PRETREATMENT AND OPTIMIZATION STUDIES ON THE EXTRACTION OF ANTIOXIDANT COMPONENTS FROMPHALERIA MACROCARPA FRUIT

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Abstract

Phaleria macrocarpa, one of many indigenous Indonesian plants, possesses potent antioxidant activity and hence may serve as natural food preservative, cosmetic ingredients, as well as food supplement. This study is the second stage of a three-year project to isolate antioxidant components in *Phaleria macrocarpa* for natural preservative application. The objective of this study is to investigate the effect of pretreatment process on the oleoresin and bioactive content, and to optimize three parameters in the extraction process, i.e. ethanol concentration, F/S ratio, and temperature. The selected pretreatment method was solid-liquid extraction using a non-polar solvent (n-hexane) and a semi polar solvent (ethyl acetate). The pretreatment process produced less sticky oleoresin, especially with n-hexane, due to significant removal of tannin, and had only a little effect (less than 4%) on the antioxidant activity and flavonoid content. However, the pretreatment indeed reduced the yield of oleoresin by around 10%. Three important parameters of the extraction process were optimized using Central Composite Experimental Design by varying operation temperature from 27.5 °C to 64.5 °C, F/S ratio from 1 : 11.5 g/mL to 1 : 53.5 g/mL, and ethanol concentration from 14 %-v/v to 93 %-v/v. The response surface analysis showed a maximum point at ethanol concentration of 30 %-v/v, F/S ratio of 1 : 45 g/mL, and temperature at 57 ºC which gave the oleoresin yield of 1.00 g oleoresin/g dry feed and antioxidant activities of 2.99 μmol DPPH/mg oleoresin.

Keywords: Antioxidant, Extraction, Optimization, Phaleria macrocarpa, Phytochemical

Introduction

Phaleria macrocarpa is one of Indonesian indigenous plants from Papua. The tree may grow up to 1.5 – 2.5 meter high with trumpet-shaped white flowers and green leaves with smooth surface. Its fruit has a ball shape and green color which turns red upon ripening. The fruit consists of exocarp, mesocarp, endocarp, and seed. The fruit is widely used for the treatment of various diseases, but the seed should not be consumed because of its toxic nature [1, 2].

P. macrocarpa has been used as an anticancer, antihyperlipidemia, antibacterial and anti-fungal, antiinflammatory, antioxidant, vasorelaxant, and antihyperglycemic agent [3-17]. Due to the high content of antioxidant, its extract may also serve as natural food preservative, cosmetic ingredient, as well as food supplement. *P. macrocarpa* fruit has a

higher antioxidant activity than berry fruits. Lay et al. [18] reported that IC_{50} concentration of *P. macrocarpa* fruit extract was 0.008 mg/mL compared to 0.04, 0.017, 0.038, and 0.04 mg/mL for bilberry, blackberry, strawberry, and raspberry fruit extract, respectively [19].

Altaf et al. [3], Tri et al. [10], and Hendra et al. [11, 12] discovered that certain components in *P. macrocarpa* could inhibit the growth of bacteria and fungi. Flavonoid, saponin, polyphenols, and tannin in *P. macrocarpa* were found to be effective for inhibiting the growth of gram-positive bacteria, such as *Bacillus ceresum, Bacillus subtilis, Enterobacter aerogenes, Escheria coli, Klebsiella pneumonia, Micrococcus luteus, Pseudomonas aeroginosa,* and *Staphylococcus aureus* [3, 10]. Moreover, kaempferol might impede the growth of *S. aureus, Enterococcus faecalis, Escherichia coli,* and *P. aeroginosa* [3], while phorboester showed inhibition effect to some fungi such as *Aspergillus niger*, *Fusarium oxysporum, Ganoderma lucidium,* and *Mucor indicus* [3, 11, 12].

Those bacteria and fungi may be found in contaminated food and consequently cause serious illnesses. *S. aureus* is the bacteria that can be found in cheese and sausage, while *E. coli* is one of many pathogen bacteria. *Pseudomonas sp* breaks down protein and lipid in food and *Aspergillus sp* produces hazardous mycotoxin and aflatoxin in contaminated food. The bioactive components in the fruit of *P. macrocarpa* can be used not only to inhibit those microorganisms, but also serve as antioxidant to prevent the oxidation of oily or fatty foods. Therefore, *P. macrocarpa* can be potentially used as natural food preservative.

