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Conditions For Extraction, Concentration And Purification of Steviol Glycosides From Dried Stevia Leaves

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Abstract: The research investigates the extraction of Steviol Glycosides (SG), a natural, low alorie sweetener, from the stevia rebaudiana plant using hot water. The study aims to address the gap in literature regarding the comparative efficiency of the extraction methods and the physio-chemical and organoleptic properties of the produced steviol glycosides. The methodology involves varying the solute (stevia leaves) to solvent (water) ratio, temperature and the duration of extraction process. The impact of these variables on the yield of SG was evaluated, providing a comparative analysis of the efficiency of water in the extraction process. Results indicate that the choice of solvent and its ratio to the solute significantly influence the yields of SG. The highest and optimized yield of 39% was obtained at run 12 at reaction temperature of 90ºC, solute to solvent ratio 1:25 and reaction time of 120 min. The analysis of variance (ANOVA) results shows that all variables: solvent-solute ratio, temperature and time are highly significant being less than 0.05. The equation generated indicates that all the variables have positive influence on Steviol glycoside yield. Proximate analysis was carried out on the extract and the concentrate and the findings provides significant insight for production of homemade stevia concentrate.

Keywords: Stevia rebaudiana, Proximate Analysis, Sweeteners, Organoleptic, Steviol glycosides, Response Surface Methodology, ANOVA

INTRODUCTION

The plant Stevia rebaudiana grows naturally in South America. It is variously known as sweet leaf, honey leaf, candy leaf, sweet weed or sweet herbs. Stevia is gaining significant popularity in different parts of the world and is expected to be a major source of high potency sweetener. (Muhammed et al, 2019). Substance produced by extraction from Stevia are about 300 times sweeter than sucrose. Stevia has been used for centuries by indigenous Guarani tribe as a sweetener and a herbal tea to treat heartburn and other complaints. The plant belongs to Asteraceae family. More than 230 species belong to the Stevia, but only the species phlebophylla and rebaudiana produce components with sweet taste The sweet taste is caused by accumulation of diterpene glycosides. In addition, beside its sweet components, the plant contains a number of other important compounds, eg tannin, flavonoid, alkaloids, vitamins, minerals, and essential oils (Cardello, 1999; Mohammed et al., 2019).

The leaves of this popular plant are sweet and ideal for people who are conscious of sugar and carbohydrate intake. With zero calories, the plant is being recognized as a great replacement for sugar and other sweeteners. Worldwide, 32,000 hectares have been under Stevia cultivation and china has a major chunk of 75%. It is a natural sweetener plant and is grown commercially in many parts of Brazil, Paraguay, Central America, Thailand, Korea and China. Japan is currently using large amounts of Stevia. Several countries have now started its commercial cultivation (Le, Robin, & Roger, 2016; Kearney, 2010). Several countries have now started its commercial cultivation. Nigeria has not been producing significant quantities in the international market. The dried leaves are sold currently in its dried form at the international price of $4 per kilogram (Adeniyi, 2023). There are other valuable products obtainable by processing these dried Stevia leaves into liquid extract. Currently there are no reported extraction or processing plant for Stevia glycoside in Nigeria.

The food industry has currently shown great interest in the use of low-caloric sweeteners instead of sugars and its derivatives because of the consumer awarenesss with respect to their implication in the pathogenesis of diseases such as obesity and diabetes (Ashwell,2015). Nowadays, the most marketed sweeteners are synthetic compounds (eg., saccharin, acesulfame K, aspartame), but there is increasing controversy over their impact on health, so regulatory agencies have established maximum allowed contents to limit their potential toxicity problems. Thus, the use of natural sweeteners instead of artificial ones is emerging as an alternative with aim of producing healthier foods. The United States Food and Drug Administration (FDA) says stevia glycosides, such as Reb-A, are “generally recognized as safe.

