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OPTIMIZATION, EVALUATION AND STABILITY STUDY OF NUTRACEUTICAL POWDER FORMULATIONS DEVELOPED FROM EDIBLE SEED AND LEAFY- VEGETABLE SOURCES

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ABSTRACT: Nutraceutical term is a combination of terms nutrient and pharmaceutical. There is a wide scope in applying pharmaceutical formulation parameters to nutraceuticals. Amongst the variety of nutraceuticals available, powder form of nutraceuticals are most popular. The nutraceutical powder mixtures can contain many ingredients, of which the nutrient source contents are generally present in more than 50% of whole formula. Optimization of the powder formulations for pharmaceutical and organoleptic parameters is the needed approach. Under this study optimization, evaluation, and stability study of nutraceutical powder formulations developed from edible seed and leafy vegetable sources is done. The research done involves development of two nutraceutical powder formulations one from edible seed sources namely - *Cucumis melo, Punica granatum* and *Linum usitatissimum* in 1:1:1 proportion, and second from green leafy vegetable sources namely *Trigonella foenum graecum, Coriandrum sativum, Raphanus raphanistrum* and *Anethum graveolens* in 1:1:1:1 proportion. The seeds and leafy vegetables selected were screened for macro and micronutrient content, prebiotic and antioxidant potential. Its *in-vivo* effect is measured as blood parameters and antiproliferative (anticancer potential) activity. The combination of the seeds and leafy vegetables proved more potent than individual source, and so the combination was used to develop a nutraceutical powder mixture formula. Excipients were added to improve taste, flavor and dispersing agents. These formulations were optimized using design expert software and three-level factorial designs. The accelerated stability study of the optimized batch showed that the formulations were stable.

INTRODUCTION: Nutraceutical is a term derived from "nutrition" and "pharmaceutics." The term is applied to products that are isolated from herbal products, dietary supplements (nutrients), specific diets, and processed foods such as cereals, soups, and beverages that other than nutrition are also used as medicine [1].

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But over the years this definition has expanded. Nowadays, nutraceuticals have received considerable interest due to potential nutritional, safety and therapeutic effects. Market research recently proposed that the worldwide nutraceutical market is expanding [2]. There is wide scope in research in the area of nutraceuticals not only in selection of natural ingredients but also how to develop them as acceptable consumer pharmaceuticals.

This research was done on these two levels and this paper particularly presents the pharmaceutical aspects of the two powder nutraceuticals. Here optimization, evaluation, and stability study of

nutraceutical powder formulations developed from edible seed and leafy vegetable sources is addressed. Under this research two nutraceutical powder formulations are developed, one from edible seed sources namely - *Cucumis melo, Punica granatum* and *Linum usitatissimum* taken in 1:1:1 proportion, and second from green leafy vegetable sources namely *Trigonella foenum-graecum, Coriandrum sativum, Raphanus raphanistrum,* and *Anethum graveolens* taken in 1:1:1:1 proportion.

Each seed and leafy vegetable used was selected after studying four parameters namely- nutrient content-determined by the proximate analysis, *in-vivo* study using Wistar rats for various parameters like hemoglobin and lipid profile, DPPH-antioxidant potential determination assay, and prebiotic potential determination. All nutraceutical ingredients qualified above parameters, and their combination proved more potent in these qualifications.

This study presents the pharmaceutical parameter-based approach and evaluation of nutraceutical powder mixtures. The nutraceutical powder mixtures can contain many ingredients and are generally present in more than 50% content of the whole formula. Optimization of the quantities for pharmaceutical and organoleptic parameters like taste is needed approach, which is addressed here.

MATERIALS AND METHODS- PART I

Preformulation Evaluation: Seed powder mixture of *Cucumis melo, Punica granatum,* and *Linum usitatissimum* 1:1:1 was evaluated for nutrient content which was determined by the proximate analysis, *in-vivo* study on Wistar rats for various parameters like hemoglobin and lipid profile, DPPH antioxidant potential determination assay, and prebiotic potential determination. The mixture proved to be effective in all above evaluations but the combination proved to be more effective than individual seed powder and so was selected as basic nutraceutical mixture I for powder formula development [3].

Formulation Development- Dispersible Powder Formula to be Mixed with Warm Water / Milk: Nutraceutical powder mixture as a dispersible powder formula using seed powder mixture of *Cucumis melo, Punica granatum,* and *Linum usitatissimum* 1:1:1. The excipients selected were-milk powder, cocoa powder, and sucrose, which are the most common excipients used in any marketed health drink formula to be mixed with milk [4].

TABLE 1: DISPERSIBLE POWDER FORMULA (N1) OPTIMIZATION

S. no.	Ingredient	Qty in g
1	*Cucumis melo, Punica granatum* and *Linum usitatissimum* 1:1:1	To be optimized
2	Milk powder	
3	Cocoa powder	
4	Sucrose	
5	Total	100

Rationale for the Proportion and Excipients-The Dispersible Powder to be Developed: Dispersible powder formula to be mixed with warm milk using (*Cucumis melo, Punica granatum, Linum uisitatissimum*) seed mixture was developed taking into consideration milk as dispersion medium before consumption so that milk powder, cocoa powder, and sucrose were the most appropriate excipients [5].

