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Full Length Research Paper

Response surface optimization of the process conditions for anti-diabetic compounds from *Cucumis sativus*

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Presently, there is a growing interest in herbal remedies due to the side effects associated with the use of insulin and oral hypoglycaemic agents for diabetic patient. Therefore, an investigation is required in a bionetwork rich and industrially-developed country like Malaysia to use alternative approaches to treat diabetics, such as plant based medicine. In this study, *Cucumis sativus* was examined on the basis of its use in traditional medicines throughout Southeast Asia, to develop an understanding of the distribution and to give an assessment of the diversity present in the selected plant. β-Glucosidase inhibitory activity reached the highest value of 96.81% at the optimum conditions of temperature, 25.66°C; incubation time, 22.30 h; agitation speed, 125 rpm and volume of solvent, 15.6 ml. High performance liquid chromatography (HPLC) analysis identified five compounds, out of which two were identified as p-coumaric and syringic acids, while the other three were unknown. These two phenolic acids are already known to have anti-diabetic properties from previous study. This potential plant with β-glucosidase inhibitory activity could be a hope for millions for treatment of diabetes and will also help in reducing the dependence on synthetic drugs in the future.

Key words: Anti-diabetic, *Cucumis sativus*, β-glucosidase inhibitor, optimization, phenolic acids.

INTRODUCTION

Diabetes mellitus is one of the major diseases which have affected human population in the world. Throughout the world, many traditional plants are used for treating diabetes and therein lay a hidden wealth of potentially useful natural products for diabetes control (Bailey and Day, 1989; Swanston-Flatt et al., 1991; Gray and Flatt, 1997). Despite this, few traditional anti-diabetic plants have received scientific or medical scrutiny, and the World Health Organization recommended accordingly that this area warrants further evaluation (WHO, 1980).

One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycaemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase, in the digestive tract (Ali et al., 2006). Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose rise (Rhabasa-Lhoret and Chiasson, 2004). Examples of such inhibitors which are in clinical use are acarbose, miglitol and voglibose (Bailey, 2003).

Many researchers had identified potential species from 725 genera, 183 families of plants, and a review identified more than 800 plant species as potential treatments for diabetes mellitus (Marles and Farnsworth, 1994; Perez et al., 1998). Another recent review, stated that more than 1123 plant species have been used ethnopharmacologically or experimentally to treat symptoms of diabetes (Grover et al., 2002). Therefore, the aim of this study was to investigate antidiabetic compound in *Cucumis sativus* and to optimize the extraction process conditions for the compound by using response surface methodology.

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Table 1. Experimental range and level of independent process variables.

Independent Variables	Symbols]	Levels of Independent Variables				
		-2	-1	0	+1	+2
Temperature (°C)	[A]	20	25	30	35	40
Time incubation (h)	[B]	15	20	25	30	35
Agitation speed (rpm)	[C]	50	75	100	125	150
Solvent (ml)	[D]	5	10	15	20	25

MATERIALS AND METHODS

The leaves of *C. sativus* were collected fresh from its natural habitat in the state of Perlis, Malaysia on February, 2009. The specimen was authenticated by the Department of Biotechnology Engineering, IIUM, Malaysia.

Pre-treatment of plant samples

The plant leaves were washed using tap water three times and one time with distilled water to clean it completely from contaminants. Then, it was dried in the drying oven (50°C) for several days. The dried samples were grinded into powder form using Warring blender.

Plant extraction

The dried powdered material of plant was successively extracted with distilled water as a solvent according to the conditions suggested by central composite design as shown in Table 1. Then, the plant extracts were filtered through filter paper to separate the plant residual from the extracts. Excess solvent was removed from plant extracts through evaporation process. All the extracts were kept at 4°C prior to the analysis of enzyme inhibition activity.

Optimization of extraction process conditions

A statistical optimization study was done for extraction process conditions to obtain maximum value of β-glucosidase inhibitory activity. According to the results obtained in preliminary stage of this study, a combination of plant sample and solvent that exhibits the highest β-glucosidase inhibitory activity was selected. Four independent variables at different levels were selected to optimize the maximum productivity of β-glucosidase inhibition. The process variables used were temperature, incubation time, agitation speed and the amount of solvent / g of plant sample. Central composite design (CCD) under response surface methodology (SRM) using Design Expert v.6.0.8 (Stat-Ease Inc. Minneapolis) was used to determine the maximum productivity of β-glucosidase inhibition.

