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Valorisation of bioactive compounds from Bentong ginger (*Zingiber officinale* Roscoe var. Bentong) using ultrasound-assisted extraction

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Abstract

The present work demonstrated the bioactive compound recovery from Bentong ginger (*Zingiber officinale* Roscoe var. Bentong) using ultrasound-assisted extraction (UAE) in terms of crude yield (CY), as well as total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and ferric reducing-antioxidant power (FRAP), which reflected the antioxidant properties. These response variables were evaluated as functions of the independent variables; temperature (30 - 60°C), time (10 - 30 min), and ultrasonic intensity (1768 - 5304.10 W/cm^2), using response surface methodology (RSM). The variables were optimised using quadratic polynomial through the correlations calculated from the regression models fitted to the experimental data. The predicted values of CY, TPC, FRAP, and DPPH under the optimal conditions (40°C, 3536.78 W/cm^2, and 25 min) were 23.42%, 778.08 mg GAE/g, 636.08 µmol Fe^{2+}/g, and 150.01%, respectively. These experimental values were well fitted with the predicted values, except for DPPH. With UAE set at the optimal conditions, a pre-leaching step (PLS) was added to the process to investigate its effect on the extraction. It was found that PLS-UAE produced higher extraction yields (30.15%); however, the antioxidant activity of the extracts was significantly ($p < 0.05$) higher at the optimised condition for UAE only treatment. The PLS application led to structural cell damage which increased CY, and this was validated through scanning electron microscopy (SEM).

Introduction

Ginger (*Zingiber officinale* Roscoe) is a tropical and subtropical plant commonly found in Southeast Asia (Mohd *et al.*, 2015). It is widely consumed in various forms including beverages (Ahammed *et al.*, 2015). It is also popularly used for medicinal purposes, for example, to treat nausea. Ginger contains a variety of bioactive compounds (Ghafoor *et al.*, 2020). Ghasemzadeh *et al.* (2010) reported that the high antioxidant content of ginger rhizomes might be of medicinal benefits such as anti-inflammatory and anticancer properties (Yadav *et al.*, 2016), and these functional properties are generally attributed to its bioactive compounds such as gingerol and shogaol (Kamaliroosta *et al.*, 2013). Bentong ginger, a variety of *Z. officinale*, has a large, dull yellowish rhizome that is less fibrous than that of other varieties. It is reported to be spicier, more aromatic, and of higher fibre content than other varieties cultivated in Malaysia (Mohd *et al.*, 2015). The significant quantities of phenolic substances and antioxidant activity of Bentong ginger (Suhaimi *et al.*, 2014) make it particularly suitable for healthcare and disease prevention. Moreover, the use of natural supplements, including phenolic compounds from plants, has grown rapidly to replace synthetic supplements.

The conservative industrial methods to recover the valuable compounds from plants include hydro-distillation and Soxhlet extraction (Azmir *et al.*, 2013). Nevertheless, these widely used methods are somewhat controversial as major drawbacks have been reported. For instance, high temperatures employed in hydro-distillation cause product loss, aroma loss, and the degradation of important volatile compounds (Ghafoor *et al.*, 2010). In addition, even a long extraction time yields a relatively small amount of product, which can make the process uneconomical. The Soxhlet extraction method is also time-consuming, as it takes from several hours up to several days, causing the degradation of unsaturated or ester compounds, and volatilisation of the bioactive compounds through thermal or hydrolytic effects (Ghafoor *et al.*, 2010). In general, the limitations of the conventional methods involve the

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thermolability of the desired compounds, which undergo various chemical alterations due to high temperatures, and the prolonged exposure times that can reduce the quality of the extracts (Selvamuthukumaran and Shi, 2018). Therefore, a better approach is necessary to overcome these drawbacks concerning product quality. Furthermore, if higher yield can be obtained in a shorter time, the profitability of the industrial process can also be increased.

Ultrasound-assisted extraction (UAE) is a promising technology for the recovery of bioactive compounds from Bentong ginger. UAE is a relatively efficient method as compared to the conservative methods, as well as economically viable (Tabaraki *et al.,* 2012). UAE employs acoustic energy that generates in the solvents to rupture the cell walls, and extracts the target compounds from a variety of samples (Aguilar-Hernández *et al.,* 2019). Technically, the cavitation phenomena involve bubble collapse and generation of high temperature, which lead to the propagation of the pressure waves in the mechanism behind cell wall breaks, thus releasing the interest compounds into the solvent (Ebringerová and Hromádková, 2010; Chemat *et al.,* 2017). The process of softening the cell walls through hydration which leads to higher solvent penetration into the sample is attributed to the mechanical effect of ultrasound, thus improving the rate of mass transfer of soluble polysaccharides due to the break-up of sample cell walls (Ebringerová and Hromádková, 2010). Therefore, UAE is a relatively efficient extraction method as compared to conservative methods such as stirring and thermal decoction, by way of enhancing the amount of extracts, reducing the time and energy consumption, while preserving the quality of the compounds (Chemat *et al.,* 2017; Aguilar-Hernández *et al.,* 2019). In short, the entire extraction process could significantly be improved by powerful ultrasound.

