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SUPERCRITICAL FLUID EXTRACTION AND BIOASSAY IDENTIFICATION OF PRODRUG SUBSTANCES FROM NATURAL RESOURCES

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Abstract – For arbitrarily chosen thirty types of natural resources which have been widely used in oriental traditional herb medicine, supercritical CO_2 extraction (SFE) and organic liquid solvent extraction (LSE) with *n*-hexane, chloroform and methanol were carried out to extract pharmaceutical substances. To evaluate relative advantages and shortcomings between the SFE and LSE, five types of bioactivity assays as well as gas- and thin layer-chromatographic analysis were performed for all the extracts obtained by the two extraction methods. Types of bioassays performed included cytotoxicity, bleb forming, DNA binding, oxygen free radical scavenger and Xanthine oxidase inhibitor tests. To evaluate economic viability of the SFE over the traditional LSE, extractability of prodrug substances was evaluated as the functions of extraction temperature and pressure. SFE was proven to be a feasible alternative over LSE. Also, the optimum SFE conditions which provided maximum extraction and cytotoxicity for each selected sample were presented.

Key words: *Supercritical Extraction, Carbon Dioxide, Pharmaceutical Substances, Natural Plant Materials, Bioassays, Cytotoxicities*

INTRODUCTION

Worldwide until present, more than eighty percent of pharmaceutical substances are being obtained or at least originating from natural products. To obtain such substances conventional separation techniques such as multi-step steam distillation or cascaded organic liquid solvent extraction have been utilized to date. However, when one adopts such traditional separation processes, it is very difficult to prevent the compositional or thermal degradation of solvents and final products. Also, high-purity removal of the residual toxic solvent in the final products still remains a less tractable problem [King and Bott, 1993].

As an alternative technology in food and pharmaceutical industries, the potential utilities of the supercritical fluid extraction (SFE) have been studied extensively during the last decades. By virtue of numerous evaluation studies of SFE for replacing traditional separation methods, the SFE becomes a new separation process for the extraction of biological materials from natural products [Rizvi, 1994]. However until present main interests of SFE studies in the isolation of biological substances have been limited to the separation of relatively low-molecular materials such as fats and fatty acids [Stahl et al., 1981].

In recent years, attention also has been placed on the replaced use of the SFE over the traditional separation techniques in pharmaceutical industry [Larson et al., 1986]. For example, relatively high molecular substances such as the cardiac glycoside (i.e., ouabain) and pyrrolizidine alkaloids from the natural products can be extracted by using the supercritical carbon dioxide [Bicchi, 1994]. Also, based on the SFE, an extensive exploring study of new and existing chemotherapeutical substances from natural plants has been done by such research bodies as the National Cancer Institute (NCI) in the United States [McHugh and Krukonis, 1994]. Also, various selective separations of bioactive substances from natural plant materials based on the use of SFE were carried out by numerous investigators [Smith and Burford, 1991; Ma et al., 1991; Liu et al., 1995; Modey et al., 1996; Bevan and Marshall, 1994; Joo et al., 1994].

However, most of existing experimental SFE information in the pharmaceutical field seemed insufficient for reliable uses in engineering process design purposes and, thus, further systematic extractability studies of SFE are required. Besides, the compositional contents of the target bioactive extractant obtained by the SFE is usually very low due to the coextractants of significant amounts of chlorophyll and wax materials. In this case a comparative and quantitative selectivity study of SFE must be followed for a designated substance [Choi et al., 1996].

Also, the cytotoxicity and separation yield of a designated pharmaceutical extractant from natural products are highly dependent upon both the kind of pretreatment for the raw materials is employed even for the same SFE study and on the type of extraction techniques such as SFE and organic liquid solvent extraction (LSE) is used. Thus, in order to draw any reliable conclusion of whether any SFE study can be a practical alternative over the conventional LSE, further systematic comparative studies should be made [Lilian, 1986; Han, 1992]. In this regard, the present authors have studied in recent years on the two-fold critical evaluations of the SFE for the possible replacement of the traditional LSE techniques to obtain pharmaceutical extracts from natural resources: one is the establishment of the optimum SFE condition for each sample resource which gives maximum extraction yield and cytotoxicity, and the other

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is the high-purity isolation of some specific compounds from the total SFE extracts.

To figure out comparatively whether the SFE is practically advantageous or not, the traditional Soxhlet LSE with *n*-hexane, chloroform and methanol was carried out. Also, simple but reliable *in vitro* bioactivity tests were established and applied to the extracts. The bioassays included cytotoxicity, bleb forming, DNA binding, oxygen free radical scavenger and Xanthine oxidase inhibitor tests.

EXPERIMENTAL

1. Selection of Natural Samples and Pretreatment

Among numerous natural resources which have long been used in oriental folk medicine, thirty types of the sample raw materials were selected in the present study based on the information of relative frequency in use and importance in natural pharmacy. The list of these sample materials is summarized in Table 1. The samples were purchased from the domestic market and they were cut by 0.59-0.71 mm and dried for 24 hours in an oven at 313.15 K. The sample prepared in this way was used for both the SFE and the conventional LSE.

