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Extraction of *Zingiberaceae family Rhizomes (C. longa, C. xanthorrhiza* and *Z. officinale)* Using Subcritical Water Extraction and its Characterization Study

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Abstract: Extracting bioactive compounds from plant materials proves to be a challenging process. The demand for a non-toxic extraction, high bioavailability and stability urges for an efficient and greener extraction method. Therefore, in this study, *Curcuma longa, Curcuma xanthorrhiza* and *Zingiber officinale* were extracted using subcritical water extraction (SWE) as a new green extraction and separation technology.The extracts are characterized in terms of its physical, chemical and structural properties. Physical properties of the samples were studied through pH and colour analysis. As for chemical properties, three analyses were performed; Total phenolic content (TPC), Total anti-inflammatory activity (TAI) and Total antioxidant activity (TAA). Finally, for functional group analysis, the rhizome extracts were passed through FTIR. The bioactive compounds were successfully extracted using SWE for all three rhizomes (temperature = 100 °C; pressure = 11 bar; sample to solvent ratio = 1:3). The pH of the extracts were between 5.2 – 6.2 and the colour analysis indicated *C. longa* is the brightest yellow in comparison to the other two samples. Total phenolic contents of all 3 samples were highest at 10 min of extraction time with *C. longa* (0.402 mg GAE/g DW), *Z. officinale* (0.392 mg GAE/g DW), and *C. xanthorrhiza* (0.38 mg GAE/g DW). Meanwhile, the maximum total anti-inflammatory activities are achieved at min 5, 15 and 10 for *C. longa* (96.3%), *C. xanthorrhiza* (94.4%) and *Z. officinale* (81.5%) respectively. The total antioxidant activity activities were achieved at min 5, 20 and 10 for *C. longa* (89.4%), *C. xanthorrhiza* (79.3%), and *Z. officinale* (76%). It is hoped that the established green extraction method will spearhead the research, innovation and commercialization in green technology and wellness product developments in Malaysia.

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Keywords: *Curcuma longa*, *Curcuma xanthorrhiza*, *Zingiber officinale,* Subcritical Water Extraction (SWE)

1. Introduction

Malaysia has long benefitted from an abundance of natural resources of herbal and plant materials with reported potential health-benefits, such as from *Zingiberaceae* family members of *Curcuma longa*, *Curcuma xanthorrhiza*, and *Zingiber officinale*. Bioactive constituents from this plant could be extracted for consumption to promote wellbeing. These plants are anti-allergic, anti-cancer, anti-diabetic, anti-inflammatory, anti-venom, cardio-protective, digestive stimulant, hepatoprotective, hypolipidemic, and neuroprotective, among other therapeutic properties [1]. Almost all *Curcuma sp.* that have been studied so far have curcuminoids and volatile oils in their chemical characteristic [2]. 32 *Curcuma sp.* have so far yielded 719 components, including terpenoids, flavonoids, phenylpropene derivatives, alkaloids, diphenylalkanoids, steroids, and other compounds [3].

Extraction and isolation of target constituents is the most important step of the plant raw material processing [4]. In most industries, the conventional methods of extractions being used are maceration, reflux and Soxhlet extractions. These methods are easy to perform, need no special equipment, applicable for extraction of large number of samples and lead to high yield of the extract obtained. However, the main concerns of using these methods are large volume of organic solvents used, long processing time, high energy consumption and degradation of thermally instable [5].

In addition, the extracts require tedious purification steps to remove toxic organic solvents residues and often resulted in non-compliance against consumer regulatory body if the extracts are to be used as medicinal or food supplements. This situation triggers for the need of better extraction methods that will reduce energy consumption, allows the use of alternative solvents (water or agro-solvents), and ensure a safe and high-quality extract. These are the principles underlying "green extraction" method [6]. Driven by the green extraction conceptual objective, green extraction methods have been developed as a result of industrial challenges to be ecological, environmental and economic impacts. Subcritical water extraction (SWE) is one of the green extraction methods because it meets all requirements of the green extraction [7].

Therefore, this study evaluates the extraction of *Zingiberaceae* family members using subcritical water extraction (SWE) and its effect on the characteristics of the extracts. The findings is hoped to boost herbal industry in Malaysia via advancing green technology as well as enhancing wellbeing of the nation.

2. Materials and Methods

2.1 Materials

Curcuma longa (Kizaherbs, Pahang), *Curcuma xanthorrhiza* (Kizaherbs, Pahang), *Zingiber officinale* (Kizaherbs, Pahang), Folin-Ciocalteu reagent (R&M Chemicals, Malaysia), Sodium carbonate (Na_2CO_3) solution (ChemAR, Malaysia), Gallic acid solution (Bio Basic Canada Inc), DPPH reagent (Sigma Aldrich, Germany), L-ascorbic acid (Sigma Aldrich, Germany), Bovine Albumin (Sigma Aldrich, Germany).

