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Enhancement of gaharu oleoresin quality by process optimization using response surface methodology

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ABSTRACT

Gaharu is the most expensive resinous wood fragrance making it a very highly valuable product. Its oleoresin was produced using ethanol reflux extraction method. In optimization study, the influence of process parameters on the extraction of oleoresin from dried gaharu particles was investigated. The parameters include particle sizes, raw material to solvent ratio and extraction duration. With the aid of regression analysis using response surface methodology (RSM), the results suggested that the optimal set of extraction condition to be consists of particle size of 0.5–1.0 mm (S3), raw material to ethanol ratio of 1:29.9 g/ml and extraction time of 4.97 h. Under this condition, the highest gaharu oleoresin yield was obtained, i.e. 7.63% (w/w) with total resin content of 6.90% (w/w). In brief, higher yield of oleoresin can be extracted from smaller particle size of dried gaharu in shorter extraction time and lower raw material to solvent ratio. In the comparison study, the optimized reflux extraction produced higher quality of gaharu oleoresin compared to reflux using WHO protocol, nevertheless just as comparable to the conventional Soxhlet extraction. Further investigation on the quality of extracts resulted to six chemical compounds determined in gaharu oleoresin using GC-MS. The outcomes of this study indicated an improvement of 62.97% of overall chemical compositions in gaharu oleoresin extract which includes 4-phenyl-2-butanone, β-guaiene, agarospirol, α-bisabolene epoxide, alloaromadendrene oxide and aromadenderence oxide compared to the conventional Soxhlet method.

1. Introduction

Gaharu or agarwood is a resin impregnated material usually found in the plants of the Thymelaeaceae family belonged to the Aquilaria genus. In Peninsular Malaysia, *A. malaccensis, A. microcarpa, A. hirta, A. rostrata and A. beccariana* are amongst the five types of *Aquilaria* species recorded with the ability to produce oleoresin (Blanchette, 2006; Barden et al., 2000). The tree's special reaction to damage causes by injury, cutting, microorganism and insect disturbance results to the formation of the resinous of gaharu (Dai et al., 2009). However, gaharu rarely occurs in the naturally healthy, wild and young trees causing its resinous wood to be extremely expensive.

Aside from differences in color, density, unique scent and its formation; the grading of gaharu wood can also be done based on its oleoresin content (Nor Atikah et al., 2015). There are four grades of gaharu wood namely A, B, C and D in descending order from the highest to the lowest of quality and price. The price of gaharu products are extremely high in the market nowadays. For instance, the price of grade A gaharu wood can reach up to RM 16,000–20,000 per kilogram. Therefore, lower gaharu grades are usually preferred for its oil (Nor Azah et al., 2013). In Malaysia, gaharu wood of grade C and D with physical appearances of natural dark yellow color with stripes and whitish yellow, respectively are mostly used (Nor Azah et al., 2013).

Gaharu has been used worldwide since the time immemorial for medicinal, aromatic and religious purposes. The demand for gaharu increases each year especially in the global market. Lipophilic compounds are often referred to as wood resin such as wax, glycerides and steryl esters, which can be produced by the extraction process (Qin et al., 2009). Gaharu oleoresin was mainly produced through a single step extraction which is a direct process with the additional of solvents (Khasanah et al., 2017). The available techniques for gaharu oleoresin extraction include Soxhlet (Sulaiman et al., 2017) and reflux extractions (WHO, 1998; Nor Azah et al., 2013). Reflux extraction is a solid-liquid extraction process under repeatable solvent evaporation and condensation at a constant temperature for a particular period of time without the loss of solvent (Chua et al., 2016). Meanwhile, oleoresin

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from other plants were extracted using different methods such as the maceration of *Cinnamonmum burmannii* leaves (Khasanah et al., 2017); the Soxhlet of *zingiber zerumbet* (Rosnani et al., 2011) and turmeric (Haldar et al., 2016); the supercritical CO_2 of leaves and flowers *Brunfelsia uniflora* (Jorge1 et al., 2017); and the microwave-reflux extraction of *Piper nigrum* (Olalere et al., 2017). Although various methods has been applied for the extraction of oleoresin, these methods have some limitations such as time consuming, low oleoresin yield, costly operating process and limited for the pilot-scale production purpose.

Therefore, the objective of the current work is to enhance the oleoresin yield through the identification of optimum parameters for gaharu oleoresin using ethanol reflux extraction. The oleoresin extraction is considered one of the way to improvise the quality of the graded gaharu due to its quality can be classified based on resin content (Nor Azah et al., 2013). The outcome is then compared to results obtained from the different extraction method to determine the extraction efficiency. In addition, the quality of gaharu extract is also determined by total phenolic content (TPC) and percentage of some chemical compounds using gas chromatography–mass spectrometry (GC-MS). In this report, the identification of six compounds in gaharu oleoresin from different extraction methods were conducted to evaluate its quality. These monoterpenes and sesquiterpene compounds have been reported as the main active components which play the important role in providing the characteristics of the pleasant aroma of gaharu oil (Nurlaila et al., 2013; Chen et al., 2011).

2. Materials and methods

2.1. Materials

The natural inoculated plant wood sample from the species of *Aquilaria malaccaensis* (grade D with resin content of lower than 9% w/w) was obtained from local supplier in Tangkak, Johor, Malaysia. The gaharu wood was dried in an oven using a tray dryer at 55 °C for 16 h to lower the moisture content in order to extend its shelf life. Then, the dried wood was grinded in a hammer mill and separated using mechanical sieving separator into the desired particle sizes (S1 (1.4–2.0), S2 (1.0–1.4), S3 (0.5–1.0) mm). The sample was stored in a closed glass jar for the next chemical analysis. The analytical grade ethanol was used as a solvent in the extraction (Nor Azah et al., 2013).

2.2. Reflux extraction of gaharu oleoresin

Gaharu oleoresin was extracted using a reflux system of extraction (Fig. 1) at constant temperature which was set according to boiling temperature of ethanol (solvent), i.e. 79 °C. Firstly, 6 g of gaharu particles and 95% ethanol were inserted into 300 ml round bottom flask. The manipulated parameters include extraction duration, raw material to solvent ratio and particle size. After the extraction process, the extract solution was collected and separated from the solid residue by filtration using an 11 μm pore size filter paper. Each experiment was employed in triplicate for reproducible results.

