

Study on Antidiabetic Effect of Herbal Mixtures (Aju Mbaise) on Reproductive Hormones and Renal Function in Induced Type 2 Diabetes Mellitus in Female Wistar Rats Using Response Surface Methodology

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Abstract. This study was performed to determine the effect of ‘Aju Mbaise’ herbal mixture on the female reproductive hormones as well as the kidney of diabetic Wistar Rats. The study was carried out from June - December, 2019 in the Research Laboratory of the Department of Biochemistry, Faculty of Science, University of Port Harcourt, Nigeria. The experiment involved a total number of twenty-seven (27) rats weighing between 40g-50g. Samples were induced with diabetes type 2 using high-fat diet for about 8 weeks and streptozotocin at 35mg/kg body weight. Consequently, samples were administered the cocktail extract at three different concentrations of 500mg/kg, 250mg/kg, and 100mg/kg respectively. Renal function and reproductive hormones tests were conducted accordingly after twelve weeks of treatment using their specific test kits. Response surface methodology was applied based on central composite design (CCD). Results obtained showed progressive increase in the concentrations of estrogen and progesterone, and reduction in urea and creatinine concentrations as treatment progressed. Also optimum responses were obtained for studied electrolytes. This study established that the cocktail of ‘Aju Mbaise’ herbal blend demonstrated a significant hypoglycaemic proclivity in diabetic female rats.

Keywords: Diabetes Mellitus, Herbal Mixture, Plant, Hormones, Kidney.

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1. Introduction

Diabetes mellitus (DM) is one of the autoimmune chronic diseases due to inherited and/or acquired deficiency in insulin production by the pancreas, or by the inability of the insulin produced to function properly. Insulin inefficiency as found in type-2 DM stimulates constant hyperglycemia with disorder of car-

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bohydrate, fat, and protein metabolism. Also, deficiency in insulin production increases blood glucose resulting to destruction of many body systems, prevalently the veins and nerves (American Diabetes Association, 2014). DM is however, seen as a global disease, prevailing throughout the world, although with variation in the rate of prevalence which differs from country to country (Chen *et al.*, 2017). The undeniably ageing populace, utilization of carbohydrate rich diet, obesity, and sedentary life style has led to increase in the number of diabetics globally. The impacts of DM include long term damage, dysfunction and failure of different organs. Symptoms of confirmed hyperglycemia include polyuria, polydipsia, polyphagia, blurred vision and weight loss. As per American Diabetes Association (2014), its severe forms, such as ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma, and without successful treatment, death. Persons with DM are at high risk of developing cardiovascular, peripheral vascular and cerebrovascular diseases. Thus, it is important to use plants and its product for the treatment and/or control of DM. The use of herbal products for medicinal benefits has played a vital role towards achieving good health worldwide. The consumption of a variety of local herbs and vegetables (medicinal plants) by man is believed to contribute significantly to the prevention and cure of some diseases (Gunjan *et al.*, 2015; Dhanik *et al.*, 2017). Most natural herbal medicines have potential for the development of newer therapeutics for anti-hyperglycemic and anti-hyperlipidemic agents (Kurihara *et al.*, 2006; Adisakwattana *et al.*, 2009) and has gained significant acceptance in developing and developed countries due to their natural origin and less side effects (Prasanna and Ravi, 2013). According to Zhang *et al.* (2018), DM is still a major health problem prompting to significant increased cardiovascular diseases and serious morbidity and mortality that is related to the onset of nephropathy, neuropathy and retinopathy. Despite great efforts that have been made in the understanding and management of diabetes, its prevalence continues to grow immensely. Thus, the use of herbal mixtures (Aju Mbaise) is imperative due to its active compound composition, low cost and less side effect. Hence, the study aim to model antidiabetic effect of herbal mixtures (Aju Mbaise) on reproductive hormones by evaluating plasma progesterone and estrogen level; and kidney by evaluating the serum renal markers, such as urea, creatinine and electrolytes (sodium, potassium, chloride and bicarbonate) on female diabetic Wistar albino rats.