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It may also drop blood pressure too low or interact with medications that lower blood sugar. Although stevia is considered safe for people with diabetes, brands that contain dextrose or maltodextrin should be treated with caution (Stones,2011; Misra et al., 2011). Liquid Stevia Extract is available as a clear liquid extract with a water, glycerin, alcohol or grapefruit base. The glycosides are extracted from the leaves using either water or alcohol and membrane filtration. Because it evaporates, no alcohol remains in the finished product. The liquid becomes clear rather than green because the extraction process removes the chlorophyll, and white glycosides remain (Puri, M et al., 2012). Water is the most selected solvent for the recovery of GS from stevia leaves as it is preferable for the development of drugs and food (Xu et al., 2019; Yilmaz et al., 2020).

SIGNIFICANCE OF THE RESEARCH

The significance of this research lies in its potential to contribute to the sustainable production of natural sweeteners, a field of increasing importance due to the rising concerns associated with synthetic sweeteners and high-calorie sugars. By comparing the efficiency of water and food-grade ethanol in the extraction of SG from dry stevia leaves, this research could help optimize the extraction process, thereby enhancing the commercial viability of stevia cultivation. Furthermore, the research could have significant implications for countries like Egypt, Nigeria, where Stevia cultivation is seen as a potential solution to the country's sugar production deficit. By improving the efficiency of the SG extraction process, this research could help make stevia cultivation a more viable and profitable venture, thereby contributing to the country's agricultural and economic development. It is therefore the objective of this project to study the conditions that favours the extraction of the natural sweetener and concentrate the extract for domestic use as a substitute for refined sugar. The research could also provide valuable insights into the physio-chemical and organoleptic properties of the produced SG, which is crucial for the application of stevia sweeteners in various food and beverage products.

EXPERIMENTAL PROCEDURE

Materials and methods

Plant material

Stevia rebaudiana Bertoni leaves were harvested from a garden in Kaduna-Nigeria (latitude 10.609311 and longitude 7.429504). The garden is promoting the cultivation of Stevia in the Northern part of the Country. The leaves were sun dried for 3-4 days at an ambient air temperature of 25- 30 °C . The dried leaves was separated from the stems and then stored at room temperature in air tight plastic bags to maintain constant quality raw material.

EXTRACTION OF STEVIOL GLYCOSIDES

(i)Preliminary Experimental Procedure

Hot water extraction

The natural sweetener was extracted from the dried stevia leaves with solvent: water, using water bath. A solute-solvent ratio of 1:12.5, 1:25 and 1:35 was used for the extraction. 20grams of stevia leaves was used for the extraction with 250ml, 500ml and 750ml respectively of solvent (water) at 40°C temperature for an extraction time of 2hrs, 4hrs and 5hrs respectively which was known during a series of trial runs conducted, stirring was done at intervals and the solute-solvent checked at every 10mins using hand held refractometer. The liquid extract was filtered using a filter paper after which the liquid extract was kept at room temperature and the solute-solven checked after 12hrs before proceeding to refrigerate.

The same procedure was further used to carry out runs at 60^0C and 90^0C. Results were obtained for the effect of temperature, solute to solvent ratio and time duration on the rate of sweetener extraction by the use digital and hand held refractometer expresses in terms of percentage solute-solvent and brits. The data obtained were subjected to ANOVA analysis and the results are presented in table 1-5.

(ii) Experimental design for Steviol Glycosides extraction using Surface Response Methodology

In this study, we aim to optimize the recovery of steviol glycosides from dried stevia leaves under laboratory condition using purely hot water. The dried leaves were used for extraction instead of the crushed or powdered leaves in order to reduce the impurities pigment in the extract. The multiple batch extractions were carried out employing laboratory scale water bath with shaker and combination of soxhlet apparatus. The temperature of the bath was controlled by a thermostat system with circulating heating water. The measured amount of leaves were placed in covered flasks and placed in the water bath. The extract was concentrated using soxhlet apparatus . To study the solute to solvent ratio a range of 1:125, 1:1;250 and 1:375 were used and temperature effects was investigated from 40^0 - 90^0C. The measured

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variable is the brix or percentage extraction as measured by refractometer. Surface response methodology was applied in generating the number of runs or experimental design

(iii) Proximate analysis

Proximate analysis is the quantitative analysis of macronutrients in food samples using combination of different techniques such as extraction, Kjeldahl, NIR, are used to determine protein, fat, moisture content, and carbohydrates levels. Proximate analysis was carried out on the liquid extracts and the results presented in table 9.