2.2 Preparation of samples

The rhizomes were first inspected for any damages such as rotten or presence of pests. Next, the rhizomes were washed with distilled water and were trimmed off to remove all fine foreign materials. Then, the rhizomes were cut into small pieces. The rhizomes cubes were then weighted to specification (12 kg for each run). About 5 L of distilled water was added to the rhizomes. The rhizomes were blended

into fine course by using food blender. Water was added to the rhizomes due to assist extraction process. Without water, the sample will begin to clog in the pipes during the SWE process.

2.3 Extraction of *Curcuma sp.* using Subcritical Water Extraction (SWE)

The subcritical water extraction (SWE) is built from the prototype on a laboratory scale into a 70 L subcritical water extractor industry scale (AM Zaideen Sdn. Bhd.) (Figure 1). The sample after processing was weighed and embedded in the pressure vessel, and the ratio of sample to water as a solvent is 1:2 [8]. The pressure tank was then pressurized with air until 2.0 bar purged with N_2 gas to remove the dissolved oxygen. The temperature was fixed to remain at 100 °C, the pressure inside the pressure tank was increased to 11 bar and the ratio of sample to solvent was kept to 1:2. The only manipulated variable of the SWE process is the extraction time (duration of the sample to be in subcritical condition). The sample was left in subcritical condition for 5 min, 10 min, 15 min and 20 min. Once the extract was cooled down, the extracts were removed from the collection tank and stored in the freezer at 4℃ until further analysis.

Figure 1: The 70 L subcritical water extractor

2.4 pH analysis

The pH scale shows how many hydrogen ions develop in a specific quantity of water. The pH analysis was conducted by using a pH probe (HANNA Instruments). The probe was dipped into each plant extract to obtain the data. The probe was cleaned using distilled water and tissue. Samples of *C. longa, C. xanthorrhiza, and Z. officinale* pH were measured using the pH meter. The findings were recorded in triplicates. The data was tabulated and analysed.

2.5 Colour analysis

The sample colour was analysed using a Hunters Lab Colorimeter. The value was taken in the form of L*a*b. The following values were noted: L* (lightness), a* (redness and greenness), and b* (yellowness and blueness). L* stands for the lightness, which ranges from 0 (black) to 100 (white); a* denotes redness with a plus sign (+) and greenness with a minus sign (-); and b* denotes yellowness with a plus sign (+) and blueness with a minus sign (-) [9]. The findings were recorded in triplicates. The data was tabulated and analysed.

2.6 Total Phenolic Content

Total phenolic compound content was determined using the Folin-Ciocalteu method. To measure total phenolic content, gallic acid was used as a standard. The calibration curve was made using gallic acid with different concentration (100 – 500 µg/mL). 10 mg of gallic acid was diluted in 10 mL of 95%

ethanol until dissolved. The solution was then marked up with distilled water until the solution was 100 mL. To make 7.5% of Na_2CO_3, 7.5 g of sodium carbonate was diluted into 100 mL of distilled water. Finally, 10 mL of 2N Folin-Ciocalteu reagent was diluted in 90 mL distilled water to make 0.2 N Folin-Ciocalteu reagent.

A serial dilution was performed in which 1, 2, 3, 4 and 5 mL of gallic acid stock solution was taken and mixed with distilled water until the total solution was 5 mL in order to make various concentration of gallic acid (100, 200, 300, 400 and 500 µg/mL) respectively. 0.25 mL of Folin-Ciocalteu reagent was also added. Finally, 2.5 mL of 7.5% Na_2CO_3 was then added. The solution was then left to incubate in the dark for two hours. The absorbance for each concentration of gallic acid was using UV-Vis spectrophotometer at the wavelength of 765 nm. The mean of the absorbance was used to plot the calibration curve to determine the level of phenolic compound in the samples. Total phenolic content of the samples was expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g).

0.25 mL of plant extract was diluted with 2.25 mL of distilled water. 0.25 mL of Folin-Ciocalteu reagent was then added, continued with 2.5 mL of 7.5% Na_2CO_3 solution. Total phenolic content was expressed in mg which is equivalent to gallic acid per gram of extract. The concentration of phenol in the extracts were extrapolated for the calibration curve. The total phenolic content was then calculated using the formula:

$$C_1 = \frac{C_0 \times V}{m} \quad \text{Eq. 1}$$

Where C_1 is the total phenolic contents (mg GAE/g), C_0 is concentration of gallic acid obtained from the standard curve (mg/mL), V is volume of extract (mL) and m is mass of extract (g).

2.7 Total anti-inflammatory activity

With 0.1 M phosphate buffer saline (pH-6.4), the stock solution of test sample extracts and standard samples was pipetted in various test tubes. The reaction mixture (5 mL) was made up of 0.02 mL of extract, 4.78 mL of phosphate-buffered saline (PBS, pH 6.4), and 0.2 mL of 1 percent bovine albumin. The reaction mixture was combined, heated to 70 °C for 5 minutes, and then incubated for 15 minutes in a water bath (37°C). A UV-VIS spectrometer was used to quantify turbidity at 660 nm after cooling the reaction mixture. The percentage inhibition of protein denaturation was calculated utilizing the following equation:

$$Anti - denaturation\ activity\ (\%) = \frac{A_1 - A_0}{A_1} \times 100 \quad \text{Eq. 2}$$

Where, A_1 is the absorbance of the control and A_0 is the absorbance of the sample.

