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# Chromatography of separation and qualitative, quantitative analysis biflavonoids from crude extract of *Selaginella tamariscina*

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Previous investigations reported that the class of biflavonoids represents a great pharmacological activity, which remained to be fully exploited, since most of them have not yet been found in nature or else have not been synthesized. Owing to its significant bioactivities and the quality control of medicine herb, effective methods for the extraction, isolation and purification of biflavonoids from *S. tamariscina* become necessary. In order to evaluate the performances of the optimal extract method, four extraction solvent and five extraction methods (Accelerated solvent extraction, SFE-$CO_2$ extraction, reflux extraction, Soaking extraction and Ultrasonic extraction)were used to extract the bioactive compounds from *Selaginella tamariscina* (Beauv) Spring. HPLC method was attempted for quantitative analysis and comparison with different extract by determining the content of amentoflavone. The result indicates that extraction temperature has significant impact on the extraction efficiency, and reflux extraction is proved to be the effective technique for extracting biflavonoid of *S. tamariscina*. Reverse-phase medium-pressure liquid chromatography(RP-MPLC) was first applied to separate and isolate biflavonoid from extract of *S. tamariscina*. High-purity biflavonoid was obtained and at over 98% purity as determined by HPLC. Furthermore, electrospray ion source mass spectroscopy (ESI-MS) was employed for rapid identification of the obtained compound from extract of *S. tamariscina*. Amentoflavone were identified based on the detection of the molecular ions, and the fragment ions of the molecular ion obtained in the CID experiments with those of the data reported in the literature. The chromatography technologies we employed were effective and feasible for the extraction, separation, qualitative and quantitative analysis of biflavonoids from *S. tamariscian*.

**Keywords:** *Selaginella tamariscina* (Beauv) Spring; amentoflavone; RP-MPLC; ESI-MS; extraction

## INTRODUCTION

*Selaginella tamariscina*(Beauv) Spring belongs to the family Selaginellaceae, the genus of *Selaginellace* contains about 80 species in the world, of which about 50 species are distributed in China. *S. tamariscina* is a traditional medicine, which was first recorded by "ShenNong Ben Cao Jing" around 1700 years ago. It has been used in oriental medicine to treat amenorrhea, dysmenorrheal, metrorrhagia, hematuria, prolapse of the anus, chronic hepatitis and hyperglycaemia.

Data from preliminary research has shown that different classes of natural products have been isolated from these species, including flavonoids, lignans, anthraquinone, alkaloids [1-2]. Investigation of the phytochemical constituents of *S. tamariscina* revealed that the major constituents in *Selaginella tamariscina*are flavonoids, especially a rich source of biflavonoids. Biflavonoids are flavonoid dimmers connected with a C–C or a C–O–C bond, which exhibited several biological activities, including cytotoxic [3], antiallergenic, anti-inflammatory [4-5], antiviral, antioxidant [6-7], inhibition of nuclear factor-k B activation [8], hypoglycemic and hypolipidemic [9], antiplasmodial and leishmanicidal activity [10]. In addition, sometimes the bioactivity of a biflavonoid is greater than that of the corresponding monomers. Amentoflavone is the main biflavonoid of *S. tamariscina*, with higher content in crude extract, and represents a great pharmacological activity.

Owing to bioflavonoids' significant bioactivities and the quality control of medicine herb, effective methods for the extraction, isolation and purification biflavonoids of *S. tamariscina* become necessary. However, extract and separate analysis of chemical components from *S. tamariscina* has been reported less. Traditionally, the extraction method of biflavonoids almost applied the conventional method, such as organic solvent extraction and reflux extraction; in recent years, a series of modern technology has been widely used to extract, contained accelerated solvent extraction (ASE), SFE-$CO_2$ extraction, microwave and Ultrasonic extraction, etc. But the technology is more suitable for the extraction of active substances need to be experimental investigation. The conventional preparative separation and purification of flavonoids employed column chromatograph, such as silica gel, Sephadex LH-20 and macroporous adsorptive resin, which are often involve repeated chromatographic

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steps on different columns. Comparing preparative HPLC separation, the MPLC has high flow rate and low pressure, the analytical grade solvents were used as elution solvent, so it is time consuming and lower costs. MPLC has enabled a significantly improved efficiency in separation and purification of the compounds of interest in short time.

