DEVELOPMENT OPTIMIZATION FORMULA OF THROAT SPRAY WITH IVY LEAF EXTRACT AND *BACILLUS* SPORES

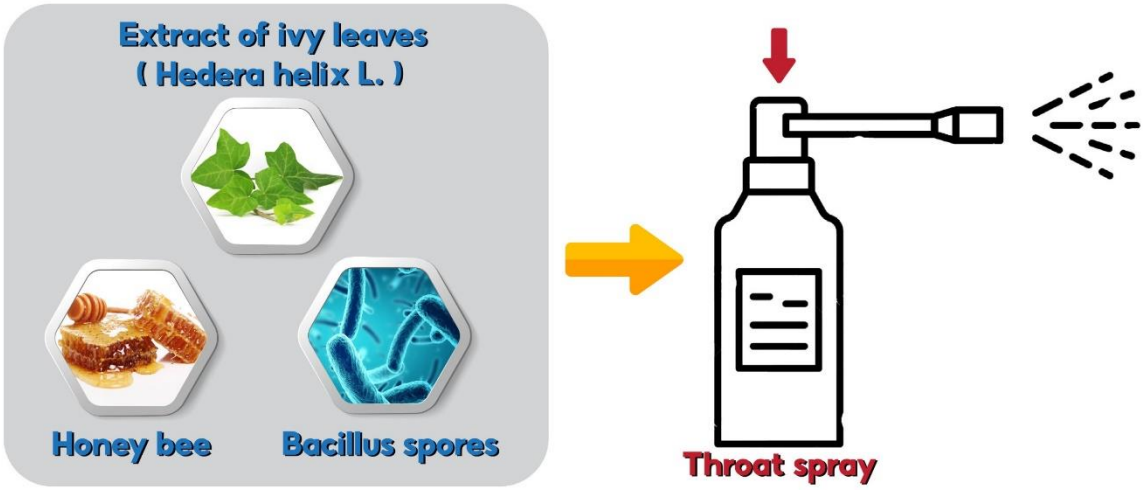
Development Optimization Formula of Throat Spray with Ivy Leaf Extract and Bacillus Spores

Anh Ngo[1], Pharmaceutical Science, Department of Pharmaceutical and Pharmaceutical Technology, Haiduong Central College of Pharmacy, Hai Duong, Vietnam.
Ba Kien Tran[2], Pharmaceutical Science, Department of Pharmaceutical Administration, Haiduong Central College of Pharmacy, Hai Duong, Vietnam.
Cong Hung Mai[3], Pharmaceutical Science, Department of Pharmaceutical and Pharmaceutical Technology, Haiduong Central College of Pharmacy, Hai Duong, Vietnam.
Corresponding author: Anh Ngo[1] (qanhk63hup@gmail.com)

Abstract

The goal is to develop a throat spray containing *Bacillus* and ivy leaf extract that prevents and aids in the treatment of pharyngitis and upper respiratory tract infections. JMP 14 Pro software was used to create an experimental model using 16 formulas. The percentages of ivy leaf extract, honey, and *Bacillus* spores are independent variables, while *Bacillus* survival and mean beam width spray are dependent variables. Based on the variance analysis of the dependent variables and the effect of the independent factors on the dependent variable, the computer creates optimum equations with various desired values. The assessment of appropriate formulae by examining anti-inflammatory efficacy as well as quality standards such as organoleptic, pH, qualitative ivy leaf extract, and *Bacillus* survival quantification. The findings demonstrated that JMP 14 Pro suggested an ideal formula with an index that was the most preferred and contained 1,65% ivy leaf extract. The survey approach showed that the formula had good anti-inflammatory power when compared to the reference drug. The anti-inflammatory potential was evaluated using the mouse paw edema test on white mice. The end product is a suspension with a mild and sweet flavor. It achieves a constant spray volume, with a pH range of 5,5 to 6,5, and a round or almost circular spray shape that measures 7,2 to 9,3 cm in diameter. It also matches the standard's Rf on thin-layer chromatography (HPTLC). Strong anti-inflammatory properties are present in the optimal formulation of the Bacillus-containing throat spray, and the quality criteria are studied as a basis for the eventual establishment of the product's quality standards.

Keywords: *Bacillu*s spores; ivy leaf extract; throat spray



1. Introduction

Everyone can have pharyngitis, which is a common illness. Viruses, bacteria, and fungi are the primary culprits of the illness. The majority of the time, sore throats resolve on their own without the need for therapy, but because the symptoms are frequently unpleasant, it is unavoidable to utilize treatments to ease the discomfort. In particular, local treatment is becoming more and more common since it is not only very effective because it has a direct influence on the inflammatory region but also because it reduces the negative effects when using systemic medications [1].

