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Research Paper

Optimization and comparative study of different extraction methods of biologically active components of Indonesian propolis *Trigona* spp.

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ABSTRACT

Application of propolis as a medicinal agent is not usually in the form raw material, but it must be purified by extraction with solvents. This extraction process should remove the inert material and preserve the polyphenolic (flavonoid and other phenolic compounds) fraction, which is considered to contribute more to the observed healing effects than the other propolis constituents. Aim of present study was to compare three methods of extraction: maceration, reflux, and microwave-assisted extraction (MAE), to extract polyphenolic fraction from Indonesian propolis *Trigona* spp. All of the methods were carried out in optimal conditions. Response surface methodology (RSM) was used to optimize the parameters of extraction such as extraction time, the concentration of solvent (ethanol), and microwave power. Total phenolic and flavonoid contents were determined by spectrophotometric method. The results showed that the relationship between the response (flavonoid and total phenolic yields) and the parameters of extraction followed significantly a second order polynomial models ($P<0.05$). Under optimal conditions, the method of maceration and reflux gave a similar yield, ie about 0.2% and 4% of flavonoid and total phenolic, respectively. The increase of yield was observed in MAE method that was 0.4% and 5.8% of flavonoids and total phenolics, respectively. On the basis of yield, extraction time and solvent consumption, MAE method was more efficient and selective in extracting flavonoid and total phenolic than those of two other methods.

Keywords: Optimization; Extraction; Polyphenolic fraction; Propolis.

INTRODUCTION

Propolis is a natural substance collected by bees from various plant sources which have been used since ancient times, such as traditional medicine, bio-cosmetics, and food supplementary material for human health (Bankova, et al., 2000). The composition of propolis varies depending on the source, in general, propolis contains 50% resin, 30% wax, 10% essential oils and aromatic, 5% pollen, and 5% other materials (Burdock, 1998; Sforcina, 2007). Propolis has a biological activity with a

very broad spectrum, including antimicrobial, antiviral, antioxidant, anticancer, anti-inflammatory, immunomodulatory, and agents anticaries (Burdock, 1998; Sforcina and Bankova, 2011). Flavonoids and other phenolic derivatives have been considered as the main biologically active compounds in propolis (Burdock, 1998; Ghisalberti, 1979). Banskota et al., (2001) studied Brazilian propolis in order to identify the substances with hepatoprotective activity and those active against *Helicobacter pylori*. They found that these activities were due mainly to phenolic components, but diterpenic acids also contributed to hepatoprotective activity. In a study of Brazilian propolis, it discovered a new antibacterial compounds with the main compounds in the form of phenolic derivatives, such as the 3,5-diprenyl-*p*-coumaric acid, lignans, diterpenic hydroxylated acid, and the other was non-phenolic compounds, such as acid diterpenat with labdane skeleton (Bankova, 2000; Bankova, et al., 2000).

The chemical composition (quantitative and qualitative) of propolis plays an important role in its biological activity. Therefore, the extraction methods should be developed not to damage the bioactive compounds, especially flavonoids and other phenolic. The most often utilized solvent is a aqueous solution of alcohol (ethanol or methanol) with various concentrations (Park, et al., 1998; Cunha, et al., 2004). The 70% ethanol was found to extract most of the active components of propolis but not waxes (Bankova, et al., 1992). Because propolis might contain up to 20-30% of wax, this solvent has been applied in many studies. Water has also been used in many occasions; however, it is important to note that in general, water dissolves a small part of propolis constituents, about 10% of its weight, where as 70% ethanol may dissolve 50–70% of it, depending on the wax amount.

