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Total Phenol, Flavanoid and Antioxidant Activity of *Physalis angulata* Leaves Extract by Subcritical Water Extraction

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Abstract

Physalis angulata, having familiar name in Indonesia as "*Ceplukan*", is wellknown empirically in folk medicine to treat several diseases such as hepatitis, malaria, boil, liver problem, diuretic etc. Clinically several researchers have revealed the activity of *Physalis angulata* extract as anticancer, antitumor, antimycobacterial, immunosuppresion etc. So far, the common method to obtain *Physalis angulata* extract is by hot water extraction (HWE) and maceration using organic solvent such as methanol or ethanol. Meanwhile, stricker regulation of organic solvent residue to the pharmaceutical product encourages the research to replace organic solvent by environmentally benign solvent. The objective of this research is to investigate the potential of Physalis angulata leaves extract obtained by Subcritical Water Extraction (SWE) method as antioxidant

The *Physalis angulata* leaves were extracted by water in subcritical condition. Water in this condition may have polarity similar with organic solvent, so it can extract the phytochemical in plant material. Three variables were investigated including pressure (100-200 bar), temperature (100-250^{o}C) and extraction time (15-45 min). After evaporating the water, the extracts were analyzed for antioxidant activity, total phenol and flavanoid using spectrophotometer. Water content in extract was analyzed by karl fischer titrator. The result revealed that pressure has negligible effect, while temperature has significant effect to the antioxidant activity, total phenol and flavanoid content. The results also compared with that of obtained by conventional methods such as maceration (water and ethanol), HWE and soxhlet.

Keywords: *Physalis angulata* leaves, subcritical water extraction, antioxidant, total phenol, total flavonoid

1. Introduction

Physalis angulata grows well in tropical regions such as the Pacific America, Australia, and Asia, including Indonesia. *Physalis angulata* or ceplukan grows wild in the vacant lot, yard, and other places that are not water logged. It is one of nature product which can be used for traditional medicine. Overall, *Physalis angulata* plants (roots, flowers, leaves, leaf and stem) are used as herbs (drugs). For instance, traditionally, it is used for asthma medication (Shu-jing, et al., 2003; Chang, et al., 2008), hepatitis (Chang et al., 2008; Abdia 2011), malaria and dermatitis (Chang et al., 2008). In addition, it can be used for modern medicine because of its properties as antihiperglikemi, antiviral, anti-inflammatory, antimicrobial, antiseptic, diuretic, expectorant, febrifuge, antinociceptive (Susanto, 2009; Budi, 2008; Bastos et al., 2006). However, there are limited research about the antioxidant potential of *Physalis angulata.* Antioxidant itself is the chemical compounds which can prevent oxidation inside body. Free radicals come into the body, can cause oxidation process and weaken the immune system. Antioxidant intake can neutralize that free radical and prevent damage or death of the cells. In terms of chemical, antioxidant compounds are electron donating compounds (electron donors). Biologically, the antioxidant compound is a compound that can counter actor reduce the negative impact of oxidants in the body. Antioxidants work by donating an electron to the oxidant compounds or free radicals so that the activity of oxidant compounds that can be inhibited (Winarsi, 2007). Electrons from free radicals unpaired electron is very easy to draw from other molecules, so that the free radicals become more reactive and easier to attack healthy cells in the body. Most of phytochemical in plants work as an antioxidant. The leaves part of *Physalis angulata* contains phytochemicals such as flavonoid, alkaloid, saponnin, tannin, steroid (Nnamani et al., 2009; Rengifo-Salgado & Vargas-Arana, 2013). Composition of Physalis angulata leaves obtained from phytochemical

screening were reported by (Nnamani et al., 2009) and are shown in table 1 while the bioactive constituents from Physalis angulata leaves are summarized by (Rengifo-Salgado & Vargas-Arana, 2013). Those bioactives are Physalin, Withangulatin, Physagulin, Oleanolic acid, Myricetin 3-O-neohesperidoside. By looking these opportunity, the needs of knowledge for optimizing treatment condition are needed so *Physalis angulata* can be used widely.