In this work, five different extraction technologies, containing ASE, SFE-CO2 extraction, reflux extraction, soaking extraction and ultrasonic extraction were applied to extract the biflavonoids from *S. tamariscian*. The extraction technologies were optimized with the extraction quantity of amentoflavone as investigated by HPLC-DAD. Then, RP-MPLC was selected to separate the crude extract of *S. tamariscina* and enrich the interested fraction containing amentoflavone,whicn was further determined by HPLC-DAD. Finally, ESI-MS was employed for rapid identification of main bioactive compounds from extract of *S. tamariscina*. Fig.1 shows the structure of amentoflavone.

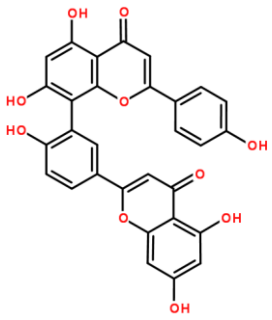


**Fig.1.**The strcture of the amentoflavone

## EXPERIMENTAL

### *Chemicals and reagents*

*S. tamariscian* was purchased from HebeiKanghui Medicinal Company (Hebei, China); all organic solvents used for extraction and RP-MPLC separation were of analytical grade and purchased from Beijing Chemicals (Beijing, China); Methanol and acetic acid were HPLC grade and purchased from Fisher chemical Ltd. (USA). Water was purified on a Milli-Q water purification system (Millipore, France).

### *Sample preparation*

In order to compare the extraction effects of different methods, four different extract solvent and five extraction methods were carried out to extract the biflavonoids from *S. tamariscian*.

### *Different solvent extraction*

Four *S. tamariscina* powder (5.001, 5.002, 5.006, 5.007 g) was accurately weighed and place in 100 mL erlenmeyer flask, respectively, then extracted with 50 mL of 70% aqueous ethanol, hexane, n-butanol, ethyl acetate by sonication for 2 h at room temperature. Four extraction mixtures were filtered, concentrated to dryness in vacuo at 55 ℃, dissolved in 10 mL volumetric flask with methanol and filtered through a 0.45 μm filter prior to use for following analysis.

### *Supercritical fluid extraction*

In this work, the SFE apparatus used was performed on a Speed™ SFE Prime device (Applied Separations Inc). Carbon dioxide (99.99% purity) obtained from an oxygen factory (Changchun, China) was used as the extraction solvent. The operating methodology was as follows: Accurate weighing 5.006 g of *S. tamariscina* power was placed inside the 24 mL extractor equipped with filter film, filter wool on both ends to prevent the particles being flushed out. The loaded extractor was then introduced into the extraction vessel and $CO_2$ was let in. The device was cooled for 1 h before being set the desired pressure and temperature. Experimental conditions: extractor temperature at 60 ℃, pressure 200 bar, static extraction time for 0.5 h and dynamic extraction time for 1 h, outlet temperature at 90 ℃, entrainment agents was 70% ethanol 40mL. The crude extract was collected after the desired time and concentrated to dryness in vacuo at 55℃. The sample obtained was dissolved in 10 mL volumetric flask with methanol and filtered through a 0.45 μm filter prior to use for following HPLC analysis.

### *Accelerated solvent extraction（ASE）*

Accelerated Solvent Extraction 150 System (Dionex, Sunnyvale, CA, USA) with 100 mL stainless steel ASE vessels was used for the pressurized liquid extraction. Accurate weighing 5.005 g of *S. tamariscina* powder was placed into the extraction cell (34 mL). The extraction cells were placed into the ASE system and the extraction conditions and process were as follows: 70% ethanol was used as the extract solvent, firstly, static time of 4 min, followed by a flush elution with 60% volume, and followed by the nitrogen purge of 60 s, and extracted three time. The extraction temperature was 80 ℃, and pressure was below 1500 psi. The ethanol extract was concentrated to dryness in vacuo at 55℃. The sample obtained was dissolved in 10 mL volumetric flask with methanol and filtered through a 0.45 μm filter prior to use for following analysis.