2.3. OFAT technique for preliminary study

One-factor-at-a-time or OFAT experiment was performed using ethanol reflux extraction mainly to assess the effect of important parameters on the yield of oleoresin extract from gaharu and to identify their suitable ranges for optimization study. The experimental works were run as follows;

1) As for effect of raw material to solvent ratio, gaharu oleoresin was extracted using ratios of 1:6, 1:10, 1:20, 1:30 and 1:40 g/ml. The extraction duration was fixed at 9 h with varied particle sizes (i.e. S1: 1.4–2.0 mm and S3: 0.5–1.0 mm).
2) As for effect of extraction duration, gaharu oleoresin was extracted by varying duration, i.e. 1, 3, 5, 7, and 9 h. The best raw material to solvent ratio determined in the first step was used with varied particle sizes (S1: 1.4–2.0 mm and S3: 0.5–1.0 mm).

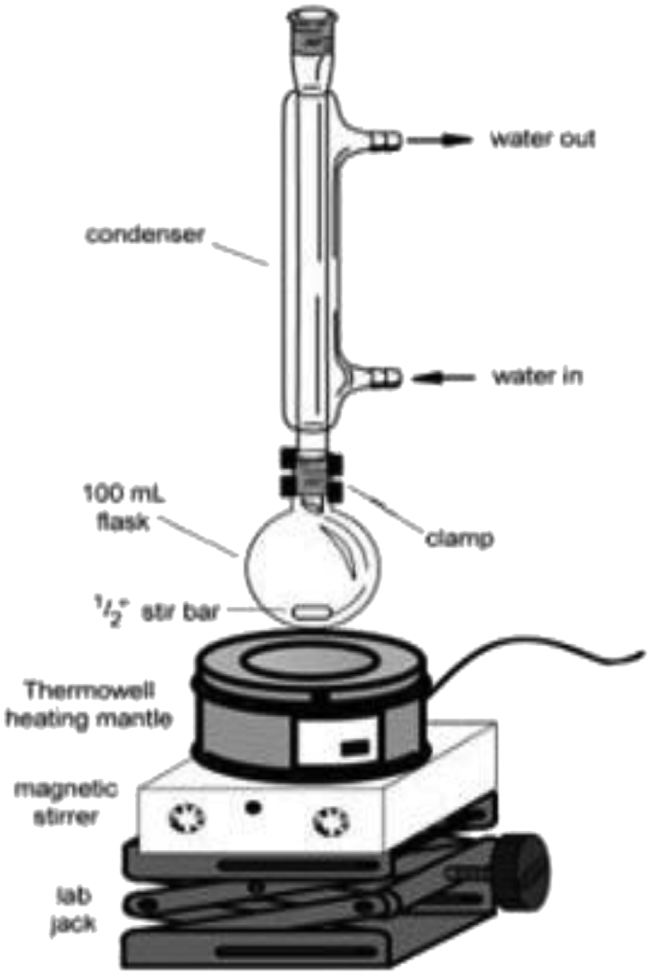

Fig. 1. The schematic representation of reflux extractor.

2.4. Experimental design by RSM

A central composite design (CCD) of response surface methodology (RSM) was employed to determine the optimal conditions of gaharu oleoresin extraction. The experiments were performed using ethanol reflux extraction. Three independent variables were used at specified levels and coded as shown in Table 1. Aside from preliminary studies, the maximum and minimum values of the independent variables were also based on the ability of the reflux solvent extractor used in this study. A set of 33 runs was conducted at five levels with axial point (at selective $\alpha = 1.21421$) on two responses (or dependent variables) of oleoresin yield and total resin content. The experimental design was arranged with the aid of Design Expert software version 6.0.8. Data obtained was then analyzed by multiple regression in order to fit the polynomial equation.

2.5. Comparison methods of gaharu oleoresin extraction

2.5.1. Reflux extraction of gaharu oleoresin according to WHO protocol

The extraction was done according to the protocol developed by WHO (1998) and Nor Azah et al. (2013). Ground gaharu sample (8 g) was accurately weighed and 200 ml of 95% ethanol were added. The mixture was shaken and allowed to stand for 1 h. It was then proceeded with the reflux system for 1 h at temperature of 79 °C.

Table 1
The independent variables coded levels in CCD for gaharu oleoresin extraction.

Category	Factor	-α	−1	0	+1	+ α
Numerical	Duration (hr)	2.57	3	5	7	7.43
	Raw material to solvent ratio (g/ml)	1:7.86	1:10	1:20	1:30	1:32.14
Categorical	Particle size (mm)	S1 (1.4–2.0), S2 (1.0–1.4), S3 (0.5–1.0)				

2.5.2. Soxhlet extraction of gaharu oleoresin

Soxhlet extraction was performed by introducing 10 g of gaharu powder sample into the thimble followed by the addition of 250 ml of ethanol into a round bottom flask. Then, the extraction chamber was inserted with the sample-filled thimble and connected together with both round bottom flask and condenser. The extraction temperature was carried out according to the boiling point of ethanol (boiling temperature of is 79 °C). In this study, in order to achieve maximum extraction yields, the extractions were carried out at substantial extraction duration of 8 h. The extraction was carried out until a clear extract was obtained (Sulaiman et al., 2015).

2.6. Analytical procedure

2.6.1. Determination of oleoresin yield

The filtrate solutions of oleoresin extract were primarily evaporated using vacuum rotary evaporator (Laborata 4000, Heidolph, Germany) at 70 °C. Then, the dried extract obtained was weighed and calculated as the oleoresin yield. The dried oleoresin obtained were kept in air-tight amber bottles and stored at 5 °C for further analysis (Bhuiyan et al., 2009). The oleoresin yield was calculated according to equation (1);

$$\text{Oleoresin yield \% } ({}^{W}/_{W}) = \frac{\text{Weight of dried oleoresin (g)}}{\text{Weight of gaharu particle used (g)}} \text{ x 100 \%} \quad (1)$$

2.6.2. Determination of total resin content

The resin content analysis was done according to the method proposed by Nor Azah et al. (2013) with modification. The total resin was determined based on the evaporated oleoresin obtained (in prior step) which is further weighed into a crucible dish. Then, the sample was dried in a convection oven at 105 °C for 6 h and cooled in a desiccator for 30 min for reweighed. At the end, the resin content was calculated in % (w/w) as in the following formula;

$$\text{Total resin \% } ({}^{W}/_{W}) = \frac{\text{Weight of dried resin (g)}}{\text{Weight of gaharu particle used (g)}} \text{ x 100\%} \quad (2)$$

