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*Full Length Research Paper*

# Optimization of ultrasound-assisted extraction of total phenol from betel (*Areca catechu* L.) nut seed and evaluation of antioxidant activity *in vitro*

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**Optimization of the ultrasound-assisted extraction technology for total phenol from betel (*Areca catechu* L.) nut seed was carried out. On the basis of one factor tests, the method of response surface analysis with 3 factors including extracting temperature, time and solvent-material ratio on the content of total phenol was adopted. The optimal extracting conditions are as follows: extraction temperature 58°C, extraction time 42 min and solvent-material ratio 53 ml/g. The predicted value and measured value of total phenol was 164.74 mg catechin equivalents/g of betel nut seed (mg CE/g BNS) and 160.95 mg CE/g BNS under this condition, respectively. The results indicate that the obtained model developed by response surface methodology is feasible for practical prediction. The experiments of antioxidant activity showed that the betel nut seed extract presents the strong antioxidant activities to the DPPH and ABTS radical and the $EC_{50}$ is 113.42 and 105.69 µg/ml, respectively.**

**Key words**: Betel nut seed, total phenol, ultrasound extraction, antioxidant activity.

## INTRODUCTION

Betel nut (*Areca catechu* L.) is a rare medicinal material in China, which is popularly chewed in many Asian areas including India, China, Malaysia, Thailand, Indonesia and Vietnam. Most of the Areca was planted in Hainan Province of Mainland China, but the habit of betel nut chewing is prevalent in Hunan Province particularly (Zhang and Reichart, 2007). Betel nut contains many active substances, such as arecoline, arecaidine and tannins. The main tannins in betel nut are condensed tannin, which is combined with arecoline. Many research showed that areca quid chewing is the major risk factor associated with oral squamous cell carcinoma (Wang et al., 2007; Shiuan-shinn et al., 2007; Lin et al., 2006; Tseng et al., 2007; Jeng et al., 2001), but the recent research discovered that the extracts of betel nut possessed a lot of efficacy including antibacterium, antifungal and antivirus (Anthikat et al., 2009; Zhang et al., 2009), anti-aging (Lee et al., 2001), lower cholesterol (Byun et al., 2001) and antioxidant activity (Ashawa et al., 2007).

The betel nut seed (*BNS*) was the byproduct of betel nut-processing and in this study, we used response surface methodology to optimize the extraction conditions for the total phenolics content from betel nut seed, simultaneously, the antioxidant activity of the extract from betel nut seed was evaluated by different methods including DPPH radical scavenging activity, ABTS radical scavenging activity and reducing power. This study could provide a new train of thought for the comprehensive utilization and development of high value-added products of betel nut.

## MATERIALS AND METHODS

Betel nut seed was obtained from the Areca (*Areca catechu* L.) plant grown in Hainan, China. The areca fruit was harvested in March, 2010. The samples were cleaned and dried in an air dryer at 40°C, then milled and passed through a 0.45 mm sieve. The moisture of betel nut seed powder was found to be 5.32%. DPPH (1, 1-diphenyl-2-picrylhydrazyl) and catechin were purchased from Sigma Chemical Company (St. Louis, MO) and ABTS was

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**Table 1.** Factors and levels of central composite test on extraction of total phenols from BNS.

| Level | Factor | | |
|---|---|---|---|
| | $X_1$ Extraction temperature (℃) | $X_2$ Extraction time (min) | $X_3$ Solvent-material ratio (ml/g) |
| -1.68 | 33.2 | 23.2 | 23.2 |
| -1 | 40 | 30 | 30 |
| 0 | 50 | 40 | 40 |
| 1 | 60 | 50 | 50 |
| 1.68 | 66.8 | 56.8 | 56.8 |

purchased from Amersco Company. All chemicals and solvents used were of analytical grade.

