PAPER

Investigation of new functional ingredients obtained from various *Angelica species* leaves using supercritical carbon dioxide extraction method

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■Abstract

Nihon Yamaninjin, classified in *Umbelliferae Angelica genus*, is a perennial plant grown wild in Japan, and it has been recognized as herb medicine for all ailments in folklore. For the extraction of natural compounds, supercritical carbon dioxide (SC-CO_2) has been widely used. Carbon dioxide is the most preferred because of its low critical temperature (31℃) suitable especially for thermally labile components such as bioactive compounds. SC-CO_2 extraction method was expected as good solvent to recover the high grade crude drag. SC-CO_2 extraction was carried out under the conditions at temperatures of 40, 60 and 80℃, pressures of 30 MPa on the leaves of *Angelica*. Functional ingredients in the extracts were analyzed using a HPLC and GC-MS. Bioactive compounds ferulic acid and phthalides such as ligustilid and butylidenephthalides were observed. Especially Kaempferol which is one of functional flavonoids was extracted only in leaves. It was revealed that composition of the extracted components varies depending on the species. In addition, they contained the other bioactive compounds such as flavonoids.

セリ科（*Umbelliferae Angelica*）に属する日本山人参（*Angelica frucijuga*）は、日本に生息する野生の多年生植物で、民間伝承の病気の薬草として認識されている。日本山人参の根は日本薬局方に登録され、その薬効成分および効能が明らかにされている。一方で、その葉についての既往研究は過少なものであり、含有成分などがわかっていない。

そこで本研究では日本山人参の葉を対象として超臨界二酸化炭素抽出実験を行った。超臨界二酸化炭素はグリーン溶媒の一種である。超臨界二酸化炭素は環境負荷低減に寄与する抽出溶媒であり、これは植物などの天然物に含有される機能性成分抽出法としてしばしば用いられる手法である。抽出実験の結果、葉にはトウキ類の根と同様にフェルラ酸およびフタライド類が含有されていることが明らかとなった。また、ケンフェロールに関しては葉のみに含有していることがわかり、葉特有の機能性成分であることが示唆された。

Key Words: Medicinal herb; Yamninjin; *Angelica sinensis; Angelica acutiloba; Angelica furcijuga*; Supercritical carbon dioxide; Ferulic acid; Ligustilid; Butlidenephthalide; Kaemperol

キーワード：薬草、山人参、カラトウキ、ヤマトトウキ、ヒュウガトウキ、超臨界二酸化炭素、フェルラ酸、リグスチリド、ブチリデンフタライド

Introduction

Angelica sinensis is famous material of Chinese medicines. It has been used as an analgesic in treatment of menstrual disorders, menorrhalgia, and rheumatalgia [1][2]. There are several subspecies of *Angelica*“Nihon yamaninjin”that is grown in Japan. *Angelica actiloba* is major species of Japanese *Angelica*. It has been studied well. Recently, *Angelica frucijuga*, called“Nihon yamaninjin”, attracts attention as a very important crude drag.“Nihon yamaninjin”was called locally“Hyuga Touki”. Figure 1 shows the appearance of“Hyuga Touki”fields located in Kawasaki Town, Fukuoka Prefecture.“Hyuga Touki”has a harvest time twice a year. The harvested Hyuga starch is shown in Figure 2.

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Figure 1 ***Angelica furcijuga*** field

Figure 2 Nature ***Angelica furcijuga sp.***

Hyuga means Miyazaki prefecture. Originally, Nihon yamaninjin has been secretly cultivated in the southern part of Kyushu islands Japan. However, cultivation is sufficient even in Fukuoka prefecture which is not in the southern part of Kyushu if it is mild climate. Nihon Yamaninjin roots have been certified as crude drag in the Japanese Pharmacopoeia. It has been suggested that the root have relieving pain effect and apoptotic effect on human cancer cell lines and alzheimer's prevention effect [2]. Other part of the Nihon Yamaninjin such as leaf has been valued highly as a health tea. There is still no comparative report, about the functional ingredients that is contained in each kind of part such as leaves of Nihon Yamaninjin.

