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Optimization of Insulin Yield from Extract of Stevia Leave (*Rebaudiana bertoni*)

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ABSTRACT

Insulin extraction from plants is still experimental and not a common method. For the first time, this study tries to extract insulin from stevia leave (*Rebaudiana bertoni*). Stevia is a natural sweetener that had gained popularity as a sugar substitute due to its zero-calorie and its potential benefits for individuals looking to reduce their sugar intake. In order to optimize the production of insulin from stevia leave (objective or desired outcome), three factors namely, A: mass of stevia, B: volume of n-hexane and C: volume of ethyl acetate were defined under a response surface design (RSM) in Design Expert software 10.0. A, B and C values for a maximum yield of insulin (5.36%) discovered are 5.668 g, 49.107 mL and 32.386 mL respectively. An R^2 value of 0.9971 obtained, is in line with 5.253% predicted insulin yield, which then gives a fitted correlated plots against the experimental data. The elliptical shape of the 3D contour lines for a 3D plots of insulin yield against A & C as well as B & C, shows an interaction between those parameters. Temperature and other variables effects on the extraction process may be included in the expert design in future works. This work will spur the utilization of tobacco, *Costuspictus*, corn, rice, lettuce, *Costus igneus* and *Arabidopsis thaliana* plants, among others (containing high level insulin), to extract insulin. Concerns on a novel insulin extraction method from the suggested plants should be devised in future studies.

Keywords: Stevia leave, *Rebaudiana bertoni*, Insulin extraction, *Costus igneus*, *Diabetes mellitus*

INTRODUCTION

Stevia (*Rebaudiana bertoni)* is an ancient perennial shrub containing glycoside and phytochemicals, and is about 300 times sweeter than sucrose (Chang et al., 2005; Ilca et al., 2017; Lemus-Mondaca et al., 2012; Theophilus et al., 2015; Witono & Chandra, 2020). Stevia extracts including stevioside, have therapeutic properties, antioxidant effect, antimicrobial and antifungal activity (Abou-Arab et al., 2010; Abubakar, et al., 2023a). Lemus-Mondaca *et al.* (2012) reported different techniques for the extraction of stevioside from stevia leaves which include solvent extraction, ultrasonic assisted extraction, chromatographic adsorption, enzymatic extraction, ion exchange, pressurized fluid extraction (PFE) method, selective precipitation, microwave assisted extraction, membrane processes and supercritical fluids (Abubakar et al., 2023b; Mathur et al., 2017; Puri et al., 2012; Yildiz-Ozturk et al., 2015). Notably, Karásek *et al.* (2007) uses PFE method to extract stevioside from stevia utilizing water and methanol as solvent, where they compared the extraction effectiveness of both solvents. The numerous importance attached to insulin had warranted a serious search for its presence in several plants. Literature sources reviewed from 1976-2023 in this work, however, did not reveal any instances where insulin was extracted from stevia leave. Insulin is a polypeptide hormone obtained from animal pancreas. It is the main anabolic hormone of the animal's body, responsible for promoting the absorption of glucose from the blood into the liver, fat and skeletal muscle cells (Koona et al., 2010; Xavier-Filho et al., 2003). Insulin plays a vital role in enhancing carbohydrate, fat and protein metabolism and the regulation of certain gene expression (Anwer et al., 2012; Kootstra & Huurman, 2017; Sangeetha & Vasanthi, 2009).