2.8 Total anti-oxidant activity

The DPPH free radical scavenging activity was used to assess the antioxidant activity. Ascorbic acid was used as a standard. 0.1 mM DPPH was prepared by diluting 4 mg of DPPH with 100 mL methanol. The purple coloured methanolic DPPH solution was then stored in dark and cool environment until further usage [10].

10 mg of L-ascorbic acid was dissolved in 10 mL of methanol and the volume was marked up to 100 mL with distilled water. Different concentration of ascorbic acid was made (10, 20, 30, 40 and 50 µg/mL). 1 mL of each concentration was pipetted into a test tube. 1 mL of methanolic DPPH and 3 mL of methanol was added into the tube. The tubes were covered and left to incubate in dark environment for 30 minutes. Finally, the absorbance of the solutions was measured by using a UV-Vis spectrophotometer at 517 nm wavelength.

Different concentrations of samples were prepared (10 – 50 µg/mL). Methanol was added to the tubes until it reached a total of 10 mL. For each concentration, 1 mL of solution was extracted. 1 mL of methanolic DPPH and 3 mL of methanol was added to make a total volume of 5 mL. The test tubes were covered and left to incubate in dark environment for 30 minutes. The absorbance reading was obtained through UV-Vis spectrophotometer at 517 nm. The percentage of inhibition was calculated as the following equation:

$$Radical\ Scavenging\ (\%) = \frac{A_1 - A_0}{A_1} \times 100 \qquad \text{Eq. 3}$$

Where, A_1 is the absorbance of the sample and A_0 is the absorbance of the control.

2.9 Functional group analysis by Fourier-Transform Infrared Spectroscopy (FTIR)

The functional group or chemical bonds of the extracts was determined via Fourier-transform infrared spectroscopy (FTIR). The characteristic of the chemical bond shown in the annotated spectrum is the wavelength of light absorbed. The infrared absorption spectra can be used to identify the chemical bonds or functional groups. Extracts from *C. longa, C. xanthorrhiza, and Z. officinale* fed onto a Pekin Elmer infrared spectroscope with a 400–4000 cm^{-1} frequency range.

3. Results and Discussions

3.1 Physical characterization (pH analysis)

Table 1 illustrates the pH of each extract at different extraction time. For *C. longa* (turmeric), the pH was the highest (pH = 5.91) when the sample was placed in subcritical condition for 15 min, while the lowest pH (pH = 5.21) is when the sample is placed in subcritical condition for 20 min. As for *C. xanthorrhiza* (temulawak), the highest pH obtained was when the sample was placed in subcritical condition for 15 min (pH = 5.86) and the lowest pH was during 20 min (pH = 5.34). Finally, for *Z. officinale* (ginger), the highest pH obtained was when the sample was placed in subcritical condition for 20 min (pH = 6.22) and the lowest is for 5 min (pH = 5.92). Different extraction time shows different pH of the extract. However, all extracts indicated a slight acidic condition.

Table 1: pH analysis of *C. longa, C. xanthorrhiza* and *Z. officinale* rhizomes

Plant Sample	Extraction time (min)	Plant Denotation	pH
Curcuma Longa (Turmeric)	5	CL 05	5.56
	10	CL 10	5.80
	15	CL 15	5.91
	20	CL 20	5.21
Curcuma Xanthorizza (Temulawak)	5	CX 05	5.51
	10	CX 10	5.44
	15	CX 15	5.86
	20	CX 20	5.34
Zinger Officinale (Ginger)	5	ZO 05	5.92
	10	ZO 10	5.92
	15	ZO 15	6.09
	20	ZO 20	6.22

3.2 Physical characterization (Colour analysis)

Table 2 shows the colour characterization of *C. longa, c. xanthorrhiza* and *Z. officinale* against the *curcumin* standard. The L* reading of plant extracts differ from each other even though some are from the same plant. This can be assumed that the duration of the sample being extracted in subcritical condition changes the physical properties of the sample. Sample with the highest and lowest value of L* are turmeric (64.22 ± 0.48) and temulawak (22.89 ± 0.53) at 10 mins respectively.

As for a* value, most of the plant samples indicated a slight tendency to redness except for CL 10 (-0.22 ± 0.02) and CX 15 (-0.44 ± 0.06) have a slight tendency to greenness. For b* value, all *C. longa* samples show high yellowness followed by the sample of *C. xanthorrhiza* and then *Z. officinale.* The yellow colour was determined by the content of bio-active ingredient in the samples, in this case is *curcumin.* Based on the b* value of the samples *C. longa* has the highest content of *curcumin* followed by *C. xanthorrhiza* and then *Z. officinale* [11].