Table 1. Percentage composition of phytochemicals in leaf of *Physalis angulata* (Nnamani et al., 2009)

Phytochemicals (%)	Leafy
Alkaloid	2.0
Cyanide	0.39
Flavonoid	15.5
Phytate	0.02
Saponnin	2.0
Tannin	0.05

The solvents commonly used to extract *Physalis angulata* are typical organic solvents such as methanol, ethanol, and hexane (Manggau et al., 2010). Organic solvents are well known because of its toxicity, carcinogenic and environment hazards (Queensland Goverment, 2014). Because of some physical or chemical barriers, it can not be removed completely from the product by separation such as drying or evaporation. Eventhough some organic solvents are allowed of daily exposures of around 50 mg/day and less, the possibility of the residual solvents in acceptable limit may induce phase transformation and jeopardize the psicoxhemical stability of an active substance and finally the quality of the dossage form may exist, for instance the effect of methylene chloride on the crystallinity of ampicillin tryhydrate and the redsidual ethanol on phase transformation of orthorhombic paracetamol (Grodowska & Parczewski, 2010; Nojavan et al., 2005; Al-Zoubi et al., 2002). Therefore, pharmaceutical companies constantly attempt to eliminate the usage of organic solvents with those more environmentally friendly such as water, supercritical CO_2, ionic liquids etc as a potential of reaction media.

In this report, subcritical water was used as a solvent. Water in room condition behaves as very polar solvent, however its polarity can be adjusted to be similar with organic solvent by arranging the temperature and pressure. The condition where water temperature is above the boiling point of water and below its critical temperature under pressure to maintain its liquid state is called subcritical water (Nakajima, 2013). Subcritic water (near critical water) is chosen as solvent in this experiment because water is abundant, non-toxic and environmental friendly with adjustable polarity. The development of subcritical water as a new solvent need to be deepened so can be used commercially in Indonesia.

The objective of this research is to investigate the potential of *Physalis angulata* leaf extract obtained by Subcritical Water Extraction (SWE) method as antioxidant. The variables studied are temperatures (100, 150, 200, and 250^{o}C), pressure (100 and 200 bar), and extraction time (15, 30, and 45 minutes).

2. Method

The research method is divided into 5 stages : collection and identification test plant, pretreatment of *Physalis angulata* leaf, extraction of *Physalis angulata* leaf with subcritical water (main experiment) as a comparison with conventional extraction (maceration, Soxhlet, and hot water extraction (HWE)), separation of the solvent from the extraction, and analysis of the resulting extract.

2.1 Pretreatment

Physalis angulata plants were collected and then identified at the Faculty of Pharmacy, University of Gadjah Mada. After collection, leaves of *Physalis angulata* were washed and dried in tray dryer for 24 hours. The dry leaves were ground and clasified based on the size using screen. In this experiment, the size of -20+30 mesh were used. The water content was analyzed using moisture analyzer (Mettler Toledo, HR83, USA)

2.2 Subcritical Water Extraction

The extractions were conducted in Subcritical Water Extraction batch apparatus (Hanyang Accuracy, Korea). The batch reactor system has volume of 150 mL, equipped with electric heating for heating the reaction to the desired temperature and cooling jacket for decreasing the reactor temperature once the reaction finished. The temperature and pressure of the system was controlled using control box. Ten gram of the sample was put in the reactor basket. The reactor then was closed and purged with nitrogen for about 15 minutes to remove the oxygen

in the reactor and lines. The distilled and deionized water (DDI) water was also purged to remove the dissolved oxygen. Then, DDI water was delivered to the reactor using high pressure metering pump (HKS-600, Hanyang Accuracy, Korea) and brought to the desired pressure. The solution then was heated to the desired extraction temperature and keep for specific time, called as static time. After the time fulfilled, the reactor then was cooled by opening the insulation and flowing cooling water. The extract then was then filltered out with fine grade filter paper (No 393, Sartorius, Germany) to make sure no residue in the extract. For further analysis, water was evaporated from the extract using rotavapor (RE-3000A, Xi an HEB Biotechnology Co. Ltd, Shanghai, China) and the water content was checked by Karl Fischer analyzer (Mettler Toledo, Switzerland). The dry extracts were stored at refrigerator until use.