2.6.3. Determination of gaharu oil content

Approximately 2 g of oleoresin samples were extracted for oil determination using Soxhlet extraction with n-hexane as solvent at volume of 250 ml. The sample was inserted into the thimble and then introduced into a 300 ml round bottom flask which was connected to a condenser. The temperature was set at 68.7 °C (boiling point of n-hexane) and the reaction time was fixed at 8 h. At the end of extraction, the sample was filtered by a vacuum filtration using a filter paper to remove suspended solids. The filtrate solutions were separated using vacuum rotary evaporator at 70 °C, which subsequently was gathered in the receiving flask. The obtained dried samples was weighed and calculated as gaharu oil content as expressed in equation (3). All experiments were repeated thrice.

$$\text{Gaharu Oil yield \% } ({}^{W}/_{W}) = \frac{\text{Weight of oil obtained (g)}}{\text{Weight of gaharu oleoresin (g)}} \text{ x 100 \%} \quad (3)$$

2.6.4. Determination of total phenolic content (TPC)

Folin-Ciocalteu spectrophotometric method with some modification was used to determine the TPC of the gaharu extract (Kadir and Hale, 2017; Chigayo et al., 2016). Gallic acid solution was prepared for standard calibration curve in concentrations of 12.5, 25, 50, 100, and 200 ppm (mg/ml).

1 g of oleoresin was diluted in methanol to give final concentration of 1.0 mg/ml. Then, 0.5 ml of diluted oleoresin and standard solutions were mixed with 2.5 ml of Folin-Ciocalteau reagent (10-fold dilute with DI water) and 2 ml of 7.5% w/v sodium carbonate. The solution was allowed to stand at room temperature for 30 min for color development. Next, the total phenolic was measured using UV–Vis spectrophotometer (Shimadzu 1800; Shimadzu Corporation, Kyoto, Japan) at a wavelength of 765 nm. Each extract was analyzed in triplicate and the results were expressed in milligrams of gallic acid equivalents per 1 g of oleoresin (mg GAE/g) and calculated using the formula as shown in equation (4);

$$TPC = \frac{C \; x \; V}{M} \quad (4)$$

where C is concentration of gallic acid established from the calibration curve (mg/ml), V is volume of the extract (ml) and M is mass of the extract of the oleoresin.

2.6.5. Gas chromatography – mass spectrometry (GC-MS)

The main chemical compounds of gaharu oleoresin were analyzed by GC-MS (series of II/5971A Hewlett-Packard GCMSD 5890). The identification of the chemical compounds was assisted by mass spectral library (HPCH2205.L; Wiley7Nist05.L; NIST05a.L). The results of the peak areas were expressed as peak area counts (Mailina et al., 2010; Sulaiman et al., 2015). The analysis was carried out using a fused silica capillary column on DBJ (J&W Scientific 30 m × 0.25 mm, 0.25 µm film thickness), helium as gas carrier and electron energy.

3. Results and discussion

3.1. Effect of raw material to solvent ratio and particle size on oleoresin yield

Based on the preliminary experiments, the effect of raw material to solvent ratio and particle size on yield of gaharu oleoresin for specified particle sizes was investigated. The results pointed out the significant increment of yields from 1:6 to 1:30 (w/v) ratio but remain unchanged (constant) until 1:40 (w/v) ratio for both particle sizes of S1 and S3. This indicates that there is an optimal ratio at which the maximum yield can be obtained. According to Xi et al. (2009), dissolution of plant bioactive components into the solvent is a physical process. The increment of oleoresin yield at lower ratios (between 1:6 to 1:30) was due to higher volume of solvent that leads to more active compounds being diffused out from the plant matrix, which is consistent with the mass transfer theory. However, the oleoresin yield started to become slightly constant beyond the ratio of 1:30 which is basically due to the saturation phenomenon of active compounds concentration. In fact, as the solvent is saturated on solutes, the cellular diffusion halts with the existence or decrement of the extracted compounds stabilization rate (Nyamien et al., 2013).

Moreover, size reduction is a breaking process of the cell structure in order to maximize the surface accessibility hence minimizes the mass

transfer resistance in gaharu wood passageways for ease of oleoresin release. Particle size reduction is important to improve the path of solvent diffusion and active compounds within the solid matrix. The decrement of the average particle diameter of the introduced raw material (i.e. gaharu wood) from S1 (course particle) to S3 (fine particle) into the extractor resulted to final oleoresin recovery increment from 6.00 ± 0.02 to 7.66 ± 0.12% (w/w) and 5.91 ± 0.11 to 8.08 ± 0.12% (w/w) at 1:30 (w/v) ratio respectively. In brief, the results clearly demonstrated that oleoresin recovery can be increased more than 36% by particle size reduction, without having any detrimental impact on the purity of the final gaharu oleoresin extract within the covered ranges (i.e. 0.5–2.0 mm). Nevertheless, an excessive small particle size is unwanted as it can cause agglutination thus is difficult for solvent permeation, and contributes to clogging issues in real practice of mixing extractor.

In the other hand, raw material to solvent ratios ranging between 1:30 to 1:40 reached beyond the equilibrium phase at solid-liquid interfaces and started to slowly decrease. Thus, the best range of raw material to solvent ratio is decided from 1:10 to 1:30 for the gaharu oleoresin extraction.

3.2. Effect of extraction duration and particle size on oleoresin yield

The raw material to solvent ratio was fixed at 1:30 and the duration was in the range of 1–9 h. As expected, longer extraction time generally led to a higher percentage yield of gaharu oleoresin. The extraction achieved the highest yield at 5 h, and slowly remain constant until the end of 9 h duration. During the extraction time of 1–5 h, gaharu oleoresin yield for particle size of S1 increased from 4.75 ± 0.02% to 6.00 ± 0.02% w/w, while for S3 increased from 7.08 ± 0.02% to 7.66 ± 0.12% w/w. This could have been due to the longer contact time between solute and solvent, which permits more diffusion of active compounds from the plant matrix. However, excessive extraction time would be unnecessary as the active compounds and solvent would be in the final equilibrium after certain duration as stated by the Fick's second law of diffusion (Gertenbach, 2002). By then, the rate of extraction of compounds would decelerate. Beside that the final oleoresin extract was also significantly impacted by the initial average particle size by 27% increment of oleoresin yield for particle size S3 (i.e. 0.5–2.0 mm) compared to S1 (i.e. 1.4–2.0 mm). Other than economic point of view, finding the best length of extraction time is highly important in order to avoid incomplete extraction process which would affect the final yield of gaharu oleoresin. Therefore, for this study, the best range of extraction duration is from 3 to 7 h.