Nutraceutical products, unlike the products containing APIs are designed to have more than 50% nutraceutical ingredients. Applying 3^2 factorial design 60% nutraceutical mixture was decided to be level 1, 50% -1 and 70% +1 : as X1(Seed mixture) and 40% excipient as level 1, 30% as -1 and 50% as +1. Quantity of sucrose was kept constant. (Here, it is used as coating and wetting agent along with sweetening agent).

TABLE 2: DISPERSIBLE POWDER FORMULA (N1) OPTIMIZATION LEVELS

N-X1 – Nutraceutical product I seed mixture	-1(50%)	1(60%)	+1(70%)	
E-X2- Mixture of milk powder and cocoa powder in equal proportion	-1(30)	1(40%)	+1(50%)	Independent variables
Dispersion time		Dependent variables		
% Compressibility				

Nine batches were prepared and their dispersion time and % compressibility was determined as dependent variables.

% Compressibility / Carr's Index: It was determined as per the following formula. The resultant reading of 9 mixtures is shown in **Table 3** as Y2.

Bulk density (poured density) = m / V_0, in g per cm^3

Tapped density = m / V_t g per cm^3

M = weight of the powder
V_0 = bulk volume and V_t = tapped volume

Carr's index (%) = (Tapped density – Bulk density) / Tapped density × 100 [6]

Dispersion Time: 10 g mixture was put in 100 mL warm water, and the beaker was held in a bath sonicator (immersed 2/3 deep) in the bath. Time taken for the entire mixture to disperse and soak in was taken as dispersion time.

The proportion of N and E was decided as independent variables and resultant Dispersion time and % Compressibility as dependent variables. With three levels mentioned above the 3^2 factorial design was applied as per the following table.

TABLE 3: DISPERSIBLE POWDER FORMULA OPTIMIZATION 3^2 FACTORIAL DESIGN

N-X1	E-X2	Y1 Dispersion time in seconds	Y2 % Compressibility
50	30	40	25
50	40	35	23
50	50	50	28
60	30	35	26
60	40	40	27
60	50	50	28
70	30	35	30
70	40	40	31
70	50	55	33

Using Design expert software (Statease) various responses / observations were obtained which are interpreted in result and discussion

Stability Study: [8] Nutraceutical mixture of three seeds was kept for stability studies at 40°C/75% RH in stability chamber. After three months the sample was analyzed for antioxidant potential and compared with zero reading (DPPH assay of fresh seed powder mixture). The prebiotic potential was determined and compared with zero reading (of fresh seed powder mixture).

MATERIALS AND METHODS PART II:

Formulation Development: Formulation of Dispersible Powder using leaf powder mixture (to be dispersed in hot water and consumed) was prepared by mixing *Trigonella foenum-graecum, Coriandrum sativum, Raphanus raphanistrum,* and *Anethum graveolens* using formula shown in **Table 4**, where the quantities were optimized. The excipients selected were- *Zingiber officinale* (ginger powder), *Solanum lycopersicum* (tomato powder) Sodium starch glycolate (SSG), and sucrose.

Out of these quantity of SSG and sucrose were kept constant and the formula was optimized for the proportion of basic nutraceutical mixture of equal quantities of *Trigonella foenum-graecum, Coriandrum sativum, Raphanus raphanistrum,* and *Anethum graveolens* as N2 and mixture of equal quantities of *Zingiber officinale* (ginger powder), *Solanum lycopersicum* (tomato powder) as E2.

TABLE 4: FORMULA FOR POWDER TO BE MIXED WITH WARM WATER

S. no.	Ingredients	Use	Qty. in g
1	*Trigonella foenum graecum* leaf powder	Active nutraceutical ingredient	
2	*Coriandrum sativum* leaf powder	Active nutraceutical ingredient	
3	*Raphanus raphanistrum* leaf powder	Active nutraceutical ingredient	To be optimized
4	*Anethum graveolens* leaf powder	Active nutraceutical ingredient	
5	*Zingiber officinale* (ginger powder)	For flavor and taste	
6	*Solanum lycopersicum* (tomato powder)	For taste	
7	Sodium starch glycolate	Dispersing agent	2
8	Sucrose	Sweetener	8
9	Total		100

Rationale for the Proportion and Excipients: The Dispersible Powder to be Developed: Dispersible powder formula to be mixed with hot water using (*Trigonella foenum graecum, Coriandrum sativum, Raphanus raphanistrum,* and *Anethum graveolens* leaf powders) was developed

taking into consideration hot water as dispersion medium before consumption so that *Zingiber officinale* (ginger powder) as flavoring and taste enhancer and *Solanum lycopersicum* (tomato powder) for the taste were considered as appropriate excipients, which are compatible with green leafy vegetable taste and flavor.

The leaf powders did not disperse instantly so sodium starch glycolate (SSG) in concentration 2% was needed. Sucrose as a sweetener in 10% fixed concentration was used which enhanced the taste and its mixing order with leaf powder mixture made difference in dispersion time for the final formula.