2. Supercritical CO_2 Extraction

2-1. Experiment by Large-scale Apparatus

In most cases the content of bioactive substances in a sample natural product is extremely low. As a result, in order to obtain a total extract which contains at least necessary amount of target biosubstances, in the first place the SFE was performed in a relatively large scale apparatus. The schematic of the self-designed and constructed flow-type extractor is shown in Fig. 1. The internal volume of the extractor was 1.5 *l*. Optimum pressure in the extractor was obtained by a gas booster (HASKEL 75/15, USA) and controlled by a back pressure regulator (TESCOM 26-1700, USA). To prevent any entrainment from the extractor, porous layers were attached to both ends of the extractor column. Also, to mitigate pressure fluctuation, a relief reservoir tank was installed between the regulator and extractor. The extractor was heated by a heating tape and the temperature of the extractor was controlled within ± 0.5 K by a PID con-

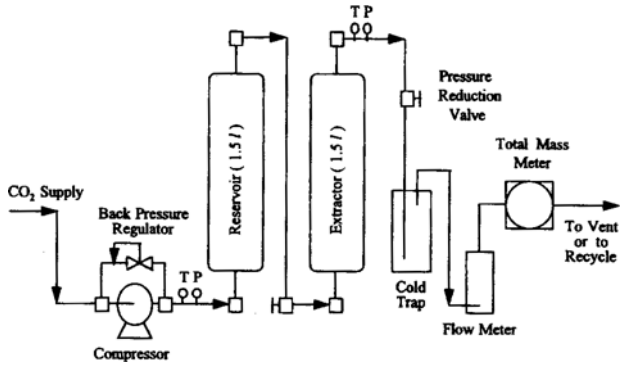

Fig. 1. Schematic diagram of the large-scale SFE apparatus.

Table 1. Specific names of the natural resources tested in the present experiment

Code no.	Specific name	Part used	Name of herb medicine	Origin
1	*Lycium chinese*	fruit	Lycii Fructus	Korea
2	*Schizandre chinensis*	fruit	Schizandraea Fructus	Korea
3	*Citrus unshiu*	fruit bark	Aurantii Nobilis Pericaripium	Korea
4	*Angelica gigas*	root	Angelicae gigas Radix	Korea
5	*Cornus officinalis*	fruit	Corni Fructus	Korea
6	*Cnidium officinale*	rhizome	Cnidii Rhizome	Korea
7	*Ginkgo biloba*	leaf	Ginkgo Folium	Korea
8	*Aralia cordata*	root	Duhuo Radix	Korea
9	*Evodia officinalis*	fruit	Evodiae Fructus	China
10	*Crataegus pinnatifida*	fruit	Crataegi Fructus	China
11	*Paeonia lactiflora*	root	Paeoniae Radix	Korea
12	*Leonurus sibricus*	all	Leonuri Herba	Korea
13	*Sophora japonica*	flower	Sophora Flos	China
14	*Artemisia capillaris*	all	Artemisiae capillaris Herba	Korea
15	*Platago asiatica*	seed	Plantaginis Semen	China
16	*Ephedra sinica*	all	Ephedra Herba	Korea
17	*Aconitum carmichaeli*	tuber	Aconiti Tuber	China
18	*Scolopendra subspines*	all	Scolopendra	Korea
19	*Paeonia suffruticosa*	root	Moutan Cortex	China
20	*Pueraria thunbergiana*	root	Puerariae Radix	Korea
21	*Polygala tenuifolia*	root	Polygalae Radix	China
22	*Coptis japonica*	rhizome	Coptidis Rhizoma	Korea
23	*Astragalus membranaceus*	root	Astragali Radix	Korea
24	*Eucommia ulmoides*	stem bark	Eucommiae Cortex	China
25	*Bupeuri falcatum*	root	Bupleuri Radix	Korea
26	*Acanthopanax sessiliflorum*	bark	Acanthopanacis Crtex	Korea
27	*Epimedium koreaum*	all	Epimedii Herba	China
28	*Morus alba*	root bark	Mori Cortex Radicis	Korea
29	*Artium lappa*	fruit	Arcii lappae Fructus	China
30	*Spirodela polyrhiza*	all	Spirodelae Herba	Korea

troller (HY-AT96, Korea).

From the extractor an effluent extract dissolved in supercritical CO_2 was made to pass through a heating-jacketed metering valve (HIP 60-11-HFV-V, USA) and a two-step cold methanol trap in which the separated extract solute was dissolved. At this stage the flow rate and cumulative consumption of CO_2 at ambient state were measured by a mass flowmeter (Sierra 8810, USA) and a calibrated wet testmeter (GCA/Precision Scientific, USA).

For each extraction, 150 g of sample was used. Experimental conditions in this large scale extractor were fixed at 313.15 K and 15 MPa. The volumetric velocity of fluid in the apparatus was controlled at 0.5 *l*/min and in each experiment about 800 *l* of CO_2 at ambient state (298.15 K and 0.1 MPa) was consumed. All the extracts obtained by this extractor were analyzed by gas chromatography (GC) and thin layer chromatography (TLC) and five types of cytotoxicity tests were performed as discussed in the later section.