Propolis extracts are prepared by maceration or some cases with Soxhlet extraction. Ultrasonic-assisted extraction appears to give excellent results, spectacularly accelerating the process, while the microwave treatment can cause a decrease in phenolic content due to the oxidation processes (Trusheva, et al., 2007). On the basis of this description, it indicated that the need for optimization of extraction process prior to further study the bioactivity of the propolis was required. Response surface methodology (RSM) is a collection of statistical and mathematical techniques, which is effective for the optimization process that is influenced by many factors and their interactions (Myers and Montgomery, 1995). Many reports have been published on the extraction of polyphenols from natural materials using this method (Kim, et al., 2009; Bai, et al., 2010; Singh, et al., 2011). Study of bioactive compounds of propolis Trigona spp from Baten-Indonesia has not been reported. Therefore, the selection of an effective method is important for expression of bioactivity of the propolis sample in optimal condition. In this study, three extraction methods ie without heating (maceration), with heating (reflux) and microwave (MAE) were evaluated. All the method was carried out in an optimal conditions to extract of flavonoid and other phenolic substances from propolis *Trigona* spp which are often found in Indonesian forests.

MATERIALS AND METHODS

***Raw Materials and Chemicals*:** Propolis samples collected from the area Pandeglang Banten, West Java province of Indonesia in July of 2011, stored in a plastic container and was kept in a refrigerator (-10°C) before used. The following analytical grade chemicals were used: ethanol, sodium bicarbonate, aluminum nitrate, potassium acetate and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Gallic acid and quercetin were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA).

***Maceration extraction*:** Extraction of propolis was carried out using a method described by Trusheva et al., (2007) with minor modifications. The propolis sample (5g) was extracted with 50ml of ethanol at a variety of concentrations (55-85%, v/v), and was periodically shaken at room temperature for various times (14-82h). The extract was separated from the residue by centrifugation at 1500g for 5 min. The residue was washed with 2 X 10ml of extracting solution, centrifuged and combined with the initial supernatant. The samples were preserved at 4°C until analysis.

***Reflux extraction*:** Reflux extraction was performed using a method described by Park et al. (1995) and Alencar et al., (2007) with minor modifications. The propolis sample (5g) was added to 50ml of ethanol 55-85% v/v. The sample was then refluxed in a water bath (70°C) for 10-140 min. The extract was separated from the residue by centrifugation at 1500xg for 5 min. The residue was washed with 2 X 10ml of extracting solution, centrifuged and combined with the initial supernatant. Furthermore, the samples were preserved at 4°C until analysis.

***Microwave-assisted extraction (MAE)*:** Microwave-assisted extraction was performed using a method of Trusheva et al. (2007). About 5g of the propolis sample was added 50ml of extracting solvent (ethanol 55-85%, v/v) in an extraction vessel and was then irradiated with microwaves at different power levels (420 – 600 watts) over different periods of time (5-30 min). The extract is separated from the residue by centrifugation at 1500xg for 5 min. The residue was washed with 2 X 10ml of extracting solution, centrifuged and combined with the initial supernatant. Furthermore, the samples were preserved at 4°C until analysis.

***Determination of flavonoid content*:** Flavonoid content in the extracts were determined according to the method used by Park et al. (1995), with some modifications. The sample (0.5ml aliquot 1/10) was mixed with 4.3ml of 80% ethanol, 0.1ml of 10% aluminum nitrate and 0.1ml of 1M potassium acetate. After 40 min at room temperature, absorbance was measured at 415nm with a UV-VIS spectrophotometer (Shimadzu UV-1700 Pharma Spec). The flavonoid content was calculated as quercetin equivalents (gQE/100 g sample) from a calibration curve.

***Determination of total phenolic*:** Total phenolic content in the extracts of propolis was determined by Folin-Ciocalteau colorimetric method (Singleton et al, 1999) by mixing 0.5ml aliquot (1:25) with 2.5ml of Folin-Ciocalteau reagent diluted to 1:10 and 2.0ml of 4% Na_2CO_3. Absorbance was measured at 740nm after two hours of incubation in the dark at room temperature. Total phenolics content expressed as gallic acid equivalents (g GAE/100g acid sample).

***Experimental design and statistical analysis*:** Optimization of extraction parameters for maceration and reflux was performed on the basis of response surface methodology with central composite design. The concentration of ethanol as a solvent and extraction time were taken as the variables tested in a 10-run experiment. As shown in Table 1, The two factors chosen for this study were designated as x_1 and x_2, and were prescribed into five levels, coded with -1.414, -1, 0, +1 and +1.414 from lowest to highest, respectively. The response used to determine the effect of both variables on the extraction process was a total phenolic and flavonoid content of the resulting extract.

