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1   **Optimization of phenolic antioxidants extraction from *Fucus vesiculosus* by pressurized**
2   **liquid extraction**

3

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13

14   **Abstract**

15   This study aimed to optimize pressurized liquid extraction (PLE) of phenolic compounds from
16   brown seaweed *Fucus vesiculosus* to obtain extract with high yield, rich in phenolic compounds,
17   good radical scavenging and metal chelating ability. The extraction was optimized with Box-
18   Behnken design experiment and response surface tool. Optimal condition were found at
19   137.18°C, 58.65% v/v ethanol in water and 4.68 min extraction time which resulted in yield of
20   31.16% dry basis, total phenolic content (TPC) of 3.69 g gallic acid equivalent (100 g dry
21   seaweed)⁻¹, effective concentration for 50% inhibition (EC₅₀) of DPPH radical (92.6 µg mL⁻¹
22   ¹), ABTS radical (2.35 mg mL⁻¹) and metal chelating (1.10 mg mL⁻¹). The TPC value and
23   radical scavenging activity of PLE extract were insignificantly different from the one obtained
24   from conventional solvent extraction, which proved PLE advantages in terms of shorter time
25   and less solvent requirement. Gallic, protocatechuic and gentisic acids were identified in the
26   PLE extracts.

27

28   **Keywords:** *Fucus vesiculosus*; phenolic antioxidants; pressurized liquid extraction;
29   antioxidant activity

30   **Introduction**

31   Seaweeds are known to contain a lot of bioactive compounds such as proteins, lipids, pigments,
32   iodine, halogenated compounds and phenols (Holdt and Kraan 2011). These compounds can
33   be extracted for their functionalities in food or pharmaceutical products. One brown seaweed
34   species that has a great potential is *Fucus vesiculosus*. Although there is no big scale cultivation
35   and harvesting of this species recorded in Denmark, availability of this species is very high
36   since it is the most common canopy forming species in the Baltic Sea (Wikström and Kautsky
37   2007). Harvesting of this species has been done exclusively at the Atlantic coast of Ireland and
38   France, while its cultivation is still in experimental stage (Meichssner et al. 2020). This species
39   contains high amounts of phenolic compounds with antioxidant activity, with content varying
40   from <0.4-12.2% of dry weight gallic acid equivalent (GAE) (Holdt and Kraan 2011; Hermund
41   et al. 2018; Ummat et al. 2020).

42

43   These phenolic compounds are possible alternatives for replacing synthetic antioxidants in
44   delaying lipid oxidation and prolonging food shelf life. They have been proven to be good
45   antioxidants when applied in granola bars (Karadağ et al. 2017), cod mince and cod liver oil-
46   in-water emulsion (Jónsdóttir et al. 2016) and facial cream (Hermund et al. 2018). Phenolic
47   compounds in *F. vesiculosus* are accumulated in a membrane-bound cytoplasmic vesicles
48   called physodes on the surface cells (Jacobsen et al. 2019). Although these phenolic compounds
49   could be as potent as synthetic ones, they co-exist amongst other compounds which might not
50   be active in managing oxidation. Therefore, selective extraction of these phenolic compounds
51   is needed to obtain antioxidant compounds with high activity. Beside the selectivity,
52   maximizing antioxidant yield from the extraction is important for these antioxidants to be
53   economically viable for competition with synthetic antioxidants since natural antioxidants are
54   more expensive than the synthetic ones (Berdahl et al. 2010).

55

56 Currently, main conventional technique used for extracting bioactive compounds from
57 seaweed is solvent extraction (SE), where seaweed is exposed to organic solvents (Joana Gil-
58 Chávez et al. 2013). This traditional method has low efficiency and needs large volumes of
59 environmentally unfriendly organic solvents (Saha et al. 2017). Pressurized liquid extraction
60 (PLE) is a novel extraction method that utilizes solvent in subcritical condition to change the
61 physical properties in order to be more favorable for recovering hydrophobic secondary
62 metabolites from their matrix (Plaza and Marina 2019). In subcritical zone, London dispersion
63 forces (induced dipole-induced dipole forces) becomes more favorable than hydrogen bonding
64 and thus solvent turns less polar (Marcus 2018). Therefore, generally recognized as safe
65 (GRAS) solvents that are considered as environmentally friendly, such as ethanol, can be used
66 in PLE (Alvarez-Rivera et al. 2020).

67

68 PLE is also a faster process for extraction compared to traditional SE (Marcus 2018) due to the
69 following reasons. High pressure used increases diffusivity of solvent and enables better
70 penetration of solvent into matrix particles to reach the analytes (Teo et al. 2010). At higher
71 temperature, surface tension becomes lower which improves sample wetting and creates larger
72 contact area between solvent and sample matrix (Plaza and Turner 2017). Higher temperature
73 used in PLE compared to SE also decreases cohesive forces between solute molecules and
74 adhesive forces between solute and matrix molecules by lowering the requirement of activation
75 energy to desorb (Teo et al. 2010). Utilizing a dual mixture of solvents also gives another
76 advantage, where one solvent could act to improve analyte solubility, while the other could
77 enhance the analyte desorption (Mustafa and Turner 2011).

78

79 Until now, to the best of authors' knowledge there has been no publications on application of
80 PLE for extracting phenolic compounds from *F. vesiculosus*. Therefore, the objective of this
81 study is to investigate the effect of some PLE parameters, i.e. extraction time, temperature and
82 ratio of ethanol to water; on total phenolic compounds (TPC), dry matter yield, radical
83 scavenging activity (measured by diphenylpicrylhydrazyl/DPPH and 2,2'-azino-bis(3-
84 ethylbenzothiazoline-6-sulfonic acid)/ABTS) and also metal chelating ability. Furthermore, the
85 extraction process was optimized using mathematical models obtained from response surface
86 methodology. Optimization was done to obtain maximum amount of phenolic compounds with
87 good antioxidant properties. The extracts obtained from optimized condition were compared
88 to those produced with SE. Lastly, phenolic acids in the extracts were identified.

89

90 **Materials and methods**

91 **Sample preparation**

92 Brown seaweed *F. vesiculosus* were harvested on May 2018 from Bellevue beach,
93 (55°46'17.4"N 12° 35'48.4"E), Denmark. After harvesting, the seaweed was rinsed with
94 distilled water and its holdfasts and epiphytes were removed. The samples were frozen at -20°C
95 prior to experiment. The seaweed was freeze dried in Christ Freeze Dryer Beta 1-8 (Osterode,
96 Germany) for 2 days and then powdered with CB15VXE Waring blender (Torrington, USA)
97 and sieved to remove large particles. The powdered seaweed was stored at -20°C after being
98 purged with nitrogen to avoid oxidation.

99

100 **Pressurized Liquid Extractions**

101 *Single parameter effects screening*

102 The effects of extraction temperature [T] (75, 100, 125, 150, 175°C), extraction time [t] (5, 10,
103 15 min) and ethanol concentration in solvent [E] (40, 60, 80, 100% v/v in water) on total

phenolics content (TPC) were firstly investigated using single-factor experiment. Each parameter was optimized in triplicate experiments while other parameters were kept constant. Temperature range was chosen based on a study of PLE to extract phenolic compounds from another species of brown seaweed (*Saccharina japonica*) (Vo Dinh et al. 2018). This study concluded that maximum TPC was achieved at 125°C. Ethanol concentration range was selected based on a PLE study on *Sargassum muticum*, a brown seaweed species (Sánchez-Camargo et al. 2016). The response surface experiment of this study gave 95% ethanol as optimum for extracting phenolic compounds. Extraction time range was picked between 5 and 15 min because the first study was operated at 5 min, while the latter was run at 20 min.