*Reflux extraction*

*S. tamariscina* powder (5.009 g) was accurately weighed and place in 100 mL round-bottomed flask, then extracted with 50 mL of 70% aqueous ethanol for 1 h at 90 ℃. The extraction mixture was filtered, concentrated to dryness in vacuo at 55℃, dissolved in 10 mL volumetric flask with methanol and filtered through a 0.45 μm filter prior to use for following analysis.

*Soaking extraction*

*S. tamariscina* powder (5.007 g) was accurately weighed and place in 100 mL erlenmeyer flask, then extracted with 50 mL of 70% aqueous ethanol for 2 h at 40 ℃. The extraction mixture was filtered, concentrated to dryness in vacuo at 55 ℃, dissolved in 10 mL volumetric flask with methanol and filtered through a 0.45 μm filter prior to use for following analysis.

*Ultrasonic extraction*

*S. tamariscina* powder (5.005 g) was accurately weighed and placed in 100 mL erlenmeyer flask, then extracted with 50 mL of 70% aqueous ethanol by sonication for 1 h at room temperature. The extraction mixture was filtered, concentrated to dryness in vacuo at 55 ℃, dissolved in 10 mL volumetric flask with methanol and filtered through a 0.45 μm filter prior to use for following analysis.

*RP-MPLC Separation*

RP-MPLC Separation was carried out in an Isolera one MPLC system (Biotage Switzerland). The separation was carried out with a Biotage® SNAP Cartridge KP- C18-HS column (12 g). The binary mobile phase consisted of methanol (solvent A) and water containing 0.2% acetic acid (solvent B). A binary gradient elution program with the flow-rate of 4 mL/min was used for the separation. The system was run with a gradient program: 20 mL 80% B; 50 mL 80-70 % B; 10 mL 70-40% B; 30 mL 40-15% B; 10 mL 15% B. The column was first equilibrated with 20 mL 80% B, the 1.024 g *S. tamariscian* extracted by ultrasonic dissolved in 2 mL methanol was then injected into the column. Peaks of interest were monitored at 337 nm by a DAD detector. The fractions were collected into test tubes per 10 mL with a fraction collector, and further analyzed by HPLC-DAD. The fractions were determined at over 98% purity by HPLC were combined, which was prepared for further ESI-MS analyses, then freeze-dried. The purified compounds were stored at -20 ºC before HPLC quantitative analysis.

*HPLC Analysis*

HPLC equipped with DAD detector was used for the analysis of biflavonoids of *S. tamariscina*. The separation was carried out in an Agilent Extend-$C_{18}$ column (250×4.6 mm, 5μm) with a $C_{18}$ guard column. The binary mobile phase consisted of methanol (solvent A) and water containing 0.2% acetic acid (solvent B). All the solvents were filtered through a 0.45 μm filter prior to use. The sample injection volume was 10 μL. The flow-rate was constantly kept at 1.0 mLmin-1 with a total run time of 20 min. Column temperature: 30 ℃. The system was run with a gradient program: 0–5 min: 70% B to 40% B; 5–10 min: 40% B to 20% B; 10–18 min: 20% B to 10% B; 18–20 min: 10% B to 70% B; detection wavelength: 337 nm.

Seven amentoflavone methanol samples of different concentrations (0.1 mg/mL~2.5 mg/mL) were loaded into HPLC system for construction of the calibration curve by plotting the peak areas (Y) versus the quality (X). Each extract sample was loaded into HPLC system for identification and quantification of amentoflavone in *S. tamariscina*, respectively.

*ESI-MS experiments*

The mass spectrometry experiments were performed on a Thermo Scientific LCQ Fleet mass spectrometer with an electrospray ionization (ESI) interface (ThermoFisher, USA). The flow-rate was constantly kept at 10 μL/min. The sheath gas and auxiliary flow rates were set at 40 and 10 (arbitrary unit), respectively. The capillary voltage was set at 4.5 V and its temperature was controlled at 350 °C. The isolation width for ESI-MSn was 2.0 Da, and the collision energy (%) was 20-25%. Full scan of ions ranged from 100 to 1500 molecular weights in the negative ion mode.