2-2. Experiment by Micro-scale Apparatus

After five types of cytotoxicity tests as discussed in the later section were carried out for extracts obtained from the large-scale extractor, further extraction for each sample which was identified to contain noticeable amount of target pharmaceutical substances was carried out by a micro-scale extractor. The schematic diagram of the flow-type micro-scale apparatus is shown in Fig. 2. Through experiments in this extractor, optimum conditions which provide maximum yields and cytotoxicities were intended to establish.

The internal volume of the micro-scale extractor was 60 ml. A syringe pump (ISCO 260DM, USA) was used for feeding CO_2 and the pressure was measured by a Heise gauge (HEISE MM-43776, USA). Temperature in the extractor was maintained by an air-bath and controlled within ± 0.1 K by a PID controller. Extracts from the apparatus were passed through a heating jacket, a metering valve, and were collected by a two-step cold trap which contains methanol.

Among 30 extracts obtained by the large scale SFE extractor from raw materials as shown in Table 1, five sample materials (*i.e., Angelicae Gigas Radix, Duhuo Radix, Bupeuri Radix, Acanthopanacis Cortex and Spirodelae Herba*) were representatively selected and extraction experiments were performed in the micro-scale extractor and optimum conditions were established for each substance which give maximum extraction yield and cytotoxicity. For each 5 g sample, about 30 *l* of CO_2 at ambient condition was consumed. The range of extraction temperatures employed in this experiment was 308.15-328.15 K and the range of pressures was 10-30 MPa.

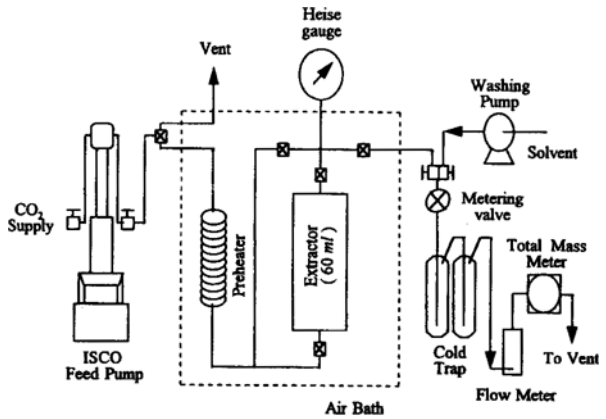

Fig. 2. Schematic diagram of the micro-scale SFE apparatus.

3. Liquid Solvent Extraction

To provide a criterion of comparison of the SFE with the traditional LSE, the Soxhlet LSE with methanol and chloroform was carried out for 30 types of samples. In each experiment, 100 g sample was extracted, respectively, by a 500 ml solvent for 9 hours [Trevor, 1991; Dey and Harbone, 1991].

After the cytotoxicity tests were carried for the extracts obtained by both methanol and chloroform, samples which showed noticeable cytotoxicities were selected, and the LSE by *n*-hexane was additionally performed for each 5 g of each sample.

4. Analysis of Extract

TLC is an auxiliary analyzing tool for a quick identification of the composition of an extract. Also, it can be used to isolate compositional distribution of an extract easily with respect to the variation of mobile phase [Baugh, 1993]. And the GC is a convenient tool to be used for analyzing residence time of mobile phase. Thus, in the present study both the TLC and GC methods were used for the analysis of all the extracts obtained by both SFE and LSE.

For TLC, precoated silica gel 60 F_{254} plate (layer thickness; 0.25 mm, 20×20 cm, Art 5715, Merck, Germany) was used and anisaldehyde-H_2SO_4 solution was used as a coloring reagent. The distribution of nonpolar substances was analyzed by applying extract to the TLC plate and saturated the sample with a mobile phase mixed solvent (toluene : ethylene=93 : 3). The polar substances in an extract was analyzed by saturating sample on a plate with the mobile phase or mixed solvent (ethyl acetate: formic acid : acetic acid : water=100 : 10 : 10 : 20) [Wagner, 1984].

For GC analysis, an extract was diluted as 1 ml/mg by chloroform and 1 ml/mg cholesterol was added as an internal standard substance. Thus, for every chromatogram, the same magnitude of cholesterol peak appeared and was used as a standard datum for compositional distribution for both SFE and LSE, respectively. The GC used was HP 5890 series II model. The column was HP-5 (5% diphenylpolysiloxane, Hewlette Packard, USA) and FID detector was used. The column temperatures were in the range of 453.15 to 553.15 K, the temperature at the injector was 563.15 K, and at the detector was 573.15 K.

BIOACTIVITY ASSAYS

In general bioactivity of a natural pharmaceutical material is due to the enhanced interaction among pharmaceutical substances which exist in the material rather than the separated pharmaceutical activity of each substance. Also, the effective activity of a natural pharmaceutical material is frequently based on the traditional therapeutic experiences. In general, it is extremely difficult to analyze quantitatively the actual pharmaceutical activity solely in terms of modern pharmaceutical approaches. At the same time even in the case where the modern pharmaceutical methodology is applicable, the establishment of a general procedure of the identification of active substances in the material is apt to vary with respect to types of activity test adopted. Also, the natural pharmaceutical substances show a great

variety in their chemical structures and activities. Thus, isolated substances from natural products were frequently used directly or used as a model substance in the development of a new drug or as intermediate substances in synthetic pharmacy[Han, 1992].