Experiment started by varying temperature and maintaining extraction time of 5 min and 80% v/v ethanol. While performing extraction time variation, temperature and ethanol concentration were kept constant at 125°C and 80% v/v respectively. Lastly, temperature and time were maintained at 125°C and 5 min for ethanol concentration optimization. PLE was performed with Dionex ASE 350 Accelerated Solvent Extractor (Thermo Scientific, Leicestershire, UK). For each experiment, 1 g of dried seaweed powder and 1 g of Ottawa sand were mixed and loaded into a 10 mL extraction cell equipped with a cellulose filter on its bottom. Each experiment used maximum operating pressure of 10 bar, 50% rinsing volume and purge time of 100 s. Samples were stored at -20°C prior to characterization.

Response surface for optimization

A Box-Behnken factorial experimental design with three repetitions at the central point was used to observe the effect of each variable in single parameters screening experiments on yield, TPC and several antioxidant activities (DPPH, ABTS and metal chelating). Three levels of each variable were tested, and the values were set according to results of single parameter

screening. A total of 15 different experiments (**Table 1**) were conducted in a randomized order. In this experiment, different configuration of seaweed and Ottawa sand was used to avoid filter blockage by seaweed swelling in presence of water, where 1 g of seaweed was sandwiched between 1.5 g of sand on the bottom and top of it without any mixing. JMP Pro 15 statistical software (SAS, Cary, USA) was used for analysis of variance (ANOVA) and response surface modelling with second-order polynomial equations. Model adequacy was evaluated using the coefficient of determination (R^2) and lack-of-fit test with a 5% level of significance. Regression coefficients were generated with method of least squares to produce lowest possible residual. Optimization was done with top N pareto front search add-in in JMP with objectives of maximum TPC, DPPH and metal chelating ability. Fifty-one random sets of condition around the optimum for each antioxidant characteristic were generated by JMP and selection of the most optimum condition was done with helps of scatter plot, mixture plot and parallel plot.

Models validation and comparison with conventional solvent extraction

Validation of the optimum condition was done by conducting triplicate PLE experiments. As a comparison, conventional solvent extractions were also done in triplicate. Two kinds of solvents were used for this extraction, i.e. 80% v/v ethanol in water solution (best solvent for extracting antioxidants (Wang et al. 2012)) and ethanol solution with the same concentration as optimum PLE.

One gram of dried seaweed was submerged with 25 mL of solvent and incubated for 24 h at dark condition and room temperature on a shaker board (Heidolph Unimax 2010, Schwabach, Germany) with speed of 150 rpm. Afterwards, the mixture was centrifuged (Sartorius Sigma 4K15, Göttingen, Germany) at $2900\times g$ for 10 min. The residue was re-extracted under the same condition as above. The supernatant of re-extraction was mixed with supernatant from the day

before reaching total volume of 50 mL. Lastly, the mixture of supernatants was filtered with Whatman filter no. 4. Both extracts from validation and conventional solvent extraction were analyzed with the same assays as response surface experiments.

Extraction yield

Five mL of each extracted samples were weighed and then evaporated with nitrogen gas purging. The solution was dried further in a freeze dryer (Christ Freeze Dryer Beta 1-8, Osterode, Germany) for 1 day after being frozen in a -20°C freezer. The powder products were weighed again, and those data were used for yield calculation on dry matter basis.

Total phenolic compounds (TPC)

TPC was determined with Folic-Ciocalteu assay based on method from Farvin and Jacobsen (Sabeena Farvin and Jacobsen 2013). 100 µl of diluted extract was mixed with 750 µL of Folin–Ciocalteu reagent (10% v/v in water) in a plastic microcuvette. The mixture was incubated for 5 min and then 750 µL of sodium carbonate (Na_2CO_3 , 7.5% w/v in water) was added. This mixture was incubated at room temperature in dark condition for 90 min more. Afterwards, absorbance of this mixture was measured at 725 nm using a spectrophotometer (Shimadzu UV mini 1240, Duisburg, Germany). TPC value of each extract was measured in triplicate and reported as mean with standard deviation of all measurements. Amount of total phenolics were calculated as milligrams of gallic acid equivalents (mg GAE) per 100 g of dry seaweed based on a standard curve for gallic acid solution with concentrations from 7.8125 µg mL⁻¹ to 250 µg mL⁻¹.

DPPH radical capacity scavenging

Method for the assay was adopted from Hermund et al. (Hermund et al. 2018). An aliquot (100 μL) of extract at a concentration of 0.5 mg mL^{-1} were poured into a well in a microtiter plate. 100 μL of DPPH (0.1 mM in ethanol) was added to each well. These mixtures were incubated for 30 min at room temperature in dark condition. Afterwards, absorbance of each mixture was measured at wavelength of 517 nm in a BioTek Eon Microplate Spectrophotometer (Bad Friedrichshall, Germany). Effective concentrations for 50% inhibition (EC_{50}) were calculated with Origin 2019b software (Northampton, USA) using dose-response model.

ABTS radical capacity scavenging

ABTS assay was done using modified protocol from Stengel and Connan (Stengel and Connan 2015). 10 mL of ABTS solution (7 mM) was mixed with 10 mL of potassium persulfate (2.45 mM) and incubated at 4°C for 24 h to form ABTS^+ radical. This solution was diluted once more with ethanol until reaching absorbance of $0.7 \pm 0.02 \text{ AU}$ at 734 nm. 990 μL of this solution was poured into a 1.5 mL plastic Eppendorf tube and mixed with 10 μL of extract sample at concentration of 0.5 mg mL^{-1} . The mixture was vortexed and incubated for 6 min at 30°C and dark condition. The absorbance of each sample was measured with BioTek Eon Microplate Spectrophotometer (Bad Friedrichshall, Germany) at 734 nm by pipetting 200 μL of each solution into a well in a microtiter plate. All experiments were done in triplicate. EC_{50} values were calculated with Origin 2019b software (Northampton, USA) using dose-response model.

Metal chelating capacity

The method for measuring metal chelating ability was based on Hermund et al. (Hermund et al. 2018). In brief, 100 μL of antioxidant extracts with concentration of 1.5 mg mL^{-1} were

202 pipetted into a well of a microtiter plate. 110 μ L distilled water and 20 μ L ferrous chloride
203 solution (0.5 mM) were mixed into the samples. After 3 min of incubation, 20 μ L ferrozine
204 solution (2.5 mM) was added into each well. The plate was kept in dark for 10 min of incubation
205 at room temperature. The absorbance of each solution was measured at 562 nm in a BioTek
206 Eon Microplate Spectrophotometer (Bad Friedrichshall, Germany). Effective concentrations
207 for 50% chelating activity (EC50) were calculated with Origin 2019b software (Northampton,
208 USA) using dose-response model.

210 **Phenolic acid identification**

211 Method for phenolic acid identification was modified from Farvin and Jacobsen (Sabeena
212 Farvin and Jacobsen 2013). The extracts from model validation and conventional solvent
213 extraction were passed through a PVDF type syringe filter 0.22 μ m before being injected into
214 HPLC at a concentration of 9 mg/mL. The column used for this purpose was Prodigy 5 μ m
215 ODS-3 100 Å. The equipment consisted of an Agilent 1100 series HPLC (Agilent Technologies,
216 Waldbronn, Germany) and a diode array detector (Agilent G1315A).

