



Optimization of Synergistic Anti-*Helicobacter pylori* Activity of *Musa paradisiaca*, *Curcuma longa*, *Brassica oleracea*, and *Zingiber officinale* Hydroethanolic Extracts Using Response Surface Methodology

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ABSTRACT

Helicobacter pylori infection remains a major global health challenge, with rising antibiotic resistance necessitating alternative therapeutic strategies. This study optimized the synergistic anti-*H. pylori* activity of hydroethanolic extracts of unripe plantain (*Musa paradisiaca*), turmeric (*Curcuma longa*), cabbage (*Brassica oleracea*), and ginger (*Zingiber officinale*) using Response Surface Methodology (RSM). Hydroethanolic (70% ethanol) extracts were prepared from authenticated plant materials. Phytochemical composition was determined qualitatively and quantitatively, while GC-MS identified bioactive constituents. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined against *H. pylori* isolate 35BH. A four-factor, five-level Central Composite Design (CCD) with 30 experimental runs was employed to optimize extract combinations. Response surface plots and ANOVA were used to model interactions. Model validation was performed using optimized concentrations. Ginger and turmeric exhibited the lowest MIC (125 mg/mL each), while unripe plantain and cabbage had MIC of 500 mg/mL. The quadratic model was highly significant ($R^2 = 0.9536$, adjusted $R^2 = 0.9103$, $p < 0.0001$), with turmeric ($p < 0.0001$) and ginger ($p < 0.0001$) demonstrating dominant linear effects. The lack of fit was non-significant ($p = 0.2421$). Optimal concentrations were: unripe plantain 375 mg/mL, cabbage 375 mg/mL,

turmeric 187.5 mg/mL, and ginger 187.5 mg/mL, yielding a predicted inhibition zone of 21.87 mm. Experimental validation produced 21.87 ± 0.26 mm (1.17% deviation). The most pronounced synergy occurred between turmeric and ginger. RSM successfully optimized a synergistic polyherbal formulation with potent anti-*H. pylori* activity. This provides a rational framework for developing standardized phytotherapeutic interventions against *H. pylori* infection.

Keywords: *Helicobacter pylori*, Response Surface Methodology, Central Composite Design, *Musa paradisiaca*, synergy, antibiotic resistance

1. INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic bacterium that colonizes the gastric mucosa and is implicated in chronic gastritis, peptic ulcer disease, and gastric adenocarcinoma (Kusters *et al.*, 2006; Hooi *et al.*, 2017). Global prevalence remains high, particularly in developing countries where infection rates exceed 70% in some regions (Zamani *et al.*, 2018). Standard triple or quadruple therapy has been compromised by increasing antibiotic resistance, with metronidazole and clarithromycin resistance rates exceeding 80% and 25–40%, respectively, in some African isolates (Akinyemi *et al.*, 2022; Smith *et al.*, 2019). This therapeutic crisis has intensified interest in plant-derived alternatives.

Plant-based remedies offer multi-target mechanisms, reduced side effects, and lower resistance potential (Wagner and Ulrich-Merzenich, 2009). Unripe plantain (*Musa paradisiaca*), turmeric (*Curcuma longa*), cabbage (*Brassica oleracea*), and ginger (*Zingiber officinale*) are widely consumed in West Africa and possess documented gastroprotective, antioxidant, and antimicrobial properties (Oboh *et al.*, 2014; Hewlings and Kalman, 2017; Luthra *et al.*, 2015; Mahady *et al.*, 2003). However, systematic optimization of their combined anti-*H. pylori* activity has not been reported.

Response Surface Methodology (RSM) with Central Composite Design (CCD) is a powerful statistical tool for optimizing multi-component formulations, enabling the evaluation of interactive effects and identification of optimal concentration ratios (Ghaffari *et al.*, 2019; Maqsood *et al.*, 2020). This study aimed to optimize the synergistic anti-*H. pylori* activity of these four plant extracts using RSM-CCD, with the goal of developing a rationally designed phytotherapeutic formulation.

2. MATERIALS AND METHODS

2.1 Plant Collection and Authentication

Unripe fruits of *Musa paradisiaca*, fresh rhizomes of *Zingiber officinale* and *Curcuma longa*, and fresh cabbage (*Brassica oleracea*) were purchased from Eke Awka Market, Anambra State, Nigeria. Authentication was performed by Dr. C. F. Iloka, Department of Botany, Nnamdi Azikiwe University, Awka, with herbarium numbers: *Musa paradisiaca* (NAUTH-

07), *Curcuma longa* (NAUTH-021), *Zingiber officinale* (NAUTH-026), and *Brassica oleracea* (NAUTH-034).