This study is the second stage of a three-year project to isolate antioxidant components in *P. macrocarpa* for natural preservative application. Previous studies showed that ethanol-water mixture with proper extraction conditions resulted in much better for oleoresin yield as well as antioxidant activity, in comparison with other less polar solvents, such as acetone and ethyl acetate [20-23]. However, the oleoresin produced was sticky and paste-like. Unfortunately, this kind of oleoresin will make the isolation stage of the bioactive components difficult. Moreover, it is highly unlikely to get the desired crystallized final product.

Therefore, several pretreatment steps prior to extraction are needed to remove unwanted components, such as tannin and resin, which may also cause allergic reaction due to its moderate toxic attribute. Resin may be considered as terpene components which have thick and sticky appearance [24]. Tannin in *P. macrocarpa* is hydrolysable tannin which are not useful as antioxidant, and thus it needs to be removed [17]. The selected pretreatment method is solid-liquid extraction using a non-polar solvent and a semi polar solvent. In this investigation, n-hexane is chosen as the non-polar solvent to extract particular contaminants such as fat, oil, and resin [24-29]. Meanwhile, semi polar solvent which may be utilized to remove tannin component is ethyl acetate [30].

The objectives of this investigation are to study the effect of pretreatment process on the oleoresin characteristic and bioactive, especially antioxidant, content, and to optimize three extraction process conditions, i.e. temperature, feed to solvent (F/S) ratio, and ethanol concentration.

Material and Methods

Material

Fresh *P. macrocarpa* fruits were supplied from a local plantation in Subang, West Java, Indonesia. Fresh fruits were dried using a tray drier at 40 °C until its moisture content was below 10%, and then subsequently grinded prior to extraction step. Solvents for

extraction were technical grade ethanol, ethyl acetate, and n-hexane which were purchased from Brataco Chemicals (Bandung, Indonesia). Chemicals for analyses, i.e. Karl Fischer Reagent, methanol, ferric chloride, sodium hydroxide, hydrochloric acid, Mayer reagent, acetic anhydrate, sulphuric acid, chloroform, gallic acid, Folin-Ciocalteu, sodium carbonate, casein, rutin, pyridine, acetic acid, aluminium chloride, and DPPH, were purchased from Merck, Aldrich, and Emsure.

Pretreatment

Pretreatment experiments were carried out at room temperature using solid-liquid extraction technique in a 1 Liter laboratory scale batch extractor for 4 hours. Solvents in the pretreatment stage were n-hexane and ethyl acetate. After pretreatment, raffinate from subsequent filtration was then extracted with ethanol 70 %-v/v at room temperature for 4 hours. Ethanol-extracted oleoresin with and without pre-treatment were then compared. The existence of phytochemicals such as phenol, tannin, phytosterol, flavonoid, saponin, and alkaloid were identified qualitatively. The total phenol components, tannin, and flavonoid content in oleoresin were determined using quantitative analyses. The antioxidant activity was measured using DPPH method. The details of these analyses methods are described below.

Extraction

The experiments were carried out in a 1-Liter laboratory scale batch extractor (Figure 1) which equipped with an immersion thermostat, an agitator, and a condenser. Each extraction lasted for 4 hours since preliminary study [23] showed that equilibrium for extraction using ethanol 70%-v/v had been reached after 3 hours. Extraction was carried out with variations in extraction temperature, feed to solvent ratio, and concentration of ethanol. These variations were determined through Central Composite Design which would be explained further in the optimization section. *P. macrocarpa* and 500 mL solvent put into extractor in accordance with ethanol concentration and feed to solvent ratio variation. Extraction temperature was set using the thermostate in accordance with temperature variation. After the extraction process (4 hours), the extract was then separated from the solid by filtration using Whatman 41 filter paper. Further purification was carried out by centrifugation at 6000 RPM for 15 min (for water-ethanol and n-hexane) and another filtration using Whatman 42 filter paper. The filtrate was then evaporated using a rotary vacuum evaporator (Buchii, Switzerland) at 50 °C with gradual depressurization from boiling point of solvent to 5.5 kPa.

