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Subcritical Water Extraction of Bioactive Compounds from Ginger (*Zingiber officinale* Roscoe)

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ABSTRACT

Ginger is one of the commonly used spices that has been exhibited to have pharmaceutical activities. These therapeutic properties are mainly attribute to gingerols and shogaols. To extract these bioactive compounds, subcritical water extraction (SWE) was employed as a green method. The influence of adding co-solvents, temperature, retention time and particle size on extraction yield were investigated. In addition the impact of ultrasonic and enzyme pretreatment was studied. Enzyme-assisted SWE with 2% ethanol as co-solvent and using ginger having particle size of 1mm, operated at 130°C and 20 bars for 30 min, is approved as optimized condition. At this condition, the total obtained polyphenol content and the concentration of total gingerols and shogaol were 5325 µg GAE/g dried ginger and 2990.5 µg bioactive/g dried ginger, respectively. Pretreatment of ginger powder with α-amylase prior to SWE, resulted in a 2.8 and 2.22 folds increase in total polyphenol content and concentration of total gingerols and shogaol, respectively. SEM analysis was conducted to evaluate the effect of pretreatment on morphology of ginger and analyze the extraction process.

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1. INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) that belongs to the family Zingiberaceae, is indigenous to South East Asia, mainly India and China. The ginger plant has tuberous rhizome that used in both forms of fresh and dried in food preparation [1, 2]. From ancient times, this spice has been used as medicine to treat headaches, colds, sore throat, diarrhea, nausea, digestive disorder, rheumatism and muscular discomfort [2, 3].

The main components of ginger rhizome contain essential oil and oleoresin which are responsible for its aroma and pungent taste, respectively. Essential oil mainly composed of sesquiterpene hydrocarbons and monoterpenoid while oleoresin comprises gingerols, shogaols, paradols and zingerone [3]. The medicinal properties of ginger are mostly attribute to gingerols, a group of phenolic compounds, of which the major content is 6-gingerol. These thermolabile compounds are convert to their dehydrated form, shogaols, at high temperature [2, 4]. The chemical structure of these compounds with phenolic ring and additional active functional groups are represented in Figure 1. Extensive studies have proved that gingerols and shogaols have pharmaceutical activities such as antioxidant, anti-inflammatory, anticancer, antitumor, analgesic, antiemetic and antifungal [4-6]. Research revealed that among bioactive compounds of ginger, 6-shogaol shows the strongest antioxidant and anti-inflammatory activities were due to α,β-unsaturated ketone section. In addition, the main functional group is attributed to carbon chain length, 10-gingerol, which is the most efficacious between gingerols [7].

It is significant to select an appropriate method to extract bioactive compounds from plants. Conventional methods like soxhlet, maceration and hydrodistillation have deficiencies such as long extraction time, necessity of a large volume of solvent, low selectivity and thermal degradation of compounds.

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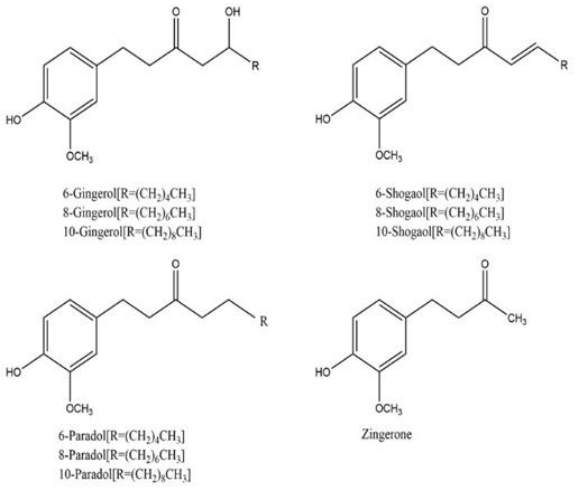

Figure 1. Chemical structure of bioactive compounds of ginger

In order to raise these difficulties, new green techniques such as microwave-assisted extraction (MAE), Ultrasound-assisted extraction (UAE), enzyme-assisted extraction, supercritical fluid extraction, subcritical water extraction (SWE) have been developed in recent years [8, 9].

SWE which so-called as pressurized hot water extraction is an environmentally-safe method that is generally applied for medicinal plants extraction. Subcritical water refers to water in liquid state at condition between boiling point (T=100°C, 0.1MPa) and critical point (374°C, 22.1MPa) [10, 11]. At atmospheric condition, water is a polar solvent with high dielectric constant owing to presence of hydrogen bonds in its structure. Hence, it is not a proper solvent for dissolving non-polar compounds. The growth in temperature, led to change in the physicochemical characteristics of water. The dielectric constant decline is the most important parameter in solvency property of water. This phenomena is because of the weakening of hydrogen bonds that led to decrease waters dielectric constant to the value close to those of organic solvents. Therefore, under this condition, water act the same as organic solvents and can dissolve compounds with low and medium polarity [12, 13].

In the present study, subcritical water extraction set up was used in order to extract bioactive compounds from ginger. The influence of different parameters such as adding co-solvent, temperature, extraction time and particle size on extraction yield were investigated. In addition, the influence of enzymatic and ultrasonic pretreatments on the extraction yield was studied. These environmentally friend methods were compared with traditional methods.