β-Glucosidase inhibition assay

The β-glucosidase inhibition assay was performed using modified method adapted from previous study (Brueggeman and Hollingsworth, 2001; Workman and Donal, 1982). Sweets almond β-glucosidase was dissolved in 1 ml ice-cold of 0.05 M Tris-HCl buffer (pH 7.8) and then diluted with 0.2% bovine serum albumin (BSA) solution in 0.01 M phosphate buffer (pH 7.0) to obtain a concentration of 0.022 unit/ml solution. This assay was based on the reaction of p-nitrophenol formed during the reaction, which was determined by spectrophotometer at 400 nm.

First, 1 ml of acetate buffer (0.1 M, pH 5.0) and 0.5 ml of p-nitrophenol-beta-D-glucopyranoside (PNPG) solution (0.02 M) were mixed in a test tube and equilibrated in the water bath at 37°C for 15 min. The reaction was started by addition of 200 µl of enzyme solution and 300 µl of plant extract (10 mg/ml in dimethylsulphoxide (DMSO). The tubes were incubated for a total of 15 min in the same condition. Then, 2 ml of Na_2CO_3 solution (0.2 M) was added to stop the reaction.

At the same time, control incubations which represent 100% of enzyme activity were conducted in identical fashion replacing plant extract with DMSO (300 µl). For blank incubations, which allow for absorbance produced by the plant extract, the enzyme solution was replaced with BSA solution (200 µl) and the same procedure was used as stated earlier. β-Glucosidase activity was determined by measuring the absorbance of the mixture at 400 nm. The inhibition activity was calculated by using the following Equatio (1):

$$\text{Inhibition (\%)} = [A - (B - C)] \times 100 \quad (1)$$

Where, A is the control; B is the OD test sample and C is the OD blank.

High performance liquid chromatography (HPLC) analysis

Sample extracts were analyzed by using Waters 2998 HPLC equiped with Waters 600 controller, integral vacuum degasser, Waters 717plus auto sampling injector, Waters 2998 dual λ photodiode array detector (PDA) and column thermo regulator. Empower software was used for data processing. Operating conditions were according to the suggested method by Nardini and Ghiseli (2004) with slight modification. The column temperature and the flow rate were set at 30°C and 0.4 ml/min, respectively. The injection volume was 50 µl and the detection was performed at 280 nm.

RESULTS AND DISCUSSION

Design of experiment and statistical analysis

The effect of four process variables such as temperature, incubation time, agitation speed and the volume of extraction solvent were evaluated to determine the maximum productivity of β-glucosidase inhibition. Table 2 shows the diagnostics case statistics with regard to the comparison between the actual response (experimental data) and the predicted response obtained by the design expert software employed for all 30 runs. Data were fitted by the following quadratic polynomial Equation (2):

$$Y = 79.32 - 12.93A - 7.48B + 12.11C - 6.00D - 8.22A2 - 10.89B2 - 4.63C2 - 7.77D2 + 4.91AB + 1.25AC - 6.08AD - 11.65BC + 4.56\,BD - 7.88CD \quad (2)$$

Table 2. Central composite design (CCD) with actual values of factors and β-glucosidase inhibition activity as a response.

Standard	Run	Variables				Enzyme Inhibition (%)		Residual
		[A] (°C)	[B] (h)	[C] (rpm)	[D] (ml)	Actual (response X)	Predicted (response Y)	
3	1	25	30	75	10	25.57	36.64	-11.06
4	2	35	30	75	10	45.57	30.26	15.31
5	3	25	20	125	10	89.34	108.02	-18.68
6	4	35	20	125	10	96.39	86.97	9.42
20	5	30	35	100	15	14.43	20.83	-6.40
19	6	30	15	100	15	58.36	50.73	7.63
25	7	30	25	100	15	95.74	79.32	16.42
10	8	35	20	75	20	18.95	3.66	15.29
28	9	30	25	100	15	82.13	79.32	2.81
30	10	30	25	100	15	78.98	79.32	-0.34
12	11	35	30	75	20	29.84	30.97	-1.14
17	12	20	25	100	15	91.80	72.30	19.50
7	13	25	30	125	10	54.10	50.81	3.29
23	14	30	25	100	5	49.84	60.25	-10.41
22	15	30	25	150	15	92.13	85.03	7.10
24	16	30	25	100	25	47.87	36.23	11.64
15	17	25	30	125	20	29.84	44.33	-14.49
9	18	25	20	75	20	24.92	54.00	-29.09
21	19	30	25	50	15	30.71	36.58	-5.87
26	20	30	25	100	15	76.62	79.32	-2.70
14	21	35	20	125	20	29.18	37.93	-8.75
16	22	35	30	125	20	19.85	18.63	1.23
27	23	30	25	100	15	68.52	79.32	-10.80
18	24	40	25	100	15	2.30	20.57	-18.27
11	25	25	30	75	20	70.82	61.66	9.16
13	26	25	20	125	20	86.56	83.29	3.27
29	27	30	25	100	15	73.93	79.32	-5.39
1	28	25	20	75	10	64.59	47.23	17.36
8	29	35	30	125	10	58.69	49.42	9.27
2	30	35	20	75	10	15.88	21.20	-5.32