RESULTS AND DISCUSSION

1. Determining the effect of temperature on the rate of extraction of the sweetener from 40^0C – 90^0C

ANOVA was used to determine the individual variable of temperature and combined effects. In the combined effect the equation generated produced the f value and the confidence levels

Table 1.0: .Univariate Analysis of Variance Between Subjects Factors

		Value Label	N
Temperature	1.00	40 degrees	12
	2.00	60 degrees	12
	3.00	80 degrees	12

Table 2.0: Tests of Between-Subjects Effects Dependent Variable: Solute-solvent

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	152.390[a]	3	50.797	86.541	.000
Temperature	152.390	3	50.797	86.541	.000
Error	19.370	33	.587		
Total	171.760	36			
a. R Squared = .887 (Adjusted R Squared = .877)					

From table 2.0, it could be observed that the F value of 86.541 and significance level of 0.000 all indicated that the parameter under consideration (temperature effect on the rate of extraction have significant effects. The contributory effect at each level of temperature is shown in table 3 below

Table 3.0: Multiple Comparisons of the Dependent Variable: Temperature on the rate of extraction

						95% Confidence Interval	
	(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
LSD	40 degrees	60 degrees	-.3667	.31278	.249	-1.0030	.2697
		80 degrees	-2.0167*	.31278	.000	-2.6530	-1.3803
	60 degrees	40 degrees	.3667	.31278	.249	-.2697	1.0030
		80 degrees	-1.6500*	.31278	.000	-2.2863	-1.0137
	80 degrees	40 degrees	2.0167*	.31278	.000	1.3803	2.6530
		60 degrees	1.6500*	.31278	.000	1.0137	2.2863

Based on observed means.

The error term is Mean Square(Error) = .587.

*. The mean difference is significant at the 0.05 level.

From table 3.0, when comparing two temperatures of 40-60 and 40-80, it indicated that at such temperature, the significant levels is not pronounced at a temperature of 40-60, that is why the value of 0.249>0.05 significant level. In other words, performing the extraction at either of 40 or 60 degrees, one can conclude that they are not significant and their effects are not well felt. On the other hand, when the extraction is performed at a temperature of between 40-80 or 60-80, the rate of extraction is highly felt. That is why the level of significance is 0.000<0.05 level of significance.

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2. Determining the effect of solute-solvent ratio using hot water extraction

Table 4.0:Univariate Analysis of Variance Between-Subjects Factors

		Value Label	N
StoSratio	1.00	1:37.5	26
	2.00	1;25	23
	3.00	1:12.5	12

Table 5.0: Tests of Between-Subjects Effects Dependent Variable: Solute-solvent

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	36.237[a]	3	12.079	89.671	.000
StoSratio	36.237	3	12.079	89.671	.000
Error	7.813	58	.135		
Total	44.050	61			

a. R Squared = .823 (Adjusted R Squared = .813)

From table 5.0 above, it shows that the combined effect of solvent ratio of between 1:37.5, 1:25 and 1:12.5 are highly significant at a level of significance of 0.05. their contributory effects of each of the solvent ration is shown below

Post Hoc Tests

Table 6.0: Multiple Comparisons Dependent Variable: Solute-solven

	(I) StoSratio	(J) StoSratio	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LSD	1:37.5	1;25	-.1184	.10506	.264	-.3287	.0919
		1:12.5	-1.4423*	.12809	.000	-1.6987	-1.1859
	1;25	1:37.5	.1184	.10506	.264	-.0919	.3287
		1:12.5	-1.3239*	.13070	.000	-1.5855	-1.0623
	1:12.5	1:37.5	1.4423*	.12809	.000	1.1859	1.6987
		1;25	1.3239*	.13070	.000	1.0623	1.5855
Dunnett t (2-sided)[b]	1:37.5	1:12.5	-1.4423*	.12809	.000	-1.7291	-1.1555
	1;25	1:12.5	-1.3239*	.13070	.000	-1.6166	-1.0313

Based on observed means.

