

## Optimum Extraction Condition of *Anacardium occidentale* on Antiurolithiatic Activities (*in-vitro*)

(Keadaan Pengekstrakan Optimum *Anacardium occidentale* pada Aktiviti Antiurolithiatik (*in-vitro*))

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### ABSTRACT

Urolithiasis or kidney stone has become a worldwide problem including Malaysia. Its management is depending on surgical procedures that are quite costly and can cause reoccurrence. *Anacardium occidentale* (cashew) has been used traditionally to treat urolithiasis but no scientific data has been recorded that proven its efficiency particularly on its extraction parameters. Therefore, this study aims to identify the optimum extraction parameters of *A. occidentale* leaves extracts on *in-vitro* antiurolithiatic activities using Response Surface Methodology (RSM). The effect of three extraction parameters (solvent concentration,  $X_1$ ; extraction temperature,  $X_2$ ; extraction time,  $X_3$ ) on antiurolithiatic activities (turbidity or nucleation assay,  $Y_1$ ; titrimetric or calcium oxalate dissolution assay,  $Y_2$ ) were determined by Central Composite Design (CCD). The results showed that each parameter and its interaction significantly affect the antiurolithiatic activities ( $p < 0.05$ ). The quadratic model was closely fitted for all responses as the  $R^2$  values obtained were 0.9786 (turbidity assay) and 0.9956 (titrimetric assay). The optimum extraction condition of *A. occidentale* leaves extract suggested by the predicted model was at 0.4% ethanol concentration, temperature 31.5 °C and for 30 min of extraction time, exhibited antiurolithiatic activities of  $85.57 \pm 0.43\%$  (turbidity) and  $96.48 \pm 0.70\%$  (titrimetric). The phytochemical screening was done on the presence of phenols, tannins, flavonoids, alkaloids, saponins as well as terpenoids and all phytochemicals were identified in the optimized *A. occidentale* leaves extract. This study has given basic scientific evidence that optimum extraction conditions are necessary to obtain optimum antiurolithiatic activity.

Keywords: *Anacardium occidentale*; antiurolithiatic; extraction parameters; *in-vitro*; RSM

### ABSTRAK

Urolithiasis atau batu karang telah menjadi permasalahan di seluruh dunia termasuk Malaysia. Pengurusannya bergantung pada prosedur pembedahan yang agak mahal dan boleh menyebabkan pembentukan semula. *Anacardium occidentale* (gajus) telah digunakan secara tradisi untuk merawat urolithiatik tetapi tiada data saintifik yang direkodkan bagi membuktikan keberkesanannya terutama pada parameter pengekstrakannya. Oleh itu, kajian ini bertujuan untuk mengenal pasti parameter pengekstrakan yang optimum bagi ekstrak daun *A. occidentale* secara *in-vitro* terhadap aktiviti antiurolithiatik melalui Kaedah Permukaan Tindak Balas (RSM). Kesan tiga parameter pengekstrakan (kepekatan pelarut,  $X_1$ ; suhu pengekstrakan,  $X_2$ ; masa pengekstrakan,  $X_3$ ) terhadap aktiviti antiurolithiatik (kaedah kekeruhan atau nukleasi,  $Y_1$ ; kaedah titrimetrik atau pembubaran kalsium oksalat,  $Y_2$ ) ditentukan oleh Pusat Reka Bentuk Komposit (CCD). Hasil kajian menunjukkan bahawa setiap parameter dan interaksinya secara signifikan mempengaruhi aktiviti antiurolithiatik ( $p < 0.05$ ). Model kuadratik telah dipilih untuk semua respons kerana nilai  $R^2$  yang diperolehi adalah 0.9786 (kaedah kekeruhan) dan 0.9956 (kaedah titrimetrik). Keadaan pengekstrakan optimum ekstrak daun *A. occidentale* yang dicadangkan oleh model ramalan adalah pada kepekatan etanol 0.4%, suhu 31.5 °C dan 30 min masa pengekstrakan, menunjukkan aktiviti antiurolithiatik  $85.57 \pm 0.43\%$  (kekeruhan) dan  $96.48 \pm 0.70\%$  (titrimetrik). Ujian fitokimia dilakukan bagi menentukan kehadiran fenol, tanin, flavonoid, alkaloid, saponin serta terpenoid dan kesemua fitokimia telah dapat dikenal pasti dalam ekstrak daun *A. occidentale* yang dioptimumkan. Kajian ini telah memberikan bukti saintifik asas bahawa keadaan pengekstrakan optimum diperlukan untuk menghasilkan aktiviti antiurolithiatik yang optimum.