This innovative technology is also considered as 'green' technology by minimising the amount of solvent used (Turrini *et al.,* 2019), hence positively influencing the environmental impact, particularly in term of energy. Chemat *et al.* (2017) reported that 8 kW.h energy was needed to extract fat and oil from oleaginous seeds using the Soxhlet method which is relatively higher than UAE (0.25 kW.h). In addition, the CO_2 emission is significantly reduced to 200 g CO_2/100 g of extracted solid material when using UAE as compared to Soxhlet (6,400 g CO_2/100 g extracted solid material) (Chemat *et al.,* 2017). Santos *et al.* (2010) stated that the solvent extraction process using ethanol is categorised under "GRAS" (generally recognized as safe) which offers a good manufacturing practice. In addition, ethanol used in solvent extraction is also considered environmentally friendly and efficient for valuable compound recovery from herbal sources (Bimakr *et al.,* 2016). In this regard, ethanol is considered as a "green" solvent that could potentially replace other non-environmentally friendly solvents such as hexane. The capability and efficiency of ethanol in extracting oils has been reported in radish seed (Stevanato and da Silva, 2019) and ginger (Murphy *et al.,* 2020).

Response surface methodology (RSM) is an empirical model platform by collecting a set of mathematical and statistical techniques for a simultaneous solution to the multivariate equations. This can be used to evaluate the variables, for instance, amplitude, solid-to-solvent ratio, power, time, and ultrasonic wave distribution that affect the UAE process (Tabaraki *et al.,* 2012; Sharayei *et al.,* 2019). In turn, an accurate evaluation of the effects of such variables, individually or in combination, allows the UAE process to be optimised. In the present work, the main goal was to evaluate and optimise the effects of UAE temperature, time, and intensity of ultrasound with a three-level Box-Benkhen design on crude yield, total phenolic content, and scavenging activity of Bentong ginger extracts. Thereafter, the effect of adding the pre-leaching step to the UAE process was investigated in terms of the amount of the extracts and secondary metabolites of Bentong ginger extracts.

Materials and methods

Sampling of Bentong ginger

Fresh Bentong ginger rhizomes were harvested from Bentong, Pahang. Samples were packed and stored in a chiller to maintain the freshness of the ginger rhizomes until processing. The ginger rhizomes were pre-treated as described by Ghasemzadeh *et al.* (2010). Briefly, the fresh rhizomes were thoroughly washed to remove any contaminants, drained, and cut into thin cylindrical slices with average thickness of 5 mm. The slices were then freeze-dried in a freeze drier (Virtis Genesis Freeze Dryer; SP Scientific, New York, USA) at -60°C and 0.1 - 1 mbar for approximately 96 h. The dried ginger slices were then ground at room temperature using a food processor (MX-GM1011;

Panasonic, Malaysia), and sieved through an aperture of 500 µm to obtain particles of similar size. The dried ginger powder was kept at 5°C in vacuum-sealed plastic bags until further analysis.

Chemicals and reagents

All chemicals and reagents used in the present work were of analytical grade. Folin-Ciocalteu reagent, gallic acid, and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were purchased from Merck (Darmstadt, Germany). Sodium hydroxide, sodium acetate trihydrate, and iron (III) chloride hexahydrate were purchased from QRec (Malaysia). 1,1-Diphenyl-2-picrylhydrazyl free radical scavenging (DPPH), ferrous sulphate, and quercetin were purchased from Sigma Aldrich (USA). Acetic acid, hydrochloric acid, and aluminium chloride were purchased from Friendemann Schmidt (USA). Ethanol, sodium bicarbonate, and sodium nitrite were purchased from Systerm ChemAR (Malaysia), J. Kollin (UK), and ACROS Organics (Sweden), respectively.

Ultrasound-assisted extraction (UAE)

The UAE unit (model: CL-334) used in this present work had a maximum power of 500 W, and a 20 kHz fixed frequency was equipped with an immersible probe (3 mm diameter of a horn microtip). The UAE probe set was placed in 100 mL cylindrical jacket glass vessel with a circulated water bath to maintain the desired temperature. The probe was dipped in the vessel containing a sample and a solvent, while emitting the sound of vibration into the sample.

The UAE experiments were performed by placing 10 g of Bentong ginger powder and 200 mL of ethanol (1:20, w/v) in the extraction unit. The mixture was conducted at different UAE conditions including times, ultrasound intensities, and temperatures (controlled by placing the extraction flask in the water bath). A nylon membrane filter paper (0.45 µm) and a vacuum rotary evaporator (model: RV10) was used to vacuum-filtered the mixture and evaporate the solvent, respectively. The extracts were refrigerated in amber-coloured glass bottles (5°C ± 1) until further analysis.

Following UAE condition optimisation, in subsequent experiments, a pre-leaching step was added to the optimised UAE process (SS-UAE), which was conducted from 20 to 60 min to investigate whether it would enhance the recovery of bioactive compounds from the Bentong ginger.

Intensity of ultrasonic estimation

The probe microtip of the UAE produced the intensity of ultrasonic (I) which was calculated using Eq. 1:

$$I\ (\frac{w}{cm^2}) = \frac{P}{\Pi r^2} \quad \text{(Eq. 1)}$$

where, P (W) = input power, and r (cm) = probe microtip radius. The total input power of 500 W was adjusted to 25, 50, and 75% which corresponded to 125, 250, and 375 W, respectively. The corresponding ultrasonic intensities were 1,768.39, 3,536.78, and 5,305.17 W/cm^2, respectively.