Traditionally, insulin is isolated from animal pancreas. But recent research had revealed the presence of extractable insulin in several plants, bacteria and fungi (Polez et al., 2016). Some of the advantages of isolating insulin from plant sources than isolating it from animals' pancreas includes scalability and sustainability, reduced risk of contamination and disease transmission, lower production costs, elimination of cruelty to animals, customization and modification, consistency and purity, reduced allergic reactions, and fewer regulatory hurdles. Plant-based insulin production can be scaled up more easily than relying on animal sources. Plants can be cultivated in large quantities under controlled conditions, ensuring a consistent and reliable supply of insulin. In addition, plant-based insulin production could potentially be more cost-effective than traditional methods that involve extracting insulin from animal sources. Scientists can manipulate the genetic makeup of plants to improve the efficiency of insulin production and potentially create variants of insulin with improved therapeutic properties. Previously, Khann *et al.,* (1976) uses *Memordica charantia* Linn fruit as source for the extraction of insulin using Thin Layer chromatography (TLC). Using cowpea (*Vigna unguiculata (L.) Walp*.) seeds of the Epace 10 genotype, Venâncio *et al.,* (2003) extracted insulin, adopting the method of Khann *et al.,* (1976). Apart from cowpea seed and Linn fruit, tobacco (*Nicotiana tabacum*), *Arabidopsis thaliana*, maize (corn), rice (*Oryza sativa*), *Costus igneus*, spiral ginger (*Costuspictus*) and lettuce (*Lactuca sativa*) are the major plants containing significant amount of insulin. Among them, *Costus igneus* and *Costuspictus*, are acknowledged as the 'insulin plant' because they help lower glucose levels for some patients with diabetes (Fallabel & Wood, 2022; Hegde et al., 2014; Kale et al., 2019; Mathew & Varghese, 2019; Vishnu et al., 2010).

Current manufacturing technologies would be unable to meet the rising demand of inexpensive insulin due to limitation in production capacity and high production cost (Baeshen et al., 2014; Ju-Bong et al., 1991). This study adopts a laboratory scale method to isolate insulin from stevia leave. The fact that extensive review of papers published between 1976-2023 shows the absence of any form of research on insulin extraction from stevia leave, instigated this particular study. A significant yield of insulin, if obtained in this study utilizing stevia leave, will draw the attention of medicinal or pharmaceutical experts to the potential of the plant in deriving insulin for diabetic treatment. The study further utilizes Design Expert software tool, commonly used in industries such as engineering, chemistry, and manufacturing for the experimental design, optimization, and statistical analysis of the insulin production data. In the process, a model that takes three input factors and gives an output response was generated and analyzed. In Design Expert software (Kumar, 2013), response surface design can be created, analyzed and optimize with ease; because the software offers features like experimental design, regression analysis, graphical visualization, and predictive modeling, all tailored to help users effectively apply Response Surface Methodology (RSM) principles in their research and projects. RSM is a statistical and mathematical technique used to optimize and improve processes, products, or systems by studying the relationships between input variables (factors) and the output response (performance).