Kata kunci: *Anacardium occidentale*; antiurolithiatik; *in-vitro*; parameter pengekstrakan; RSM

## INTRODUCTION

The incidence of urolithiasis or kidney stones in these current years has become a worldwide problem as it is a long term ailment that gives consequences throughout a patient's lifetime (Liu et al. 2018). This disease is generally known as the third common illness of the urinary system after urinary tract infection and prostate disease with estimated occurrence in approximately more than 1/10th of population (Bahmani et al. 2016; Shukla et al. 2017). Urolithiasis is defined as non-metallic minerals in the urinary tract which major by 75% calcium stones (Bahmani et al. 2016). In Malaysia, the pattern of incidence was risen for period of year in 1962 to 1981 per 100,000 populations (Liu et al. 2018; Sreenevasan 1990). To the best of our knowledge, there were lack of documentation on this disease in Malaysia and last published was in 1990 for overall states in Malaysia and recent one is in 2018 for only Kelantan state which dominated by Malay ethnicity with 91.1% (Nouri & Hassali 2018; Sreenevasan 1990). The prevalence of urolithiasis in Malaysia may be due to the subtropical climate that tends to accelerate body dehydration process caused by exposure to hot temperature which indirectly cause urine concentration and lead to stone formation (Hussein et al. 2013).

In this era of modernization, the management of urolithiasis is through interventional techniques, for instance, extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS) and percutaneous nephrolithotomy (PNL) that are quite expensive and can cause adverse effect to some individuals. This includes renal injury, kidney dysfunction and reoccurrence (Tiwari et al. 2012). These procedures are applicable to the stones that are larger than 5 mm or stones failed to pass through the urinary tract (Mikawlawng et al. 2014). Besides that, pharmaceutical drugs available for urolithiasis meditation mostly have side effects such as nausea, anxiety, and kidney damage that compromise their long-term use (Ankur et al. 2010). Hence, traditional remedies using medicinal plants are highly recommended as those plants have high margin of safety, cost-effective, readily available and environmental-friendly. As Malaysia has wide variety of medicinal plant sources, traditional practitioners have acknowledged many antiurolithiatic plant remedies that could inhibit and disintegrate the stone formation (Ong & Norzalina 1999).

*Anacardium occidentale* (cashew) or locally known as 'gajus' in Malaysia is from Anacardiaceae family. It is commonly distributed in the tropics, subtropical and temperate countries such as Brazil, Nigeria, Indonesia, and Malaysia. In Malaysia, the young leaves are usually consumed raw as condiment or 'ulam' (Chan

et al. 2017). *A. occidentale* plant has been extensively used for medicinal purposes as the plant family is rich with important phytochemical compounds such as tannins, saponins, alkaloids, and flavonoids with diverse biological activities that may include antiurolithiatic potential (Abu-reidah et al. 2015). Previous studies have reported that *A. occidentale* exhibits antioxidant, anti-inflammatory, anti-bacterial and anti-ulcerogenic properties (Chan et al. 2017). In relation with kidney stone, the traditional practitioners mixed paste of young or tender cashew leaves and cumin seed in coconut water and consumed it to remove stones (Kumar et al. 2014).

Extraction process is the initial step in evaluating properties of herbal plants in order to discover the potential compounds. There are many factors that may influence the extraction efficiency of the plant bioactive compounds such as extraction technique, type of solvent and its concentration, temperature, time, solid-to-liquid ratio, pH and particle size (Pandey & Tripathi 2014). One-factor-at-a-time (OFAT) is an outdated method where only a single factor is varying at a time while the other factors are constant. However, this method is laborious as it requires a lot of experimental runs, time consuming and could lead to false conclusion (Kharia & Singhai 2013). Thus, Response Surface Methodology (RSM) is used to overcome the traditional limitations as it can evaluate several factors at a time (Che Sulaiman et al. 2017). It can also evaluate the effect of independent variables and their interaction effect that is graphically represented as response surfaces (Ilaiyaraja et al. 2015).

RSM has been applied in various experiments to optimize certain experimental conditions particularly on extraction of phytochemical compounds from plant extracts (Belwal et al. 2016; Ilaiyaraja et al. 2015). Previous studies have proven that extraction condition of certain plant extracts has been successfully optimized by using RSM. Recent studies had achieved maximum phenolic and flavonoid compounds and also anti-gout activity of *Euphorbia hirta* extracts at optimum extraction condition that generated by RSM and Central Composite Design (CCD) (Abu Bakar et al. 2020). Another study by Belwal et al. (2016) had concluded that optimum condition to achieve good antioxidant activities from *Berberis asiatica* fruit extraction occurs at 80 °C, 30 min, 1:50 sample to solvent ratio, pH 3 and methanol concentration at 80%. To the best of our knowledge, there were no scientific evidence regarding the optimum extraction parameters of *A. occidentale* leaves extracts on antiurolithiatic activities are reported in the literature data. Hence, this research intended to optimize the solvent concentration, extraction temperature and extraction time towards antiurolithiatic

activities (*in-vitro*) from *A. occidentale* leaves extracts by using RSM.