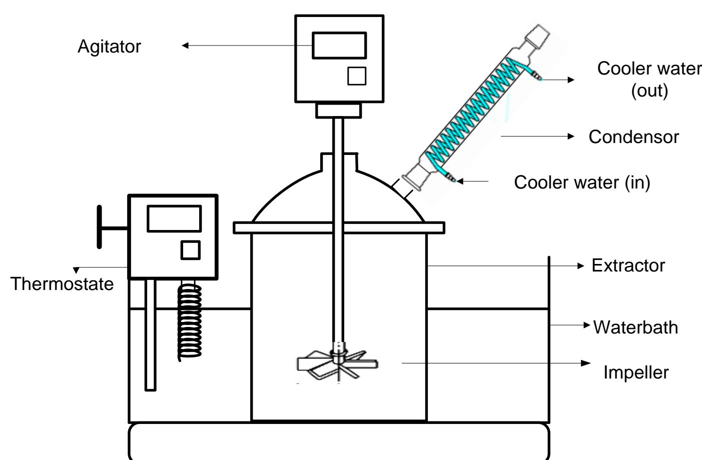


Figure 1. Batch extractor set up [20-23]

Optimization

The optimization of three important extraction variables was carried out using Response Surface - Central Composite Experimental Design. The method used in response surface for optimization was steepest accent. In central composite design, each variable was varied into 5 levels and coded –α, -1, 0, 1, and α which represent minimum, low, center, high, and maximum level, respectively. This low and high level for each variable were determined using experimental data from first year (2013) project [20-23], in which the extraction temperature ranging from 27.5 to 64.5 ^{0}C, feed to solvent ratio from 1 : 11.5 g/mL to 1 : 53.5 g/mL, and ethanol concentration from 14 %-v/v to 93 %-v/v. Experimental design points were arranged with the assistance of Design Expert software version 7.00, and presented in Table 1, together with the experimental results.

Table 1. Central Composite Design with Experimental Results

Run	Ethanol Concentration (%-v/v)	F/S ratio (g/mL)	T (°C)	The Yield of Oleoresin (g oleoresin/ g dry feed)	Antioxidant Activity (μmol DPPH/ mg oleoresin)
1	53.5	1 : 32.5	46.0	0.511	3.12
2	53.5	1 : 53.5	46.0	0.507	3.15
3	53.5	1 : 32.5	46.0	0.527	3.12
4	93.0	1 : 32.5	46.0	0.505	3.13
5	30.0	1 : 45.0	35.0	0.648	3.10
6	53.5	1 : 11.5	46.0	0.340	3.14
7	53.5	1 : 32.5	46.0	0.474	3.13
8	77.0	1 : 45.0	57.0	0.513	3.14
9	53.5	1 : 32.5	46.0	0.498	3.13
10	30.0	1 : 45.0	57.0	1.06	3.04
11	77.0	1 : 20.0	35.0	0.472	3.12
12	77.0	1 : 20.0	57.0	0.452	3.14
13	53.5	1 : 32.5	27.5	0.457	3.11
14	30.0	1 : 20.0	57.0	0.739	2.86
15	53.5	1 : 32.5	46.0	0.500	3.12
16	30.0	1 : 20.0	35.0	0.443	3.01
17	14.0	1 : 32.5	46.0	0.812	2.05
18	77.0	1 : 45.0	35.0	0.512	3.13
19	53.5	1 : 32.5	64.5	0.726	3.13

Qualitative Phytochemicals Analyses

Qualitative phytochemicals analyses were performed in duplicate for each extract to detect the component of interest. These qualitative methods were conducted according to Raaman, Mdlolo, and Sasidharan et. al. and Uzochukwu [31-34]

Phenol and Tannin Identification

The identification of phenol and tannin was carried out using ferric chloride test [31-34]. The solvent-free extracts were diluted in 5 mL distilled water and then a few drops of 5 %-w/v ferric chloride solution were added. The formation of green color indicated the presence of phenol component and tannin.

Flavonoid Identification

Alkaline reagent test was used to identify the flavonoid [31-34]. A few drops of 2 M sodium hydroxide solution were added to the solvent free extracts. The yellowish color turned colorless after the addition of 2 M hydrochloric acid solution indicated the existence of flavonoid components.