218 Two types of mobile phases were used for this analysis: phosphoric acid in deionized water
219 with pH value of 3 (A), and 1:1 mixture of methanol and acetonitrile (B). The gradient elution
220 analysis program was as follows: 0–2 min, 5% (B); 2–20 min, increasing to 40% (B); 20–35
221 min, increasing to 100% (B); 35–37 min, decreasing to 5% (B), with 3 min of posttime at a
222 flow rate of 0.9 mL min⁻¹. Injection volume of sample was set at 20 μ L and the column was
223 maintained at 25°C. Detection with the diode array detector was done at four different
224 wavelenghts, i.e. 235, 255, 280 and 325 nm with reference wavelenght of 360 nm.
225 Quantification and identification of each phenolic acid was done by producing calibration
226 curve by injecting phenolic acids mixture standards with concentration of each phenolic acid

(gallic, caffeic, chlorogenic, gentisic, vanillic and protocatechuic acid) from 5.21 to 166.67 $\mu\text{g mL}^{-1}$.

Table 1

Results

Single parameter effects on TPC

Effect of each single parameter effect on TPC value of extract is shown in **Fig. 1**. The shortest extraction time which was 5 min gave significantly higher TPC (2.82 ± 0.1 g GAE (100 g dry seaweed) $^{-1}$) than 10 and 15 min ($p < 0.05$). Furthermore, TPC value reached maximum at 100 and 125°C with significantly lower values at higher and lower temperature points tested ($p < 0.05$). When ethanol concentration in solvent was varied, the significant highest phenolic content was gained at 60% v/v ethanol with a value of 5.17 ± 0.28 g GAE (100 g dry seaweed) $^{-1}$ ($p < 0.05$).

Fig. 1

These results of single parameter effects were utilized to define levels of each parameter in experimental design. For temperature, optimization around 125°C was done with narrower range from 110 to 140°C. Extraction time less than 5 min was investigated for a hypothesis of giving higher TPC value due to less phenolic compounds degradation. Therefore, time optimization was done between 3 and 7 min. Range for ethanol concentration chosen was between 40 to 80% v/v because antioxidant activity on these points should be inspected although the TPC values were high. The assigned experimental design for response surface experiments are shown in **Table 1**.

Response surface model results

Experimental results of extraction yield and antioxidant properties using Box-Behnken design are shown in **Table 1**. Analysis of variance (ANOVA) was done to screen the significance of each independent variable and their derivations for overall models built as shown in **Table S.1**. The models were refitted without statistically insignificant effects to obtain more precise estimate of the model parameters. Extraction time was not significant, but still be included in the model to be optimized. The developed mathematical models for predicting extraction yield and each antioxidant property are shown in Equation (1) through (5). These models were used to predict each parameter value and the results were compared with actual experimental data (**Table S.2**). Little differences were found between them which showed the adequacy of the models. ANOVA test for each equation (**Table S.3**) proved that all models were statistically significant with p -values lower than 0.05. On the other hand, p -values of lack of fit in each model were higher than 0.05 which showed that lack of fit, relative to pure error, were not significant.

$$Yield(\%) = -236.8104 + 4.8423 E + 1.4988 T + 0.5187 t - 0.0188 E^2 - 0.0209 E.T \quad \dots(1)$$

$$TPC = -39.3426 + 0.8207 E + 0.2542 T + 0.0175 t - 0.0028 E^2 - 0.0037 E.T \quad \dots(2)$$

$$DPPH = 113.7723 - 0.3457 E - 0.2125 T - 0.0563 t + 0.0018 E^2 - 0.0022 E.T \quad \dots(3)$$

$$ABTS = 65.8006 - 0.1324 E - 0.5158 T - 0.725 t - 0.0052 E^2 + 0.0066 E.T \quad \dots(4)$$

$$Fe^{2+}Chelating\ Ability = 366.6935 - 4.9714 E - 2.4633 T - 0.7125 t - 0.0020 E^2 + 0.0382 E.T \quad \dots(5)$$

Fig. 2 presents response surface results of the effect of extraction temperature, time and ethanol concentration in solvent on extraction yield, TPC value, DPPH inhibition activity, ABTS inhibition activity and metal chelating activity. **Fig. 2** a and b shows that highest extraction

yield could be achieved by higher extraction temperature and lower ethanol concentration. As shown in three-dimensional response surface graphs in **Fig. 2 c** and **d**, high temperature and low ethanolic solution were preferred to obtain highest TPC, which was similar to findings for yield. Interaction between temperature and ethanol concentration was observed here, where increasing temperature on low ethanol concentration can raise TPC value, while increasing temperature on high ethanol concentration will reduce TPC value.

Maximum DPPH activity of phenolic compounds in extracts can be achieved when higher ethanol concentration and lower temperature were applied to the seaweed powder (see **Fig. 2 e** and **f**). These graphs also show that extract with high TPC value did not possess good DPPH radical scavenging activity. Higher temperature and lower ethanolic content in solvent gave negative effect on the ABTS inhibition activity as shown in **Fig. 2 g** and **h**. On the other hand, **Fig. 2 i** and **j** depicts that highest activity of metal chelating could be accomplished with lower temperature and lower ethanol concentration. However, extract with high TPC value or DPPH scavenging activity was not a good metal chelating agent.

Fig. 2

Optimization

Based on the mathematical models for each parameter, 51 combinations of temperature, extraction time and ethanol concentration which gave near-maximum value of each parameter were generated (**Table S.4**). Four potential solutions were chosen from these combination sets with the aid of mixture plots and a parallel plot (**Fig. S.1**, **Fig S.2** and **Fig. S.3**). These four potential sets were then plotted in a scatter plot together with other solution sets as shown in

Fig. 3, while the independent variables, predicted values of antioxidant properties and desirability of all these potential solutions were listed in **Table 2**. In this study, objectives of optimization were maximum values of TPC, DPPH and metal chelating ability. Yield were not considered on the optimization since it had the same profile as TPC, while ABTS radical scavenging activity could be represented by DPPH. As the aim for this study was to extract maximum amount of phenolic compounds, optimization was more emphasized on TPC than other antioxidant properties.

Fig. 3

Table 2

The first potential solution (colored pink in **Fig. 3**) had the highest TPC value, but DPPH value of this solution was one of the lowest and the metal chelating ability was also quite low with desirability of 0.52. The second potential solution (blue dot in **Fig. 3**) possessed one of the highest DPPH scavenging ability, but it was extract with the lowest metal chelating ability and intermediate TPC value. The third potential solution which was indicated by green dot in **Fig. 3** achieved the highest total desirability among them. It owned one of the highest metal chelating capability and moderate DPPH inhibition value. This extract contained nonetheless one of the lowest phenolic compounds with desirability value of 0.59. The last potential solution which colored in orange was one of the extracts with highest extraction yield of phenolic compound. Metal chelating ability of this extract was in average value, but the antioxidant capability of scavenging DPPH radical was one of the lowest with desirability value of 0.31. However, the DPPH inhibition percentage range of all combination sets was quite narrow. The DPPH inhibition activity of this last combination was 87.57% which indicated

good antioxidant radical scavenging ability, and together with high value of TPC and moderate value of metal chelating ability, this solution was chosen for the optimized condition. Therefore, optimized PLE conditions was found to be temperature of 137.18°C, ethanol concentration of 58.65% v/v and extraction time of 4.68 min.

Models validation and comparison with SE

Optimized condition was validated by performing triplicate experiments whose results were represented in **Table 3**, as well as comparison with the predicted value from the models. The validation suggested that the mathematical models produced were quite accurate and robust to predict all antioxidant properties with little error. The results of comparison of the this optimized PLE condition with SE were shown in **Table 4**. In comparison, SE with the same ethanol concentration as PLE yielded nearly the same amount of extract as the PLE process and gave nearly the same TPC value ($p > 0.05$). Meanwhile, SE with 80% ethanol resulted in lower phenolic content and powder yield compared to the two previously aforementioned extraction methods. For DPPH and ABTS radical scavenging ability, EC50 values of PLE were higher than SE with the same ethanol concentration. This finding means that more antioxidant extract obtained with PLE was needed to achieve the same activity as SE with optimized ethanol concentration extract. Moreover, in terms of metal chelating ability, SE extract with optimized ethanol concentration was found as a worse metal chelator than the one extracted using 80% ethanol as observed in the EC50 value. Extraction with pressurized liquid was also found to lower the metal chelating ability compared to SE.