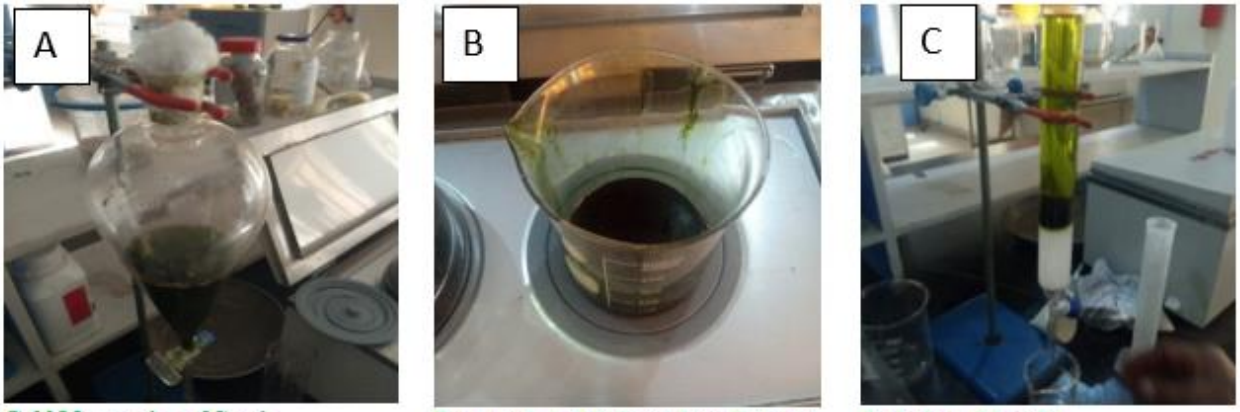
MATERIALS AND METHOD

Feedstock, Equipment and Reagents

The main feedstock for this research is fresh *Rebaudiana bertoni*, usually consisting of 11.6 wt% dry matter, as reported by Kootstra & Huurman (2017) and a moisture content of 5.37% on dry weight basis (Abou-Arab et al., 2010). Equipment used are beaker, measuring cylinder, separating funnel, conical flask, glass rod, mortar, pestle, and spatula, manufactured by Tianjin Glass Instrument Factory, China. Also included are water bath XMTD-4000 (Zhengzhou Keda Machinery Co., Ltd), furnace SX 25 10 (PEC Medical USA), MT-5000D digital weighing balance (Metlar, India), 75 mm long capillary tube (Smart Diagnosis, China) and 60 F254 TLC plate (E. Merck, Darmstadt Germany). A.R ethyl acetate (JHD Chemical Reagent Co. Ltd.), 99% pure n-hexane (BDH, VWR International Limited), silica gel 60-120 mesh (Qualikems Laboratory Reagent) and Tween 20, manufactured by Sigma Aldrich.

Extraction

In the NLNG Chemical Engineering Laboratory, UNIMAID, Nigeria, the cold maceration technique described by Okoduwa et al. (2016) was adopted. In that case, 25 g of the powered sample was put in a separating funnel, mixed with 250 mL of ethyl acetate as shown in Plate 1 (A) and left for 48 hours. The extract was collected in a 1000 mL beaker and heated to 64.7℃ to evaporate the solvent as shown in Plate 1 (B). A batch of 4.2g of the extract was mixed with 10g of silica gel 60-120 mesh and dropped into the glass column shown in Plate 1 (C). A 7:3 n-hexane and ethyl acetate solvent system was used. Thin Layer Chromatography as used by Khann *et al.*, (1976), can as well be used to extract insulin from stevia leave.



Cold Maceration of Stevia | Evaporation of Solvent from Extract | Isolation of Insulin

Plate 1: Empirical Steps Followed

RSM Optimization

Design Expert software 10.0 was used to design the experimental data for the optimization of insulin yield from stevia. The product (insulin) yield is desired to be maximized and thus, was defined as the objective variable. Custom design with three factor and one response was applied. The ‘factor’ in question is the independent variable which might influence the objective variable. The independent variables specified were amount of stevia powder (g), volume of n-hexane (mL) and volume of ethyl acetate (mL). The response (or % insulin yield) was calculated using Equation (1).

$$Y_{Insulin} = \frac{W_{Insulin}}{W_{Stevia}} \times 100 \qquad (1)$$

Where, $Y_{Insulin}$ = insulin yield (%), $W_{Insulin}$ = weight of insulin (g) and W_{Stevia} = weight of stevia powder (g).

Table 1 shows the experimental data or levels and the 3 design factors specified in the software.

Table 1: Experimental Data for Optimization of Insulin Yield

Run	**Factor 1** Amount of stevia (g)	**Factor 2** Volume of n-hexane (mL)	**Factor 3** Volume of ethyl acetate (mL)
1	4.5	50	30
2	4.5	30	50
3	4.5	50	50
4	4.5	70	50
5	3	50	50
6	4.5	50	50
7	4.5	50	70
8	3	70	70
9	3	30	30
10	4.5	50	50
11	3	30	70
12	4.5	50	50
13	6	70	70
14	3	70	30
15	6	70	30
16	6	30	70

17	4.5	50	50
18	6	50	50
19	6	30	30
20	4.5	50	50

Each combination of factors and levels represents a unique experiment, which is aided by the Design Expert tool.

Next, the experiments were carried out as defined by the software and the responses (outcomes) for each experiment was recorded, as shown in Table 1. The experimental data was then inputted into Design Expert to allow the software analyze the data statistically, in order to determine the relationships between the factors and responses. Design Expert was used to build a mathematical (quadratic) model based on the specified data to represent how the factors affects the responses. To know which 'factor' have the most influence on the outcome, the model was analyzed to identify significant factors and interactions. In furtherance of the process, the built-in optimization tool can be used to find the optimal combination of factor levels that achieves the objective; either maximizing or minimizing the response. Additional experiments using the suggested optimal conditions was performed to validate the predicted outcomes against the experimental results. The outcome of the optimization was then adequately reported and interpreted using relevant plots.