Table 2: Colour analysis of *C. longa, C. xanthorrhiza* and *Z. officinale.*

Sample	Mean values ± SEM		
	L*	a*	b*
Standard Curcumin	64.74 ± 0.37	15.62 ± 0.33	54.77 ± 0.39
CL 05	53.11 ± 0.40	13.56 ± 0.04	76.50 ± 0.36
CL 10	64.22 ± 0.48	-0.22 ± 0.02	42.66 ± 0.38
CL 15	45.48 ± 0.36	22.18 ± 0.07	75.09 ± 0.29
CL 20	55.26 ± 0.50	7.77 ± 0.19	52.00 ± 0.34
CX 05	60.55 ± 0.49	1.86 ± 0.49	33.7 ± 0.29
CX 10	22.89 ± 0.53	11.88 ± 0.28	36.91 ± 0.47
CX 15	47.21 ± 0.35	-0.44 ± 0.06	28.90 ± 0.37
CX 20	33.87 ± 0.14	6.89 ± 0.22	34.83 ± 0.25
ZO 05	33.47 ± 0.14	7.06 ± 0.41	31.64 ± 0.36
ZO 10	41.69 ± 0.41	3.77 ± 0.29	25.27 ± 0.37
ZO 15	38.89 ± 0.35	5.68 ± 0.34	27.61 ± 0.53
ZO 20	37.76 ± 0.20	7.02 ± 0.43	30.44 ± 0.36

3.3 Total Phenolic Content (TPC)

The calibration curve of standard gallic acid against absorbance measured at 765 nm is shown in Figure 2. The regression equation is obtained from the calibration curve ($y = 12.449x - 0.1887$) with the R^2 value of 0.9939, the total phenolic content of the extracts were calculated and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The results were then plotted and represented in Figure 3.

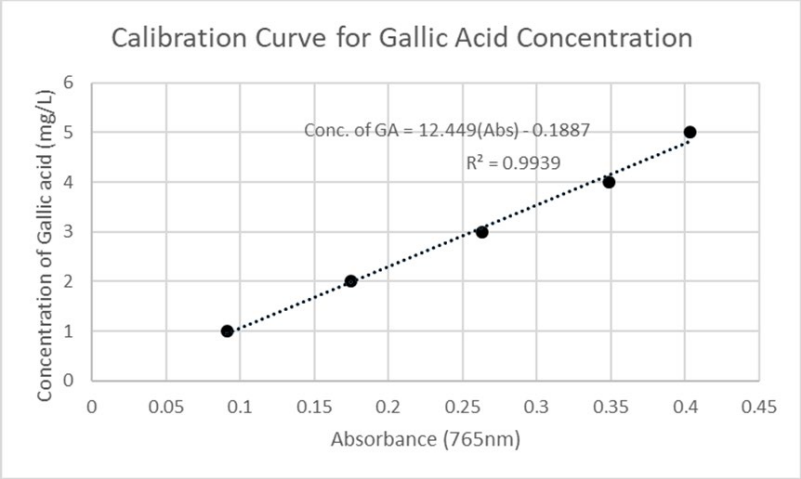

Figure 2: Standard curve of gallic acid

In Figure 3, *C. longa* has the highest amount of total phenolic content followed by *Z. officinale* and finally *C. xanthorrhiza* for every extraction time. It was observed that the total phenolic content for the three plant extracts was the highest when the sample was placed in subcritical condition for 10 min. Again the value was led by *C. longa* followed by *Z. officinale* and *C. xanthorrhiza* (0.402, 0.392, and 0.38 respectively mg/L). In another extraction method, Akinola et al. also found that *C. longa* has the highest amount of total phenolic content followed by *Z. officinale* and *C. xanthorrhiza* (42.71 mg/GAE/g DW, 22.03 mg/GAE/g DW and 17.85 mg/GAE/g DW respectively) [12].

Any plant's ability to act as an antioxidant is closely correlated with its phenol concentration. Phenolic compounds can scavenge free radicals and function as hydrogen donors and reducing agents. The antioxidant capabilities of *C. longa, C. xanthorrhiza*, and *Z. officinale* may be strongly influenced by the presence of a sizable quantity of phenolic in these plants. These plants have been employed in various conventional herbal medicines because of their therapeutic qualities.

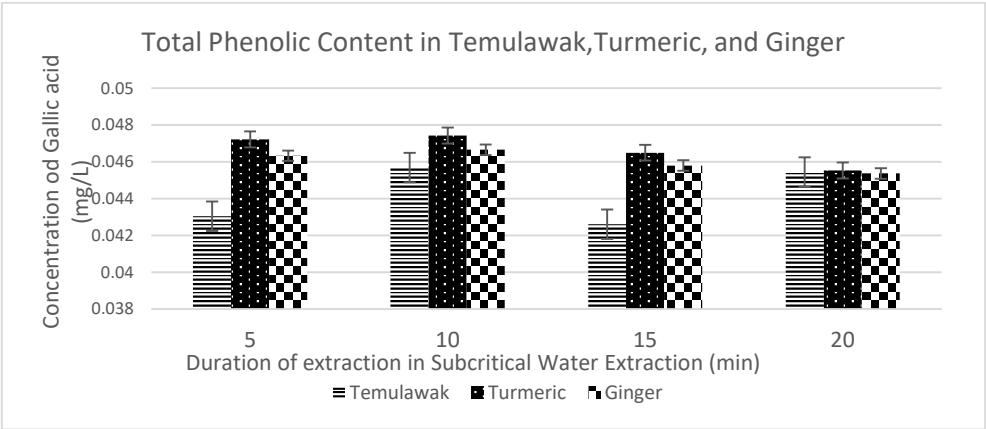

Figure 3: Total phenolic content in Temulawak, Turmeric and Ginger

3.3 Total anti-inflammatory activity (TAI)

The BSA denaturation assay was done to explore the anti-inflammatory activity of *C. longa*, *C. xanthorrhiza* and *Z. officinale* against different extraction time (duration) in the subcritical water condition. Aspirin is one of the most used nonsteroidal anti-inflammatory drugs (NSAIDs) in the current