Nutraceutical products unlike the products containing APIs, are designed to have more than 50% nutraceutical ingredients. Applying 3^2 factorial design 60% nutraceutical mixture was decided to be level 1, 50% -1 and 70% +1: as X1 (vegetable leaf powder mixture) and 40% excipient mixture as level 1, 30% as -1 and 50% as +1. Quantity of sucrose (here it is used as coating and wetting agent along with sweetening agent) and SSG were kept constant

TABLE 5: APPLYING 3^2 FACTORIAL DESIGN TO THE FORMULA IN TABLE NO. 4 DISPERSIBLE POWDER FORMULA II (N2) OPTIMIZATION

N2 – Nutraceutical product II vegetable leaf mixture	-1(50%)	1(60%)	+1(70%)	Independent
E2- Mixture of *Zingibe rofficinale* and *Solanum Lycopersicum* in equal proportion	-1(30)	1(40%)	+1(50%)	variables
Dispersion time Y1		Dependent variables		
% Compressibility Y2				

Nine batches were prepared and their dispersion time and % compressibility was determined as dependent variables.

% Compressibility / Carr's Index: It was determined as per the following formula. The resultant reading of 9 mixtures is shown in **Table 6** as Y2.

Bulk density (poured density): m/V_0, in g per cm^3

Tapped density: m/V_t g per cm^3

M = weight of the powder
V_0 = bulk volume and V_t = tapped volume

Carr's index (%) = (Tapped density – Bulk density) / Tapped density × 100 [6]

Dispersion Time: 10 g mixture was put in 100 mL warm water and the beaker was held in a bath sonicator (immersed 2/3 deep) in the bath. Time taken for the entire mixture to disperse and soak in was taken as dispersion time.

The proportion of N and E are decided as independent variables and resultant Dispersion time and % Compressibility as dependent variables. With three levels mentioned above, the 3^2 factorial design can be as per the following table.

TABLE 6: DISPERSIBLE POWDER FORMULA II (N2) OPTIMIZATION LEVELS

		Factor 1	Factor 2	ResponseY1	Response Y2
Std	**Run**	**N2**	**E2**	**DT(Sec)**	**Compressibility (%)**
7	2	-1	1	60	30
1	3	-1	-1	45	26
4	9	-1	0	52	27
5	5	0	0	60	27
2	7	0	-1	50	26
8	8	0	1	65	28
3	1	1	-1	55	26
6	4	1	0	65	27
9	6	1	1	72	29

Using Design expert software (Statease) various responses/ observations were obtained which are interpreted to decide the optimum formulation [7].

Accelerated Stability Study: [8] Nutraceutical mixture of four leaves was kept for stability studies at 40°C/75% RH in stability chamber. After three months the sample was analyzed for antioxidant potential and compared with zero reading (DPPH assay of four-leaf powder mixture). Prebiotic potential was determined and compared with zero reading (of fresh leaf powder mixture).

RESULTS AND DISCUSSION PART I:

TABLE 7: FORMULATION AS DISPERSIBLE POWDER -FACTORIAL MODEL 3^2 *CUCUMIS MELO, PUNICA GRANATUM, LINUM UISITATISSIMUM* 1:1:1

Dispersible powder formula (*Cucumis melo, Punica granatum, Linum uisitatissimum* 1:1:1) NI with Excipients EI			
NI-X1	**EI-X2**	**Y1 Dispersion time in seconds**	**Y2 % Comressibility**
50	30	40	25
50	40	35	23
50	50	50	28
60	30	35	26
60	40	40	27
60	50	50	28
70	30	35	30
70	40	40	31
70	50	55	33

TABLE 8: RESPONSE SUMMARY FOR Y1

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0156		0.6667	0.3575	Suggested

Model Suggested for N-X1, E-X2 as Independent Variables and Dispersion Time in Seconds as Dependent Variable is Linear: The mixture of nutraceutical powder and excipients (milk powder and cocoa powder) has no chemical interaction, it's a physical mixture and thus the relationship with a combination of nutraceutical seed powder with excipients is linear. Linear polynomial where the additional terms are significant is selected. Here Adjusted R^2 and Predicted R^2 are optimum.

TABLE 9: RESPONSE 1-D.T. (SEC) ANOVA FOR RESPONSE SURFACE LINEAR MODEL ANALYSIS OF VARIANCE TABLE [PARTIAL SUM OF SQUARES - TYPE III]

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	341.67	2	170.83	9.00	0.0156	significant

The Model F-value of 9.00 implies the model is significant. There is only a 1.56% chance that a "Model F-value" this large could occur due to noise. The Linear model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant.

Final Equation in Terms of Coded Factors:

D.T.(Sec) = + 42.22 + 0.83 * A + 7.50* B

Final Equation in Terms of Actual Factors:

D.T. (Sec) = + 42.22222 + 0.83333 * N1 + 7.50000 * E1

Proportion of nutraceutical seed mixture N1 and proportion of excipient mixture E1 both have positive coefficient for dispersion time. Effect of excipient proportion on dispersion time is more indicated by the coefficient 7.5 as compared with 0.8333 coefficients for seed powder proportion.