Table 3

Table 4

Phenolic acid identification

Identification of phenolic acid was done for six phenolic acid compounds which were reported in previous study to exist in *F. vesiculosus* (Sabeena Farvin and Jacobsen 2013). The quantification results for different extraction method and solvent are shown in **Fig. 4**. Chlorogenic, vanillic and caffeic acids were not detected in all samples. More gallic acid was extracted using SE with 58.65% ethanol ($335.1 \pm 14.4 \mu\text{g (g DW)}^{-1}$) compared to PLE with the same ethanol concentration and SE with 80% ethanol.

Fig. 3

Discussion

F. vesiculosus as a brown seaweed species contains polyphenolic compounds, dominantly in the form of phlorotannins (polymer of phloroglucinol) (Wang et al. 2012). Phenolic compounds in polymer forms are more prone to degradation (De Paepe et al. 2014). These polyphenol compounds could degrade into soluble compounds and reversibly bonded phenolic were broken because of protein precipitation (Pangestuti et al. 2019). Therefore, high temperature might decrease TPC value due to degradation of these phlorotannins compounds as shown in **Fig. 1** where TPC decreased after reaching highest value at 100 and 125°C. However, the optimum temperature obtained for phenolic extraction in this study was a value high enough to enhance extraction yield but low enough to prevent degradation of the phenolic compounds (Catarino et al. 2019). Meanwhile, ethanol in a concentration of 40% v/v performed better for phenolic extraction with PLE which was different from SE. In SE, pure water is better for phenolic extraction compared to 80% ethanol in water (Koivikko et al. 2005). Solvent physical

properties change a lot in subcritical condition, and the properties of 60% ethanol which gave maximum result might be similar to the best solvent for SE, which was 70% acetone in water (Koivikko et al. 2005).

The profile of obtaining higher yield by applying higher PLE temperature and lower ethanol concentration was also observed by several studies on PLE extraction of phenolic compounds from olive pomace (Pavez et al. 2019), goji berry (Tripodo et al. 2018) and jabuticaba skin (Santos et al. 2012). High temperature can enhance yield by improving wetting of sample matrix, disrupting analyte-matrix interactions and boosting diffusivity. However, the high yield on high temperature might also show co-extraction of some undesired compounds with the phenolic compounds (Plaza and Turner 2017). For obtaining highest TPC, the same condition for highest yield was preferred. Interaction between extraction temperature and ethanol concentration found in this study was also discovered by another study on PLE of flavonoid from spinach (Howard and Pandjaitan 2008). They examined that TPC linearly increased in ethanolic extracts over the temperature range of 90 to 170°C, and then declined from 170 to 190°C. In water extracts, TPC kept increasing linearly over the temperature range of 110 to 190°C. Extraction time was not a significant parameter for TPC as also observed by Luthria (Luthria 2012).

In terms of DPPH radical scavenging activity, higher ethanol concentration and lower temperature were better to gain higher activity. The similar effect of ethanol concentration was discovered while extracting phenolic compounds from beetroot leaves where ethanol/water mixture gave less antioxidant reducing capacity than pure ethanol solvent (Battistella Lasta et al. 2019). Reducing DPPH activity trend when escalating temperature was also observed on another extraction experiment from walnut seed (Kamali et al. 2018). This profile can be

explained by lower selectivity of extraction in higher temperature at temperature range of 110-140°C and lower ethanol concentration in solvent. Although TPC value for these extracts were high, water in the solvent and high temperature enabled co-extraction of some water-soluble compounds from the sample matrix due to higher diffusivity of these contaminant compounds and increasing polarity of solvent. Amarante et. al. found out that phlorotannin amount in *F. vesiculosus* extracts obtained with microwave-assisted extraction with high temperature was lower than the ones collected from conventional extraction at room temperature (Amarante et al. 2020). They suggested that some lipophilic compounds such as fatty acids, sterols and fucoxanthin pigments were co-extracted at high temperature.

TPC assay might also detect other compounds than phenolic that have reducing capabilities (Jacobsen et al. 2019) such as laminarin (Olatunji 2020; Yugay et al. 2020) and fucoidan (Yugay et al. 2020). Laminarin and fucoidan capability of inhibiting DPPH radical was found to be lower than phenolic compounds extracted here, with inhibition of 1.09% for laminarin and 66.13% for fucoidan at a concentration of 1 mg/mL (Moroney et al. 2015). Although some simple sugars and ascorbic acid might also be co-extracted into the extracts, these compounds were proven to be unable to significantly interfere Folin-Ciocalteu causing overestimation of TPC values (Castro-Alves and Cordenunsi 2015).

When Ferreira et al. (Ferreira et al. 2019) extracted bioactive compounds from *F. vesiculosus* using SE with water as solvent, they also observed lower antioxidant ABTS inhibition for hot-water extracts which might be caused by phlorotannin degradation because of high temperature. ABTS cation is able to react with both lipophilic and hydrophilic antioxidant because it is soluble in aqueous and organic solvents (Stengel and Connan 2015). This fact often makes its activity higher than DPPH. However, ABTS inhibition activities in this study were always

lower than the DPPH ones for the same extract (**Table 1**). This phenomenon was also noticed for fucose-containing sulphated polysaccharides extracts from *F. vesiculosus* with higher difference of ABTS and DPPH scavenging activity on extracts with higher TPC value (Imbs et al. 2014). The possible reason for this could be antioxidant compounds in the extracts were not very reactive towards ABTS radical cation or not enough time for complete reaction during the incubation time (Ilyasov et al. 2020).

Water extract of *F. vesiculosus* was previously demonstrated to possess higher metal chelating ability than ethanolic extract (Sabeena Farvin and Jacobsen 2013; Hermund et al. 2018). This profile might indicate that phenolic compounds were not the major contributors to the metal chelating properties (Wang et al. 2012; Sabeena Farvin and Jacobsen 2013). Hence, metal chelating abilities of the extracts produced in this study might come from co-extracted compounds in low ethanolic solvent. A good metal chelator forms σ -bonds with metal which can reduce redox potential and thereby stabilizing the complex formed, and usually they are indicated by functional groups such as $-\text{OH}$, $-\text{SH}$, $-\text{COOH}$, $-\text{PO}_3\text{H}_2$, $\text{C}=\text{O}$, $-\text{NR}_2$, $-\text{S}-$ and $-\text{O}-$ (Yuan et al. 2005; Terpin et al. 2012). Most of the phenolic compounds have only hydroxyl groups among these potential functional groups which explains the low metal chelating ability.

The equations generated to predict the values of extraction yield, TPC, DPPH and ABTS radical scavenging activities at 0.5 mg mL^{-1} , and metal chelating ability at 1.5 mg mL^{-1} were proven by validation experiments to be quite accurate with little error. These errors might come because of the models' uncertainty as well as equipment limitation where decimal values could not be inputted, consequently extractions were done at temperature of 137°C and time of 5 min.