Carbon dioxide is the most preferred to extract the natural compounds because of its low critical temperature (31.1℃) suitable especially for thermally labile components [3]. Besides, it is readily available and non-toxic. In addition, SC-CO_2 is possible to dissolve ethanol limitlessly by pressurizing. Therefore, by adding the small amount of ethanol, it is possible to extract the polar components while performing dehydration of raw material.

In this research, we focused on the leaves that are not registered in the Japanese Pharmacopoeia. Because the root has already been registered in the Japanese Pharmacopoeia, its medicinal effect is revealed. Besides Hyuga touki, extraction experiment by SC-CO_2 extraction method was conducted on *Angelica sinensis* and *Angelica acutiloba*. After that, the functional ingredients of various angelica subspecies were analyzed. *Angelica sinensis* is called "Kara Touki", and *Angelica acutiloba* is called "Yamato Touki" in Japan.

2. Experiment

2.1 Materials and chemicals

In this research, leaves were used as the raw material to examine the difference of individual part. Samples of *Angelica sinensis, acutiloba and furcijuga* were harvested in Fukuoka prefecture. These roots and leaves were cut, placed inside an air-tight wrapping bag, and then stored in a freezer at −20℃ until extraction experiments were performed. Figure.3 shows freeze-drying treatment apparatus (FDU-1200, EYELA, Japan) and figure 4 is the sample after freeze dried. Prior to extraction experiments, the sample was gently thawed to room temperature.

Chemicals for SC-CO_2 extraction were as follows: carbon dioxide (Uchimura Sanso Co., Japan.), ethanol

Figure 3 Samples on freeze-dring

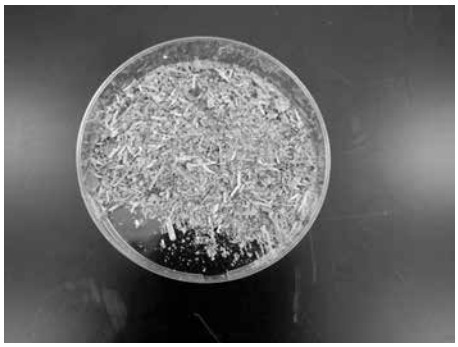

Figure 4 Sample of after freeze-drying

(Wako Pure Chem. Ind., Ltd., Japan). Regents used as standard compounds obtained SC-CO_2 extraction for GC-MS analysis were as follows: ferulic acid, Z-ligstilide (Wako Pure Chem. Ind., Ltd., Japan), n-Butylidenephthalide (Santa Cruz Biotech., Inc., USA), Kaemperol (Wako Pure Chem. Ind., Ltd., Japan). Reagents for HPLC analysis were as follows: acetic acid, acetonitrile (Wako Pure Chem. Ind., Ltd.).

2.2 Solvent extraction

A 2 g of raw material was extracted with methanol to investigate medicinal ingredients. Raw material (2 g) was placed in a flask and 100 ml of methanol was added. The mixture was homogenized with polytron homogenizer (8000 rpm, 3 min). Then, the mixture was treated with ultrasonic waves for 30 min and centrifuged (3000rpm, 5min). After centrifugation, the supernatant was filtered through Minisart RC15 (Sartorius stedim biotech, German) and used in various analysis.

2.3 GC/MS analysis of extract

GC-MS was performed with a Shimadzu GC-2010 gas chromatography instrument coupled to a Shimadzu GCMS-QP-2010 mass spectrometer. The GC conditions were as follows: the oven temperature was initially at 50℃ for 2 min, and then allowed to ramp up to 190℃ at a rate of 5℃ min^{-1} for 4 min, then to 250℃ at a rate of 10℃ for 5 min. The injector and detector temperatures were set at 250℃. The split ratio was 5:1; with a total carrier gas (helium) flow rate of 6.2 mL/min; and ionizing energy of 70 eV. The injection volume was 2 μ L. For the identification of the peaks in the chromatograms, the probability-based matching algorithm was employed for finding the most probable match in the reference library (NIST library of mass spectra and subsets, Shimadzu NIST05).

