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Research Paper

Supercritical Fluid Extraction of Flavonoid from Achillea wilhelmsii in Pilot Scale

Sonia Jafari, Ahmad Riahi *, Seyed Mojtaba Mostafavi

Department of Chemistry, Faculty of Science, Payam Noor University, Tehran, Iran

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Abstract

Microdesmis puberula is a plant that is used in traditional medicine for the treatment of diarrhea, stomachache, intestinal worms, menstrual complaints, sterility, miscarriage, loss of virility and venereal diseases, treat skin conditions, renal pain, severe headache, erectile dysfunction, and snake bite. In this work, methanol and petroleum ether extracts of the stem of Microdesmis puberula were evaluated for phytochemical constituents, antimicrobial activity and antioxidant activity. DPPH scavenging assay and total antioxidant capacity were used for the determination of the antioxidant activity. The agar well diffusion method was used to determine the antimicrobial activities of the extracts against the test organisms, Klebsiellapneumonia, Bacillussubtilis, Salmonella typhi, Enterococcus faecalis, Neisseria gonorhoeae, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pyrogenes, Staphylococcus aureus and Candida albicans. The broth dilution method was used to determine the minimum inhibitory concentrations (MIC) of the methanol and petroleum ether extracts. The methanol extract exhibited antimicrobial activity against both Gram-positive and Gram-negative test organisms with zones of growth inhibition ranging from 12 to 16 mm in the agar well diffusion test, but the petroleum ether did not exhibit antimicrobial activity as it recorded no zone of growth inhibition. The methanol extract was active against the test organisms with MIC range of 6.25 to 12.5 mg/mL and that of petroleum ether ranged from 50 to 200 mg/mL. The reference drug showed activity between 1.56 to 25 mg/mL. The IC50 of the methanol and petroleum ether extract, and the reference drug with regard to the DPPH scavenging activity, were 1.1µg/mL, 1.2 µg/mL and 0.2 µg/mL respectively. Both the methanol and petroleum ether extracts exhibited antimicrobial and antioxidant activity.

Keywords: Achillea wilhelmsii, Flavonoid, Supercritical fluid extraction, separation science

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1. Introduction

Flavonoids are compounds found in fruits, vegetables and certain beverages that have diverse beneficial biochemical and antioxidant effects. Although this plant is used more traditionally there is less study about its physiological property and the best way of its extraction. Supercritical fluid extraction (SFE) is a new and powerful developing technique in separation process that produces bioactive compounds [1]. The extraction of flavonoid compounds using supercritical fluids is important due to the high purity of the final compounds, which increases the added value of the final products and their price in the international market [2,3,4]. The supercritical fluid is usually CO2 because is not toxic, with low cost, operation under mild conditions (p>74 bar and T>310 ◦C) and it can be easily removed from products. Nowadays, supercritical fluid extraction, is used as an attractive extracting method to conventional liquid extraction in wide variety areas including the industries of food, pharmacy, environmental engineering, chemical and oil industries [3,5,6]. Optimization of the experimental conditions is a critical step in the development of a successful supercritical fluid extraction process due to the effect of various variables on the extraction efficiency [8,9]. There are three extraction modes with supercritical fluids: dynamic, static and static-dynamic [10]. In this research the static-dynamic mode was used. The combined liquid-like solvating capabilities and gas-

like transport properties of supercritical fluid make them particularly suitable for the extraction of diffusion-controlled matrices such as plant tissues. Moreover, the solvent strength of supercritical fluid can be manipulated by changing pressure (P) and/or temperature (T); therefore, it may achieve a remarkably high selectivity. This tunable solvation power of supercritical fluid is particularly useful for the extraction of complex samples such as plant materials [11]. One good example in the selective extraction by using supercritical fluid was a vindoline component extraction from among more than 100 alkaloid compounds from the leaves of Catharanthus roseus [12].