2. MATERIALS AND METHODS

2. 1. Material Dried ginger was obtained from local market (Babol, Iran). It was powdered in a hammer mill and acquired different size dispersion in mesh 18-40 using brass sieves. External standard nonanoic acid vanillyl amide (NVA) was purchased from Sigma-Aldrich (USA). Methanol, acetonitrile and water (HPLC grade) were supplied by Merck (Germany). Ethanol and acetone were purchased from Scharlau (Spain). α-amylase was obtained from SERVA (Germany). All other reagents and chemicals which employed in this study were of analytical grade from Merck.

2. 2. Conventional Extraction In order to evaluate the capability of subcritical water extraction, the maceration and soxhlet method were performed. 2 g of ginger powder was extracted with 300 ml ethanol for 8 h using soxhlet extractor. Extraction temperature was confirmed at 85 °C. To do the maceration extraction, 1g ginger was put in a 100 ml volumetric flask and was diluted to the mark with methanol. The flask was shaken vigorously. Repeatedly after 2 h was shaken vigorously and then it was left for 12 h. Some of the solution was taken by pipette. These extracts were kept aside for further analysis.

2. 3. Subcritical Water Extraction (SWE) SWE was accomplished using the assembled experimental rig that was built in "Biotechnology laboratory of Babol Noshirvani University of Technology" as the schematic diagram is presented in Figure 2 [14]. 40 g ground ginger was put into extraction cell which is made of stainless steel and is covered by heating jacket. Deionized water was purged with nitrogen for 1h in water tank to remove dissolved oxygen. Water is delivered to extraction cell by high pressure pump. After the temperature reaches to the set number and the extraction time is given, after specific time duration, the extracts passed through heat exchanger and collected for further analysis. Achieving optimal conditions was conducted by one-factor-at-a-time method. To determine the effect of addition of co-solvents, one time SWE was performed by adding 2% ethanol and again by 2% acetone to deionized water. Amount of 2% was selected using the research that estimated the solubilities of ginger bioactive compounds in hot compressed water with ethanol as entrainer [15]. The extractions were carried out at constant temperature of 130 °C, with particle size of 0.71 mm, at 20 bars, for 20 min. In order to study the effect of temperature on extraction yield, the extraction was accomplished at 110, 120, 130, 140 and 150°C; with appropriate co-solvent, at constant pressure 20 bars, with particle size of 0.71 mm, for 20 min.

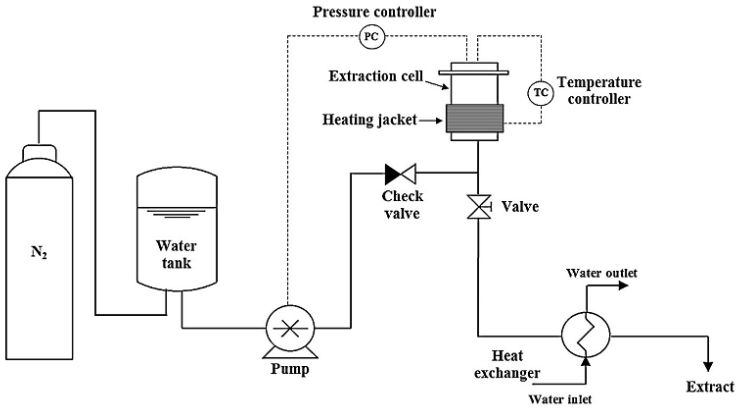

Figure 2. Schematic flow diagram of SWE experimental setup

To investigate the effect of retention time, SWE was run for 10, 20 and 30 min with appropriate co-solvent, at optimum temperature and constant pressure of 20 bars, with particle size of 0.71 mm. In order to consider the influence of particle size, ginger was extracted with particle size of 0.6, 0.71 and 1mm at optimum conditions determined from previous steps. Each experiment was run twice to ensure the reputability of the results.

2. 4. Enzyme-assisted SWE Enzyme pretreatment was carried out with the help of data reported in the literature [16]. 25 mg α-amylase with activity of 6 U/mg was dissolved in water and sprayed on 40 g ginger powder. pH of the sample was conformed to 4.5-5 by adding citric acid in water. The material was incubated at 50°C for 30 min. The prepared sample was extracted by SWE at optimum condition.

2. 5. Ultrasonic-assisted SWE To treat ginger powder, an ultrasonic bath (Elmasonic easy 30 H, Germany) with 50/60 Hz frequency and power of 280 W, was operated. Flat bottom flask containing the sample was placed in ultrasonic bath and the pretreatment was done at 40°C for 30 min. the sonicated ginger was extracted by SWE at optimum condition.