In terms of MAE, the optimization of extraction parameters was carried out using a full factorial design involving three independent variables, namely the concentration of ethanol (x_1), extraction time (x_2), and the power of microwave irradiation (x_3) were tested in a 27-run experiment. These three variables were formulated into three levels, coded with +1, 0, -1 for the highest, intermediate and low

value, respectively. The range and level of independent variables in the optimization of the method were presented in Table 2.

All experiments were performed in duplicate and the averages of total phenolic and flavonoid yields were take as response.

Minitab 14 software was used for design and analysis of experimental data. To predict the optimal point, second-order polynomial models fitted to correlate relationship between independent variables and the response (total phenolic and flavonoid yields) as shown in the following equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{1 \le i \le j}^{k} b_{ij} x_i x_j + \sum_{i=1}^{k} b_{ii} x_i^2 \qquad (1)$$

- Where Y was the total phenolic or flavonoid levels and k was the number of variables (k = 2 for maceration and reflux mehods, and k = 3 for MAE).
- The regression coefficients of variables were intercept (b_o), linear (b_i), quadratic (b_{ii}), and interaction between variables i and j (b_{ij}).
- The independent variables were x_i and x_j ($i \neq j$).

The quality of the developed model was determined by the coefficient of determination (R^2), while the statistical significance of the model was evaluated using two way analysis of variance (ANOVA). Model and the regression coefficient was considered significant if the *P* value was less than 0.05.

RESULTS AND DISCUSSION

Optimization of extraction parameters with RSM: In all three extraction methods the ratio of propolis/solvent used was constant (1:10) refers to Trucheva at al. (2007) which stated that the use of propolis/solvent ratios larger than 1:10 (w/v) was unnecessary, leading only to solvent and energy loss.

Optimization of extraction parameters of maceration: The response values at different experimental combination of variables were presented in Table 3. The range of total phenolic and flavonoid yielded from 3.24% to 4.56% and 0.14% to 0.22%, respectively.

By applying multiple regression analysis on experimental data, the response (total phenolic, Y_1, and flavonoids, Y_2) and the test variables were related to the following second-order polynomial equation:

$$Y_1 = 4.49 + 0.103x_1 + 0.06x_2 - 1.09x_1^2 - 1.10x_2^2 + 0.26x_1x_2 \qquad (2)$$

$$Y_2 = 0.21 + 0.01x_1 + 0.01x_2 - 0.05x_1^2 - 0.06x_2^2 + 0.01x_1x_2 \qquad (3)$$

Significance and suitability of the model could be evaluated using analysis of variance (ANOVA) (Fu, et al., 2007). ANOVA results (Table 4) showed that the regression model was significant ($P<0.05$). The coefficient of determination (R^2) and the adjusted determination coefficient (Adj R^2) for total phenolics were 0.930 and 0.843, respectively and for flavonoid 0.952 and 0.893, which were suggested that there were high degree of correlation between the observed and predicted values. Moreover, a low value of coefficient of the variation (CV<10%) illustrated that the model was considered to be reproducible. In addition, the value of P for the lack of fit ($P>0.05$) implied that the model of correlation between variables and the response was significant.

Equation (2) and (3) allowed the prediction of the effects of ethanol concentration and extraction time on total phenolic and flavonoid content in the extract of propolis samples. Under this design, the optimum conditions to obtain maximum total phenolic were as followed: ethanol concentration 70.72% and extraction time 49.21 hours with the predicted total phenolic content 4.50%. In the case of optimum parameters to obtain maximum yield of flavonoid were as: ethanol concentration 71.99% and the extraction time 50.03 hours with the predicted flavonoid content 0.21% .