Soxhlet extraction

Soxhlet apparatus containing 5 g of dried powder of Bentong ginger was placed in a covered thimble with wool. The extractor was connected to the extraction flask which contained 100 mL of ethanol. During the process, the condensed fresh ethanol from a distillation flask was filled in the thimble. Two replications were conducted for 8 h. The solvent's boiling point was used as a reference for the extraction temperature.

Experimental design and statistical analysis

A three-level Box-Behnken design (BBD) response surface methodology (RSM) tool and Minitab software (Version 18.0) were employed in the present work. Accordingly, 15 experiments with three levels (-1, 0, and +1) of the coded independent variables were performed for the optimisation. The triplicate experiments at the centre of the design were used to estimate the sum of square and pure error (Sharayei *et al.*, 2019). A response surface regression procedure was used to analyse the effect of the independent variables [temperature (x_1), ultrasonic intensity (x_2), and time(x_3)] on the crude yield (CY; %), total phenolic content (TPC; mg gallic acid equivalents (GAE)/g), ferric reducing-antioxidant power (FRAP; µmol Fe^{2+} /g), and DPPH free radical scavenging (DPPH; %), and fitted to a second-order polynomial model (Eq. 2):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_j^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j + \varepsilon \quad \text{(Eq. 2)}$$

where, Y = predicted response, β_0, β_i, β_{ii}, and β_{ij} = constant, linear, squared, and interaction coefficients, respectively, X_i and X_j = independent variables, and ε = noise or error. Statistically, the significant value

(p-value < 0.05) of the process variables in the model could be determined based on the F-ratio and p-value by ANOVA (Poodi *et al.,* 2018). Model analysis, lack-of-fit test, coefficient of determination, and R^2 were used to evaluate the quality of the fitted models.

Determination of extraction yield

The total yield from the extraction of Bentong ginger was estimated using Eq. 3:

$$Crude\ yield = \frac{Weight\ of\ crude\ extract}{Weight\ of\ sample} \times 100 \quad \text{(Eq. 3)}$$

Total phenolic content (TPC)

As previously described by Bursal and Koksal (2011) with slight modification, Folin-Ciocalteu reagent was used to analyse the total phenolic content. To 25 µL of the sample solution made from 1 mg of sample (ginger extract) in 1 mL of ethanol, 75 µL of Folin-Ciocalteu phenol reagent was added. After 3 min, the solution was mixed with 150 µL of 2% Na_2CO_3. The mixture was mixed well, and stored at ambient temperature in darkness for 30 min before absorbance measurement at 760 nm. TPC was expressed as milligram gallic acid (GA) equivalent per gram. Gallic acid was used as a standard, and the calculation was based on the standard curve of gallic acid.

Antioxidant activity (DPPH)

The DPPH radical-scavenging assays were conducted according to Mensor *et al.* (2001). Test extracts dissolved in ethanol (1 mg/mL) were mixed with 140 µL of DPPH ethanolic solution. The mixture was mixed well, and left for 30 min in the dark, at ambient temperature, before absorbance measurement at 517 nm. Ascorbic acid (25 µM) was used as a standard, and the calculation was based on the standard curve of ascorbic acid. The percentage inhibition was calculated using Eq. 4:

$$\%\ inhibition = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100 \quad \text{(Eq. 4)}$$

where, Abs sample = absorbance of DPPH radical + sample, and Abs control = absorbance of DPPH radical + ethanol.

Antioxidant activity (FRAP)

The FRAP reagent was prepared by mixing 500 mL of acetate buffer containing 1.6 g of sodium acetate and 8 mL of acetic acid, 10 mM TPTZ solution in 40 mM HCL, and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively (Benzie and Strain, 1996). To prepare 200 µL of this FRAP reagent, 50 µL of the ginger extract was added, and the mixture was mixed well. Microplate reader spectrophotometer (ELISA Plate Reader) was used for absorbance measurement at 593 nm following 4 min incubation. Three replications of the sample were measured. A similar procedure was applied to prepare a standard curve of iron (II) sulphate solution (200, 400, 600, 800, and 1000 ppm). The results were expressed as µmol Fe^{2+}/g.

Results and discussion

Model fitting

The effects of extraction temperature, ultrasound intensity, and extraction time on the quantity and quality of Bentong ginger extracts (*i.e.* CY, TPC, FRAP, and DPPH) were evaluated using RSM. The CY, TPC, FRAP, and DPPH of Bentong ginger extracts ranged from 18.0 to 30.7%, 129.06 to 1081.64 mg GAE/g, 318.86 to 1062.59 µmol Fe^{2+}/g, and 12.47 to 520.14%, respectively. This indicated the ability of UAE to extract bioactive compounds from Bentong ginger.