2. 6. HPLC analysis of Ginger Extracts Concentration of bioactive compounds of ginger was determined in a manner that was presented in ISO13685 protocol[17]. For this purpose, HPLC (Smartline, Knauer, Germany) equipped with Eurospher I 100-5 C18 column (250×4.6 mm) by UV detection at 280 nm was employed. Mobile phase was acetonitrile with water containing 1% acetic acid at a ratio of 65/35 at flow rate of 1 ml/min. nonanoic acid vanillylamide (NVA) was used as external standard. The retention time is comparable to that of 6-gingerol and has a similar UV absorption spectrum. Standard solution with concentration of 1 mg/ml was prepared by dissolving NVA in methanol and was injected to HPLC. The response factor for NVA was calculated by eqs 1 and then the values for each gingerols and shogaols was determined.

$$K_{NVA} = \frac{C_{NVA} \times 100}{A_{NVA}} \text{ mg/100ml/unit area} \quad (1)$$

$$K_{6-gingerol} = K_{NVA} \times \frac{\text{MW of 6-gingerol}}{\text{MW of NVA}} = K_{NVA} \times 1.003 \text{ mg/100ml/unit area} \quad (2)$$

$$K_{8-gingerol} = K_{NVA} \times 1.099 \text{ mg/100ml/unit area} \quad (3)$$

$$K_{10-gingerol} = K_{NVA} \times 1.194 \text{ mg/100ml/unit area} \quad (4)$$

$$K_{6-shogaol} = K_{NVA} \times 0.942 \text{ mg/100ml/unit area} \quad (5)$$

Where C_{NVA}, is the concentration of NVA (mg/ml), A_{NVA} is the mean area of its peak.

As an example concentration of 6-gingerol in sample can be calculated as follows, where c is the concentration of sample solution.

$$\text{Concentration of 6-gingerol} = \frac{A_{6-gingerol} \times K_{6-gingerol}}{c} \% \quad (6)$$

2. 7. Total Polyphenol Content To evaluate total polyphenol content in extracts, Folin-Ciocalteu method was used [18]. One ml Folin-Ciocalteu reagent solution, which was diluted in ratio of 1:10 with deionized water, was added to 0.2 ml of extract. 4 min later, 0.8 ml aqueous solution of sodium carbonate with concentration of 75mg/ml was added. The mixture was incubated at ambient temperature for 2 h. The absorbance was measured at 765nm using UV-vis spectrophotometer (Analytik Jena AG, Germany). The results was presented as mg of gallic acid equivalent per gram of ginger.

2. 8. Radical Scavenging Assay by DPPH Method Radical scavenging activity (RSA) of the extracts was measured using 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical. The sample was obtained from enzyme-assisted SWE was used in this analysis. 5 ml methanolic solution of DPPH with concentration of 0.1 mM was added to test tubes containing 1 ml various concentrations of the extracts. To prepare blank solution, 1ml of methanol was used instead of the extract. The mixtures were shaken well and incubated at room temperature in dark for 1 h. The absorbance was determined at 517 nm against the absorbance of methanol. % RSA was calculated as follows:

$$\%RSA=(1-\frac{\text{sample absorbance}}{\text{blank absorbance}})\times 100 \quad (7)$$

2. 9. Scanning Electron Microscopy (SEM) To study the morphology of ginger powder, enzyme and ultrasonic pretreated ginger and the subcritical water extracted one, SEM imaging was accomplished. The samples were freeze dried before analysis. For SEM analysis, VEGA\\TESCAN-XMU electron microscope was employed at 15 KV. The samples were coated with thin layer of gold before analysis.

3. RESULTS AND DISCUSSION

Figure 3 represents the chromatogram of NVA and the ginger extract. It is obvious that the external standard and the extracted 6-gingerol exhibit similar peaks at almost the same retention time. The peaks related to other bioactive compounds were determined according to ISO13685 protocol.

3. 1. Effect of Adding Co-solvents on Extraction yield Addition of some co-solvents to water can change its physicochemical properties. They can improve the solubility of compounds and can modify the interactions of water and solutes. Figure 4 and 5 shows the effect of addition of 2% ethanol and 2% acetone in water. The results indicated that addition of these co-solvents led to increase in mass of extraction and total gingerols and shogaol concentrations. As observed in the Figure 4, addition of ethanol had desired effect in compare to acetone. Total gingerols and shogaol concentrations significantly increased from 535.32 to 1081 µg bioactive/g dried ginger in presence of ethanol.

3. 2. Effect of Temperature on Extraction Yield Temperature is the most important factor affecting on the yield of SWE. As shown in Figure 6, with raising the temperature, the total mass of extract increased. Figure 7 reveals that temperature has significant effect on the bioactive compounds extraction. Increasing the temperature from 110 to 130°C led to enhance the yield of gingerols and shogaol extraction. This enhancement was rapid from 120 to 130 °C. Kinetic of extraction is modified at high temperature due to alteration of some properties of water. An elevated temperature results in reducing viscosity and surface tension of water.