Alkaloid Identification

Mayer's test was performed to detect alkaloid [31-34]. The solvent-free extracts were diluted in 2 M hydrochloric acid solution. Into this solution a few drops of reagent Mayer were added. White creamy precipitate formed showed the alkaloid components within the extract.

Steroid Identification

The identification of steroid was conducted using Libermann-Buchard's test [31]. The solvent-free extracts were diluted in 2 mL of acetic anhydrate. Into this solution a few drops of concentrated sulphuric acid were slowly added. The formation of green color proved the steroid content.

Terpenoid Identification

The solvent-free extracts were diluted in 1 mL of chloroform. Into this solution a few drops of concentrated sulphuric acid were slowly added. The formation of redish brown layer identified the presence of terpenoid [33].

Saponin Identification

The foam test was used to identify saponin [31-34]. The solvent-free extracts were diluted in 20 mL distilled water and shaken in a test tube for 15 minutes. The saponin content coused the formation of stable layer of foam.

Quantitative Phytochemicals Analyses

Quantitative phytochemicals analyses were performed in duplicate for each extract to quantify the component of interest.

Quantification of Total Phenol and Tannin

The quantification of total phenol and tannin was conducted according to Amorim et. al. [35]. 500 µL extract solution (1 mg/mL solvent-free extract in 80%-v/v methanol) was transferred to a test tube. Into this solution, 500 µL of Folin-Ciocalteu solution (10 %-v/v), 1 mL of sodium carbonate solution (7.5 %-w/v), and 8 mL of distilled water were added. Blue solution was produced and then stored at room temperature for 30 minutes. Subsequently, its absorbance was measured at a wavelength of 760 nm to get the total phenol content. Meanwhile, to quantify the waste phenol, 500 mg of casein was added to 5 ml of extract solution to remove tannin. After two hours, the mixture was filtered and the waste phenol concentration in the filtrate was then measured. Tannin content was calculated as the difference between the total and waste phenol content. The phenol content was expressed as milligrams of gallic acid equivalents per gram of extract.

Quantification of Flavonoid

The quantification of flavonoid was conducted according to Amorim et. al. [35]. 500 µL extract solution (1 mg/mL solvent-free extract in 80 %-v/v methanol) was transferred to

a test tube. Into this solution, 500 μL of acetic acid solution (60 %-v/v in 80 %-v/v methanol), 2 mL of pyridine solution (20 %-v/v in 80 %-v/v methanol), 1 mL reagent aluminium chloride solution (5 %-w/v in 80 %-v/v methanol) and 6 mL of 80 %-v/v methanol were added. Yellow solution was stored at room temperature for 30 minutes. Afterwards, its absorbance was measured at a wavelength of 420 nm. The flavonoid content was expressed as milligrams of rutin equivalents per gram of extract.

Antioxidant Activity Analysis

Antioxidant activity was measured by DPPH method [36]. This method measured the ability of plant extracts to donate hydrogen or electrons by changing the color of DPPH solution from purple to yellow. Extract solution (2.5 ml of 30 ppm extract solution in methanol p.a) was transferred to a tube test. Futhermore, 2.5 mL of 0.1 mM DPPH-methanol solution was added. The mixture was stirred and stored at room temperature in dark room for 4 hours. The solution's absorbance was measured at a wavelength of 517 nm. Antioxidant activity was expressed as μmol DPPH equivalencies/mg extract.

Calculation of Yield

Yield of oleoresin was calculated as follow:

$$Yield = \frac{mass\ of\ oleore\sin}{mass\ of\ feed \times (1 - feed\ moisture\ content)} \quad (1)$$

Results and Discussion

Pretreatment with n-Hexane and Ethyl Acetate

Pretreatment process was carried out to investigate the effect of n-hexane and ethyl acetate pre extraction on the ethanol extract in terms of physical appearance, yield, and antioxidant activity. The extraction in pretreatment experiments were carried out with F/S ratio = 1:40 (g/mL) at room temperature for 4 hours. The existence of phytochemicals such as phenol, tannin, phytosterol, flavonoid, saponin, and alkaloid were determined qualitatively (Table 2). The total phenol components, tannin, and flavonoid content in oleoresin were determined using quantitative analyses as previously described. The antioxidant activity was determined using DPPH method. The results of quantitative analyses are summarized in Table 3.