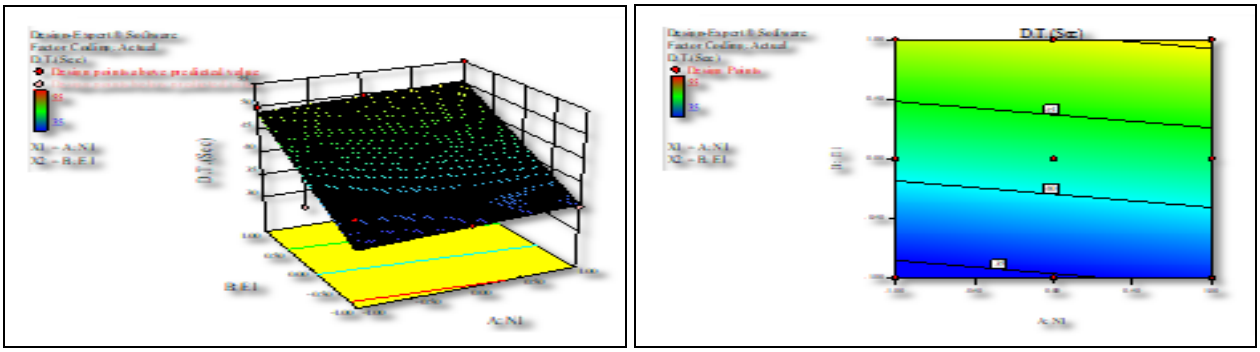

FIG. 1: THE RESPONSE SURFACE GRAPH FOR D.T. PRODUCT 1

Shows if 55 seconds is maximum dispersion time and 35 minimum, then the surface response graph shows 45 seconds as predicted value, and there is a linear relation between N1 and E1 for Dispersion time

TABLE 10: FACTORIAL MODEL 3^2 *CUCUMIS MELO, PUNICA GRANATUM, LINUM ISITATISSIMUM* 1:1:1 FOR Y2

Dispersible powder formula (*Cucumis melo, Punica granatum, Linum uisitatissimum*)			
N-X1	**E-X2**	**Y1 Dispersion time in seconds**	**Y2 % Comressibility**
50	30	40	25
50	40	35	23
50	50	50	28
60	30	35	26
60	40	40	27
60	50	50	28
70	30	35	30
70	40	40	31
70	50	55	33

TABLE 11: RESPONSE SUMMARY FOR Y2

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0040		0.7881	0.5979	Suggested

The model suggested for N-X1, E-X2 as independent variables, and % compressibility in as dependent variable is Linear. The mixture of nutraceutical powder and excipients (milk powder and cocoa powder) has no chemical interaction, it's a physical mixture, and thus the relationship with combination of nutraceutical seed powder with excipients is linear. Linear polynomial where the additional terms are significant is selected. Here Adjusted R^2 and Predicted R^2 is optimum.

TABLE 12: ANOVA FOR LINEAR MODEL RESPONSE 2 C (%): R2 ANALYSIS OF VARIANCE TABLE [PARTIAL SUM OF SQUARES - TYPE III]

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	64.67	2	32.33	15.87	0.0040	significant

The Model F-value of 15.87 implies the model is significant. There is only a 0.40% chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Final Equation in Terms of Coded Factors:

$$C\ (\%) = +27.89 + 3.00 * A + 1.33 * B$$

Final Equation in Terms of Actual Factors:

$$C\ (\%) = +27.88889 + 3.00000 * N1 + 1.33333 * E1$$

The proportion of nutraceutical seed mixture N1 and proportion of excipient mixture E1 both have positive coefficient for % compressibility. The effect of seed powder mixture proportion on % compressibility is more indicated by the coefficient 3.00 as compared with 1.3333 coefficient for excipient proportion.

Box-Cox Plot for Power Transformations: The response surface graph shows that if 33% is maximum % compressibility and 23% minimum, then the surface response graph shows 28% as predicted value, and there is a linear relation between N1 and E1 for % compressibility.