The extraction yield of the optimized PLE condition was higher than polyphenols yield of PLE from *Fucus spiralis* using 80% ethanol in water (23.67% dry weight) (Tierney et al. 2013). This discrepancy might come from the different composition between different species and lower temperature that they used (100°C). The amount of water in the solvent used in this study was also higher, which enabled co-extraction of some water-soluble compounds. This fact shows the superiority of PLE compared to SE because the latter required longer extraction time (48 h compared to 5 min for PLE) and higher solvent requirement (solvent to solid ratio of 50 mL:1 g compared to 17 mL:1 g for PLE). Even though PLE requires energy to heat and pressurize the system, SE also requires energy to agitate the mixture for a long time. The fact that SE with lower ethanol concentration could produce more extract than the higher one was also observed in other macroalgae (Monteiro et al. 2020).

Phenolic content of the SE extract with 80% ethanol was lower than the value reported for polyphenols extraction from *F. vesiculosus* harvested from the same place and the same extraction method as this study (16.5 g GAE/100 g) (Hermund et al. 2018). This can be explained by the difference of harvesting month, where this study used seaweed harvested in May whereas the former harvested in September. TPC value of *F. vesiculosus* varied during the season with the highest value achieved in early autumn (September) (Connan et al. 2004). On the other hand, SE using the same solvent as PLE gave nearly the same TPC value ($p > 0.05$), again confirming the advantages of PLE.

Radical scavenging ability of the phenolic PLE extracts in this study were lower than some literatures as shown with DPPH and ABTS EC50 comparison (Sabeena Farvin and Jacobsen 2013; Hermund et al. 2018; Ferreira et al. 2019). This study reported higher EC50 value for both stable radicals which mean more antioxidant was needed to achieve the same inhibition

level. The variation might be resulted from the lower selectivity of PLE compared to SE which was already shown by low DPPH inhibition activity for sample with high yield and TPC value. In comparison, DPPH and ABTS EC₅₀ values for PLE were higher than SE with the same ethanol concentration as also observed on another study (Tierney et al. 2013).

The metal chelating EC₅₀ value of extract from SE on another study was reported to be 87.7 $\mu\text{g mL}^{-1}$ for 80% ethanolic extract which was quite similar to the result of the same experiment in this study (Hermund et al. 2018). Some example of good natural chelators are citric acid, phospholipids and amino acid/peptides/proteins (Berdahl et al. 2010). Aqueous extract of *F. vesiculosus* has been proven to contain small peptides, with molecular weight between 204 and 740 Da (André et al. 2020). Phospholipids are best extracted using organic solvent with high polarity such as ethanol, acetone and ethyl acetate (Zhou and Rakariyatham 2018). Therefore, SE extract using 80% ethanol had highest metal chelation ability because more phospholipids and peptides with similar polarity extracted. PLE samples might have low metal chelating ability because large molecules of phospholipids and peptides require longer times to be extracted.

In a previous study, vanillic and caffeic acids were only identified in extract produced by SE using pure water, while chlorogenic acid was detected in ethanolic extract but in a much smaller amount than other phenolic acids (Sabeena Farvin and Jacobsen 2013). Caffeic acid solubility decreases monotonically with increasing concentration of ethanol in water-ethanol mixture (Zuorro et al. 2019) which explains absence of caffeic acid in all extracts which were extracted with ethanolic solvents. It was also reported in previous study that ethanolic extracts generally contained higher concentration of phenolic acids but lower number of different phenolic acids

when compared to water extracts (Sabeena Farvin and Jacobsen 2013). This phenomenon was not found in this study due to smaller range of polarity difference between the solvent used.

The discovery that SE with 58.65% ethanol extracted more gallic acid than PLE with the same ethanol concentration and SE with 80% ethanol showed that gallic acid polarity is closer to the polarity of 58.65% ethanol solution rather than other solutions studied. Gallic acid is classified as a polar phenolic compound; thus it is more soluble in solvent of higher polarity (Yeop et al. 2017). Polarity of solvent in PLE decrease because of diminishing hydrogen bond strength in subcritical area (Marcus 2018), which explained lower concentration of gallic acid in PLE for the same solvent. The polarity of 58.65% ethanol in PLE condition of this study might be similar to 80% ethanol in unpressurized condition, which was shown by similar amount of gallic acid extracted for these two conditions.

Conclusions

In this study, phenolic compounds with high antioxidant activity have been successfully extracted from *Fucus vesiculosus* with PLE method. Effect of temperature, ethanol concentration and extraction time on extraction yield, TPC, DPPH and ABTS radicals scavenging activity and metal chelating activity have been modelled using Box Behnken experimental design and proved to be statistically significant with ANOVA analysis. These models can act as a cornerstone for different aims, either for maximizing TPC or maximizing antioxidant properties, with a little error. However, this study focused on maximizing the extraction of phenolics with average antioxidant activities. The models could predict phenolic compounds yield and antioxidant activities values with little error, which showed the robustness and accuracy of the mathematical models. This study also observed that PLE could extract phenolic compounds with higher yield and TPC compared to SE although SE required

longer extraction time and higher solvent requirement. The extract obtained from PLE had the same radical scavenging ability and lower metal chelating ability in comparison with SE extract. Moreover, when the PLE extract was analysed with HPLC, gallic, protocatechuic and gentisic acids were detected.

This study was one of the first to apply PLE for extracting phenolic compounds from *F. vesiculosus*. Therefore, many further studies are still needed to give better understanding especially to justify the low selectivity of PLE with analysis of polysaccharides and proteins in the samples. For implementation of PLE in a larger scale, a study is needed to avoid filter blockage observed in this study due to water presence in solvent. It is interesting to study PLE as one stage of biorefinery to extract more than one product together with other extraction method, e.g. enzymatic assisted extraction, and observe the effect of this combination on the antioxidant properties. Finally, application of antioxidant extract from PLE in food or pharmaceutical products are needed to study the efficacy of this extract to inhibit lipid oxidation.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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Figure Captions

Fig. 1 Effect of extraction time, temperature and ethanol concentration in solvent on TPC value of extracts (mean $\pm$ SD; n = 6)

Fig. 2 Effect of extraction temperature, time and ethanol concentration in solvent on (a) and (b) extraction yield; (c) and (d) TPC value; (e) and (f) DPPH inhibition activity at 0.5 mg mL⁻¹; (g) and (h) ABTS inhibition activity at 0.5 mg mL⁻¹; (i) and (j) metal chelating activity at 1.5 mg mL⁻¹

Fig. 3 Scatter plot for all 51 randomized set of solutions, pink color: first potential solution, blue color: second potential solution, green color: third potential solution, orange color: fourth potential solution.

Fig. 4. Amount of six phenolic acids in extracts (mean $\pm$ SD; n = 3) obtained from PLE and SE

Fig. 1

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

Fig. 2

Fig. 3

Fig. 4

726 **Table 1.** Box-Behnken experimental design and experiment results (mean $\pm$ SD; n=3) of extract antioxidant properties with variations of ethanol
727 concentration, temperature and extraction time