**Ultrasound-assisted extraction of phenol compounds from betel nut seed**

Ultrasound-assisted extraction was performed in an ultrasonic cleaning bath (WF-300EH type, 40 kHz, 300W, Ningbo Haishu Wufang Ultrasonic Instrument Co. Ltd., China). Samples were soaked with 70% (v/v) ethanol solvent (varying solvent-material ratio from 20 to 60, v/w) and sonicated for different times at the required temperature. The extracts were filtered through Whatman No.4 paper under vacuum, to constant volume and then used for the determination of the total phenolics content.

**Effect of extraction temperature on extraction of total phenolics compounds**

One gram of betel nut seed was soaked with 70% (v/v) ethanol (40 ml) and sonicated for 40 min at different temperatures ranging from 30 to 70℃. The extract was filtered under vacuum and the filtrates were used for the determination of the total phenolics content.

**Effect of extraction time on extraction of total phenolics compounds**

One gram of betel nut seed was soaked with 70% (v/v) ethanol (40 ml) and sonicated for different times ranging from 20 to 60 min at 50℃. The extract was filtered under vacuum and the filtrates were used for the determination of the total phenolics content.

**Effect of solvent-material ratio on extraction of total phenolics compounds**

Betel nut seed (1.0 g) was soaked with 70% (v/v) ethanol (varying solvent-material ratio from 20 to 60, v/w) and sonicated for 40 min at 50℃. The extract was filtered under vacuum and the filtrates were used for the determination of the total phenolics content.

**Experimental design**

A five level, three variable central composite rotatable designs was applied to determine the best combination of extraction variables for the total phenolics content from betel nut seed (Wang et al., 2008). 70% ethanol was found to be the optimum concentration for the extraction solvent. The effects of temperature ($X_1$), time ($X_2$) and solvent-material ratio (v/w) ($X_3$) on the yield of polyphenols were evaluated and the yield of total phenols ($Y$) from betel nut seed was taken as the response.

Regression analysis was performed based on the experimental data and the general equation of the second degree polynomial equation:

$$Y = b_0 + \sum_{n=1}^{5} b_n X_n + \sum_{n=1}^{5} b_{nn} X_n^2 + \sum_{n=1}^{4} \sum_{m=n+1}^{5} b_{nm} X_n X_m \quad (1)$$

Where, Y is the response variable, $b_0$ , $b_n$, $b_{nn}$ *and* $b_{nm}$ are the regression coefficients of variables for intercept, linear, quadratic and cross-product coefficients, respectively. $X_n$ and $X_m$ are independent variables.

The responses obtained from the experimental design set (Table 1) were subjected to multiple nonlinear regressions using the software SAS 9.0, to obtain the coefficients of the second polynomial model. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination $R^2$ and its statistical significance was checked by an *F-test*. The significances of the regression coefficient were tested by a *t-test*.

**Determination of total phenolics content**

The total phenolics content of the betel nut seed extracts was determined by the Folin-Ciocalteu method with some modifications (Duan et al., 2006). Briefly, 0.05 ml of sample was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and the volume adjusted to 10 ml with distilled water. The reaction was kept in the dark for 60 min; the absorbance was then read at 725 nm (UV-2450 spectrophotometer). Catechin was used to produce a standard curve (2.0 to 12.0 µg/ml; y = 69.232x+0.0443; $R^2$ = 0.9976) and the results were expressed as mg of catechin equivalents/g of betel nut seed (mg CE/g BNS). The experiment was repeated in triplicate and the data were reported as means±SD.

**Determination of phenol compounds antioxidant activity**

**DPPH radical scavenging activity**

To evaluate their free radical-scavenging activity, the extracts were allowed to react with a stable free radical, 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sun and Ho, 2005). Different concentrations (50, 100, 150 and 200 ppm) of betel nut extracts (0.1 ml) and BHT (0.1 ml) were added to 3.9 ml solution of DPPH (25.61 mg/l) in 70% ethanol. The mixture was shaken vigorously and left to stand for 60 min in the dark at room temperature (until stable absorbance values were obtained). A control sample was prepared as earlier described without any extracts and 70% (v/v) ethanol was used for the baseline correction. Changes in the absorbance of the sample were measured at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of the

