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A Comparative Study on the Compositions of Hwangryeonhaedok-tang's Essential Oils Obtained by Supercritical Carbon Dioxide Extraction and Hydrodistillation Methods

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Abstract

초임계추출법과 수증기증류법을 이용하여 황련해독탕의 정유성분을 추출하여 성분 패턴을 비교하였다. 이때 초임계추출조건은 압력 200 atm, 45℃ 그리고 추출시간 25분 이었다. 이때의 추출된 성분의 93.9%인 37개의 성분을 가스크로마토그래피/질량분석기로 확인하였다. 주요성분으로는 tetradecenoic acid (11.7%), Vanillin (5.9%), dl-Limonene (5.5%) 및 Eicosane (4.6%)으로 나타났다. 수증기증류법으로 추출한 정유에서는 34개 성분을 확인할수 있었다. 주요성분으로는 tetradecenoic acid (8.9%), Vanillin (5.8%)및 Eicosane (4.7%)를 확인 할 수 있었다. 또한 구강내의 12균주를 이용하여 항균효과를 측정하여 최소엑제농도(MIC)와 사멸농도(MBC) 0.025 - 12.8 ㎎/㎖와 0.05 - 12.8 ㎎/㎖ 각각 나타났다.

Keywords : Hwangryeonhaedok-tang, herbal acupuncture, supercritical fluid extraction, hydrodistillation, antimicrobial action

Introduction

Herbal acupuncture refers to a kind of acupuncture technique that one injects the extract of herbal medicine into the region for acupuncture. It differs from the ordinary acupuncture in the sense that medicinal substances are injected. It also differs from the injection therapy in the western medical clinic because injection is made exactly onto the region for acupuncture.

Hwangryeonhaedok-tang (H-tang) has been widely used as a medicinal substance for herbal acupuncture for the treatment of fever, inflammation, toxin etc. in Oriental Medicine. The compound H-tang recipe is officially recorded in Bang-Yak-Hap-Pyun[1], and has the efficacy in removing toxic heat and reducing inflammation. H-tang is composed of four herbs: *Scutellariae Radix, Coptis Rhizome, Phellodendri Cortex and Gardenia Fructus*.

Scutellariae Radix is from the root of

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Scutellaria baicalensis Georgi, family Labiatae. It is dug up and collected in spring and autumn and usually cut into pieces. It can be used as is crude, stir-baked with wine or carbonized. The medicinal properties of *Scutellariae Radix* are bitter in flavor, cold in nature and attributive to lung, stomach, gallbladder and large intestine meridians. Its major actions include clearing away heat, removing dampness, purging sthenic fire, removing toxic materials, cooling blood and stopping bleeding to prevent miscarriage[2].

Coptis Rhizome is gathered from Rhizome of *Coptis chinensis* Franch. The medicinal material is dug and collected in autumn and dried. It can be used as is crude or stir-baked with wine. Its medicinal properties are bitter in flavor, cold in nature and attributive to heart, liver, stomach and large intestine meridians. The major actions of *Coptis Rhizome* are clearing away heat and removing dampness, purging the sthenic fire and eliminating toxic materials[2].

Phellodendri Cortex is collected from the bark of a deciduous arbor, *Phellodendrou amurense* Rupr. or *P. chinense* Schneid, family Rutaceae. Usually the crude one is cut into thin pieces or further stir-baked with salt solution or with wine for medication. Its medicinal properties are bitter in flavor, cold in nature and attributive to the kidney, bladder and large intestine meridians. The major actions of Phellodendri Cortex are clearing away heat and removing dampness, purging sthenic fire and eliminating toxic materials, reducing asthenia-heat and bone steaming[2].

Gardenia Fructus is extracted from a fruit of *Gardenia jasminoides* Ellis var. radicans(Thunb.) Makino, family Rubiaceae. Its medicinal properties are bitter in flavor, cold in nature and attributive to heart, liver and triple energizer meridians. Its major actions include purging sthenic fire to relieve vexation, clearing away heat, removing dampness, cooling blood, removing toxic materials, and subsiding swelling to alleviate pain[2].