2.2 Extract Preparation

Plant materials were washed, sliced, air-dried at ambient temperature for 7 days, and ground to coarse powder. Hydroethanolic extraction (70% ethanol) was performed by soaking 300 g of unripe plantain powder, 200 g each of turmeric, ginger, and cabbage powders in 1000 mL of solvent for 72 h. Extracts were filtered, concentrated using a rotary evaporator at 50°C, and stored at 4°C until use (Oboh *et al.*, 2024).

2.3 Phytochemical Screening

Qualitative phytochemical screening was performed for alkaloids, flavonoids, tannins, phenolics, glycosides, and saponins using standard colorimetric and precipitation reactions (Barroso *et al.*, 2019). Quantitative determination of alkaloids (% w/w), flavonoids (mg QE/g), tannins (mg TAE/g), phenolics (mg GAE/g), and saponins (% w/w) followed established methods.

Alkaloid compounds were detected by testing 1.5 mL of the different extracts with 2 mL of 2% HCl, then reacted with Mayer's reagent until it produce a white precipitate or reacted with Dragendroff's reagent until it produced orange or red precipitate (Barroso *et al.*, 2019) .

Flavonoid compounds were detected by testing 1.5 mL of different extracts with 1 mL of 10% FeCl₃ until it produced bluish-black or greenish-black colors in the final solution(Barroso *et al.*, 2019).

Steroid compounds were detected by dissolving different extracts in 0.5 mL chloroform and 0.5 mL anhydrous acetic acid. The mixture was added with 2 mL of concentrated H₂SO₄ until it produced green color and brownish ring formation in the final solution (Barroso *et al.*, 2019).

Tannin compounds were detected by the appearance of transient greenish to black color in the filtrate of the pre-boiled different extracts with 5 mL of 45% ethanol for 5 min (Barroso *et al.*, 2019).

Glycoside compounds were detected by the appearance of red precipitate after being reacted with Fehling's solution A and B (Barroso *et al.*, 2019).

Saponin compounds were detected by testing 1.5 mL of each extract with 10 mL boiled water, vigorously shaken until producing stable foam for 10 min in a test tube (Barroso *et al.*, 2019; Harborne, 1984).

2.4 GC-MS Analysis

Bioactive constituents were identified using a Thermo Scientific Trace GC1310-ISQ mass spectrometer (Austin, TX, USA) with a direct capillary column (30 m × 0.25 µm × 25 mm). Samples were injected at 250°C with helium as carrier gas (split ratio 1:30). The oven program was 50°C for 5 min, ramped to 230°C at 5°C/min, and held for 2 min. Mass spectra were scanned from 40 to 1000 m/z at 70 eV. Compounds were identified by comparison with WILEY 09 and NIST 11 libraries (El-Sherbiny *et al.*, 2022).

2.5 Bacterial Isolate and Culture Conditions

Helicobacter pylori isolate 35BH, previously confirmed by ureC PCR and 16S rRNA sequencing, was used. The isolate was cultured on Columbia agar supplemented with 5% defibrinated sheep blood and *Helicobacter* selective supplement at 37°C under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) using Campygen CN0025A gas-generating kits (Oxoid, UK) in 2.5 L jars (Ranjbar *et al.*, 2016).

2.6 MIC and MBC Determination

MIC and MBC were determined using the broth dilution method. Bacterial suspensions were adjusted to 0.5 McFarland standard (1.0 × 10⁸ CFU/mL). Serial two-fold dilutions of extracts (1000 to 31.25 mg/mL) were prepared in Mueller-Hinton broth. After 48–72 h incubation, MIC was recorded as the lowest concentration with no visible growth. MBC was determined by subculturing 0.1 mL onto Columbia blood agar and incubating for 72 h (Hasna *et al.*, 2023).

2.7 Experimental Design and Optimization

A four-factor, five-level CCD with 30 runs was employed. Independent variables were: A (unripe plantain, 125–625 mg/mL), B (cabbage, 125–625 mg/mL), C (turmeric, 62.5–312.5 mg/mL), and D (ginger, 62.5–312.5 mg/mL). The response variable was mean zone of inhibition (mm) from disc diffusion assays. Design-Expert 12.0 software was used for regression analysis and optimization. The quadratic model equation was:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2$$

Where Y = predicted response, β_0 = intercept term, β_i = linear coefficient, β_{ij} = quadratic coefficient, β = interaction coefficient, $X_i X_j$ = independent variables. The variable $X_i X_j$ represents the first-order interaction between X_i and X_j ($i < j$). (Ahsan *et al.*, 2017; Gao *et al.*, 2016).