market and was used as the positive control in this study. The drug exhibits the highest amount of anti-inflammatory activity compared to the other extracts (98.15%). The highest anti-inflammatory activity of the extracts was achieved by CL 05 (96.30%) and closely followed by CL 10 (95.68%). Based on the result, *C. longa* anti-inflammatory activity is comparable to current market nonsteroidal anti-inflammatory drugs. ZO 05 was seen to have the lowest amount of anti-inflammatory activity, 66.05%. The data was then plotted into a bar chart, as shown in Figure 4. Protein denaturation occurs by an unexpected mechanism that alters hydrophobic, disulphide, and electrostatic hydrogen bonds. In inflammatory diseases such rheumatoid arthritis, cancer, and diabetes, protein denaturation results in the generation of auto-antigens. So, it is possible to decrease inflammatory activity by preventing protein denaturation. To conclude, the best time for extraction for each sample are as follow: *C. longa* (5 min), C. *xanthorrhiza* (15 min) and *Z. officinale* (10 min).

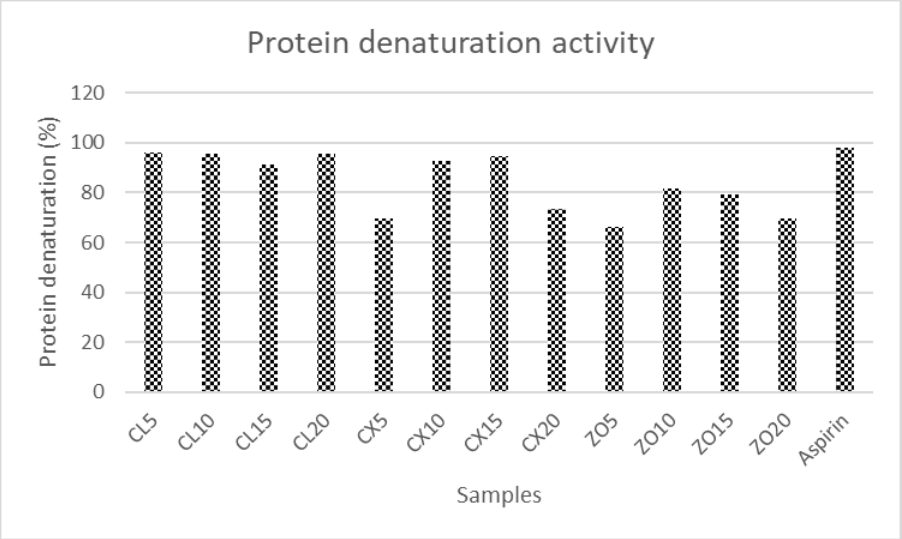

Figure 4: Protein denaturation activity comparison

3.4 Total antioxidant activity

The antioxidant properties of *C. longa, C. xanthorrhiza* and *Z. Officinale* were examined using the DPPH technique, and the percentage based on the scavenging activity of the DPPH in the plant extract. Ascorbic acid was used to measure the antioxidant activity and create a standard-calibration graph with various concentrations. It was discovered that when the concentration of the standard rose, the DPPH scavenging activity will also rise (Figure 5).

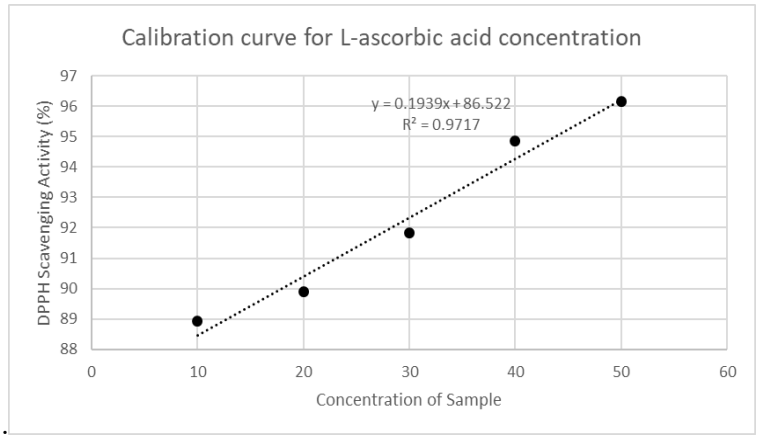

Figure 5: Calibration curve for L-ascorbic acid concentration

From Figure 6, the scavenging activity increases as the concentration of extract increases. This is true except for sample CL 15, the scavenging activity began to drop after 30 µL of sample concentration. The highest scavenging activity obtained for *C. longa* is 89.4% which is the value of CL 5 at 50 µL concentration. While the lowest scavenging activity value is 35.6% by CL 10 at the lowest concentration of 10 µL.