## MATERIALS AND METHODS

### CHEMICALS AND REAGENTS

Cystone tablet (Himalaya, India) and potassium citrate were acted as positive control. Ethanol, sodium oxalate, calcium chloride dihydrate, Trizma® pre-set crystals, potassium permanganate, ammonium hydroxide, hydrochloric acid, sulphuric acid, chloroform, Mayer's reagent, sodium hydroxide pellets and iron (III) chloride were used in this research. All the chemicals and reagents were used of analytical grade.

### PLANT MATERIALS

*Anacardium occidentale* leaves was collected from Kampung Sri Lukut, Kluang, Johor in January 2019 and authenticated by Assoc. Prof. Dr. Alona Cuevas Linatoc, a botanist from Universiti Tun Hussein Onn Malaysia (UTHM). The voucher specimen was consigned in the herbarium of the same institute under the number NYM030-0001.

### SAMPLE EXTRACTION

The leaves of *A. occidentale* was washed and air-dried at room temperature (26 - 30 °C) until the moisture reduced at about 10% to avoid fungal and mould

contamination. Next, it was grounded and stored in a desiccator before proceed to the extraction process as stated by Belwal et al. (2016). The powdered plant extract sample (10 g) was mixed with ethanol (100 mL) at various concentration (0 - 100%) in a beaker. These samples were mixed properly for 1 to 2 min and left in a water bath at different temperatures (30 - 80 °C) for varying durations (30 - 90 min) and stirred constantly for every 10 min. Next, filter paper grade no. 1 is used to filtered the extracts and dried to room temperature (26 - 30°C). Then, it was stored at -20 °C for further testing.

### EXPERIMENTAL DESIGN

Central Composite Design (CCD) coupled with Response Surface Methodology (RSM) were developed to obtain the optimum extraction condition for maximum antiurolithiatic effect on *A. occidentale* leaves extracts. The independent variables were solvent concentration ( $X_1$ ), extraction temperature ( $X_2$ ), and extraction time ( $X_3$ ) while dependent variables were *in-vitro* antiurolithiatic assays, turbidity ( $Y_1$ ), and titrimetric ( $Y_2$ ). The independent variables were analyzed in three coded levels (-1, 0 and 1). The experimental runs were generated using statistical software package, Design Expert® (Version 6.0.4, State Ease, Inc., Minneapolis, USA). It results with 17 experimental runs as shown in Table 1 which includes three (3) central points, one (1) axial and factorial point respectively. The experimental values were fitted to second-order polynomial model to obtain the regression coefficients (Belwal et al. 2016).

TABLE 1. Design layout for the optimization process using RSM

Standard order	Solvent concentration, $X_1$ (%)	Extraction temperature, $X_2$ (°C)	Extraction time, $X_3$ (min)
1	0	30	30
2	100	30	30
3	0	80	30
4	100	80	30
5	0	30	90
6	100	30	90
7	0	80	90
8	100	80	90
9	0	55	60
10	100	55	60
11	50	30	60
12	50	80	60
13	50	55	30
14	50	55	90
15 <sup>a</sup>	50	55	60
16 <sup>a</sup>	50	55	60
17 <sup>a</sup>	50	55	60

<sup>a</sup> indicates center point

ANTIUROLITHIATIC ACTIVITY - TURBIDITY  
(NUCLEATION ASSAY)

Turbidity or nucleation assay was conducted as according to Khare et al. (2014) with a slight modification. The control experiment is done without any inhibitor which only contained 1 mL calcium chloride dihydrate (0.025 M) and 2 mL of Tris-buffer (pH 7.4) in a test tube. Then, 1 mL of sodium oxalate (0.025 M) was added. The turbidity was determined immediately at wavelength 620 nm by using UV-Vis spectrophotometer after mixing the solutions up to period of 5 min (300 s). It was performed in triplicates. The study was proceeded on standard drugs, cystone (Khare et al. 2014) and chemical drug, potassium citrate (Sharifa et al. 2012) as well as *A. occidentale* extract. Three sets of test tubes with 1 mL CaCl<sub>2</sub> (0.025 M), 2 mL Tris-buffer and 1 mL (10 mg/mL concentration) of drugs and plant extract were taken. The induction time in the presence of the drugs and plant extract were compared with control in order to calculate the nucleation rate. The results were expressed in percentage. The changes in turbidity of the solution were measured up to 5 min post mixing. Percentage of inhibition was evaluated by the graphical method using the formula as in (1):

$$\text{Inhibition (\%)} = [1 - (S_i / S_c)] \times 100 \quad (1)$$

where  $S_i$  is the slope of graph in the presence of inhibitor (drugs/extracts); and  $S_c$  is the slope without inhibitor (negative control).

