4. DISCUSSIONS

4.1. Statistical Analysis of Response Surface Method on Ultrasound-Assisted Extraction

In Central Composite Design analysis, p-value indicates the significance of each coefficient in the polynomial regression model constructed, where the lower the p-value is, more significant the coefficient towards the overall regression model is (Zhong & Wang, 2010). Table 2 shows that the linear coefficient temperature and time, also quadratic coefficients temperature^2 and time^2 have values below 0.05 which means that they are significant. The rest of the coefficients have p-value of higher than 0.05 which means that these are insignificant coefficients in the model (Y. Wang, Liu, & Hu, 2014). However, even though there are some coefficients that are not significant, some experts argue that these coefficients are not to be removed and the regression model is viewed as a whole (Anderson & Whitcomb, 2017).

To check if the response surface quadratic formula can be used to describe the data distribution correctly, analysis of variance was done. Table 5 and Table 8 each show the ANOVA of the 3 extraction factors on total phenolic content and antioxidant activity respectively. Since both ANOVA for total phenol content and antioxidant activity showed probability of <0.0001 ($p<0.05$), the response surface quadratic models for both responses (dependent variables) were significant and can be used to optimize the extraction factors. This is backed up by the summary of fit shown in Table 4 and Table 7 for total phenol content and antioxidant activity respectively. The R^2 of regression model of total phenol content was 0.961397 while the R^2 of regression model of antioxidant activity was 0.962376, which means that the two regression models are significant and adequately represents the data distribution (Y. Wang et al., 2014).

4.2. Effect of Ultrasound-assisted Extraction on Total Phenolic Content and Antioxidant Activity

In this study, the efficacy of UAE on betel leaves is indicated by the amount of total phenolic content and antioxidant activity present in the crude extract of betel leaves. Figures 6a, 6b and 6c show the effect of different combinations of UAE factors on total phenolic content while Figures 8a, 8b and 8c show the effect of different combinations of UAE factors on antioxidant activities. The pattern of both sets of figures looks similar, this is due to the fact that antioxidant activity from crude extract comes from the phenolic content of the extract (Pin *et al*., 2010)(Murata *et al*., 2009). In this paper, only the effect of temperature and duration of extraction will be studied on comprehensively as the experimental result in Table 2 showed that power is not a significant factor.

4.2.1. Effect of Temperature on Total Phenolic Content and Antioxidant Activity of Crude Extract

In studying the phenolic compound stability during extraction, temperature becomes the main factor to be taken into consideration (Setyaningsih *et al*., 2019). Based on Table 2, the p-value of temperature as a coefficient in regression model was 0.00000. This value put temperature as the most significant factor out of all factor. Since temperature is a linear coefficient, this means that there is a linear, directly proportional correlation between temperature and the value of total phenolic content and antioxidant value. In the same table, the p-value of quadratic coefficient temperature*temperature also shows a value below 0.05 which means that this coefficient is a significant coefficient and there is a significant quadratic correlation between temperature and total phenolic content and antioxidant activity. This correlation was depicted in Figures 6a, 6b and 6c. From these surface plots, there is a positive trend where the higher the temperature is, there is an increase in the value of total phenolic content. The same trend can be seen from Figures 8a, 8b and 8c where an increase in the temperature is followed by an increase in the antioxidant activity. In this experiment, the range of extraction temperature used in UAE was 45^{o}C-55^{o}C.

Both sets of surface plots are in accordance with several studies on UAE with temperature as one of the factors studied. Vuong *et al.*, 2015, reported similar effect of temperature where an increase in efficiency of euphol extraction. Similarly, Muruganandam *et al.*, 2017 who studied about optimization of betel leaves' phytochemicals extraction also reported that an increase in the extraction temperature would lead to an increase in yield of phytochemical components. However, despite the overall positive trend of temperature, from prediction plot in Figure 9 there was a slight downward trend beyond 51^{o}C at higher extraction time value. Some studies have concluded that at higher temperature, phenolic compounds can be damaged (Bahadur & Hathan, 2017). However, this degradation of phenolic compound should be occurring only at 72^{o}C or above, therefore there is a deviation in the experimental result. This is further backed up by a study by (Setyaningsih et al., 2019) in which the stability of phenolic compound was studied and concluded that until temperature of 70^{o}C, phenolic compounds remain stable. Since this only happens at longer extraction duration, the reduction in total phenolic content and antioxidant activity at higher temperature level can be attributed to a prolonged extraction at a high temperature which causes degradation to phenolic compounds (Wahida *et al*., 2012).

Increasing extraction temperature has several effects on the efficacy of extraction process in general, not only in UAE. Higher heat value subjected to solvent used during extraction can break cell wall easier which assist in the mass transfer of phenolic compounds out of the cell (S. Wang *et al*., 2007). Furthermore, an increase in the extraction temperature was found to be able to promote extraction of active compounds, especially phenolic compounds by increasing the diffusion coefficient and solid to solvent solubility which means now per volume unit of solvent used, there are more solutes extracted (Al-farsi & Lee, 2008).