## RESULTS AND DISCUSSION

*HPLC quantification of amentoflavone content of different extract*

In this study, we prepared seven amentoflavone methanol samples of different concentrations to construct the calibration curve and quantified the *S. tamariscina* extracts with different extraction method by HPLC. The linear regression equation was calculated as Y= 254190X - 52.189, (r=0.9998), which showed good linear regression within the test ranges (1~25 μg). The amentoflavone content in different *S. tamariscina* extracts was calculated as shown in Table 1 and Table 2.

*Comparison of different extraction solvent*

It is known that biflavonoid are flavonoid dimmers connected with a C–C or a C–O–C bond, most of them have lower polarity than flavonoid containing many hydroxyl groups. Hence, 70% aqueous ethanol, hexane, n-butanol, ethyl acetate had been tested. As shown from the result of Table 1, amentoflavone content of 70% aqueous ethanol extract was higher (14.051 mg/g) than others, and that of hexane extract was the lowest (0.404 mg/g). It is not significantly different from those obtained by n-butanol (1.718 mg/g) and ethyl acetate extracts (1.707 mg/g). The reason may be the polarity of 70% aqueous ethanol is similar with the amentoflavone. So 70% ethanol was proved to be most efficient to extract the biflavonoids from the *S. tamariscina.*

*Comparison of different extraction methods*

In order to evaluate the performances of the optimal method, five extraction methods were also applied. The chromatogram of the extract obtained by the ultrasonic extraction method was shown in Fig. 2A.

The amentoflavone content of different crude extract (Table 2) obtained by five methods mentioned above were significantly different; the extraction yields obtained by the reflux method are higher than those obtained by the other four methods. As shown from the result, we should know that extraction temperature has significant impact on the extraction efficiency. Increasing the temperature could improve the yield of amentoflavone, the reflux extraction with 90 ℃ obtained the highest extraction amount (33.006 mg/g). If the extraction temperature was lower, the content of amentoflavone would be reduced obviously. Followed by accelerated solvent extraction（ASE） with 80 ℃ (27.880 mg/g) , SFE-$CO_2$ extraction with 60 ℃ (20.181 mg/g), respectively. From the result, it is not significantly different from those obtained by soaking extraction and ultrasonic extraction, the reason may be the extraction temperature was similar (40 ℃ and room temperature), the both methods obtained the lower content (Table 2).

Compared with temperature, the extraction pressure probably influenced less on the extraction yield, reflux extraction with atmospheric pressure got the highest content of amentoflavone, ASE and SFE with higher pressure but lower content than reflux extraction. The extraction time of reflux method is longer than that of ASE (12 min). ASE extraction process included injecting extract solvent, elevating pressure, static time of 4 min, flushing elution with 60% volume, and purging the nitrogen of 60 s, extracting three times, relieving pressure. It would take more time to complete the extraction, not much shorter than that of reflux extraction. The results indicated that the reflux method was suitable for extracting amentoflavone from *S. tamariscina*.

*Separation amentoflavone by RP-MPLC*

MPLC has enabled a significantly improved efficiency in separation and purification of the compounds of interest, and enriched some lower content compounds in the herb in short time. The theory of MPLC separation is much similar with HPLC， so we selected the separation condition depended on the condition of HPLC. In RP-MPLC Separation, a Biotage® SNAP Cartridge KP-C18-HS column (10 g) was used to separate biflavonoids in the extract of *S. tamariscina* and the RP-MPLC chromatogram is given in Fig.2. As shown in Fig.2,

**Table 1.**Amentoflavone content of different extraction solvent

| Extraction solvent | Crude drug (g) | Amentoflavone (mg/g) |
|---|---|---|
| 70% aqueous ethanol | 5.001 | 14.051 |
| n-butanol | 5.002 | 1.718 |
| Ethyl acetate | 5.006 | 1.707 |
| Hexane | 5.007 | 0.404 |