The bioassay tests necessary in the process of an exploration or extraction of pharmaceutical substances from natural products are different from those tests necessary in a high-level chemotherapeutic experiment. Also, the bioassay methods usually required in conjunction with natural product must be simple, quick and, furthermore they should require a small amount of sample and should have a good reproducibility [Hostettmann, 1991]. Based on such preliminary requirements, five types of bioassay methods were selected. Employed bioassays included cytotoxicity, bleb forming, DNA binding, oxygen free radical scavenger and xanthine oxidase inhibitor tests.

1. Cytotoxicity [Hostettmann, 1991]

The cytotoxicity assay employed in this work is a cell-level anticancer assay for searching a substance which gives toxicity to cancer cell. In this assay, the cell used was a typical P388 cell which is a representative leukemia cell of mouse. The effective dose, ED_{50} (μg/ml), was defined as an extract concentration at which 50 percent of the cell growth was inhibited. It denotes that the lower is this value, the more cytotoxic is the extract.

In the assay, the 100 μl substrate which contained certain number of cells was added into each well of 96 plate at 310.15 K. Then the plate was cultured in an incubator with an atmosphere of 5 percent CO_2 for 24 hours. The known sample extract obtained by SFE or LSE was dissolved into mixed solvent (*n*-hexane : isopropanol=1 : 1) of 4, 20, and 100 μg/ml, diluted as 100 ml, and cultured again for 48 hours. After the two-step culture, 75 μl of microculture tetrazolium (MTT) solution was added to each well to dye the cell. Then after 4-6 hours later, each well was centrifuged at 2000 rpm for 20 min and the upper phase solution was removed. Formazan produced by living cells in microculture tetrazolium (MTT) solution was dissolved by dimethylsulfoxide (DMSO) solution and an absorbency was measured at 540 nm. ED_{50} was calculated with a calibrated curve.

2. Bleb Forming [Hostettmann, 1991]

Bleb forming assay was used for finding an agent which prevents an arbitrary production of active protein kinase C (PKC) which is supposed to excel the cancer cell propagation. In this assay, 10 μl solution of a sample extract was diluted by 1000 μg/ml of DMSO solution and placed it into a 96 well plate. 90 ml of K562 cell solution with a concentration of 1.1×10^5 cells/ml which was prepared by a culturing at RPMI 1640 substrate that contained 10 % fetal calf serum was added into each well and, subsequently 10 μl of PDBU of a concentration of 0.1 μg/ml was added to the well and cultured for 10 min in incubator. Then, blebs appearing in the surface of K562 cell was observed. For a reference of the degree of bleb forming, a value of bleb forming observed without adding staurosporine as a standard substance into the K562 cell was taken as 0 %. Also, the value of bleb forming with the standard substance added into the K562 cell was assumed as 100 %.

3. DNA Binding [Hostettmann, 1991]

This assay is used to evaluate whether an extract contains a resisting substance for the duplication and propagation of DNA by incorporating with DNA of anticancer cells. A sample extract was dissolved into ethanol and three different concentrated solutions of 0.1, 0.5 and 1.0 mg/ml, were prepared. Each of these solutions was transferred into 96 well plate and solvent was removed under reduced pressure. Then, 20 ml of 0.05 M DNA-MG suspended solution which contained 7.5 mM $MgSO_4$ was added into 100 ml of Tris-buffer solution of pH of 7.5 and it was stirred for 24 hours at 310.15 K. This DNA-MG reagent was, then, added into each well plate, absorbency was measured at 630 nm by ELISA reader to prepare a calibration curve. ED_{50} was calculated using this calibration curve.

4. Oxygen Free Radical Scavenger [Hostettmann, 1991]

This assay can be classified as one of anti-gout tests and it is used to check an existence of a substance of deactivating oxygen free radicals which accelerates oxidation reaction with a substrate and destroys living-cell membranes and DNA strands. In this assay, amount of formazan produced by the reduction reaction of NBT with superoxide radical were measured.

After 100 μM Xanthile and 600 μM NBT were added into 0.1 M phosphate buffer solution of pH 7.4, 0.07 units/μl Xanthine oxidase was added into the solution. Again, each diluted sample extract prepared by dissolving into 1 mg/ml DMSO was added into the solution previously prepared, cultured for 10 min at 298.15 K, and light absorbency was measured at 560 nm, and subsquently the degree of inhibition was calculated. In this calculation, measured value of light absorbency for solution to which the sample extract was not added was used as a reference.