RESULTS AND DISCUSSION

Design Expert Optimization

Table 2 shows the yield of insulin (Response 1) obtained after running the extraction process, as used also in running the RSM optimization.

Table 2: Design of Experiment

Run	**Factor 1** A: Amount of Stevia (g)	**Factor 2** B: n-hexane (mL)	**Factor 3** C: Ethyl acetate (mL)	**Response 1** Insulin Yield (%)
1	4.5	50	30	4.36
2	4.5	30	50	3.66
3	4.5	50	50	4.44
4	4.5	70	50	3.11
5	3	50	50	0.13
6	4.5	50	50	4.31
7	4.5	50	70	5.01
8	3	70	70	0.66
9	3	30	30	0.33
10	4.5	50	50	4.28
11	3	30	70	0.83
12	4.5	50	50	4.50
13	6	70	70	5.36
14	3	70	30	1.66
15	6	70	30	3.33
16	6	30	70	5.15
17	4.5	50	50	4.47

18	6	50	50	3.89
19	6	30	30	3.87
20	4.5	50	50	4.42

It is difficult to ascertain the behaviour of the response with the amount of insulin used as well as Factor 2 and 3. In summary n-hexane and ethyl acetate volume between 30-70 mL and the weight of stevia used between 3-6 g yielded 0.13-5.36 % of insulin. The highest insulin yield of 5.36% was achieved using 6g stevia leave and 70 mL each of ethyl acetate and n-hexane. It shows that the higher the amount of stevia taken for the experiment, the higher the solvent requirement resulting in the highest insulin yield.

Table 3 shows the design summary/information that was used in the RSM optimization and Table 4 gives the experimental intervals.

Table 3: Design Summary

File Version	10.0.6.0				
Study Type	Response Surface	**Subtype**	Randomized		
Design Type	Central Composite	**Runs**	20		
Design Model	Quadratic	**Blocks**	No Blocks	**Build Time (ms)**	25.00

Table 4: Design of Experiment Intervals

Factor	**Name**	**Units**	**Minimum**	**Maximum**
A	Amount of Stevia	g	3	6
B	n-hexane	mL	30	70
C	Ethyl acetate	mL	30	70

Common experimental design in Design Expert are factorial designs, response surface designs, and mixture designs. In this analysis, the response surface design (as shown in Table 3) was chosen for the 20 runs of experiment. RSM helps explore the design space efficiently by reducing the number of experiments required compared to full factorial design. It reveals interactions and quadratic effects that may not be immediately apparent. The levels or range as assigned to each factor is shown in Table 4. These range are however hypothetical. Lower or higher boundary values of A, B and C can be specified.

Generated Mathematical Model and Statistical Analysis

Analysis of variance (ANOVA) was carried out to determine the significance and the fitness of the quadratic model. The ANOVA for the quadratic model is presented in Table 5. It is obvious, that RSM provides the statistical tools to assess the significance of factors and their interactions.

Table 5: ANOVA for Response Surface Quadratic Model

Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	72.77	9	8.09	377.75	< 0.0001	significant
A-Amount of Stevia	32.36	1	32.36	1511.95	< 0.0001	

B-n-hexane	0.45	1	0.45	21.00	0.0010	
C-Ethyl acetate	1.06	1	1.06	49.65	< 0.0001	
AB	1.89	1	1.89	88.37	< 0.0001	
AC	1.81	1	1.81	84.77	< 0.0001	
BC	1.24	1	1.24	57.94	< 0.0001	
A^2	18.36	1	18.36	857.87	< 0.0001	
B^2	4.02	1	4.02	187.81	< 0.0001	
C^2	10.90	1	10.90	509.22	< 0.0001	
Residual	0.21	10	0.021			
Lack of Fit	0.17	5	0.035	4.44	0.0637	not significant
Pure Error	0.039	5	7.867E-003			
Cor Total	72.99	19				