2. Materials and methods

2.1 Experimental Design

Aju Mbaise herbal mixture is composed of combination of leaves, roots and trunk of medicinal tree wrapped together. According to Nnadiukwu *et al.* (2019), the composition of Aju Mbaise herbal cocktail includes *Sphenocentrum jollynum*, *Cnestis ferruginea*, *Xylopia aethiopica*, *Uvaria chamae*, *Palisota hirsuta*, *Scleria sp.*, *Napoleona sp.*, *Dialium guineense*, *Combretum racemosum* and *Heterotis rotundifolia*. These plants are well known for their individual therapeutic actions and as such, possess greater medicinal potential when combined.

A total of twenty-seven (27) female rats were utilized for this experiment. They were procured from the Department of Veterinary Medicine, University of Nigeria, Enugu State, Nigeria. The experimental animals were about 3 months old and weighs between 40g-50g at the inception of the experiment. The animals were left for about seven (7) days to adapt to the experimental conditions before the experiment proper, which involves the induction and treatment of type 2 DM with the herbal extract. A single dose intraperitoneal injection of 35mg/kg bodyweight of STZ was used to induce type-2 DM in the experimental animals (Nnadiukwu *et al.*, 2019). Respective doses of the STZ were dissolved in 0.2ml normal saline before administration to each animal, and DM was confirmed after 7 days of the induction.

Fasting blood sugar (FBS) was ascertained before the commencement of treatment with a standard antidiabetic drug (metformin) and 3 different concentrations of the herbal extract. This was done daily for a period of twelve (12) weeks. At the end of every 4 weeks, 3 animals were fasted overnight, anaesthetized, sacrificed and blood samples collected for the various biochemistry analyses. The collected samples were properly centrifuged at 3000 rpm for about 10 minutes at room temperature, and the individual serum collected for the analyses.

2.2 Test Procedure

Before the addition of the samples and reagent, the microplate wells for both the reference and samples were formatted. A volume of 50 μ L of the reference and samples were dropped into the assigned wells after which 100 μ L of the enzyme reagent (estrogen or progesterone depending on the hormone been analyzed) solution were added to the wells. The microplate was whirled gently for 20-30 seconds to mix the contents, and then covered and incubated for 60 minutes at 20-25°C. The contents of the microplates were discarded by decantation and the plate blotted dry with absorbent paper. The plate was washed thrice with 350 μ L of wash buffer before the addition of 100 μ L of working substrate solution which was added accordingly to minimize the reaction time differences between the wells. The microplate was incubated again at 20-25°C but for 15 minutes, followed by the addition of 50 μ L of stop solution. The wells were moderately mixed for 15-20seconds and the absorbance read at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader within 30 minutes after the addition of the stop solution.

Also, assay of urea concentration was performed with Urea Randox test kits as indicated by the Urease-glutamate Dehydrogenase technique depicted by Berthelot (Weatherburn, 1967; Nnadiukwu *et al.*, 2017). The assay of creatinine concentration was performed with Creatinine Randox test kit according to the modified method of Jaffé as described by Bartels and Bolmer (Bartels and Bohmer, 1971; Nnadiukwu *et al.*, 2017). Finally, sodium and potassium ion concentration were performed with sodium and potassium reagent (Teco Diagnostics) kit, and as indicated by the technique by Henry (Henry, 1974; Armstrong *et al.* (2005). The chloride ion concentration was performed with Chloride reagent (Teco Diagnostics) kit according to the method described by Tietz, (Tietz, 1976; Uboh *et al.*, 2009). The HCO₃⁻ concentration was determined ac-

cording to the back-titration method described by Van Slyke (Van Slyke, 1919; Amsterdamska, 2003).