3.3. Optimization of gaharu oleoresin extraction

3.3.1. Model fitting and statistical analysis

According to RSM, the gaharu oleoresin extraction was successfully carried out using a reflux system as shown in Table 2. Based on the CCD design matrix results, the oleoresin yield were produced in the range of 3.33–8.00% (w/w), while the total resin content varied from 2.67 to 6.92% (w/w). The experimental data were well-fitted to the second-order polynomial equations for all responses. By analysis of variance (ANOVA), the regression models were highly significant at 95% confidence interval as indicated in Table 3. Models also show better reproducibility by having the coefficient of determination (R^2) of 0.9569 and 0.9785 for oleoresin yield and total resin content, respectively. This also agrees with the plots of predicted values versus actual data as shown in Fig. 2. Moreover, each model was statistically acceptable due to having low value of variation coefficient, i.e. $CV < 10\%$ (Table 3). Owing to particle size as a categorical factor, only the combined effects of two factors of duration, and raw material to solvent ratio were observed and visualized through the generated 3D response surface and contour plots for each response variable.

Table 2
The CCD design matrix and corresponding response values.

Run	Variables code			Responses	
	Extraction duration (hour) [X_1]	Raw material to ethanol ratio (g/ml) [X_2]	Particle size of gaharu [X_3]	Oleoresin yield (% w/w)	Total resin content (% w/w)
1	3	1:10	S1	3.50	2.67
2	7	1:10	S1	5.00	3.83
3	3	1:30	S1	5.00	4.33
4	7	1:30	S1	6.67	4.58
5	2.57	1:20	S1	4.83	3.83
6	7.43	1:20	S1	6.33	4.50
7	5	1:7.86	S1	3.33	3.00
8	5	1:32.14	S1	5.67	4.67
9	5	1:20	S1	5.83	4.33
10	5	1:20	S1	5.92	4.17
11	5	1:20	S1	5.75	4.5
12	3	1:10	S2	4.08	3.67
13	7	1:10	S2	4.83	4.17
14	3	1:30	S2	7.08	5.42
15	7	1:30	S2	7.67	5.83
16	2.57	1:20	S2	6.17	5.00
17	7.43	1:20	S2	6.08	5.67
18	5	1:7.86	S2	4.17	3.83
19	5	1:32.14	S2	7.17	5.75
20	5	1:20	S2	6.00	5.33
21	5	1:20	S2	6.50	5.50
22	5	1:20	S2	6.92	5.64
23	3	1:10	S3	4.92	4.08
24	7	1:10	S3	4.83	4.17
25	3	1:30	S3	6.83	6.75
26	7	1:30	S3	7.25	6.33
27	2.57	1:20	S3	6.58	6.17
28	7.43	1:20	S3	7.08	6.08
29	5	1:7.86	S3	4.83	4.33
30	5	1:32.14	S3	8.00	6.92
31	5	1:20	S3	7.17	6.50
32	5	1:20	S3	7.25	6.08
33	5	1:20	S3	7.33	6.57

Table 3
Analysis of variance for oleoresin yield and total resin content.

Model	SS	DF	MS	*F*-Value	*p*-value
Oleoresin yield (R^2 = 0.9569; CV = 5.31%)					
Regression	46.57	11	4.23	42.39	< 0.0001
Residual	2.10	21	0.10		
Total	48.67	22	–		
Total resin content (R^2 = 0.9785; CV = 4.06%)					
Regression	39.05	11	3.55	86.87	< 0.0001
Residual	0.86	21	0.041		
Total	39.91	32	–		

3.3.2. Effect of variables on oleoresin yield

As for oleoresin yield, the model showed high significance at low probability of *p*-value ($p < 0.0001$) and high *F*-value of 42.39. Furthermore, the standard deviation of 0.32 also implies the model is significant. The obtained R^2_{adj} is 0.9343, which verifies the model as significant for the percentage of extraction yield. All linear, quadratic and interactions of the model were significant except for the interaction of duration, and raw material to solvent ratio. By removing the non-significant variables ($p > 0.05$), the polynomial model for oleoresin yield for particle (S3) is as showed in equation (5).

$$\text{Oleoresin yield} = 0.599 + 0.579X_1 + 0.371X_2 - 0.055X_1^2 - 6.627 \times 10^{-3}X_2^2 \quad (5)$$

According to the regression model, extraction duration represents a major positive impact on the extraction yield, followed by raw material to solvent ratio. It implies that oleoresin yield increases with increasing

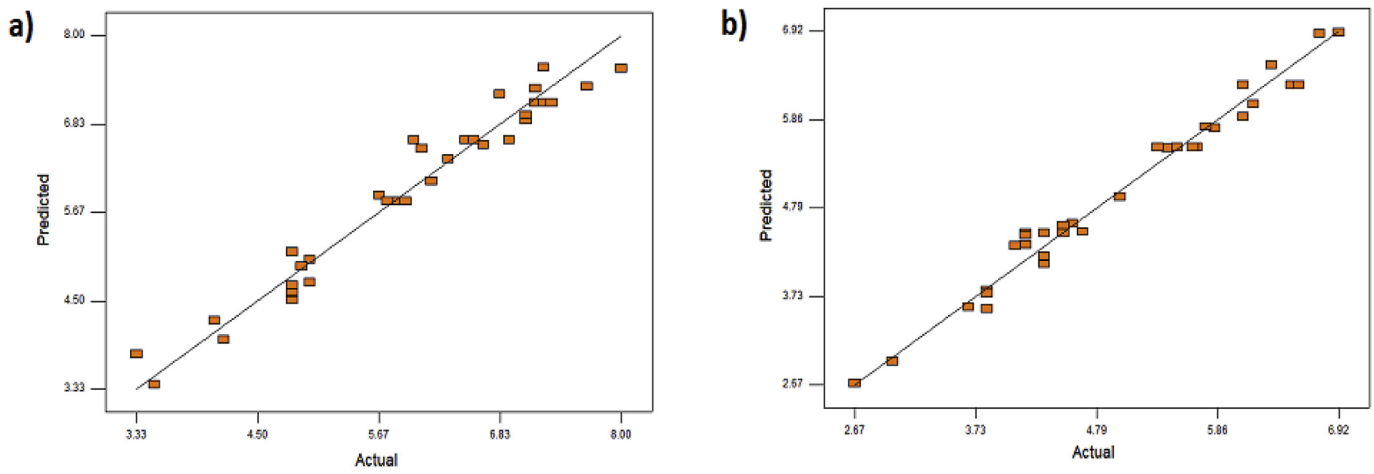

Fig. 2. Correlation between predicted values versus measured data for responses (a) oleoresin yield and (b) total resin content.

values of each variable.