Where, Y is the predicted response (β-glucosidase inhibitor activity) and A, B, C and D are the coded parameters for temperature, incubation time, volume of extraction solvent and agitation speed, respectively. β-Glucosidases inhibitor activity reached the highest value in run 4 (Table 2) at temperature = 35°C, incubation time = 20 h, volume of solvent extraction = 10 ml and agitation speed = 125 rpm.

Analysis of variance (ANOVA) was done to test the significance and adequacy of the model. The mean squares were calculated by dividing the sum of square of each of the two sources of variation, the model and the error variance, by the respective degrees of freedom. The Fisher variance ratio, the *F*-value, is a measure of how well the factors describe the variation in the data about its mean. The greater the *F*-value from unity, the more certain the factors explained adequately the variation in the data about its mean, and the estimated factor effects are real.

The quadratic regression model was highly significant, as evident from the Fisher's *F*-test with a very low probability value (P_{model} > *F*=0.0020). The *P* value (Prob>F) were used as a tool to check the significance of each coefficient, which in turn are necessary to understand the pattern of the mutual interaction between the test variables. The smaller the magnitude of the *P*-value, the more significant is the corresponding coefficient. Values of "Prob > F" less than 0.0500 indicated that the model terms are significant. The statis-tical analysis of the coefficients of the model revealed that mean and quadratic terms were significant. The model (quadratic) *F*-value of 4.94 implies the model is significant. There is only a 0.20% chance of a "model *F*-value", this could occur due to noise (Table 3).

It was observed that the significant variables were the square term of temperature (A^2), incubation time (B^2) and

Table 3. ANOVA for response surface quadratic model.

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value > F
Model	19638.45	14	1402.746	4.942263	0.0020*
A	4014.436	1	4014.436	14.14397	0.0019*
B	1341.059	1	1341.059	4.724921	0.0462*
C	3519.938	1	3519.938	12.40172	0.0031*
D	865.4079	1	865.4079	3.049072	0.1012
A^2	1854.268	1	1854.268	6.5331	0.0219*
B^2	3250.465	1	3250.465	11.45229	0.0041*
C^2	587.7933	1	587.7933	2.070958	0.1707
D^2	1656.504	1	1656.504	5.836321	0.0289*
AB	386.4159	1	386.4159	1.36145	0.2615
AC	24.85355	1	24.85355	0.087566	0.7714
AD	590.8793	1	590.8793	2.081831	0.1696
BC	2173.024	1	2173.024	7.656166	0.0144*
BD	333.1137	1	333.1137	1.173652	0.2958
CD	992.4199	1	992.4199	3.49657	0.0811
Residual	4257.4	15	283.8267	-	
Lack of Fit	3827.024	10	382.7024	4.446132	0.0567
Pure Error	430.3767	5	86.07535	-	
Corrected Total	23895.85	29		-	

*P<0.05 indicate the model terms are significant.

solvent (D^2). Furthermore, the linear effect of temperature (A) and agitation speed (C) were significant at the level of $P<0.01$ and the incubation time (B) was significant at the level of $P<0.05$. The interactive term between incubation time and agitation speed was significant at the level of $P<0.05$. Values greater than 0.1000 indicate the model terms are not significant. Linear and quadratic effects of parameters were significant which indicated that they can act as limiting condition and little variation in their magnitude would alter the inhibition activity of β-glucosidases. The ANOVA showed that there was a non-significant lack of fit (0.0567) that further validates the model (Table 3). The lack of fit *F*-value of 4.45 implies that there was 5.67% chance that this value could occur due to noise. The goodness of the model fitting was checked by the determination of the coefficient (R^2). The closer the value of *R* to 1, the better is the correlation between the observed and the predicted values. In this case, the value of R^2 was 0.8218 which indicates that only 17.82% of variation was not explained by the model.