2.3 Experimental Design and Statistical Analysis

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques that can be useful for modeling and analyzing situations in which responses of interest are influenced by several variables and the objective to optimize these responses (Montgomery, 2019). RSM is a useful technology in developing processes and optimizing their performance (Raissi, 2009). A central composite design (CCD) was employed in this study. The total number of experimental combinations in the CCD is equal to $2k + 2k + \eta_0$, where k is the number of independent factors and η_0 is the number of repetitions of the experiments at the center point (Iwundu and Oturu, 2019).

A CCD with two independent factors (X_1 , concentration (mgkg⁻¹) and X_2 , duration (weeks)) at three levels was used, the diabetic animals were assembled into three groups of nine animals each, although these three levels for the independent variables are not standard. The three level of duration were considered to explain the short and long term effect of concentration (Nnadiukwu and Oturu, 2020), while three level of concentration were chosen based on standard for curative treatment (WHO, 2010). The independent variables were coded according to (1).

$$x_i = \frac{X_i - X}{\Delta X_i} \quad (1)$$

where, x_i is the coded value of the i^{th} independent factor, X is the natural value of the i^{th} independent factor, the natural value of the i^{th} independent factor at the center point and ΔX_i is the value of step change. By using (1) the independent variables were coded and their levels are presented in Table 1.

Table 1: Independent Variables Levels

Independent Variable (Unit)	Levels		
	-1	0	1
x_1 , Herbal Mixture Concentration (mgkg ⁻¹)	100	250	500
x_2 , Duration (weeks)	4	8	12

Each independent variable had three levels which were coded as -1, 0 and +1. The experimental design was a central composite design (CCD) with three replications of a factorial point, stars points and center points in a total of 27 runs. The order of the experiments was randomized to minimize the effects of unexplained variability in the observed responses (due to extraneous variables). The independent variables and the responses are shown in Table 2.

The empirical model was developed for the responses by applying a second order polynomial equation (Chen *et al.*, 2011). The model can be represented as:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

where 'Y' is the response, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficients and β_{ij} is the interaction coefficient, k is the number of variables, x_i, x_j , are the coded values of the independent variables and the terms $x_i x_j$, x_i^2 and ε represent the interaction, quadratic and error terms respectively. Analysis of variance (ANOVA) test was applied to evaluate the adequacy (by applying the lack-of-fit test) of different models and to evaluate the statistical significance of the variables in the model. In order to examine the goodness and evaluate the adequacy of the fitted model, coefficient of determination (R^2) was computed. Simultaneous optimization of multiple response processes was applied, aided by a global desirability function (Montgomery, 2019). All independent variables were kept within range while responses were either maximized or minimized.

A global desirability function, $D(Y)$ is a geometric mean of n individual desirability functions ($d_i(y_i)$), one for each element, y_i of Y . Each $d_i(y_i)$ value is converted from associated response y_i and scaled to be between 0 and 1. With a value of zero indicating no effect and 1 indicating acceptable effect (Rouissi *et al.*, 2013). A general form of mathematical relationship of responses with desirability function is as follows;

$$Max D(Y) = (d_1(y_1)^{k_1} \times d_2(y_2)^{k_2} \times \dots \times d_n(y_n)^{k_n})^{\frac{1}{\sum_{i=1}^n k_i}} \quad (3)$$

where y_i denote the determined value of response i , $d_i(y_i)$ is the converted desirability value of i^{th} response and k_i represent the relative importance of response i compared to others. If all responses have the same importance, then $D(Y)$ become a geometric mean of all n transformed responses without weights. Overall desirability value can only be close to 1 if all of the responses are close to their optimal values, because $D(Y)$ is a geometric mean of the $d_i(y_i)$'s. Likewise, $D(Y)$ will be small if any of the $d_i(y_i)$'s are sufficiently close to zero. In consequence, to optimize responses simultaneously, one seeks to find values of x to maximize $D(Y)$ (Khuri, 2006; Montgomery Runger, 2011; Hakan and Sermet, 2013).

Non-programing interface software (computer codes are not required) called Design Expert 8.0.1 Software (Stat-Ease, Inc., Minneapolis, USA) was used for experimental design and data analysis.

