Impact of Ultrasound-Assisted Extraction on Supercritical Recovery of Valuable Compounds from Dry Pine Needles

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***Abstract*—Pine (Pinus pinea L.) have been used as a folk medicine for various health problems. As sustainable resource utilization methods become increasingly important, alternative methods have being investigated for evaluation of agricultural residues. The aim of this study is to recover phenolic substances that constitute the majority of antioxidants from dry pine needles by using sub-critical water extraction with and without ultrasound-assistance. The experiments were carried out at pressure of 10 to 20MPa and the effects of extraction temperature (60–200 ℃) and water flow rate (1–5 ml/min) were investigated. The results showed the most suitable extraction condition to determine highest amount of phenolic compounds was found to be at 180 ℃ and at the flowrate of 3 ml/min. Hereby, new process alternatives were projected and results were discussed. Hence, the present study investigated the influence of ultrasound assistance on extraction of phenolic compounds and their physico-chemical characteristics.**

***Index Terms*—pine needles, extraction, supercritical fluids, ultrasound extraction, phenolic compounds**

I. Introduction

Pine (Pinus pinea L., PP), also called stone pine and peanut pine in Turkey, belongs to the Pinaceae family [1], [2]. Pious pinea L., which attains its widest distribution in Turkey, naturally grows in the Mediterranean, Aegean, and Black Sea regions from sea level to 1000 m [3]. PP needles have been used in various areas as a food supplement, drink or herbal medicine. It is also known to be used in the treatment of some blood pressure disorders, diabetes, bronchitis, stomach and cardiovascular diseases [4], [5]. Pine needles are also recognized as a food flavoring and coloring agent [6], [7]. Pine leaf extracts have become a focus of attention in the academic world day by day, with its use in daily life, and also because of its effects in the field of pharmacy [8].

Phenolic compounds and anthocyanins have been studied extensively not only for their medicinal and sensory properties but also their antioxidant capacity and bioactive properties. They could be found in many kind of vegetables and in food process residuals. But it is important to ensure availability of these compounds by different preservation techniques [9].

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Dry pine needles, being agro-residual compounds, are a good sources of antioxidants for functional food industry among antioxidants in plant materials, phenolics and anthocyanins are one of the richest ones belonging to the polyphenolic class of compounds. Table I gives information on the main food constituents and the presence percentages found in the pine needles.

TABLE I. Chemical Characteristics (MGG-1 Dry Weight) of Pine Needles

Name of Component	mg/g dry e-weight
Soluble carbohydrates	96.3
Polyphenols	27.2
Hemicellulose	127.4
Cellulose	171.6
Holocellulose	298.9
Lignin	239.8
C	483.8
N	17.1
P	1.7
C	10.8
Mg	1.6
K	7.4

TABLE II. Main Chemical Components (% of Dry Weight) of Pine Needles

Component	Sample
Extractives	41.1
Solid residue	65.4
Sum of fractions	106.5
Ash	2.3
Crude protein	5.3

TABLE III. Composition (% of Dry Weight) of the Main Groups of Extractable Components

Component	Sample
Light petroleum soluble	29.7
Ethyl acetate soluble	12.2
2-Butanone soluble	9.7
Water soluble	48.4
Sum of fractions	100.0

It is also possible to classify the pine needle components in different classes such as solid waste, extractable fraction, ash and protein values (Table II) [10].

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In this classification, it is seen that the extractable fraction has a significant share. As stated in Table III, it is noteworthy that the water-soluble components have the most share within this extractable part [11], [12]. This would be considered as an explanation for the choice of water as a supercritical fluid in the study.

Generally, conventional techniques have been used for the extraction of phenolic compounds and anthocyanins from natural matrices [13]-[15]. But the conventional methods are quite expensive and mostly organic solvents are used for long operation time. Also, they may deteriorate the structure of bioactive materials in which phenolic compounds have great percentage due to high temperature [16]-[18].

In addition, it is very difficult to eliminate organic solvents used in conventional methods in such a way as to cause zero damage to the environment [19], [20]. Generally, acidified water in citric or lactic acid is used to extract phenolic compounds from pine needles. But solute deterioration occurs due to inconvenience of the system for higher temperatures over 200 ℃. New alternative methods have been searched for higher recovery of phenolic compounds and anthocyanins.

