AENSI Journals

Advances in Environmental Biology

ISSN-1995-0756 EISSN-1998-1066

Journal home page: http://www.aensiweb.com/aeb.html

Development and Improvement of Anti-Gout Property from Aqueous-Methanol Extract of *Morinda elliptica* using Central Composite Design

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ARTICLE INFO

Article history:
Received 14 Feb 2014
Received in revised form 24 February 2014
Accepted 29 March 2014
Available online 14 April 2014

Key words:
Morinda elliptica, Xanthine oxidase inhibitor, Gout, Optimisation, Central composite design.

ABSTRACT

Xanthine oxidase (XO) is a key enzyme in hyperuricemia, catalyzing the oxidation of hypoxanthine to xanthine and then to uric acid. Excess serum accumulated with uric acid leads to a type of arthritis known as gout. In this study, development of process conditions for XO inhibitory activity from the leaves of *Morinda elliptica* was performed by using 70% methanol. Optimization of process parameters such as extraction temperature (ºC), extraction time (h), agitation speed (rpm) and ratio of sample to solvent (1g/ml) at five levels was carried out using central composite design (CCD) for the improvement of activity to treat gout. The analysis of variance demonstrated that the model F-value of 18.31 showed the significance of the model with R^2 of 97.71%. The analysis revealed that the percentage of XO inhibitory activity was improved at 32 ºC, 30 h, 125 rpm and 1 g/15 ml of solvent. The optimized conditions were verified and the percentage of XO inhibitory activity obtained was 88.93%. The results are encouraging to formulate food, nutraceutical or pharmaceutical products incorporating natural xanthine oxidase inhibitor (XOI), an alternative to irresponsive synthetic XOI.

To Cite This Article: Parveen Jamal, Saiful Mohammad Nizam Azmi, Azura Amid, Hamzah Mohd. Salleh and Yumi Zuhanis Has-Yun Hashim., Development and Improvement of Anti-Gout Property from Aqueous-Methanol Extract of Morinda elliptica using Central Composite Design. ***Adv. Environ. Biol., 8(3),*** *734-743, 2014*

INTRODUCTION

Xanthine oxidase (XO) is a metabolic pathway for uric acid formation, in which, it catalyzes the enzymatic degradation of hypoxanthine and xanthine, and xanthine to uric acid [1]. XO generates superoxide (O_2^-) during oxidation of substrates [2] and reactive oxygen species are considered to be the main contributor to oxidative stress, which has been linked to diseases like atherosclerosis, tissue damage in rheumatoid arthritis, cancer [3, 4], various forms of ischemic injuries, inflammatory diseases, and chronic heart failure [5].

Elevated level of serum urate in human body leads to gout [6]. Gout causes inflammation of the joints and its typical symptoms include acute recurrent gouty arthritis, a tophinodular collection of monosodium urate crystals and uric acid urolithiasis. Gout can also develop as co-morbidity of other diseases such as polycythaemia, leukemia, obesity, diabetes, hypertension, renal disorders, and hemolytic anemia [7]. Gout is dominance of more than 2% in men older than 30 years and in women older than 50 years [8], and the prevalence increases with increasing age, reaching 9% in men and 6% in women older than 80 years [9].

The cornerstone of the clinical management of gout is anti-hyperuricemic therapy, either by uricosuric drugs or by XO inhibitors (XOI), such as allopurinol [5]. However, there are many adverse reactions associated with allopurinol [10], ranging from mild skin allergy to a concerted allopurinol hypersensitivity syndrome which can sometimes be life-threatening [10, 11]. Definite progress on drug development led to the discovery of new powerful XOI including purine analogs, imidazole and triazole derivatives [12]. One of these very potent new compounds is febuxostat [13], a non-purine inhibitor of XO that has a favorable toxicology profile, high bioavailability, more potent and longer-lasting hyperuricemia action than allopurinol [14]. However, it is also important to note that these synthetic XOIs, although inhibiting the activity of the XO, they are actually reduce the enzyme by transfer of an electron to oxygen, thus generating superoxide [15].