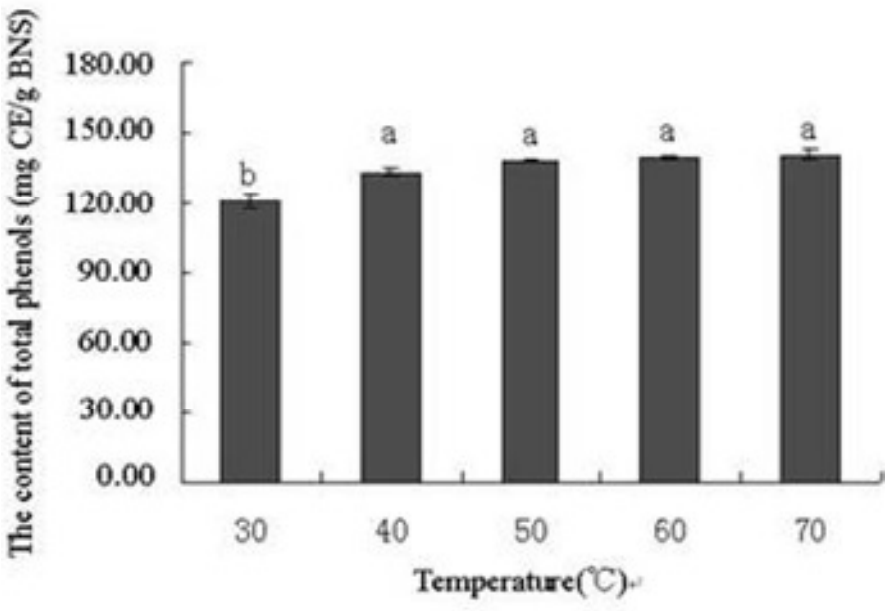


**Figure 1.** Effect of extraction temperature on the total phenols content extracted from BNS.

DPPH discoloration, using the equation:

$$\% \text{ RSA} = [(A_C - A_S)/A_C] \times 100 \quad (2)$$

Where, $A_C$ is the absorbance of the control without any extracts and $A_S$ is the absorbance with extracts.

#### ABTS radical scavenging activity

The ABTS free radical scavenging ability of betel nut seed extracts was carried out from a modified method as described by Re et al. (1999) and Fang et al. (2009). Briefly, 5.0 ml, 7.0 mM ABTS was reacted with 88.0 µl 140 mM potassium persulfate overnight in the dark to yield the $ABTS^{\cdot+}$ radical cation at room temperature. Prior to use in the assay, the $ABTS^{\cdot+}$ radical cation was diluted with 70% ethanol for an initial absorbance of 0.700±0.020 at 734 nm. Free radical scavenging activity was assessed by mixing 3.9 ml diluted $ABTS^{\cdot+}$ radical cation with 0.1 ml extracts and 0.1 ml BHT. The reaction mixture was kept at room temperature for 6 min and the absorbance was recorded at 734 nm on the UV-2450 spectrophotometer. The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100 \quad (3)$$

Where, $A_B$ is the absorbance of the blank sample and $A_E$ is the absorbance of the extract.

#### Reducing power

The reducing power assay of betel nut extracts was determined according to the method of Oyaizu (1986). Various concentrations (50, 100, 150 and 200 ppm) of the extracts (2.5 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 30 min. Following the addition of 2.5 ml 10% trichloroacetic acid (w/v), the mixture was centrifuged at 1000 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml deionised water and 1 ml 0.1% of ferric chloride and the absorbance was measured spectrophotometrically at 700 nm (Barros et al., 2007). A blank sample was prepared using distilled water instead of extract. The values are presented as the means of triplicate analyses.

#### Statistical analysis

The software SAS (SAS9.0 for windows) was used to handle the results. Analysis of variance was performed by ANOVA procedure. P values < 0.05 were regarded as significant and P values < 0.01 as very significant. The experimental results obtained were expressed as means ± SD. All analyses were performed in triplicate.