ANTIUROLITHIATIC ACTIVITY - TITRIMETRIC  
(CALCIUM OXALATE DISSOLUTION ASSAY)

The titrimetric assay was done as described by Atodariya et al. (2013) with minor modifications. Homogenous precipitation method was applied to prepare experimental kidney stones, calcium oxalate (CaOx). Solution of calcium chloride dihydrate in distilled water and sodium oxalate in 10 mL of 2 N sulphuric acid were allowed to react to form precipitate. Then, the precipitate was washed with ammonia solution to remove traces of sulphuric acid, rinsed with distilled water and dried at 60 °C for 4 h. Meanwhile, a semi-permeable membrane was obtained from farm eggs by decalcifying the eggs in hydrochloric acid (2 M) until complete decalcification process. The contents of the decalcified eggs were squeezed out completely and washed thoroughly with distilled water. The membrane is then placed in ammonia solution for a while before rinsing them again with distilled water.

The dissolution percentage of calcium oxalate was determined by packing experimental calcium oxalate (1 mg) and extract (10 mg) in the semi-permeable membrane. This was allowed to suspend in a conical flask containing 100 mL of Tris buffer (0.1 M). For negative control, it was contained only 1 mg of calcium oxalate while positive control contained calcium oxalate and cystone and potassium citrate, respectively. The conical flasks of all four groups were kept in an incubator that was pre-heated to 37 °C for about 2 h and left it for 7 to 8 h. The contents of semi-permeable membranes from each group were removed into separate test tubes. Approximate 2 mL of sulphuric acid (1 N) was added to each test tube and titrated with potassium permanganate (0.9494 N) until a light pink colour end-point obtained. The percentage dissolution was calculated using following formula in (2).

$$\text{Dissolution (\%)} = [(C - T)/C] \times 100 \quad (2)$$

where C is the precipitate of calcium oxalate remained in control; and T is the precipitate of calcium oxalate remained when test solution is used.

PHYTOCHEMICAL QUALITATIVE ANALYSIS

*A. occidentale* leaves extract was screened qualitatively for detection of the phytochemical constituents using standard methods (Madike et al. 2017). The extract was prepared at concentration of 10 mg/mL.

*Test for Phenol:* Screening of phenols and tannins were done using Ferric chloride test where crude extract (1 mL) was treated with 5% solution of iron (III) chloride (2 mL). A black or blue-green colour indicates the presence of phenols and tannins.

*Test for Tannin:* Screening of phenols and tannins were done using Ferric chloride test where crude extract (1 mL) was treated with 5% solution of iron (III) chloride (2 mL). A black or blue-green colour indicates the presence of phenols and tannins.

*Test for Flavonoid:* Alkaline reagent test was applied for screening of flavonoids. Sodium hydroxide solution (10%) was taken for about 1 mL and added to plant extract (3 mL). The formation of an intense yellow colour indicates the presence of flavonoids.

*Test for Alkaloid:* Mayer's test was done for alkaloid content where the extract (2 mL) was treated with

concentrated hydrochloric acid (2 mL) and few drops of Mayer's reagent. Presence of white precipitate implies the presence of alkaloids.

*Test for Saponin:* Detection of saponin was done by foam test where a few drops of the crude extract were tested with distilled water (5 mL) and shaken vigorously. The formation of stable bubble froth for about 60 s indicates the presences of saponin.

*Test for Terpenoid:* Salkawski test was applied where chloroform (2 mL) and concentrated sulphuric acid (3 mL) were added to the crude extract to form a layer. A reddish brown colouration denotes the presence of terpenoids.

#### STATISTICAL ANALYSIS

The results were expressed as means  $\pm$  standard deviation (SD) to show variations in the various experiments. Differences are considered significant when  $p < 0.05$  (Alara et al. 2020). One-way analysis of variance (ANOVA) was performed on mean data percentage inhibition (turbidity assay) and dissolution

(titrimetric assay) of calcium oxalate crystals using commercial statistical software IBM SPSS Statistics (Version 20.0, USA) of Tukey- LSD.

## RESULTS AND DISCUSSION

### FULL MODEL FITTING

Fitting the models are essential in indicating the effectiveness of the RSM mathematical models for optimization of extraction parameters on both antiurolithiatic activities (turbidity and titrimetric) from *A. occidentale* leaves extract (Abu Bakar et al. 2020). The outcomes of the independent variables for the 17 runs following the experimental design are shown in Table 2. As recommended by the CCD, a quadratic polynomial model was selected and closely fitted for all three factors and responses. In terms of coded values, the predicted responses for both antiurolithiatic activities could be expressed by the second-order polynomial equation by multiple regression analysis as shown in Table 3. It should be noted that positive sign in the equation referring to synergistic effect while negative sign indicates antagonistic effect (Rahim et al. 2018).