Table 1 presents the ANOVA results and regression coefficients of the independent variables on the linear, quadratic, and interaction terms. The experimental data were in good agreement with a second-order polynomial model. The models for CY, TPC, FRAP, and DPPH of process variables are given in Eqs. 5 to 8, respectively:

$$CY\ (\%) = 24.80 + 2.36X_a + 1.79X_b + 1.18X_c - 1.27X_a^2 - 2.37X_b^2 + 1.15X_c^2 - 0.25X_aX_b + 0.73X_aX_c - 0.23X_bX_c \quad \text{(Eq. 5)}$$

$$TPC\ (\%) = 748.60 - 35.20X_a + 0.60X_b + 212.70X_c - 113.80X_a^2 + 68.10X_b^2 - 116.20X_c^2 + 74.60X_aX_b - 132.10X_aX_c + 133.70X_bX_c \quad \text{(Eq. 6)}$$

$$FRAP\ \left(\frac{\mu mol}{g}\right) = 399.00 - 72.70X_a - 19.80X_b + 123.80X_c + 70.20X_a^2 + 140.80X_b^2 + 79.80X_c^2 + 8.10X_aX_b - 34.50X_aX_c + 180.90X_bX_c \quad \text{(Eq. 7)}$$

$$DPPH\ (\%) = 202.40 - 46.60X_a - 26.80X_b - 25.90X_c + 71.60X_a^2 - 21.70X_b^2 - 147.30X_c^2 + 101.30X_aX_b - 33.80X_aX_c - 12.90X_bX_c \quad \text{(Eq. 8)}$$

Table 1. Adequacy of the models in term of regression coefficients and significance value of the effects for the independent variables on CY, TPC, FRAP, and DPPH.

Source	CY (%)			TPC (mg GAE/g)			FRAP (μmol Fe^{2+}/g extract)			DPPH (%)		
	β	***F*-ratio**	***p*-value**	**β**	***F*-ratio**	***p*-value**	**β**	***F*-ratio**	***p*-value**	**β**	***F*-ratio**	***p*-value**
Constant	24.8	7.2	< 0.05*	748.6	9.53	< 0.05*	399	1.61	< 0.05*	202.4	0.46	> 0.05
X_a	2.36	11.87	< 0.05*	-35.2	0.76	> 0.05	-72.7	1.22	> 0.05	-46.6	0.84	> 0.05
X_b	1.79	6.79	< 0.05*	0.6	0	> 0.05	-19.8	0.09	> 0.05	-26.8	0.28	> 0.05
X_c	1.18	2.94	> 0.05	212.7	27.82	< 0.05*	123.8	3.53	> 0.05	-25.9	0.26	> 0.05
X_{aa}	-1.27	1.6	> 0.05	-113.8	3.68	> 0.05	70.2	0.53	> 0.05	71.6	0.92	> 0.05
X_{bb}	-2.37	5.54	> 0.05	68.1	1.32	> 0.05	140.8	2.11	> 0.05	-21.7	0.08	> 0.05
X_{cc}	1.15	1.3	> 0.05	-116.2	3.83	> 0.05	79.8	0.68	> 0.05	-147.3	3.89	> 0.05
X_{ab}	-0.25	0.07	> 0.05	74.6	1.71	> 0.05	8.1	0.01	> 0.05	101.3	2.02	> 0.05
X_{ac}	0.73	0.56	> 0.05	-132.1	5.37	> 0.05	-34.5	0.14	> 0.05	-33.8	0.22	> 0.05
X_{bc}	-0.23	0.05	> 0.05	133.7	5.5	> 0.05	180.9	3.77	> 0.05	-12.9	0.03	> 0.05
Regression coefficients (R^2)	0.86			0.91			0.70			0.64		
Lack-of-fit		19.56	0.049		4.97	0.172		3.45	0.233		0.91	0.561

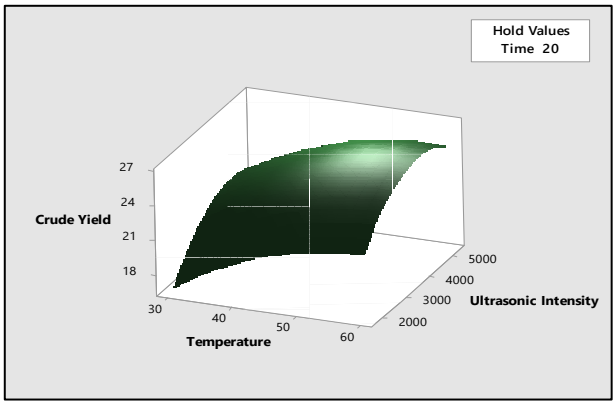
X_a: extraction temperature; X_b: intensity of ultrasound; X_c: extraction time; X_{aa}, X_{bb}, X_{cc}: quadratic effect of extraction temperature, intensity of ultrasound, and extraction time, respectively; X_{ab}, X_{ac}, X_{bc}: interaction effect of extraction temperature, intensity of ultrasound, and extraction time, respectively. * Significant at *p*-value < 0.05.

where, X_a, X_b, and X_c = independent variable extraction temperature, ultrasound intensity, and extraction time, respectively. The models were all statistically significant ($p < 0.05$).

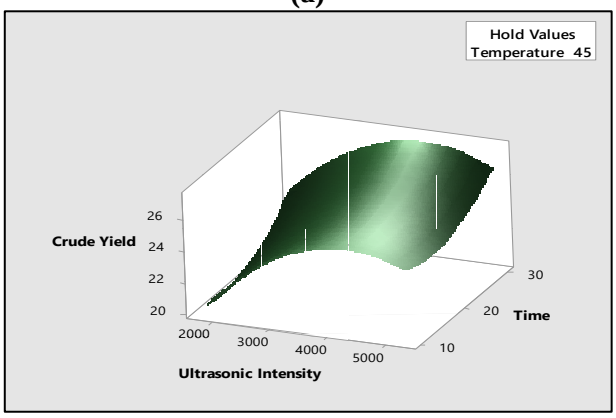
Effect of independent variables on crude yield (CY)