The Model F-value of 377.75 implies that the model is significant. There is only a 0.01% chance that an F-value of this magnitude could occur due to noise. Values of "Prob > F" less than 0.0500 indicate that the model terms are significant. In this case, A, B, C, AB, AC, BC, A^2, B^2 and C^2 are significant model terms. Values greater than 0.1000 indicate that the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve the model. The "Lack of Fit F-value" of 4.44 implies there is a 6.37% chance that a "Lack of Fit F- value" of that size could occur due to noise. Lack of fit is bad -- we want the model to fit. The yield of insulin in terms of coded factor is given by Equation 2.

$$Y = +4.48 + 1.80A - 0.21B + 0.33C - 0.49AB + 0.48AC - 0.39BC - 2.58A^2 - 1.21B^2 + 1.99C^2 \quad (2)$$

Where; Y = yield of insulin; A = amount of stevia; B = volume of n-hexane and; C = volume of ethyl acetate.

Equation 2, in terms of coded factors, can be used to make predictions about the response for a given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels of the factors are coded as –1. The coded equation is useful in identifying the relative impact of the factors by comparing the factor coefficients. Another statistical parameter, R^2 or coefficient of determination can be described. Table 6 shows the values of the R^2 and its adjusted value.

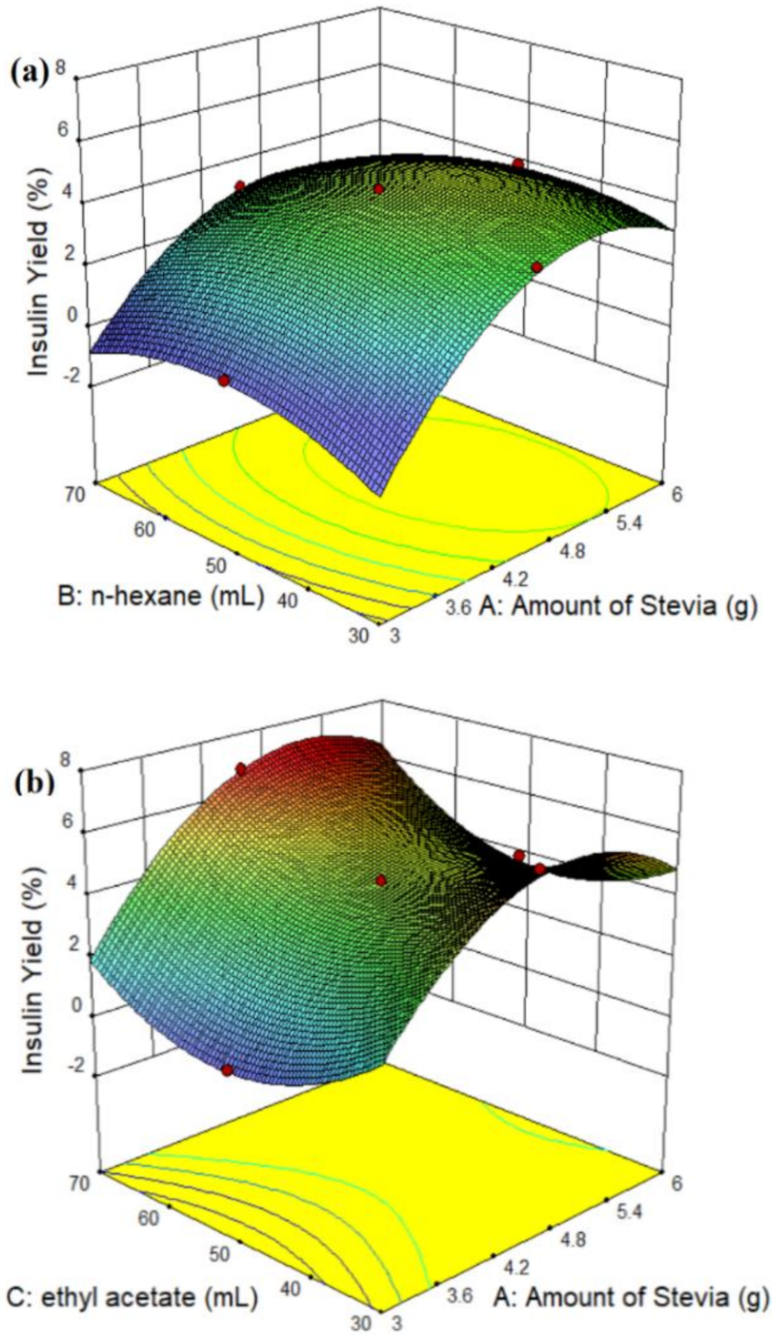
Table 6: R-Square Values

