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Extraction and purification of eupatilin from *Artemisia princeps* PAMPAN recycling preparative HPLC

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Abstract−A recycling preparative HPLC was used to extract and separate the eupatilin contained in *Artemisia princeps* PAMPAN, and the optimum operating condition was experimentally determined. Eupatilin was extracted by ethanol solvent from the leaf and trunk of *Artemisia princeps* PAMPAN. The resulting solution was further partitioned with n-hexane, chloroform and ethyl acetate. The solution containing eupatilin was collected using a preparative as well as analytical column, and it was identified by LC-CE-MS. In the experiment, the mobile phase consisted of water/acetonitrile/TFA=50/50/0.5 (vol%), and UV wavelength measured was 370 nm. For analytical chromatography, the injection volume was 20 μL and the flow rate was fixed at 1.0 mL/min. The measured retention time for eupatilin was approximately 8.6 min in the above operating condition. For recycling preparative HPLC with commercially available GSA 310A column at 1.5 mL/min of the flow rate and 2 mL of injection volume, the purity of the eupatilin was almost 100% after recycling twice.

Key words: Eupatilin, *Artemisia princeps* PAMPAN, Recycling HPLC, Extraction

INTRODUCTION

The availability and stability of medicines has become more important with the concern for increasing a patient's lifespan. Developed countries have validated the economic merits of using both the crude plants and new function materials from these plants all over the world. The crude plant extracts are relatively important for the development of new medicines as a basis resource. Therefore, it has become more important to investigate how to extract the active material from crude plants.

Artemisia is herb that includes many useful compounds, and there are more than 400 classes of artemisia worldwide. In Korea alone, almost 40 classes of artemisia have been reported [Lee, 1975]. Previous studies revealed that *Artemisia asiatica*, a medicinal plant that is widely used in traditional oriental medicine, has strong anti-inflammatory/antioxidative activity [Seo et al., 1999; Ahn et al., 1997; Lee et al., in press; Oh et al., 1997] and also induces apoptosis in cultured cells [Seo et al., 1999; Hahm et al., 1998]. Artemisia is classified as follows: plants only used for food, plants only used for medicine or a combination of both for food and medicine. Artemisia contains various enzymes, chloroplasts, dietary fiber, protein, good quality minerals and various vitamins [Koshihara et al., 1983]. It is not only a strongly alkaline food for improving the basic physical constitution and cleaning the blood, but also a medicine for ending disharmony in the body, helping promote digestion, as well as oppressing and excreting harmful materials. There is an abundance of vitamins A and C in artemisia, which can improve the energy balance and prevent colds. The eupatilin in the artemisia can control the mutation of the plants and is involved in the increase and decrease in blood sugar [Seo et al., 2001]. It was recently reported that the stomach protection action of eupatilin is approximately 3.5 times stronger than cimetidine and it has a gastric ulcer effect when injected into white mice [Kim et al., 1997]. The chemical name of eupatilin is 5,7-dihydroxy-3,4,6-trimethoxyflavone. The chemical structure of eupatilin is shown in Fig. 1. Eupatilin has been reported to inhibit the activity of 5-lipoxygenase in cultured mastocytoma cells [Koshihara et al., 1983].

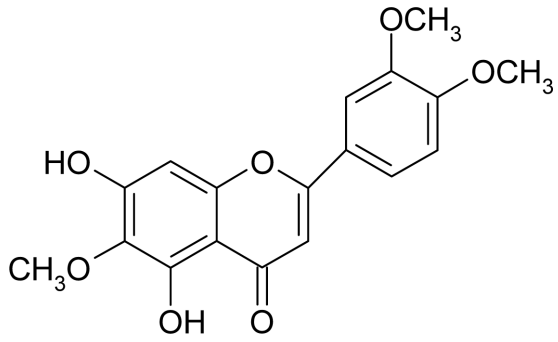

Fig. 1. Chemical structure of eupatilin.