The extraction using n-hexane, ethyl acetate and ethanol 70 %-v/v resulted in oleoresin which had yellow, cream, and brown color, respectively. Phytochemical analyses revealed that the oleoresin extracted using n-hexane had steroid and terpenoid component and the oleoresin from ethyl acetate extraction showed phenol, tannin, flavonoid, steroid and terpenoid content. Meanwhile, oleoresin extracted using ethanol possessed the most complete phytochemical components which were phenol, tannin, flavonoid, alkaloid, terpenoid, and saponin.

Table 2. Phytochemical Screening Result

Oleoresin	Phenol	Tannin	Flavonoid	Alkaloid	Phytosterol		Saponin
					Steroid	Terpenoid	
n-hexane	-	-	-	-	+	+	-
Ethyl acetate	+	+	+	-	+	+	-
Ethanol 70%	+	+	+	+	-	+	+
n-hexane followed by ethanol 70%	+	+	+	+	-	+	+
Ethyl acetate followed by ethanol 70%	+	+	+	+	-	+	+

Table 3. Total Phenol, Tannin, Flavonoid Content, Yield and Antioxidant Activity

Oleoresin	Total Phenol[1]	Tannin Content[2]	Flavonoid Content[3]	Yield[4]	Antioxidant Activity[5]
n-hexane	0±0.10	0±1.50	0±0.30	0.0476	0.724±0.95
Ethyl acetate	1.74±0.90	0.528±3.45	0.778±3.10	0.0540	2.06±0.05
Ethanol 70%	5.95±0.10	3.43±0.65	10.3±0.10	0.541	2.96±0.15
n-hexane followed by ethanol 70%	5.52±0.80	2.90±1.60	10.5±0.00	0.484	2.90±0.55
Ethyl acetate followed by ethanol 70%	5.45±0.45	3.11±0.90	10.6±0.40	0.478	2.92±0.35

[1] Total phenol is expressed in mg gallic acid equivalencies/g oleoresin
[2]Tanin content is expressed in mg gallic acid equivalencies/g oleoresin
[3] Flavonoid content is expressed in mg rutin equivalencies/g oleoresin
[4] Yield is expressed in g oleoresin/g dry feed
[5] Antioxidant activities is expressed in μmol DPPH equivalencies/mg oleoresin

Oleoresin came from ethanol extract contained flavonoid, saponin, phenol, tannin, terpenoid, and alkaloid. Flavonoid, saponin, phenol, and tannin content in *P. Macrocarpa* can be used as antibacterial [3, 10]. In addition, flavonoid and terpenoid components can serve as anti-fungal [3, 12]. Meanwhile, flavonoid, alkaloid, phenol, and saponin can be utilized as antioxidant [3, 7, 13]. Therefore, it can be potentially used as natural preservative.

The pretreatment processes produced less sticky oleoresin (especially with n-hexane by visual observation) and did not show any effects on the phytochemicals content of the oleoresin. Rheometer measurements showed that the oleoresin obtained from the extraction without and with pretreatment using n-hexane and ethyl acetate had viscosity of 12.00 cP, 6.00 cP, and 10.00 cP, respectively. Experimental results in Table 2 revealed that both n-hexane and ethyl acetate extracts contained steroid. Steroid would react with acetic anhydrate forming pentaenylic cationic which appeared as green color [37]. Its absorbance could be measured at a wavelength of 626 nm [37]. N-hexane extract had darker green color, indicating higher steroid content. In other words, n-hexane was a more selective solvent for steroid elimination. Steroid consists of long chain and saturated fatty acids, pack tightly beside one another, which causes the membrane cell less fluid [38]. As a result, due to the more steroid eliminated in pretreatment process, the n-hexane extracted oleoresin was less sticky.