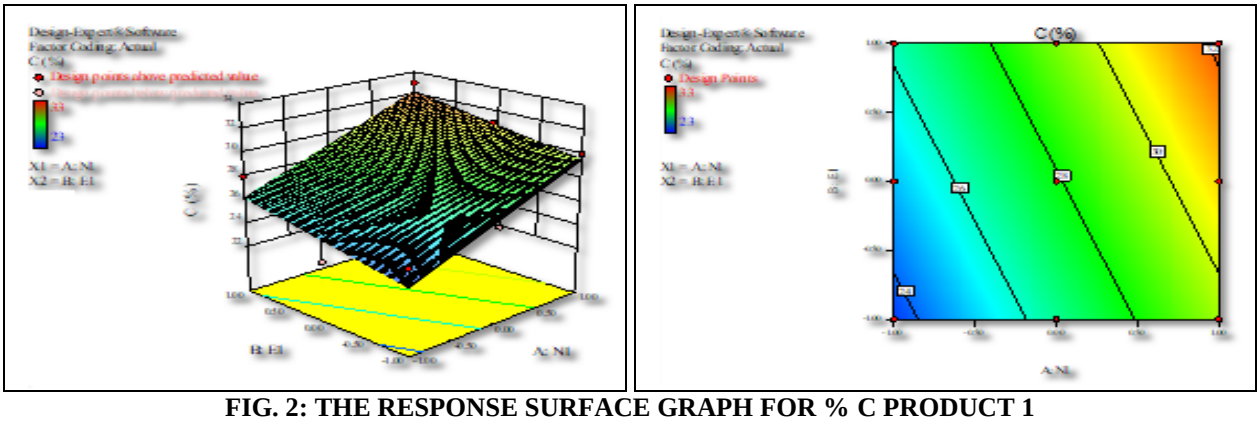

FIG. 2: THE RESPONSE SURFACE GRAPH FOR % C PRODUCT 1

TABLE 13: OPTIMIZED BATCH PRODUCT I

Dispersible powder formula (*Cucumis melo, Punica granatum, Linum uisitatissimum* 1:1:1)							
B. no.	**N-X1**	**E-X2**	**Y1 Dispersion time (D.T.) in seconds**	**Acceptability For (D.T.)**	**Y2 (%C)**	**Acceptability For (%C)**	**Organoleptic preference**
1	50	30	40	Good	25	Poor	+
2	50	40	35	Very good	23	Passable	++
3	50	50	50	Fair	28	Poor	+++
4	60	30	35	Very good	26	Poor	+
5	60	40	40	Good	27	Poor	++
6	60	50	50	Fair	28	Poor	+++
7	70	30	35	Very good	30	Poor	+
8	70	40	40	Good	31	Poor	++
9	70	50	55	Poor	33	Very Poor	++

Batch 2 is suggested optimum by the software as has least D.T. and % C, but since the concentration of nutraceutical mixture is 50% in it, batch 5 is considered optimum with slight higher D.T. and % C. This is a dispersible powder mixture and would taste like hot health drink and is expected to have good agreeable taste.

This powder is not expected to be free-flowing and can be scooped out to be dispersed in hot water/milk.

The score is given to dispersion time and taste. As per the concentration of excipients (milk and cocoa powder) in final formula is given taste score as +++/ ++/ +. Any nutraceutical product is supposed to have more than 50% of nutraceutical ingredients and so batch no 5 is considered to be optimum. As per software batch no 2 is considered optimum based on lowest dispersion time and best result of % C. Since, batch 2 has low taste score batch 5 can be considered optimum.

Results of Stability Study of Nutraceutical Product Mixture I:

TABLE 14: STABILITY STUDY OF NUTRACEUTICAL PRODUCT MIXTURE I -DPPH ASSAY -FRESH SEED MIXTURE AND AFTER 3 MONTHS [9, 10]

	% inhibition Concentration in µg/ml					Equation (Squared co relation coefficient) R^2	IC_{50} (µg/ml)
	10	**20**	**30**	**40**	**50**		
Powder mixture (fresh)	45.35	62.21	74.35	86.25	92.65	y = 68.436x - 24.729 R^2 = 0.9902	2.3402
Powder mixture (after accelerated stability of three months)	42.56	59.36	73.5	84.56	88.35	y = 68.118x - 26.775 R^2 = 0.9909	2.19

TABLE 15: STABILITY STUDY, THE PREBIOTIC POTENTIAL OF THE SEED MIXTURE AFTER THREE MONTHS OF STABILITY STUDY USING *LACTOBACILLUS ACIDOPHILUS* ATCC 4356 PREBIOTIC POTENTIAL DETERMINATION FOR INDIVIDUAL SEED POWDER SAMPLE USING *LACTOBACILLUS ACIDOPHILUS* ATCC 4356 ON DE MAN, ROGOSA AND SHARPE (MRS) AS POSITIVE CONTROL AND PLAIN AGAR AS NEGATIVE CONTROL (AFTER 3 MONTHS) [11, 12]

Sample	Type	No of colonies fresh sample	No of colonies after stability
0.2 g Seed powder of *Cucumis melo* with plain agar	Test	67	52
0.2 g Seed powder of *Punica granatum* with plain agar	Test	41	32
0.2 g Seed powder of *Linum usitatissimum* with plain agar	Test	57	46
0.2 g 1:1:1: seed powder mixture	Test	94	63
0.2 g Chicory powder with plain agar	Positive control	59	32
De Man, Rogosa and Sharpe (MRS) agar	Positive control	47	45
Plain agar	Negative control	0	0

The prebiotic potential of the seed powder mixture is not affected much in comparison with standard chicory powder. If the product is stored at a cool place, then the potential of prebiotic effect will stay long. The prebiotic potential depends on the stability of prebiotic food ingredients (oligosaccharides and soluble fibers) present in the mixture. The positive control MRS agar and *Bifidobacterium* agar are used fresh, so the number of colonies essentially remained the same.