Plants have played a dominant role as a source of highly effective conventional drugs for the treatment of many diseases [16]. Consumers prefer safe and more palatable products [17], therefore, the substitution of

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currently used synthetic drugs by natural ones demands food technologists to formulate food or nutraceutical products [18], containing natural products. Our screening study revealed that an aqueous-methanol extract of *Morinda elliptica* leaves was one of the positive samples that had potent XO inhibitory effect. Two reasons accounting for the higher XO inhibitory activity of methanolic extract may be due to the nature of biological components (flavonoids, alkaloids, essential oil, terpenoids, etc), which was enhanced in the presence of methanol [19] and also the stronger extraction capacity of methanol may have produced a large number of active constituents responsible for various biological activities [20, 21].

Morinda elliptica of *Rubiaceae* family is a shrub or small tree in Peninsular Malaysia. It has been used traditionally in folk medicine for a number of health problems and ailments including loss of appetite, headaches, cholera, diarrhea and hemorrhoids [22], as anti-viral, anti-microbial, cytotoxic [23], anti-tumor-promoting and anti-oxidant [24]. The aim of this study was to look for the experimental conditions for extraction leading to maximum production of XOI from *Morinda elliptica* leaves using an aqueous-methanol as extraction solution. As many factors can influence the extraction yield, central composite design was employed to model the optimization study and response surface methodology (RSM) was applied to fit and exploit a mathematical model representing the relationship between the response (%XOI) and variables (extraction temperature, extraction time, agitation speed and sample to solvent ratio).

MATERIALS AND METHODS

Chemicals and Reagents:

Allopurinol, xanthine and xanthine oxidase (buttermilk) were purchased from Sigma-Aldrich Chemicals (St. Louis, USA). Dimethylsulphoxide (DMSO), methanol and other reagents of analytical grade were obtained from Merck (Darmstadt, Germany). Potassium di-hydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were of the highest purity.

Plant Materials:

The leaves of *Morinda elliptica* were collected fresh from its natural habitat in the state of Selangor, Malaysia on June, 2009. The plant material was authenticated by the Department of Biotechnology Engineering, IIUM, Malaysia. The leaves were soaked in water, washed to get rid of any adhering dust and impurities, and then oven dried at 40°C for 72 hours. The dried leaves were ground into powdered-like particles and stored in a –20°C freezer prior to optimization process.

Preparation of the Extract:

Aqueous-methanol (70%) was added to the ground leaves triturate (v/w) according to the design of experiment (DOE), capped with aluminum foil, and placed in a shaker incubator. The process conditions for extraction were conducted following the DOE. The mixture of plant material and solvent was filtered using Whatman No. 1 filter paper and the filtrate was collected, concentrated by vacuum rotary evaporator and dissolved in DMSO, subjected to the XO inhibitory activity assay spectrophotometrically at 295 nm.

Xanthine Oxidase Inhibitory Activity Assay:

The inhibitory effect on XO was measured spectrophotometrically at 295 nm under aerobic condition, with some modifications, following previously reported methodology [25, 26]. Allopurinol (100 µg/ml), a known XOI was used as a positive control for the inhibition test. The reaction mixture consisted of 300 µl of 50 mM sodium phosphate buffer (pH 7.5), 100 µl of sample solution dissolved in distilled water or DMSO, 100 µl of freshly prepared enzyme solution (0.2 units/ml of xanthine oxidase in phosphate buffer) and 100 µl of distilled water. The assay mixture was pre-incubated at 37 °C for 15 min. Then, 200 µl of substrate solution (0.15 mM of xanthine) was added into the mixture. The mixture was incubated at 37 °C for 30 min. Next, the reaction was stopped with the addition of 200 µl of 0.5 M hydrochloric acid (HCl). The absorbance was measured using UV/VIS spectrophotometer against a blank prepared in the same way but the enzyme solution was replaced with the phosphate buffer. Another reaction mixture was prepared (control) having 100 µl of DMSO instead of test compounds in order to have maximum uric acid formation.

The equation reported in [27], was used to evaluate the degree of XO inhibitory activity. Thus, XOI activity was assessed as the % XO inhibition = $(1 - \beta/\alpha) \times 100$, where α is the activity of XO without test extract and β is the activity of XO with test extract.