Extract No.	Independent Variables			Dry Basis Yield (%)	TPC (g GAE (100 g DW) ⁻¹)	DPPH at 0.5 mg mL ⁻¹ (%) inhibition)	ABTS at 0.5 mg mL ⁻¹ (%) inhibition)	Fe ²⁺ Chelating Ability at 1.5 mg mL ⁻¹ (%)
	Ethanol concentration (%v/v) - E	Temperature (°C) - T	Time (min) - t					
1	40 (-1)	110 (-1)	5 (0)	4.49	0.74 $\pm$ 0.02	87.5 $\pm$ 1.0	21.2 $\pm$ 5.3	56.8 $\pm$ 7.3
2	40 (-1)	140 (1)	5 (0)	22.32	4.52 $\pm$ 0.06	86.0 $\pm$ 0.3	12.4 $\pm$ 0.8	21.9 $\pm$ 3.4
3	80 (1)	110 (-1)	5 (0)	13.78	3.17 $\pm$ 0.06	92.0 $\pm$ 0.6	19.6 $\pm$ 1.7	35.0 $\pm$ 12.0
4	80 (1)	140 (1)	5 (0)	6.56	2.51 $\pm$ 0.06	93.1 $\pm$ 0.8	18.7 $\pm$ 1.7	32.6 $\pm$ 15.8
5	60 (0)	110 (-1)	3 (-1)	11.63	3.43 $\pm$ 0.11	90.7 $\pm$ 0.8	24.1 $\pm$ 2.1	31.8 $\pm$ 1.5
6	60 (0)	110 (-1)	7 (1)	14.06	4.06 $\pm$ 0.19	90.9 $\pm$ 0.5	19.6 $\pm$ 4.2	34.7 $\pm$ 5.5
7	60 (0)	140 (1)	3 (-1)	18.55	3.99 $\pm$ 0.10	86.6 $\pm$ 4.1	20.2 $\pm$ 1.4	46.9 $\pm$ 3.9
8	60 (0)	140 (1)	7 (1)	26.09	4.24 $\pm$ 0.04	85.5 $\pm$ 2.8	18.7 $\pm$ 0.7	32.8 $\pm$ 0.4
9	40 (-1)	125 (0)	3 (-1)	12.38	2.30 $\pm$ 0.06	86.8 $\pm$ 1.9	18.3 $\pm$ 0.6	52.5 $\pm$ 5.3
10	80 (1)	125 (0)	3 (-1)	12.52	3.08 $\pm$ 0.11	91.8 $\pm$ 1.0	22.4 $\pm$ 1.2	28.3 $\pm$ 2.0
11	40 (-1)	125 (0)	7 (1)	10.30	1.62 $\pm$ 0.09	86.7 $\pm$ 1.3	16.3 $\pm$ 0.8	49.0 $\pm$ 1.7
12	80 (1)	125 (0)	7 (1)	12.93	3.16 $\pm$ 0.10	91.9 $\pm$ 0.6	18.8 $\pm$ 2.8	24.1 $\pm$ 7.1
13	60 (0)	125 (0)	5 (0)	18.76	3.12 $\pm$ 0.17	89.5 $\pm$ 1.4	21.7 $\pm$ 0.7	43.4 $\pm$ 2.4
14	60 (0)	125 (0)	5 (0)	20.50	3.91 $\pm$ 0.14	89.7 $\pm$ 1.6	19.4 $\pm$ 0.1	36.7 $\pm$ 2.9
15	60 (0)	125 (0)	5 (0)	26.42	3.67 $\pm$ 0.07	88.5 $\pm$ 1.6	20.0 $\pm$ 0.5	38.7 $\pm$ 5.7

728

Table 2. Independent variables with predicted values and desirability of each antioxidant property for four selected potential solutions

Color in Fig. 3	Ethanol Conc. (% v/v)	T (°C)	Time (min)	TPC (g GAE (100 g DW) ⁻¹)	DPPH at 0.5 mg mL ⁻¹ (%)	Fe ²⁺ Chelating Ability at 1.5 mg mL ⁻¹ (%)	Desirability	TPC Desirability	DPPH Desirability	Fe ²⁺ Chelating Ability Desirability
Pink	52.49	140.00	7.00	4.420	86.25	30.78	0.5007	0.8564	0.1857	0.5179
Blue	71.59	114.02	6.82	3.741	91.11	26.22	0.6035	0.7087	0.6691	0.4029
Green	56.74	113.69	5.03	3.188	89.36	40.66	0.6154	0.5907	0.4855	0.7190
Orange	58.65	137.18	4.68	4.193	87.57	33.97	0.5991	0.8070	0.3106	0.5873

Table 3. Actual experiment results of optimized PLE (mean $\pm$ SD; n=3, except n=9 for TPC) and the predicted value from mathematical models

Parameter	Actual	Predicted
Dry Basis Yield (%)	31.16 $\pm$ 3.20	22.6
TPC (g GAE (100 g DW) ⁻¹)	3.69 $\pm$ 0.30	4.19
DPPH at 0.5 mg mL ⁻¹ (% inhibition)	86.7 $\pm$ 1.9	87.6
ABTS at 0.5 mg mL ⁻¹ (% inhibition)	16.2 $\pm$ 1.3	19.1
Fe ²⁺ Chelating Ability at 1.5 mg mL ⁻¹ (%)	46.3 $\pm$ 6.8	33.4

Table 4. Comparison of extraction yield and each antioxidant properties between PLE and SE (mean $\pm$ SD; n=3, except n=9 for TPC)

Extract	Dry Basis Yield (%)	TPC (g GAE (100 g DW) ⁻¹)	DPPH EC50 (μ g mL ⁻¹)	ABTS EC50 (mg mL ⁻¹)	Fe ²⁺ Chelating EC50 (μ g mL ⁻¹)
Optimized PLE	31.16 $\pm$ 3.20	3.69 $\pm$ 0.30	92.6 $\pm$ 11.5	2.35 $\pm$ 0.15	1100.2 $\pm$ 103.5
SE with optimized ethanol concentration	30.75 $\pm$ 2.36	3.41 $\pm$ 0.24	86.6 $\pm$ 5.6	2.11 $\pm$ 0.23	334.1 $\pm$ 152.5
SE with 80% v/v ethanol	20.35 $\pm$ 1.15	2.53 $\pm$ 0.18	97.2 $\pm$ 47.0	2.74 $\pm$ 0.74	96.6 $\pm$ 24.0

749 **Table S.1.** Analysis of variance (ANOVA) of each independent variable and their derivations for
750 overall models built

Parameter	<i>p</i> value before insignificance removal	<i>p</i> value after insignificance removal
E	0.00384	0.00014
E*E	0.00971	0.00161
E*T	0.01342	0.00172
T	0.03700	0.01475
E*t	0.18040	
t	0.21223	0.18807
T*T	0.29031	
t*t	0.33431	
T*t	0.52089	

751
752 Using *p* value of 0.05 as limit of statistical significance, terms of (E*t), (T*T), (t*t) and (T*t)
753 were not statistically significant to the models produced. They were excluded from the
754 mathematical models, while time was still included although it was not significant because it
755 needed to be optimized.

756

Table S.2. Comparison between actual experimental and predicted results from mathematical models