TABLE 16: STABILITY STUDY, THE PREBIOTIC POTENTIAL OF THE SEED MIXTURE AFTER THREE MONTHS OF STABILITY STUDY USING *BIFIDOBACTERIUM BIFIDUM* ATCC 29521 PREBIOTIC POTENTIAL DETERMINATION FOR INDIVIDUAL SEED POWDER SAMPLE USING *BIFIDOBACTERIUM BIFIDUM* ATCC 29521 ON *BIFIDOBACTERIUM* AGAR AS POSITIVE CONTROL AND PLAIN AGAR AS NEGATIVE CONTROL [13]

Sample	Type	No of colonies fresh sample	No of colonies after stability
0.2 g Seed powder of *Cucumis melo* with plain agar	Test	57	37
0.2 g Seed powder of *Punica granatum* with plain agar	Test	42	32
0.2 g Seed powder of *Linum usitatissimum* with plain agar	Test	62	49
0.2 g 1:1:1: seed powder mixture	Test	77	52
0.2 g Chicory powder with plain agar	Positive control	35	27
Bifidobacterium agar	Positive control	47	49
Plain agar	Negative control	0	0

RESULTS AND DISCUSSION PART II:

Formulation Development: For formulation as Dispersible powder, application of 3^2 Factorial design is as follows:

TABLE 17: 3^2 FACTORIAL DESIGN FOR DISPERSIBLE POWDER FORMULA FOR NUTRACEUTICAL MIXTURE II

Dispersible powder formula (*Trigonella foenum graecum*, *Coriandrum sativum*, *Raphanus raphanistrum* and *Anethum graveolens* 1:1:1:1) N2 with Excipients E2			
Factor X1 N2	**Factor X2 E2**	**ResponseY1 DT (Sec)**	**Response Y2 Compressibility (%)**
		(Sec)	%
50	70	60	30
50	50	45	26
50	60	52	27
60	70	60	27
60	50	50	26
60	60	65	28
70	50	55	26
70	60	65	27
70	70	72	29

TABLE 18: RESPONSE SUMMARY FOR Y1 PRODUCT II

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	< 0.0001		0.9834	0.9757	Suggested

Model Suggested for N-X1, E-X2 as Independent Variables and Dispersion Time in Seconds as Dependent Variable is Linear: The mixture of nutraceutical powder and excipients (Ginger and tomato powder) has no chemical interaction, it's a physical mixture and thus the relationship with a combination of nutraceutical leaf powder with excipients is linear. The highest-order polynomial (here linear) is considered optimum which is linear in this case. The Adjusted R^2 and the Predicted R^2- suggested by the software are optimum for linear model and are therefore focused on.

TABLE 19: RESPONSE 1-D.T. (SEC) ANOVA FOR RESPONSE SURFACE LINEAR MODEL PRODUCT II ANOVA FOR LINEAR MODEL RESPONSE 1: R1

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	572.33	2	286.17	237.74	< 0.0001	significant

The Model F-value of 237.74 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. Values greater than 0.1000 indicate the model terms are not significant.

Final Equation in Terms of Coded Factors (D.T. in seconds):

R1= +58.22+5.83A+7.83B

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1.

The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients (D.T. in seconds).

R1 = + 58.22222+5.83333A+7.83333B

The proportion of nutraceutical leaf mixture N2 and the proportion of excipient mixture E2 both have positive coefficient for dispersion time. The effect of excipient proportion on dispersion time is more indicated by the coefficient 7.833 as compared with the 5.8333 coefficient for nutraceutical powder proportion. The response surface graph shows that if 72 seconds is maximum dispersion time and 45 minimum, and there is a linear relation between N2 and E2 for Dispersion time.

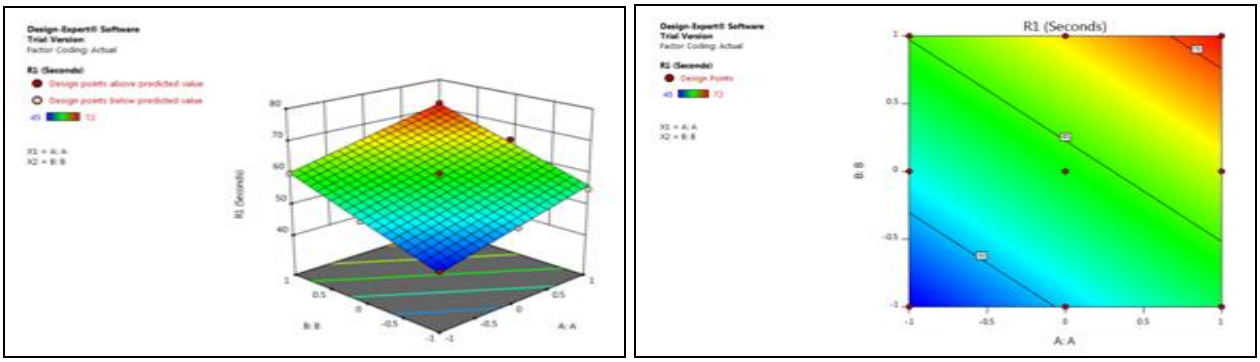

FIG. 3: THE RESPONSE SURFACE GRAPH FOR D.T. PRODUCT II

TABLE 20: FACTORIAL MODEL 3^2 FOR DISPERSIBLE POWDER FORMULA FOR *TRIGONELLA FOENUM GRAECUM, CORIANDRUM SATIVUM, RAPHANUS RAPHANISTRUM* AND *ANETHUM GRAVEOLENS* 1:1:1:1 FOR Y2 RESPONSE SUMMARY FOR Y2