2.2.1 Maceration

Ten gram of sample was dissolved in 300 mL of solvent (methanol, or DDI water). The extraction is conducted at 25^{o}C with stirring. Maceration is conducted for 24 hr without renew the solvent. The solvent was then evaporated from the extract for then analyzed.

2.2.2 Hot Water Extraction

Ten gram of sample were extracted by 240 mL of boiled DDI Water for 15 min. The extract was filtered with filter paper to separate the residue which may escape from the basket. The filtrate wass concentrated and analyzed.

2.2.3 Soxhlet Extraction

Soxhlet extractions were carried out by placing ground of samples (10 gram) in a Sartorius filter paper. The filter paper containing sample was wrapped and placed in a thimble. The thimble was then placed in a Soxhlet extraction apparatus. Approximately 300 ml of extraction solvent (methanol and water) was added into the Soxhlet apparatus. All samples were extracted with hot boiling solvent mixture until the solvent color in the timble clear.

2.3 Analysis

All analysis use colourimetric method with spectrophotometer (SP-2000UV, China).

2.3.1 DPPH Assays

The stock DPPH solution was prepared by dissolving 19.7 mg DPPH in 250 ml methanol p.a. (0.2 mM). The solution was obtained by diluting 40 mg dry extracts with 100 ml methanol p.a (400 ppm). The different concentration of solutions (20-400 ppm) were made by diluting the 400 ppm extracts solution with methanol. 2 ml of this solution was allowed to react with 2 ml of the DPPH solution for 24 h. Then the absorbance was measured at 517 nm.

2.3.2 Total Phenols Analysis

The total phenols of the extracts was analyzed by Follin-Ciocalteu methods. Accurately, 0.5 ml extracts 500 ppm (5 mg in 10 ml DDI water) were mixed with 2.5 mg Follin Ciocalteu reagent (1:10) and 2 ml of Na_2CO_3 solution (75 g/l). The mixture solution incubated at 40^{o}C for 30 minutes, and the absorbance measured at 735 nm.

2.3.3 Total Flavonoids Analysis

The total flavanoid content of extract ws analyzed using calorimetric method. As much as 0.25 ml extracts (100 mg in 10 ml DDI water) were mixed with 1.25 ml DDI water and 0.075 ml $NaNO_2$ 5%. After 5 minutes 0.15 ml $AlCl_3$ 10% added. After 6 minutes 0.5 NaOH 4% added. Immediately after water was added to the mixture to bring the final volume of 4 ml. The mixture was thoroughly mixed and allowed to stand for another 15 minutes. The absorbance measured at 510 nm.

2.3.4 DPPH Using Trolox Standard

The stock DPPH solution was prepared by dissolving 19.7 mg DPPH in 250 ml methanol p.a. (0.2 mM). 2 ml extract (0.6 mg extract in 10 ml DDI water) were mixed with 2 ml. The absorbance measured at 517 nm. Standard curve was made by diluting 40 ppm trolox in methanol into different concentrations (2-26 ppm).

2.3.5 FRAP

FRAP reagent was prepared by mixing 2.5 ml of TPTZ (10 mMTPTZ in 40 mMHCl) with 2.5 ml of $FeCl_3.6H_2O$ 20 mM, and 25 ml of acetate buffer 0.3 M (pH 3.6) and were incubated at 37^{o}C for 5 min. 1.4 ml extract (100 mg in 10 ml methanol) were mixed with 4.2 ml FRAP reagent, then incubated for 30 min. The absorbance measured at 593 nm.