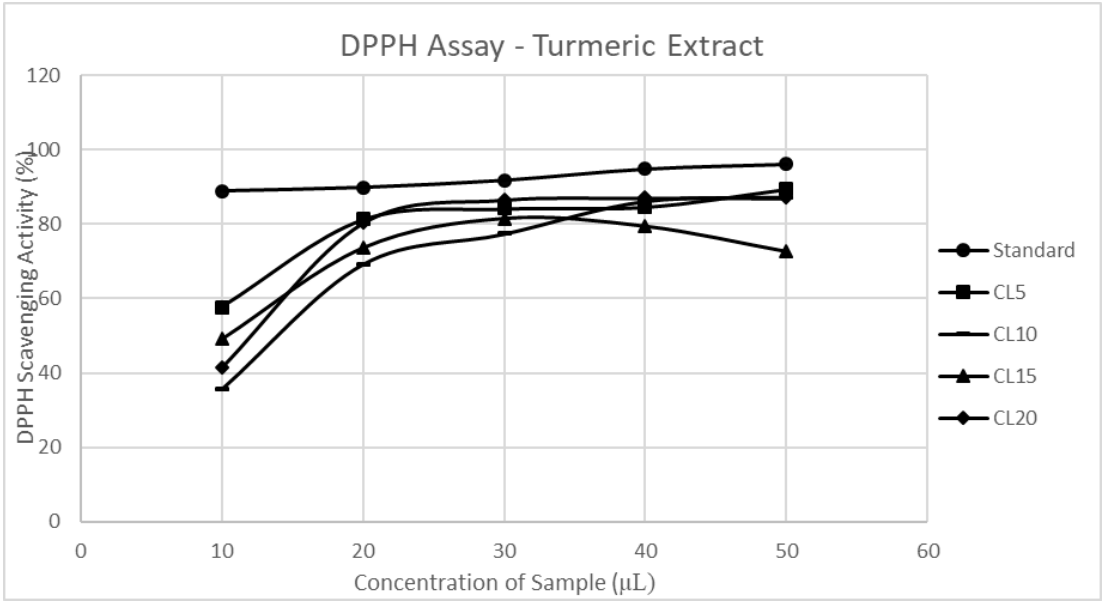

Figure 6: DPPH Assay for *C. longa* (turmeric) extract

C. xanthorrhiza extracts with different concentrations (10 – 50 µL) were analysed for the antioxidant activity by DPPH assay, where the scavenging activity of the DPPH was determined. In Figure 7, the scavenging activity increases as the concentration of extract increases. The highest scavenging activity obtained for *C. xanthorriza* is 79.3% which is the value of CX 20 at 50 µL concentration. While the lowest scavenging activity value is 0.96% for CX 5 at the lowest concentration, 10 µL.