Experimental Design for Optimization of Extraction Parameters:

A four-factor and five levels Central Composite Design (CCD) showed in Table 1, consisting of twenty-one experimental runs were employed including five replicates at the center point to estimate pure error variance [28]. The design variables were extraction temperature (X_1, ºC), extraction time (X_2, h), agitation speed (X_3, rpm), and ratio of the sample to the solvent (X_4, ml/1 g) while the response variable was the degree of XO

inhibition, which is associated with uric acid formation. For each factor, the experimental range was chosen on the basis of results of preliminary experiments. In this work, the relationship between the %XOI activity and the four selected quantitative variables was approximated by the second order polynomial function. The second-order model is widely used in RSM. The model proposed for response (*Y*) is indicated in Eq. (1), as follows:

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

where b_0 is the value for the fixed response at the central point of the experiment, b_n, b_m, b_{nn} and b_{nm} are the linear, quadratic and cross product coefficients, respectively. Data is analyzed to yield regression equations, regression coefficients and validation of the model is carried out by an appropriate analysis of variance (ANOVA). The relationship between the response and the variables (factors) is visualized by a response surface or contour plot to see the relative influence of the parameters, to find an optimum parameter combination, and to predict experimental results for other parameter combinations.

Table 1: Coded and actual levels of the design factors.

Factors	Variables Levels				
	– 2	– 1	0	+ 1	+ 2
X_1: Extraction Temperature (ºC)	25	30	35	40	45
X_2: Extraction Time (h)	20	25	30	35	40
X_3: Agitation Speed (rpm)	50	75	100	125	150
X_4: Ratio of 1 g of Sample to Solvent (1g/ml)	5	10	15	20	25

Statistical Analysis:

Statistical analysis was carried out to work out means and standard deviations (mean ± S.D.) from triplicate measurements using Microsoft Office Excel 2007. Extractions conditions were optimized using contour plots for two independent parameters while fixing remaining two at coded zero levels. The isoresponse contour plots of RSM as a function of two factors at a time, holding all other factors at fixed level, are helpful for understanding both the main and the interaction effects of these two factors.

RESULTS AND DISCUSSIONS

Optimization and empirical modeling approach was of great importance to establish the optimum conditions for the improvement of XO inhibition. The fit of the model variables were performed by regression methods. The mean experimental results obtained after the 21 trials of the statistical design and the design layout are shown in Table 2, varied from 73.88% to 87.84% inhibition. Therefore, the optimization of the process parameters for the XO inhibition was able to be defined by analyzing the effect of each factor.

Table 2: Central composite arrangement for independent variables, X_1 (extraction temperature), X_2 (extraction time), X_3 (agitation speed), X_4 (sample to solvent ratio) and their response (%XOI).

Run	X_1[a]		X_2[b]		X_3[c]		X_4[d]		% XOI	
	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	Uncoded	Actual	Predicted
1	0	35.00	0	30.00	0	100.00	0	15.00		
2	0	35.00	0	30.00	0	100.00	0	15.00	86.93	86.85
3	0	35.00	0	30.00	0	100.00	0	15.00	86.49	86.85
4	0	35.00	0	30.00	0	100.00	0	15.00	86.49	86.85
5	0	35.00	0	30.00	0	100.00	0	15.00	87.84	86.85
6	+1	40.00	+1	35.00	+1	125.00	–1	10.00	83.34	82.89
7	+1	40.00	+1	35.00	–1	75.00	–1	10.00	82.88	83.39
8	+1	40.00	–1	25.00	+1	125.00	+1	20.00	82.88	82.43
9	–1	30.00	+1	35.00	–1	75.00	+1	20.00	83.34	83.84
10	+1	40.00	–1	25.00	–1	75.00	+1	20.00	81.08	81.58
11	–1	30.00	–1	25.00	+1	125.00	–1	10.00	83.34	82.89
12	–1	30.00	+1	35.00	+1	125.00	+1	20.00	84.69	84.24
13	–1	30.00	–1	25.00	–1	75.00	–1	10.00	73.88	74.38
14	–2	25.00	0	30.00	0	100.00	0	15.00	82.43	82.40
15	+2	45.00	0	30.00	0	100.00	0	15.00	81.53	81.50
16	0	35.00	–2	20.00	0	100.00	0	15.00	79.73	79.70
17	0	35.00	+2	40.00	0	100.00	0	15.00	83.34	83.31
18	0	35.00	0	30.00	–2	50.00	0	15.00	83.78	82.80
19	0	35.00	0	30.00	+2	150.00	0	15.00	86.49	87.42
20	0	35.00	0	30.00	0	100.00	–2	5.00	78.38	78.35
21	0	35.00	0	30.00	0	100.00	+2	25.00	80.18	80.15