3. Results and Discussion

3.1 Yield

Yield is defined as the weight of dry extract obtained devided by the weight of dry feed (dry leaves). Figure 1 shows yields as a function of temperature, pressure and extraction time. In this research, it can be seen that the *Physalis angulata* leaves extract reaches the highest yield at 200°C and yield increases along an increase of temperature. Temperature can change properties of water like density, viscosity, and surface tension. The higher the temperatur, the space between molecules will increase tenuously. Therefore viscosity will be decrasing due to decreasing of cohesion and density (density is decreasing due to increasing of volume). A decrease of cohesion causes a decrease of surface tension. Those properties cause an increase in diffusivity and the rate of mass transfer (Vargaftic et al., 1983). This is the reason why increasing of temperature shows increasing the yield significantly, especially at short extraction time. In addition, an increase of temperature increases the solubility of solute (Eikani et al., 2007; Ozel et al., 2002). However, an increase of extraction temperature to 250°C leads to a decrease of extract yield. This is due to pore collapse which cause effective diffusivity decreases. The rate of mass transfer at 250°C is affected by internal diffusion while at below 250°C is affected by external diffusion. Pressure makes matrix pore swell and pushes solvent to contact with solute (Ramosa et al., 2002; Richter et al., 1996). On the other hand, an increase of pressure from 100 bar to 200 bar does not contribute to the extract yield significantly. It is because pressure does not change properties of solvent such as dielectric constant as shown in table 2. While extension in extraction time leads to a decrease in extraction yield. It may happen due to solid matrix exposed with high temperature for a long time. The matrix collapse and reduce the contact area (Richter et al., 1996; Olson et al., 1995).

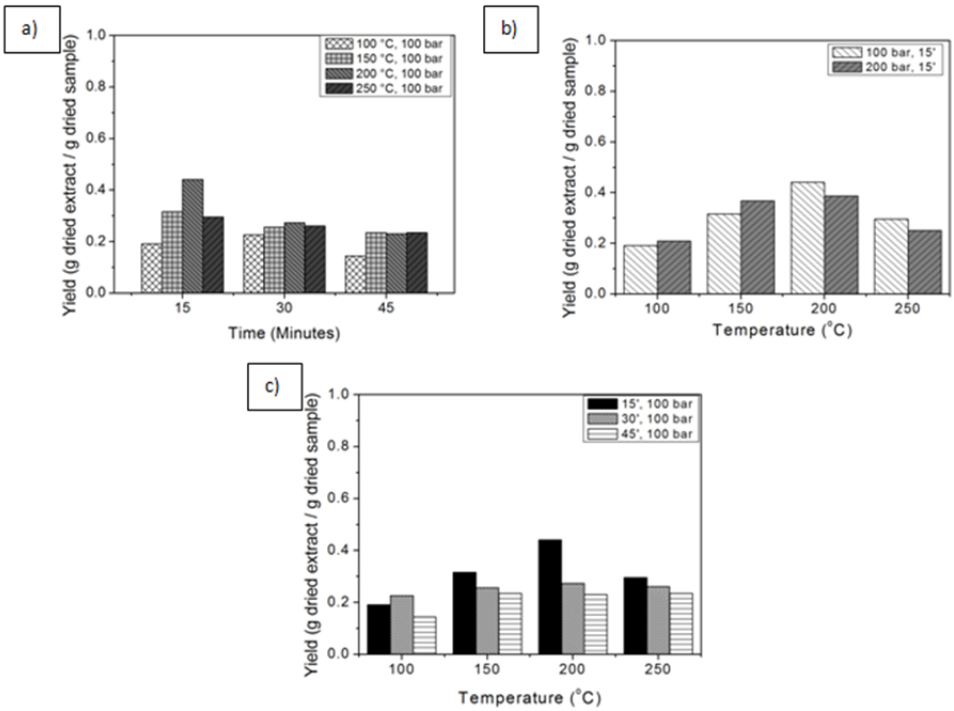


Figure 1. Yield of extract as a function of (a) temperature and extraction time, studied at a fixed pressure of 100 bar (b) temperature and pressure at a fixed extraction time of 15 min (c) extraction time and temperature at a fixed pressure of 100 bar