Extract No.	Independent Variables			Dry Basis Yield (%)		TPC (g GAE (100 g DW) ⁻¹)		DPPH at 0.5 mg mL ⁻¹ (% inhibition)		ABTS at 0.5 mg mL ⁻¹ (% inhibition)		Fe ²⁺ Chelating Ability at 1.5 mg mL ⁻¹ (%)	
	E (%v/v)	T (°C)	t (min)	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	40	110	5	4.49	2.42	0.74 ± 0.02	0.70	87.5 ± 1.0	88.6	21.2 ± 5.3	20.8	56.8 ± 7.3	58.0
2	40	140	5	22.32	22.33	4.52 ± 0.06	3.89	86.0 ± 0.3	84.9	12.4 ± 0.8	13.3	21.9 ± 3.4	29.9
3	80	110	5	13.78	14.02	3.17 ± 0.06	3.61	92.0 ± 0.6	92.8	19.6 ± 1.7	19.7	35.0 ± 12.0	17.3
4	80	140	5	6.56	8.88	2.51 ± 0.06	2.35	93.1 ± 0.8	91.6	18.7 ± 1.7	20.0	32.6 ± 15.8	35.0
5	60	110	3	11.63	14.70	3.43 ± 0.11	3.26	90.7 ± 0.8	90.1	24.1 ± 2.1	23.8	31.8 ± 1.5	39.9
6	60	110	7	14.06	16.77	4.06 ± 0.19	3.33	90.9 ± 0.5	89.9	19.6 ± 4.2	20.9	34.7 ± 5.5	37.1
7	60	140	3	18.55	22.09	3.99 ± 0.10	4.22	86.6 ± 4.1	87.6	20.2 ± 1.4	20.2	46.9 ± 3.9	34.7
8	60	140	7	26.09	24.16	4.24 ± 0.04	4.29	85.5 ± 2.8	87.4	18.7 ± 0.7	17.3	32.8 ± 0.4	31.9
9	40	125	3	12.38	11.33	2.30 ± 0.06	2.26	86.8 ± 1.9	86.9	18.3 ± 0.6	18.5	52.5 ± 5.3	45.4
10	80	125	3	12.52	10.41	3.08 ± 0.11	2.94	91.8 ± 1.0	92.3	22.4 ± 1.2	21.3	28.3 ± 2.0	27.6
11	40	125	7	10.30	13.41	1.62 ± 0.09	2.33	86.7 ± 1.3	86.6	16.3 ± 0.8	15.6	49.0 ± 1.7	42.5
12	80	125	7	12.93	12.48	3.16 ± 0.10	3.01	91.9 ± 0.6	92.1	18.8 ± 2.8	18.4	24.1 ± 7.1	24.8
13	60	125	5	18.76	19.43	3.12 ± 0.17	3.77	89.5 ± 1.4	88.8	21.7 ± 0.7	20.5	43.4 ± 2.4	35.9
14	60	125	5	20.50	19.43	3.91 ± 0.14	3.77	89.7 ± 1.6	88.8	19.4 ± 0.1	20.5	36.7 ± 2.9	35.9
15	60	125	5	26.42	19.43	3.67 ± 0.07	3.77	88.5 ± 1.6	88.8	20.0 ± 0.5	20.5	38.7 ± 5.7	35.9

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759 These mathematical models produced by response surface methodology were used to predict the yield and values of each antioxidant
760 property for variation of independent variables. The prediction values were very close to the experimental results which proved the
761 adequacy of the models.

762 **Table S.3.** Analysis of variance (ANOVA) of the fitted second-order polynomial models for all
763 antioxidant properties

	Sum of squares	DF	Mean square	F-value	p-value
Yield^a					
Model	0.0487544	5	0.00975	8.0803	0.0038
Error	0.0108608	9	0.001207		
Lack of Fit	0.0076358	7	0.001091	0.6765	0.7086
Pure error	0.003225	2	0.001612		
Total	0.070476	23			
Total Phenolic Content^b					
Model	12.563619	5	2.51272	10.0777	0.0017
Error	2.244021	9	0.24934		
Lack of Fit	1.9159548	7	0.273708	1.6686	0.4249
Pure error	0.3280667	2	0.164033		
Total	17.051662	23			
DPPH^c					
Model	75.295548	5	15.0591	10.1737	0.0017
Error	13.321786	9	1.4802		
Lack of Fit	12.495119	7	1.78502	4.3186	0.2009
Pure error	0.826667	2	0.41333		
Total	101.93912	23			
ABTS^d					
Model	90.6013	5	18.1203	14.4987	0.0004
Error	11.24804	9	1.2498		
Lack of Fit	8.401369	7	1.2002	0.8432	0.6399
Pure error	2.846667	2	1.42333		
Total	113.09738	23			
Iron Chelating Ability^e					
Model	1227.3038	5	245.463	9.1196	0.0025
Error	242.2436	9	26.9160		
Lack of Fit	218.58357	7	31.2262	2.6396	0.3021
Pure error	23.66	2	11.8300		
Total	1711.791	23			

764 a. R²: 0.817818; mean: 0.154193; adj R²: 0.716606

765 b. R²: 0.848455; mean: 3.168; adj R²: 0.764264

766 c. R²: 0.849671; mean: 89.14667; adj R²: 0.766154

767 d. R²: 0.889562; mean: 16.24; adj R²: 0.828208

768 e. R²: 0.835159; mean: 19.42667; adj R²: 0.74358

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Table S.4. Generated random points near optimum conditions for optimization

No.	Ethanol Concentration (% v/v)	T (°C)	Time (min)	Yield	TPC (g GAE (100 g) ⁻¹)	DPPH at 0.5 mg mL ⁻¹ (% inhibition)	ABTS at 0.5 mg mL ⁻¹ (% inhibition)	Fe ²⁺ Chelating Ability at 1.5 mg mL ⁻¹ (%)	Desirability	Yield Desirability	TPC Desirability	DPPH Desirability	ABTS Desirability	Fe ²⁺ Chelating Ability Desirability
1	47.18	130.92	6.17	0.203	3.580	86.59	16.71	36.48	0.5080	0.7004	0.6740	0.2180	0.5148	0.6387
2	52.56	127.10	5.41	0.196	3.622	87.63	19.06	37.82	0.5773	0.6743	0.6831	0.3160	0.6625	0.6651
3	72.74	138.77	3.29	0.149	3.295	90.13	21.32	35.43	0.6085	0.4972	0.6132	0.5652	0.7842	0.6176
4	78.86	129.47	6.86	0.126	2.950	91.68	18.69	27.91	0.5426	0.4142	0.5411	0.7298	0.6408	0.4485
5	77.87	128.29	5.56	0.129	3.071	91.59	19.72	28.58	0.5626	0.4242	0.5662	0.7206	0.6993	0.4657
6	55.69	124.90	3.66	0.185	3.620	88.30	21.11	38.75	0.6129	0.6295	0.6826	0.3812	0.7733	0.6828
7	51.78	136.57	6.69	0.241	4.195	86.52	16.35	32.56	0.5234	0.8478	0.8074	0.2105	0.4894	0.5574
8	68.34	129.59	3.78	0.176	3.704	89.80	21.34	33.58	0.6324	0.5981	0.7008	0.5309	0.7850	0.5791
9	48.35	133.51	5.12	0.214	3.832	86.50	17.23	35.43	0.5211	0.7428	0.7285	0.2088	0.5504	0.6176
10	58.00	114.48	6.17	0.170	3.333	89.35	20.93	38.56	0.6174	0.5734	0.6213	0.4853	0.7637	0.6792
11	70.81	116.14	3.50	0.160	3.670	91.06	22.22	29.80	0.6331	0.5377	0.6935	0.6630	0.8303	0.4954
12	59.31	133.24	4.22	0.212	4.033	88.03	20.01	35.10	0.6150	0.7350	0.7722	0.3549	0.7151	0.6109
13	58.80	126.24	3.54	0.190	3.770	88.59	21.34	37.18	0.6278	0.6508	0.7149	0.4091	0.7850	0.6526
14	52.22	119.65	4.25	0.159	3.130	88.39	21.13	42.27	0.5870	0.5329	0.5784	0.3900	0.7744	0.7487
15	71.19	135.00	4.12	0.165	3.503	89.98	20.84	33.84	0.6164	0.5551	0.6574	0.5495	0.7592	0.5845
16	67.44	123.61	6.69	0.190	3.768	89.88	19.64	31.11	0.6195	0.6493	0.7145	0.5388	0.6949	0.5254
17	69.89	138.41	5.70	0.183	3.618	89.43	19.47	33.54	0.6078	0.6219	0.6823	0.4933	0.6854	0.5783
18	58.66	137.18	4.68	0.226	4.193	87.57	19.07	33.97	0.5991	0.7908	0.8070	0.3106	0.6632	0.5873
19	47.61	138.39	4.64	0.234	4.164	85.91	16.41	32.68	0.4886	0.8223	0.8007	0.1530	0.4937	0.5598
20	79.86	134.74	5.55	0.102	2.601	91.75	19.59	31.58	0.5317	0.3303	0.4700	0.7383	0.6921	0.5358
21	63.99	111.06	5.32	0.169	3.560	90.35	21.81	34.15	0.6405	0.5717	0.6699	0.5884	0.8095	0.5911
22	41.65	123.74	6.58	0.136	2.405	86.93	16.65	43.32	0.4521	0.4476	0.4309	0.2496	0.5108	0.7680
23	58.31	124.15	5.32	0.193	3.710	88.60	20.27	36.60	0.6170	0.6637	0.7022	0.4104	0.7292	0.6410
24	61.66	136.65	6.81	0.227	4.130	87.98	18.04	32.57	0.5945	0.7962	0.7931	0.3503	0.6018	0.5576
25	64.70	112.01	5.36	0.172	3.606	90.37	21.65	33.42	0.6401	0.5805	0.6797	0.5900	0.8013	0.5758
26	62.97	115.01	3.50	0.166	3.562	90.04	22.79	36.21	0.6467	0.5584	0.6703	0.5550	0.8598	0.6333
27	50.88	131.49	4.76	0.208	3.805	87.02	18.46	36.55	0.5578	0.7197	0.7227	0.2588	0.6271	0.6401
28	71.64	119.38	6.71	0.175	3.680	90.82	19.66	27.71	0.6050	0.5925	0.6956	0.6378	0.6960	0.4433
29	66.83	125.69	5.40	0.187	3.770	89.72	20.43	32.54	0.6281	0.6379	0.7151	0.5219	0.7375	0.5568
30	54.78	128.05	4.53	0.199	3.757	87.85	19.89	37.34	0.5979	0.6844	0.7121	0.3374	0.7084	0.6557
31	79.23	119.55	5.40	0.131	3.272	92.24	19.66	23.27	0.5397	0.4311	0.6084	0.7912	0.6958	0.3171
32	77.13	118.19	5.39	0.145	3.453	91.91	19.99	24.14	0.5649	0.4809	0.6468	0.7550	0.7142	0.3431
33	54.16	114.36	5.47	0.153	3.013	89.02	21.29	42.29	0.5953	0.5092	0.5541	0.4519	0.7826	0.7491
34	52.65	127.54	6.69	0.205	3.675	87.52	18.07	36.68	0.5665	0.7077	0.6946	0.3059	0.6040	0.6426