## RESULTS AND DISCUSSION

### Effect of extraction temperature on extraction of total phenolics compounds

Betel nut seeds were extracted by 70% (v/v) ethanol with different temperature and the results are presented in Figure 1. The total phenolics content increased from 120.85 to 141.07 mg CE/g BNS over the extraction temperature range (30 to 70°C), but when the temperature reached to 40°C, the increase of total phenolics content was not significant. However, the extraction temperature chosen between 40 to 60°C in view of the high temperature would affect the stability of

total phenols.



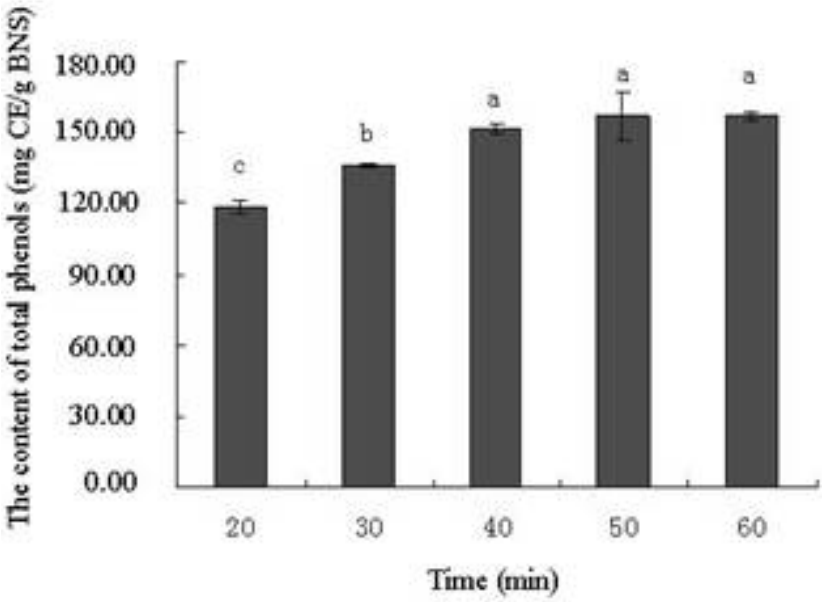


**Figure 2.** Effect of extraction time on the total phenols content extracted from BNS.

**Effect of extraction time on extraction of total phenolics compounds**

Figure 2 shows the effect of extraction time under sonication on the content of total phenolics compounds extracted from betel nut seed. A marked increase of the total phenolics content was observed up to 40 min, remaining constant until 60 min.

**Effect of solvent-material ratio on extraction of total phenolics compounds**

Figure 3 shows the effect of solvent-material ratio under sonication on the content of total phenolics compounds extracted from betel nut seed. The total phenolics content signally increased with the increase of solvent-material ratio. After the solvent-material ratio reached to 40 ml/g, the increment of total phenols was no longer markedly.

**Optimization of ultrasound-assisted extraction conditions**

In this study, ultrasound-assisted extraction was employed for total phenols extract from betel nut seed. Based on the investigations of the effects of extraction time, extraction temperature and solvent-material ratio on the content of phenolics compounds of betel nut seed, these operational parameters were optimized using central composite rotatable design combined with response surface methodology.

Table 2 shows the process variables and the results of extraction according to the factorial design. The content of phenolics compounds ranged from 117.99 mg CE/g BNS to 163.67 mg CE/g BNS. The maximum content of phenolics compounds (163.67 mg CE/g BNS) was recorded under the experimental conditions of extraction temperature 60°C, extraction time 50 min and solvent-material ratio 50 ml/g. The statistical analysis revealed that extraction time and solvent-material ratio for phenolics compounds extract are highly significant ($P < 0.01$). The most relevant variable concerning the content of total phenolics compounds was the solvent-material ratio ($P = 0.0012$) (Table 3).