Ethanol concentration and extraction time by maceration method to gave the maximal yield of total phenolics and flavonoid were relatively similar so that the optimal extraction can be performed on the same conditions. Trusheva, et al., (2007) has done extracted propolis by maceration with 70% of ethanol for 72 hours, whereas Shouqin et al., (2005) have used 70% of ethanol for 7 days. Mărghitas et al., (2007) also have been using 70% of ethanol with extraction time of 24 hours to validate analytical methods for determination of total phenols and flavonoid in romanian propolis. Miguel et al., (2011) has done extracted by maceration with 70% ethanol for 96 hours to identify the antioxidan activity of propolis from *Algarve*. All of these researchers did not give a description about the conditions used by the optimization process. However, the ethanol concentration used by these researchers was the same and also similar to the optimal solvent conditions were evaluated. In contrast, the optimum extraction time obtained in this study was shorter than the time of extraction carried out by these researchers. From this picture, the optimization of the extraction method needs to be done to get the maximal of bioactive components in order to further exploration of the bioactive component is also optimal, in addition to more efficient extraction process takes place.

Optimization of extraction parameters of reflux: Optimization data of extraction parameters by the reflux method were presented in Table 5. Regression equation that connects the experimental response (total phenolic, Y1, and flavonoids, Y2) with variable test were as followed:

$$Y_1=4.25 + 0.09x_1 + 0.04x_2 - 0.79x_1^2 - 0.86x_2^2 – 0.11x_1x_2 \quad (4)$$

$$Y_2=0.239+ 0.002x_1 + 0.006x_2 - 0.074x_1^2 – 0.074x_2^2 – 0.003x_1x_2 \quad (5)$$

ANOVA summary, presented in Table 6, indicate that the regression model was significant for the total phenolic and flavonoid (P<0.05). Likewise, the lack of fit (P> 0.05) indicates that the model of correlation between variables and the response was significant.The coefficient of determination (R^2) and the adjusted determination coefficient (Adj. R^2) for total phenolics were 0.949 and 0.886, respectively, and for flavonoid were 0.933 and 0.849. There showed a high degree of correlation between the predicted and observed values.In addition, a smaller CV value of 10% implies that the model was considered reproducible.

The optimum conditions of independent variable to obtain the maximum total phenolic yield, were as: the concentration of ethanol 70.79% and the extraction time 77.37 minutes with a phenolic content predicted 4.25%. In the case of optimum parameters to obtain the maximum flavonoid were as: ethanol concentration 70.25% and extraction time 78.76 minutes with the flavonoid content predicted 0.24%. Based on these results, it appears that the extraction conditions to obtain maximum yield of total phenolics and flavonoids were similar.

Various studies on propolis using extraction methods which are stimulated by heat showed that the extraction conditions (ethanol concentration and extraction time) were more varied than maceration. Shouqin, et al., (2005) has conducted the extraction of polyphenols from propolis by refluxing with 95% ethanol for 4h, whereas the extraction conditions that have been used by Alencar et al., (2007) were 80% ethanol for 30 minutes. As with maceration technique, various studies using heat-assisted extraction technique (reflux) did not highlight that the conditions used have been through the process of optimization.

Optimization of extraction parameters of MAE: Table 7 presents the responses to various combinations of experimental parameters of microwave-assisted extraction. Regression equation that connects the experimental response (total phenolic, Y1, and flavonoids, Y2) with variables test obtained from the optimization process were as followed:

$$Y_1 = 5.59 - 0.46x_1 + 0.32x_2 + 0.09x_3 - 1.37x_1^2 - 0.16x_2^2 - 0.27x_3^2 - 0.11x_1x_2 + 0.21x_1x_3 + 0.09x_2x_3 \quad (6)$$

$$Y_2 = - 0.358 - 0.002x_1 + 0.017x_2 + 0.005x_3 - 0.086x_1^2 - 0.015x_2^2 - 0.005x_3^2 - 0.001x_1x_2 + 0.005x_1x_3 + 0.011x_2x_3 \quad (7)$$

ANOVA summary (Table 8) showed that the regression model was significant for the total phenolic and flavonoid (P<0.05). In addition, the predicted and observed values has a high degree of correlation as indicated by the parameter R^2 and Adj. R^2 (total phenolic: R^2 = 0.917, Adj. R^2 = 0.872 and flavonoid: R^2 = 0.789, Adj. R^2 = 0.872). In addition, the value of CV<10% illustrates that the obtained models were reproducible.