It is widely used in medicine, agriculture, and industry. *Bacillus* is a helpful bacterium that is exposed to tough environmental conditions, has the best capacity to create enzymes, and is turned into probiotic goods on an industrial scale. food sector. Beneficial bacterial spores will become "inactivated" when the virus binds to them, rendering them unable to penetrate cells and spread illness. Probiotics were frequently employed in pharmacological forms such as capsules, tablets, lozenges, and suspension… in treating upper respiratory tract disorders due to its antibacterial, antiviral, and anti-inflammatory qualities. Delivering probiotics through nasal spray could be a rapid and effective symptomatic treatment for ARTIs. Because the method of interaction between Bacillus spores and the virus is non-specific, our findings suggested that nasal-spraying Bacillus spores could also be effective against other quickly growing RTI viruses like influenza virus. Overall, Bacillus spores sprayed into the nose can help with symptoms. Treatment of influenza virus-induced ARTIs is rapid and efficient, and it has the potential to be employed as a cost-effective supportive treatment for respiratory viral illness in general [2, 3, 6]. Preparations of ivy leaf (Hedera helix) extract are commonly used over-the-counter, non-antibiotic cough treatments approved by the European Medicines Agency [4].

Due to several exceptional benefits, including delivering medications directly to the site of action, local effects, convenience of administration, and avoiding liver metabolization, research into the spray form directly into the oral cavity and oropharynx is now being pursued [3, 6]. There are several nasal and throat sprays with helpful bacteria available worldwide. But as of yet,

Vietnam has no such product. The study on a throat spray containing *Bacillus* was done to advance knowledge and satisfy the market for biotechnology-derived goods used to treat and prevent upper respiratory tract disorders.

2. Materials and methods

2.1. Materials

The ivy leaf extract (*Hedera helix L.*) (France), honey (Hung Yen, Vietnam) and *Bacillus subtilis* (lyophilized powder spores – Chr. Hansen, Denmark); other products used included Tween 80, Citric acid, Sucralose, Sodium Citrate, Sodium Chloride (China); Peptone Water, Hydrochloric Acid, Monopotassium Phosphate (Sigma-Aldrich, U.S.A.), MRS agar and MRS broth (Merck, Germany).

Strains of *Bacillus subtilis* were cultured statically in MRS broth at 37°C. By centrifuging the bacteria at 2000g for 10 minutes, sterile phosphate-buffered saline (PBS) was used to wash the bacterial pellets. UV inactivation of bacteria was accomplished in a biosafety level 2 cabinet using 15 minutes rounds of UV irradiation followed by vortexing (for tests with human cells) or PBS (for antiviral activity experiments). In order to verify inactivation, bacteria were plated out.

2.2. Methods

2.2.1. Technique for preparing throat spray

Prepare: Weighing raw materials: *Bacillus* lyophilized powder (equivalent to 3.10^9 CFU/g) Sodium chloride (g); Sodium citrate (g); citric acid (g); sucralose (g).

Hot dissolving: Apply sodium chloride; Sodium citrate; citric acid; sucralose in 10ml warm water (30 ^oC-40^oC) stir completely (**DD1**)

Dissolve: Take 10ml of warm water (40^oC), add *Bacillus* and stir to homogenize (**DD2**). Pour **DD2** into **DD1**. Stirred. Add enough water 30ml

2.2.2. Design and optimize the formulation of throat spray formulation

The tissue I-Optimal model, which consists of 16 formulae, is created using the JMP 14 Pro program for the design and optimization of throat spray emulsion formulations. The percentages of ivy leaf extract (X1 - %), honey (X2 - %), Bacillus spores (X3 - %), were modified so that the formula was only 30ml, establishing four significant independent variables that impact product attributes. The number of surviving *Bacillus* (**Cb**), the average diameter of the spray beam (**D**), and other factors are all evaluated at various levels. formula choice. The JMP program suggests a desirable index for optimization; the higher the index, the more prediction values that are as near as feasible to the real value.

2.2.3. Spray shape rating

Spray shape ratings were determined using FDA regulations [14]. On the adhesive side of the paper vellum A4 size, evenly and thickly sprinkle talcum powder. Fix the paper sheet to a square plane at an angle to the ground's horizontal. Spray the bottle no more than two to three times. Place the preparation container with the mouth spray nozzle 7 cm away from the paper's plane and

perpendicular to it. By hand, apply forceful pressure to the nozzle. captured by the human eyes. On the paper surface, the spray beam form is created. Repeat 3 distinct test samples should be used for the test. The judgment relies on the size and form of the spray beam.

2.2.4. Evaluation of product quality standards

2.2.4.1. Qualitative ivy leaf extract

Silicagel GF254 (Merck): 30 minutes of activation at 105°C. Chemicals used include butanol, methanol, chloroform, distilled water, and H2SO4.