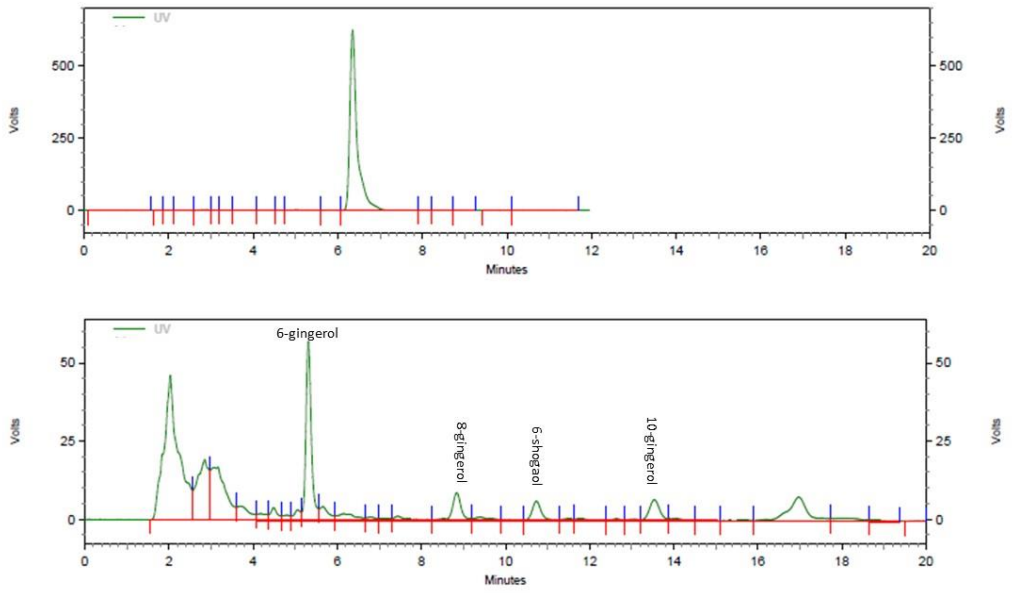

Figure 3. The HPLC profile (a) chromatogram of the analysis of NVA (b) typical chromatogram of ginger extract

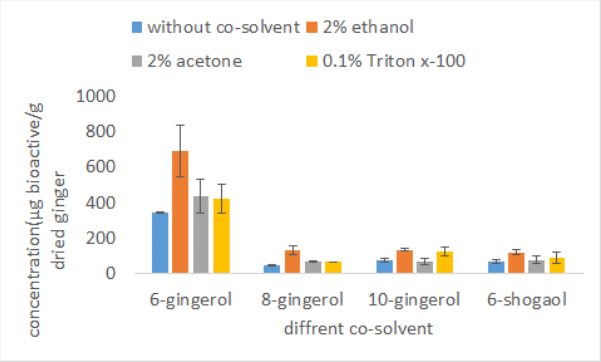

Figure 1. Effect of addition of co-solvents on ginger bioactive compounds concentration in SWE at constant temperature of 130°C, constant time of 20 min, constant pressure of 20bars and constant particle size of 0.71 mm

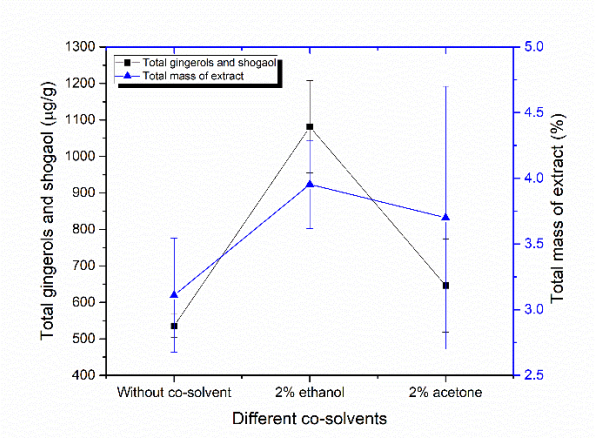

Figure 2. Concentration profiles of total gingerols and shogaol obtained from SWE at constant temperature of 130°C, constant time of 20 min, constant pressure of 20bars and constant particle size of 0.71 mm

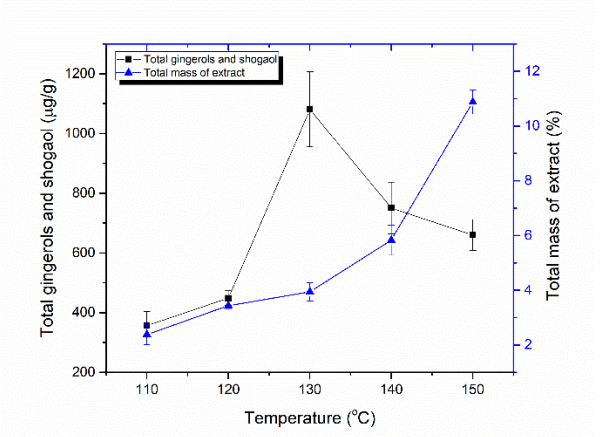

Figure 6. Concentration profiles of total gingerols and shogaol obtained from SWE with 2% ethanol at constant time of 20 min, constant pressure of 20bars and constant particle size of 0.71 mm