2.4 HPLC analysis of extract

Extracts were analyzed using a HPLC LC-10AD gradient system, equipped with Diode Array Detector SDP-M10A. Inertsil ODS-3 column was used for separation at 35℃. The mobile phase consisted of solvent A, 0.1 % acetic acid in water, and solvent B, 0.1 % acetic acid in acetonitrile (acetonitrile/water = 75/25, v/v). The flow rate was 1.0 mL/min. Peaks were measured at a wavelength of 285 nm to quantify flavonoids. The gradient elution was as follows: time 0 min A-B (88:12); time 18 min A-B (78:22); time 28 min A-B (72:28); time 35 min A-B (62:38), time 48 min A-B (52:48), time 58 min A-B (0:100); time 70 min A-B (88:12). The flow rate was 1.0 mL/min. Peaks were measured at wavelength of 285 nm to quantify flavonoids.

2.5 SC-CO_2 extraction

Fig.2 shows schematic diagram of SC-CO_2 extraction apparatus with an option of adding a co-solvent. The maximum working conditions of the apparatus are 200℃ and 45 MPa. The pressure in the extractor was controlled by a back-pressure regulator (HBP-450; Akico Co., Ltd.). The extraction temperature was monitored at oven. 5.0 g portion of raw material was charged in the extractor (10 mL). SC-CO_2 extraction was carried out at a pressure of 30 MPa and temperatures are 40℃ , 60℃ , 80℃ . The percent ratio of ethanol as a co-solvent was 10% of CO_2 flow rate. The extraction rate was defined as the weight of dried residues per weight of wet raw materials.

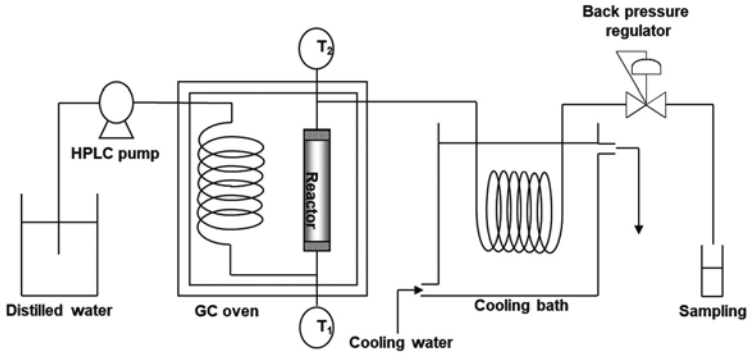

Figure 5 Schematic diagram of supercritical carbon dioxide extractor

3. Result and Discussion

3.1 GC-MS analysis of extract on solvent extraction

All obtained extract samples were injected into GC-MS for component analyses. Figure 3 shows a typical GC-MS chromatogram of extracts obtained from solvent extraction. "*A.*" in the figure is an abbreviation of *Angelica*. As a result, ferulic acid, phthalides such as Z-ligustilide and E-butylidenephthalide, and kaempferol were observed. It was suggested that Kaempferol is not contained in roots and is a component specific to leaves.

Figure 4 is a comparison of the amounts of functional ingredients contained in roots and leaves of *Angelica*. When comparing roots and leaves, the content of functional ingredients is more in root. That is, the leaves have less fragrance than the roots. This tendency is particularly strong for *A. furcijuga*. It is interesting that the content of functional ingredients is higher in roots, but kaempferol was detected only in leaves.