In recent years, number of studies have been done on evaluation on food products and recovery of bioactive compounds from plant materials. But the contrary to the importance of the matter, it is noteworthy that the small number of studies have been carried out for the evaluation of agricultural waste products. This has led to the need for orientation to new techniques that are easy to implement, economical and at the same time more efficient than conventional techniques. So, researchers have been focusing on alternative methods that can be used to assess agricultural and food waste materials [21]-[23] Traditional methods currently in use take time and require high installation and operating costs. For the reasons, new alternative directions have been formed for production of pure, high value-added products. Green extraction method is a good example for recovery of bioactive compounds from Food and plant materials [24]. Supercritical extraction technology is an outstanding example of green technology applications. It has advantages of being cheap, safe and is applicable to wide range of food materials [25]-[27]. With supercritical fluids various kinds of bioactive components such as fats, oils, antioxidants, flavor essences, etc. can be extracted by using advantages of their diffusivity power like gases and their solvating power like liquids [28]. These techniques increase the effect value with the support of some methods. Ultrasound Assisted Extraction (UAE) is a notable method for being a good candidate for increasing supercritical extraction efficiency. In the case of UAE, it is simple and easy to scale up to the industrial level. UAE is also used for extraction of bioactive compounds like phenolics from plant based products. Extraction temperature is low and it is beneficial in consistency for target compound bioactivity [29]-[33]. UAE also extends the shelf life of food products by requiring less energy and shorter periods of time. For the reason explained above, it has been investigated whether the ultrasonically assisted extraction method is more efficient than the pure supercritical extraction. Being an interdisciplinary research, this study combines the importance of bioactive compound recovery by applying an alternative method for extraction by investigating the effect of ultrasound assisted extraction to assess a new functional method. Therefore, the purpose of this study was to determine the applicability of the effect of ultrasound extraction and to determine the factors effecting the extraction efficiency. So, the technique couples subcritical water extraction with ultrasound-assisted supercritical extraction technology.

Specifically, dry pinee needle samples were extracted at different temperatures ranging from 60 ℃ to 130 ℃ using supercritical water extraction. After the most efficient conditions were determined, ultrasound-assisted extraction method was used for the same conditions and it was researched whether this subsequent process was effective on the total extraction process or not.

II. Material and Methods

Based on the significance of anthocyanins and phenolics as well, experimental procedures were investigated to determine the effect of ultrasound-assisted extraction to sub-critica water extraction on extraction yield. Total amount of extracts were calculated as anthocyanins and phenolic compounds, respectively.

A. Materials

Needles from Pinus pinea were supplied from Karabel Forest, Kemalpaşa, İzmir province, Turkey, in 2016. Prior to the raw material supplement, the necessary permits were taken from the forest management chief. Samples were collected, washed with water at room temperature and then were allowed to dry completely in an oven at 40 to 50 ℃ for 18 hours. Samples were ground in a coffee grinder until powder was obtained. These samples were stored in airtight polyethylene bags at 4 ℃ for the extraction process.

Anthocyanin standards: Cyanidin-3-diglucoside-5 gluco-side and Cyanidin 3,5 diglucoside were obtained from Carl RothGmbH D-76185 Karlsruhe, Germany. Glucose standard solution containing 0.1% (w/v) benzoic acid and having 1 mg/mL concentration was purchased from Sigma Aldrich Co. LLC Munich, Germany. The solvents including Folin-ciocalteu reagent were also purchased from Sigma Aldrich Company.

B. Methods

Sub-critical Water Extraction of Pinus pinea needles: Dried pine needle samples were cut into small particles and were dried at between 40 to 50 ℃ in an oven (Memmert, Germany). Samples were then ground to nearly 2 mm particle size. The ground particles were then placed in extraction cell which was located in sub-critical water extraction apparatus. Subcritical water extraction was carried out in a laboratory-built apparatus shown in Fig. 1. The extraction system consisted of one HPLC pump (PU 980, JASCO, Japan), a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS,

Germany), and an extraction vessel (10ml, Thar Design, USA), a pressure gauge, and a back pressure regulator valve (AKICO, Japan). All lines were connected with stainless steel capillaries (1/16 inch diameter). Water was de-oxygenated for 30 min using ultrasonic cleaner (Honda, W-211) prior to the extraction. Water was then delivered at a constant flow rate to the extractor using the HPLC pump. The extraction cell was completely filled with plant material (5.5 grams of ground pinus pinea sample) and placed vertically in extraction oven. The bottom and upper parts of extraction column was filled with glass beads to reduce the void volume in the parts and to form compact extraction area. Degassed water was brought to a constant temperature before entering to the extractor. Before starting the extraction, all connections were checked for possible leakage. The extraction pressure was controlled by adjusting the back-pressure regulator (AKICO) connected to the outlet coil.