TABLE 2. RSM and CCD layout and results of *A. occidentale* on both antiurolithiatic activities

Sample/ standard order	Extraction parameters			Responses				
	Solvent concentration, $X_1$ (%)	Extraction temperature, $X_2$ ( $^{\circ}$ C)	Extraction time, $X_3$ (min)	Turbidity assay, $Y_1$ (%)		Titrimetric assay, $Y_2$ (%)		
Cystone	-	-	-	94.28	$\pm$ 0.86 <sup>a</sup>	85.19	$\pm$ 4.24 <sup>b</sup>	
Potassium citrate	-	-	-	83.83	$\pm$ 1.14 <sup>b</sup>	71.33	$\pm$ 3.21 <sup>dc</sup>	
1	0	30	30	86.32	$\pm$ 1.72 <sup>b</sup>	95.19	$\pm$ 1.79 <sup>a</sup>	
2	100	30	30	83.58	$\pm$ 1.49 <sup>b</sup>	87.04	$\pm$ 2.12 <sup>ab</sup>	
3	0	80	30	53.73	$\pm$ 1.97 <sup>dc</sup>	74.07	$\pm$ 5.26 <sup>cd</sup>	
4	100	80	30	29.35	$\pm$ 2.62 <sup>e</sup>	51.85	$\pm$ 1.60 <sup>e</sup>	
5	0	30	90	54.73	$\pm$ 1.88 <sup>dc</sup>	88.89	$\pm$ 2.78 <sup>ab</sup>	
6	100	30	90	59.20	$\pm$ 2.83 <sup>cd</sup>	89.81	$\pm$ 1.60 <sup>ab</sup>	
7	0	80	90	54.98	$\pm$ 2.40 <sup>dc</sup>	64.81	$\pm$ 2.12 <sup>ef</sup>	
8	100	80	90	49.75	$\pm$ 3.02 <sup>ef</sup>	56.48	$\pm$ 4.32 <sup>fg</sup>	
9	0	55	60	59.70	$\pm$ 2.59 <sup>cd</sup>	95.83	$\pm$ 1.39 <sup>a</sup>	
10	100	55	60	55.47	$\pm$ 2.40 <sup>dc</sup>	89.81	$\pm$ 4.24 <sup>ab</sup>	
11	50	30	60	62.69	$\pm$ 1.97 <sup>c</sup>	90.74	$\pm$ 1.60 <sup>ab</sup>	
12	50	80	60	44.78	$\pm$ 2.59 <sup>f</sup>	59.26	$\pm$ 3.21 <sup>fg</sup>	
13	50	55	30	62.69	$\pm$ 0.75 <sup>c</sup>	74.07	$\pm$ 4.24 <sup>cd</sup>	
14	50	55	90	50.50	$\pm$ 4.11 <sup>ef</sup>	71.30	$\pm$ 3.21 <sup>dc</sup>	
15 <sup>a</sup>	50	55	60	51.74	$\pm$ 2.28 <sup>c</sup>	83.33	$\pm$ 1.39 <sup>b</sup>	
16 <sup>a</sup>	50	55	60	50.00	$\pm$ 0.75 <sup>ef</sup>	82.59	$\pm$ 1.60 <sup>bc</sup>	
17 <sup>a</sup>	50	55	60	51.00	$\pm$ 1.14 <sup>ef</sup>	83.80	$\pm$ 1.60 <sup>b</sup>	

'a' indicates center point; Different letters at each column indicates significant different ( $p < 0.05$ ). Each value is presented as mean  $\pm$  SD (n = 3)



The fitness of the quadratic models were validated by ANOVA of the response variables. The significance of each coefficient was determined by the Fisher's F-test (F-value) and *p*-value (Prob.> F). For both responses, the models were highly significant as the computed F-values were greater than tabulated F-value and the probability values were very low ( $p < 0.001$ ) (Behera et al. 2018). The lack of fit for turbidity and titrimetric assay were 0.0627 and 0.1311, respectively, which is non-significant ( $p > 0.05$ ), implying the model could adequately fit the responses data (Behera et al. 2018). Both assays also showed that all interaction between each parameter studied where  $X_1X_2$ ,  $X_1X_3$  and  $X_2X_3$  had significant effect as *p*-value less than 0.05.

In addition, the coefficient of determination interprets the correlation between the actual values and

predicted ones, which in this study was  $R^2 = 0.9786$  and  $R^2 = 0.9956$  for turbidity and titrimetric assay, respectively. This represents a satisfactory correlation between the experimental data and predicted values. Same goes to adjusted  $R^2$  and predicted  $R^2$  that is closely to 1 signifying that there is a high mutuality between the observed and predicted values (He et al. 2018). Besides that, coefficient of variance (CV) implies high degree of precision and reliability of the experimental values. Prior research suggested that CVs of 10% or higher means weak method performance (Dibazar et al. 2015). The obtained CVs was 5.17% (turbidity assay) and 1.77% (titrimetric assay). Hence, it can be concluded that the model is adequate for predicting inhibition and dissolution percentage of calcium oxalate crystal within the experimental ranges specified for the variables.