H-tang herbal acupuncture has been much studied for the past few years. Notable studies among them include the followings: the effect of H-tang herbal acupuncture at PC6(Naegwan) on the EEG[3], effects of H-tang herbal acupuncture at GB21(Kyŏnjŏng: 肩井) on the heart rate variability[4], the clinical study on acupuncture sensation in H-tang herbal acupuncture and hominis placenta herbal acupuncture[5], pharmacological effect of H-tang on experimental

triglyceride accumulated HepG2 cells[6], and a study on the physical reactions with administration of BU mixed H-tang herbal acupuncture[7].

In the above-mentioned studies, the essential oils of plants have been isolated usually by either hydrodistillation or solvent extraction. The methods, however, have exposed some disadvantages such as low yield, losses of volatile compounds, long extraction times, toxic solvent residues, degradation of unsaturated compounds, and giving off the undesirable compounds due to heat.

Supercritical fluid extraction (SFE) instead has received increasing attention in a variety of fields these days especially due to the following features:[8-12]

(a) Supercritical fluid provides high solubility and improved mass-transfer rate.

(b) Its operation can be easily controlled by changing pressure or temperature.

In the SFE method, carbon dioxide (CO_2) is used as the supercritical fluid mainly because it is a safe, noncombustible, inexpensive, odorless, colorless, tasteless, nontoxic, and readily available solvent.

The low viscosity of CO_2 also enables it to penetrate the matrix to reach the material to be extracted, and its low latent heat of evaporation and high volatility allow it to be easily removed without leaving a solvent residue. In addition, by varying the temperature and pressure of CO_2 during the extraction process, the components of specific flavor or odor can be selectively extracted[13-17].

The SFE method often involves the investigation of many variables which may affect the efficiency of extraction. The selection of the variables and the decision on their levels are critical. Several statistical techniques, such as simplex optimization and factorial design, are employed to ensure the optimization of analytical methods[18-19].

Factorial design has certain advantages over simplex optimization for the following reasons: global optimum can be provided, large amounts of quantitative information can be extracted and both discrete and continuous factors can be estimated.

One obvious disadvantage of factorial design is a number of experiments are required when several variables are examined. However, the number of the experiments can be considerably reduced by employing orthogonal array design.

Despite the obvious advantages of the SFE, no existent study on H-tang has employed the SFE method. The present study, based on the prevailing scholarly

achievements on H-tang, examines the availability and efficacy of the SFE for H-tang. In particular, it aims at a thorough investigation of the effects of different parameters, such as pressure, temperature, modifier volume and dynamic extraction of H-tang. This study, along with the essential oil obtained through the SFE method, uses the one through hydrodistillation for comparison's purpose. The antimicrobial actions of the essential oil and some of its major components are also investigated[20-25].

Materials and Methods

Preparation of Hwangryeonhaedok-tang

Each component of H-tang is listed in Table 1. All materials were obtained from the College of Oriental Medicine, Woosuk University. This prescription was prepared according to the method of Bang-Yak-Hap-Pyun.

Table 1. Component of H-tang

Medicinal Plants	Weight(g)
Scutellariae Radix	5.0
Coptis Rhizome	5.0
Phellodendri Cortex	5.0
Gardenia Fructus	5.0
Total amount	20.0

Reagents

CO_2 (99.9% purity), contained in a cylinder with an eductor tube, was obtained from Hansin Co.(Jeonju, KOREA).

Hydrodistillation Extraction

H-tang (20.0g) was powdered by a blender and then distilled for three hours in a modified Clevenger type apparatus in order to obtain essential oil. Anhydrous sodium sulphate was used to absorb the little amount of water that the essential oil contained. The oil was taken by dissolving in HPLC grade *n*-hexane(2 ㎖) and stored in a deep freezer to minimize the loss of volatile compounds.