Analysis using response surface methodology (RSM)

An elliptical response surface in the entire region was found from the second order quadratic equation for the β-glucosidase inhibitor activity with the interaction of temperature and incubation time (Figure 1). The results show that the activity of β-glucosidase inhibitor was considerably affected by varying the temperature and incubation time. The maximum inhibitor activity was predicted at given ranges of both temperature and incubation time. The activity decresed at the maximum and minimum values of ranges considered in both parameters. About 87.55% inhibition activity was obtained from the response surface as the maximum inhibition activity at the temperature (25°C), incubation time (22.5 h), agitation speed (100 rpm) and solvent (15 ml), respectively.

Figure 2 also shows the elliptical response surface plot of β-glucosidase inhibitor activity as a function of temperature and solvent. The predicted inhibitor activity decreased at the higher and lower values of ranges for both temperature and solvent values. Maximum activity was obtained near the center points of response surface. The maximum activity of β-glucosidase inhibition of about 84.41% was predicted at the temperature and solvent value of about 27.5°C and 15 ml. While incubation time and agitation speed remained constant at 25 h and 100 rpm.

Figure 3 is the response surface plot for inhibitory activity of β-glucosidase, as a function of incubation time and solvent by keeping the value of temperature and agitation speed at 30°C and 100 rpm, respectively. The activity of β-glucosidase inhibitor was affected by the variation of incubation time and solvent. Inhibition activity of β-glucosidase increased with increase in the incubation time from 15 to 22.5 h and decreased upon further increase from 22.5 to 35 h. Similarly, inhibition increased upon increasing the extraction solvent from 5 to 12.5 ml and decreased upon further increases of solvent from 12.5 to 25 ml. Maximum activity of β-glucosidase inhibitor

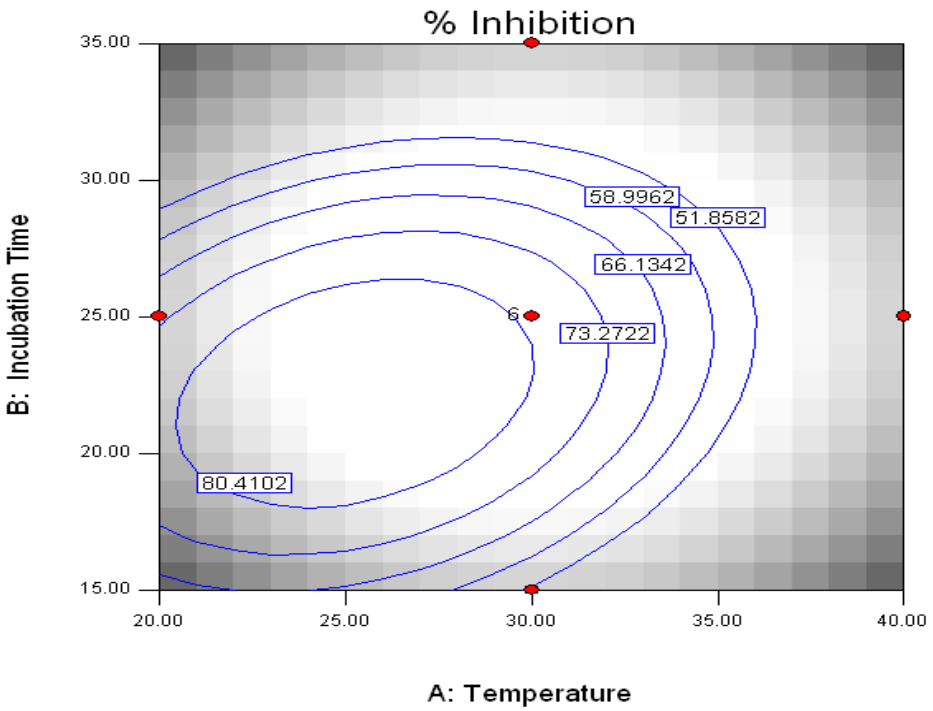

Figure 1. 2D contour plots showing the effect of temperature and incubation time, and their mutual interaction on percentage of β-glucosidase inhibition by *C. sativus.*