As high performance liquid chromatography (HPLC) is widely used as a standard analytical instrument, a number of stationary phases are commercially available [Lee et al., 1996]. In a chromatographic column, materials injected are separated based on their differences in the retention with stationary phase [Choi et al., 2004]. HPLC is used for separation, purification, collection of the single component, qualitative and quantitative analysis by adsorption effect, division effect, ion exchange effect, and an exclusion effect when the mobile

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phase is through the solid phase. The recycling of preparative HPLC has an additional recycling effect compared with normal analytical HPLC [Hong et al., 2004]. This effect can increase the resolution of an extraction material. Recycling preparative HPLC uses a larger column than analytical HPLC. In this study, eupatilin was extracted from the *Artemisia princeps* PAMPAN by recycling preparative HPLC. A qualitative analysis of the useful component collected from the peak was carried out by LC-CE-MS.

EXPERIMENT

1. Materials

Artemisia princeps PAMPAN was purchased from the Kyung Dong market in Korea. TFA (trifluoroacetic acid) was from Sigma (St Louis, MO, USA). Acetonitrile, ethanol, hexane, chloroform and ethyl acetate were all of HPLC grade and purchased from the Duksan Chemical Co. LTD. (Ansan, Korea). Double distilled water was filtered through a 0.45 μm filter membrane prior to use.

2. Instrument

The HPLC system used was a Waters 600s Multisolvent Delivery System and Waters 616 liquid chromatography (Waters Associates, Milford, MA, U.S.A.) with Rheodyne injector (20 μL sample loop). Millennium 3.2 (Waters, Milford, MA, U.S.A.) was used for the data acquisition system. The mobile phase was a mixture of water/acetonitrile/TFA=50/50/0.5 (vol%), the UV wavelength was 370 nm. The preparative column (3.9×300 mm, 15 μm) used was C_{18} (Lichrospher, Merck, Germany), and the analytical column (4.6×250 mm, 5 μm) used was C_{18} (RS-tech Co. LTD., Korea). The recycling preparative HPLC system used was LC-908-G30 (JAI, Japan Analytical Industry Co., LTD). The column was a GS310A column (6.3×500 mm, multi method, 3-10 μm JAI, Japan). The UV and RI detector were JAI UV 3702 and JAI RI RI-5, respectively. Multichro 2000 V4.2 (JAI, Japan), was used in the data acquisition system for recycling preparative HPLC. The flow rate and injection volume for the recycling preparative HPLC were 1.5 mL/min and 2 mL, respectively. The qualitative analysis of the eupatilin used LC-CE-MS (Liquid Chromatography-Capillary Electrophoresis-Mass Spectrometer), HP-1100 HPLC, HP-3D Capillary Electrophoresis, and UATTRO LC Triple Quadrupole Tandem Mass Spectrometer.

3. Extract

Two grams of *Artemisia princeps* PAMPAN dissolved in 60 mL of 100% ethanol was extracted by supersonic at 25 °C for 30 min. After the extracted solution was filtered, 60 mL of water was added to the solution, and the solution was then concentrated to 30 mL by using a rotary evaporator. The resulting solution was further partitioned with 30 mL of n-hexane. The solution was separated into a hexane and a water layer. Then water layer was then added equal amount of chloroform. Finally, the partitioned water layer was added to 30 mL ethyl acetate. The solution collected by this method then was injected into the preparative column.

4. Analytical

A mixture of water, acetonitrile and TFA was used as the mobile phase, and the flow rate was 1.0 mL/min. The UV wavelength was 370 nm. After 20 μL of the solution passed through the preparative column, useful components from artemisia were collected. These solutions were then injected into an analytical column and two peaks were obtained. The solutions collected from the two peaks were identified by LC-CE-MS. When the recycling preparative HPLC was used, the collected component from the preparative chromatography was separated into two peaks. The second peak was recycled two times and it was further separated into two peaks. The resulting two peaks were collected and then were injected into the analytical chromatograph for identification.