5. Xanthine Oxidase Inhibitor[Hostettmann, 1991]

This assay is one of extended test of oxygen free radical scavenger, and, it is used to measure the effect of resistance of the production of uric acid from Xanthine. A reagent solution as a substrate was prepared by adding 0.15 mM aqueous Xanthine solution into 0.1 M Xanthine oxidase phosphate buffer solution of pH 7.5. 1.0 ml of sample extract diluted by DMSO to 1.0 mg/ml was dissolved by 2.9 ml phosphate buffer and 0.1 ml enzyme solution was added into the reagent solution. Then the solution was cultured at 298.15 K for 15 min and to this solution 2.0 ml Xanthine solution was added. Again, the final solution was cultured at 298.15 K for 30 min and prevented further reaction by adding 1.0 ml of 1 N HCl solution. Light absorbency was measured at 290 nm. Set as zero percent inhibition for the case of a standard substance allopurinol, then, percent inhibition for each sample extract solution was calculated as a relative value.

RESULTS AND DISCUSSION

1. Bioactivity Tests of Total Extract

For thirty natural resources, total extracts were obtained by both the SFE with CO_2 at 313.15 K and 15 MPa and by both the LSE with chloroform and methanol, respectively. For all the extracts, five types of bioassays described previously were performed and the qualitative results were summarized in Table 2. Also, results of the specific degree of cytotoxic activity of both extracts by the SFE and LSE were summarized in Table 3-7, respectively. For the case of cytotoxicity assay, the extracts of *Angelica gigantis Radix, Duhuo Radix, Bupleuri Radix, Acanthopanacis Cortex and Spirodelae Herba* by the SFE showed higher cytotoxicity (each ED_{50} was lower than 30 mg/ml). Especially,

the extracts of *Angelica gigantis Radix, Duhuo Radix*, and *Bupleuri Radix* by SFE gave much higher cytotoxicity than those by the LSE. Only the extracts of *Duhuo Radix* and *Bupleuri Radix* by SFE showed the cytotoxicity. Also, for the cases of bleb forming and DNA binding assay, the SFE extracts of *Leonuri Herba, Plantaginis Semen, Ephedra Herba* and *Scolopendra* were more active than those by the LSE.

In the oxygen free radical scavenger assay, the SFE extracts of *Schizandrae Fructus* and *Moutan Cortex* were more active than the case of the LSE. In Xanthine oxidase inhibition assay, the SFE extract showed almost no bioactivity as in the case of the LSE.

Table 2. Bioassay results for the extracts obtained from the natural resources by supercritical carbon dioxide extraction

Code no.	Bioassay result				
	I†	II	III	IV	V
1	×‡	×	×	○	○
2	×	×	×	○	×
3	×	×	×	○	×
4	○‡	×	×	×	×
5	×	×	×	×	○
6	×	×	×	×	×
7	×	○	○	×	○
8	○	×	×	×	×
9	×	×	×	×	×
10	×	×	×	×	×
11	×	×	×	×	×
12	×	×	○	×	×
13	×	×	○	×	×
14	×	×	×	×	○
15	×	×	○	×	×
16	×	×	○	×	×
17	×	×	×	×	×
18	×	×	○	×	×
19	×	×	×	×	×
20	×	×	×	×	×
21	×	×	○	×	○
22	×	×	×	×	○
23	×	×	×	×	×
24	×	×	×	×	○
25	○	×	×	×	○
26	○	×	×	×	×
27	×	×	×	×	○
28	×	×	×	×	×
29	×	×	×	×	×
30	○	×	×	×	○

†I: Cytotoxicity assay(P388), II: Bleb forming assay, III: DNA binding assay, IV : Oxygen free radical scavenger assay, V: Xanthine oxidase inhibition assay.

‡×: shows no bioactivity, ○: shows bioactivity.

Table 3. Results of cytotoxicity test for extracts by the SFE with CO_2 and the LSE with chloroform and methanol

Code no.	ED_{50} values		
	SFE CO_2	LSE	
		Chloroform	Methanol
4	29.5	30.1	52.5
7	-	21.4	31.3
8	10.6	20.9	22.7
9	-	12.9	-
14	18.1	13.5	26.7
15	45.2	12.9	13.8
16	39.8	9.95	25.1
18	-	27.8	-
19	-	-	29.4
22	-	18.6	20.4
25	10.9	27.0	-
26	14.1	10.8	-
27	-	17.6	18.4
30	28.3	27.9	28.1

Table 4. Results of bleb forming test for extracts by the SFE with CO_2 and the LSE with chloroform and methanol

Code no.	Positive control (staurosporine) % Values		
	SFE CO_2	LSE	
		Chloroform	Methanol
7	90.0	70.0	-
16	-	95.0	-
30	-	80.0	-

Table 5. Results of DNA binding test for extracts by the SFE with CO_2 and the LSE with chloroform and methanol

Code no.	EC_{50} values		
	SFE CO_2	LSE	
		Chloroform	Methanol
7	0.648	0.541	0.379
12	0.358	0.592	-
13	0.783	-	-
15	0.309	0.402	0.582
16	0.470	0.514	-
18	0.592	0.751	-
21	0.198	-	-
23	0.734	-	-
25	0.592	-	-

Table 6. Results of oxygen free test radical scavenger for extracts by the SFE with CO_2 and the LSE with chloroform and methanol