Multiple regression analysis was applied on the experimental data and the coefficients of the model were evaluated for significance with a Student *t*-test (Table 3). Neglecting the non-significant terms, the regression model for total phenolics content in the extracts is presented in Equation 1. Table 4 shows the analysis of variance for the central composite rotatable design. The coefficient of determination ($R^2$) for total phenolics compounds extraction yield was 0.9745, which indicated the adequacy of the applied model.

$$Y=154.373312+10.078557X_1+7.898674X_2+19.110251X_3-7.125755X_1^2-12.014755X_2^2-10.424755X_3^2 \quad (1)$$

On the basis of Equation 1, the optimal conditions obtained by SAS analysis were as follows: extraction temperature 58°C, extraction time 42 min and solvent-material ratio 53 ml/g. The model predicted a maximum content of phenolics compounds (164.74 mg CE/g BNS)

under this optimal conditions. The experimental rechecking was done and a mean value of 160.95 mg CE/g BNS was obtained. The experimental value had a good correlation with the predicted value, so the response model was confirmed to reflect the expected optimization adequately.

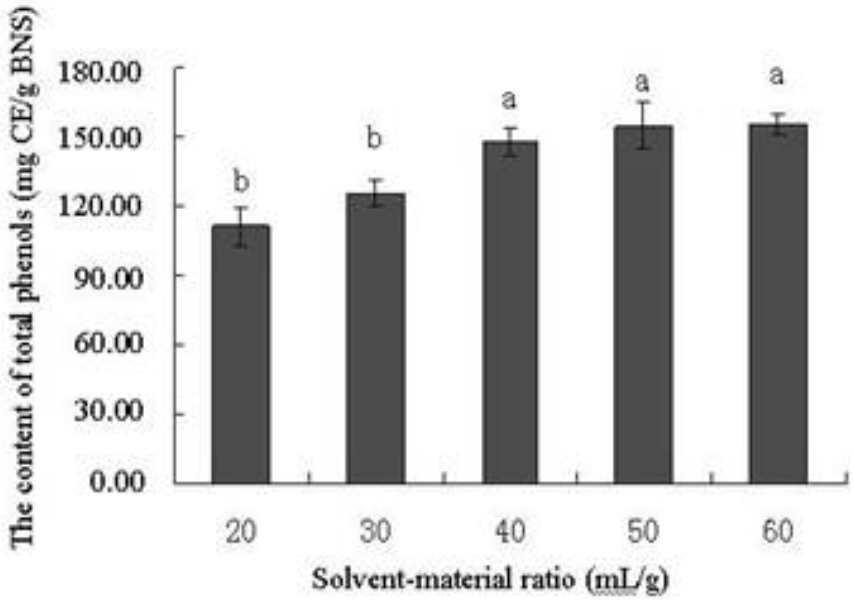


**Figure 3.** Effect of solvent-material ratio on the total phenols content extracted from BNS

**Table 2.** Experiment design and result of central composite test on extraction of total phenols from BNS.

| Run order | $X_1$ Extraction temperature (℃) | $X_2$ Extraction time (min) | $X_3$ solvent-material ratio (ml/g) | *Y* Total phenolicscontent (mg CE/g BNS) |
|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 163.67 |
| 2 | 1 | 1 | -1 | 146.27 |
| 3 | 1 | -1 | 1 | 154.95 |
| 4 | 1 | -1 | -1 | 135.24 |
| 5 | -1 | 1 | 1 | 153.47 |
| 6 | -1 | 1 | -1 | 137.12 |
| 7 | -1 | -1 | 1 | 150.24 |
| 8 | -1 | -1 | -1 | 117.99 |
| 9 | 1.68 | 0 | 0 | 157.94 |
| 10 | -1.68 | 0 | 0 | 133.81 |
| 11 | 0 | 1.68 | 0 | 147.55 |
| 12 | 0 | -1.68 | 0 | 134.42 |
| 13 | 0 | 0 | 1.68 | 163.26 |
| 14 | 0 | 0 | -1.68 | 121.89 |
| 15 | 0 | 0 | 0 | 156.44 |
| 16 | 0 | 0 | 0 | 153.20 |
| 17 | 0 | 0 | 0 | 153.97 |

To investigate the interactive effects of operational parameters on total phenolics compounds extract, three-