Table 2. Dielectric Constant (Heger, 1969)

			Temperature (°C)			
			100	150	200	250
Subcritics water	Pressure (bar)	100	55.6	44.3	34.8	27.0
		200	56.0	44.6	35.2	27.7
Water at 20°C			80.1			

3.2 Phenol

Figure 2 shows the total phenol as a function of temperature, pressure and extraction time. An increase in reaction temperature shows positive effect to the total phenol in all ranges of extraction time studied. For instance at a fixed pressure of 100 bar and extraction time of 15 min, an increase in reaction temperature from 100^{o}C to 250^{o}C increase the total phenol significantly from 80.62 mg Gallic Acid (GA)/ mg extract to 113.18 mg GA/ mg extract. The highest total phenol obtained in this research is at 250^{o}C, 100 bar, and 15 minutes with value 113.18 mg GA/ mg extract. Phenol is semi-polar and becomes non-polar when its carbon chains increase (Seager & Slabaugh, 2010). On the other hand, as temperature increases water polarity decreasing, favourable for extraction of phenol. Figure 2b shows that an increase in pressure does not change total phenol significantly. It is because there is negligible change in the water polarity as pressure, shown by the dielectric constant value in table 2.

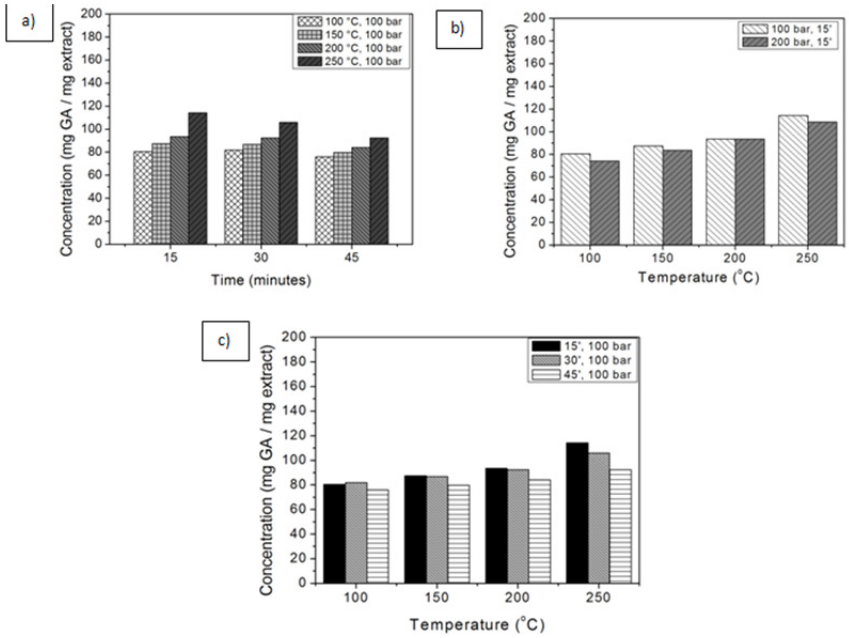
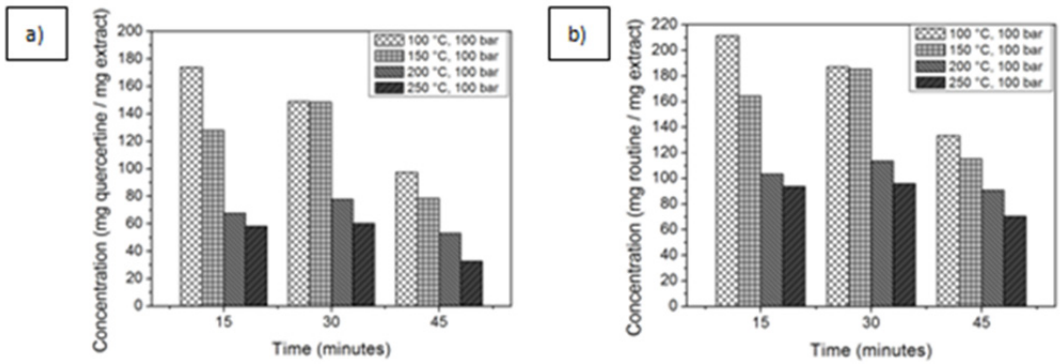