The evolving solvent system: chloroform - MeOH - Water = (65: 35: 10) is made up and allowed to stand in the developing vessel for at least 15 minutes before chromatography to completely saturate the solvent vapor.

10% H2SO4 solution in 96% ethanol is used as the color development reagent. Shake constantly as you gradually add 6 ml of sulfuric acid to 50 ml of ethanol. Cool, then fill 100 ml with ethanol. 50 mL or so of the test sample should be added to the decant shaker. Insert 30 ml of butanol. Shake thoroughly and allow the solvent layer to separate. Then, decant the top solvent and shake twice with 15 ml of ammonium hydroxide 10% to get rid of the biting. Then deliver her the fluid you've gathered for her to bite into. For chromatography, dissolve the bite in 1 ml of ethanol. The developing solvent system should be ready. Chloroform: Water = (65: 35: 10) in MeOH. Place 15 to 20 µl of the test and reference solutions on the plate separately. When the developing solvent has moved about 10 to 12cm, deploy chromatography, remove the plate, and let it dry in the air. To display the color, mist the reagent, then let it dry. When there are traces of the same color, size, and location (Rf) in the chromatograms of the standard and the test solutions, the test sample is said to be positive.

2.2.4.2. Probiotics cell count

One ml of solution was added to 9 ml of sterile water and mixed. During this process, bacterial spores were evenly distributed. Serial dilution step with sterile peptone water solution (0.1%) and using pour plate method in MRS Agar medium. Finally, the number of bacteria was counted after 37 hrs of incubation at 37°C. For free cell count, the pour plate technique was performed according to the method provided by Turano et al. (1988) with some modifications.

2.2.5. Evaluation of anti-inflammatory properties

Trials were conducted following the carrageenan-induced inflammation model due to Winter et al proposal in 1962 [7]. Male adult white rats of the Wistar train, weighing 180-200g on average, were used as test subjects. They were given by the Institute of Vaccines and Medical Biologicals in Nha Trang, Vietnam. Rats were maintained for two days to acclimate to the test field for lips. They are given enough food and drink during the test. Chemicals and test supplies, such as solutions. Before inducing inflammation, 1% carrageenan is thoroughly dissolved in physiological saline, which contains 0,9% sodium chloride. This process takes around 2 hours. Mice were divided into lots at random, with 10 mice in each batch. The measuring tool is a **Ugo Basile** plethysmometer (model number 37140).

Apply a 1% carrageenan solution to the mice's rear legs for the control group. (**Group A)**

Approximately 10^{10} CFU/kg. of *Bacillus* throat spray solution was applied to the back legs of mice as part of a drug test batch. 10^{10} CFU/kg (**Group B**).

Reference batch: 5 mg of indomethacin in 0.9% sodium chloride was applied to the rat hind legs (**Group C**)

In the comparison batch, the medication was not applied to the rats' hind legs. (**Group D**)

Conduct: Using a plethysmometer, measure the volume of the mouse leg before causing inflammation (**Vo**). Apply the medication three times daily to the rat's legs. Each time apply 50µl of reagent for 5 days straight to examine the outcome.

Stop the product's inflammatory response in the mice model of foot edema. Apply the medication for 5 days, then measure the inflammation and test the Anti-inflammatory activity: injecting 0,05 ml of a 1% carrageenan solution into the rear of the left foot of the rats causes inflammation. To avoid infection, mice were kept in plastic cages with sawdust inside to minimize irritation of their feet. When applied, measure the mouse leg volume three, five, seven, twelve, twenty-four, forty-eight, and seventy-two hours later. The volume of mouse foot with inflammation is known as **Vn**.

3. Results and discussion

3.1. Formula optimization test results

Using a software design test with variables in JMP Pro 14

High percentage of ivy leaf (1-10%), password rate (1-5%), and percentage of *Bacillus* spores (1-20%) are the input factors.

Bacillus survival rate (Cb - log CFU/ml); and average spray diameter (D - cm) are the output variables.

- Decide on a level 1 mathematical model.
- The process of using a neural network to analyze the outcomes
- 16 formulations (M1 to M16) with different excipient ratios based on the defined input factors were synthesized.