Code no.	% inhibition values		
	SFE CO_2	LSE	
		Chloroform	Methanol
1	4.9	-	-
2	8.2	-	-
3	-	-	5.0
4	-	-	3.5
9	-	-	3.6
11	-	-	0.4
12	-	-	4.4
13	-	-	24.4
16	-	-	13.8
17	-	1.5	12.6
19	10.1		-
20	-	-	3.3
21	-	-	15.7
22	-	-	3.2
23	-	-	8.1
25	-	-	7.5
26	-	-	10.4
27	-	-	18.1

2. Established Optimum SFE Conditions

Based on the bioassay tests, SFE with the microscale equipment was carried out for five sample resources such as *Angelica gigantis Radix, Duhuo Radix, Bupleuri Radix, Acanthopanacis Cortex* and *Spirodelae Herba* in the ranges of 308.15-328.15 K and 10-30 MPa. In establishing an optimum condition for each sample, emphasis was given to a condition which guarantees maximum cytotoxicity. It was found that there exists a unique optimum condition for each sample. In Fig. 3, the variations of cytotoxicity of the *Duhuo Radix* extracts with respect to the extraction methods were shown illustratively. The cytotoxicity increases with decreasing pressure for the case of SFE. In general SFE extracts show higher cytotoxicity than those by the LSE. The optimum SFE conditions which gave maximum cytotoxicity of *Angelica gigantis Radix* and *Spirodela Herba* were 328.15 K, 30 MPa and 318.15 K, 10 MPa as shown in Fig. 4 and 5, respectively.

Table 7. Results of xanthine oxidase inhibition test for extracts by the SFE with CO_2 and the LSE with chloroform and methanol

Code no.	% inhibition values		
	SFE	LSE	
	CO_2	Chloroform	Methanol
1	4.6	3.8	21.8
4	-	2.7	-
5	21.3	15.1	-
7	6.0	-	-
9	2.7	6.0	-
10	-	-	2.0
11	-	17.7	8.1
12	-	-	5.0
13	-	6.6	9.2
14	24.2	31.0	10.2
20	-	1.3	4.1
21	4.6	-	-
22	2.0	-	-
23	-	11.5	7.1
24	10.6	16.7	-
25	4.3	19.7	-
26	0.9	7.4	18.3
29	5.5	-	-
30	5.6	3.1	17.3

3. Results of Qualitative Analysis by TLC and GC

Based upon the coloring pattern appearing in the TLC, we found that there exists many types in quality of nonpolar and polar bioactive substances in every extract. For example of TLC pattern, a result of TLC for *Lyvii Fructus* (Code No. 1) and *Bupleuri Radix* (Code No. 25) are shown in Fig. 6. The first plates in Fig. 6 (a-I, and b-I) are the result of nonpolar substances and the second plates in Fig. 6 (a-II and b-II) are the cases of polar

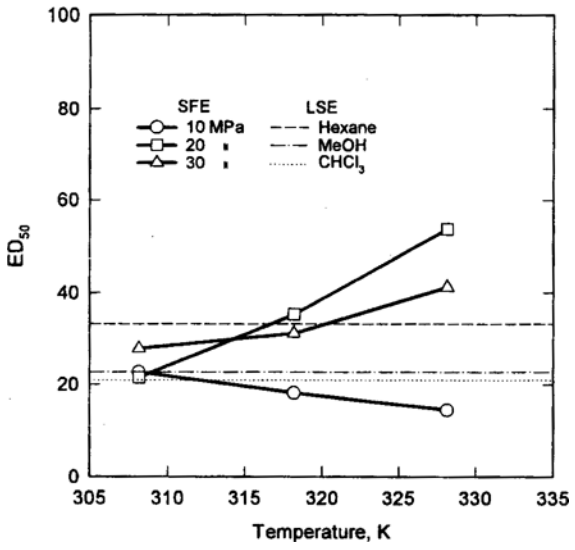

Fig. 3. Results of cytotoxicity assay (P388) for extracts obtained by the SFE and LSE from *Duhuo Radix* (LSE was performed at a fixed ambient condition).

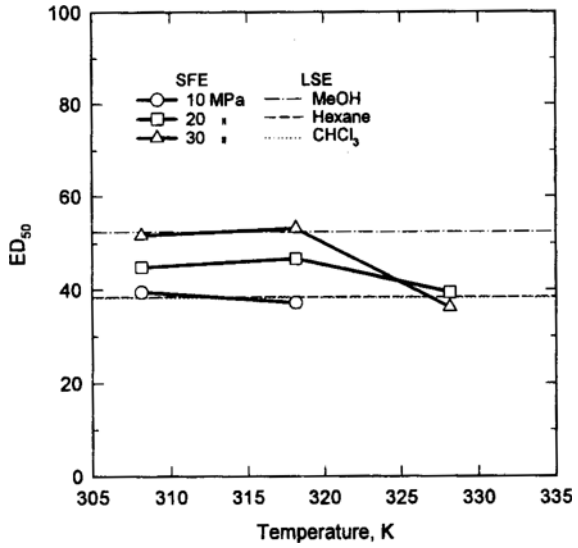

Fig. 4. Results of cytotoxicity assay (P388) for extracts obtained by the SFE and LSE from *Angelica gigas Radix* (LSE was performed at a fixed ambient condition).