Figure 2. Total phenol of extract as a function of (a) temperature and extraction time, studied at a fixed pressure of 100 bar (b) temperature and pressure at a fixed extraction time of 15 min (c) extraction time and temperature at a fixed pressure of 100 bar

3.3 Flavonoid

Figure 3 shows the total flavanoid as a function of temperature, pressure and extraction time. It analyzed using two standards, quercetine and routine. The results are presented as mg quercetine or routine per gr of dry extract. Figure 3a and 3b show the temperature effect at different extraction time.



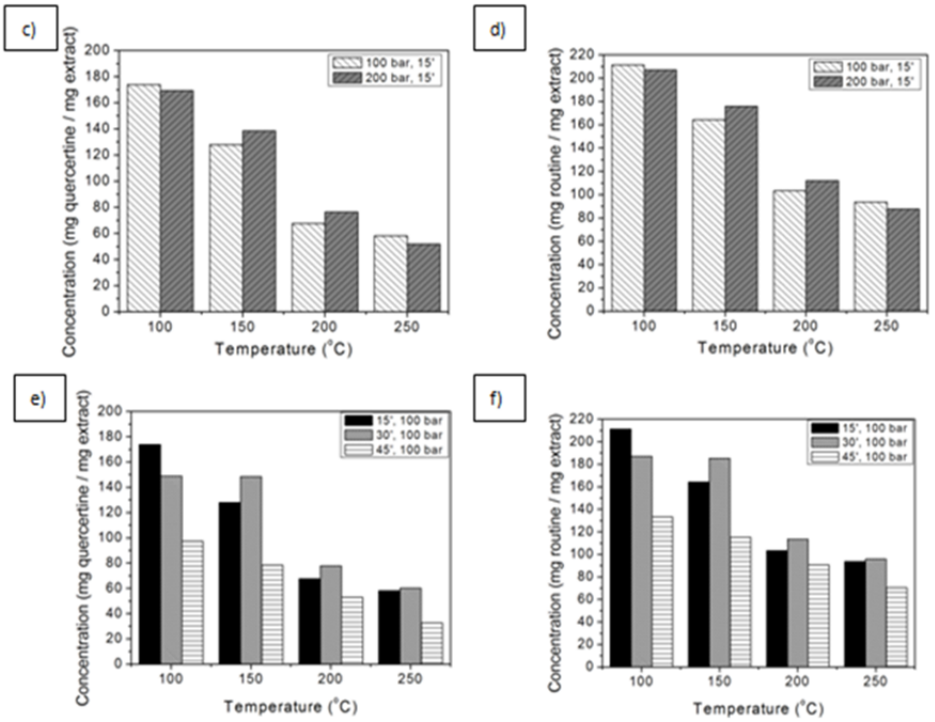


Figure 3. (a) Total flavonoid using quercertine standart of extract as a function of temperature and extraction time, studied at a fixed pressure of 100 bar (c) temperature and pressure at a fixed extraction time of 15 min (e) extraction time and temperature at a fixed pressure of 100 bar (b) Total flavonoid using routine standart of extract as a function of temperature and extraction time, studied at a fixed pressure of 100 bar (d) temperature and pressure at a fixed extraction time of 15 min (f) extraction time and temperature at a fixed pressure of 100 bar