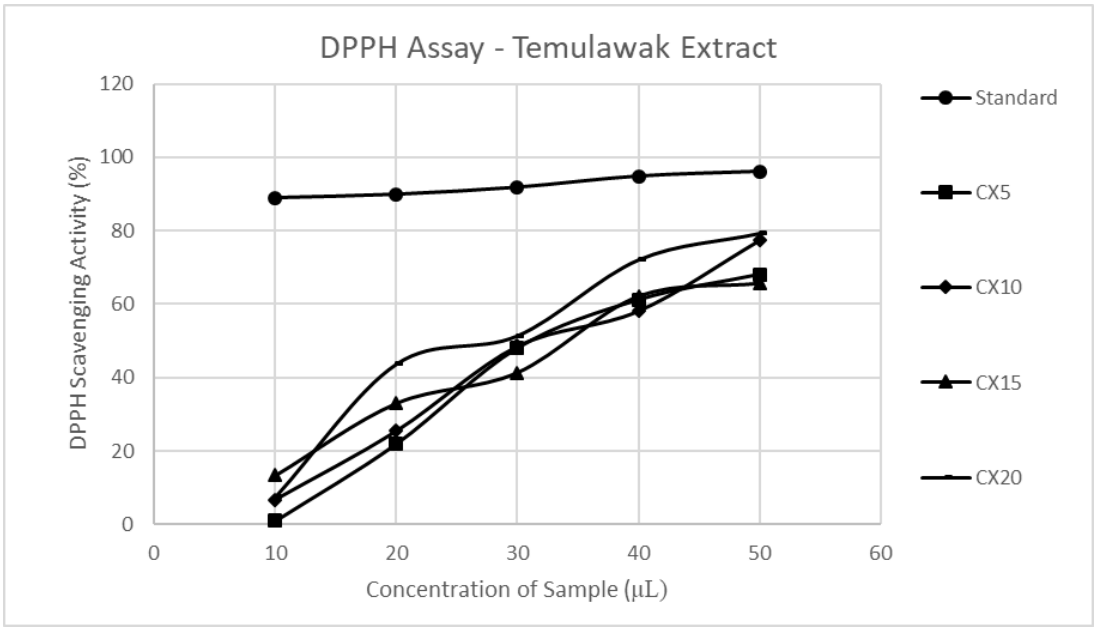

Figure 7: DPPH Assay for *C. xanthorrhiza (*temulawak) extract

Figure 8 shows the scavenging activity of *Zingiber officinale* extracts at different concentration with different extraction time. It was observed that the scavenging activity increases as the concentration of extract increases. The highest scavenging activity obtained for *Z. officinale* is 76.0% which is the value of ZO 10 at 50 µL concentration. While the lowest scavenging activity value is 14.90% for ZO 20 at the lowest concentration of 10 µL.

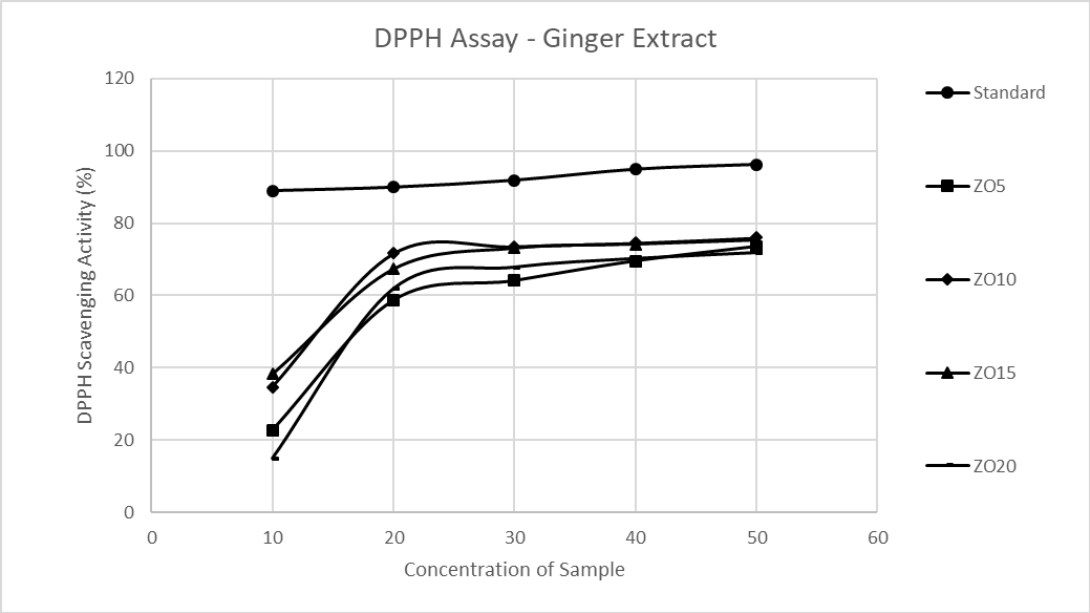

Figure 8: DPPH Assay for ginger extract

To conclude, the best extraction time for the highest total antioxidant activity for each sample are as follow; *C. longa* (5 min), C. *xanthorrhiza* (20 min) and *Z. officinale* (10 min).

3.4 Functional group characterization

The FTIR spectra of extracts from *Curcuma longa* (turmeric), *Curcuma xanthorrhiza* (temulawak), and *Zingiber officinale* (ginger) with various extraction times were shown in Figures 9, 10, and 11 respectively, each peak corresponds to different functional group. Analysis of the FTIR spectra of all the extracts from *Z. officinale, C. longa,* and *C. xanthorrhiza* reveals comparable peaks caused by shared chemical components. The extracts have variable component concentrations, which results in a little change in peak intensity (absorbance).

The most prominent functional group found in all the extracts is alkenes (Table 3). The biggest peak is at 3265 cm^{-1}. The peak was broad and tall. Meanwhile, for the second peak (1638 cm^{-1}), it was sharp and strong. The alkenes detected might be lycopene and carotenes which both are isomeric polyenes ($C_{40}H_{56}$) that give the attractive red, orange, and yellow colours to fruits and vegetables [13].

Table 3: FTIR spectral wavenumber's values, chemical bonds and functional groups identified based on peaks.

S. No	Wave Number (cm^{-1})	Frequency Range (cm^{-1})	Chemical bond	Functional Group
1.	3265	3040-3010	CH Stretching	Alkenes
2.	1638	1680-1620	C=C Stretching	Alkenes

