounds viz., engeletin, oxyresveratrol, piceid, resveratrol, scirpusin A and taxifolin-3-*O*-glycoside from ethyl acetate fraction of 95 percent ethanol extract of *S. china* rhizome by repeated column chromatography over silica gel and developed a HPLC method for the simultaneous determination of these phenolic compounds. The HPLC system contained a C_{18} (Zorbax XDB) column eluted gradiently with acetonitrile and 0.02 percent phosphoric acid and the rate of flow was 1 ml/min and detected at 300 nm.[28] Another study reported on the quantification of TA in a HPLC system with a C_{18} (Zorbax SB) column (250 mm x 4.6 mm inner dia, 5 µ) using the solvent mixture of acetonitrile (A) and 0.1 percent acetic acid solution (B) programmed in linear gradient elution of 15-20 percent (A) in 0-15 min, and 20-40 percent (A) 15-40 min with a rate of flow of 1 ml/min. The gradient method was observed in all of the conventional techniques, which used acetonitrile and acidified water solvents of changing composition.[10] A better chromatographic technique was developed using the results of several trials with different mobile phase compositions. Finally, a 70:30 (v/v) mobile phase composed of methanol and water was selected on, which showed peak asymmetry at flow rates of one millilitre per minute. For the solid phase, a 150 mm x 4.6 mm C_{18} column was used and measured the peaks at 254 nm. The compound TAR was eluted in 2.917 min, while the molecule TA took 3.924 min to elute. Before injecting the solvents into the HPLC system, they were filtered via a 0.45 µm membrane filter made up of poly tetra fluoro ethylene. The chromatographic peaks were detected, recorded and processed using the lab solution software. Methanol, unlike ethanol or hydro alcohol, was shown to be the most effective solvent for extracting these compounds.

Linearity

For five different chemical concentrations, the calibration curve was compared to the matching peak region. Acceptable correlations were found between concentration and peak area for the compounds in the range of concentrations (0.1-0.8 µg/ml). When looking at the slope and intercept, it was found that 474.32 and 8225.5 were the values for the compounds (TA and TAR) correlation coefficient, which is more significant at 0.9999. The graph of linearity is shown in Table 1 and Figure 1, respectively.

Specificity

The specificity/selectivity of the method was shown by injecting dilute solutions of standards (TA and TAR) and sample solution of *S. china* for checking the absence of co eluting peaks during the retention times of the compounds (2.917 and 3.924) (Figure 2). There were no co-eluent peaks; the form of it is symmetric and crisp, demonstrating the specificity of the improved chromatographic technique. Three replicates of the sample solutions and standard were used in the analysis.

Table 1: Calibration curve data.

Regression parameters	TA	TAR
Regression equation	72919x+1191.7	72088x-5432.1
Correlation coefficient (R^2)	0.9941	0.9963
Slope	72919	72088
Y-intercept	1191.7	5432.1
Concentration range (µg/mL)	0.1, 0.2.0.4, 0.6 and 0.8	0.1, 0.2.0.4, 0.6 and 0.8
Number of points	2	2

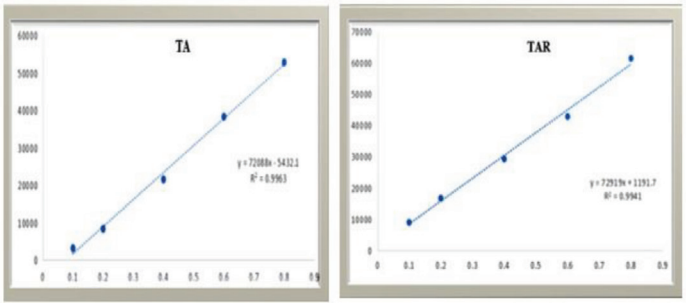

Figure 1: Linearity graph of taxifolin (TA) and taxifolin 3-O-rhamnoside (TAR).

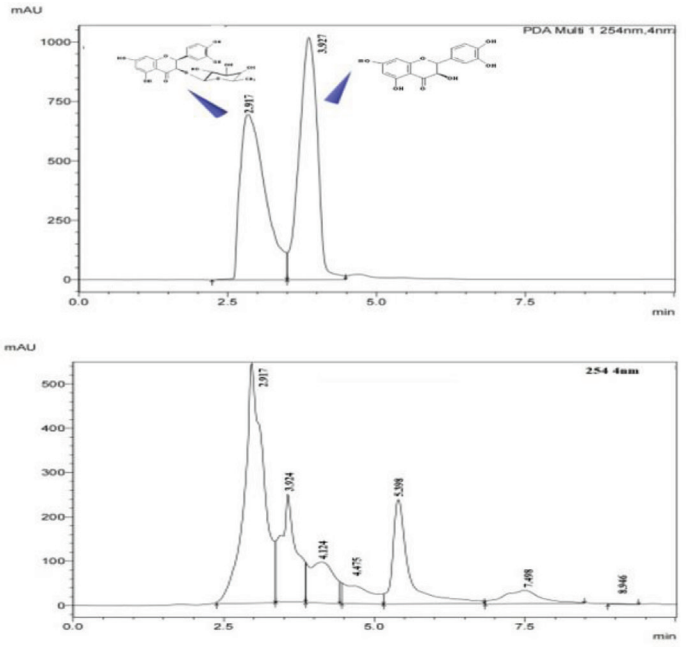

Figure 2: HPLC chromatogram of standard mixture of TAR and TA and methanol extract of *S. china*.