Unlike the method of extraction by maceration and reflux, the influence of ethanol concentration with MAE had a negative impact on response rates as shown in term x_1 in equation (5) and (6). Dielectric properties of the solvent towards microwave heating play an important role in microwave extraction (Letellier, et al., 1999; Kiss, et al., 2000). In this case, with increasing concentrations of ethanol will cause a decline in its the dielectric constant, reducing its ability to absorb microwaves and to extract the phenolic components. Two important things that need to be compromised in the solvent mixture, namely the extraction ability and dielectric properties of each individual solvent.

The optimum parameters obtained for maximum total phenolic, which is the concentration of ethanol 60.85%, extraction time 30.57 minutes, Power 495.4 watts with predicted total phenolic content 5.81%. In case of optimum parameters to obtain maximum levels of flavonoid, namely the con. of ethanol 64.66%, extraction time 24.42 min, power 520.9 watts with the predicted flavonoid content of 0.36% .

***Comparison of extraction methods*:** Summary of the three optimal conditions of extraction methods were shown in Table 9. The optimal conditions showed total phenolic and flavonoid yield relatively similar to maceration and reflux techniques, namely 0.2% to 4% for flavonoids and total phenolics. Increase in yield was observed in MAE technique that is 0.4% and 5.8% respectively for total phenolic and flavonoid.

Effect of the extraction time and concentration of ethanol factors for extraction by maceration method on the total phenolic and flavonoid yields were relatively equal. Instead, it appears that both factors influence the differences in the presence of heat stimulation (reflux) and especially microwave irradiation. In addition, the extraction time for the flavonoids in the presence of these was shorter than the extraction of total phenolic which illustrates that the flavonoid fraction was easily degraded/oxidized by thermal effects. This phenomenon was in accordance with the results found by Trucheva, et al., (2007). Nevertheless, this phenomenon can be suppressed by working at optimal conditions. Of the three methods tested, MAE method is more effective (based on the extraction yield, extraction time and solvent consumption) in extracting total phenolics and flavonoids than the two other methods. In addition, MAE method also showed high selectivity in extracting flavonoid fraction than the other methods tested.

CONCLUSION

Extraction time, ethanol concentration and microwave power factors were significantly influenced the yield of total phenolic and flavonoid in the propolis sample tested and these factors were related by the second-order polynomial model. On the basis of yield, extraction time and solvent consumption, MAE method was more efficient in extracting flavonoid and total phenolic than the two other methods. Further MAE method also showed high selectivity (through setting extraction time and ethanol concentration) than the other methods in extracting flavonoid. By working in optimal conditions, the influence of thermal and microwave irradiation on the oxidation bioactive components of propolis could be controlled.

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Table-1: Range and level of independent variables in the optimization of extraction by maceration and reflus methods.

Independent variables	Symbol	Level				
		-1.414	-1	0	+1	+1.414
Ethanol concentration, %	x_1	56	60	70	80	84
Extraction time, h (maceration)	x_2	14	24	48	72	82
Extraction time, min (reflux)	x_2	12	30	75	120	140

Table-2: Range and level of independent variables in the optimization of MAE.

Independent variables	Symbol	Level		
		-1	0	+1
Ethanol concentration, (%)	x_1	40	60	90
Extraction time, (min.)	x_2	5	15	30
Microwave power, (watt)	x_3	360	480	600

Table 3: Central composite design matrix of extraction parameters by the maceration method and the experimental response.