Fig. 3 shows the effect of variables on oleoresin yield for all particle sizes. As the raw material to solvent ratio is increased from 1:10 to 1:30, the yield increases due to high ethanol volume which enhanced the mass transfer driving force through the solute concentration gradient (Gertenbach, 2002). Particularly exists in fluid form, gaharu oleoresin contains semi-volatile and volatile compounds of terpenics acids and chromones, which are soluble in polar solvent thus can be effortlessly extracted by ethanol especially in higher volume (Naef, 2011).

It is also clearly seen that the fine particle size (S3) has the highest extract yield at higher duration, and higher raw material to solvent ratio. Consistently, a reduced particle size increase the solid surface mass transfer area, hence led to an increase in the extract yield (Cacace and Mazza, 2003). Moreover, faster extraction time was observed for particle size S3 where the highest oleoresin yield (i.e. 7.66% w/w) is achieved at only 5.8 h. The disrupted cells in fine particle have shorter distance to travel from the solid matrix into the bulk solvent thus reducing the extraction time. Consequently, more oleoresin are easily released during the extraction process.

3.3.3. Effect of variables on total resin content

In order to verify another interesting extractives quality of gaharu wood, the effect of variables on total resin content was examined. Total resin is lipid-soluble mixtures of various compounds, which is basically made up of diterpenoid and triterpenoid containing non-volatile fraction (Yarnell, 2007). In this study, according to ANOVA results, all terms of linear, quadratic and interactions of variables were significant at $p < 0.05$. The high F-value of 86.87 certainly verified the model as significant. At particle size S3, the generated model is expressed as follows;

$$\text{Total resin} = 0.169 + 0.604X_1 + 0.352X_2 - 0.051X_1^2 - 5.150 \times 10^{-3}X_2^2 - 6.292 \times 10^{-3}X_1X_2 \quad (6)$$

Based on the regression coefficients, similar to oleoresin yield, the extraction duration also showed major positive effect on the total resin content, followed by the raw material to solvent ratio. Meanwhile, the quadratic and interactions of variables exhibit the negative impacts.

The response surface and contour plots showed that the greatest amount of total resin can be found at 29.97 ratio and 4.09 h duration in particle size S3 as shown in Fig. 4. This result suggests that high total resin is achievable under a shorter extraction duration, higher raw material to solvent ratio and smaller particle size.

In contrary, other courser particle sizes (S1 and S2) demonstrate higher duration which resulted to high total resin. It could be attributed to the presence of high molecular weights of non-volatile active compounds in gaharu resin content, which leads to a longer extraction time for solubilisation process into the bulk solvent. Moreover, consistent to mass transfer theory, the high raw material to solvent ratio resulted in increment of higher content of total resin due to active compounds which have high solubility in ethanol (Naef, 2011).

3.3.4. Determination of optimal conditions and verification experiments

The optimal conditions of gaharu oleoresin extraction were determined by maximizing the desirability of all responses with a set of "is in range" for independent variables. In this study, the chosen solution of the optimal processing parameters at desirability of 0.96% resulted to extraction duration of 4.97 h, raw material to solvent ratio of 1.29.9 g/ml and particle size S3. Under these predicted conditions, the produced gaharu oleoresin yield and total resin content were expected to obtain 7.63% and 6.90% w/w respectively. In order to validate the optimized conditions, verification experiments according to the suggested model was run in triplicate, which subsequently produced 7.08 ± 0.06% and 5.75 ± 0.86% w/w for oleoresin yield and total resin, respectively. The percentage difference between predicted and verification experimental was 7.21% for oleoresin and 16.67% for total resin content yields. Higher percentage differences for total resin content indicate that there was a fluctuation in the quality of gaharu raw materials used (Nor Azah et al., 2013). Therefore, the verification results confirmed the application of RSM method with CCD design for multi-response optimization of oleoresin extraction from gaharu wood.

3.4. Comparison of different extraction methods

Reflux extraction or batch distillation is widely used in herbal industries including gaharu oleoresin extraction due to its advantages such as ease of operate, efficient and cost-effective (Wang et al., 2013). According to the desired time, the separation continues until most of volatile and semi-volatile components in the solvent boiled out of the gaharu particles mixture. Using the same practice, there is a protocol which is currently being applied for measurement of the resin amount in gaharu by most companies worldwide (WHO, 1998). This WHO protocol helps to verify the quality of many herbal product for medicinal uses. Meanwhile, Soxhlet or hot continuous extraction is a conventional method for herbal extractives at large amounts with a much smaller quantity of solvent but a lengthy process. Thus, this technique is preferred as a simply way to extract particular solid solute using variety of solvents thus often being as a benchmark in developing new extraction method. Briefly, even though several methods were used in the extraction of gaharu oleoresin but no single procedure was proved to be superior to others.

The purpose of the present study is to compare the quality of oleoresin extract from gaharu wood using three different extraction