Table 1. Experimental results of the concentration of *Bacillus* (Cb) and the typical diameter of the spray beam (D)

CT	X1 (%)	X2 (%)	X3 (%)	Test results				
				Cb (log CFU/ml)	**D (cm)**	**T**	**V**	**H (gam)**
M1	1	1	1	3,15	9,85	+	+	0,101
M2	1	1	1	3,21	9,70	+	+	0,105
M3	10	1	20	11,81	8,51	+++	++	0,112
M4	10	1	20	12,20	8,40	+++	++	0,115
M5	10	5	1	2,01	5,62	++++	+++	0,197
M6	10	5	1	2,11	6,19	++++	+++	0,189
M7	1	1	20	13,01	7,20	++	+++	0,143
M8	10	5	20	8,52	5,47	++++	++++	0,185
M9	10	1	1	2,82	8,02	+++	+++	0,120
M10	1	5	20	9,24	7,22	+++	+++	0,169

M11	1	5	1	2,07	6,91	+++	+++	0,158
M12	1	5	20	8,13	7,03	+++	+++	0,146
M13	10	1	20	12,30	8,36	+++	+++	0,127
M14	5,5	2,5	10,5	12,57	9,32	++	+++	0,105
M15	5,5	2,5	10,5	13,01	9,25	++	+++	0,107
M16	5,5	2,5	10,5	12,72	9,23	++	+++	0,108

M1 – M16: The dosage formulas change the proportions according to design suggestions

Turbidity (Throat turbidity of suspended spray): Increasing turbidity is indicated by the amount of + marks (**T**)**; Viscosity** (Throat viscosity of suspended spray): Increasing viscosity is indicated by the amount of + marks (**V**)**;** Homogeneity of the average dosage distribution (**H**)

Table 2. Results of an artificial neural network study using the M1-M16 formula.

Training

Cb

Measures	Value
RSquare	0.9963028
RMSE	0.2637994
Mean Abs Dev	0.181983
-LogLikelihood	1.1228366
SSE	0.9046713
Sum Freq	13

D

Measures	Value
RSquare	0.996718
RMSE	0.0775236
Mean Abs Dev	0.0549218
-LogLikelihood	-14.79705
SSE	0.0781287
Sum Freq	13

Validation

Cb

Measures	Value
RSquare	0.9993212
RMSE	0.1189769
Mean Abs Dev	0.1025288
-LogLikelihood	-2.129662
SSE	0.0424665
Sum Freq	3

D

Measures	Value
RSquare	0.9592052
RMSE	0.2921084
Mean Abs Dev	0.2154953
-LogLikelihood	0.5649245
SSE	0.2559819
Sum Freq	3

	Generalized RSquare	-LogLikelihood
Training	1.0000	-13.67422
Validation	1.0000	-1.564738

	Cb		**D**		**Results**	
	Training	Validation	Training	Validation	Training	Validation
R^2	0,9963	0,9993	0,9967	0,959	1,0000	1,0000
-LogLikelihood	1,12	-2,12	-14,79	0,56	-13,67	-1,56

According to the results in the table, the low common -LogLikelihood value is less than 4,4, and the common R^2 for both training and validation is very close to 1. The artificial neural network model is near to the experiment, as evidenced by the output variables' R^2 values being more than 0,99 and their -LogLikelihood values being less than 14,2.

Figure 2 below displays the outcomes of the analysis of the artificial neural network using the formula M1-M16, including the expected cross-section and the best formula.