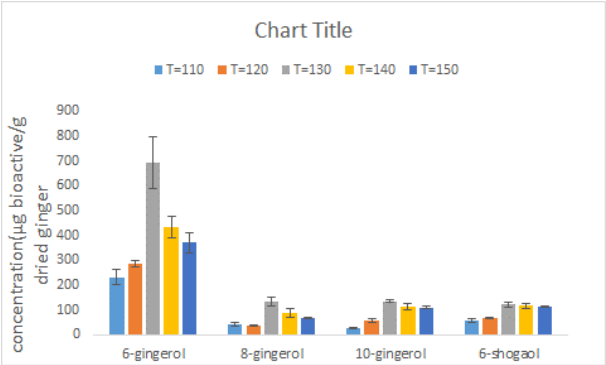

Figure 7. Effect of temperature on the ginger bioactive compounds concentration in SWE with 2% ethanol at constant time of 20 min, constant pressure of 20bars and constant particle size of 0.71 mm

The intramolecular bonds such as van der Waals, hydrogen bonds and dipole forces is broken down, so the mass transfer kinetics is developed. Anyhow the dielectric constant reduction is the most important factor affecting on elevated temperature that causes the water to behave like organic solvents. At temperature above 130°C, the concentration of total gingerols and shogaol slightly decreased while the total mass of extract was still increasing. Declining the bioactive compounds concentration is due to degradation of organic molecular structure caused by high temperature. High temperature results in low selectivity of the process because of extraction of undesirable compounds. Other chemical reactions such as oxidation, maillard and caramelization could occurred at elevated temperature. Therefore, increasing total mass of extract and decreasing concentration of total gingerols and shogaol are related to declining selectivity of the extraction. Among extracted bioactive compounds, concentration of 10-gingerol and 6-shogaol was not changed significantly. This is due to the fact that these compounds are less polar in compare to others. So water at this condition, that have lower dielectric constant, is more appropriate for extraction of these compounds. The highest yield of total gingerols and shoagol extraction was obtained at optimum temperature of 130°C.

3. 3. Effect of Retention time on Extraction Yield

SWE was performed with 2% ethanol, at 130°C, with constant pressure of 20 bars and particle size of 0.71 mm. The ginger was extracted for 10, 20, 30 and 40 min. Total mass of extract increased with longer extraction time is shown in Figure 8. Figure 9 shows that the concentration of gingerols and shogaol significantly increased from 10 to 30 min extraction time. But a slight decrease in concentration was observed for longer extraction time. This may be resulted from the molecular instability of the bioactive compounds at high temperature.

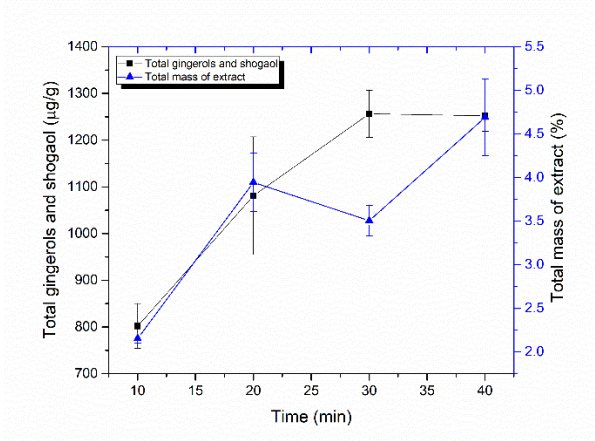

Figure 8. Concentration profiles of total gingerols and shogaol obtained from SWE with 2% ethanol at constant temperature of 130°C, constant pressure of 20bars and constant particle size of 0.71 mm

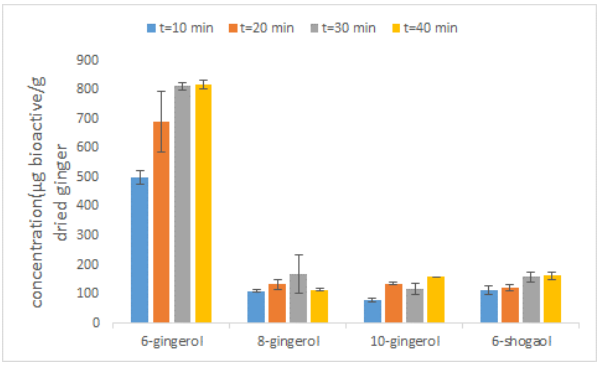

Figure 9. Effect of extraction time on the ginger bioactive compounds concentration in SWE with 2% ethanol at constant temperature of 130°C, constant pressure of 20bars and constant particle size of 0.71 mm

Concentration of total gingerols and shoagol has reached to 1256 µg bioactive/g dried ginger at optimum extraction time of 30 min.

3. 4. Effect of Particle Size on Extraction Yield

Mass transfer phenomena was facilitated using samples with small particle size due to increasing surface area. However, too much small particle size can cause channeling effects. As shown in Figures 10 and 11, using samples with size of 1mm gave a higher yield of extraction. At this condition, concentration of total gingerols and shogaol was 1668µg bioactive/g dried ginger.

3. 5. Effect of Enzyme and Ultrasonic Pretreatment on Extraction Yield

Figure 12 shows the influence of enzyme and ultrasonic pretreatment on yield of SWE. The both pretreatments resulted in higher extraction yield.