**Table 3.** Regression coefficients of predicted quadratic polynomial model.

| Parameter | Estimate | Standard error | *t* value | *P* value |
|---|---|---|---|---|
| Intercept | 154.373312 | 55.054709 | -3.67 | 0.0080 |
| $X_1$ | 10.078557 | 1.184806 | 3.23 | 0.0144 |
| $X_2$ | 7.898674 | 1.08578 | 4.56 | 0.0026 |
| $X_3$ | 19.110251 | 1.08578 | 5.27 | 0.0012 |
| $X_1^2$ | -7.125755 | 0.009812 | -2.57 | 0.0368 |
| $X_1X_2$ | -0.914105 | 0.011632 | -0.28 | 0.7887 |
| $X_2^2$ | -12.014755 | 0.009812 | -4.34 | 0.0034 |
| $X_1X_3$ | -4.052614 | 0.011632 | -1.23 | 0.2569 |
| $X_2X_3$ | -6.42343 | 0.011632 | -1.96 | 0.0913 |
| $X_3^2$ | -10.424755 | 0.009812 | -3.76 | 0.0070 |

**Table 4.** Analysis of variance (ANOVA) of the regression model.

| Regression parameter | Degree of freedom | Sum of square | R-square | *F* value | *P* value |
|---|---|---|---|---|---|
| Linear | 3 | 2558.245615 | 0.8595 | 78.78 | <.0001 |
| Quadratic | 3 | 283.710885 | 0.0953 | 8.74 | 0.0092 |
| Cross product | 3 | 58.769912 | 0.0197 | 1.81 | 0.2331 |
| Total Model | 9 | 2900.726412 | 0.9745 | 29.77 | <.0001 |

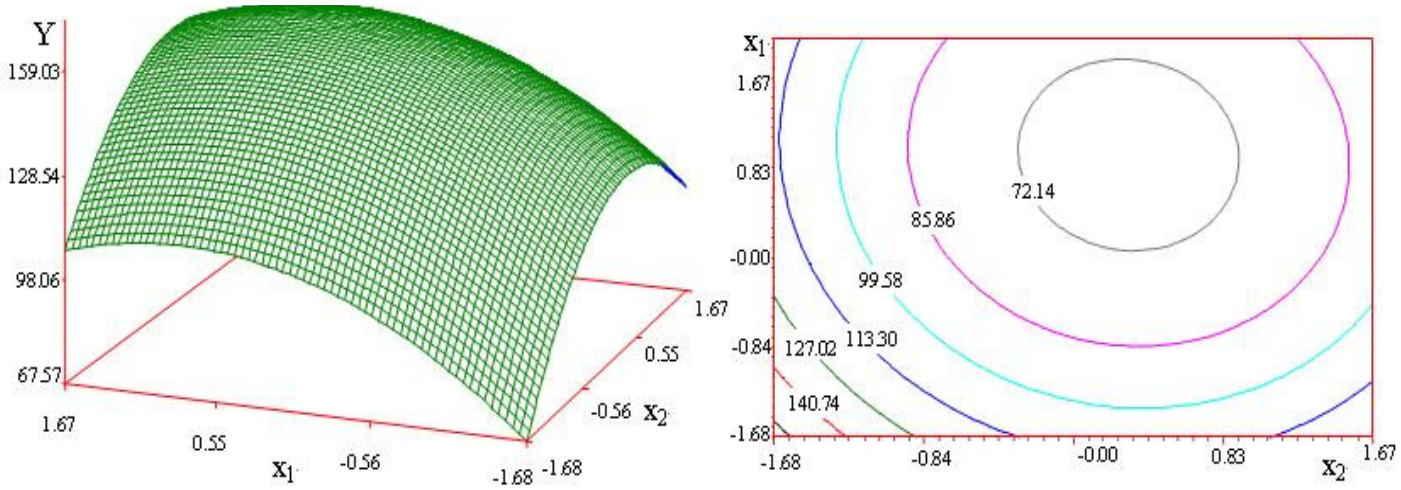


**Figure 4.** Response surface plot and contour showing the effect of extraction temperature and extracting time on the total phenols content extracted from BNS.

presented in Figures 4 to 6.