The error term is Mean Square(Error) = .135.

*. The mean difference is significant at the 0.05 level.

b. Dunnett t-tests treat one group as a control, and compare all other groups against it.

From table 6.0 above, all solvent ratio is highly significant at 0.05 level of significance (0.000) except solvent ratio of 1:25 to 1:37.5 having level of significance of 0.264. This implies that more solvent has the ability to downplay the solubility of the solute.

(iii) Response Surface Methodology

Design of experiments for the extraction of Steviol glycoside using response surface methodology with Box Behnken Design (BBD).

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Table 7.0:Design of experiments for the extraction of Steviol glycoside using response surface methodology with Box Behnken Design (BBD)

Std	Run	Factor 1 A: Temperature,℃	Factor 2 B: Solute-solvent,	Factor 3 C: Time, min	Response Yield, wt%
17	1	60.00	25.00	65.00	21.8
14	2	60.00	25.00	65.00	22.6
5	3	40.00	25.00	10.00	32.5
6	4	80.00	25.00	10.00	19.5
3	5	40.00	37.50	65.00	13.2
13	6	60.00	25.00	65.00	22.7
4	7	80.00	37.50	65.00	24
16	8	60.00	25.00	65.00	21.9
7	9	40.00	25.00	120.00	19.5
12	10	60.00	37.50	120.00	23.5
1	11	40.00	12.50	65.00	19.1
8	12	80.00	25.00	120.00	39
2	13	80.00	12.50	65.00	7
15	14	60.00	25.00	65.00	19.7
11	15	60.00	12.50	120.00	27.2
9	16	60.00	12.50	10.00	15.1
10	17	60.00	37.50	10.00	29

Table 8.0: ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	844.49	9	93.83	48.44	< 0.0001 significant
A-Temperature	3.38	1	3.38	1.74	0.2281
B-Solute-solvent	56.71	1	56.71	29.28	0.0010
C-Time	21.45	1	21.45	11.07	0.0126
AB	131.10	1	131.10	67.68	< 0.0001
AC	264.06	1	264.06	136.32	< 0.0001
BC	77.44	1	77.44	39.98	0.0004
A^2	4.17	1	4.17	2.15	0.1858
B^2	101.92	1	101.92	52.62	0.0002
C^2	199.30	1	199.30	102.89	< 0.0001
Residual	13.56	7	1.94		
Lack of Fit	7.71	3	2.57	1.76	0.2940 not significant
Pure Error	5.85	4	1.46		
Cor Total	858.05	16			

The highest and optimized yield of 39% was obtained at run 12 at reaction temperature of 80ºC, solute-solven of 25% and reaction time of 120 min.
The Model F-value of 48.44 implies the model is significant. There is only a 0.01% chance for the"Model F-Value" to be large which could occur due to errors.

Values of "Prob > F" less than 0.0500 indicate that the model terms are significant.
In this case, the factors B, C, AB, AC, BC, B^2 and C^2 are significant model terms; while A and A^2remain insignificant. Values greater than 0.1000 indicate the model terms are not significant. The model implies that solute-solvent ratio is the most influential factor, having the greatest influence on the yield of Steviol Glycoside followed by the extraction time and then temperature.

The "Lack of Fit F-value" of 1.76 implies the Lack of Fit is not significant relative to the pure error. There is a 29.40% chance that a "Lack of Fit F-value" which could occur due to noise and other errors. Non-significant lack of fit is good, confirming that the model is fit.

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The coefficient variation (CV) obtained for Steviol glycoside yield is 6.27%. The value whichisless than 10 is low enough to indicate a high level of precision and reliability of the experimental values. This also confirm the validity of the model used. Likewise, a very small P–value of 0.0001SteviolGlycosideyield established that the quadratic polynomial models are highly significant. This indicates that there is only 0.1% chance that such error was caused by noise or some sort of vibration during the course of the experiment (You et al., 2011).