[a]Extraction Temperature (ºC), [b]Extraction Time (h), [c]Agitation Speed (rpm), [d]Ratio of 1 g of Sample to Solvent (1 g/ml)

The interaction between the independent variables showed the maximum %XOI activity of 87.84% when the extraction temperature, extraction time, agitation speed and solvent: sample ratio were fixed at 35 °C, 30 h, 100 rpm, and 15 ml/g respectively. The minimum %XOI activity was recorded at 73.88% when the extraction temperature, extraction time, agitation speed and solvent: sample ratio were set at 30 °C, 25 h, 75 rpm and 10 ml/g respectively. The t-distribution (t-value) and the corresponding probability values (P-value) with the second order polynomial coefficients of the experimental design were evaluated. The t-value and P-value serve as tools for checking the significance of each of the coefficient. It has been reported that larger magnitude of t-value and smaller P-value indicates the high significance of the corresponding coefficient [29]. The predicted values of %XOI were calculated using regression model and compared with experimental values. The results for %XOI response was analysed and the analysis of variance (Table 3) showed the model F-value of 18.31 ($p <$ 0.0009), which implies that the model is highly significant; having the residuals distributed along a well randomized straight line.

Coefficient of determination (R^2), being the measure of the goodness fit and adequacy of the applied model, indicated that 97.71% of the total variation was explained by the model had a high correlation. The statistical analysis of the coefficients of the model detected by Student-*t*-test revealed that only two linear (X_2, X_3), three quadratic (X_1^2, X_2^2, X_4^2) and three interaction coefficients (X_1X_3, X_2X_3, X_3X_4) were significant (Table 3). It indicated that the extension of some of independent variables and their inter-dependency affected the response variable.

Table 3: Statistical analysis of variance of independent variables for optimization of the process conditions for extraction of XOI.

Source	Sum of Square	DF[a]	Mean Square	F Value	P-value Prob > F[b]	
Model	215.53	14	15.40	18.31	0.0009**	significant
A (X_1)	0.41	1	0.41	0.48	0.5136	
B (X_2)	6.52	1	6.52	7.75	0.0318*	
C (X_3)	21.37	1	21.37	25.41	0.0024**	
D (X_4)	1.62	1	1.62	1.93	0.2144	
A^2 (X_1^2)	37.58	1	37.58	44.70	0.0005**	
B^2 (X_2^2)	44.73	1	44.73	53.20	0.0003**	
C^2 (X_3^2)	4.75	1	4.75	5.65	0.0550	
D^2 (X_4^2)	90.48	1	90.48	107.62	< 0.0001**	
AB (X_1X_2)	1.53	1	1.53	1.82	0.2258	
AC (X_1X_3)	9.14	1	9.14	10.87	0.0165*	
AD (X_1X_4)	2.14	1	2.14	2.54	0.1618	
BC (X_2X_3)	11.16	1	11.16	13.28	0.0108*	
BD (X_2X_4)	2.83	1	2.83	3.37	0.1162	
CD (X_3X_4)	5.73	1	5.73	6.81	0.0401*	
Residual[c]	5.04	6	0.84			
Lack of Fit	3.67	2	1.83	5.34	0.0743	not significant
Pure Error	1.38	4	0.34			
Cor Total[d]	220.58	20				

[a]Degree of freedom, [b]Significant at 'Prob > F' less than 0.05, [c]Difference between experimental and predicted points, [d]Total of all information corrected for the mean. R^2= 0.9771, Adjusted R^2= 0.9238, Adequate Precision = 16.820, $^*p < 0.05$ indicate the model terms are significant, $^{**}p < 0.01$ indicate the model terms are highly significant.