At all extraction time, it shows that an increase in temperature leads to a decrease in total flavonoid. It might be because flavanoid has been degraded after 100°C (Rodriguez, 2013). Flavanoid could be degraded to quercetine-3-O-β-D-glukopiranosida (isoquersitrin), *quercetine* dan other polar compounds. (Kim, et.al, 1991) These polar compounds would be more difficult to dissolve in less polar solvent since the polarity of subcritical water dropped as temperature increase. Sharifi (Sharifi, et al., 2013) reported that total flavanoid was degraded at above 110°C at 10 min extraction time in the extraction of barberry by subcritical water extraction. However, fig 3b reveals the negligible change in total flavonoid as pressure increase from 100 bar to 200 bar which can be explained with negligible change in water polarity as pressure as shown by dielectric constant value in table 1. The effect of extraction time in different temperature concludes that the best extraction time is 30 min. Less than 30 min, flavanoid extraction does not complete yet, however longer time than 30 min leads to degradation of flavanoid due to exposure in high temperature for long time.

3.4 Antioxidant Activity

In this paper, antioxidant activity is reported from two different analysis methods, FRAP and DPPH. The difference, antioxidant activity test with DPPH is hydrogen transfer, while FRAP is ion reduction method. Figure 4 shows antioxidant activity analyzed by FRAP method and Figure 5 shows antioxidant activity analyzed by DPPH (IC 50) method. FRAP method used ion reduction from ferri (Fe^{3+}) become ferro (Fe^{2+}). FRAP calculation using trolox as its standard. The IC50 method shows the concentration of extract (ppm) needed to inhibit the DPPH radical by half. That means the smaller the IC50, the stronger antioxidant in the sample. Both calculations have the highest antioxidant activity at 200°C, 100 bar, and 30 minutes. At 250°C, antioxidant activity decrease may be caused by thermal degradation. Pressure gives uncertain trend in the antioxidant activity while time increase the antioxidant activity. Temperature shows increase ion reduction capacity.

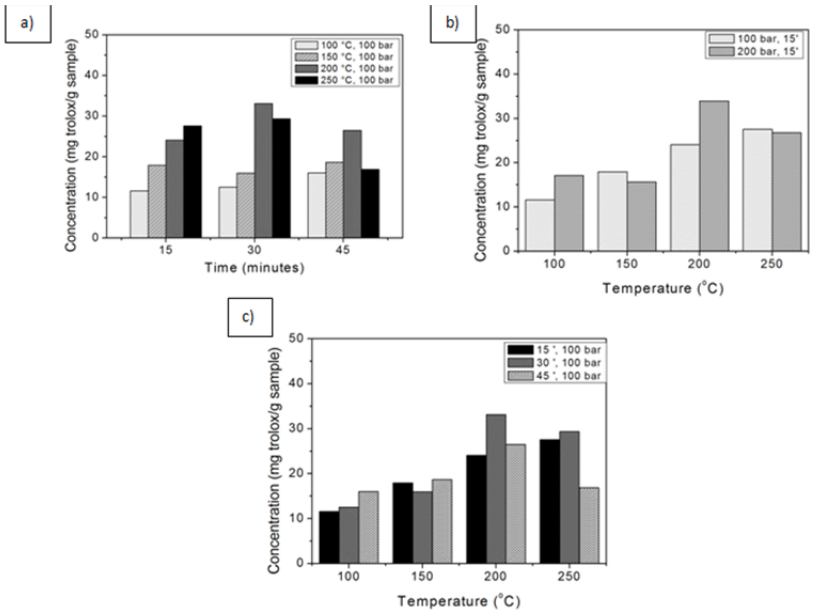


Figure 4. Antioxidant activity using FRAP method of extract as a function of (a) temperature and extraction time, studied at a fixed pressure of 100 bar (b) temperature and pressure at a fixed extraction time of 15 min (c) extraction time and temperature at a fixed pressure of 100 bar