The consistency of the fit of the model was authenticated by the coefficient of determination (R^2). El-Boulifiet al., (2014) and Abdul Manan et al., (2015) described a good fitting model to be one that attain R^2 of at least 0.80. The value of the coefficient of R^2 of the model describing Steviol Glycocide yield is 0.9842.This value obtained illustrated that the models could explain over 98% of variability for the yield obtained. This suggest that the variation of the samples of >98% for the extraction of Steviol Glycoside was ascribed to the independent variables and only <2% of the variables were not explained by the model. This reaffirms that data obtained from the experiments conducted were reliable and could represent the true relationship between the response and the significant variables. The value of the adjusted coefficient of determination Adj R^2 for methyl oleate yield was 0.9639. The high value (close to unity) show that high proportion of variation was explained by the model. (Abdul Manan et al., 2015).

The "Pred R-Squared" of 0.8456 is in reasonable agreement with the "Adj R-Squared" of 0.9639.
The relationship between Steviol Glycoside yield with the three variables investigatedis as given by the regression model of equations 1.

Final Equation in Terms of Coded Factors:

$$\text{Yield} = +21.74 + 0.65A + 2.66B + 1.64C + 5.72AB + 8.13AC - 4.40B - 0.99A^2 - 4.92B^2 + 6.88C^2$$

Eqn..... 1.0

Where A =Temperature (°C), B=Solute-solvent ratio and C=Time (min)
The equation indicates that all the variables have positive influence on Steviol Glycoside yield. Also, the interaction between all the variables, ie temperature and solute-solvent ratio, temperature and time as well as temperature and solute-solvent ratio and time show synergistic effect, implying that they all improve the yield of Steviol Glycoside and none show antagonistic effect.