**Table 2.**Amentoflavonecontent of different extraction methods

| Extraction methods | Crude drug(g) | Extraction time | Temperature | Amentoflavone (mg/g) |
|---|---|---|---|---|
| Accelerated solvent extraction（ASE） | 5.005 | 12 min | 80 ℃ | 27.880 |
| SFE-CO2 extraction | 5.006 | 1.5 h | 60 ℃ | 20.181 |
| Reflux extraction | 5.009 | 1 h | 90 ℃ | 33.006 |
| Soaking extraction | 5.007 | 2 h | 40 ℃ | 14.727 |
| Ultrasonic extraction | 5.005 | 1 h | Room temperature | 14.051 |

one major fraction was observed, and the fractions were collected into test tubes per 10 mL with a fraction collector, and further analyzed by HPLC. All fractions of the same compound at high purity determined by HPLC were combined, and freeze-dried. The present RP-MPLC Separation produced a total of 0.44 mg of fraction Ⅰfrom1.024 g of crude extract in one run.

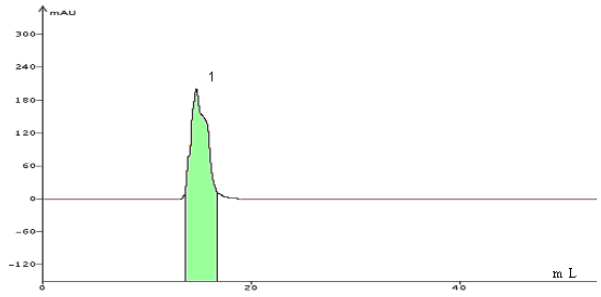


**Fig. 2.**RP-MPLC chromatogram of *S. tamariscina* extract

The crude extract of *S. tamariscina* and the fractions corresponding to each peak isolated by RP-MPLC were further analyzed by HPLC, and the results are given in Fig. 3 (A-B). Tentative identification of the compound in RP-MPLC separation was achieved by the comparison of congruent retention time with that of the crude extract of *S. tamariscina* (Fig. 3). HPLC analyses of the MPLC fraction revealed that the biflavonoid was over 98% purity.

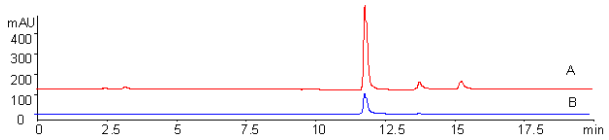


**Fig.3.** HPLC profiles of the compounds in S. tamariscina ( A. The crude extract; B. Compound 1 in RP-MPLC separation)

*Identification by ESI-MSn*

To further investigate the structure of the compound in RP-MPLC separation, ESI-$MS^n$ experiment was attempted. Compound related to the peak 1 exhibited intense molecular ions $[M-H]^-$ at m/z 537 in the negative mode, and low intensity dimer $[2M-H]^-$ ions at m/z 1075, from which the molecular weights of peak 1 was confirmed to be 538. In ESI-$MS^n$ experiment, the deprotonated molecular ion $[M-H]^-$ at m/z 537, and four main fragment ions at m/z 443, 417, 375 and 331 were observed in NI-MS.