The extract was cooled in the cooling bath to prevent degradation of the extract. The extract was collected in fractions every 10 min in sample collecting vials during the first hour and after which it was collected every 20–30 min. The extraction experiments were carried out mainly to determine the effect of temperature holding the pressure and water flow rate constant. The effect of temperature in the extraction process was studied. For this purpose, different assays were carried out between 60 to 200 ℃, working at 10 MPa and 20 MPa constant pressure values.

The flow rate used was 1 mL/min. Experiments were done in replication method. All runs were performed at least in duplicate.

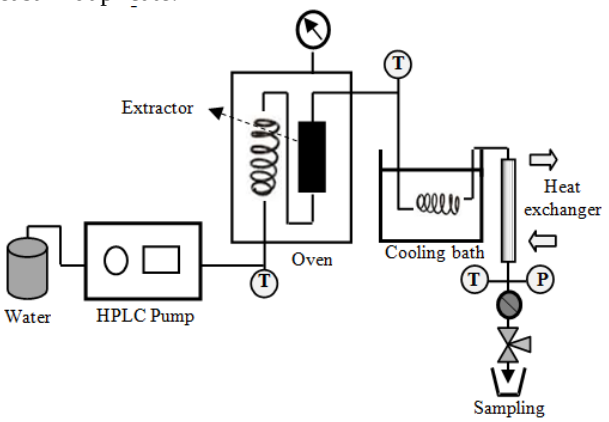

Figure 1. Subcritical water extraction flow diagram

Any doubtful results were checked and the experiments were repeated up to five times. After extraction, extracts were kept at +4 ℃ until analyzed. Following sub-critical water extraction, the ultrasound-assisted extraction method was applied to investigate the ultrasound effect.

Ultrasound-assisted Extraction of Pinus pinea needles: Phenolic compounds and anthocyanins were extracted from pinus pinea needles by ultrasound-assisted treatment of sample performed in an utrasonic generator by using the same amount of sample used in sub-critical water extraction. Ultrasound-assisted extractions (UAE) were performed in an ultrasonic extraction reactor (Elmasonic P30, Fisher Scientific Inc. St Louis USA); connected with a cooling chiller system; and a water pump (model HJ-111, submersible pump, flow rate 250 L/h, Sunsun Inc., Zhejiang, China). The transducer inside the jug was operated at 25 kHz frequency with generator output power of 120 W and tge capacity of generator was 1.7 L of water. There was a water circulation and that facilitated the control of extraction temperature. The bath temperature was set at 40 ℃, 50 ℃, and 60 ℃ by applying 40%, 50% and 60% power level. Ultrasound-assisted extraction time was set at 10min, 20min and 30min. Fig. 2 depicts the adapted ultrasound equipment for extraction.

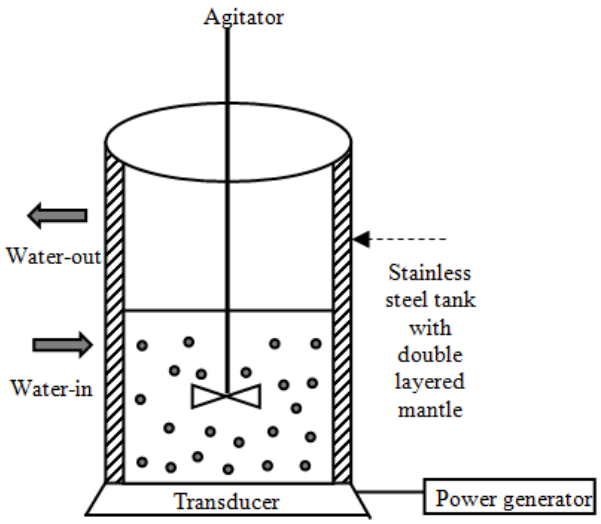

Figure 2. Ultrasound-assisted extractor

Powdered leaves were taken in a beaker and 25 mL of solvent was added. The beaker was placed in the ultra-sonic generator for maximum 30 min at 120 W exposed to ultrasonic waves to extract bioactive compounds. In other words, if the power level for ultrasonic generator was expressed in terms of W/cm^2, 75 W/cm^2 value was reached for maximum 60% power level.