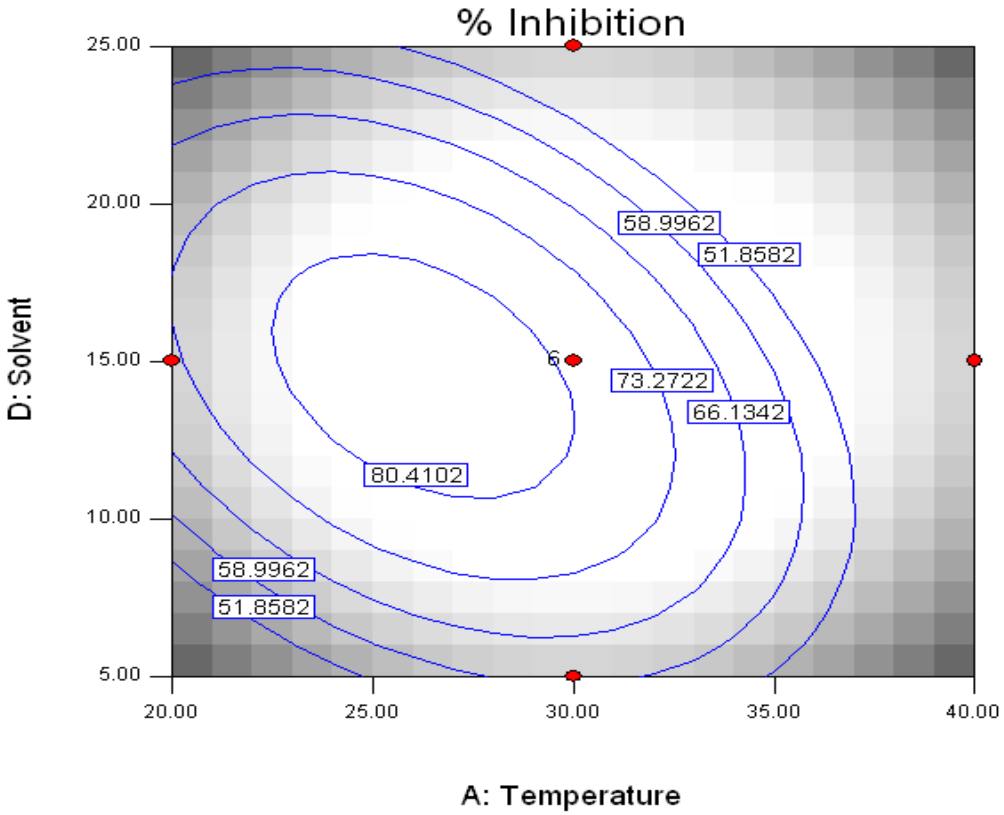

Figure 2. 2D contour plots showing the effect of temperature and solvent, and their mutual interaction on percentage of β-glucosidase inhibition by *C. sativus.*