The *p*-value of 0.0743 for the lack of fit demonstrated that the model showed no lack of fit, which further validates the model. Independent and dependent variable were analyzed to get regression equation that could predict the response under the given range. The regression equation obtained for %XOI activity (Y) is indicated in Eq. (2), as follows:

$$Y = 86.85 - 0.23X_1 + 0.90X_2 + 1.16X_3 + 0.45X_4 - 1.22X_1^2 - 1.33X_2^2 - 0.43X_3^2 - 1.90X_4^2 - 0.62X_1X_2 - 1.07X_1X_3 - 0.73X_1X_4 - 1.18X_2X_3 - 0.84X_2X_4 - 0.85X_3X_4 \quad (2)$$

When, all terms with a low significance level ($p > 0.05$) were excluded from the model then the adjusted model equation obtained for %XOI is shown Eq. (3):

$$Y = 86.85 + 0.90X_2 + 1.16X_3 - 1.22X_1^2 - 1.33X_2^2 - 1.90X_4^2 - 1.07X_1X_3 - 1.18X_2X_3 - 0.85X_3X_4 \quad (3)$$

The optimum conditions could be selected using surface graphs, contour plots or steepest ascent techniques [30, 31]. However, in the current study, both response surfaces and contour plots were employed and effect of two independent variables out of four on percentage of XOI was plotted while remaining two were held at zero level.

As the model exhibited an insignificant lack of fit with high R^2, the response was adequately explained by the regression equation. The regression model enabled the estimation of the effects of the four variables, namely, extraction temperature, extraction time, agitation speed and sample to solvent ratio. The relationship between independent and dependent variables was illustrated in three-dimensional representation of the response surfaces and two-dimensional contour plots generated by the models.

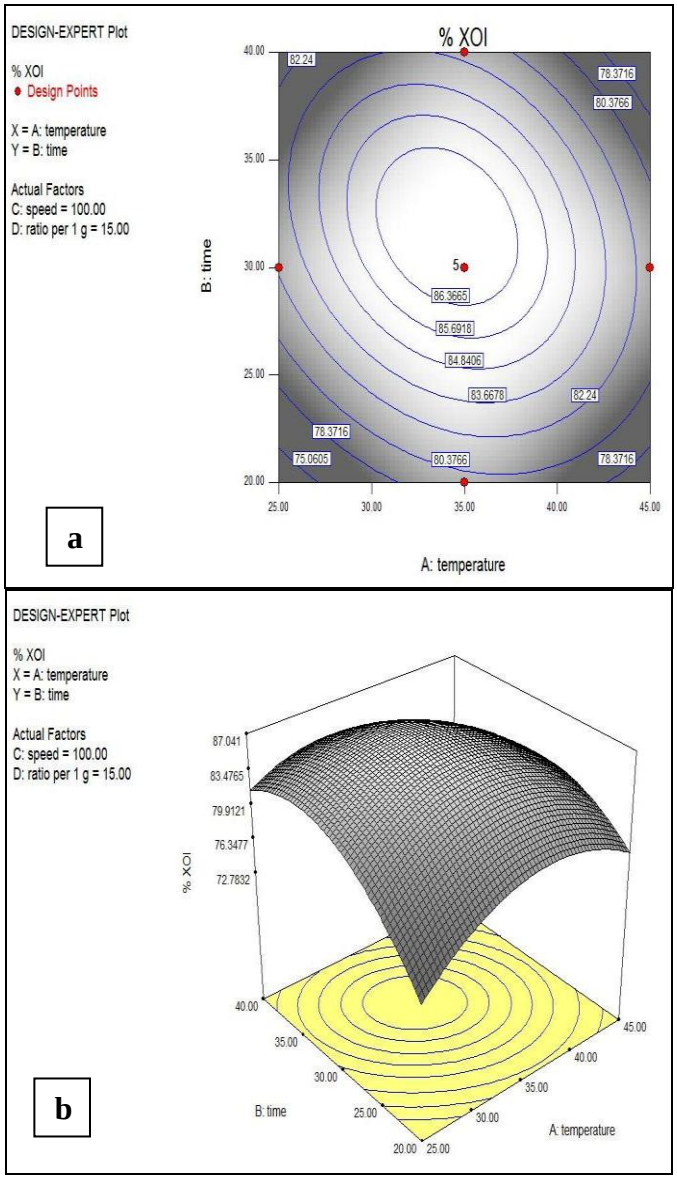

Fig. 1: (a) Contour plot and (b) 3-D plot response surface showing the effect of extraction time and extraction temperature on XO inhibitory activity (%XOI) with other variables at zero level.