3.5 Conventional Extraction (Comparison)

Conventional extractions, such as maceration (using water or ethanol), soxhlet, and HWE, are the common methods for extraction. In this research, using water maceration (MA), ethanol maceration (ME), soxhlet with water (SX1), soxhlet with ethanol (SX2), and HWE as comparison for SWE method. Tannin and saponnin are soluble in water, meanwhile flavonoid, alkaloid, and sterol soluble in ethanol (Cowan, 1999). DPPH trolox, FRAP, and flavonoid values are higher in ethanol (semi-polar) than water (polar) which shown by ethanol and water maceration. IC50 value also indicates ME is better than MA (Elsha, 2012). However, the values of yield, phenol, flavonoid, and antioxidant activity from SWE is higher than conventional method. Comparison between conventinal and SWE method are shown in Table 3.

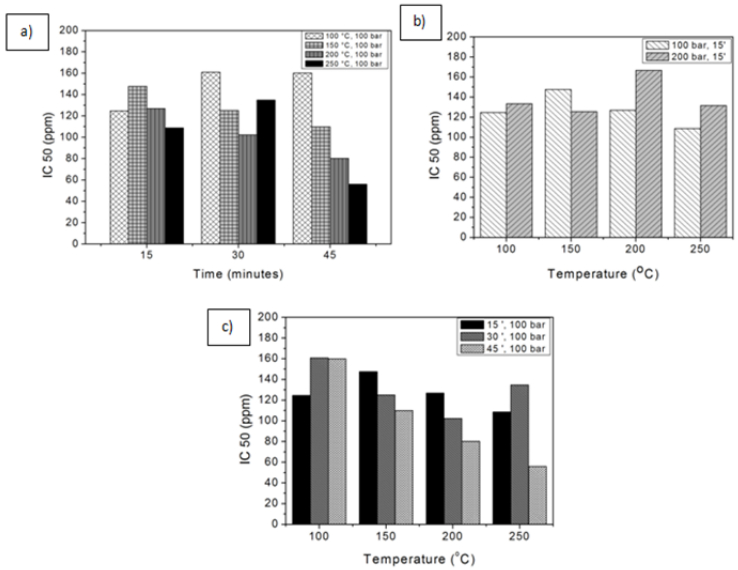


Figure 5. Antioxidant activity in the term of IC50 as a function of (a) temperature and extraction time, studied at a fixed pressure of 100 bar (b) temperature and pressure at a fixed extraction time of 15 min (c) extraction time and temperature at a fixed pressure of 100 bar using IC 50 method

Table 3. Conventional Extraction and the best result from SWE (Subcritical Water Extraction)

Sample	Condition	Yield	Phenol (mg GA/ g dried extract)	Flavonoid		DPPH IC 50	DDPH Trolox (mg Trolox/ g dried extract)	FRAP (mg Trolox/ g dried extract)
				Quercetin (mg Q/ g dried extract)	Routine (mg Routine/ g dried extract)			
MA leaf	Maceration Water, 24 hr	0.251	63.324	111.556	147.692	262.860	58.232	10.291
ME leaf	Maceration EtOH, 24 hr	0.107	41.235	117.731	153.716	180.486	84.371	86.383
L SX 1	Soxhlet Water, 54 hr	0.419	73.861	104.212	140.281	210.429	72.680	18.759
L SX 2	Soxhlet EtOH, 9 hr	0.343	62.070	208.431	244.487	118.022	129.409	64.237
L HWE	HWE, 15 min	0.286	58.944	101.804	137.902	143.785	106.239	15.476
	100 bar, 200°C, 15 min	**0.440**	93.552	67.772	103.393	105.621	142.860	29.006
L SWE	100 bar, 250°C, 15 min	0.296	**114.327**	58.252	93.892	124.038	121.591	23.410
	200 bar, 100°C, 15 min	0.209	74.281	**169.373**	**207.076**	133.348	119.770	16.526
	100 bar, 200°C, 30 min	0.273	92.486	77.820	113.629	**102.318**	**148.249**	**33.115**

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