2. Material and Methods

Material: Carbon dioxide with the purity of 99.95% was obtained from Abu-Qaddareh Company (Shiraz). The plant was used this study named Achillea wilhelmsii was collected close to the city of Marvdasht in Iran (29052'27" Northern and 52048'09" Eastern). Dichloromethane with the purity of 99.99%, purchased from Merck.

Experimental procedure: The experiments were carried out in bench scale apparatus. Carbon dioxide is feed from a tank gas and liquified by a condenser. Then pumped through a shell and tube form surge tank. So that warm water is circulated in its shell with constant temperature. The supercritical CO2 was in contact with the sample for 45 minutes and during this time, it solved the essential oil of the sample. This situation is called "static time". The dynamic extraction was started by opening the exit valve for the SC-CO2 extraction system. The static extraction allows the sample of Achillea wilhelmsii to soak in the CO2 in order to equilibrate the mixture at desire pressure and temperature. The static extraction time will be performed at supercritical condition for every run conducted by this SFE work. During the dynamic extraction time, CO2 carrying the crude extract flowed out of the extraction vessel unit and into a U-shape tube in which dichloromethane was at 0°C. The essential oil was solved in dichloromethane, and CO2 was released into the atmosphere. In the end, the essential oil+dichloromethane mixture was collected as a sample, which was used in GC analysis.

Fig.1 shows the experimental system and Fig. 2 is the schematic diagram of the extractor apparatus.

Figure 1. Laboratory extraction system for supercritical fluid extraction.

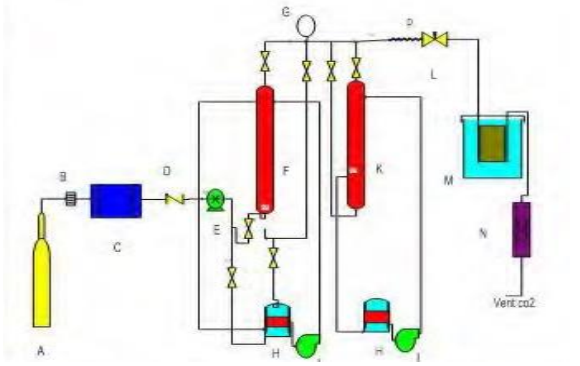

Figure 2. The schematic diagram of the extraction of flavonoid. A: CO2, B: valve, C:Condensor, D: Check valve, E: Pump, F: Surge tank, G: Pressure gauge, H: Warm bath, I: Water pump, K: Extraction column, L: Restrictor valve, M: U-shape tube, N: Gas meter, P: Temperature controlled restrictor valve.

3. Results and Discussion

3.1 Extraction of plant material

The active principles were extracted using methanol and petroleum ether separately on the pulverised stem. The yields of the extract were calculated as percentages. The yields of extracts were 20.4 % for methanol and 2.2 % for petroleum ether. The percentage yield of extracts was higher in methanol than in petroleum ether. The degree of extraction depends on the polarity of the solvents,

and that a better yield would be achieved from the solvent which is more polar [34]. This is because polar solvents are capable of dissolving the important therapeutic drug constituents which are to be extracted [35].

***Experimental conditions*:** In order to find the conditions of supercritical extraction of Achillea wilhelmsii oil that results in the higher flavonoid compounds concentration. Each experimental had 45 min of static extraction time and different dynamic extraction time with a CO_2 flow rate. Each set of conditions was tested by duplicate for the appropriate data and measuring the maximum content of oil in the samples.

***Determination of flavonoids by GC and GC-MS analysis*:** The GC-MS analysis was performed using a Variane 3400 equipped with a DB-5 column (30×25 mm internal diameter, film thickness 0.25 µm, and with helium as a carrier gas). The SFE samples (0.2µl) were injected (without any further dilution) using split mode with a split ratio of 1/60 and with split flow of 7.166ml/min. The oven temperature program at first was 60°C for 3 minutes, and then it was increased to 230°C at a rate of 3°C/min, and it remained at 230°C for 5minutes. The transfer line temperature was 280°C. The ionization energy was 69.922eV with a scan time of 1 second and mass range of 34-500amu. The injector and detector temperatures were held at 300 and 270°C, respectively.