## DPPH radical scavenging activity, ABTS radical scavenging activity and reducing power of phenolics compounds from betel nut seed

Three methods were used to evaluate the antioxidant activity of the extract from betel nut seed and the result is shown in Table 5. DPPH is a very stable organic free radical with deep violet color. Upon receiving a proton from any hydrogen donor, the color of DPPH becomes yellow because of losing chromophore (Sobhy et al., 2009). From the table, we could see the extraction from BNS revealed a good antioxidant property with the increase of mass concentration by the DPPH assay, ABTS assay and reducing power assay. In the DPPH and ABTS radical scavenging activity, the $EC_{50}$ of betel nut

seed extraction was 113.42 and 105.69 mg/ml, respectively. Meanwhile, the mass concentration of



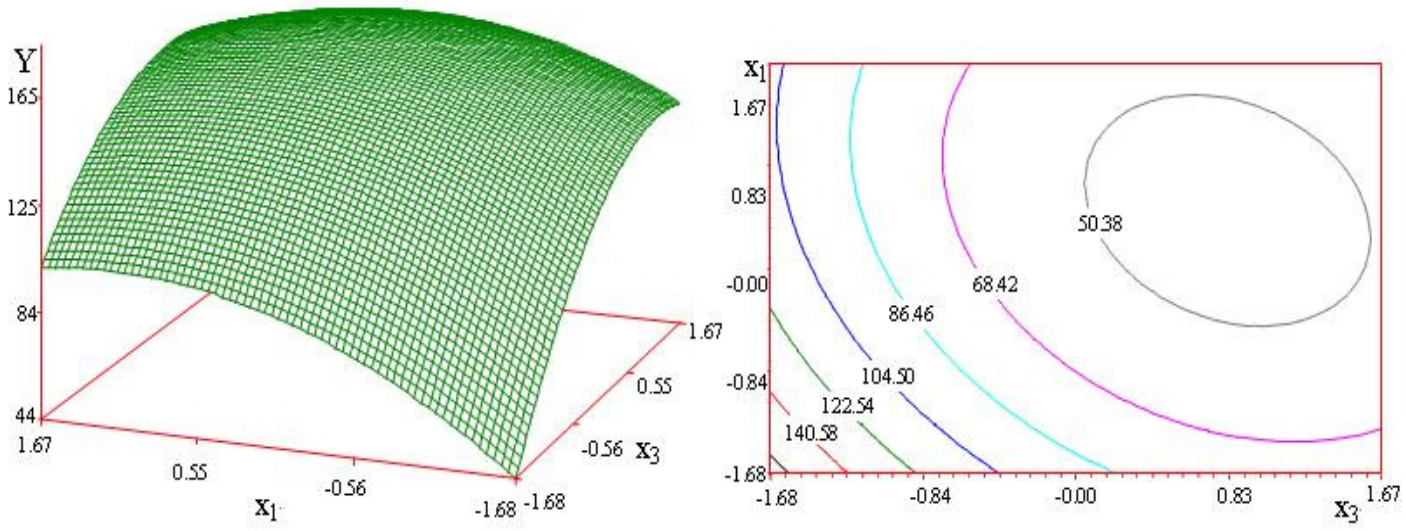


**Figure 5.** Response surface plot and contour showing the effect of extraction temperature and solvent-material ratio on the total phenols content extracted from BNS.