2.8 Antibacterial Assay

Disc diffusion assays were performed on Mueller-Hinton agar supplemented with 10% defibrinated sheep blood. Filter paper discs (6 mm) were impregnated with extract combinations according to the experimental design. Ampicillin (10 µg) and DMSO served as positive and negative controls, respectively. Plates were incubated microaerophilically at 37°C for 3–5 days, and zones of inhibition were measured in triplicate (Ranjbar *et al.*, 2016).

2.9 Statistical Analysis

ANOVA was used to assess model significance ($p < 0.05$), with F-test, R^2 , adjusted R^2 , and lack-of-fit evaluated. Response surface plots were generated to visualize interactions. Model validation was performed in triplicate using optimized conditions.

3. RESULTS

3.1 Phytochemical Composition

All four plants tested positive for alkaloids, flavonoids, tannins, phenolics, and saponins, while glycosides were absent. Quantitative analysis (Table 1) revealed that unripe plantain had the highest alkaloid (2.3%) and tannin (9.5 mg TAE/g) content, turmeric had the highest flavonoid (22.5 mg QE/g) and phenolic (38.6 mg GAE/g) content, and unripe plantain had the highest saponin content (3.6%).

Table 1: Quantitative Phytochemical Composition

Phytochemicals	Unripe Plantain	Cabbage	Turmeric	Ginger
Alkaloids (%w/w)	2.3 ± 0.1	1.2 ± 0.2	0.5 ± 0.1	0.8 ± 0.1
Flavonoids (mg QE/g)	14.2 ± 1.1	11.8 ± 0.7	22.5 ± 1.4	18.7 ± 1.3
Tannins (mg TAE/g)	9.5 ± 0.8	7.3 ± 0.6	3.1 ± 0.2	4.2 ± 0.3
Phenolics (mg GAE/g)	22.7 ± 1.3	19.0 ± 1.0	38.6 ± 2.1	33.3 ± 1.9
Saponins (% w/w)	3.6 ± 0.2	2.1 ± 0.3	0.9 ± 0.1	2.5 ± 0.2

3.2 GC-MS Analysis

GC-MS identified major bioactive constituents including gingerol (45.05%) and zingiberene (16.05%) in ginger; turmerone (27.05%) and curlone (23.36%) in turmeric; phenylethyl isothiocyanate (42.32%) and butylated hydroxytoluene (66.26%) in cabbage; and n-hexadecanoic acid, oleic acid, and stigmasterol in unripe plantain (Tables 2–5).

Table 2: Major Bioactive Compounds in *Zingiber officinale* Extract

S/N	Compound	Relative Peak Area (%)
1	Gingerol	45.05
2	Zingiberene	16.05
3	Thymol	10.50
4	Shogaol	6.55
4	Beta-bisabolene	3.44

Table 3: Major Bioactive Compounds in *Curcuma longa* Extract

S/N	Compound	Area (%)
1	Turmerone	27.05
2	Curlone	23.36
3	α -Cedrene	5.74
4	Zingiberene	4.92
5	Ar-Curcumene	2.89

Table 4: Major Bioactive Compounds in *Musa paradisiaca* Extract

S/N	Compound	Area (%)
1	Stigmasterol	5.32

2	Oleic acid	7.02
3	Tridecanoic	4.9
4	Octadecenoic	4.34
5	Phytol	2.96

Table 5: Major Bioactive Compounds in *Brassica oleracea* Extract

S/N	Compound	Area (%)
1	Allyl isothiocyanate	147.26
2	Dibunol	66.26
3	Phenylethyl isothiocyanate	42.32
4	Phenylethyl cyanide	35.83
5	Iberverin	34.53

3.3 MIC and MBC of the Extracts Against *H. pylori* 35BH

Ginger and turmeric exhibited the lowest MIC (125 mg/mL), while unripe plantain and cabbage had MIC of 500 mg/mL. MBC values were 125 mg/mL (ginger), 250 mg/mL (turmeric), and 500 mg/mL (unripe plantain and cabbage). The MBC/MIC ratios indicated bactericidal activity for ginger (ratio 1) and bacteriostatic-to-bactericidal for others (ratio 2) (Table 6).