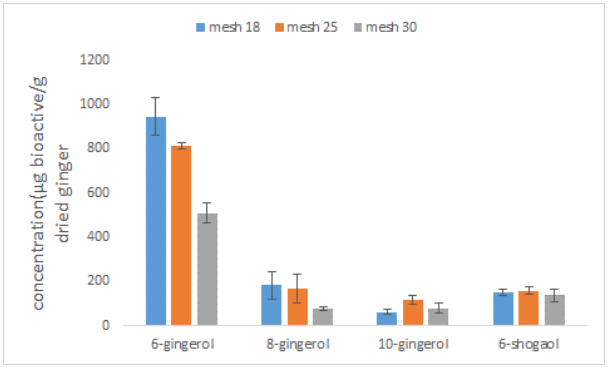

Figure 10. Effect of particle size on the ginger bioactive compounds concentration in SWE with 2% ethanol at constant temperature of 130°C, constant time of 20 min and constant pressure of 20bars

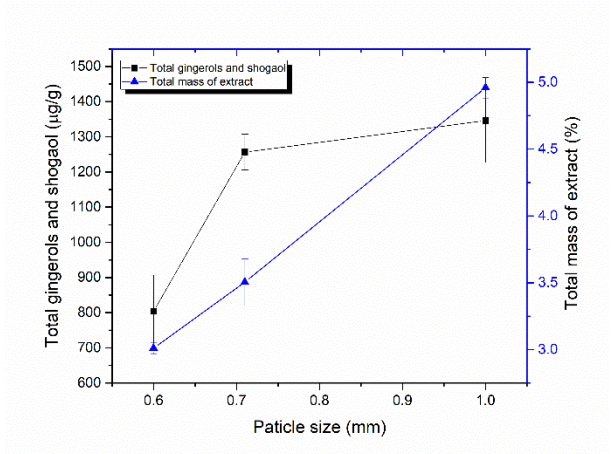

Figure 11. Concentration profiles of total gingerols and shogaol obtained from SWE with 2% ethanol at constant temperature of 130°C, constant time of 20 min and constant pressure of 20bars

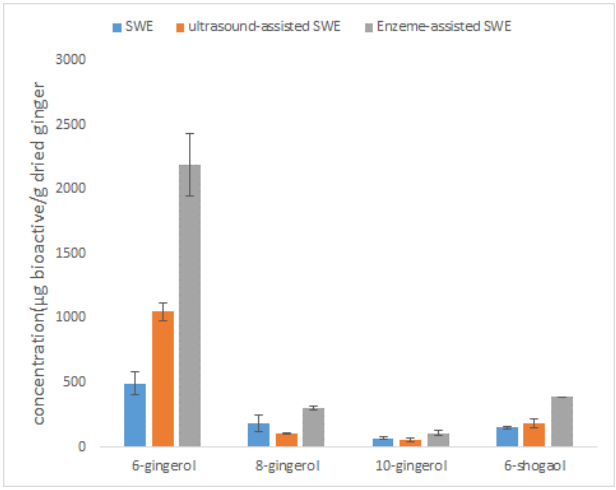

Figure 12. Effect of different pretreatments on the ginger bioactive compounds concentration in SWE with 2% ethanol at constant time of 20 min, constant pressure of 20bars and constant particle size of 1mm

This is because of weakening the intramolecular bonds and facilitating the extraction process. Certainly enzyme-assisted SWE showed much better results. Enzyme could break down the cell wall and hydrolyze the polysaccharide structure, so the subcritical water could easily penetrate into the core of ginger particles. As demonstrates in Figure 13, the concentration of total gingerols and shogaol reached to 2990.5 and 1393/5µg bioactive/g dried ginger in enzyme-assisted and ultrasonic-assisted SWE, respectively.

3. 6. Comparison of Extraction Yield in Different Methods Table 1 presents the yields of extraction by maceration, soxhlet, SWE, ultrasonic-assisted SWE and enzyme-assisted SWE. The operating conditions of these methods were compared and summary is presented in Table 1. The remarkable advantages of SWE are the lack of use of organic solvents and short operation time compared with conventional extractions. The recovery percentage of bioactive compounds in SWE based on soxhlet extraction was 16%. This value has reached to 35.8% in enzyme-assisted SWE.

3. 7. Total Polyphenol Content Table 2 shows total polyphenol content of the extracts obtained from various methods. Results indicate that the extraction of polyphenols in enzyme-assisted SWE had significantly increased compared to SWE.

3. 8. Radical Scavenging Activity of Ginger Extracts DPPH radical scavenging activity was measured for ginger extract obtained from enzyme-assisted SWE. Figure 14 presents the antioxidant activity of the various concentration of ginger extract that compared with standard antioxidant ascorbic acid. The EC50 value was 374.27 ppm. This value represents the concentration of the extract in which 50% of DPPH free radicals were inhibited. The antioxidant activity of ginger extract is owing to the presence of phenolic compounds including gingerols and shogaols.