Table 2: CCD of Independent Variables and Responses

		Independent Coded	Variables	Responses (Y_s)							
Std	Run	X_1	X_2	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8
10	1	1	1	7.17	88.33	5.63	106.67	131.67	3.97	101	28
13	2	-1	0	5.7	83.67	7.37	141.67	126.33	5.5	94.33	28
18	3	1	0	7.37	96.67	5.97	129	125	5.6	82.67	29
2	4	-1	-1	5.57	73	6.97	150.67	119.67	4.97	68	26.67
15	5	-1	0	5.7	83.67	7.37	141.67	126.33	5.5	94.33	28
12	6	1	1	7.17	88.33	5.63	106.67	131.67	3.97	101	28
8	7	-1	1	5.83	78.33	5.7	118.33	129.67	4.67	94	26.67
24	8	0	1	6.1	88.33	5.37	121.33	139	4.33	93.67	26.67
22	9	0	1	6.1	88.33	5.37	121.33	139	4.33	93.67	26.67
14	10	-1	0	5.7	83.67	7.37	141.67	126.33	5.5	94.33	28
16	11	1	0	7.37	96.67	5.97	129	125	5.6	82.67	29
4	12	1	-1	6.63	84	6.7	138.33	120.67	5.2	72.33	27.33
3	13	-1	-1	5.57	73	6.97	150.67	119.67	4.97	68	26.67
21	14	0	-1	6.03	79.67	5.7	131.67	123.33	5.33	73.67	28.33
19	15	0	-1	6.03	79.67	5.7	131.67	123.33	5.33	73.67	28.33
26	16	0	0	6.07	88	5.33	134.33	129	5.77	85.33	31.33
7	17	-1	1	5.83	78.33	5.7	118.33	129.67	4.67	94	26.67
5	18	1	-1	6.63	84	6.7	138.33	120.67	5.2	72.33	27.33
11	19	1	1	7.17	88.33	5.63	106.67	131.67	3.97	101	28
1	20	-1	-1	5.57	73	6.97	150.67	119.67	4.97	68	26.67
9	21	-1	1	5.83	78.33	5.7	118.33	129.67	4.67	94	26.67
17	22	1	0	7.37	96.67	5.97	129	125	5.6	82.67	29
6	23	1	-1	6.63	84	6.7	138.33	120.67	5.2	72.33	27.33
20	24	0	-1	6.03	79.67	5.7	131.67	123.33	5.33	73.67	28.33
25	25	0	0	6.07	88	5.33	134.33	129	5.77	85.33	31.33
23	26	0	1	6.1	88.33	5.37	121.33	139	4.33	93.67	26.67
27	27	0	0	6.07	88	5.33	134.33	129	5.77	85.33	31.33

In Table 2, X_1 and X_2 are the independent variables, Y_1 = Progesterone, Y_2 = Estrogen, Y_3 = Urea, Y_4 = Creatinine, Y_5 = Sodium, Y_6 = Potassium, Y_7 = Chloride, Y_8 = Bicarbonate.

3. Results and Discussion

Evaluation of the experimental design (CCD), the fraction of design space (FDS) graph revealed a FDS score of 1.0; hence the design is adequate to create meaningful results. Also, G-efficiency of 82.8% was obtained; this shows that the scale prediction variance (SPV) for the polynomial model and the CCD is adequate. The G-efficiency was adopted, since it is one of the commonly used optimality criterion in comparing designs. The best design is known with the largest relative G-efficiency (RG) i.e $0 \leq RG \leq 1$.