The GC-FID analysis was performed using Agilent GC-6890N gas chromatograph, which is made in the U.S., with nitrogen as the carrier gas with a velocity of 1.4mlit/s on HP-5 (Dimethylsiloxane, 5% phenyl) column (30×0.25 mm id, Film thickness 0.25µm). The SFE samples (1µl) were injected (without any further dilution) using the split mode with a split ratio of 1/10. The oven temperature program was 60°C, which was then increased to 230°C at a rate of 3°C/min, and it remained at 230°C for 5 minutes. The injector and detector temperature were held at 240 and 260°C, respectively. The percentages of components were calculated by the area normalization method, without considering response factors.

3. Results and Discussion

This experiment was designed based on Taguchi method and according to L.9 orthogonal array with Minitab 16 software. Table 1 shows the experimental conditions of SFE for Achillea wilhelmsii essential oil. In this study, the focus was placed on the main effects of three important factors namely pressure, temperature and dynamic extraction time. Other factors which may influence the extraction process such as CO2 flow rate and powder particle size are made constant.

Table 1. Three factors-three levels (L.9) orthogonal array design for SFE of Achillea Wilhekmsii.

Run	Pressure (bar)	Temperature (°C)	dynamic time (min)	Yield
1	150	35	10	0.76%
2	150	40	30	1.37%
3	150	45	40	1.74%
4	170	35	30	0.91%
5	170	35	40	1.01%
6	170	45	10	2.29%
7	190	35	40	1.89%
8	190	40	10	2.96%
9	190	45	30	3.32%

From our study, the analysis by GC-MS of the extraction yield found that there was nonexistent of any flavonoid compounds if the operating pressure was above 190 bar. Therefore, the upper limit for the pressure level was set to 190 bars. The lower limit for pressure was set at 150 bar as it is just above the critical pressure of the CO2 solvent (73 bar), as suggested by previous workers for extraction of flavonoid compounds from plant material [13,14]. Temperature of 35 ◦C is just above the critical temperature for CO2 (31.06 ◦C) and this temperature is generally used in the extraction of plant materials by SC-CO2. The selected upper limit of the temperature (60 ◦C) was low enough to avoid the damage of heat sensitive compounds [15].

Effect of pressure on extraction yield: Fig. 3 presents the effect of pressure on extraction yield of Achillea wilhelmsii in SC-CO2 at three levels namely 150, 170 and 190 bar at constant temperature. According to the results, as pressure increases from 150 to 190 bar, the extraction yield increased. At a constant temperature, increasing the pressure will increase the density of the SC-CO2. The solvent strength of SC-CO2 increases with the density of CO2. As the density increased, the distance between the molecules decreased therefore the interaction between the analytes and CO2 increased, leading to greater solubility of the analytes in CO2 [16]. Therefore, the increase in pressure will also accelerate mass transfer analytes and solvent in supercritical extractor vessel system and improve the extraction yield. This suggests that the solubility of flavonoids in SC-CO2 is proportional to the density of SC-CO2.

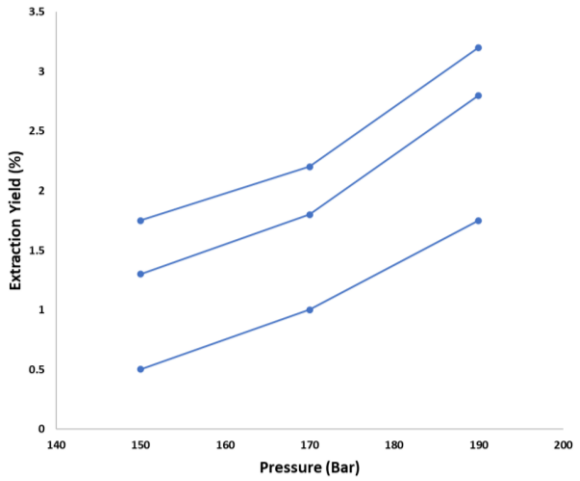

Figure 3. The effect of pressure on the extraction yield (%) at constant temperature (°C).