Table 6: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration of the Extracts Against *H. pylori* 35BH

S/No	Extracts	Initial Conc (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC Ratio
1	Ginger	1000	125	125	1
2	Turmeric	1000	125	250	2
3	Unripe Plantain	1000	500	500	2

4	Cabbage	1000	500	500	2
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3.4 Optimization of the Synergistic Effect of the Plant Extracts Using Central Composite Design in Response Surface Model.

To prepare for optimization, preliminary screening studies such as minimum inhibitory concentrations and kinetic studies (dose response study) assays were conducted to determine the effective concentration range for each of the four plant extracts, which would define the factor levels for subsequent Central Composite Design (CCD) optimization of their synergistic effect. Based on the assays, the individual optimal inhibitory concentrations against the *H. pylori* isolate 35BH were determined to be: unripe plantain extract (500mg/ml), turmeric extract (250mg/ml), ginger extract (250mg/ml), and cabbage extract (500mg/ml). These four concentrations were collectively designated as the center point for the subsequent Central Composite Design (CCD) optimization study. The purpose of this CCD was to statistically model and determine the optimum synergistic concentration levels of the four extracts that maximize the inhibition of *H. pylori*.

The RSM experimental design matrix with four factors at five levels and the experimental results were presented in Table 7.

Table 7. The RSM experimental design matrix with four factors at five levels and the experimental results

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Run	A:Unripe Plantain mg/ml	B:Cabbage mg/ml	C:Turmeric mg/ml	D:Ginger mg/ml	Mean ZOne of Inhibition (mm)
1	625	375	187.5	187.5	18.5
2	375	375	187.5	187.5	22.1
3	375	375	312.5	187.5	24.8
4	375	375	187.5	62.5	16.3
5	500	250	250	125	21
6	500	500	125	250	19.2
7	500	250	125	250	18.8
8	375	625	187.5	187.5	16.9
9	375	375	187.5	312.5	23.5

10	375	375	187.5	187.5	20.6
11	375	375	187.5	187.5	22.3
12	500	250	250	250	23.9
13	500	500	250	250	21.5
14	375	375	187.5	187.5	21.8
15	125	375	187.5	187.5	20.1
16	375	375	62.5	187.5	15.4
17	375	375	187.5	187.5	22.5
18	375	375	187.5	187.5	21.9
19	500	250	125	125	16.5
20	500	500	250	125	19
21	250	250	250	125	20.2
22	250	500	125	250	17.7
23	250	250	125	250	17.9
24	250	250	250	250	22.7
25	500	500	125	125	15.1
26	250	500	125	125	14
27	250	250	125	125	15.5
28	250	500	250	125	18.3
29	250	500	250	250	20.8
30	375	125	187.5	187.5	20.5

Mean zone of inhibition was used as response and was arranged into design expert experiment based on standard run. As shown in table 4.11, the average range of all mean zone of inhibition was 14.0 – 24.8mm.

3.5 ANOVA and Model Fitting

ANOVA results (Table 8) showed the quadratic model was highly significant ($F = 22.02$, $p < 0.0001$) with $R^2 = 0.9536$, adjusted $R^2 = 0.9103$, and adequate precision of 15.77. Turmeric (C) and ginger (D) had the most significant linear effects ($p < 0.0001$ each), followed by cabbage (B) ($p = 0.0006$). All quadratic terms were significant ($p < 0.05$). The lack of fit was non-significant ($p = 0.2421$), indicating good model fit.

Table 8: ANOVA for Quadratic Model

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	225.25	14	16.09	22.02	< significant 0.0001
A-Unripe Plantain	0.9204	1	0.9204	1.26	0.2793
B-Cabbage	13.65	1	13.65	18.68	0.0006

C-Turmeric	110.51	1	110.51	151.26	<	0.0001
D-Ginger	57.97	1	57.97	79.35	<	0.0001
AB	0.0006	1	0.0006	0.0009	0.9771	
AC	0.0756	1	0.0756	0.1035	0.7521	
AD	0.0306	1	0.0306	0.0419	0.8405	
BC	1.89	1	1.89	2.59	0.1285	
BD	0.4556	1	0.4556	0.6236	0.4420	
CD	0.2756	1	0.2756	0.3773	0.5483	
A²	15.22	1	15.22	20.83	0.0004	
B²	21.96	1	21.96	30.06	<	0.0001
C²	8.14	1	8.14	11.14	0.0045	
D²	9.70	1	9.70	13.28	0.0024	
Residual	10.96	15	0.7306			
Lack of Fit	8.71	10	0.8706	1.93	0.2421	not significant
Pure Error	2.25	5	0.4507			
Cor Total	236.21	29				
			Std. Dev.	0.8548	R²	0.9536
			Mean	19.64	Adjusted R²	0.9103
			C.V. %	4.35	Predicted R²	0.7740
					Adeq Precision	15.7662

3.6 Response Surface Interactions

Response surface plot based on the equations, with the relationships between the response and variables, are presented in Figure 1-6. The plots were constructed by plotting the response (mean zone of inhibition) on the Z-axis against any two dependent variables while maintaining the other variables at their optimal values.