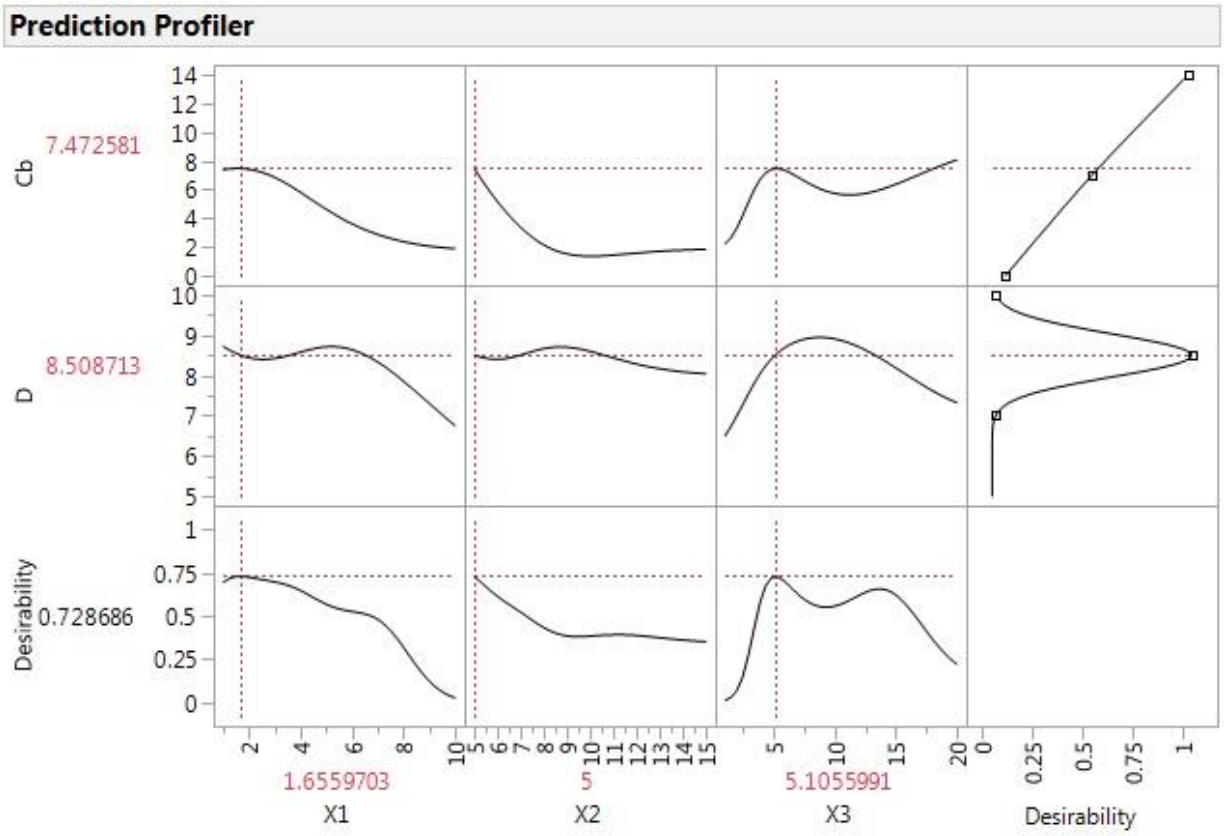


Figure 2. Results of the outcomes of the analysis of the artificial neural network using the formula M1-M16, including the expected cross-section and the best formula.

The findings from the cross-section demonstrated a distinct relationship between the output variables and the high proportion of ivy leaf extract, the proportion of honey, and *Bacillus*.

- Outside of the ideal region, the correlation between Cb and the input variables declines as X1, X2, and Cb grow. The X3 and Cb correlation graph is sinusoidal. Cb's greatest value was 7,47 with X1 = 1,65; X2 = 5; and X3 = 5,10.

- Correlation of D with the input parameters. When about X1 > 7,0; and X2 > 10, 0; the value line of D drops as X grows and X2 increases. X3 and D are connected by a parabolic graph. The value of D peaks at 8,5 for X1 = 1,65; X2 = 5,00; and X3 = 5,10.

The following is one explanation for this correlation:

When increased to a small level, the antibacterial property of ivy leaf extract (phenolic acid) prevents *Bacillus* from surviving. The high amount of ivy leaf must be balanced in the formulation if it hopes to maintain the expectorant and cough-reducer effects along with probiotics.

Honey aids in the addition of waste, the alleviation of coughing, and the expansion of *Bacillus* spores in the solution. However, due to its high density and viscosity, honey can clog spray nozzles and impair spray dispersion. Additionally, the proportion of honey must be considered within the proper range.

The spray beam and the survival concentration are also impacted by the *Bacillus* spore content. If the increase is too significant, the nozzle is simple to clog, the spray beam is irregular, it is

challenging to uniformly distribute the dose, and the average survival concentration is also decreased.