RESULTS AND DISCUSSION

1. Extract

In this study, 100% ethanol was used to extract the eupatilin from the *Artemisia princeps* PAMPAN by supersonic at 25 °C for 30 min. This is because the polarity of ethanol is 5.2. It can dissolve many

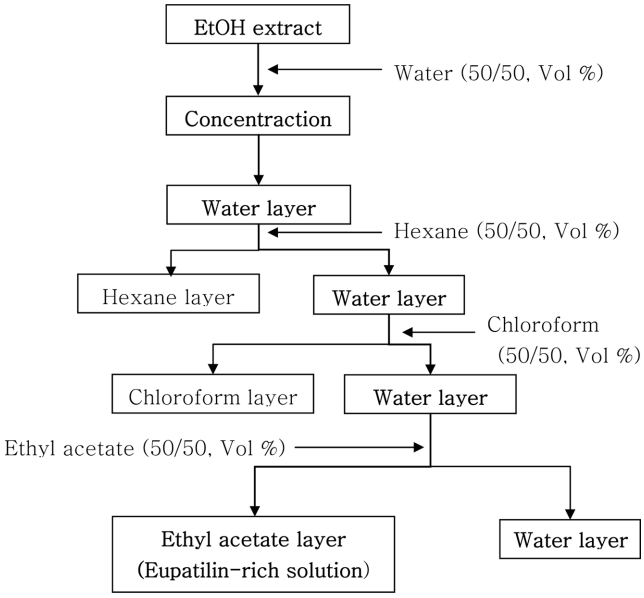

Fig. 2. Extraction steps for separating eupatilin from the *Artemisia princeps* PAMPAN.

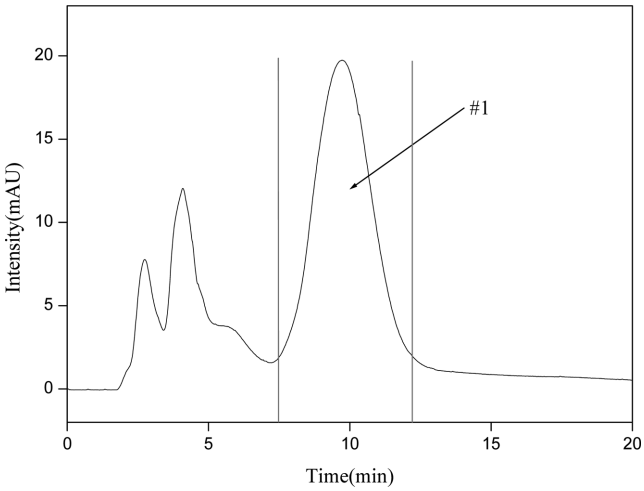

Fig. 3. Chromatogram of the solution from the *Artemisia princeps* PAMPAN by a preparative column (mobile phase: water/acetonitrile/TFA=50/50/0.5 vol%, injection volume 20 μL, flow rate: 1.0 mL/min).

polar and non-polar materials. There are two hydroxyl groups on eupatilin (Fig. 1), which means that ethanol can be used for extraction.

The resulting solution was further partitioned with n-hexane, chloroform and ethyl acetate, successively (Fig. 2). Hexane and chloroform was used to exclude the various chloroplasts, dietary fiber, protein, mineral and various vitamins, etc. This would help to separate the eupatilin from the other unnecessary materials. The partitioned ethyl acetate layer included more eupatilin than the other layer. The partition process is schematically listed in Fig. 2. The samples were analyzed from the ethyl acetate extracts.

First, by the preparative column, peak #1 was collected as shown in Fig. 3; then it was further divided by the two peaks, peak #2 and #3 by analytical column as shown in Fig. 4. The peak #2 was identified as eupatilin by LC-CE-MS.

2. Analytical HPLC

A solution of the ethyl acetate layer was injected into the preparative column, and the solution from peak #1 in Fig. 3 was collected. The retention time of peak #1 was measured as 9.6 min. The solution collected from peak #1 was injected into the analytical column, so the two solutions were then collected from the two baseline-separated peaks, peak #2 and peak #3 in Fig. 4. The retention times of peaks #2 and #3 were 8.6 and 10.5 min, respectively.