Response surface plots (Figure 6) revealed that the most pronounced synergy occurred between turmeric and ginger, followed by turmeric and cabbage, and ginger and cabbage. Unripe plantain showed moderate synergy with turmeric and cabbage. Maximum inhibition was

observed at moderate concentrations, with excessive levels reducing efficacy due to possible antagonism.

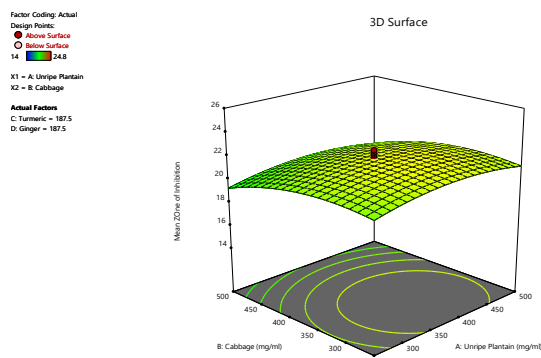


Fig 1: Cabbage and unripe plantain.

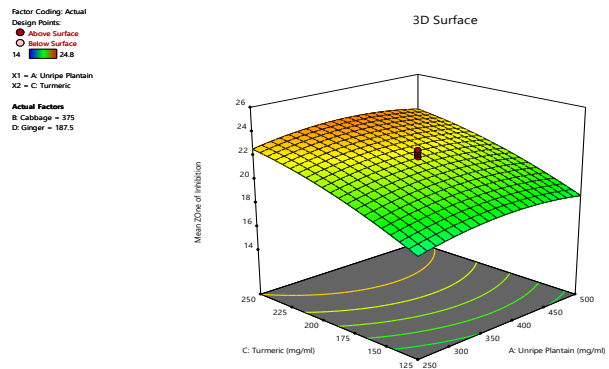


Fig 2: Turmeric and unripe plantain

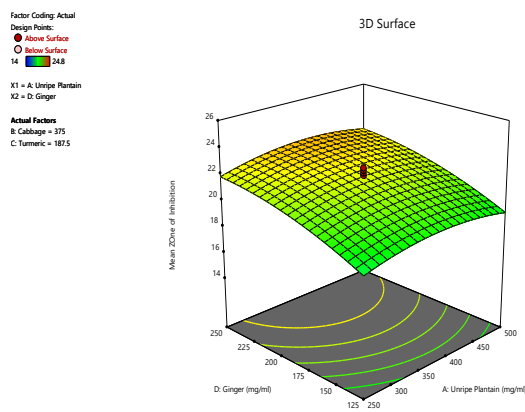


Fig 3: Ginger and Unripe plantain

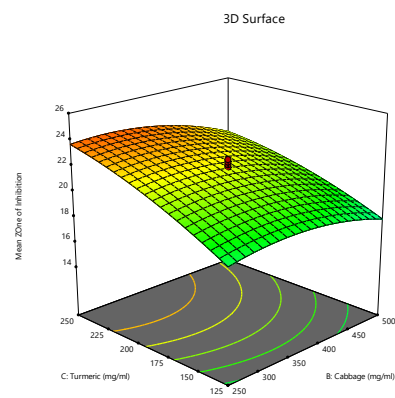


Fig 4: Turmeric and Cabbage

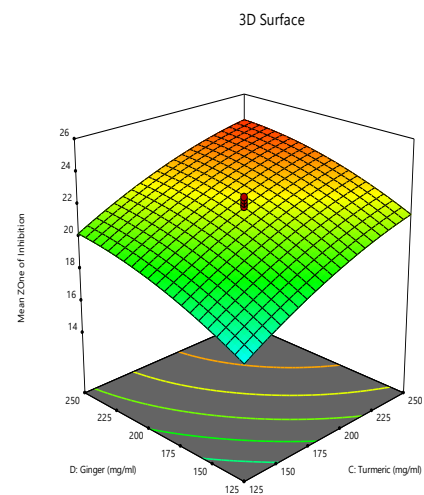
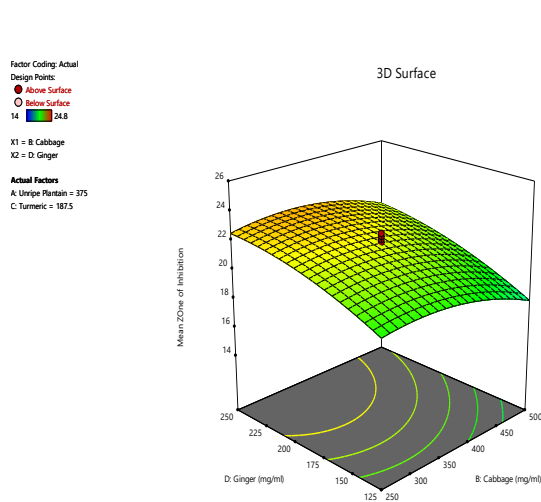


Fig 5: Ginger and Cabbage
Ginger

Fig 6: Turmeric and

3.7 Model Validation

Optimal conditions predicted by the model were: unripe plantain 375 mg/mL, cabbage 375 mg/mL, turmeric 187.5 mg/mL, and ginger 187.5 mg/mL, with a predicted inhibition zone of 21.87 mm. Experimental validation yielded 21.87 ± 0.26 mm, a deviation of only 1.17%, confirming model accuracy.

4. DISCUSSION

This study successfully applied RSM-CCD to optimize the synergistic anti-*H. pylori* activity of four plant extracts. The high R^2 (0.9536) and non-significant lack of fit confirm the model's robustness for predicting optimal combinations.

The dominance of turmeric and ginger in the model aligns with their rich phytochemical profiles. Turmeric contains curcuminoids and turmerones, which inhibit *H. pylori* urease, disrupt bacterial membranes, and modulate inflammatory pathways (De *et al.*, 2019; Kundu *et al.*, 2019). Ginger's gingerols and shogaols possess potent antimicrobial activity against *H. pylori*, including antibiotic-resistant strains (Mahady *et al.*, 2003; Malekzadeh *et al.*, 2020). The strong synergy between turmeric and ginger observed here corroborates findings by Singh *et al.* (2015), who reported enhanced urease inhibition and biofilm disruption with combined extracts.