Figure 1(a) and Figure 1(b) depicted the contour plot and response surface described by the model equation to estimate the percentage of XOI activity over independent variables; extraction temperature (°C) and extraction time (h) when the actual factors of agitation speed and solvent: sample ratio were fixed at 100 rpm and 15 ml/g. It shows that the maximum %XOI activity of could be obtained by conducting the extraction process at 35°C in between 30 to 35 h. Higher or lower temperature and longer or lesser extraction time would decrease the activity of XOI. An increase in the working temperature beyond certain value will denature or decompose some of the valuable compounds which exhibit XOI property [18, 32, and 33]. Polyphenols [34] and flavonoids including quercetin and myricetin [35, 36] have been reported to be the potent plant-based XOI, apart from their well-known antioxidant activities [37]. Extracting at high temperature showed significantly lower total polyphenol content and may cause the flavonoid glycoside to change into other forms, subsequently reducing its XO inhibitory activity. Previous study also reported that prolonged extraction time would lead to a decrease in the phenolic content of crude extract as oxidation of phenolic compounds was possible to be

occurred by prolonging the exposure to environment factors such as light and oxygen [38, 39]. These circumstances could also be well explained by Fick's second law of diffusion, which predicts that after a certain time, there will be a final equilibrium between the solute in the solid matrix (plant sample) and in the bulk solution (extraction solvent) [40]. Hence, excessive extraction time is no longer useful to extract more active compounds from *Morinda elliptica*. Similarly, lesser contact time does not allow good mixing between the samples and solvent, subsequently leads to low XOI activity.

The effect of extraction temperature (°C) and agitation speed (rpm) on %XOI activity with fixed amount of solvent: sample ratio (15 ml/g) and extraction time (30 h) was evaluated and maximum %XOI activity obtained by agitating at higher than 125 rpm with the extraction temperature of 30 °C. This was supported by other researchers that higher agitation rate results in higher rate of solubility of solute, thus, desorption of the compounds from the solid matrix increases, which leads to higher XOI activity [41]. Figure 2(a) and Figure 2(b) depicted the effect of extraction temperature and solvent: sample ratio, when the extraction time and agitation speed were fixed at 30 h and 100 rpm as the center point. It was recorded that the maximum value of %XOI activity was obtained by using the sample to solvent ratio of 1:15 and the extraction temperature of 35 °C. High amount of solvent resulted in higher yield of XOI activity. However, temperature showed greater impact on the extraction of XOI compound regardless of the amount of solvent used. This relationship is in a good agreement with the previous findings [42, 43].