Second order polynomial model was fitted for all responses. Multiple regression analysis was used to examine the relationship between each response with the two independent variables. The full-quadratic regression model for each response can be represented by following equations:

$$\hat{y}_1 = 6.17 + 0.68x_1 + 0.15x_2 + 0.07x_1x_2 + 0.31x_1^2 - 0.16x_2^2 \quad (4)$$

$$\hat{y}_2 = 90.34 + 5.67x_1 + 3.05x_2 - 0.25x_1x_2 - 1.33x_1^2 - 7.50x_2^2 \quad (5)$$

$$\hat{y}_3 = 5.61 - 0.29x_1 - 0.45x_2 + 0.05x_1x_2 + 0.92x_1^2 - 0.21x_2^2 \quad (6)$$

$$\hat{y}_4 = 133.89 - 6.11x_1 - 12.39x_2 + 0.17x_1x_2 + 1.67x_1^2 - 7.17x_2^2 \quad (7)$$

$$\hat{y}_5 = 130.07 + 0.28x_1 + 6.11x_2 + 0.25x_1x_2 - 4.94x_1^2 + 0.56x_2^2 \quad (8)$$

$$\hat{y}_6 = 5.73 - 0.062x_1 - 0.42x_2 - 0.23x_1x_2 - 0.16x_1^2 - 0.88x_2^2 \quad (9)$$

$$\hat{y}_7 = 86.67 - 0.055x_1 + 12.44x_2 + 0.67x_1x_2 + 1.17x_1^2 - 3.66x_2^2 \quad (10)$$

$$\hat{y}_8 = 30.22 + 0.50x_1 - 0.16x_2 + 0.17x_1x_2 - 1.17x_1^2 - 2.17x_2^2 \quad (11)$$

Design-Expert® Software
 Min Std Error Mean: 0.344
 Avg Std Error Mean: 0.387
 Max Std Error Mean: 0.518
 Cuboidal
 radius = 1
 Points = 100000
 t(0.05/2,21) = 2.07961

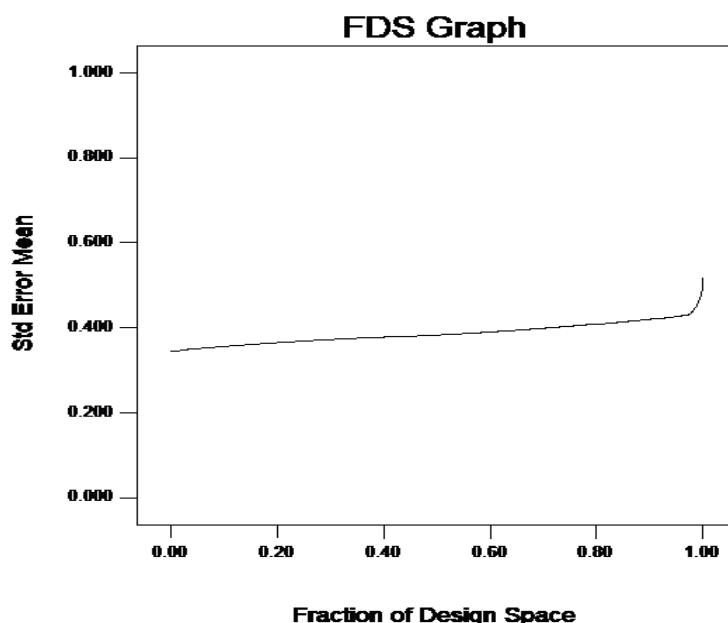


Figure 1: Fraction of Design Space

The independent variables in these equations represent coded values which were used to make predictions about the responses for given levels of each factor. The F-value revealed the model is significant ($p < 0.05$). The competence and significance of the model was justified by analysis of variance (ANOVA). The performance of the models was evaluated with a minimum value of R-Squared (0.77), adjusted R-Squared (0.71), Pred R-Squared (0.641) and Adeq precision (11.53) as shown in Table 3. Adequate prediction was obtained by subjecting each variable and response to target acceptance ranges and constraints. The surface plot for each response, explains the significant positive effect of the extract (herbal mixture) with an optimum level of concentration and duration. The contour plots show the optimum minimum level for estrogen, urea and creatinine,

and optimum maximum level for progesterone, sodium, potassium, chloride and bicarbonate (see Figure 2). The graphical optimization criteria was used to produce an over lay graph, results revealed that all responses meet the desire criteria with a variation of factors in the allowable ranges (acceptable factor settings) (see Figure 4).