TABLE 3. Quadratic polynomial equations of *A. occidentale* extracts for both responses in terms of coded factors

Responses	Equations
Turbidity assay, $Y_1$	$Y_1 = 52.5200 - 3.2100 X_1 - 11.3900 X_2 - 4.6500 X_3 + 3.8600 X_1^2 + 8.5210 X_2^2 + 2.8700 X_3^2 - 3.9200 X_1X_2 + 3.2900 X_1X_3 + 9.7000 X_2X_3$
Titrimetric assay, $Y_2$	$Y_2 = 82.7910 - 4.3800 X_1 - 14.500 X_2 - 1.0930 X_3 + 10.3658 X_1^2 - 7.4542 X_2^2 - 9.7692 X_3^2 - 2.9150 X_1X_2 + 2.8700 X_1X_3 - 0.1375 X_2X_3$

$Y$  is the predicted responses where  $Y_1$  = turbidity assay and  $Y_2$  = titrimetric assay;  $X_1$ ,  $X_2$  and  $X_3$  are the values of independent variables where  $X_1$  = solvent concentration (%),  $X_2$  = extraction temperature (°C) and  $X_3$  = extraction time (min)

#### EFFECT OF EXTRACTION PARAMETERS ON TURBIDITY ASSAY

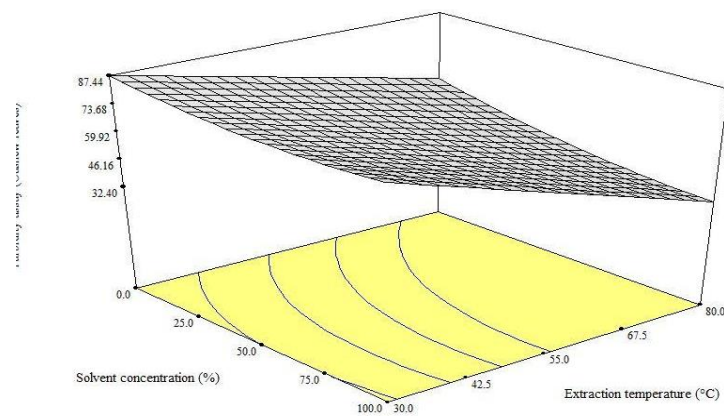
In order to investigate the interaction effect of independent variables on the optimization of extraction condition from *A. occidentale* leaves extracts, three dimensional (3D) plots were examined for turbidity and titrimetric assays. Figure 1 represents the combination effect of two parameters from the turbidity assay. As shown in Figure 1(a), a response surface plot for interaction effect of solvent concentration and extraction temperature to a fixed 30 min of extraction time on turbidity assay was illustrated. From the figure, it can be observed that using water (0% ethanol) and high extraction temperature (80 °C) was exhibited for about 60 to 74% inhibitory activity which quite good inhibition potential. This might be due the temperature being close to boiling point of water (100 °C). It was proven previously that extraction of dried plant sample at boiling point of water are able to extract more polyphenols compounds that have a large influence on inhibition activity (Nik et al. 2014).

The interaction effect of solvent concentration and extraction time on turbidity assay at fixed extraction temperature of 31.5 °C was shown in Figure 1(b). The result showed that using water (0% ethanol concentration) at 30 min of extraction able to yield up to 87% inhibition as the leaves of *A. occidentale* has hydrophilic characteristic that could release more hydrophilic compound particularly phenolic which include tannins and flavonoids (Ramos et al. 2016). Flavonoids act as anticristallo-oxalocalcic compounds which inhibit pre-formed crystal (Benalia et al. 2016). However, it was reported earlier that shorter phenolic extraction time showed not much difference to longer extraction time at room temperature (Uma et al. 2010).

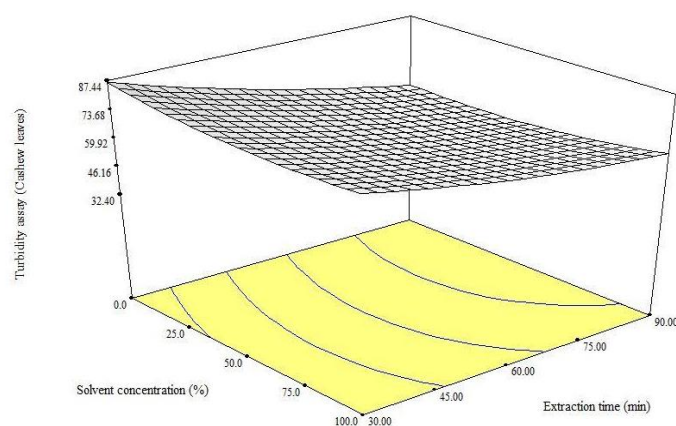
Figure 1(c) demonstrated the interaction influence of extraction temperature and extraction time at a constant ethanol concentration of 0.4%. From the figure, it was shown that at about 45 min extraction time and high extraction temperature (above 67.5 °C), showed with only 32% to 46% inhibition of calcium oxalate

crystals. This might be due to the temperature above 65 °C was reported able to degrade thermo-sensitive phenolic compound as they are easily oxidized (Kamaludin & Jaafar 2017). Besides that, high temperature for extended