S.N.	Ethanol, %, (code)	Extraction time, h, (code)	experimental response	
			Total phenolic,%	Flavonoid, %
1	56 (-1.414)	48 (0)	3.26	0.14
2	60 (-1)	24 (-1)	3.36	0.15
3	60 (-1)	72 (1)	3.83	0.15
4	70 (0)	14 (-1.414)	3.83	0.15
5	70 (0)	48 (0)	4.44	0.21
6	70 (0)	48 (0)	4.56	0.22
7	70 (1.414)	82 (1.414)	3.24	0.15
8	80 (1)	24 (-1)	3.20	0.16
9	80 (1)	72 (1)	3.86	0.18
10	84 (-1.414)	48 (0)	3.36	0.17

Tabel-4: ANOVA for response surface second-order polynomial model of maceration method.

Responce	Sources	Adj. Sum of Squares	Degree of Freedom	Adj. Mean Squares	F	P_{value}
Total Phenolic	Regression	2.0573	5	0.4115	10.69	0.020
	Linear	0.0600	2	0.0300	0.78	0.518
	Square	1.9292	2	0.9646	25.06	0.005
	Interaction	0.0681	1	0.0681	1.77	0.254
	Residual Error	0.1540	4	0.0385		
	Lack-of-Fit	0.1474	3	0.0491	7.43	0.262
	Pure Error	0.0066	1	0.0066		
	Total	2.2113	9			
Flavonoid	Regression	0.0058	5	0.0012	15.99	0.009
	Linear	0.0009	2	0.0005	6.34	0.058
	Square	0.0048	2	0.0024	32.89	0.003
	Interaction	0.0001	1	0.0001	1.47	0.293
	Residual Error	0.0003	4	0.0001		
	Lack-of-Fit	0.0002	3	0.0001	0.97	0.615
	Pure Error	0.0001	1	0.0001		
	Total	0.0061	9			

- Total phenolic: $R^2 = 0.930$, Adj.$R^2 = 0.843$, CV = 5.42%
- Flavonoid : $R^2 = 0.952$, Adj.$R^2 = 0.893$, CV = 5.10%

Tabel-5: Central composite design matrix of extraction parameters by the reflux method and the experimental response.

S.N.	Ethanol,%, (code)	Extraction time, min., (code)	Experimental response	
			Total phenolic,%	Flavonoid, %
1	56 (-1.414)	75 (0)	3.29	0.16
2	60 (-1)	30 (-1)	3.28	0.15
3	60 (-1)	120 (1)	3.56	0.18
4	70 (0)	12 (-1.414)	3.31	0.16
5	70 (0)	75 (0)	4.30	0.23
6	70 (0)	75 (0)	4.20	0.25
7	70 (1.414)	140 (1.414)	3.31	0.16
8	80 (1)	30 (-1)	3.54	0.16
9	80 (1)	120 (1)	3.59	0.18
10	84 (-1.414)	75 (0)	3.46	0.16

Tabel-6: ANOVA for response surface second-order polynomial model of maceration method.

Responce	Sources	Adj. Sum of Squares	Degree of Freedom	Adj. Mean Squares	F	P_{value}
Total Phenolic	Regression	1.1740	5	0.2348	14.97	0.020
	Linear	0.0416	2	0.0208	1.33	0.518
	Square	1.1132	2	0.5566	35.48	0.005
	Interaction	0.0131	1	0.0131	0.84	0.254
	Residual Error	0.0627	4	0.0157		
	Lack-of-Fit	0.0577	3	0.0192	3.85	0.355
	Pure Error	0.0050	1	0.0050		
	Total	1.2368	9			
Flavonoid	Regression	0.0091	5	0.0018	11.15	0.018
	Linear	0.0002	2	0.0001	0.61	0.589
	Square	0.0088	2	0.0044	27.05	0.005
	Interaction	0.0001	1	0.0000	0.07	0.802
	Residual Error	0.0006	4	0.0002		
	Lack-of-Fit	0.0004	3	0.0001	0.76	0.667
	Pure Error	0.0002	1	0.0002		
	Total	0.0098	9			

- Total phenolic: $R^2 = 0.949$, Adj.$R^2 = 0.886$, CV = 3.50%
- Flavonoid : $R^2 = 0.933$, Adj.$R^2 = 0.849$, CV = 7.13%

Table-7: Full Factorial design matrix of MAE and the experimental response.