The global desirability function $D(Y)$ was obtained by combining each response desirability as:

$$\begin{aligned} Max D(Y) &= (0.461 \times 0.079 \times 0.446 \times 0.225 \times 0.403 \times 0.330 \times 0.610 \times 0.539)^{\frac{1}{8}} \\ &= (0.000159168)^{\frac{1}{8}} = 0.335 \end{aligned} \quad (12)$$

Simultaneous optimization of responses obtained an optimum global desirability function, $D(Y)$ value of 0.335 for target settings of responses; concentration and duration (see Figure 3).

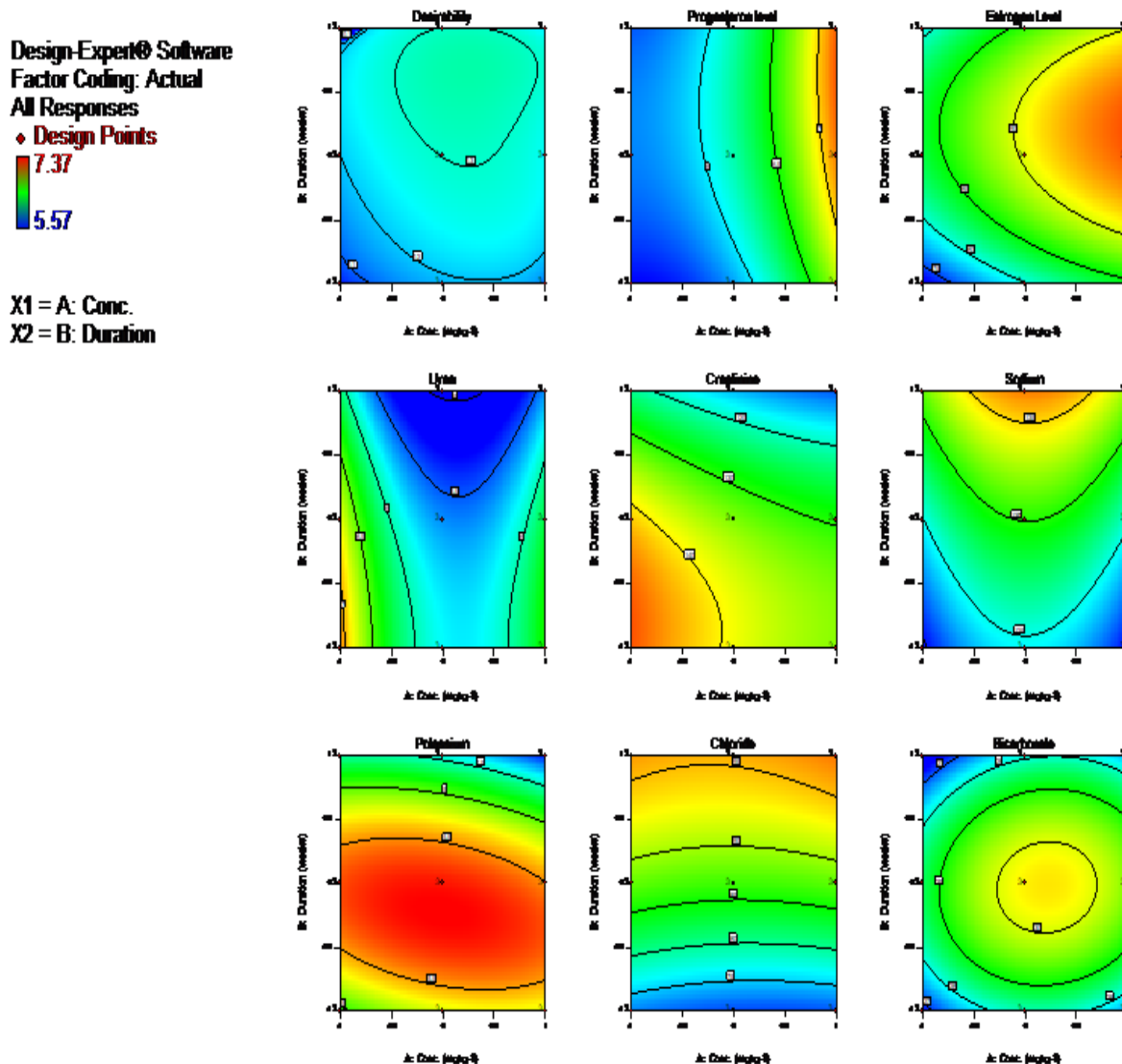


Figure 2: Contour Plot of All Responses

Design-Expert® Software
Factor Coding: Actual
Desirability



X1 = A: Conc.
X2 = B: Duration

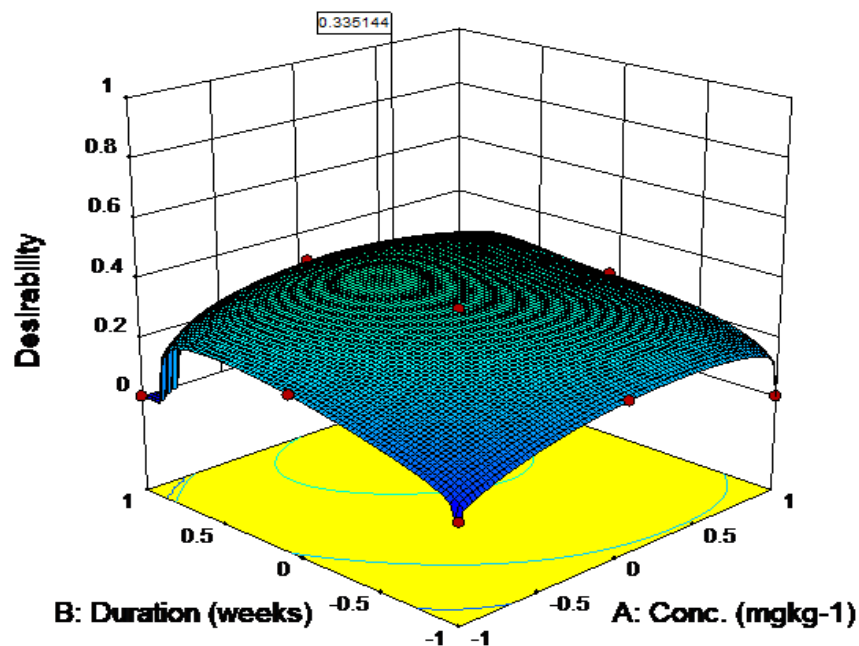


Figure 3: Surface Plot of Desirability Function

Design-Expert® Software
Factor Coding: Actual
Overlay Plot

Progesteron level
Estrogen Level
Urea
Creatinine
Sodium
Potassium
Chloride
Bicarbonate
♦ Design Points