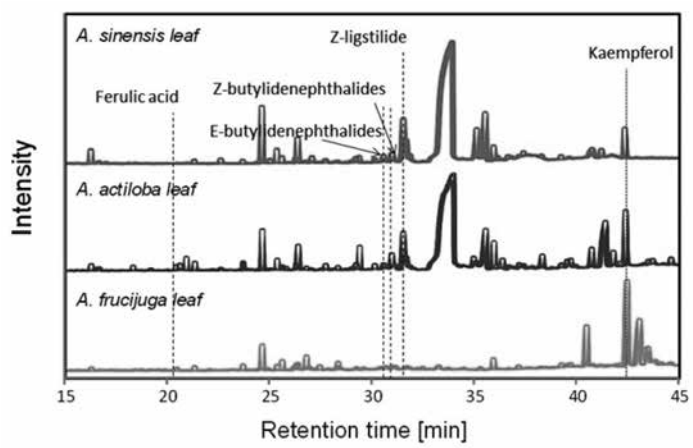

Figure 6 GC-MS chromatograms of SC-CO_2 extract obtained from each leaves

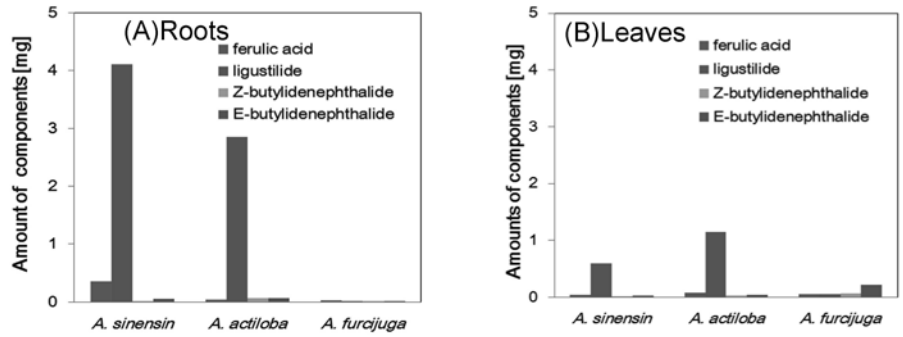

Figure 7 Comparison of functional ingredient obtained from root and leaf part of each Angelica

3.2 Extraction behavior of functional ingredient

SC-CO_2 extracts ware quantitatively analyzed by GC-MS. Figure 6 shows the yield of functional ingredients extracted from leaves of each angelica. The vertical axis shows the yield when comparing the yield of the experiment on the solvent extraction method. The horizontal axis is extraction time.

(A) is ferulic acid, (B) is E-butylidenephthalide, (C) is Z-butylidenephthalide, (D) is ligustilide and (E) is kaempferol. Considering each functional ingredient, it can be seen that in SC-CO_2 extraction method in (A), it is hardly recovered. This is probably because the structure of ferulic acid was destroyed under high pressure. (B) and (C) can be recovered to the same extent as in the solvent extraction method. However, it was found that *A. frucijuga* contains very little of these butylidenephthalides. In (D), the yield increased only for *A. frucijuga*. However, since ligustilide is also a phthalide having a structure similar to butyridenephthalide, some doubt remains as to the high yield of only ligustilide. Finally (E), *A. frucijuga* made a particularly good high yield. Since it is suggested that this kaempferol is a valuable component peculiar to the