Statistical Parameter	**Value**
R-Squared	0.9971
Adjusted R-Squared	0.9944
Predicted R-Squared	0.9860
Adequate Precision	64.763

A "Predicted R^2 " of 0.9860, as shown in Table 6, is in reasonable agreement with the Adjusted R^2 of 0.9944. This is because their difference is < 0.2. ‘Adequate Precision’ measures the signal to noise ratio. Normally, a ratio > 4 is desirable. The ratio of 64.763 obtained, indicates an adequate signal. Thus, the model can be used to navigate the design space.

Response Surface Plot

Software like Design Expert offers graphical representations of the response surface, 3D contour plots (Figure 1), and other visualizations that make it easier to interpret and communicate results. One of the independent variables was set to zero while the other two were varied accordingly in order to investigate the responses.



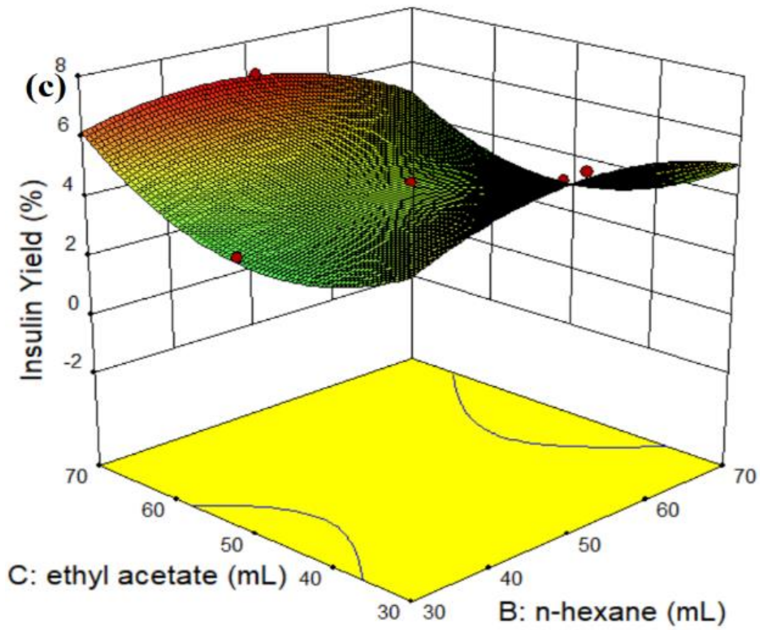


Figure 1: Insulin Yield 3D Contour

(a) N-hexane + Amount of stevia; (b) Ethyl acetate + Amount of stevia and; (c) Ethyl acetate + N-hexane

Figure 1a shows the surface plot of volume of n-hexane and amount of stevia when volume of ethyl acetate was held constant. Figure 1b shows the surface plot of volume of ethyl acetate and amount of stevia when volume of n-hexane was held constant. And Figure 1c shows the surface plot of volume of ethyl acetate and volume of n-hexane when amount of stevia was held constant.