C. Analysis

The total amount of extractable water-soluble organic com-pounds (WSOC) was determined after extractions in both ultrasound assisted sub-critical extraction and subcritical water extractions. The recovery efficiency for WSOC was determined using Total Organic Com-pounds (TOC) analysis [34], [35]. The TOC equipment was calibrated using glucose solutions prepared at various concentrations before each analysis.

The total phenolic content in the extracts was analyzed with the Folin-Ciocalteau method [36]. The extracted slurry was filtered by using No. 1 Whattman filter paper and centrifuged at 4000 rpm for 15 min to collect the supernatant. 0.1 ml of the extract was mixed with 2.8 ml of distilled water, 0.1 ml of 50% Folin-Ciocalteau reagent, and 2ml of Na_2CO_3 (2 g/100 ml). The mixture was kept for 25 min in a water bathat 40 ℃ and then cooled at room temperature. The absorbance of the samples were measured at 750 nm using a UV–vis spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight.

The percentage total phenolics yield is calculated as follows:

$$\text{Total phenolics conter } (mg_{GAE}/g) = \frac{\text{Weight of extract phenolics (mg)}}{\text{Weight of needle samples (g)}}$$

III. Results and Discussion

All fractions of pine needle extracts contain flavonoids and phenolics to a varying extent. Bioactive compounds in various plants may exist in many different combinations depending on the moity, climate conditions, the structural characteristics of the soil where the plant is grown. However, it was found that the ultrasonic assistance increased the effect of extraction for recovery of phenolic compounds. As it was stated in the literaure, flavonoids and simple phenolics like phenolic acid can be easily found in different pine species [37]-[39].

As a result of the tests carried out, the total organic carbon (TOC) level was investigated, and the proportion of the water soluble organic carbon (WSOC) component in the sample was also determined. Fig. 3 showed the effect of temperature on WSOC recovery in percentile. The lowest recovery was recorded at 60 ℃ as 6,69%. Temperature was increased up to 200 ℃ by giving 20 ℃ intervals.

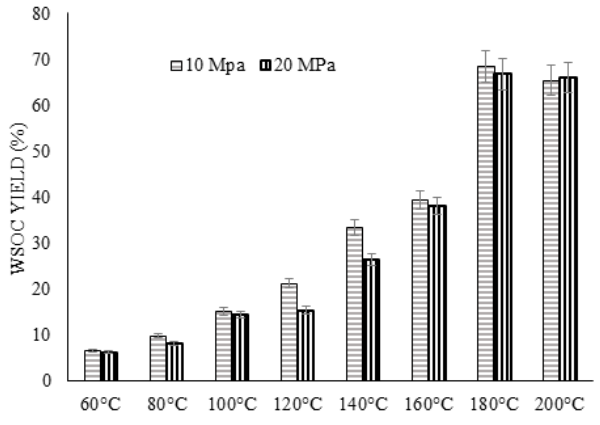

Figure 3. Effect of temperature on WSOC recovery

The WSOC recovery yield was increased starting from 9.85%, 15.2%, 21.3% to 68.54% and 65.36% for temperatures of 80 ℃, 100 ℃, 120 ℃, 180 ℃ and 200 ℃, respectively. The WSOC level was the highest at 180 ℃ for 2 h of extraction time and there is a little decrease in the efficiency at 200 ℃, this situation would be the indicator for decomposition of organic material in the sample.

At the same time, it was decided that 10 MPa pressure was more efficient when experimental data were examined. Although it provided slightly higher efficiency; a pressure of 20 MPa did not result in a significant increase in total efficiency. So, it was not advantageous to double the pressure on the system cost in order to capture a small increase in efficiency.

The effect of water flow rate was also investigated in the range of 1–5 ml/min, at a fixed extraction temperature of 180 ℃ and at 10MPa. The results were presented by plotting the total phenolic contents versus extraction time, as shown in Fig. 4. It was seen that water flow rate curves have plotted the same patterns at 180◦C. But the recovery efficiency was the best at 3 ml/min water flow rate. In addition, the rapid increase in the yield has become evident within the 2h hour extraction time for all water flow rate values.