3. 9. SEM Analysis In order to analyze the extraction process and to appraise the effect of pretreatment on morphology of ginger, SEM analysis of ginger sample was performed. This study was carried out on ginger samples before extraction, ultrasonic pretreated ginger, enzyme pretreated ginger, the ginger effluent after ultrasonic-assisted SWE and the one after enzyme-assisted SWE. Figure 15 shows a sample without porosity surface of ginger before extraction. As shown in Figure 16 and 17, ultrasonic waves were able to partly reduce the hardness of gingers surface and SWE made the ginger a little looser than before.

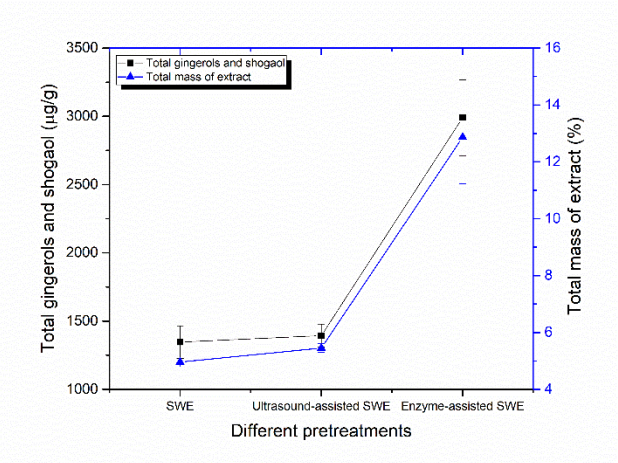

Figure 13. Concentration profiles of total gingerols and shogaol obtained from SWE with 2% ethanol at constant time of 20 min, constant pressure of 20bars and constant particle size of 1mm

TABLE 1. Total polyphenol content of extract obtained from different methods

Extraction	Total polyphenols contents GAE(mg/g)
soxhlet	11.0403
SWE	1.895
Ultrasonic-assisted SWE	2.87
Enzyme-assisted SWE	5.325

TABLE 2. Comparison of extraction yield by different extraction methods

Extraction method	Concentration of total gingerols and shogaol (µg bioactive/g dried ginger)	Operation time(min)	Organic Solvent consumption	Recovery of bioactives (bioactive concentration/ concentration using soxhlet) (%)
Meceration	7084	14 h	100ml methanol/1 g ginger	85
Soxhlet	8335	8 h	150 ml ethanol / 1 g ginger	100
SWE	1346	0.5 h	0.5 ml ethanol/1 g ginger	16
Ultrasonic-assisted SWE	1393.5	0.5 h+0.5 h=1h	0.5 ml ethanol/1 g ginger	16.7
Enzyme-assisted SWE	2990.5	1 h+0.5 h=1.5 h	0.5 ml ethanol/1 g ginger	35.8

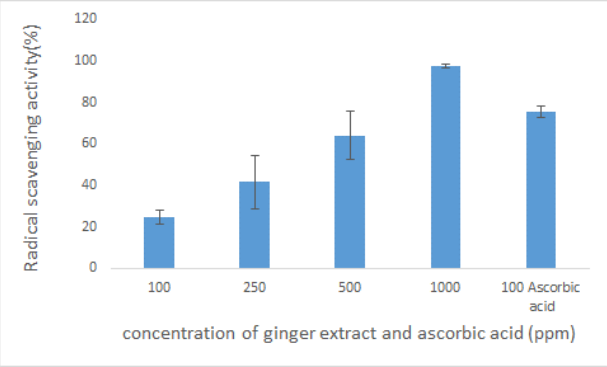

Figure 14. Radical scavenging activity of ascorbic acid and ginger extract obtained from enzyme-assisted SWE

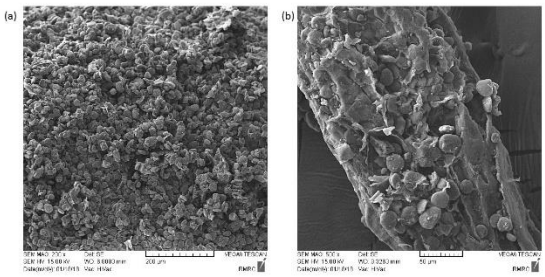

Figure 15. The SEM images of ginger before extraction (a) magnitude of 200 x (b) magnitude of 500 x

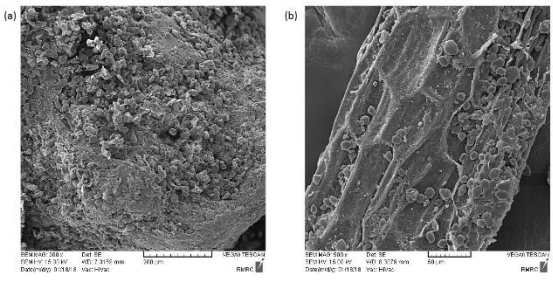

Figure 16. The SEM images of ultrasonic pretreated ginger (a) magnitude of 200 x (b) magnitude of 500 x

Therefore, the low yield of ultrasonic-assisted SWE is justifiable by these images. Figure 18, demonstrate the effect of enzyme pretreatment and SWE which was done after that, on ginger. Rupturing the cell wall is visible in Figure 18. This phenomena can facilitate SWE. The hot water could diffuse to the core of material easier and the compounds could be extracted from the porous surface. The loose structure of ginger with effluent is shown in Figure 19. This image illustrates that the extraction was well accomplished.