Ultrasound intensity is a measure of both amplitude and frequency. Technically, the ultrasonic intensity increases at a higher amplitude of a given frequency, or a higher frequency of a given amplitude (Sharayei *et al.,* 2019). The increase in ultrasonic wave through compression and rarefaction cycles will increase the amplitude, thus improving the extraction efficiency (Chemat *et al.*, 2017). As illustrated in Figures 1a and 1b, CY increased with increasing ultrasound intensity. Carrera *et al.* (2012) found significant differences between wave amplitudes of 20 and 50% for the extraction of tannin and anthocyanin. They suggested that greater ultrasonic amplitude could lead to higher ultrasonic intensity, which induces greater cavitation, and improves the extraction of anthocyanin and tannin. Entezari *et al.* (2004) reported that high intensity of ultrasound will generate and collapse more bubbles within a liquid. This has been supported by Ghafoor *et al.* (2009) who also obtained the same trends in extraction improvement. The cavitation phenomenon which improved the ultrasound-assisted extraction was also reported by Sharayei *et al.* (2019). However, the increase in CY in Figures 1a and 1b was only up to a certain threshold before the intensity of ultrasound began to have a negative effect. This could likely be due to the distraction on the propagation of ultrasound waves due to excess bubble formation. Wang *et al.* (2015) also reported the same phenomenon for pectin extraction from grapefruit peel using UAE at 20 kHz. Therefore, the intensity of ultrasound needs to first be optimised, rather than simply maximised, to obtain the highest yields (Poodi *et al.*, 2018).

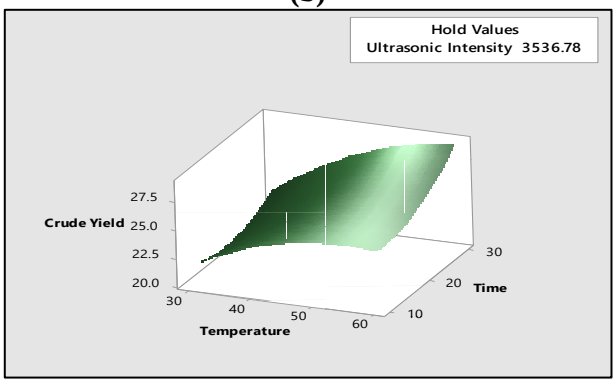
Figures 1b and 1c show that when extraction time was increased from 10 min to 30 min, CY also increased. The bioactive compounds are released from the broken cells into the exterior solvent by the action of the ultrasound. Herein, 15°C was set as the interval for 30 to 60°C range. Temperatures above 60°C are unsuitable as the bioactive compounds are heat-sensitive. Figure 1c shows that extraction temperature positively influenced CY. The highest CY values were extracted at the highest extraction temperature, 60°C. These findings are supported by two important phenomena in UAE namely cavitation and thermal effects. The effects of cavitation and thermal will loosen the cell structure through the bubbles collapsing violently and bubble swelling, respectively.



(a)



(b)



(c)

Figure 1. 3-D plots of CY (%) as a function of **(a)** temperature (°C) and intensity of ultrasound (W/cm^2) at 20 min extraction time, **(b)** intensity of ultrasound (W/cm^2) and time (min) at 45°C extraction temperature, and **(c)** temperature (°C) and time (min) at 3536.78 W/cm^2 intensity of ultrasound.

Effect of independent variables on the bioactive compounds

The bioactive compounds of Bentong ginger extract, in terms of TPC (mg GAE/g) and antioxidant

capacity of the extracts, including DPPH (%) and FRAP (µmol Fe^{2+}/g), as a function of the independent variables and their interactions, are shown in Figure 2. Figures 2a - 2c (ii) illustrate the response variables of TPC, DPPH, and FRAP as a function of ultrasound intensity and extraction temperature at a set time of 20 min (0 levels). It is apparent that the TPC and antioxidant capacity decreased as the intensity increased at lower temperature (40°C). This findings was supported by Figures 2a - 2c (i), whereby TPC and antioxidant capacity decreased with increasing ultrasound intensity. Even though there is an interruption of the cell walls and bubble collapse by the intensity of ultrasound (Chemat *et al.,* 2017), it can still cause chemical decomposition due to the acoustic cavitation, thus yielding hydroxyl radicals (Li *et al.,* 2004). To some extent, further decomposition of bioactive compounds at stronger ultrasound intensity is attributed to the explosion of bubbles which generate extremely high local temperatures and pressures during extraction. Suslick and Price (1999) reported that the generation of high temperatures (5,000 K) and pressure (50 – 1,000 atm) was due to the bubble collapse. Da Porto *et al.* (2009) found that very close quantified volatile compounds could be obtained by hydro-distillation and powerful ultrasound.