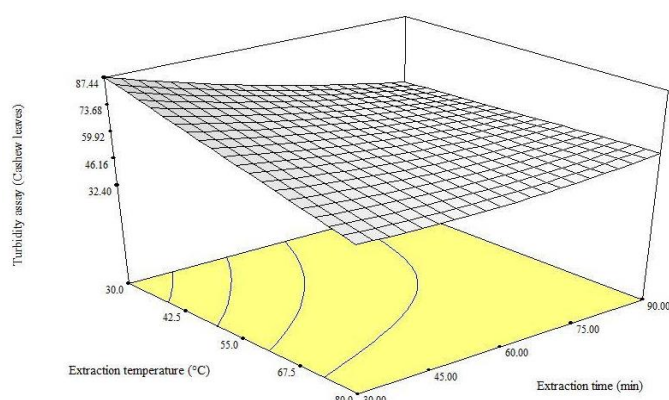
time may encourage solvent loss through vapourisation process (Uma et al. 2010). Therefore, it can be concluded that both factors had a simultaneous effect on turbidity assay.



(a)



(b)



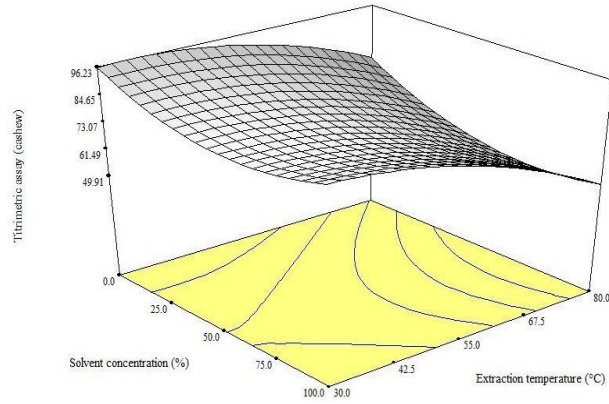
(c)

FIGURE 1. Response surface plots of *A. occidentale* showing the effect of (a) solvent concentration and extraction temperature, (b) solvent concentration and extraction time, and (c) extraction temperature and extraction time on turbidity assay

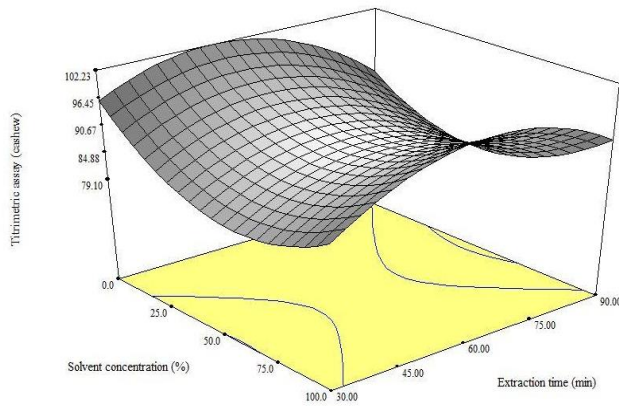
EFFECT OF EXTRACTION PARAMETERS ON  
TITRIMETRIC ASSAY

Figure 2 represents the combination effect of two extraction parameters on the titrimetric assay. As shown in Figure 2(a), a response surface plot for interaction

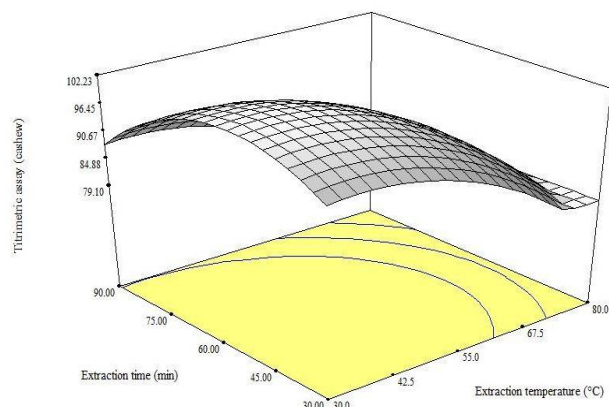
effect of solvent concentration and extraction temperature to a fixed 30 min of extraction time on titrimetric assay was presented. The percentage of dissolution was increased up to 84% at 80 °C with water. Previous study reported that hot water extraction of polyphenols works



(a)



(b)



(c)

FIGURE 2. Response surface plots of *A. occidentale* showing the effect of (a) solvent concentration and extraction temperature, (b) solvent concentration and extraction time, and (c) extraction temperature and extraction time on titrimetric assay



well for tea extracts (Banerjee & Chatterjee 2015). This is supported by another study that stated heating at 90 °C for 10 min significantly enhanced extraction yield of polyphenols as compared to lower temperatures. However, for a better quality of tea, 80 °C was suggested to ensure more compounds were extracted rather than 90 °C (Shi et al. 2003). This is concurrent with the finding in this study for *A. occidentale* leaves extract.