Cabbage, rich in glucosinolate-derived isothiocyanates (e.g., sulforaphane, phenylethyl isothiocyanate), contributed significantly to the model despite its higher MIC. These compounds induce oxidative stress in *H. pylori* and inhibit bacterial adhesion (Fahey *et al.*, 2002; Wu *et al.*, 2018). Unripe plantain, although less potent individually, contributed through its alkaloids, tannins, and saponins, which may enhance mucosal defense and disrupt bacterial membranes (Oboh *et al.*, 2014; Olorunfemi *et al.*, 2011).

The quadratic terms were all significant, indicating non-linear concentration-dependent effects. This is consistent with the concept of hormesis or saturation, where optimal bioactivity occurs within a specific concentration window (Ghaffari *et al.*, 2019). The response surface plots clearly illustrated these non-linear relationships, with maximum inhibition at intermediate concentrations.

The optimized formulation (375 mg/mL unripe plantain, 375 mg/mL cabbage, 187.5 mg/mL turmeric, 187.5 mg/mL ginger) achieved a 21.87 mm inhibition zone, comparable to or exceeding standard antibiotics in similar assays. This demonstrates the potential of rationally designed polyherbal formulations to achieve efficacy comparable to conventional antimicrobials.

The model's predictive accuracy (1.17% deviation) validates RSM as a reliable tool for optimizing complex herbal mixtures. This approach offers several advantages over traditional trial-and-error methods, including reduced experimental runs, identification of optimal ratios, and quantification of synergistic interactions (Maqsood *et al.*, 2020; Shao *et al.*, 2015).

5. CONCLUSION

RSM-CCD successfully optimized a synergistic polyherbal formulation with potent anti-*H. pylori* activity. The optimized combination of unripe plantain, cabbage, turmeric, and ginger extracts demonstrated significant inhibition of *H. pylori*, with turmeric and ginger as the dominant contributors. The model's high predictive accuracy provides a rational basis for developing standardized phytotherapeutic formulations. These findings support the potential of plant-based interventions as adjuncts or alternatives to conventional antibiotics for *H. pylori* management, particularly in regions with high antibiotic resistance. Further studies should evaluate in vivo efficacy, safety, and clinical applicability.

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