Dispersible powder formula (*Trigonella foenum graecum*, *Coriandrum sativum*, *Raphanus raphanistrum* and *Anethum graveolens* 1:1:1:1) N2 with Excipients E2			
Factor X1 N2	**Factor X2 E2**	**ResponseY1 DT(Sec)**	**Response Y2 Compressibility (%)**
50	70	60	30
50	50	45	26
50	60	52	27
60	70	60	27
60	50	50	26
60	60	65	28
70	50	55	26
70	60	65	27
70	70	72	29

TABLE 21: RESPONSE SUMMARY FOR Y2 PRODUCT II R2-RESPONSE 2 Y2- C (%)

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0031		0.8056	0.6239	Suggested

The model suggested for N-X1, E-X2 as independent variables and % compressibility in the as dependent variable is Linear. The mixture of nutraceutical powder and excipients (Ginger and tomato powder) has no chemical interaction, it is a physical mixture, and thus the relationship with combination of nutraceutical leaf powder with excipients is linear.

TABLE 22: ANOVA FOR LINEAR MODEL RESPONSE 2 C (%): R2

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	13.67	2	6.83	17.57	0.0031	significant

The Model F-value of 17.57 implies the model is significant. There is only a 0.31% chance that an F-value this large could occur due to noise. P-values less than 0.0500 indicate model terms are significant.

In this case, B is a significant model term. Values greater than 0.1000 indicate the model terms are not significant.

Final Equation in Terms of Coded Factors:

R2 =+27.33-0.1667A+1.50B

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1, and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Final Equation in Terms of Actual Factors:

R2 = +27.33333-0.166667A+1.50000B

The proportion of nutraceutical leaf mixture N2 and proportion of excipient mixture E2 both have positive coefficient for % compressibility. The effect/ relative impact of Leaf powder mixture proportion on % compressibility is less indicated by the coefficient -0.166667 as compared with +1.5000 coefficients for excipient proportion. The response surface graph shows that if 30% is maximum % compressibility and 26% minimum, then the surface response graph shows 27% as predicted value, and there is linear relation between N2 and E2 for % compressibility.

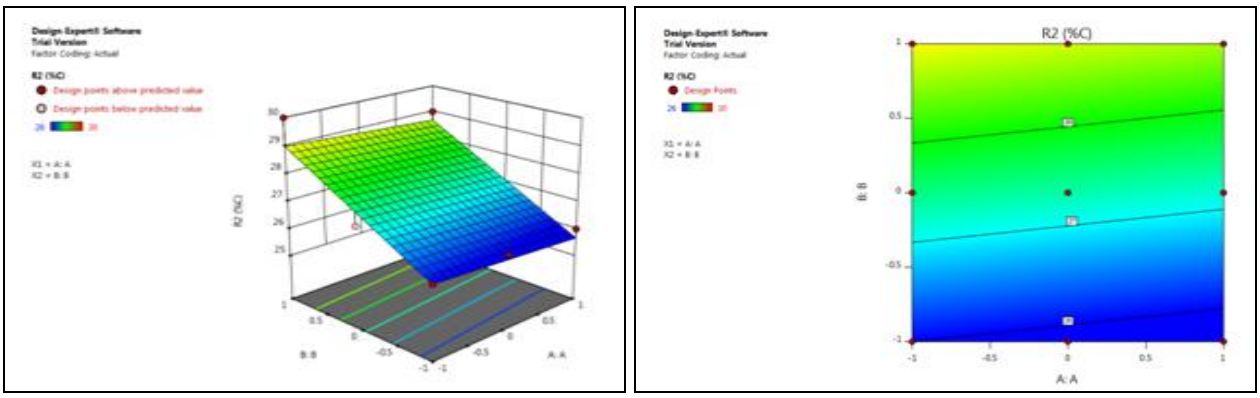

FIG. 4: THE RESPONSE SURFACE GRAPH FOR %C PRODUCT II

TABLE 23: OPTIMIZED BATCH PRODUCT II

Dispersible powder formula (*Trigonella foenum graecum*, *Coriandrum sativum*, *Raphanus raphanistrum* and *Anethum graveolens* 1:1:1:1) N2 with Excipients E2							
B. no.	**N-X1**	**E-X2**	**Y1 Dispersion time (D.T.) in seconds**	**Acceptability For (D.T.)**	**Y2 (%C)**	**Acceptability For (%C)**	**Organoleptic preference**
1	50	70	60	Fair	30	Very Poor	+++
2	50	50	45	Very good	26	Passable	+
3	50	60	52	Good	27	Poor	++
4	60	70	60	Fair	27	Poor	+++
5	60	50	50	Good	26	Passable	+
6	60	60	65	Fair	28	Poor	++
7	70	50	55	Good	26	Passable	+
8	70	40	40	Good	31	Poor	++
9	70	50	55	Poor	33	Very Poor	++

This is a dispersible powder mixture and is expected to have good agreeable taste like hot vegetable soup. This powder is not expected to be free-flowing and will be scooped out to be dispersed in hot water. So, importance is given to dispersion time and taste score. As per the concentration of excipients (tomato powder and ginger powder) in final formula is given taste score +++/ ++/ +. Any nutraceutical product is supposed to have more than 50% of nutraceutical ingredients and so batch no 4 is considered to be optimum. Whereas per software batch no 2 is optimum but since has low score of taste as per the experiments batch 4 is considered optimum.