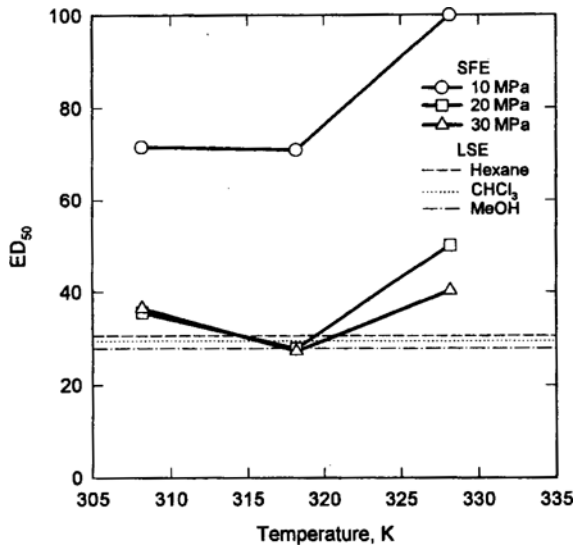

Fig. 5. Results of cytotoxicity assay (P388) for extracts obtained by the SFE and LSE from *Spirodelae Herba* (LSE was performed at a fixed ambient condition).

substances for *Lyvii* and *Buleuri Radix*, respectively. Also, the columns in each plate denote the extracts obtained by SFE (S), LSE with chloroform (C), and LSE with methanol (M), respectively, and the number in each spot in the TLC chart represent the relative concentration of coloring.

The difference of coloring pattern in the TLC charts with respect to the extraction by SFE and LSE could be observed. Also, we found that a different degree of coloring of the extracts by the SFE and LSE. Here the degree of coloring was usually linearly dependent on the concentration of the same substances in each extract by the SFE or LSE, and thus the qualitative difference of the distribution of substances with respect to the extraction methods could be observed. From this result we concluded that the distribution of extracted substances from the same sample by the SFE and LSE were significantly different from each other. Besides, we found that the SFE could be used to extract a target substances selectively. In this way, the TLC tests and the qualitative analysis were performed for all the thirty samples.

After the TLC tests, the GC analysis were carried out subsequently for all the extracts obtained by both the SFE and LSE. For example, the GC chart for the extracts by the SFE and the LSE with chloroform were comparatively shown in Fig. 7 (a, b) for the case of *Duhuo Radix* (Code No. 8). The same peaks near retention time (RT. 30 min) in both the charts (Fig. 7a and b) are the standard curves for the case of cholesterol (S). As one can see, there exists a significant difference of the distribution of substances for the extracts by the SFE and LSE. Especially, in the early retention time, a significantly different distribution of extracted components between the SFE and LSE was observed. In general, it is known that the supercritical CO_2 can be used to extract only lipophilic substances such as fatty acids and lipids appearing at the short RT in GC charts. However, based on the GC examination for all the samples obtained by the SFE and LSE, we found that the SFE with CO_2 also can be an efficient tool for the selective extraction of fatty acids and lipids existing at the long RT. For example, the gas chromatogram obtained for the SFE and LSE of *Corni Fructus* (Code No. 5) were shown in Fig. 8. As one can see in this figure, substances which showed longer retention time (i.e., near RT. 40) were extracted more by the SFE than by the LSE.

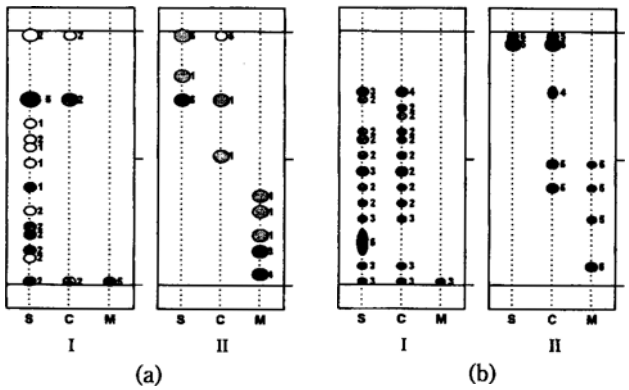

Fig. 6. TLC charts for extracts of *Lycii Fructus* (a) and *Bupleuri Cortex* (b) (S: CO_2, C: chloroform, M: methanol, Mobile phase I; toluene : ethylene=93 : 3, Mobile phase II; ethylene acetate : formic acid : acetic acid : water=100 : 10 : 10 : 20).

4. Selective Isolation of Target Compounds

To develop new prodrug materials, it is often necessary to isolate certain substances from the total extract in a high degree of purity. To evaluate such possibility with the total extracts obtained from the thirty natural resources, a column chromatographic study was carried out. Based on the examination of the TLC and the gas chromatograms for all the extracts by the SFE and LSE, some illustrative target substances were arbitrarily selected for a high-purity isolation. For the total extracts containing those target substances, a column chromatographic separation was performed.