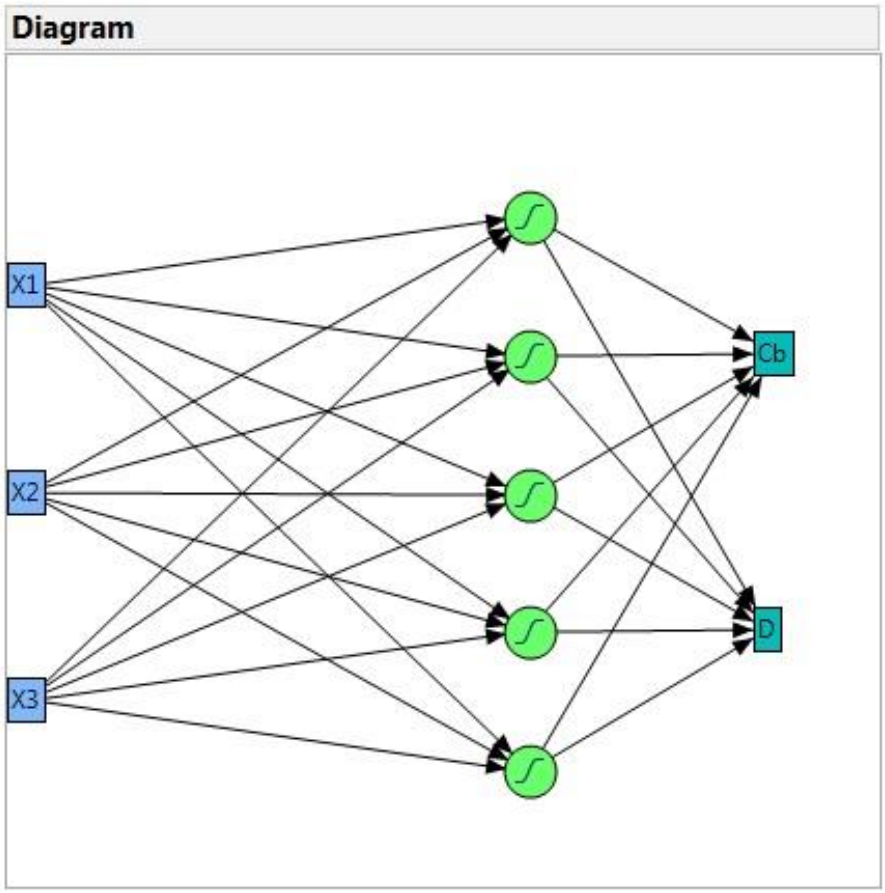


Figure 3. The artificial neural network demonstrates the relationship between variables.

The design space in the study of an artificial neural network is comprised of the values of the variables and the outcomes, as shown below.

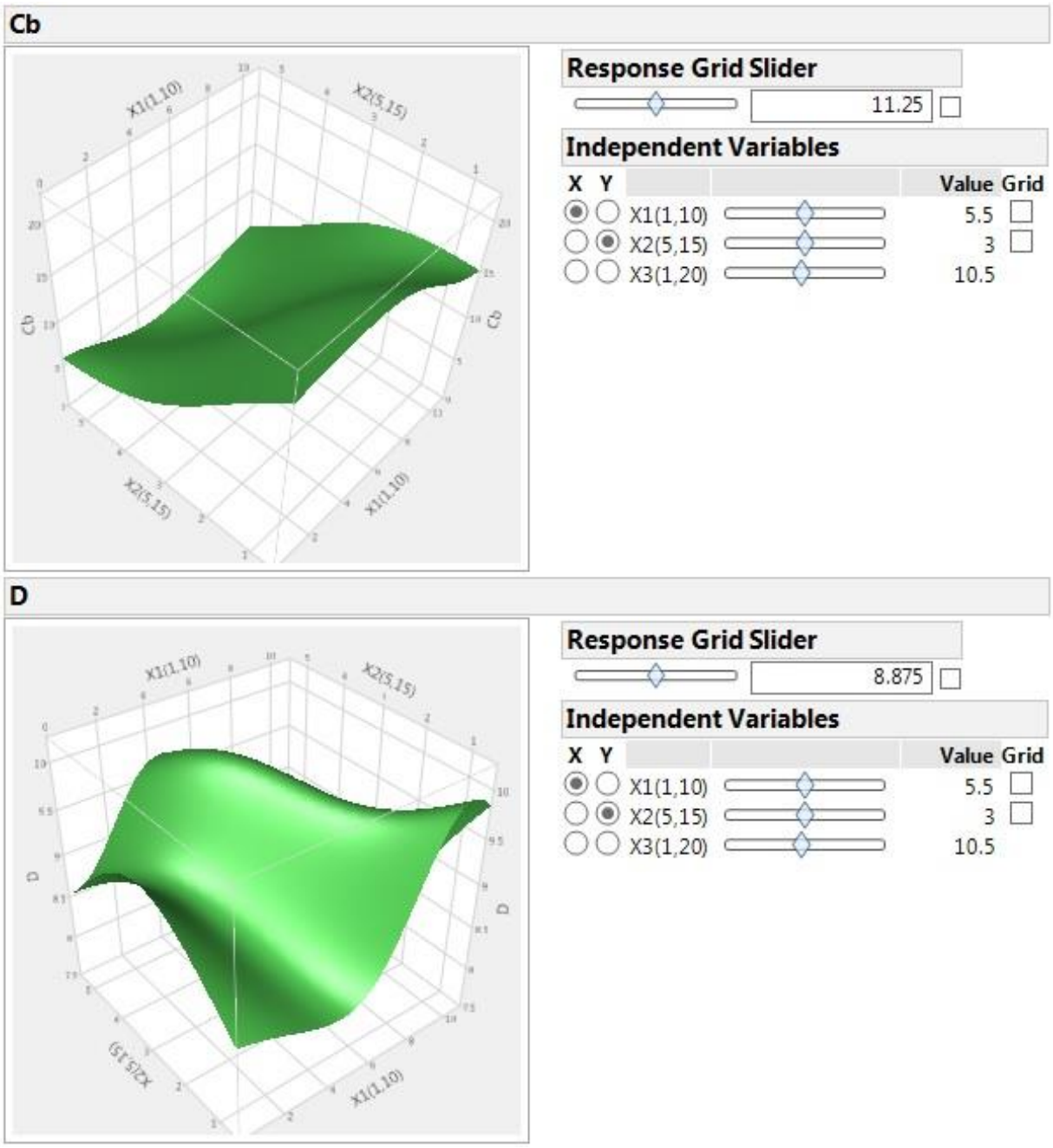


Figure 4. Results of the design space in the analysis of artificial neural networks with the formula M1- M16