The pretreatment with n-hexane and ethyl acetate reduced the yield of oleoresin approximately by 10.5% and 11.7%, respectively. However, the pretreatment process had

only slight effect (less than 4%) on the antioxidant activity and flavonoid content. The pretreatment also decreased total phenol components approximately by 7.2% (with n-hexane) and 8.3% (with ethyl acetate). As expected, the pretreatment process using n-hexane and ethyl acetate could remove the tannin content significantly by 15.39% and 9.18% pretreatment, respectively. Therefore, the pretreatment process especially with n-hexane showed promising results, in terms of tannins and resin removal, without significant loss of both yield and antioxidant content.

Optimization of the Extraction Process

Three important factors in the extraction process, i.e. ethanol concentration, F/S ratio and extraction temperature, were optimized using Central Composite Experimental Design (with 5 center points). The experimental design and results of these experiments are presented in Table 1. An analysis of variance was also conducted based on the data from Table 1.

Along with a decrease in ethanol concentration, F/S ratio, and an increase in temperature, the yield of oleoresin was increased. The decreasing concentration of ethanol (or increasing water proportion) resulted in the amount of impurities extracted were increased and hence elevated the yield of oleoresin. Water as a universal solvent was capable of extracting certain impurities such as starch and polypeptides [39-40] which were shown by gel-like oleoresin at low ethanol concentrations.

Meanwhile, lower F/S ratio translated into increasing amount of solvent used and therefore would increase the extraction driving force (the differences of solute concentration in solvent and the solid phase) [40]. As a result, the yield of oleoresin at lower F/S ratio was significantly higher.

Increasing extraction temperature would improve the yield of oleoresin (especially at low ethanol concentrations). At high temperatures, the solute solubility would increase and the interface film thickness would also decrease, minimizing the mass transfer resistance [42].

ANOVA (Table 4) confirmed that the ethanol concentration, F/S ratio, and temperature gave significant effects (p-value < 0.05) on the yield of oleoresin. However, from interaction curve, the interaction between ethanol concentration and F/S ratio occurred only at low temperature. In addition, the interaction between ethanol concentration and temperature was observed at medium to high F/S ratio.

Table 4. ANOVA of Ethanol Concentration, F/S Ratio, and Extraction Temperature on the Yield of Oleoresin

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_0	p-value
Ethanol concentration (%-v/v)	0.15	1	0.15	102.31	<0.0001
F/S ratio (g/mL)	0.060	1	0.060	39.63	0.0001
Temperature (°C)	0.095	1	0.095	62.63	<0.0001
Ethanol concentration-F/S	0.022	1	0.022	14.79	0.0039
Ethanol concentration-Temperature	0.066	1	0.066	43.54	<0.0001
F/S-Temperature	2.27×10^{-3}	1	2.27×10^{-3}	1.50	0.2515
(Ethanol concentration)^2	0.057	1	0.057	37.73	0.0002
(F/S)^2	4.62×10^{-3}	1	4.62×10^{-3}	3.05	0.1145
(Temperature)^2	0.023	1	0.023	15.06	0.0037
Error	0.014	9	1.51×10^{-3}		
Total	0.50	18			

Increasing ethanol concentration produced oleoresin with high antioxidant activities, since lower water content caused fewer impurities would be extracted, and therefore the quality of oleoresin was improved. Phenolic and flavonoid compounds extraction was favoured by the increase in ethanol concentration, and hence would increase the antioxidant activity [43]. Meanwhile, within the experimental conditions, the F/S ratio and temperature (27.5 – 64.5 °C) did not give any effect because the F/S ratio and temperature would only improve the oleoresin yield. It should be noted however, excessively high temperature might affect the antioxidant activity because some bioactive component would be degraded [43-44]. Previous study showed that temperature between 26°C and 65.3°C did not cause the degradation of any phytochemical components, however the degradation indeed occurred at temperature above 72°C [23].

ANOVA (Table 5) confirmed only the ethanol concentration gave significant effect (p-value<0.05) on antioxidant activity, while both F/S ratio and temperature gave a little or insignificant effect. Moreover, from interaction curve, there was an interaction between ethanol concentration and F/S ratio on antioxidant activity observed at all extraction temperature level.