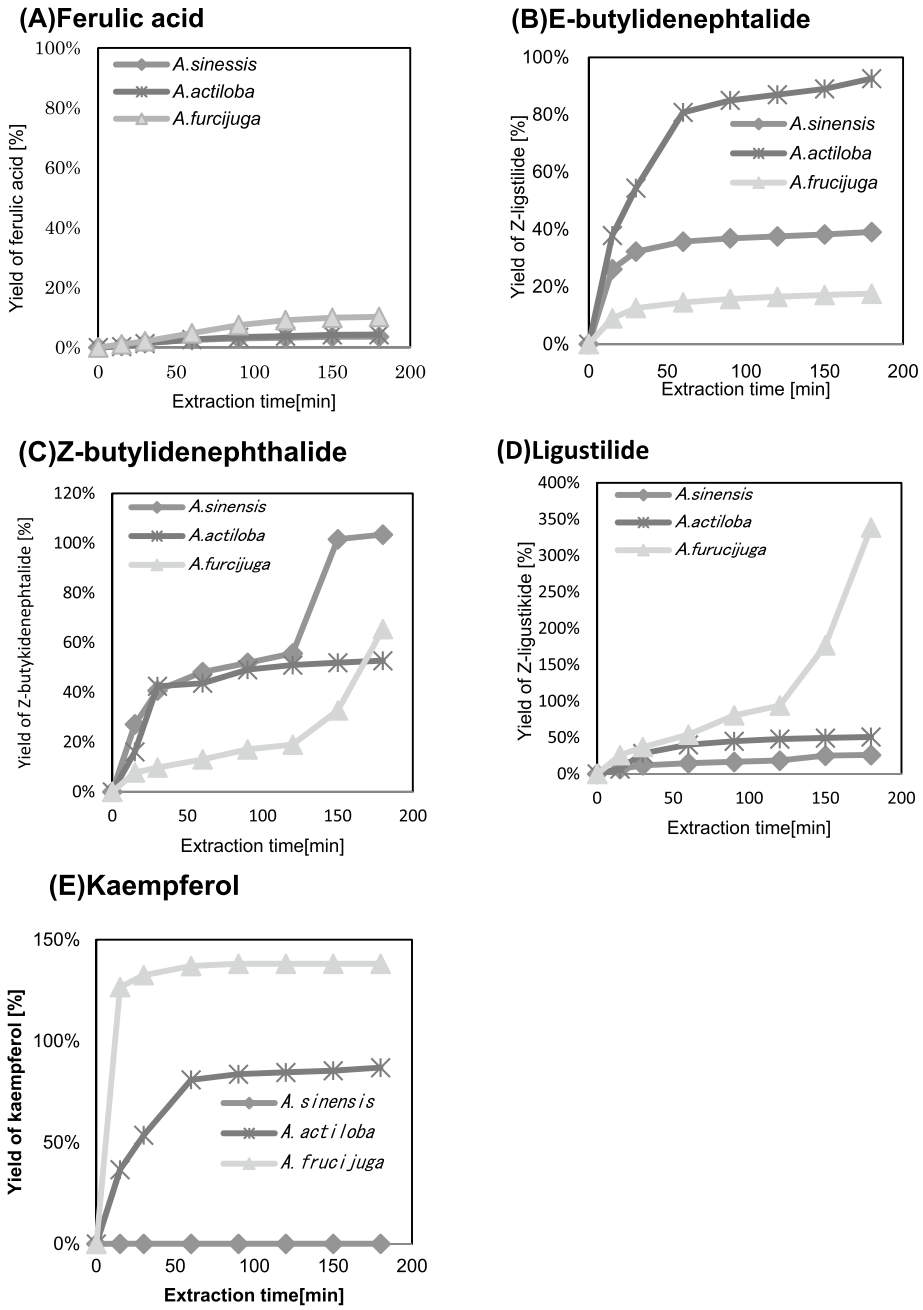

Figure 8　Extraction behavior of functional ingredients

leaves, it is necessary to investigate so that more stable extraction can be carried out.

3.3 Effect of temperature on the extraction rate of SC-CO_2 with ethanol extraction

Finally, the effects of temperature on the extraction rate of total extract were studied under temperatures at 40℃ , 60℃ , 80℃ . In general, it is thought that increasing CO_2 density increases extraction rate [4]. However, on the contrary, results in Fig. 5 show that the extraction rate was obviously increasing with decreasing SC-CO_2 density. For example, at temperatures of 40, 60, and 80℃, the density of SC-CO_2 is 0.910, 0.830, and 0.746 g/cm^3, respectively, under a pressure of 30 MPa.

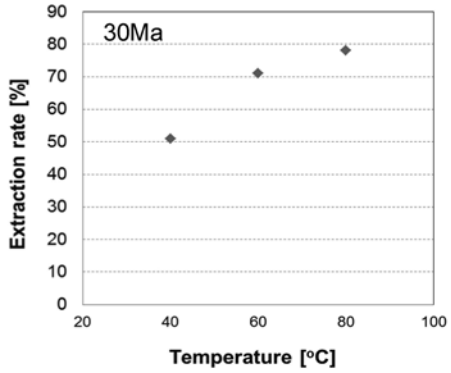

Figure 9 Effect of temperature on the extraction rate

Conclusions

SC-CO_2 was used as a solvent for extracting functional components of each angelica. Ethanol was also used as co-solvent. As a result, phthalides, which are aromatic components peculiar to the family Seriaceae, were extracted from *A. sinensis* and *A. actiloba*, but were hardly extracted from *A. frucijuga*. On the other hand, Kaempferol, a functional component peculiar to the leaves, was recovered in high yield from *A. frucijuga*. From the above, it was found that component extraction is possible in the SC-CO_2 extraction method as well as the conventional solvent extraction method in this experiment. In the future, if these are taken orally as health foods such as supplements, it can be said that it is an excellent method that is friendly to the human body and the environment. As a future task, it is necessary to stabilize the yield of functional ingredients and to examine the experimental conditions in order to obtain more yields.

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