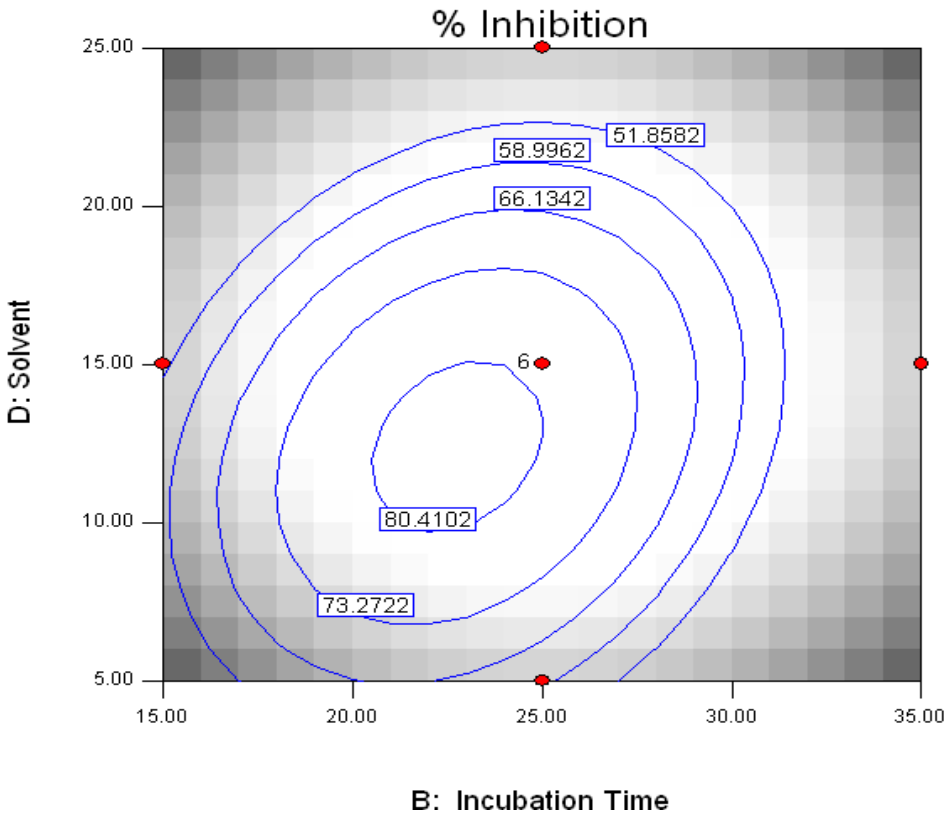

Figure 3. 2D contour plots showing the effect of incubation time and solvent, and their mutual interaction on percentage of β-glucosidase inhibition by *C. sativus*.

Table 4. Validation of developed quadratic model and optimum conditions of extraction process.

Run	Variables				Actual response	Predicted response	Residual
	[A] (°C)	[B] (h)	[C] (rpm)	[D] (ml)			
1	25.66	22.30	125	15.6	96.81	98.64	1.83
2	28.04	20.46	116	13.75	95.01	97.36	2.35
3	28.1	22.09	119	14.85	95.42	94.65	0.77

(82.47%) was obtained when incubation time was about 22.5 h and solvent was about 12.5 ml.

In order to verify the optimization results and to validate the model developed, a set of experiments with three replicates were performed according to the suggested conditions (Table 4). From the validation experiment, the highest β-glucosidase inhibitory activity of 96.81% was obtained at optimum conditions of temperature, 25.66°C; incubation time, 22.30 h; agitation speed, 125 rpm and volume of solvent, 15.6 ml.

High performance liquid chromatography (HPLC) analysis

HPLC was performed to identify compounds present in the plant extract which gave higher inhibition percentage in anti-diabetic assay. Retention times of five compounds were 48.132, 49.092, 63.600, 69.903 and 77.151, respectively. Three compounds were determined as phenolic acid based on chromatogram obtained from standard phenolic acid samples. One compound was identified as syringic acid and the other two as p-coumaric and ferullic acids. From the calibration curve of three phenolic acids involved, concentrations of syringic, p-coumaric and ferullic acids in this sample were 0.97, 1.76 and 117.98 µg/ml, respectively.

However, another two compounds in the chromatogram were unknown since the retention time for those compounds did not match with standard chromatogram. Mixture of these five compounds gave positive response for β-glucosidase inhibition assay. In order to determine its individual and synergistic effect on the compounds response, isolation and purification must be done to

separate each compound.

Benalla et al. (2010) reported that syringic acid isolated from medicinal plants show strong activity to inhibit α-glucosidase. p-Coumaric acid from *Stereospermum suaveolens Roxb.* also has anti-diabetic as well as good antioxidant properties (Srivastava et al., 2009). Hence, the presence of these two compounds in the mixture fractions would have contributed towards β-glucosidase inhibition activity.

Conclusion

In this study, distilled water was chosen as main solvent for extraction process, and C*. sativus* as the potential plant. Through optimization, β-glucosidase inhibitory activity reached the highest value of 96.81% at the optimum conditions of temperature (25.66°C), incubation time (22.30 h), agitation speed (125 rpm) and volume of solvent (15.6 ml). High performance liquid chromatography analysis determined that five compounds were present in the extract, which contributed to inhibit β-glucosidase activity. Two identified compounds: Syringic and *p*-coumaric acids were already approved by other researchers as a source to reduce blood glucose level.

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