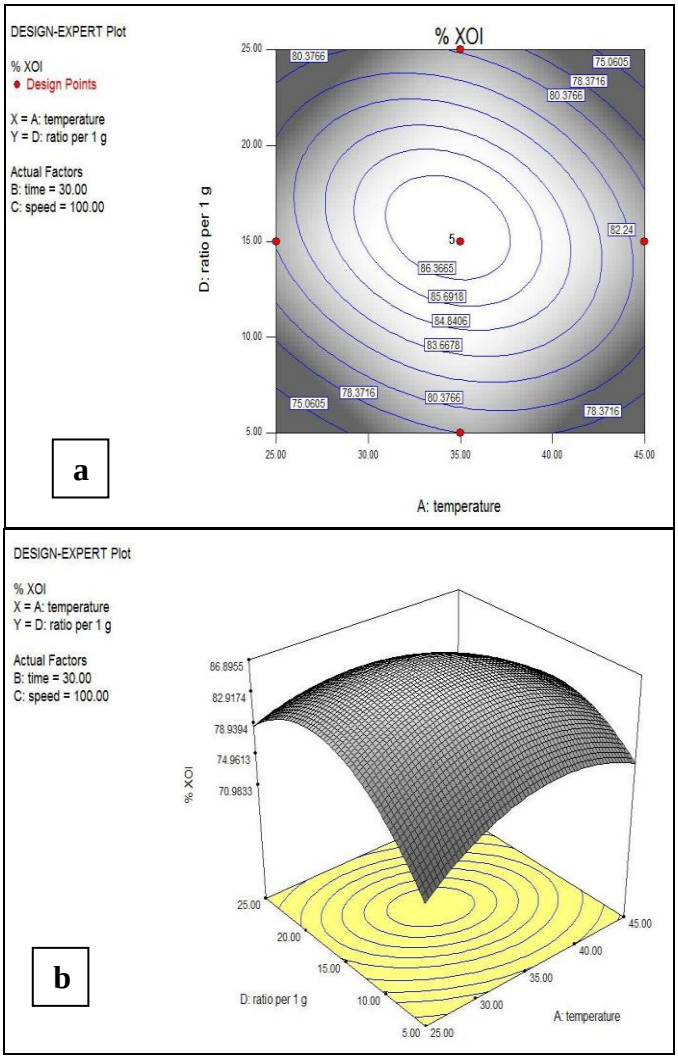

Fig. 2: (a) Contour plot and (b) 3-D plot response surface showing the effect of ratio of 1 g of sample to solvent and extraction temperature on XO inhibitory activity (%XOI) with other variables at zero level.

The effect of the variation of extraction time and the agitation speed on %XOI activity at fixed extraction temperature (35 °C) and the solvent to sample ratio (15 ml/g) was also studied. The maximum %XOI activity could be achieved by agitating higher than 125 rpm in between 25 to 30 h. Oxidation of the active compounds during the prolonged contact time could also reduce the XOI activity. Analysis on the effect of variation of

extraction time and the ratio of sample to solvent on %XOI activity is shown in Figure 3(a) and Figure 3(b). The maximum %XOI activity was obtained by conducting the extraction process with sample to solvent ratio of 1:15 for 30 h, when the actual factors of extraction temperature and agitation speed were fixed at 35 °C and 100 rpm respectively. Lastly, the effect of agitation speed and sample to solvent ratio at fixed extraction temperature (35 °C) and time (30 h) showed that the maximum %XOI activity could be achieved with 15 ml of solvent/ g of sample by using an agitation speed between 125 to 150 rpm.