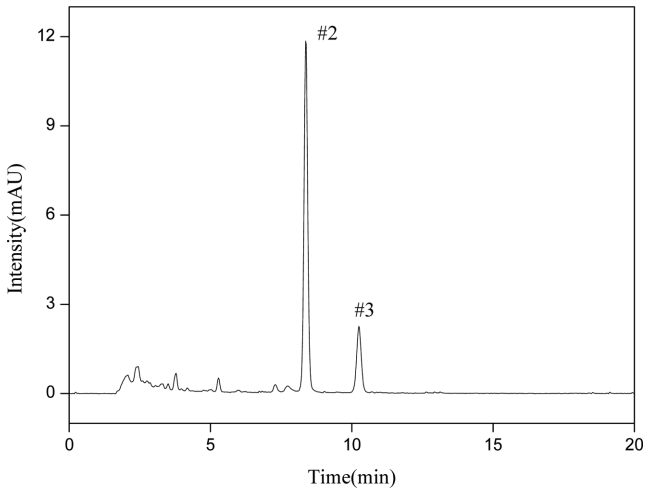

Fig. 4. Chromatogram of the peak #1 in Fig. 3 by the analytical column (the same experimental condition as in Fig. 3).

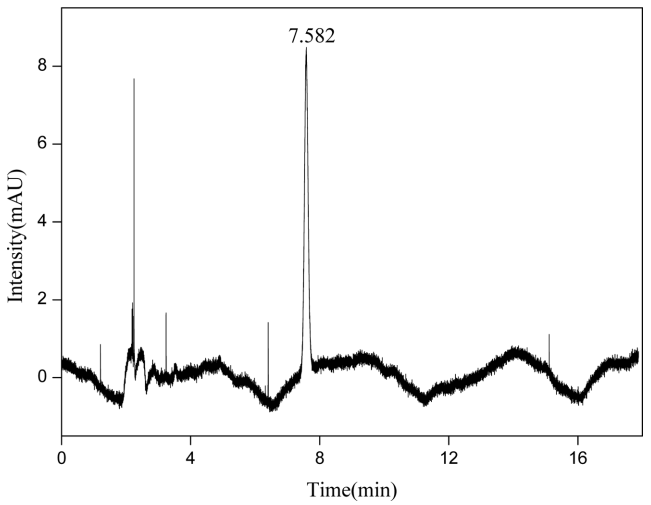

Fig. 5. Chromatogram of eupatilin from peak #2 in Fig. 4 (mobile phase: water/acetonitrile/TFA=50/50/0.05 (vol%), injection volume 20 μL, flow rate: 1.0 mL/min).

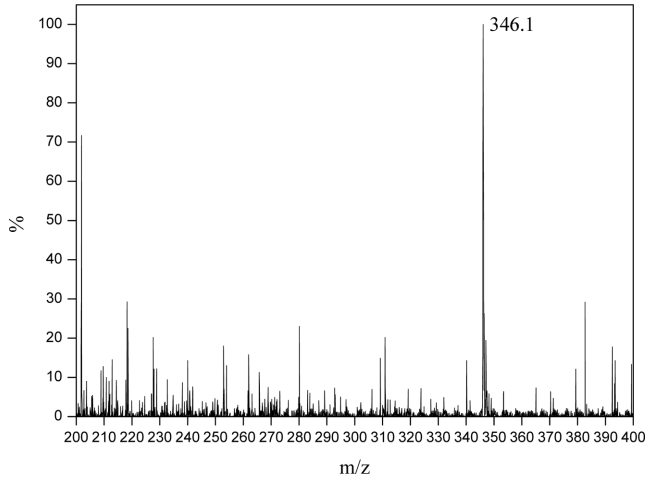

Fig. 6. Mass spectrum of peak #2 in Fig. 4 (the same experimental condition as in Fig. 5).