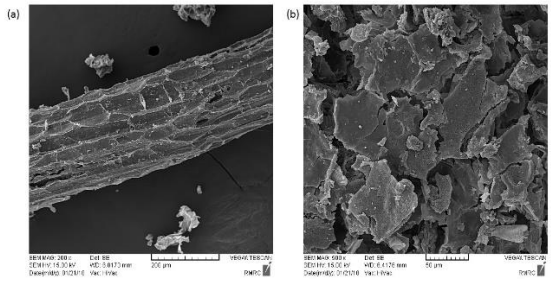

Figure 17. The SEM images of the ginger effluent after ultrasonic-assisted SWE (a) magnitude of 200 x (b) magnitude of 500 x

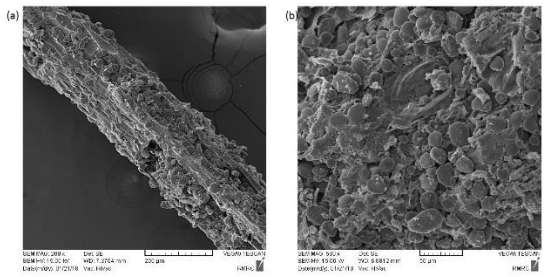

Figure 18. The SEM images of enzyme pretreated ginger (a) magnitude of 200 x (b) magnitude of 500 x

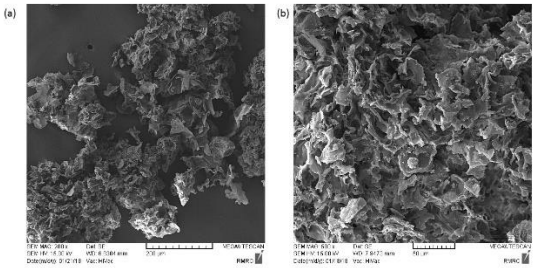

Figure 19. The SEM images of the ginger effluent after enzyme-assisted SWE (a) magnitude of 200 x (b) magnitude of 500 x

4. CONCLUISION

The SWE experimental set up was used in this study, to extract the bioactive compounds from ginger. Using water as a suitable, nontoxic and green solvent, instead of organic solvents; that is an important advantage for the developed extraction method. Also, the short extraction time is a significant superiority over conventional extractions. In this study, the effect of

adding co-solvents, temperature, time and particle size on extraction yield was investigated. Also the influence of ultrasonic and enzyme pretreatment was studied. Using 2% ethanol as co-solvent results in higher yield of extraction. The optimum temperature and retention time was obtained at 130°C and 30 min, respectively. Also, the best particle size was 1mm. enzyme pretreatment led to significant increase in yield. At the optimum condition the concentration of total gingerols and shogaol was 2990.5 5 µg bioactive/g dried ginger. The obtained extract from enzyme-assisted SWE had shown good antioxidant activity. SEM analysis represent the changes in the morphology of ginger before and after the extraction process.

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Subcritical Water Extraction of Bioactive Compounds from Ginger (*Zingiber officinale* Roscoe)

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چکیده

زنجبیل از ادویه جات پرکاربردی است که خواص دارویی مختلفی از خود نشان می‌دهد. این خواص دارویی، عمدتا به ترکیبات جینجرول و شوگول موجود در آن مربوط می‌شود. در پژوهش پیش رو ، از آب زیر بحرانی به عنوان یک حلال سبز جهت استخراج این ترکیبات استفاده شد. اثر افزودن کمک حلال، دما، زمان ماند و سایز ذرات بر بازده استخراج مورد مطالعه قرار گرفت و تاثیر پیش تیمار آلتراسونیک و آنزیمی نیز بررسی شد. استخراج همراه با پیش تیمار آنزیمی، با افزودن ۲٪ اتانول به عنوان کمک حلال، در دمای ۱۳۰ درجه سانتی گراد،فشار ۲۰ بار و زمان ماند ۳۰ دقیقه روی زنجبیل با سایز ذرات ۱ میلی متر، به عنوان شرایط بهینه انتخاب شد. در این شرایط مجموع ترکیبات فنولی و مجموع جینجرول و شوگول استخراج شده به ترتیب به میزان ۵۳۲۵ میکروگرم گالیک اسید/ گرم زنجبیل و ۲۹۹۰/۵ میکروگرم /گرم زنجبیل حاصل گشت. پیش تیمار زنجبیل با آنزیم آلفا آمیلاز قبل از استخراج توسط آب زیربحرانی سبب افزایش ۲/۸برابری مجموع ترکیبات فنولی و ۲/۲ برابری مجموع جینجرول و شوگول استخراجی شد. آنالیز SEM جهت بررسی اثر پیش تیمار بر مورفولوژی سطح زنجبیل و تحلیل فرآیند استخراج انجام شد.

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