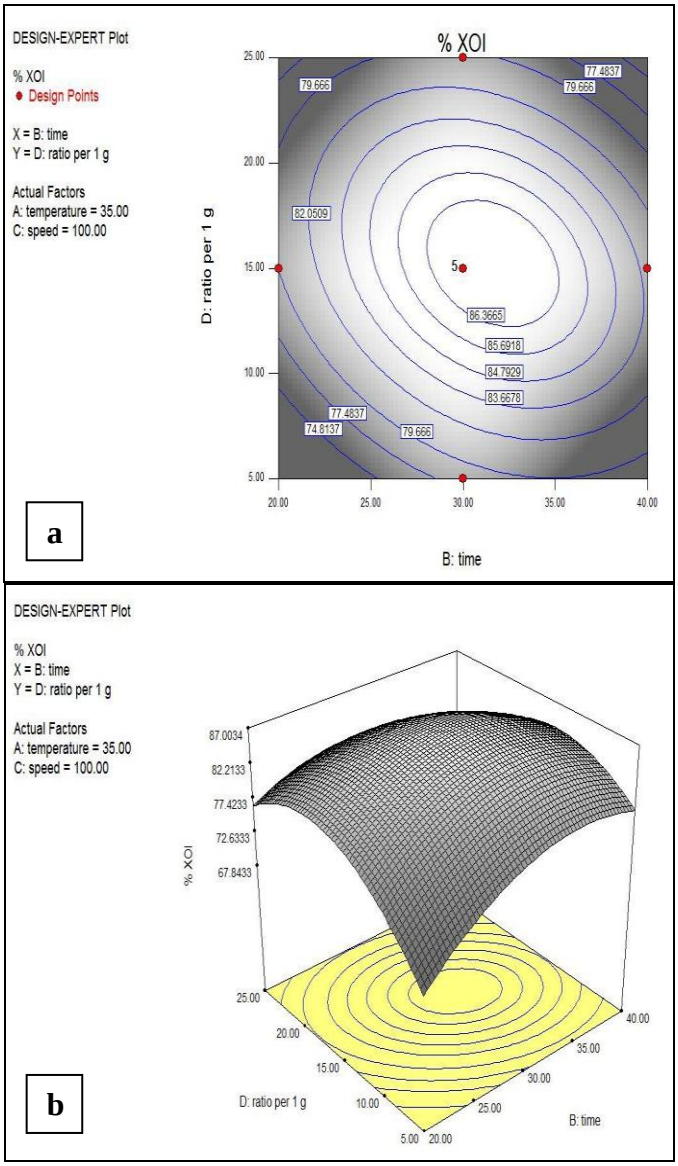

Fig. 3: (a) Contour plot and (b) 3-D plot response surface showing the effect of ratio of 1 g of sample to solvent and extraction time on XO inhibitory activity (%XOI) with other variables at zero level.

Optimum process conditions for the extraction of maximum percentage of XOI activity from aqueous-methanol *Morinda elliptica* leaves were obtained using the predictive equations of RSM. The experimental and predicted values were compared in order to determine the validity of the model. The experiment was run at the optimum conditions obtained from the above study. The suggested optimum conditions were 32 ºC (X_1), 30 h (X_2), 125 rpm (X_3) and 1 g/15 ml (X_4).

The calculated amount of %XOI activity with these parameters using regression model was 90.13%. However, the experimental %XOI activity at the optimum level was 88.93%. It was observed that experimental optimal value was slightly lower than the computed value by the regression model. Earlier studies have also demonstrated the same pattern [31].

The %XOI activity of allopurinol at 100 µg/ml was computed as 93.69%. The %XOI activity of optimized aqueous-methanol extract of *Morinda elliptica* was comparable with allopurinol as there was only 5% difference

in the inhibition studies. Methanol was considered as a good solvent by other researchers as well because it showed the capability of extracting considerable amount of total phenolic content from tropical food residues [44], which could contribute to the total XO inhibitory activity.

Conclusion:

The Response Surface Methodology was used to estimate the main synergic effects of the extraction temperature, extraction time, agitation speed and sample: solvent ratio on the extraction of XOI from *Morinda elliptica* leaves extract using aqueous-methanol as the extraction solvent. Response surfaces were drawn from the empirical models that helped to determine the optimal conditions by graphic optimization, which was performed by the Design Expert® software to visualize the method robustness. The experimental results were in good agreement with those predicted by the model. Experimental results for the %XOI activity was 73.88% to 87.84% following 21 selected combinations of extraction temperature, extraction time, agitation speed and ratio of sample: solvent. The model developed for %XOI activity exhibited non-significant lack of fit and an R^2 value of 97.71%. The surface graphs indicated that high XO inhibition could be obtained by using the following conditions: 32 ºC (X_1), 30 h (X_2), 125 rpm (X_3) and 1g/15 ml (X_4). The selection of natural products for ethno-medicine and pharmacological activity may provide identification of newer medicaments for the treatment of various ailments, especially gout. Further research on aqueous-methanol extract of *Morinda elliptica* leaves for improving the existing method or identifying the active constituents that exhibit a significant XO inhibitory activity should be well supported, as the anti-gout compound produced from natural sources could be marketed at large scale, as a substitute to the current irresponsive medicine. Nevertheless, the data presented provide important information for the discovery of new natural XOI from medicinal plants, and the findings is encouraging to plan clinical studies in hyperuricemic patients.

ACKNOWLEDGMENTS

The research was supported by a research grant approved by the Research Management Center (RMC), International Islamic University Malaysia (IIUM). The authors are grateful to the RMC and Department of Biotechnology Engineering, Kulliyyah of Engineering, IIUM for supporting and providing the laboratories facilities.

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