Supercritical Fluid Extraction

A Suprex MPS/225 system (Pittsburgh, PA, Fig. 1) in the SFE mode was used for all the extractions. The extraction vessel was a 10ml stainless steel vessel. Supercritical fluid extractions were conducted at three pressure levels of 100, 200 and 300 atm by alternating different three temperature levels of 35, 45 and 55℃ for each pressure level, for the duration of 25 min, static, followed by each duration of 15, 25 and 35 min, dynamic, respectively.

A Duraflow manual variable restrictor was used in the SFE system to collect the extracted analysis. In order to prevent

sample plugging, the restriction point was warmed electrically. The supercritical carbon dioxide flow rate through the Duraflow restrictor was approximately 0.3-0.4 mℓ/min (compressed). Plant powder (50 g) was well mixed with 2 ㎜ diameter glass beads, and then charged into the 10 mℓ extraction vessel.

The essential oil was extracted from the plant by using supercritical CO_2 under various conditions according to the Taguchi method[26]. Table 2 shows the experimental conditions under which to run each SFE. In order to improve the collection efficiency, a 5.0 mℓ volumetric flask was placed in an ice bath during the dynamic extraction stage.

Four mℓ of solution were poured into a 20 mℓ beaker. The solution was bubbled by adding argon gas in order to evaporate the solution. Then the weight of essential oil was measured and finally the extraction yield was calculated.

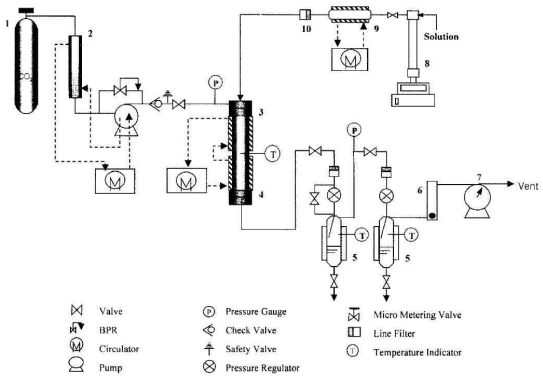

Fig. 1. Diagram of supercritical fluid extractor apparatus.

Table 2. SFE experimental conditions and extraction yields for H-tang.

Run No.	Pressure (atm)	Temperature (℃)	Dynamic time (min)	Extraction yield (w/w)	Refractive Index(nD20)
1	100	35	15	1.01	1.3465
2	100	45	25	1.19	1.3501
3	100	55	35	0.82	1.4573
4	200	35	15	2.31	1.4891
5	200	45	25	3.66	1.4937
6	200	55	35	1.82	1.5122
7	300	35	15	2.23	1.5156
8	300	45	25	3.50	1.5232
9	300	55	35	3.65	1.5221

Measurement of Refractive Index

The refractive index of chemical compounds is considered important because it indicates characteristic physical properties. I determined the index of the oil extracted at each extraction using an Abbe refractometer equipped with a sodium lamp[27,28].

GC and GC-MS Analyses

The essential oil was analyzed with GC using a Perkin-Elmer Sigma-115 gas chromatograph with a data-handling system and FID. A DB-5 fused silica capillary column (30 m × 0.25 ㎜ i.d., film thickness 0.25 μm) was used, with He as carrier gas (1 mℓ/min). The operating conditions were as follows: injector and detector temperature, 240 ℃ and 250 ℃, respectively; oven temperature program, 5 min isothermal at 40 ℃, subsequently rising at 2 ℃/min to 240 ℃, then held

isothermally at 240 ℃ for 20 min; injection mode, split-less (1μl 1 : 1000 *n*-hexane solution).

Linear retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{22}) under the same operating conditions. GC-MS analyses were performed by employing the same chromatographic conditions as described above, and using a Hewlett-Packard 5890 A apparatus linked on-line with a HP Mass Selective Detector (MDS 5970 HP). The GC-MS instrument was equipped with a DB-5 fused-silica column (30 m × 0.25 ㎜ i.d., film thickness 0.25 μm) with He as carrier gas (1 ㎖/min). MS was recorded at 70 eV; electron multiplier energy, 2000 V; mass range, 28-400 m/z.