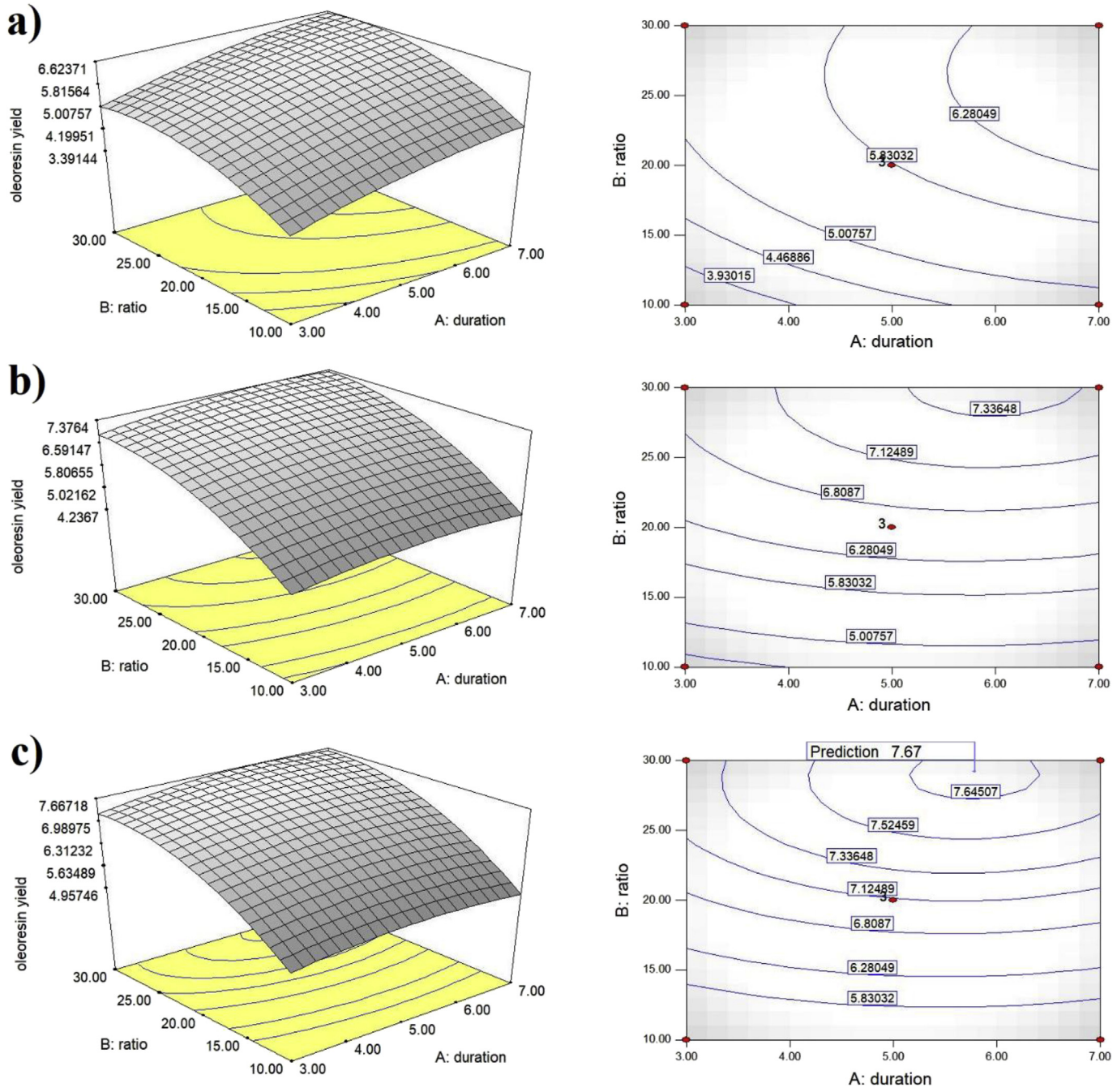

Fig. 3. The plots of response surface and contour for oleoresin yield at particle size of (a) S1, (b) S2 and (c) S3.

practices as depicted in Table 4. The same batch and grade of gaharu wood was used for comparison study to avoid any variation in intrinsic factors. It can be clearly seen that the optimized results from the study showed a much better quality compared to the reflux method according to WHO protocol. The gaharu oleoresin quality was referred to the oleoresin yield, total resin amount, gaharu oil content and TPC (mg GAE/1 g oleoresin). The WHO protocol produced the lowest values with oleoresin yield of 5.10%, total resin amount of 5.53%, gaharu oil content of 0.86% and TPC of 64.25%. Meanwhile, the higher oil content in extracts by optimized reflux method may due to higher solubility of volatile fraction from the gaharu oleoresin into the surrounding solvent as the raw material to solvent ratio (1:29.9 g/ml) and extraction duration (4.97 h) were increased compared with that obtained using the WHO protocol, i.e. raw material to solvent ratio of 1:25 g/ml and duration of 1 h. On the other hand, the Soxhlet extraction gave the lowest gaharu oil content, i.e. 0.69% which possibly due to still lack of time required for efficient azeotropic mixture in completing the oleoresin extraction.

Nevertheless, the study shows that most of the values obtained using the conventional Soxhlet method were slightly higher than the values obtained using the optimized reflux method except for the total resin (5.33%) and gaharu oil (0.69%) contents. Meanwhile, the total phenolic content (TPC) was found the highest in extracts using the Soxhlet technique, i.e. 67.84% which may due to the higher mass transfer of phenolic compounds with similar polarity to solvent (ethanol). TPC such as phenolic acids and flavonoids posses antioxidants, which play important roles in health benefits. Interestingly, the optimized reflux extraction gave the highest values of total resin amount (5.75%) and gaharu oil content (0.89%). In short, aside from lesser time consumption of the method practice, the extractives quality obtained in