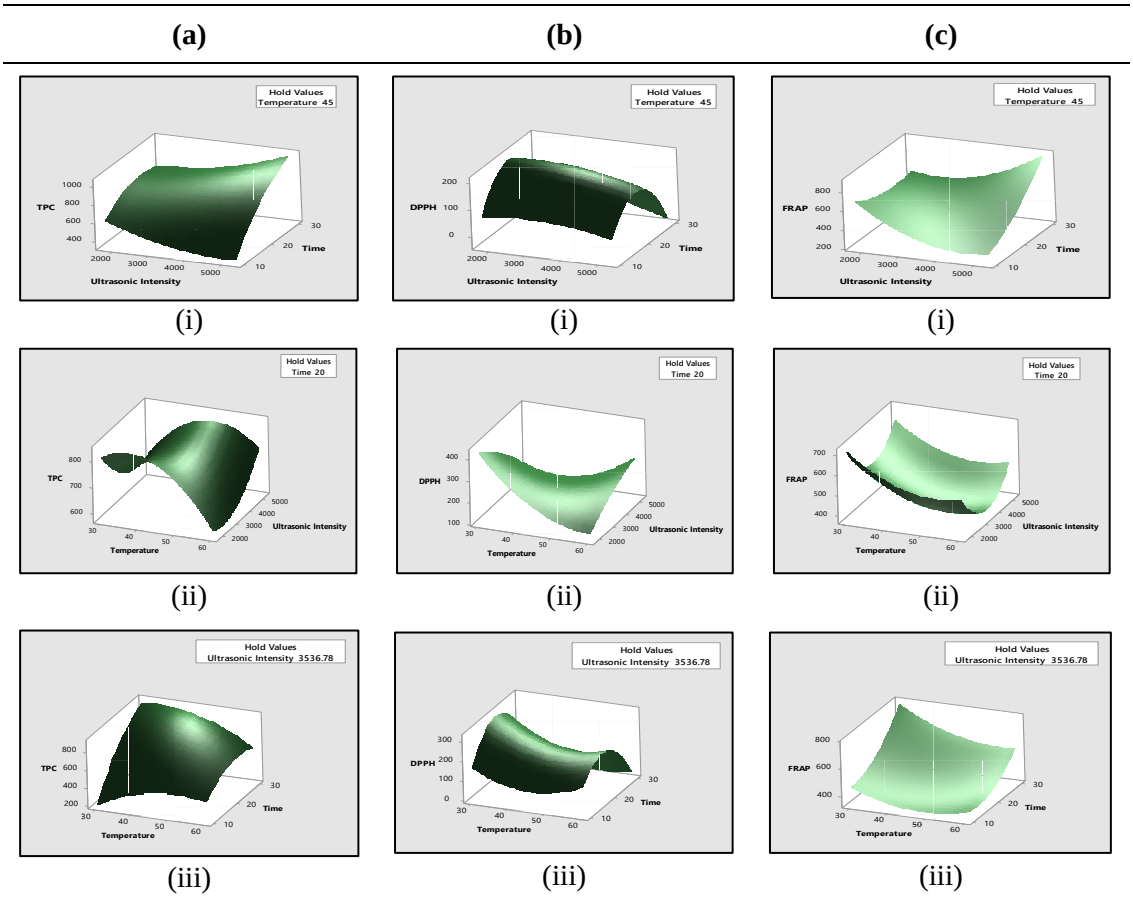


Figure 2. 3-D plots of **(a)** TPC (mg GAE/g), **(b)** DPPH (%), and **(c)** FRAP (µmol Fe^{2+}/g) as a function of (i) intensity of ultrasound (W/cm^2) and time (min) at 45°C; (ii) temperature (°C) and intensity of ultrasound (W/cm^2) at 20 min; and (iii) temperature (°C) and time (min) at 3536.78 W/cm^2.

Figures 2a - 2c (i and iii) show that TPC and FRAP of the Bentong ginger extracts positively increased with increasing extraction time. The result for TPC well agreed with the findings of Sharayei *et al.* (2019) who reported that an increase in the extraction time led to a gradual increase in TPC. This could be due to the longer exposure of the sample to the solvent, thus allowing for greater diffusion of the target compounds. There was slightly different trend for DPPH; extending duration from 10 to 20 min resulted in an increase in the DPPH values, but decreased, thereafter. Wang (2011) reported that DPPH had a linear relationship to the TPC of pomegranate peel extract. Phenolic compounds consist of one or more aromatic rings and hydroxyl groups (Sharayei *et al.,* 2019). The number of free

hydroxyl groups in the sample molecule is the main factor that influences the phenolic acids and their ester reduction activity via donating protons in a high capacity, hence stabilising the DPPH radical (Rice-Evans *et al.*, 1996). In principle, longer exposure to ultrasound will enhance the release of the active species (free radicals) from broken cells into the solvent, hence, up to a point, increasing the yield. However, an extended extraction/exposure time might destroy the conjugated double bonds, thus decreasing the free radical scavenging activity through the degradation of those compounds (Sharayei *et al.*, 2019). Fu *et al.* (2010) reported that the possibility of polysaccharide degradation in mushroom samples caused a decrease in DPPH as a result of prolonged exposure to ultrasound, with IC_{50} values of 8.9, 15.3, and 30.0 mg/mL at 50, 60, and 70 min of exposure, respectively.

Results showed that TPC, DPPH, and FRAP were positively affected at a lower temperature as compared to a higher temperature (Figures 2a - 2c (iii)). Figure 2a (iii) shows that the TPC of the extracts increased with increasing extraction temperature, and started to decrease beyond 40°C. In another work, the highest antioxidant activity (69.00%) of orange peel was obtained at 40°C, which indicated an optimum temperature. There are two possible reasons for this: increasing vapour pressure and decreasing the microbubble surface tension will result in the ultrasonic wave damping and thermal degradation of the bioactive compounds (Chemat *et al.*, 2017). The solubility of extracted compounds will increase with temperature by lowering the viscosity of extracting medium, hence improving the compound mass transfer rate due to the softened and swollen sample materials (Zhang *et al.*, 2009). Nevertheless, further increase in temperature may reduce the surface tension, and increase the vapour pressure of cavitation bubbles, which lead to a decrease in the intensity of cavitation.