Table 5. ANOVA of Ethanol Concentration, F/S Ratio, and Extraction Temperature on Antioxidant Activity

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_0	p-value
Ethanol concentration (%-v/v)	0.59	1	0.59	88.63	<0.0001
F/S ratio (g/mL)	6.27×10^{-3}	1	6.27×10^{-3}	0.94	0.3542
Temperature (°C)	2.04×10^{-3}	1	2.04×10^{-3}	0.31	0.5912
Ethanol concentration-F/S	8.81×10^{-3}	1	8.81×10^{-3}	1.33	0.2762
$(\text{Ethanol concentration})^2$	0.35	1	0.35	53.25	<0.0001
$(\text{F/S ratio})^2$	0.016	1	0.016	2.35	0.1562
$(\text{Temperature})^2$	0.010	1	0.010	1.51	0.2475
Ethanol concentration-$(\text{F/S})^2$	0.22	1	0.22	32.76	0.0002
Error	0.066	9	6.64×10^{-3}		
Total	1.13	18			

The model derived from Design Expert for the yield of oleoresin and antioxidant activity are shown in Equation (2) – (3) and 3D surface plot are shown in Figure 2 – 3. The optimization of extraction factors is a multi variabel non linear optimization which may have no solution or many solutions [45]. The optimum conditions were determined simultaneously with the help of software Design Expert version 7.0 which gave more than one solutions. The appropriate solution was the one which had the highest combined level of desirability (shown in Table 6). Figure 2 and 3 showed that the optimization of oleoresin yield would provide a saddle point, while the optimization of the antioxidant activity would give a maximum stationary point [46].

$$Y = 0.1535 + 4.9378\times10^{-3}\ \%E + 0.0169\,\frac{1}{R} - 8.6907\times10^{-3}\ T - 1.8000\times10^{-4}\ \%E\,\frac{1}{R} - 3.5097\times10^{-4}\ \%E\ T + 1.2255\times10^{-4}\,\frac{1}{R}\,T + 1.1708\times10^{-4}\ \%E^2 - 1.1772\times10^{-4}\left(\frac{1}{R}\right)^2 + 3.3756\times10^{-4}T^2 \quad (2)$$

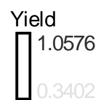
$$D = 5.9944 - 0.0251\ \%E - 0.2490\,\frac{1}{R} - 0.0217\ T + 4.4235\times10^{-3}\ \%E\,\frac{1}{R} - 2.9150\times10^{-4}\ \%E^2 + 3.9503\times10^{-3}\left(\frac{1}{R}\right)^2 + 2.2392\times10^{-4}\ T^2 - 6.9791\times10^{-5}\ \%E\left(\frac{1}{R}\right)^2 \quad (3)$$

whereas:

Y = the yield of oleoresin (g oleoresin/g dry feed)
D = Antioxidant activity (µmol DPPH/mg oleoresin)
%E = Ethanol concentration (%-v/v)
R = F/S ratio (g/mL)
T = temperature (°C)

Design-Expert® Software

Yield



X1 = A: % Ethanol
X2 = B: F:S

Actual Factor
C: Temperature = 57.00

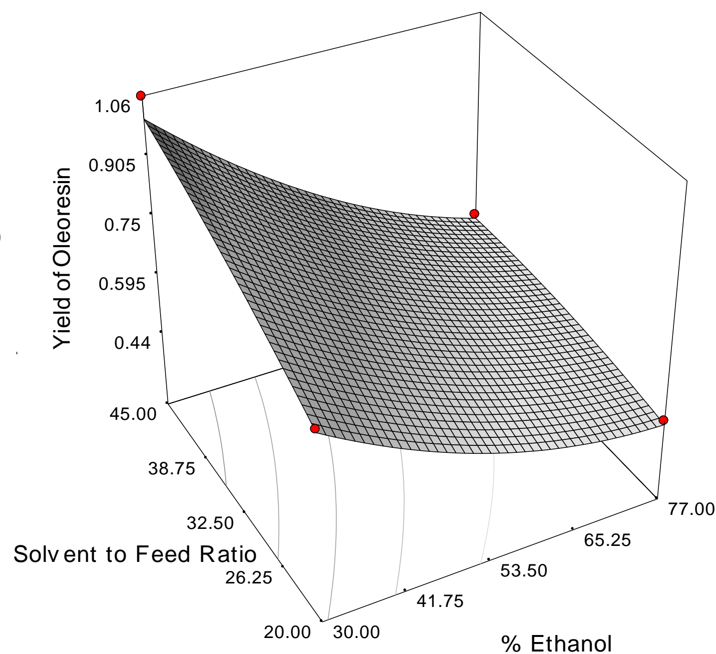


Figure 2. Oleoresin yield 3D surface plot[6]