4.2.2. Effect of Time on Total Phenolic Content and Antioxidant Activity of Crude Extract

In this study, time has a similar effect as temperature. Time is again, one of the main factors to be considered in doing ultrasound-assisted extraction (Vuong *et al*., 2015). This

statement was in accordance with the experimental result, especially effect summary of response surface shown by Table 2. The significance of time linear coefficient and time*time quadratic coefficient were lower than 0.05 which means that these two coefficient are significant in the regression model. Figures 6a, 6b, 6c, and 8a, 8b, 8c depict the effect of time and time*time coefficient on response total phenolic content and antioxidant activity respectively. In this experiment, the range of extraction duration used was 20-30 minutes.

In a study about extraction of bioactive components from arecanut using ultrasound-assisted extraction, sonication time of up to 35 minutes increases the amount of total phenolic content. Above 35 minutes of extraction at 30^{o}C, there is a decrease in the total phenolic content in the extract (Chavan & Singhal, 2013). As seen in both surface plots (Figures 6a, b, c and Figures 8a, b, c) there is an upward trend of total phenolic content and antioxidant activity when extraction time is increased while keeping the other two variables constant. At higher time and temperature combination (ie. Time = 30 minutes, temperature = 55^{o}C), there is a slight drop in both TPC and antioxidant as mentioned above. This observed phenomena, while previously unable to be explained through single factor (temperature), now can be explained. At a fixed temperature, as extraction proceeds, the TPC and antioxidant activity increase but after a certain extraction duration, the value of TPC and antioxidant activity decreases. This is due to the decomposition of some phytochemical contained in the sample (Chavan & Singhal, 2013)(J. Wang *et al.* , 2008).

The degradation of polyphenolic compounds and subsequently, antioxidant activity can be achieved through different mechanisms. One of such mechanism is through photo-oxidation where UV light can damage the structure of some polyphenolic compounds. A study conducted by Volf *et al.*, 2013 showed that irradiating gallic acid, catechin and vanillic acid with UV-C significantly reduces the antioxidant activity of those compounds due to structural degradation. Another way of losing polyphenolic compounds is through thermal degradation in which phenolic compounds are subjected to heat. The same research done by Volf *et al.*, 2013 studied thermal degradation of catechin, gallic acid and vanillic acid. When subjected to a same temperature and duration of heating, catechin

showed the worst thermal degradation followed by gallic acid and lastly vanillic acid. Nevertheless, in natural plant extract, the rate of degradation is slower as compared to pure phenolic compound. This observed effect could be due to higher concentration of phenolic compounds in natural samples which can resist thermal degradation better than pure single phenolic compound (Fischer *et al.*, 2013). To find the characteristic of phenolic compound degradation in different sample, a study exclusively conducted for each sample is needed as the complexity of phenolic compounds in each sample is different, thus affecting the overall stability of polyphenolic compounds

4.2.3. Effect of Power on Total Phenolic Content and Antioxidant Activity of Crude Extract

The effect of sonication power was proven to be the least significant factor out of all three factors. This can be seen from Table 2 where p-value of 0.23544 ($p>0.05$) which mean that power is not a significant coefficient. This same conclusion can be seen from quadratic coefficients involving power too, such as power*power, power*time and power*temperature whereby none of these quadratic coefficients were significant. This observation is backed up by some studies about optimization of ultrasound-assisted extraction using RSM, sonication power often has insignificant effect on the extraction process (Chavan & Singhal, 2013)(Vuong et al., 2015). Therefore setting sonication power at 70 Watt would be the best alternative as shown by Figure 9 where sonication power of 73.03 Watt gives out the maximum values for both response. This value is further ascertained by Wang *et al.*, 2014, where they studied the effect of sonication power on extraction of polysaccharides from *Trametes robiniophila*. When sonication power gets too high, may cause some active components to be hydrolyzed.

4.3. Optimization of Extraction Factors and Suggestions to Improve the Result

Based on Figure 9, the most optimum combinations of factors to achieve the maximum values of TPC and antioxidant activity are extraction temperature of 55^{o}C, extraction time of 27.55 minutes and sonication power of 73.04 Watt. This result was in accordance with the experimental result obtained in Table 1 where the highest and second highest values

of total phenolic content and antioxidant activity were recorded at extraction time of 25 minutes, extraction temperature of 55^{o}C and sonication power of 70 Watt, and extraction time of 30 minutes, extraction temperature of 55^{o}C, and sonication power of 90 Watt respectively. As shown in Figure 9, the optimum extraction condition was in between the extraction conditions which gave the highest and second highest total phenolic content and antioxidant activity. However, as seen in the prediction profiler in Figure 9, extraction temperature hits a maximum of 55^{o}C without reaching a peak or plateau which means that the value of both response could still be increasing with increasing extraction temperature. However, as shown by the application through prediction profiler, the standard deviation grows increasingly large the further away the point is from the experimental range, therefore, to find the exact value of responses beyond the experimental range, it is advised to conduct similar experiment with wider treatment range.

Table 3 and 6 showed lack of fit test which looks into the adequacy of the regression model to represent the data distribution. This can be attributed to the significance of the factors in the experiment. Sonication power and all coefficients related to it were found to be insignificant, thus causing the constructed model to not fit the data distribution. One way to tackle this issue is to remove the insignificant coefficients from the effect summary thus these coefficients will not be considered in the regression. However, to remove or not to remove these coefficients is still a debatable topic among experts (Anderson & Whitcomb, 2017). The choice to not remove these insignificant coefficients is has been adopted by some studies (Bawa, 2008)(Vuong *et al*., 2015)(Y. Wang *et al.*, 2014).