Identification of Compounds

The identification of oil components was established on the basis of the comparison of the retention indices and MS spectra with those reported in literature[29]. In order to identify oil components, this study also employed computer matching with the NIST 98 and Wiley 5 libraries, as well as, whenever possible, co-injections with authentic compounds available in the laboratory.

Bacterial Strains

Antimicrobial actions of the essential oil against some bacteria and reference strains were determined with the broth dilution method. The oral bacterial strains used in this study were: *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus sobrinus* (ATCC 27607), *Streptococcus anginosus* (ATCC 31412), *Streptococcus gordonii* (ATCC 10558), *Actinobacillus actinomycetemcomitans* (ATCC 43717), *Fusobacterium nucleatum* (ATCC 10953), *Prevotella intermedia* (ATCC 25611), and *Porphylomonas gingivalis* (ATCC 33277). The reference strains used in this study were: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), and *Streptococcus pyogenes* (ATCC 21059). Brain-Heart Infusion broth supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI) was used for all bacterial strains except *P. intermedia and P. gingivalis*. For *P. intermedia* and *P. gingivalis*, Brain-Heart Infusion broth containing hemin and menadione was used.

Minimum Inhibitory Concentrations / Minimum Bactericidal Concentrations Assay

The minimum inhibitory concentrations

(MICs) were determined for the essential oil by the broth dilution method, and were carried out in triplicate. The antibacterial actions were examined after incubation at 37 ℃ for 18 h (facultative anaerobic bacteria), for 24 h (microaerophilic bacteria), and for 1-2 days (obligate anaerobic bacteria) under anaerobic conditions. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth.

Following anaerobic incubation of MICs plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of the essential oil that kills 99.9 % of the test bacteria by plating out onto each appropriate agar plate. Ampicillin and Gentamicin were used as standard antibiotics in order to compare the sensitivity with the essential oil and some of its major compounds against test bacteria.

Result and Discussion

Optimization for the Extraction of Essential Oil from H-tang by the SFE method.

The first step for the extraction of the essential oil by the SFE method was to optimize the operating conditions (especially pressure and temperature) to obtain an efficient extraction of terpenic compounds that are responsible for the aroma and to avoid the co-extraction of undesired compounds such as fatty acid and their esters. In addition, obtained essential oil extractions in the nine SFE conditions were investigated for their physico-chemistries (refractive index).

As can be concluded from Table 1, Run 5 was the best extraction condition (T = 45 ℃, P= 200 atm, and dynamic time 25 min) with a resultant essential oil yield of 3.66 w/w. The pressure and temperature conditions of Run 5 were most recommendable for essential oil extraction with the SFE method, because their solubility is sufficient under the conditions, whereas the solubility of fatty oils, resins and waxes present in the plant material is negligible under the same conditions. The results revealed that the oil extraction would be performed most efficiently under the conditions of the pressure of 200 atm, the temperature of 45 ℃ and the dynamic extraction time of 25 min.

Chemical Composition of Essential Oil of H-tang Identified by GC and GC-MS Analyses.

The chemical compounds of the essential oil identified by GC and GC-MS analyses are listed in Table 3. Thirty-seven compounds were identified in the SFE oil, which is equal to 93.9 % of the total oil. The main compounds with concentrations higher than 3 % as percentage peak area of GC analysis included tetradecenoic acid(11.7 %), Vanillin(5.9 %), *dl*-Limonene(5.5 %) and Eicosane(4.6 %). In the hydrodistilled oil, however, only thirty-four compounds were identified. The major compounds in the hydrodistilled oil were tetradecenoic acid(8.9 %), Vanillin(5.8 %) and Eicosane(4.7 %).