Contours are the curved lines on the plot. Each contour line represents a constant value of the response. If the contours are circular and concentric (as in Figure 1a), it suggests there's no interaction between the variables. By implication factor A and B interacted poorly with the insulin yield. If the contours are elliptical or distorted, in semblance with Figure 1(b & c), there's likely an interaction. Especially between factor B & C and A & C. The spacing between the contour lines indicate the gradient of change. Closer lines indicate a steeper gradient, the case of Figure 1(a & b), while wider gaps suggest a more gradual change, as in Figure 1c. The point where the contours are closest together usually represents the optimal region, where the response is maximized or minimized, depending on the context. If the contours are sloping upwards to the right, it means increasing both variables simultaneously increase the response. If a flat contour (parallel to the axes) is encountered, it suggests that changes in one variable do not affect the response while the other variable changes. Where contours intersect or cross, can provide insights into the relationships between variables. If contours cross, it implies a more complex interaction.

Figure 2 is a linear comparison graph between the predicted insulin yield and the actual insulin yield.

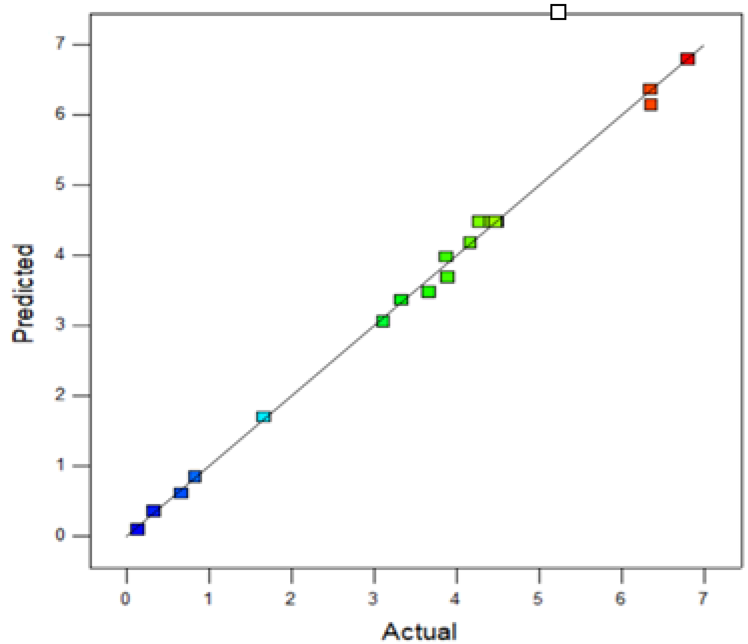


Figure 2: Correlation of Insulin Yield Data with Forecasted Values

The optimized factors and response based on central composite design prediction for the optimum extraction was found for 5.668 g of stevia powder, 49.107 mL of n-hexane and 32.386 mL of ethyl acetate with insulin yield of 5.253 %. The experimental yield was found to be 5.36 % while the predicted value was 5.253 %, with an error limit of 0.1 %. These results revealed that the statistical prediction and experimental results are almost in agreement, based on an approximately 100% fit showcased by Figure 2.

CONCLUSION AND RECOMMENDATIONS

A predictive regression model was developed using Design Expert 10.0 analysis software. ANOVA was further used to optimize extraction conditions that gives the optimum yield of the insulin. The optimum extraction conditions with the optimum yield were found to be 5.668g of stevia powder, 49.107 mL of n-hexane and 32.386 mL of ethyl acetate. This condition yielded 5.36% of insulin from stevia leave. It is also essential to test factors like temperature etc. as well as several other methods like solvent extraction, ultrasonic assisted extraction, chromatographic adsorption, enzymatic extraction, ion exchange, pressurized fluid extraction (PFE) method, selective precipitation, microwave assisted extraction, membrane processes and supercritical fluids for insulin yield. Insulin production currently consists of numerous small steps that involves lengthy processing time and high cost. Producing insulin is very traditional in process and technologies, so hopefully new technologies will come along to reduce costs. Such research would be a stepping stone to actualizing an advanced and improved technology of insulin extraction from stevia leave. Characterization of the feedstock (e.g., stevia leaves and other plants) as well as the extract (insulin) using GC-MS, AAS, and FTIR is essential in understanding the properties that demonstrate the best anti-diabetic effect.

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