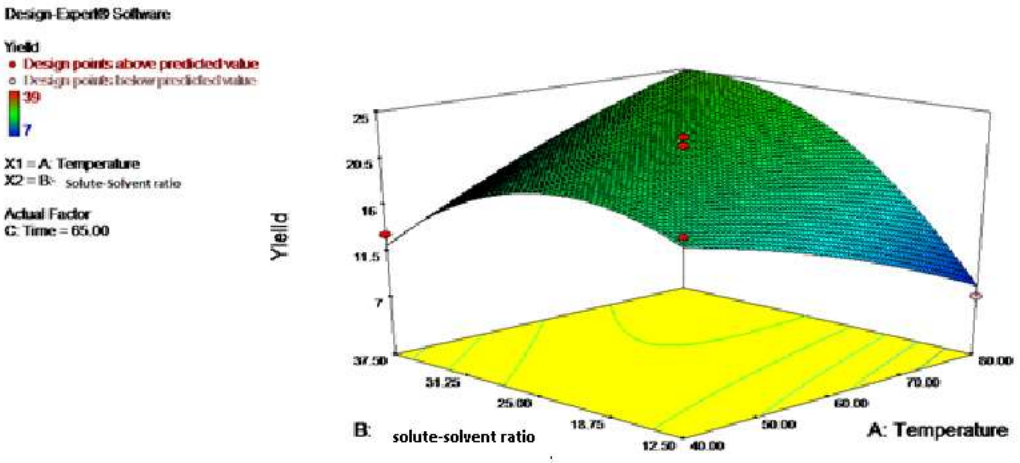

Fig 1: Interaction between solute-solvent ratio and temperature

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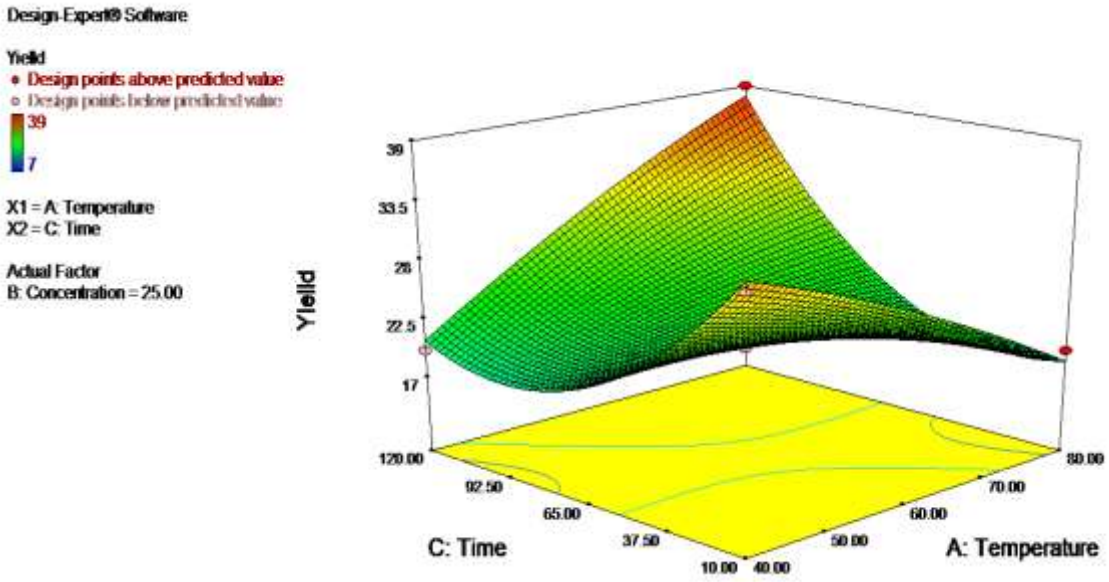

Fig 2: Interaction between time and temperature

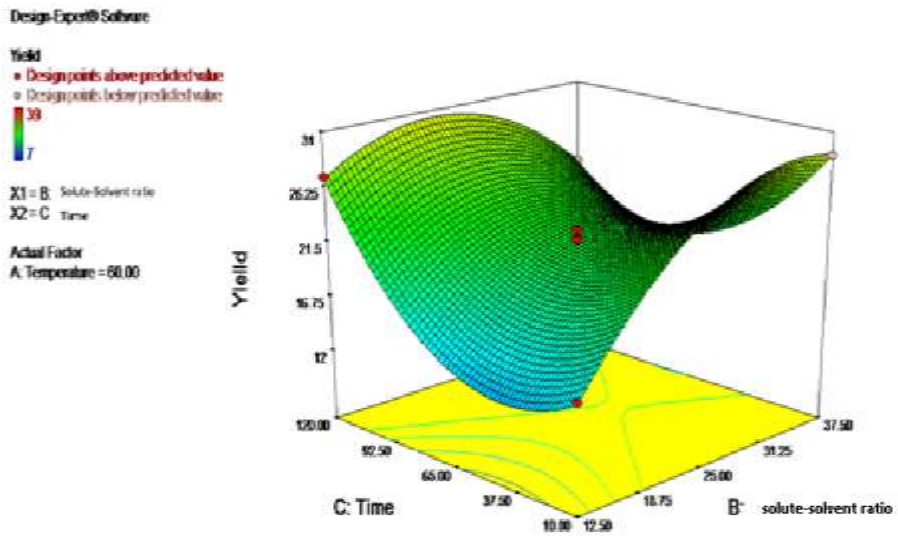

Fig 3: Interaction between time and solute-solvent RATIO

Fig 1 shows the interaction between solute-solvent and temperature. From the 3D diagram, it can be visualized that the yield increased steadily and tend towards its maximum as solute-solvent and temperature are increased.

Fig 2. Shows the interaction between time and temperature which indicates an increase in yield up to about 98% as the reaction time and temperature are increased.

Fig 3. Shows the interaction between reaction time and solute-solvent where about 98% yield was obtained with simultaneous increase in time and feed solute-solvent respectively.