X1 = A: Conc.
X2 = B: Duration

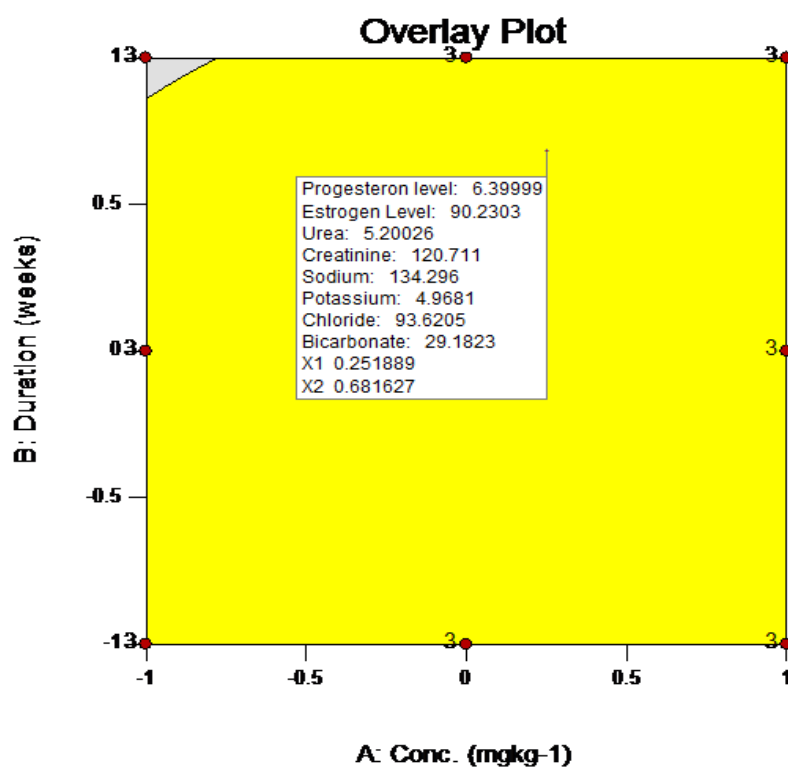


Figure 4: Overlay Plot of All Responses

Table 3: Model Validation

Measures	Y1	Y2	Y3	Y4	Y5	Y6	Y7
F-value	99.17	79.65	16.09	33.54	81.35	204.22	33.07
(p-value)	(< 0.0001)	(< 0.0001)	(< 0.0001)	(< 0.0001)	(< 0.0001)	(< 0.0001)	(< 0.0001)
Std. Dev.	0.14	1.66	0.36	4.74	1.42	0.092	4.17
Mean	6.27	84.44	6.08	130.22	127.15	5.04	85.00
C.V.%	2.20	1.96	5.94	3.64	1.12	1.83	4.91
PRESS	0.65	90.98	4.42	766.14	68.50	0.29	594.58
-2 Log	-37.08	97.15	14.84	153.81	88.86	-58.84	147.01
Likelihood							
R-Squared	0.9594	0.9499	0.7930	0.8887	0.9509	0.9798	0.8873
Adj.	0.9497	0.9380	0.7437	0.8622	0.9392	0.9751	0.8605
R-Squared							
Pred.	0.9345	0.9211	0.6663	0.8190	0.9208	0.9673	0.8169
R-Squared							
Adeq.	25.299	28.324	12.648	16.576	25.643	40.283	13.550
Precision							
BIC	-17.31	116.92	34.62	173.59	108.64	-39.06	166.78
AICc	-20.88	113.35	31.04	170.01	105.06	-42.64	163.21

4. Conclusion

Diabetes mellitus (DM) is one of the autoimmune chronic diseases globally. There is an increase prevalence and effect of this disease worldwide, even with continuous development of treatment and/or control mechanisms. This study therefore seeks to proffer locally based solution or herbal product (plants extract) for treatment and/or control of DM. Hence, this study applied response surface methodology (RSM) in optimizing the effect of herbal mixture (Aju Mbaise) concentration on reproductive hormones and kidney. The combination of RSM and optimization technique (desirability function) enables the determination of the best combination of all responses and controllable variables. The proposed extract (herbal mixture) concentration of 288mg/kg-1 and duration of administration (11 weeks) maximizes progesterone (6.38) and minimizes estrogen (90.23), as well as minimizes electrolytes such as urea (5.20), creatinine (120.7) and maximizes sodium (134.2), potassium (4.96), chloride (93.62) and bicarbonate (29.18). The optimized effect of herbal mixtures (Aju Mbaise) on reproductive hormones and kidney are within the standard level recommended by WHO for these parameters. Thus, herbal mixture (Aju Mbaise) is an efficient solution for the treatment of DM, as well as the improvement of reproductive hormones and kidney problem. Also, this study proves the efficacy of herbal medicine for the treatment of diseases. However, further study is needed to verify the negative effect of this herbal mixture.

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