3. Qualitative Analysis

The qualitative analysis of the solution collected from peak #2 by LC-CE-MS is shown in Figs. 5 and 6. When the ratio of TFA is above 0.05%, it would do damage to the LC-CE-MS instrument. So 0.05% TFA in the mobile phase was recommended. Under this condition, the retention time of the solution from peak #2 was 7.582 min. The reduction of retention time from 8.6 to 7.582 is attributed to the decreased proportion of TFA. The molecular weight of the solution from the peak #2 was 346.1 (Fig. 6). This is because the natural molecular weight equals to the measurement value minus 1, namely the molecular weight of the substance from peak #2 was 345.1. Considering the molecular weight of eupatilin was 344.3, this implies that the collected solution from peak #2 was found as eupatilin.

4. Recycling Preparative HPLC

From the above analysis, it can be seen that eupatilin was the main compound of peak #1. Therefore, the solution collected from peak #1 was injected into the recycling preparative HPLC column. In analytical chromatography, the solvent from the pump and the injected sample from the manual injector were mixed together, and the useful component was fractioned and detected by a detector before discharge from a column. However, recycling preparative HPLC has a recycling system with an auto recycler that recycles the unseparated sample. Because it uses a larger column than analytical chromatography, the recycling preparative system could be used for larger quantity and higher purity separating work. In this study, a preparative column of JAI GS310A was used. It can be seen that the column includes the advantages of GPC, GFC and RP-HPLC. When the compounds collected from peak #1 went through recycling preparation HPLC, the two compounds of peak #4 and peak #5 were obtained. Recycling peak #5 two times enabled the resolution of peak #5-1 and peak #5-2 from peak #5 as shown in Fig. 7.

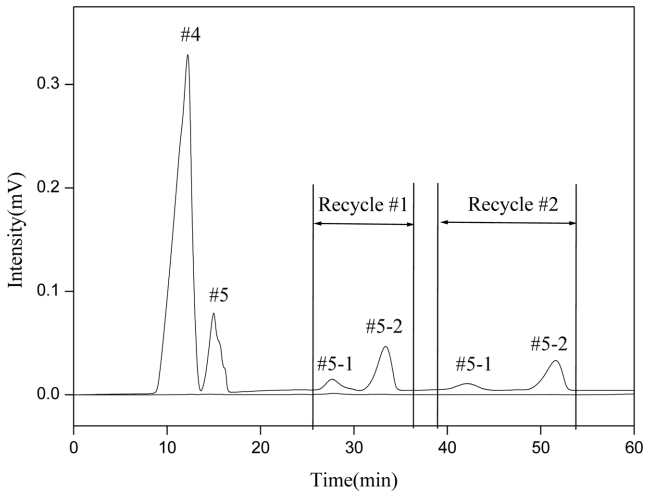

Fig. 7. Chromatogram of eupatilin by recycling preparative HPLC (mobile phase: water/acetonitrile/TFA=50/50/0.5 (vol%), injection volume 2 mL, flow rate: 1.5 mL/min).

The collected components from peak #5-1 and #5-2 were injected into the analytical HPLC. Then the resulting component from the peak #5-2 was indicated as eupatilin based on the retention time. The retention time equals the retention time of eupatilin at the same flow rate. It is concluded that the collected component from peak #5-2 was eupatilin. This indicates that the purity of eupatilin from *Artemisia princeps* PAMPAN was almost 100% by recycling preparative HPLC.

CONCLUSION

Eupatilin was extracted from *Artemisia princeps* PAMPAN by using 100% ethanol with ultrasonification at 20 °C for 30 min; then the resulting solution was further partitioned with n-hexane, chloroform, and ethyl acetate, successfully. The solution of ethyl acetate layer was injected into the preparative column followed by analytical column, and the effluents collected from the column were identified by LC-CE-MS. The solution containing eupatilin was injected into the recycling preparative HPLC and eupatilin was obtained after recycling twice. The purity of the eupatilin extracted from *Artemisia princeps* PAMPAN was almost 100%.

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