Accelerated Stability Studies of Product II: Accelerated stability studies were carried out at 40°C/75% RH for three months, which is an exaggerated condition of storage. If cool and dry place storage is recommended then the product will be stable for a reasonable time period. Most of the marketed nutraceutical formulations have 9-12 months expiry. This product may comply with it as per the preliminary stability studies. As method evaluation of stability -antioxidant activity and prebiotic potential of nutraceutical powder mixture were studied.

TABLE 24: STABILITY STUDY OF NUTRACEUTICAL PRODUCT MIXTURE II-DPPH ASSAY-FRESH LEAF MIXTURE AND AFTER 3 MONTHS [9, 10]

	% inhibition Concentration in µg/ml					Equation (Squared co relation coefficient) R^2
	10	20	30	40	50	
Powder mixture (fresh)	54.7	54.7	54.7	54.7	54.7	y = 54.91x - 1.466 R^2 = 0.984
Powder mixture (after accelerated stability of 3 months)	52.7	52.7	52.7	52.7	52.7	y = 54.91x - 1.466 R^2 = 0.984

Prebiotic potential determination for individual leaf powder sample using *Lactobacillus Acidophilus* ATCC 4356 on De Man, Rogosa and Sharpe (MRS) as positive control and plain agar as a negative control (after 3 months) [11, 12].

TABLE 25: STABILITY STUDY, THE PREBIOTIC POTENTIAL OF THE LEAF MIXTURE AFTER THREE MONTHS OF STABILITY STUDY

Sample	Type	No of colonies fresh sample	No of colonies after stability
0.2 g Leaf powder of *Trigonella foenum graecum* with plain agar	Test	74	61
0.2 g Leaf powder of *Coriandrum sativum* with plain agar	Test	102	89
0.2 g Leaf powder of *Raphanus raphanistrum* with plain agar	Test	47	32
0.2 g Leaf powder of *Anethum graveolens* with plain agar	Test	05	02
0.2 g 1:1:1:1: leaf powder mixture	Test	64	42
0.2 g Chicory powder with plain agar	Standard	59	42
De Man, Rogosa Sharpe (MRS) agar	Positive control	47	44
Plain agar	Negative control	0	0

Prebiotic potential determination for individual leaf powder sample using *Bifidobacterium Bifidum* ATCC 29521 on *Bifidobacterium* agar as positive control and plain agar as negative control [13].

TABLE 26: STABILITY STUDY, THE PREBIOTIC POTENTIAL OF THE LEAF MIXTURE AFTER THREE MONTHS OF STABILITY STUDY USING *BIFIDOBACTERIUM BIFIDUM* ATCC 29521

Sample	Type	No of colonies fresh sample	No of colonies after stability
0.2 g Leaf powder of *Trigonella foenum graecum* with plain agar	Test	67	52
0.2 g Leaf powder of *Coriandrum sativum* with plain agar	Test	94	77
0.2 g Leaf powder of *Raphanus raphanistrum* with plain agar	Test	42	32
0.2 g Leaf powder of *Anethum graveolens* with plain agar	Test	04	2
0.2 g 1:1:1:1: leaf powder mixture	Test	57	39
0.2 g Chicory powder with plain agar	Standard	35	29
Bifidobacterium agar	Positive control	47	52
Plain agar	Negative control	0	0

The prebiotic potential of the leaf powder mixture is not affected much in comparison with standard chicory powder. If the product is stored at a cool place, then the potential of prebiotic effect will stay long. The prebiotic potential depends on the stability of prebiotic food ingredients (oligo-saccharides and soluble fibers) present in the mixture.

The positive control MRS agar and *Bifidobacterium* agar are used fresh, so the number of colonies essentially remained the same.

CONCLUSION: *Cucumis melo*, *Punica granatum* and *Linum usitatissimum*, edible seed mixture and *Trigonella foenum graecum*, *Coriandrum sativum*, *Raphanus raphanistrum*, and *Anethum graveolens*

green leafy vegetable mixture are rich in nutrients, antioxidants and have prebiotic content. Both mixtures can have applications in treatment and prevention of many illnesses that can be helped with prebiotic and antioxidant content. Being indigenous nutraceuticals these can be economical and could be promising products. The preformulation results of the individual ingredients were promising and the combination proved more beneficial than individual ingredient. So, two mixtures were developed into two dispersible nutraceutical powder formulae to be mixed with warm milk/water before consumption. The two formulations were optimized for excipient proportions using design expert software and evaluated for pharmaceutical parameters. The stability study shows that antioxidant and prebiotic potential did not decrease significantly.

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