Analyses of Physicochemical Parameter

The physiochemical properties of essential oil were d^{20}=0.9856, and $[\alpha]_D^{22}$ = 24.8 ($CHCl_3$, *c* 0.28). The refractive index (n_D^{20}) of essential oils extracted from H-tang is presented in Table 3, which ranged from 1.3465 to 1.5232 as determined by the D-line of a sodium lamp 20 ℃. Variation in the refractive index was observed between each set of SFE condition. As was the case in Table 2, Run 5(n_D^{20} = 1.4937) was the best extraction condition. The refractive index of liquid oil or fatty acids depends on the numbers of carbon and double bonds and

Table 3. H-tang obtained (%) by SFE and hydrodistillation.

No	Compounds	R.I.[a]	SFE	HD[b]
27	9-Eicosyne	706	1.1	2.7
24	Chrysandemic acid	711	2.9	3.2
25	*trans*-Chrysanthermal	720	6.4	2.2
12	cis-Ocimene	741	2.8	2.3
28	5-Eicosyne	744	0.9	0.7
21	2(3H)-Furanone, dihydro-	771	0.6	1.0
26	Menthol	775	0.6	1.1
30	Guaiazolene	777	0.4	t
1	3,3-Dimethoxy-1-phenyl-1,2-propanedione	813	1.9	t
6	2-Pentanol, 3-methyl	823	1.1	0.5
31	E-9-Tetradecenoic acid	833	0.6	t
35	Oleic acid	836	0.6	0.9
11	2,4-Cycloheptadiene-1-one	838	3.1	0.7
9	α-Pinene	844	0.8	0.5
33	1-Octadecene	846	0.7	1.1
18	Bornyl acetate	856	2.0	0.8
5	3-Hexanal	864	1.2	2.3
8	α-Thujene	874	0.6	1.1
16	5-Pentyl-3h-furan-2-one	877	1.1	0.5
13	Cyclohexane, isothiocyanato	887	2.1	0.8
23	Vanillin	887	5.9	5.8
17	α-Fenchyl acetate	889	0.7	t
19	2-Methoxy-4-vinylphenol	890	3.3	2.5
4	Octane	893	0.6	1.1
22	Phenol,4-(ethoxymethyl)-	894	3.5	2.7
10	Campene	898	2.1	1.2
34	Oxacyclohexadecan-2-one	904	0.6	1.6
37	Eicosane	905	4.6	4.7
14	dl-Limonene	912	5.5	2.9
36	Hexadecanoic acid, methyl-	914	3.4	2.1
15	Phenol,3-methyl-	914	2.1	1.1
29	1-Hexadecene	918	4.8	5.1
3	2-Hexanone	920	0.9	0.4
7	Cyclotrisilioxane, hexamethyl-	934	0.5	0.6
2	3-hexanone	939	0.7	1.2
32	Tetradecenoic acid	948	11.7	8.9
20	5-Pentyl-2(5H)-furanone	955	4.6	3.1

[a]Kovats retention indices on DB-5 column.
[b]Hydrodistillation
[c]Percent of component based on the area normalization

the presence of ketone or hydroxyl groups. Therefore the variation in the refractive index can provide the basis for simple measurements to predict in vivo activity and to discern between pure and mixed oils. This will also illustrate the variation of essential oil composition between sites and species and even in individual oils[30-32].

The Antimicrobial Action

The results of the antimicrobial action (Table 4) showed that the essential oil of H-tang exhibited antimicrobial actions against all the bacteria tested (MICs, 0.025 to 12.8 ㎎/㎖; MBCs, 0.05 to 12.8 ㎎/㎖). The essential oil showed the strong antimicrobial action against all bacteria (MICs, 0.025 to 0.8 ㎎/㎖; MBCs, 0.05 to 1.6 ㎎/㎖), excepted E. coli (MICs/MBCs values 12.8/12.8 ㎎/㎖).

These results also indicate the possibility of exploitation of the essential oil of H-tang as an effective inhibitor of oral bacteria. However, for medicinal purposes, the safety and toxicity of this essential oil need to be addressed.