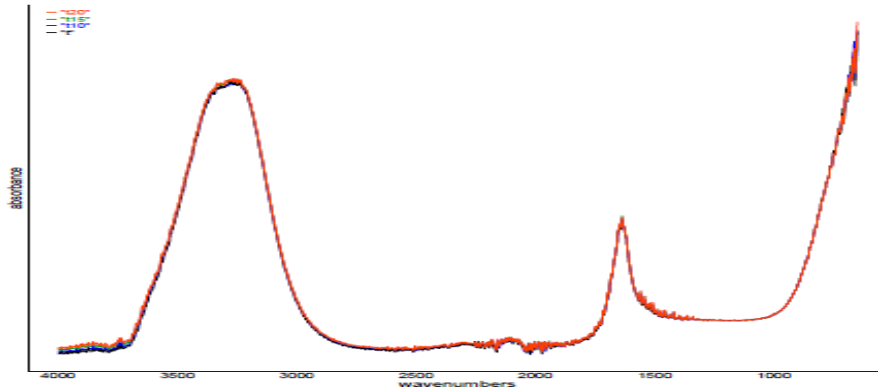

Figure 9: FTIR spectrum analysis of *Curcuma longa* rhizome extracts

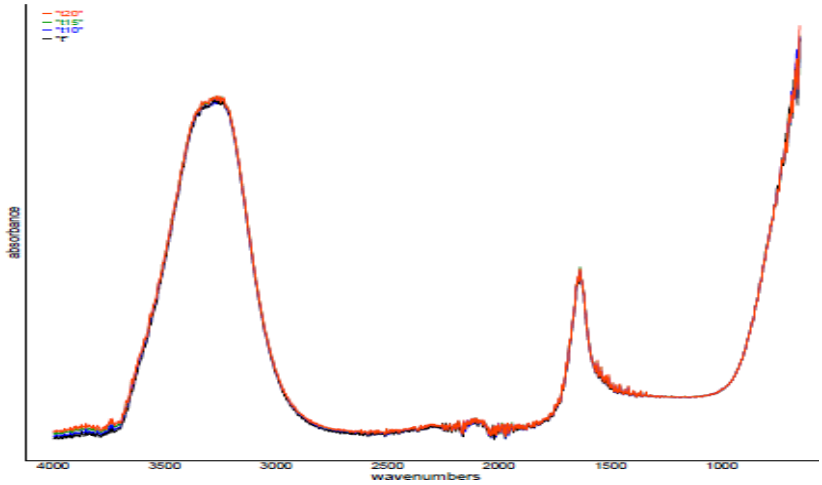

Figure 10: FTIR spectrum analysis of *Curcuma xanthorrhiza* rhizome extracts

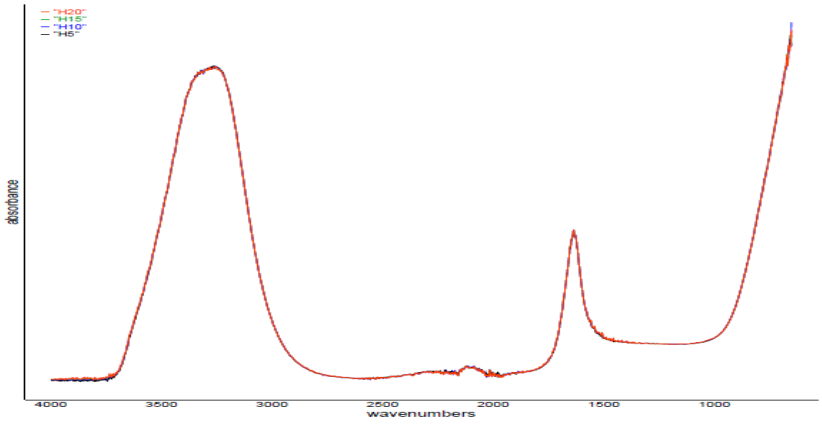

Figure 11: FTIR spectrum analysis of *Zingiber officinale* rhizome extracts

4. Conclusion

Overall, this research proposed the extraction of *C. longa, C. xanthorrhiza* and *Z. officinale* using subcritical water extraction (SWE) method and its characterization study. Bioactive compounds were successfully extracted using SWE for all three rhizomes (temperature = 100°C; pressure = 11 bar; sample to solvent ratio = 1:3) at different extraction times (5 min, 10 min, 15 min and 20 min).

The pH of the extracts were between 5.2 – 6.2 and the colour analysis indicated *C. Longa* contains the brightest yellow in comparison to the other two samples. As for functional group analysis, all three samples are having alkenes group with carbon-carbon chemical bond. Finally, the total phenolic content (TPC) of all 3 samples were highest at 10 min of extraction time with *C. longa* (0.402 mg GAE/g DW), *Z. officinale* (0.392 mg GAE/g DW), and *C. xanthorrhiza* (0.38 mg GAE/g DW).

The functional activities of *C. longa, C. xanthorrhiza* and *Z. officinale* extracts were evaluated for its total anti-oxidant activity (TAA) and total anti-inflammatory activity (TAI). The maximum TAA were achieved at min 5, 20 and 10 for *C. longa* (89.4%), *C. xanthorrhiza* (79.3%), and *Z. officinale* (76%). Meanwhile, the maximum TAI are achieved at min 5, 15 and 10 for C. longa (96.3%), *C. xanthorrhiza* (94.4%) and *Z. officinale* (81.5%) respectively.

Based on these functional activities, the best SWE extraction time for *C. longa* is 5 min, *C. xanthorrhiza* is 10-20 min and *Z. officinale* is 10 min.

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