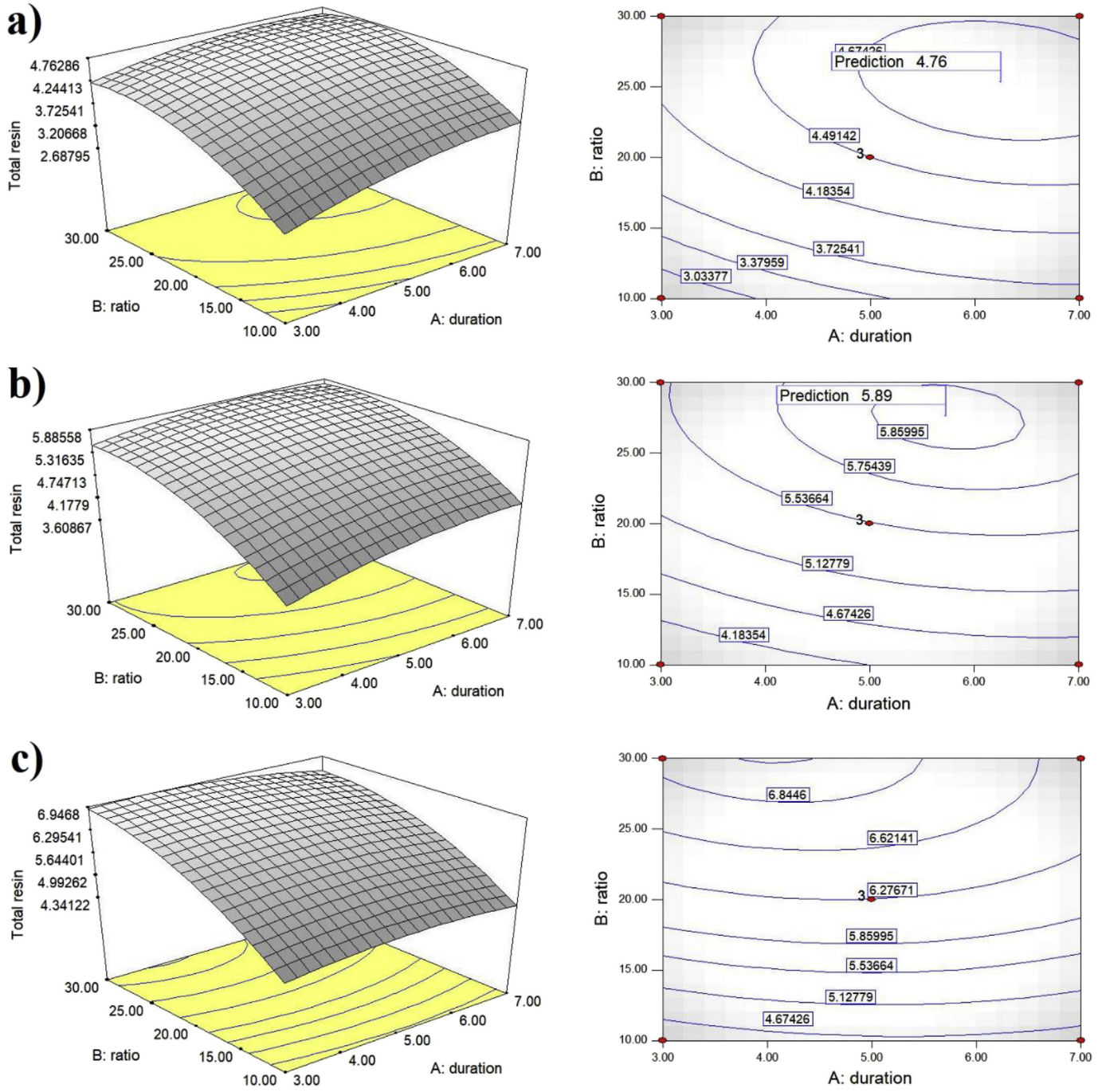

Fig. 4. The plots of response surface and contour for total resin content at particle size of (a) S1, (b) S2 and (c) S3.

Table 4
The comparison of gaharu quality from different extraction methods.

	Optimized Reflux	WHO Reflux	Soxhlet
Oleoresin yield (% w/w)	7.08 ± 0.06	5.10 ± 0.00	7.10 ± 0.00
Total resin amount (% w/w)	5.75 ± 0.83	5.53 ± 0.00	5.33 ± 0.00
Oil content (% w/w)	0.89 ± 0.01	0.86 ± 0.02	0.69 ± 0.00
TPC (mg GAE/g oleoresin)	65.23 ± 1.19	64.25 ± 4.64	67.84 ± 3.95

optimization study of reflux extraction is also highly comparable to the soxhlet method as shown in Table 4.

Though, using the same technique of reflux extraction and taken from the similar natural inoculation of agarwood species (i.e *Aquilaria malaccaensis*) but at different location (i.e. Indonesia), another study has shown that higher total resin amount was obtained compared to this study, i.e. 5.75 ± 0.83% (Balfas, 2008). Extracted by ethanol solvent, their experimental works produced resin amounts of 8.196 ± 0.15% and 7.77 ± 0.22% w/w for low grade woods originated from Jambi and Banjarmasin, respectively. The variation of the yield obtained might be due to the different grade and origin of the raw material used as well as the extraction methods applied in the experiment (Sulaiman et al., 2015). For instance, the oleoresin was dried at 80 °C for 1 h during the concentration process (Balfas, 2008). Meanwhile, the WHO protocol method of this study implemented the oleoresin that dried in a convection oven at 105 °C for 6 h and then cooled in a desiccator for 30 min before weighed. This possibly the cause of the resin amount showed a decrease in moisture content where

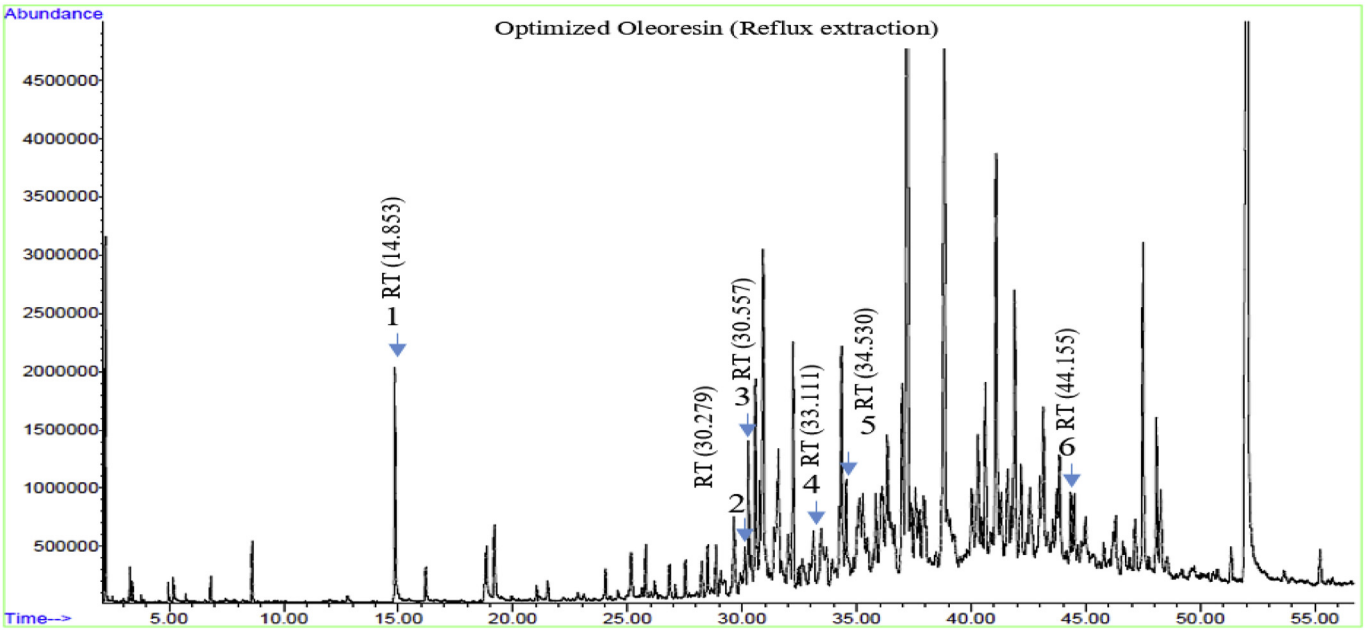

Fig. 5. GCMS Chromatogram of the gaharu oleoresin obtained via optimized reflux ethanol extraction method.

higher drying temperature and longer drying time that eventually contributed to the lower final weight of resin for each experiment (WHO, 1998; Fudholi et al., 2011).