[6] Yield is expressed in g oleoresin/g dry feed, solvent to feed ratio in mL solvent/g feed, and % ethanol in %-v/v

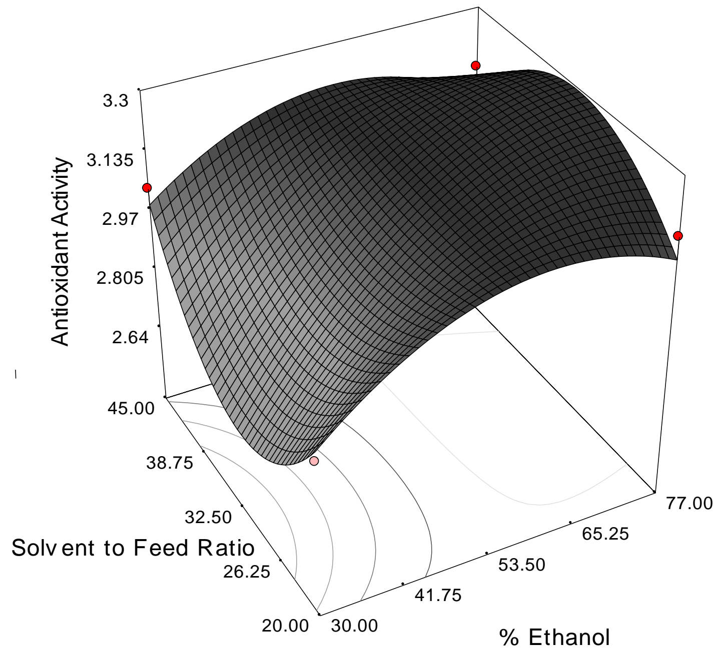


Figure 3. Antioxidant activity 3D surface plot[7]

Table 6. Optimum Condition of Extraction

Extraction Parameter	Operation Condition			Response	Individual Desirability	Combined Desirability
	Ethanol Concentration	F : S	Temperature			
Oleoresin Yield[8]	30 %-v/v	1 : 45	57 °C	1.00	0.925	0.892
Antioxidant Activity[9]				2.99	0.859	

[7] Antioxidant activity is expressed in μmol DPPH equivalencies/mg oleoresin
[8] Yield is expressed in g oleoresin/g dry feed
[9] Antioxidant activity is expressed in μmol DPPH equivalencies/mg oleoresin

Response surface with a maximum response point is achieved at ethanol concentration of 30 %-v/v, F/S ratio of 1 : 45 g/mL, and temperature at 57 ºC. At his conditions, the oleoresin yield was 1.00 g oleoresin/g dry feed and the antioxidant activities of 2.99 μmol DPPH/mg oleoresin was obtained. Those optimum conditions were validated using additional runs which gave the yield of 0.91 g oleoresin/g dry feed and the antioxidant activities of 2.71 μmol DPPH/mg oleoresin, with differences of 8.7% for oleoresin yield and 9.3% for the antioxidant activity. Therefore, the model and the experimental data showed an excellent agreement, indicating that the model was appropriate, for both yield and antioxidant activity.

Conclusion

The pretreatment process, especially with n-hexane, showed promising results in terms of tannins and resin removal without significant loss of both yield and antioxidant content. Based on ANOVA, the ethanol concentration, F/S ratio, and temperature gave significant effect on yield of oleoresin. Meanwhile, the effect of the interaction between ethanol

concentration and F/S ratio and between ethanol concentration and temperature were also significant on the yield. Furthermore, only the ethanol concentration gave significant effect on antioxidant activity. The response surface analysis showed a maximum point at ethanol concentration of 30 %-v/v, F/S ratio of 1 : 45 g/mL, and temperature at 57 ℃ which gave the yield of oleoresin of 1.00 g oleoresin/g dry feed and antioxidant activities of 2.99 μmol DPPH/mg oleoresin. The validation of optimum extraction conditions showed that the model was reliable for the oleoresin yield as well as the antioxidant activity.

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