Optimisation of UAE and verification

The process variables (extraction time, extraction temperature, ultrasound intensity) were adjusted to optimise the output, namely CY, TPC, FRAP, and DPPH. The parameters were limited to economical ranges such as time, temperature, and ultrasonic intensity with an optimum target of responses. The overall optimum conditions for the extraction of Bentong ginger calculated from the above models was to be at the combined level of 40°C extraction temperature, 3,536.78 W/cm^2 ultrasonic intensity, and 25 min extraction time. Under these processing conditions, the experimental output of CY was 0.3 percentage points more than the predicted value, and the TPC, FRAP, and DPPH experimental outputs were 9.5, 14.3, and 40.8% of the predicted values, respectively.

Table 2 compares the experimental values for UAE bioactive compounds from Bentong ginger at the highest levels used in the experiments (60°C, 5305.17 W/cm^2, and 30 min) of each parameter with those obtained under the optimum operating conditions (40°C, 3536.78 W/cm^2, and 25 min). It can be seen that optimum processing condition extracted more bioactive compounds. Hence, the determination of optimum conditions is of great importance to enhance the efficiency of UAE.

Table 2. Effect of extraction conditions on CY, TPC, FRAP, and DPPH of Bentong ginger.

Extraction condition		CY (%)	TPC (mg GAE/g)	FRAP (μmol Fe^{2+}/g extract)	DPPH (%)
UAE	Type 1[1]	23.45 ± 0.92^a	704.10 ± 20.91^a	544.90 ± 33.73^a	88.80 ± 6.36^a
	Type 2[2]	23.95 ± 0.21^a	631.43 ± 3.57^a	550.00 ± 15.7^a	182.50 ± 8.43^b
PLS	Type 1[3]	27.75 ± 0.50^b	426.64 ± 3.21^b	371.10 ± 92.80^b	234.80 ± 44.70^c
	Type 2[4]	30.15 ± 0.92^c	404.60 ± 35.20^b	186.60 ± 39.00^c	566.00 ± 60.90^d
CSE*	Ethanol	16.72 ± 0.81^d	413.91 ± 4.29^b	184.16 ± 2.50^c	40.40 ± 40.00^e

Means that do not share a lowercase superscript in the same column are significantly different ($p < 0.05$). [1]Optimum parameter studied (40°C, 3536.78 W/cm^2, 25 min). [2]Maximum parameter studied (60°C, 5305.17 W/cm^2, 30 min). [3]Pre-leaching of 20 min prior to UAE at optimum conditions. [4]Pre-leaching of 60 min prior to UAE at optimum conditions. *CSE: conventional Soxhlet extraction.

Effect of pre-leaching step on phenolic compounds and antioxidant activity

A pre-leaching step or known as static before the dynamic extraction at various time intervals from 20 min up to 60 min was then incorporated into the optimised UAE process. A similar solvent in the UAE process was used in the pre-leaching step of dried Bentong ginger. This step was before UAE, which was done under optimised conditions. It was then experimentally determined whether the pre-leaching step enhanced the recovery of bioactive compounds from Bentong ginger by increasing the permeability of cell walls. It was observed (Table 2) that the pre-leaching step significantly enhanced the recovery of CY and DPPH. The combination of UAE and 60 min of the pre-leaching step yielded the highest CY and DPPH. As the pre-leaching time increased, the CY and DPPH also significantly increased. This indicated that the pre-leaching step could increase CY and DPPH by improving cell wall permeability, and by increasing the swelling of the plant sample, thus providing a larger surface area for the ultrasound waves to affect sample cell walls (Chemat *et al.*, 2017). This has been supported by Jadhav *et al.* (2009) who found that a pre-leaching of 30 min improved the yield of vanillin extracts from vanilla pods after 60 min of UAE. Poodi *et al.* (2018) reported a similar observation on the crude yield extracts from Feijoa leaves; 40 min of pre-leaching significantly increased the yield of extracts.

TPC of Bentong ginger was reduced when the pre-leaching time increased from 20 to 60 min. It must be noted that longer exposure time in the pre-leaching step at a set temperature resulted in the loss of TPC due to the susceptibility of heat-sensitive phenolic substances to leach from a sample and be degraded (Babbar *et al.*, 2014). This finding has been supported by a longer extraction time of the Soxhlet method of up to 8 h, thus resulting in the lowest TPC recovery (413.91 mgGAE/g). Silva *et al.* (2020) reported that the recovery of TPC could likely happen at the beginning of the extraction process, hence, TPC tends to degrade when exposed for a longer time.