Of further interest, the active compounds from gaharu oleoresin were identified by GCMS, and later were compared between the two better methods as above mentioned. Eventually, the identifications of six chemical compounds were revealed by matching their retention indices and masses with library databases as illustrated in Figs. 5 and 6. The results were then summarized and compared as shown in Table 5. It was found that the amount of total compounds in extracts by optimized reflux method (6.47% area) is higher compared to the Soxhlet method (3.97% area). Overall, the enhancement of total chemical compounds in optimized reflux extraction conducted in this study was 62.97% compared to the Soxhlet method.

Out results of optimized reflux extraction showed that the main

Table 5
The chemical compounds in the gaharu oleoresin from optimized reflux extraction and Soxhlet method.

Components	Peak	RT	Optimized Reflux (% area)	Soxhlet Method (% area)
4-phenyl-2-butanone	1	14.875	2.07	1.09
β-Guaiene	2	30.307	1.46	0.18
Agarospirol	3	30.605	1.38	0.84
α-bisabolene epoxide	4	32.751	0.67	0.36
Alloaromadendrene oxide	5	34.816	0.64	0.87
Aromadenderence oxide	7	44.710	0.25	0.63
Total percentage compounds			6.47	3.97

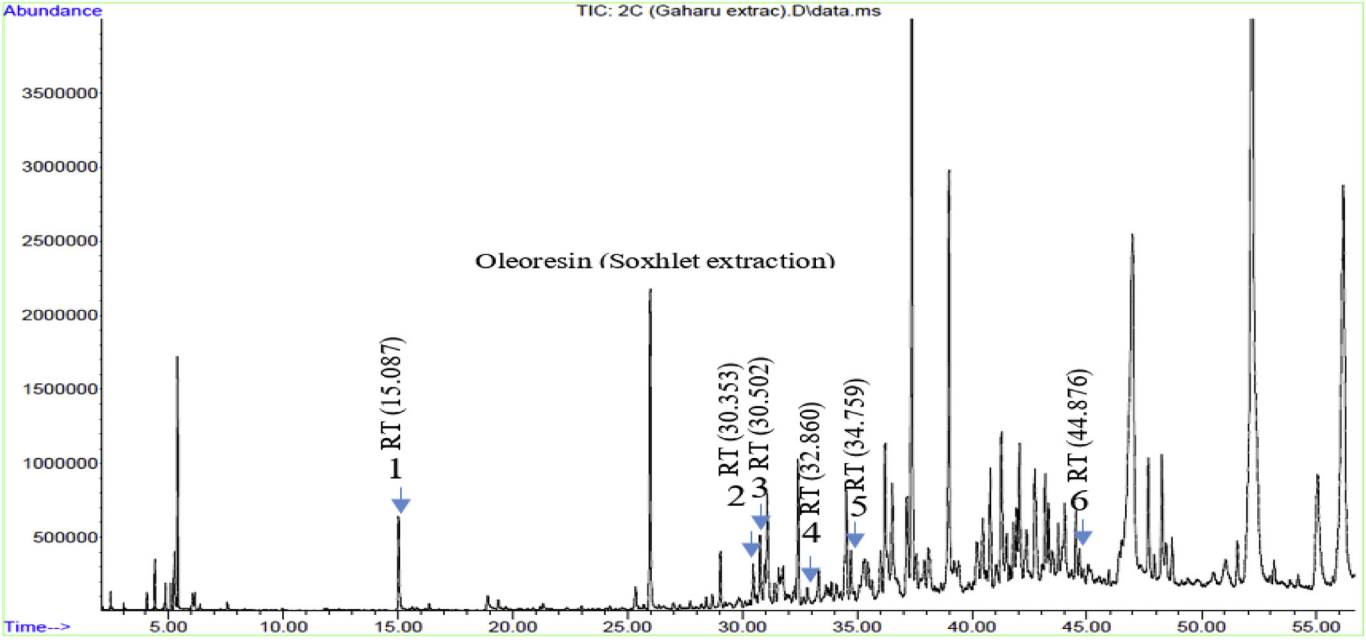

Fig. 6. GCMS Chromatogram of the gaharu oleoresin obtained via Soxhlet ethanol extraction method.

compounds presence in oleoresin have been identified as the monoterpenes and sesquiterpenes. Several studies have been done to investigate the quality of gaharu oil affected by several different factors which include species/origin of raw material, method of resin inducement and extraction method (Hashim et al., 2014; Chong et al., 2015; Bhuiyan et al., 2009). There are several chemical compounds commonly detected in high grade gaharu oil which includes 4-phenyl-2-butanone, β-guaiene, agarospirol, α-bisabolene epoxide, allo aromadendrene oxide and aromadenderence oxide. (Nor Atikah et al., 2015; Chong et al., 2015; Nurlaila et al., 2013). The similar chemical compounds were detected in the in oleoresin extract from this study show that the high quality gaharu oleoresin successfully produced. This study gives benefits to the local farmers due to the utilization of the low-grade raw material by using the proposed processing step that produces good oleoresin yield and quality.

4. Conclusion

The extraction of grade D gaharu oleoresin was successfully optimized and produced a better extractives quality using a reflux system. Process optimization by response surface methodology resulted in oleoresin yield of 7.08% and total resin content of 5.75% at a set of conditions: extraction duration of 4.97 h, raw material to solvent ratio of 1: 29.9 g/ml and particle size of 0.5–1.0 mm. Aside from a quicker practice than Soxhlet extraction (i.e. 9 h), employing the optimized reflux method also provides higher amount of gaharu oleoresin extract in terms of chemical compounds identification. The optimal conditions of ethanol reflux method enhanced the extractives quality of oleoresin from gaharu wood by 62.97% compared to the conventional technique. In brief, the outcomes from the study proved that the method used is feasible in producing better quality of gaharu oleoresin extract with less time consumption. It helps to improve the current industries practice facing the low yield and varied extractives of gaharu oleoresin products in the market.

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