3.2. Re-evaluate the optimal formula

Prepare **Mi** throat spray using the following ingredients using the optimal recipe predicted above: 1,65% ivy leaf extract; 5,0% honey bee extract; 5,10% Bacillus using the procedure described in section 2.2.1. Evaluation of **Cb** and **D**.

Table 5 shows a comparison of the optimal formula's output variable results with the predictive formula's outcomes.

Table 5: Results of the optimal formula's output variable and the predictive formula's outcomes.

	Mi.1	Mi.2	Mi.3	$\overline{Mi}$	SD	Prediction
Cb	7,52	7,48	7,46	**7,486**	0,03	**7,47**
D	8,46	8,49	8,52	**8,490**	0,03	**8,50**

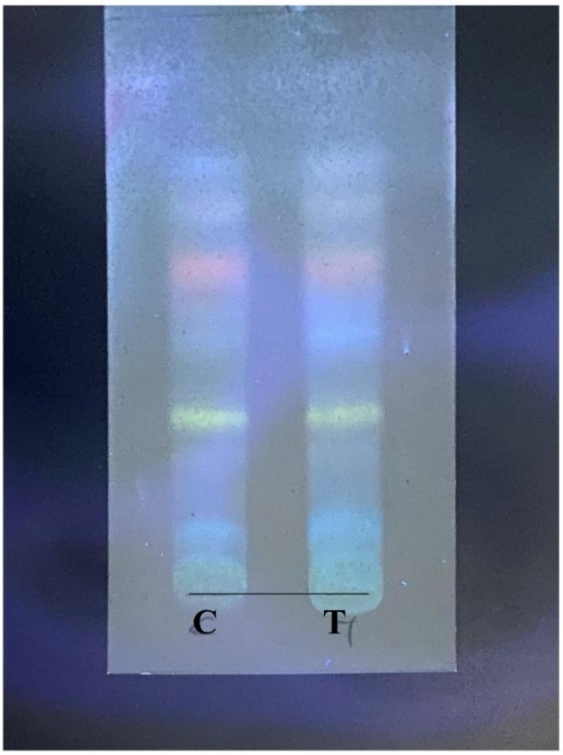
Mi.1, Mi.2, Mi.3: Optimization formula for test batches 1, 2, 3.

The concentration of surviving *Bacillus* and the mean diameter of the actual spray beam were both close to the expected value (deviation less than 1%), indicating that the artificial neural network model is accurate.

3.2. Evaluation of product quality standards

3.2.1. Qualitative ivy leaf extract

Prepare the developing solvent system as follows. Chloroform: MeOH: Water = (65: 35: 10). Place 15 to 20 µl of test solution and reference solution on the plate separately. When the solvent travels about 10-12 cm, stop chromatography and remove the thin plate to dry in the air. Spray with color development reagent and let dry. Results of the ivy leaf extract calculation were positive which displayed below.



C: Standard solution
T: Sample solution

UV 366 nm

- **The evolving based solvent system: Chloroform - MeOH - Water = (65 : 35 : 10)**
- **Reagent for color appearance: 10% solution of H2SO4 in ethanol 96%**

Figure 5. Image of ivy leaf extract thin layer chromatography test results

We discovered that there was a match in the chromatographic spectrum between the test solution and the reference solution by looking at the order of zones contained in the generated chromatograms. At the top of the plate, there was a green zone was α-hederin. In the purple zone had a broad very faint purple (α-hederin), a broad yellow zone and 2-3 purple or green zones. At the bottom of the plate was Hederacoside-C in the purple zone. This result is consistent with research by Miao Yu et al (2015) [5].

3.2.2. Analyze the finished product's capacity to reduce inflammation.

After using a rat carrageenan edema model, per Winter [13]. The analysis of alterations in rat paw edema yielded the following findings.