The proposed fragmentation route is, main daughter ion at m/z 433 produced directly from m/z 537 by two γ-H of the A-ring in flavonoid part Ioccurred McLafferty rearrangement, losses of $C_3O_2$, followed by the cleavage of the C-ring in flavonoid part Ⅰ at positions 0/4 （0,4A）, losses of $C_2H_2$. The $MS^2$ spectra of the $[M-H]^-$ ions also showed one daughter ion of m/z 417, which indicated the loss of $C_7H_5O_2$ via the cleavage of the C-ring in flavonoid part II at positions 0/2 （0,2A）. In the $MS^3$ spectra, low abundance fragment ion at m/z 331 also be found, which could be obtained by two γ-H of the Ⅰ-A-ring in m/z 417 undergone McLafferty rearrangement, losses of $C_3O_2$, and lose a $H_2O$ molecule via intramolecular dehydration between –OH on the IB4' and IIA7''. The fragment ion at m/z 375, which corresponded to the loss of a $C_9H_6O_3$ unit from the cleavage of the C-ring in flavonoid part II at positions 0/4 in the parent ion m/z 537, it is similar with biflavonoids mass spectra of previous report studied [11-12]. Generally, fragmentation routes involving the cleavage of the C-ring in flavonoid part II at positions 0/2 and 0/4 are the primary pathways of amentoflavone-type biflavonoids in our experiment. By comparing the retention time, peak order and molecular weights information of ESI-MS data with above LC-ESI-MS experiment, the compound 1 was confirmed as amentoflavone. Based on the LC peak cases, detection of the molecular ions and the fragment ions of the molecular ion obtained in the CID experiments with those of the data reported in the literature, the compound corresponding to peak 1 was therefore identified as amentoflavone [13-15]. The structure of the amentoflavone (peak 1) is showed in Fig. 1. Amentoflavone is a biflavonoids consist of two flavonoids connected by IC3'-IIC8''.

## CONCLUSION

The present paper describes several chromatography methods were joint applied to analyze and separate the bioactive compounds of *S.tamariscina*.To establish the quantitative analysis HPLC method to compare with different extract by determining the content of amentoflavone. The result indicates that extraction temperature has significant impact on the extraction efficiency, and reflux extraction is proved to the more effective technique for extracting biflavonoid than ASE, SFE-$CO_2$ extraction, soaking extraction and ultrasonic extration. RP-MPLC was successfully applied to separate biflavonoid from extract of *S. tamariscina*. High-purity amentoflavone was obtained and at over 98% purity as determined by HPLC. ESI-$MS^n$ method could also be applied to rapid identification of obtained compound based on the detection of the molecular ions, and the fragment ions of the molecular ion with those of the data reported in the

literature. The research demonstrates that chromatography technologies we employed were effective and feasible for the extraction, separation, qualitative and quantitative analysis of biflavonoids from *S. tamariscian*or other medicinal plants.

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## ХРОМАТОГРАФИЯ НА РАЗДЕЛЯНЕТО, КАЧЕСТВЕНИЯ И КОЛИЧЕСТВЕНИЯ АНАЛИЗ НА БИФЛАВОНОИДИ В СУРОВИ ЕКСТРАКТИ ОТ *Selaginella tamariscina*

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(Резюме)

Известно е от предишни изследвания, че класът бифлавоноиди има голяма фармакологична активност, която трябва да се използва по-пълно, тъй като много от тях не се срещат в природата. Поради тяхната голяма биологична активност и качествения контрол на медицинските растения е необходимо създаването на ефективни методи за екстракция, изолиране и пречистване на бифлавоноидите от растението *S. tamariscina*. За оценяването на оптимален метод за екстракция са изпитани четири разтворителя, пет метода за екстракция (ускорена течна екстракция, свръх-критична екстракция SFE-$CO_2$, екстракция с рецикъл, с промиване и ултразвукова екстракция ) за извличане на биологично-активни вещества от *Selaginella tamariscina*. Изпитан е BETX-метод за количествен анализ и е направено сравнение с определянето на амантофлавон в различните екстракти. Резултатите показват, че температурата на екстракция има значително влияние върху ефективността на екстракция, а екстракцията с рецикъл предлага най-ефективен способ за извличане на бифлавоноиди от *S. tamariscina*. Течната хроматография с обратна фаза и умерено налягане (RP-MPLC) е приложена на-напред за разделянето и изолирането на бифлавомоиди от екстракти от *S. tamariscina*. Получени са бифлавоноиди с висока чистота (над 98%), както е определеноч чрез BETX. Освен това, електроспрей-йонна мас-спектрометрия (ESI-MS) е приложена за бързата идентификация на съединенията получени от екстрактите от *S. tamariscina*. Аменофлавонът е идентифициран чрез детекция на молекулни йони, а фрагментните йони, получени при CID-експериментите са сравнени с данни от литературата.

Приложените хроматографски технологии са ефективни и подходящи за екстракция, разделяне, количествен и качествен анализ на бифлаоноидите от растението *S. tamariscina*.