No	Ethanol,%, (code)	Time,min, (code)	Power, watt, (code)	experimental response	
				Total phenolic,%	Flavonoid, %
1	40 (-1)	5 (-1)	360 (-1)	4.02	0.25
2	40 (-1)	15 (0)	360 (-1)	4.64	0.28
3	40 (-1)	30 (1)	360 (-1)	4.32	0.23
4	40 (-1)	5 (-1)	480 (-1)	4.09	0.26
5	40 (-1)	15 (0)	480 (-1)	4.20	0.23
6	40 (-1)	30 (1)	480 (-1)	5.57	0.29
7	40 (-1)	5 (-1)	600 (1)	3.93	0.24
8	40 (-1)	15 (0)	600 (1)	4.00	0.23
9	40 (-1)	30 (1)	600 (1)	4.59	0.32
10	60 (0)	5 (-1)	360 (-1)	4.80	0.29
11	60 (0)	15 (0)	360 (-1)	5.95	0.37
12	60 (0)	30 (1)	360 (-1)	5.29	0.36
13	60 (0)	5 (-1)	480 (-1)	5.13	0.33
14	60 (0)	15 (0)	480 (-1)	5.41	0.37
15	60 (0)	30 (1)	480 (-1)	5.58	0.36
16	60 (0)	5 (-1)	600 (1)	4.68	0.31
17	60 (0)	15 (0)	600 (1)	5.17	0.33
18	60 (0)	30 (1)	600 (1)	5.92	0.34
19	90 (1)	5 (-1)	360 (-1)	2.85	0.21
20	90 (1)	15 (0)	360 (-1)	2.97	0.27
21	90 (1)	30 (1)	360 (-1)	3.23	0.25
22	90 (1)	5 (-1)	480 (-1)	3.27	0.25
23	90 (1)	15 (0)	480 (-1)	3.89	0.28
24	90 (1)	30 (1)	480 (-1)	3.94	0.22
25	90 (1)	5 (-1)	600 (1)	3.52	0.23
26	90 (1)	15 (0)	600 (1)	3.75	0.27
27	90 (1)	30 (1)	600 (1)	3.73	0.31

Tabel -8: ANOVA for response surface second-order polynomial model of MAE.

Responce	Sources	Adj. Sum of Squares	Degree of Freedom	Adj. Mean Squares	F	P_{value}
Total Phenolic	Regression	18.8656	9	2.0962	20.76	0.000
	Linear	5.8104	3	1.9368	19.18	0.000
	Square	10.8231	3	3.6077	35.73	0.000
	Interaction	0.7843	3	0.2614	2.59	0.087
	Residual Error	1.7166	17	0.1010		
	Total	20.5822	26			
Flavonoid	Regression	0.0491	9	0.0055	7.08	0.000
	Linear	0.0056	3	0.0018	2.41	0.103
	Square	0.0414	3	0.0138	17.92	0.000
	Interaction	0.0017	3	0.0006	0.74	0.540
	Residual Error	0.0131	17	0.0008		
	Total	0.0622	26			

- Total phenolic: $R^2 = 0.917$, Adj.$R^2 = 0.872$, CV = 7.24%
- Flavonoid : $R^2 = 0.789$, Adj.$R^2 = 0.678$, CV = 9.73%

Table-9: Summary of the total phenolic and flavonoid levels in the optimal conditions of maceration, reflux and MAE.

Methods	Extraction time		Ethanol conc., %		Power, watt		Flav (%)	TP (%)
	Flav	TP	Flav	TP	Flav	TP		
Maceration	50.03 h	49.21 h	71.99	70.72	-	-	0.21	4.50
Reflux	78.76 m	77.37 m	70.25	70.79	-	-	0.24	4.25
MAE	24.42 m	30.57 m	64.66	60.85	520.9	495.4	0.36	5.81

- Flav : Flavonoid, TP: Total phenolic, h: Hour, m: Minute