Table 6: Evaluate the effectiveness of reducing rat paw edema between test batches

Group	Average rat paw edema						
	3h	5h	7h	12h	24h	48h	72h
A	72,52 ± 4,23	67,61 ±6,33	64,629 ±5,18	52,31 ± 3,90	45,19 ±3,25	50,19 ±4,25	43,12 ±3,35
B	79,58 ±6,19	59,53 ±5,70	53,46 ±5,91	51,45 ± 2,51	49,73 ±6,71	42,40 ±4,15	34,12 ±4,10
C	76,87 ±6,89	75,68 ±2,81	72,45 ±5,32	66,68 ±3,85	62,61 ±2,89	56,51 ±3,60	52,54 ±3,16
D	50,16 ±9,70	47,32 ±3,72	48,52 ±3,87	60,52 ±3,88	55,46 ±3,89	42,54 ±3,90	35,60 ±2,18

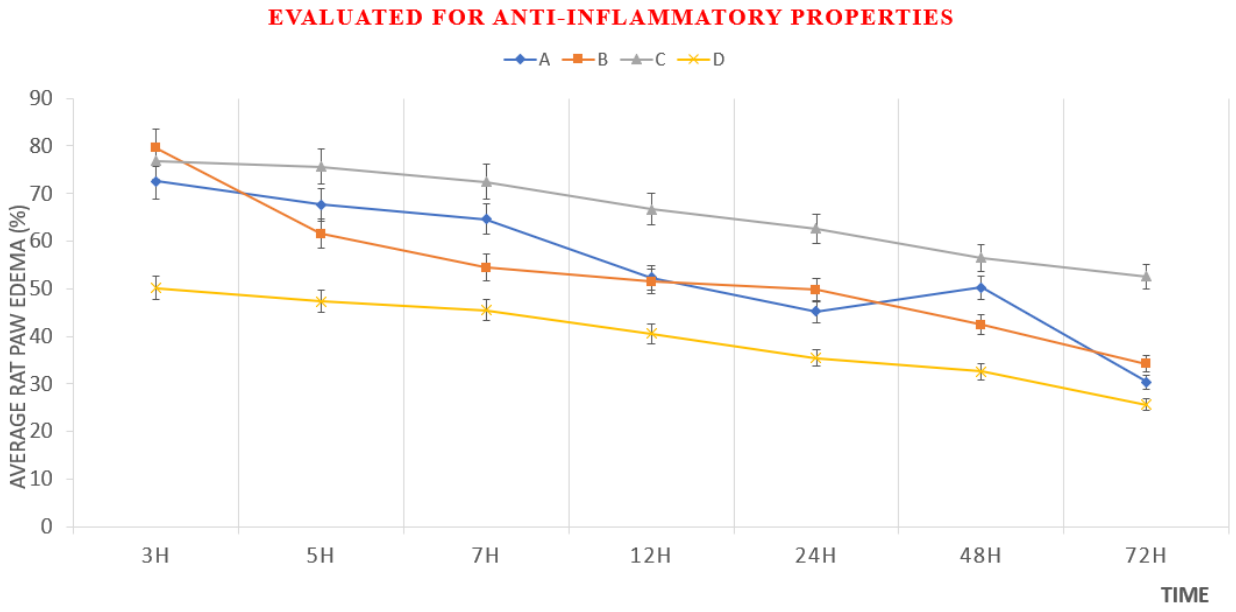


Figure 6. Results of test for anti-inflammatory properties

A. Control group; B. Test group; C. Reference group; D. Comparison

Excipients show no anti-inflammatory action, according to Table 6 and Figure 6. At 3 hours after injection of carrageenan, the comparative medicine indomethacin has a good edema-reducing effect (P<0,05), with an edema reduction impact of 50,16%. However, according to the amount of carrageenan metabolized and removed, the swelling of the mouse's paws steadily

increased after 24 hours and dropped significantly after 72 hours. Thus, the reference medicine provides good anti-inflammatory effects after 3 days of administration.

After 72 hours, the completed product exhibits pretty good anti-inflammatory properties, with a low and constant reduction in rat paw edema at all times. Mouse paw edema has now decreased from 79,80% to 34,12%. The efficiency of edema reduction improved significantly with time, peaking at 42,40% after 48 hours (Table 6). The difference in rat paw edema between the completed product batch and the control batch was statistically significant at $P<0.05$. As a result, the completed substance has a strong anti-inflammatory impact. The throat spray's anti-inflammatory properties will aid in easing cough symptoms. This outcome is in line with earlier investigations into the effects of *Bacillus* spores and ivy leaf extract [3, 4, 10]

4. CONCLUSION

A throat spray comprising *Bacillus* spores and ivy leaf extract has been developed to help prevent and treat sore throats. The best formula was discovered through research with a ratio of 1,65% ivy leaf extract and 5,10% Bacillus spore. Bacillus throat spray provides good anti-inflammatory benefits in mice, according to the results of a rat study. This research will lay the groundwork for future potential topical treatments incorporating Bacillus or other probiotics and appropriate dosing forms and methods. Combining probiotics with medicinal extracts is both safe and effective.

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