The interaction effect of solvent concentration and extraction time on titrimetric assay at fixed extraction temperature of 31.5 °C was shown in Figure 2(b). The results demonstrated that dissolution activity of calcium oxalate crystals is in between 79 and 85% of dissolution when extracted with 25% ethanol concentration at 75 min. Theoretically, escalating the water content in the solvent system can cause puffiness in the plant materials. This increases the surface in contact with the plant matrix, leading to extraction of more phytochemical compounds (Alara et al. 2020). Previous research had demonstrated that minimum extraction time have a slight effect on antioxidant activity (Che Sulaiman et al. 2017). Thus, combination of ethanol and water at a longer extraction time was highly suggested for a better dissolution of calcium oxalate crystals.

Figure 2(c) demonstrated the interaction effect between extraction temperature and extraction time at a constant solvent concentration of 0.4%. From the figure,

it was illustrated that the extraction temperature of 80 °C and 90 min caused dissolution activity happened at the range 84 to 90% which can be concluded quite high dissolution potential. However, it could not go any higher as high temperature may encourage solvent loss through vapourization process since boiling point of water is 100 °C (Uma et al. 2010). Therefore, it can be concluded that both factors are dependent to each other.

#### VALIDATION OF THE MODEL

The optimum condition was determined by exploiting the desirability of the responses using the statistical software. The optimum condition was used for the extraction process and later the responses was determined and validated according to the above-mentioned procedure of antiurolithiatic activities. The optimum condition for determining both antiurolithiatic activities on *A. occidentale* leaves extracts were solvent (ethanol) concentration (0.4%), extraction temperature (31.5 °C) and extraction time (30 min). Under this optimum condition, the experimental values agree with the predicted values with the percentage of errors obtained below 5% as in Table 4 (Che Sulaiman et al. 2017). Thus, it can be concluded that the model from RSM coupled with CCD was precise and reliable enough to predict the antiurolithiatic activities (*in-vitro*) of *A. occidentale* leaves extracts.

TABLE 4. Experimental data of the validation of predicted values at optimal extraction conditions for *A. occidentale* leaves extract

Responses	Predicted value	Experimental value	Percentage of error (%)
Turbidity assay (%)	86.34	85.57 ± 0.43	0.89
Titrimetric assay (%)	95.84	96.48 ± 0.70	0.67

Values were presented as mean ± standard deviation

#### PHYTOCHEMICAL SCREENING

Phytochemical screening was performed to justify the inhibitory and dissolution activity of calcium oxalate crystals on optimized *A. occidentale* leaves extracts. Prior studies had established that antiurolithiatic activities were influenced by the active compounds presence in the plant extracts particularly phenols, alkaloids, saponins, flavonoids, tannins, and terpenoids (Mumtaz et al. 2014; Phatak & Hendre 2015). Based on Table

5, it was observed with the strong presence of phenol, alkaloid, saponin, tannin, and terpenoid compounds found in optimized *A. occidentale* leaves extract. This might be because the optimized condition used was 0.4% ethanol concentration and this promotes extraction efficiency as addition of water cause swelling in the plant materials and led to increase surface in contact with the plant matrix. Hence, more phytochemical compounds were extracted (Alara et al. 2020).

TABLE 5. Phytochemical screening of optimized *A. occidentale* leaves extracts

Optimized sample	Qualitative phytochemicals assessment					
	Phenols	Alkaloids	Saponins	Flavonoids	Tannins	Terpenoids
<i>A. occidentale</i>	+++	+++	+++	+	+++	+++

'+' indicates traces; '++' indicates moderately presence; '+++ indicates strongly presence

### CONCLUSION

Optimization of extraction condition of *A. occidentale* leaves extracts on antiurolithiatic activities (*in-vitro*) was successfully employed using RSM and CCD that proven to be a useful tool for the optimization of studied extraction parameters. The optimized extraction condition was verified and it was found to be fitted with the experimental values. The best combination of solvent concentration ( $X_1$ ), extraction temperature ( $X_2$ ), and extraction time ( $X_3$ ) were found to be 0.4% ethanol concentration, 31.5 °C and 30 min, respectively. At this condition, *A. occidentale* leaves extract exhibited  $85.57 \pm 0.43\%$  inhibition (turbidity) and  $96.48 \pm 0.70\%$  dissolution (titrimetric) of calcium oxalate stones through this *in-vitro* study. In addition, phytochemical screening on the optimized *A. occidentale* leaves extract was presented with phenols, alkaloids, saponins, flavonoids, tannins and terpenoids. In conclusion, this optimum condition could be benefited for future upscale extraction of *A. occidentale* leaves extract by considering the solvent concentration, temperature and time for economical evaluation. This study could be helpful in the development of nutraceutical products containing extract of *A. occidentale*. For future recommendation, *in-vivo* studies are required to strengthen the work and prove their therapeutic usefulness.

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