For example, squalene (MW. 410) with high-purity was isolated from the extract of *Spirodelae Herba*. From 1.5 g total extract, obtained by the large scale SFE apparatus (Fig. 1), 10 mg of squalene (purity 98 %) was separated. Also, for 1 g sample of *Spirodelae Herba*, the LSE with *n*-hexane and the SFE (Fig. 2) were carried out over a wide range of operating conditions

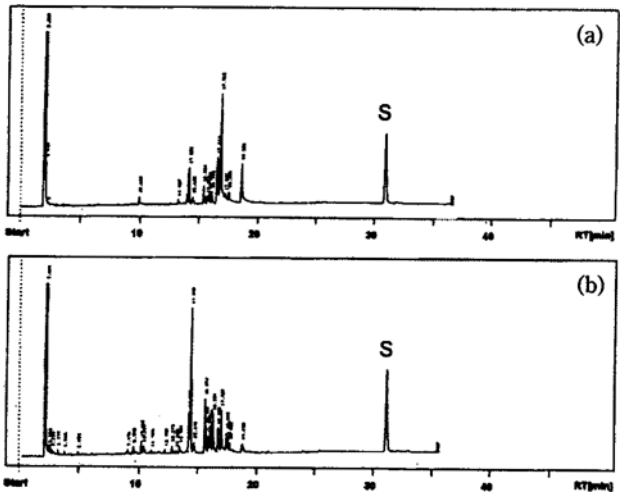

Fig. 7. Gas chromatogram for extracts of *Duhuo Radix* [(a) resulted by SFE with CO_2, (b) LSE with chloroform and S denotes the standard substance cholesterol].

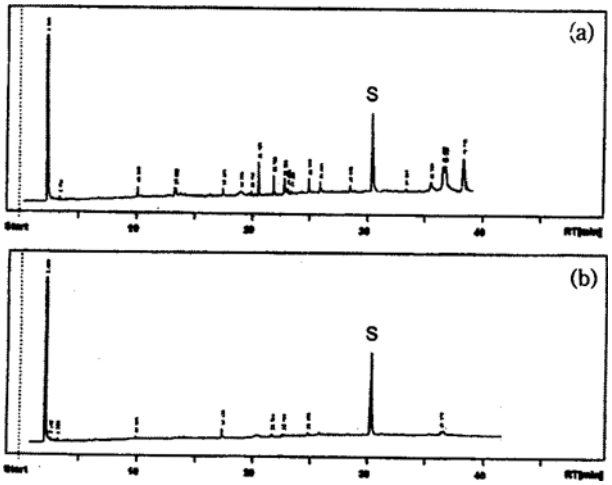

Fig. 8. Gas chromatogram for extracts of *Corni Fructus* [(a) resulted by SFE with CO_2, (b) LSE with chloroform and S denotes the standard substance cholesterol].

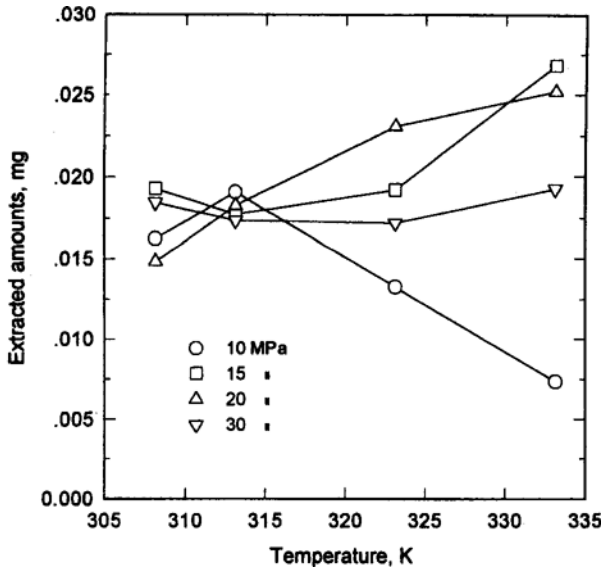

Fig. 9. Extracted amounts of squalene from *Spirodelae Herba* at different SFE conditions.

(308.13-333.15 K and 10-30 MPa). In Fig. 9, the variation of extracted amount of squalene as the functions of temperature and pressure is shown. Except for the case at 10 MPa, extracted amount of squalene tends to increase with increasing temperature.

At the room temperature (308.15 K), the amounts of squalene obtained by the SFE from the 1 g sample of *Spirodelae Herba* were in the range from 0.014 to 0.018 mg. However, for the case of LSE with the same sample with *n*-hexane, the extracted amount of squalene was 0.0011 mg.

CONCLUSION

For natural pharmaceutical products, supercritical fluid extraction with carbon dioxide and traditional liquid solvent extraction with chloroform, methanol and *n*-hexane were carried out in order to obtain both the total extracts of prodrug substances and some high-purity target substances. Also, to evaluate quantitatively the effect of supercritical fluid extraction conditions on the bioactivities of the extracts, five types of bioactivity tests were performed. We found that the supercritical solvent extraction can be an alternative separation tool over the conventional organic liquid solvent extraction. Especially we found that the supercritical extraction shows better selective separation of the target substances than the liquid solvent extraction.

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