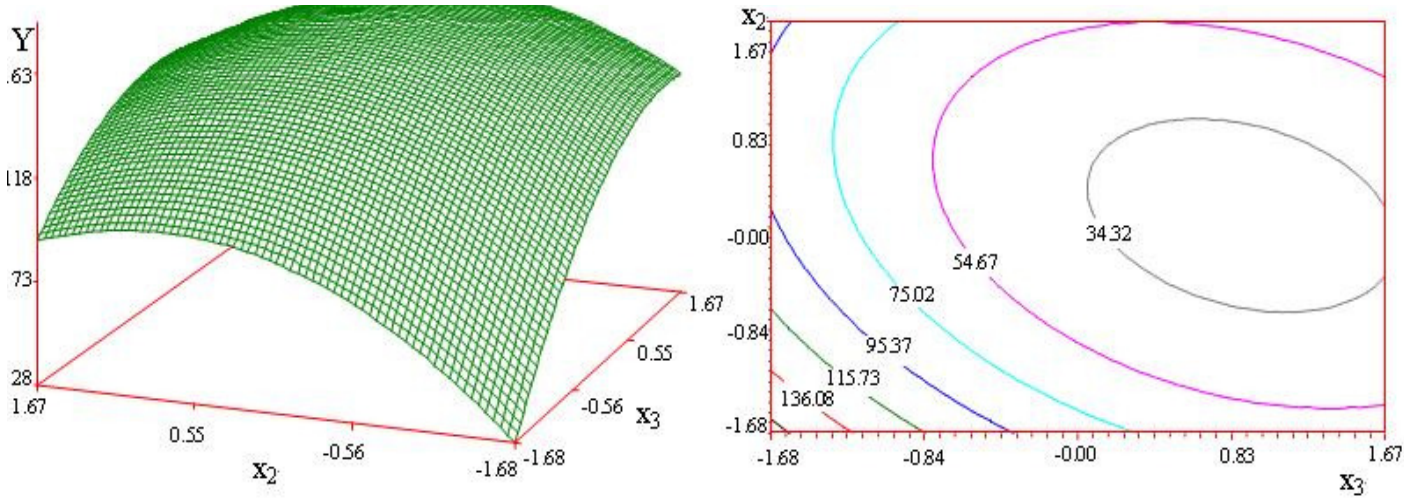


**Figure 6.** Response surface plot and contour showing the effect of extraction time and solvent-material ratio on the total phenols content extracted from BNS.

**Table 5.** The result of antioxidant activity of extraction from BNS.

| Mass concentration of extract (μg/ml) | DPPH radical scavenging activity (%) | ABTS radical scavenging activity (%) | Reducing power |
|---|---|---|---|
| 50 | 28.30 ± 1.91$^{c}$ | 24.28 ± 2.33$^{d}$ | 0.53 ± 0.06$^{d}$ |
| 100 | 49.84 ± 1.77$^{b}$ | 45.27 ± 1.28$^{c}$ | 1.24 ± 0.02$^{c}$ |
| 150 | 65.04 ± 0.29$^{a}$ | 72.18 ± 4.42$^{b}$ | 1.38 ± 0.01$^{b}$ |
| 200 | 69.72 ± 2.50$^{a}$ | 95.39 ± 1.63$^{a}$ | 1.50 ± 0.00$^{a}$ |
| Linear equation | y = 0.2789x + 18.366 | y = 0.4805x-0.7819 | y = 0.0061x+0.3977 |
| $R^2$ | 0.9311 | 0.998 | 0.82 |
| $EC_{50}$ | 113.42 | 105.69 | — |

| | | | |
|---|---|---|---|
| $EC_{50}$ of BHT | 61.98 | 127.92 | — |



extraction from BNS showed a good linear relationship with the clearance rate of DPPH and ABTS radical, as well as the reducing power, the $R^2$ were 0.9311, 0.998 and 0.82, respectively.

## Conclusion

The betel nut seed can be considered a good source of phenolics compounds. The solvent-material ratio strongly affects the phenolics content in the extracts. The optimal extracting conditions are as follows: extraction temperature 58°C, extraction time 42 min and solvent-material ratio 53 ml/g.

This study suggests that the extraction from BNS under ultrasonic revealed a good antioxidant property with the increase of mass concentration by the DPPH assay, ABTS assay and reducing power assay. Compared with the antioxidant activities of reflux extraction and ultrasonic extracting, we found that the extract which was extracted by ultrasonic extraction was stronger than reflux extraction (Zhang et al., 2009). In our subsequent study, we will separate and identify the antioxidant active compounds in the betel nut seed. More so, the toxicological experiments will be done next.

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