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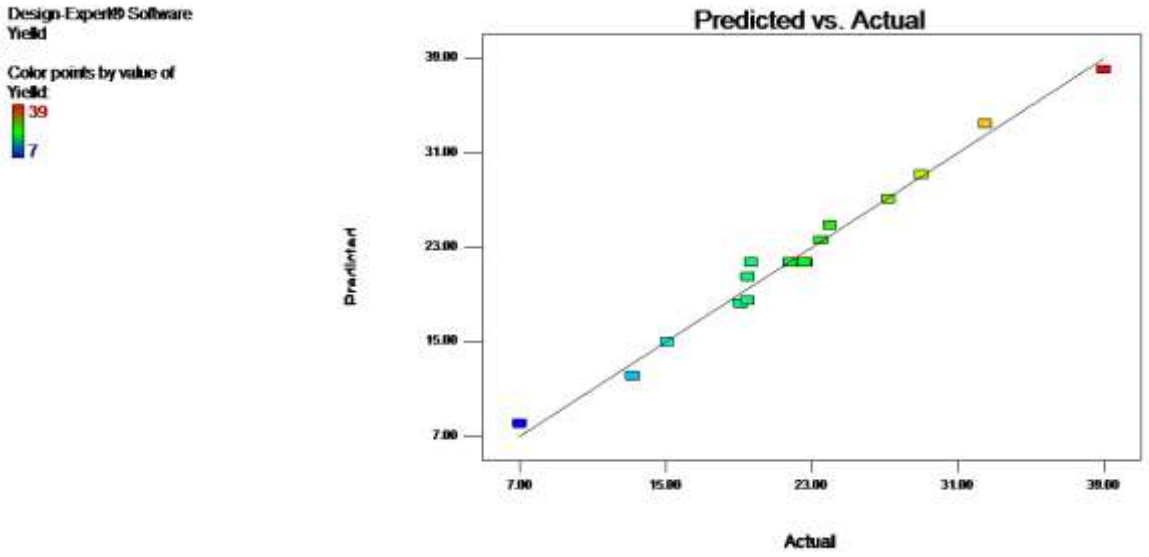

Fig 4: Diagnostic plots of the quadratic model for the actual versus predicted response

Fig 4 shows the closeness between the actual and predicted values measured during the experiments which shows reliability of the model

(iii) PROXIMATE ANALYSIS AND CHEMICAL PROPERTIES OF STEVIA EXTRACT USING ETHANOL SOLVENT

Proximate analysis deals with the chemical composition of food and the quality of food is based on the natural composition, combined nutrients in it, quantity of anti-nutrients and synergistic or qualitative balance between the nutrient amino acids. Proximate which comprises ash, moisture, protein (Kjeldahl protein), fat, fibre and carbohydrate or nitrogen-free extracts (digestible carbohydrate) which is gotten by difference is one of the methods of identifying nutrient composition of foods. Food proximate analysis was carried out on the liquid in obtained to determine its nutritional properties and given in table 9.0 are the results obtained from the food proximate analysis. The extracts were obtained to have a dark color with a pleasant small and sweet taste.

TABLE9.0: Physiochemical Parameters For Hot Water Extract At Various Temperatures

S/N	PARAMETERS	UNIT	40^0C	50^0C	90^0C
1	Moisture Content	%	80	76	70
2	Crude Fat	%	3.1	2.7	1.8
3	Carbonhydrate	%	13.38	18.04	24.34
4	Crude Protein	%	3.49	3.53	3.83
5	Nitrogen	%	0.5576	0.5648	0.612
6	Sugar	%	1.8	1.5	1.1
7	Titrable acidity	%	0.315	0.288	0.27
8	Ash Content	%	0.01	0.01	0.01
9	Fibre	%	0.02	0.02	0.02
10	Ph	-	6.8	6.2	6.1

CONCLUSIONS

Model predicting the yield of steviol glycosides (SG) was developed using RSM. The model was analyzed and found to be sufficient in determining the effect of extraction temperature, solute to solvent ratio and time on the yield with all showing independent and bifactor significance to the model except solute to solvent ratio of 1:37.5 due to solvent capability of producing more dilute solution.

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The regression model generated also revealed that all the variables have positive effects on the SG yield. The interactive effects of the variables as revealed by the surface plots indicated that all variables shows synergistic effect, implying that all improve the yield of SG and none showed antagonistic effect. The diagnostic plot of the quadratic model showed closeness of the actual and predicted responses, implying that the model is reliable.

The proximate analysis carried out on the extract and the concentrate provides significant insight for the production of home made stevia concentrate.

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