Conclusion

In this study, the supercritical fluid extraction of H-tang was examined, and the extraction results by the SFE method were compared with the essential oil composition obtained by hydrodistillation. This study discovered that the SFE method has many important advantages over hydrodistillation. The SFE requires shorter extraction time (25 min vs. 4 hr for hydrodistillation). The energy cost required for performing hydrodistillation is much higher than that for reaching the SFE conditions. The possibility of manipulating the composition of the oil, by changing the parameters of the extraction (pressure, temperature and

Table 4. MICs and MBCs (㎎/㎖) of essential oil and its major components of H-tang for some oral bacteria with a few reference strains.

Strains	Essential oil (MIC/MBC)	Ampicillin (MIC/MBC)	Gentamicin (MIC/MBC)
Escherichia coli (ATCC 25922)	12.8/12.8	$256/256\times10^{-3}$	$8/16\times10^{-3}$
Staphylococcus aureus (ATCC 29213)	0.2/0.4	$16/16\times10^{-3}$	$2/4\times10^{-3}$
Staphylococcus epidermidis (ATCC 12228)	0.8/1.6	$32/64\times10^{-3}$	$1/2\times10^{-3}$
Streptococcus pyogenes (ATCC 21059)	0.2/0.2	$4/8\times10^{-3}$	$8/16\times10^{-3}$
Streptococcus mutans (ATCC 25175)	0.1/0.2	$4/4\times10^{-3}$	$8/8\times10^{-3}$
Streptococcus sanguinis (ATCC 10556)	0.1/0.1	$32/32\times10^{-3}$	$8/16\times10^{-3}$
Streptococcus sobrinus (ATCC 27607)	0.1/0.1	$2/2\times10^{-3}$	$4/8\times10^{-3}$
Streptococcus anginosus (ATCC 31412)	0.1/0.2	$4/4\times10^{-3}$	$16/16\times10^{-3}$
Streptococcus gordonii (ATCC 10558)	0.025/0.05	$1/2\times10^{-3}$	$2/4\times10^{-3}$
Fusobacterium nucleatum (ATCC 10953)	0.05/0.1	$0.25/0.25\times10^{-3}$	$16/32\times10^{-3}$
Prevotella intermedia (ATCC 25611)	0.05/0.1	$32/32\times10^{-3}$	$0.5/1\times10^{-3}$
Porphylomonas gingivalis (ATCC 33277)	0.025/0.05	$0.5/1\times10^{-3}$	$256/512\times10^{-3}$

dynamic extraction time), is more attainable in the SFE. The SFE and hydrodistillation, as the methods of oil extraction, may not have qualitative difference but do have quantitative difference. I obtained higher selectivity in the SFE than by the hydrodistillation method.

The flexibility in the management of the variables involved in the SFE process allows one to optimize the experimental conditions, considering the selectivity of a substance or classes of substances of interest. The selectivity of supercritical CO_2 also allows a maximization of the concentrations of selected compounds: the SFE process proved to be more advantageous than hydrodistillation, as demonstrated in the case of H-tang. As presented above, the optimum SFE condition was obtained in the following experiment condition: pressure = 200 atm, T = 45 ℃, extraction times = 25 min.

Thirty-seven compounds were identified in the oil obtained by the SFE method, and the identified compounds account for 93.9 % of the total oil. The main compounds with concentrations higher than 3 % as percentage peak area of GC analysis were as follows: tetradecenoic acid (11. %), Vanillin (5.9 %), dl-Limonene (5.5 %) and Eicosane (4.6 %). In the meanwhile, thirty-four compounds were identified in the hydrodistilled oil. The major compounds of the hydrodistilled oil were tetradecenoic acid (8.9 %), Vanillin (5.8), α-Thujene (6.1) and Eicosane (4.7 %). Also, essential oil was tested for antimicrobial action against 12 different genera of oral bacteria. The essential oil of H-tang exhibited considerable inhibitory effects against all bacteria tested (MICs, 0.025 to 0.05 ㎎/㎖ MBCs, 0.05 to 0.1 ㎎/㎖).

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