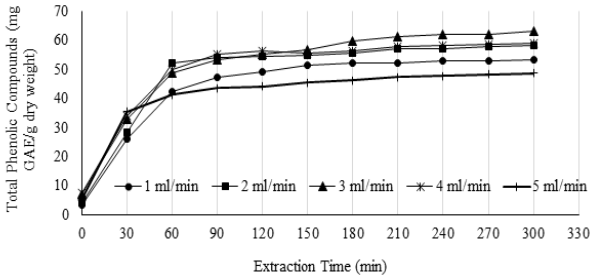

Figure 4. Total phenolic contents versus time in different water flow rate values (180 ◦C, 10MPa).

Extracts rich in water soluble phenolic compounds including anthocyanins were obtained best from from the semi-continuous processing at 180 ℃, 10 MPa at 3 mL/min water flow rate for 120 min. The efficiency was also depended on the extraction method. So, ultrasoound-assisted extraction was applied for the same experimental conditions by setting the time at constant value. There was a significant increase in total extraction efficiency being from 74.21% to 83.85% for subcritical water extraction and ultrasound-assisted extraction, respectively (Fig. 5).

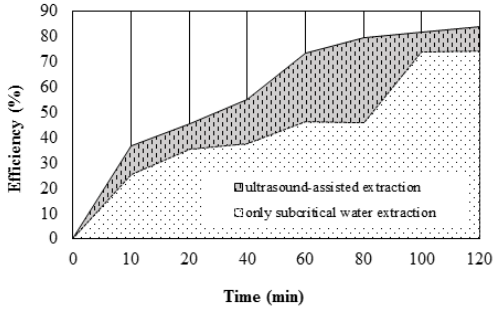

Figure 5. Effect of ultrasound-assisted sub-critical water extraction on total extraction efficiency

Ultrasound frequency was also an important parameter in extracting bioactive compounds from pine needles. The results showed that better responses were recorded at 75 kHz frequency level. As a result of an increase in diffusion rate, the organic compound solubility of solutes and the mass transfer, anthocyanin and phenolic compound content increased by increasing the temperature from 60 ℃ to 200 ℃ at 10 MPa. There was a little decrease in phenolic compound and anthocyanin content at 200 ℃ due to the decomposition of organic compound at elevated temperatures.

So, increasing extraction temperature has led to a turbid or cloudy appearance in the extracts, whereas the extract was clear at 180 ℃. On the other hand, pressure did not have a significant effect on the extraction of phenolic compounds from sample material. This result

could be due to the fact that a favorable condition that has been caused by high pressure (20 MPa) for the co-extraction of other analytes (Fig. 6).

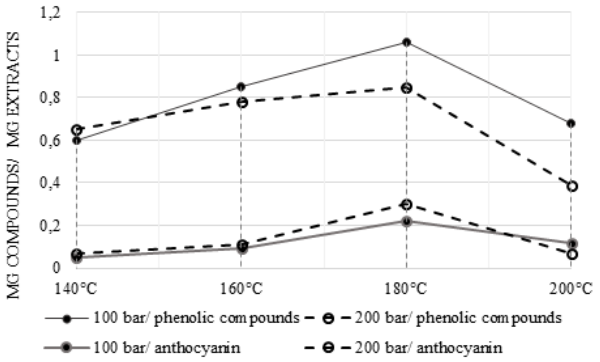

Figure 6. Effect of temperature, pressure and extraction method on phenolic compound/ anthocyanin recovery.

IV. Conclusion

This study showed an alternative and practical way to extract bioactive compounds from pine (Pinus pinea L.) needles. It was demonstrated that pine needle extracts contain a reasonable amount of phenolic compounds and anthocyanins. Furthermore, importance of sub-critical water to recover total phenolic compounds in extraction process was emphasized. The research also combined the ultrasound-assisted extraction with sub-critical water extraction process. The best condition to extract phenolic compounds and anthocyanins was detected as 180°C at 10 MPa for 2 hours of extraction time. In other part of the study, conditions were set to synchronize sub-critical water extraction with ultrasound-assistant extraction. Compared with sub-critical water extraction, ultrasound assistance was found to be a promising alternative for extraction of the phenolic compounds from pine needles. This finding has been a sign of success of new promising methods to recover heat labile compounds at moderate conditions that is suitable for raw material.

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