Effect of temperature on extraction yield: Fig. 4 shows the effect of temperature on extraction yield of Achillea wilhelmsii in SC-CO2 at three levels namely 35, 40 and 45 ◦ C at constant pressure. The influence of temperature on the yield extraction was studied. Density of CO2 at constant pressure decreases with increasing temperature and hence reduces the solvent power for SC-CO2. On the other hand, the increase of temperature can increase the vapor pressure of analytes. Therefore, the tendency of compounds to be extracted passing through the supercritical fluid will increase [17]. Amoderate increase in temperature can lead to a large decrease in fluid density, with a consequent reduction in solute solubility [18]. In this study the dual effect was clearly shown at the three constant pressures. Results showed that the extraction yield increased as temperature was increased from 35 to 45 ◦ C. This can be explained in a way that increasing temperature affected the enhancement of vapor pressure of analytes which is greater than the reduction of density of CO2.

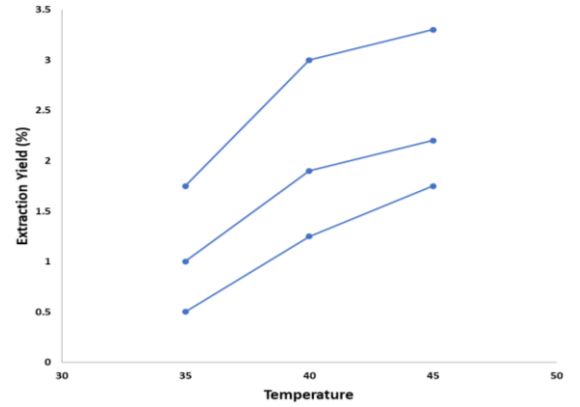

Figure 4. The effect of temperature on the extraction yield (%) at constant pressure.

Experimental data: Table 2 shows the selectivity of SFE, two runs (5, 7) were chosen randomly. And the components of their essential oil were reported. The components are identified by GC-MS analysis.

Table 2. Percentage composition of the Achillea wilhelmsii samples extraction with supercritical fluid at randomize runs.

Compounds	Run 5 Essential oil compounds (%)	Run 7 Essential oil compounds (%)
Carvacrol	8.73	10.01
Camphor	38.09	43.10
Thymol	6.22	8.19
α-Pinene	9.25	20.27
Pinocarvone	3.42	4.06
Borneol	3.52	3.89
Camphene	-	-
Caryophyllene oxide	-	0.03
Artemisyl acetate	0.10	0.05
Terpinene-4-ol	-	0.02
Eugenol	0.15	0.21
Myrtenyl acetate	-	0.086
Carvacrol methyl ether	0.067	0.171

4. Conclusions

According to our results, the optimum conditions of SC-CO2 for Achillea wilhelmsii flavonoid compounds were pressure at 190 bar, temperature at 45 ◦ C and dynamic time at 30 min. Based on mean value, it can be shown that the effect of extraction variables on extraction yields decreased in the following order: pressure, temperature and dynamic extraction time. The extraction pressure played a dominant role in the yield of the sample while the effect of time could be ignored. Under the optimum conditions, highest flavonoid compound content was at 3.32% and several flavonoid compounds were identified. From identification of flavonoid compounds by GC-MS in this study, it clearly revealed that temperature at 45 ◦ C is more convenient to be selected for SC-CO2 extraction, in order to avoid thermal degradation of the sample.

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