Precision and Accuracy

The accuracy of the developed method was represented as the average recovery for four distinct concentration levels (0.1, 0.4, 0.6, 0.8 µg/ml). At each stage, a three-fold analysis was performed. The percentage

Table 2: Mean recovery of TA and TAR.

TA			TAR		
Amount injected (µg/mL)	Amount recovered (µg/mL)	% Recovery	Amount injected (µg/mL)	Amount recovered (µg/mL)	% Recovery
0.1	0.097	97	0.1	0.098	98
0.4	0.395	98.75	0.4	0.388	97.0
0.6	0.613	102.1	0.6	0.598	99.6
0.8	0.807	100.8	0.8	0.801	100.12

Table 3: Summary of validation parameters.

Parameter	TA	TAR
Linearity (R^2)	0.9941	0.9963
Intra−day precision (% RSD)	1.945	1.622
Inter−day precision (% RSD)	1.715	1.812
LOD (µg/mL)	0.156	0.077
LOQ (µg/mL)	0.473	0.234
Accuracy (%)	99.66	98.68
Robustness	Robust	Robust
Specificity	Specific	Specific

average recovery was computed (Table 2). The accuracy was between 97 and 102.1 percent. The developed approach was utilized for chemical analysis (TA and TAR). The percent RSD for the assay experiments performed on actual samples was less than 2%, indicating the method's accuracy. The intraday and interday precision experiments revealed the percentage RSD value less than 2 indicating the accuracy of the method.

Limit of Detection and Quantification

The limit of detection and quantification shows the sensitivity of the developed method. The LOD and LOQ were determined as 0.156 and 0.473 µg/ml for the TA component and 0.077 and 0.234 µg/ml for the TAR component, showing the sensitivity of the procedure.

Robustness and Ruggedness

The flow rate (1 ml/min), pH, column temperature and organic phase composition were all varied to see the robustness of the technique. As a result of these differences, the chromatographic parameters didn't alter much even after varying the experimental settings. A fast and sensitive reverse phase HPLC method has been established to quantify the compounds TA and TAR, with run times of 2.917 and 3.924 min, respectively. The sample's retention time were unaltered (TA and TAR).

System suitability

The compatibility of the system was evaluated by executing the tests and noting changes in dissociation, retention time and peak distortion with four replicating injections of the standard at the working concentration. System appropriateness has been incorporated through permissible limits which are shown in Table 3.

CONCLUSION

An RP-HPLC technique that is quick, affordable, sensitivity, accurate, and precise has indeed been established and validated for method as per ICH criteria. In this case, the technique accuracy was shown by a relative standard deviation of 0.9982. Compounds TA and TAR exhibited excellent linearity in the concentration range (0.1-0.8 µg/ml). During the retention period of the chemicals in the sample solution, no interference was found (TA and TAR). The current verified technique has a recovery rate of 97% – 102% on average. To sum up, a quick reverse phase HPLC technique was developed and validated for measurement of components TA and TAR in *S. china* extracts and other dietary supplements. This approach is accurate, precise, as well as prompt.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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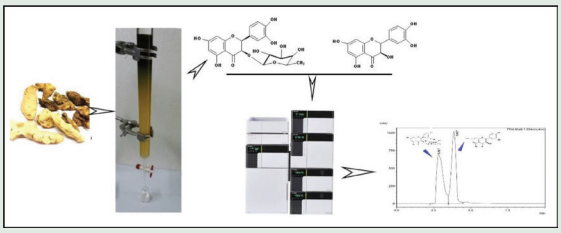
GRAPHICAL ABSTRACT



SUMMARY

The taxifolin and taxifolin 3-O-rhamnoside were isolated from the hydro alcoholic extract of *S. china* rhizome. RP-HPLC method was developed to quantitatively estimate taxifolin and taxifolin 3-O-rhamnoside. Rt of the compounds were 2.917 (taxifolin 3-O-rhamnoside) and 3.924 min (taxifolin). The linearity was achieved in the range of 0.1-0.8 µg/ml; the linear relationship r2 > 0.9963 and 0.9941 respectively; < 2% relative standard deviation; percentage recoveries were between 97 and 102.1. TA and TAR had detection limits of 0.156 and 0.077 µg/ml; quantification limits of 0.473 and 0.234 µg/ml respectively.

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