No.	Ethanol Concentration (% v/v)	T (°C)	Time (min)	Yield	TPC (g GAE (100 g) ⁻¹)	DPPH at 0.5 mg mL ⁻¹ (% inhibition)	ABTS at 0.5 mg mL ⁻¹ (% inhibition)	Fe ²⁺ Chelating Ability at 1.5 mg mL ⁻¹ (%)	Desirability	Yield Desirability	TPC Desirability	DPPH Desirability	ABTS Desirability	Fe ²⁺ Chelating Ability Desirability
35	73.31	134.08	3.22	0.146	3.312	90.51	21.54	33.93	0.6099	0.4848	0.6169	0.6047	0.7955	0.5864
36	46.79	131.12	3.12	0.187	3.516	86.70	18.79	38.59	0.5312	0.6380	0.6603	0.2284	0.6467	0.6798
37	69.72	114.86	5.59	0.173	3.721	90.85	20.87	28.95	0.6252	0.5854	0.7045	0.6411	0.7608	0.4749
38	40.16	136.07	3.64	0.191	3.460	85.45	15.30	34.56	0.4065	0.6539	0.6484	0.1090	0.4005	0.5998
39	56.74	113.69	5.03	0.158	3.188	89.36	21.83	40.66	0.6154	0.5282	0.5907	0.4855	0.8102	0.7190
40	58.25	121.35	3.28	0.175	3.564	88.95	22.12	38.76	0.6305	0.5928	0.6707	0.4448	0.8251	0.6831
41	43.69	122.98	5.85	0.141	2.567	87.21	17.85	43.59	0.4862	0.4659	0.4631	0.2761	0.5899	0.7728
42	56.39	114.98	4.92	0.160	3.218	89.21	21.71	40.63	0.6140	0.5371	0.5970	0.4710	0.8044	0.7185
43	71.59	114.02	6.82	0.175	3.741	91.11	19.82	26.22	0.6035	0.5944	0.7087	0.6691	0.7048	0.4029
44	56.65	128.21	6.95	0.213	3.862	87.94	18.36	35.00	0.5896	0.7406	0.7350	0.3459	0.6215	0.6087
45	63.78	129.15	4.60	0.196	3.867	89.02	20.61	34.34	0.6303	0.6723	0.7361	0.4522	0.7470	0.5952
46	42.14	126.28	5.31	0.148	2.693	86.73	17.09	41.85	0.4671	0.4928	0.4885	0.2304	0.5407	0.7410
47	52.36	126.08	3.60	0.182	3.518	87.80	20.52	39.66	0.5892	0.6206	0.6608	0.3332	0.7422	0.7002
48	46.79	131.12	6.54	0.205	3.576	86.51	16.30	36.14	0.4981	0.7068	0.6731	0.2101	0.4856	0.6319
49	46.03	110.93	4.31	0.082	1.788	88.84	22.03	52.13	0.4844	0.2597	0.3112	0.4336	0.8206	0.9277
50	41.25	132.84	5.96	0.188	3.267	85.82	14.78	35.84	0.4149	0.6411	0.6074	0.1441	0.3503	0.6258
51	52.49	140.00	7.00	0.256	4.420	86.25	15.70	30.78	0.5007	0.9092	0.8564	0.1857	0.4368	0.5179

Fig. S.1. Mixture plot for weight value of metal chelating = 0

Mixture plot uses greyscale to show top potential solutions across all evaluated weight objectives combinations. Horizontal axis of this plot is the weight of TPC with a scale of zero to one because the sum of weigh of TPC, DPPH, and metal chelating is one. Vertical axis of the graph is the number assigned for each set of condition as shown in **Table S.4**. Darkest colour on the plot shows highest desirability for the selected weight of criteria set. This mixture plot was produced with no emphasize in metal chelating (weight = 0). For maximum value of TPC without focusing on other parameters, weight value of one for TPC and zero for DPPH was selected. Solution set number 51 gave highest desirability and thus this set was chosen as the first potential solution. While more weight was put to DPPH (DPPH weight value of 0.4 and TPC weight value of 0.6), solution set number 43 had highest desirability and could be considered as the second potential solution.

Fig. S.2. Parallel plot for all set of solutions

Parallel plot visually illustrates the trade-offs of one solution over another in terms of the scaled criteria values. Best values of each criterion are on the top of the plot. Flatter lines in the graph indicate less trade-off between the criteria. This plot shows that high values of TPC always had a trade-off of low DPPH values. Contrarily, high values of DPPH had a trade-off of low metal chelating activity. The best set of solution from this graph was indicated with the flattest gradients of line (red line) and hence this set was chosen as the third potential solution.

Fig. S.3. Mixture plot for weight value of metal chelating = 0.2

Mixture plot uses greyscale to show top potential solutions across all evaluated weight objectives combinations. Horizontal axis of this plot is the weight of TPC with a scale of zero to one because the sum of weigh of TPC, DPPH, and metal chelating is one. Vertical axis of the graph is the number assigned for each set of condition as shown in **Table S.4**. Darkest colour on the plot shows highest desirability for the selected weight of criteria set. As metal chelating ability had different profile than the other parameters which was shown in the parallel plot, this parameter was included in a mixture plot with a weight of 0.2. In this case, weight of DPPH was set on 0.2 and weight of TPC was on 0.6. The best desirability value for this condition was achieved by solution set number 18 which was selected as the fourth potential solution.