On the other hand, inconsistent results have been observed for different antioxidant assays namely DPPH and FRAP. Findings showed apparent increasing trend for DPPH assay, but showed irregular trend for FRAP assay. Although both methods were used to measure the capacity of antioxidants, different mechanisms were inviolved particularly on the response of antioxidants and oxidant substances to different radicals in each assay. It has been reported that thiol antioxidants, which are important components of the antioxidant defence system, cannot be determined using FRAP assay (Ivanova *et al.*, 2015). In this regard, the trends for all the extraction conditions on the responses are summarised as follows:

CY: PLS60 > PLS20 > UAE > CSE
TPC: UAE > PLS20 > PLS60 > CSE
FRAP: UAE > PLS20 > PLS60 > CSE
DPPH: PLS60 > PLS20 > UAE > CSE

Surface characterisation of Bentong ginger by scanning electron microscopy

The bioactive compounds extracted as a function of the independent variables were further analysed using scanning electron microscopy (SEM). The microstructure of Bentong ginger after extraction under various conditions is presented in Figures 3a - 3d. It can be seen that the breakage of cells was greater after the application of ultrasound (Figure 3b) when compared with a non-treated sample (Figure 3a). This indicated that the ultrasound improved the extraction of the bioactive compounds from Bentong ginger due to the cavitation phenomenon which imploded the bubbles on the surface of the samples, thus leading to the breakage of sample cells and the release of the compounds (Chemat *et al.*, 2017). A similar observation was reported by Mohammadpour *et al.* (2019) who found that UAE treatment disrupted the plant tissues of *Moringa peregrina* seeds. According to Toma *et al.* (2001), ultrasound increased the surface area of the sample material, thus, the mass transfer rate of the target compounds also increased. This improved the extraction from the Bentong ginger sample to the solvent (Table 2). The addition of a pre-leaching step lasting for 20 or 60 min (Figures 3c - 3d) before UAE increased the breakage of the sample cells to an extraordinary degree. Poodi *et al.* (2018) studied the effect of pre-leaching step on the UAE of bioactive compounds from Feijoa leaves. They reported that the addition of 40 min pre-leaching step before UAE enhanced the extract yield and DPPH. This has been supported by the swelling index results of Feijoa leaves which were influenced by the pre-leaching. The swelling index indicated that the pre-leaching improved the UAE process via enhancing the permeability of cell walls, thus making it easier for the ultrasound waves to break the cell walls (Poodi *et al.*, 2018).

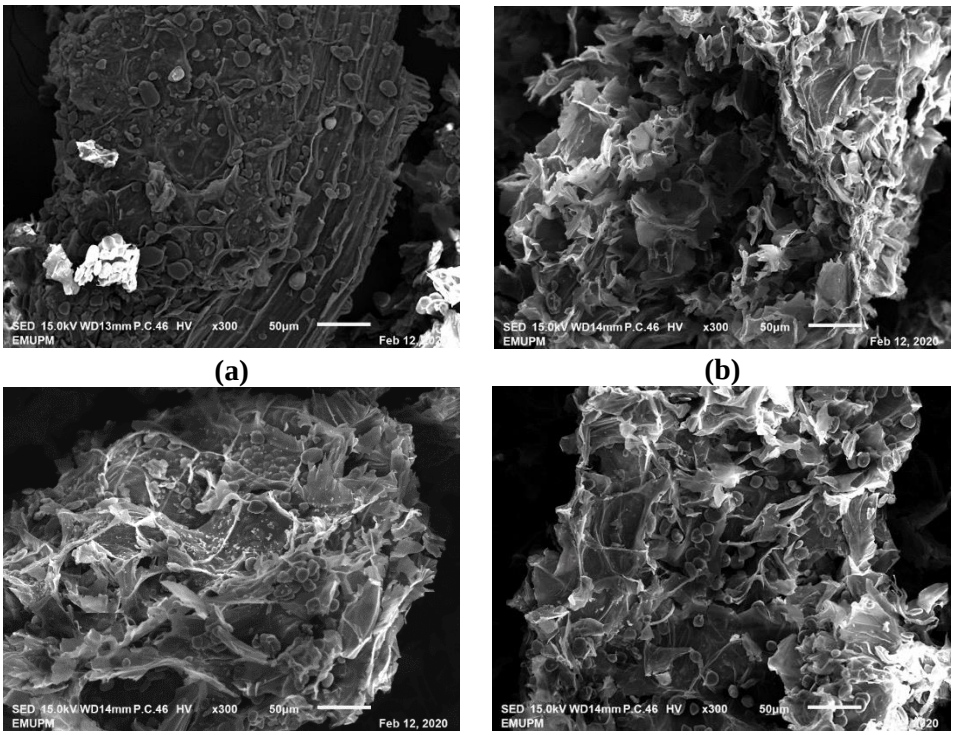


Figure 3. SEM images of Bentong ginger: **(a)** non-treated sample; **(b)** UAE sample under optimum conditions; **(c)** PLS at 20 min, and **(d)** PLS at 60 min and continued with UAE at optimum conditions.

Conclusion

The present work demonstrated that Bentong ginger could be a potential source of bioactive compounds, such as TPC, that have antioxidant properties. Results also indicated that UAE could be a promising technique to extract these bioactive compounds, which requires less extraction time than conservative techniques. The optimal conditions for UAE were found by RSM to be 3,536.78 W/cm^2 of ultrasonic intensity for 25 min extraction time at 40°C. The experimental values of CY, TPC, DPPH, and FRAP under these optimal processing conditions were 23.45%, 704.10 mg GAE/g, 88.80%, and 544.90 µmol Fe^{2+}/g, respectively. A relatively low extraction temperature and a relatively short extraction time could be beneficial in terms of the quality and quantity of the bioactive compounds extracted. The addition of a pre-leaching step before the optimised UAE significantly increased the quantity of extract, but not on the quality of the extracts, particularly the capability of the antioxidant activity to scavenge free radicals. SEM images showed the breakage of cell structures by UAE and especially by SS-UAE.

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