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Evaluation of the antimicrobial potential of *Astragalus fasciculifolius* gum extract against *Clostridium perfringens* in meatball formulations using response surface methodology

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ABSTRACT

Astragalus fasciculifolius Boiss is one of the native medicinal plants of Iran that has a special place in Iranian medicine. We investigated the phenolic compounds profile of ethanolic gum extracts, antimicrobial activity (MIC and MBC), and modeling and optimization of Clostridium perfringens growth dynamics in meat matrices. The results showed that the highest phenolic composition in the ethanolic extract was hesperidin (17.61%). Ethanolic *A. fasciculifolius* gum extract had antimicrobial activity. The MIC and MBC of Clostridium perfringens were reported as 156 and 78 (mg/g extract). The ethanolic gum extract caused shrinkage and changes in bacterial membranes. Dynamic modeling of bacterial growth in the meat matrix in the presence of the ethanolic *A. fasciculifolius* gum extract was performed as a quadratic equation. It was found that the lowest number of bacteria would be observed at 7200.8 ppm of extract, a storage time of 14.29 hours, and a storage temperature of 4°C. This study showed that *A. fasciculifolius* gum has important active ingredients that can be used in the food, cosmetics, and drug industries.

1. Introduction

Maintaining food safety and quality throughout its life has attracted the attention of food industry experts and national health officials, and neglect or insufficient attention to this issue could lead to irreparable harm to society (1). Diseases caused by eating contaminated food are a significant problem worldwide. Therefore, the need to reduce or eliminate food pathogens using new methods is quite noticeable (2). Meat is a food with high perishability. Various factors, such as storage temperature, the amount of oxygen in the atmosphere, internal enzymes, light, moisture, and especially microorganisms, are involved in its spoilage (2). Predictive microbiology models the growth, survival, and death responses of important microbes in food by considering the primary and influential controlling factors. If mathematical models for a wide range of foods are developed, the need for specific and case-specific microbiological tests for new foods will be significantly reduced (3). Therefore, modeling the growth response of pathogenic bacteria in the food matrix under the influence of natural compounds can be substantial; consumers welcome natural preservatives, including natural compounds that can be used as preservatives in food, plant extracts, and essential oils. These compounds have antibacterial, antifungal, antioxidant, and anti-cancer properties and can control the growth of pathogens and the production of toxins by microorganisms (4). The World Health Organization says that nearly 80% of people use herbal medicines for primary and preventive health care (5). In addition, more than 50% of newly approved drugs are derived directly from modified herbs or their active ingredients (6). *Astragalus fasciculifolius* Boiss belongs to the legume family and is also used in traditional Iranian medicine (7). This plant has anti-inflammatory, anti-viral, anti-diabetic, anti-cancer, and anti-poisoning effects due to its compounds such as flavonoids; phthalides are another group of effective compounds of this plant, which is considered a dietary supplement and a chemical preventive agent against cancer and gastric wounds and protects the liver (8). This study aimed

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to investigate the antimicrobial properties of the ethanolic gum extract of *A. fasciculifolius*. Also, the growth dynamics of *Clostridium perfringens* bacteria in meatballs in the presence of the ethanolic extract of *A. fasciculifolius* were modeled and optimized using Design-Expert software.

2. Materials and Methods

2.1. Materials

Astragalus fasciculifolius was collected from the cultivation pastures of this plant in the north of Hormozgan-Iran (25° 24′ 28°.53″N 52° 44′ 59.14″E), and a Botanist from Hormozgan University approved the genus and species of this plant. Plants were harvested on 18 July 2020, and the root, aerial parts, and gum of the plant were separated; after that, the *A. fasciculifolius* were cleaned and dried in the shade at room temperature with proper airflow for 72 h and were ground into adequate powder particle size using an industrial mill. The plants were stored in the refrigerator at 4 to 6 °C.

2.2. Preparation and extraction of A. fasciculifolius extract

For ethanolic extraction, ethanol was used as solvent at a rate of 1:20 (solids: solvent) for 24 hours by the maceration method, and the remaining organic solvents were evaporated using an evaporator (9). The extracts were labeled and kept at 2-8 °C until needed.

2.3. Phenolic profile in the ethanolic extract

The resulting extract was diluted with hexane and injected into a gas chromatograph (GC) model (Hewlett Packard-Sunnyvale, USA-GC 5890 Series II). The most suitable thermal programming was performed to separate the components of the extract. The chromatography device was equipped with a BPX5 column (column length: 30 m, internal diameter: 0.33, and stationary phase thickness: 0.25 µm). The extract's constituents were identified by comparing the standard compounds' mass spectra and inhibition indices using the Wiley 275. L database in the GC/MS device. The relative percentage of each extract constituent was obtained according to their sub-curved surface in the relevant chromatogram (10).

2.4. Antimicrobial efficacy: MIC and MBC determination

The dilution broth method was used to determine the MIC. 12 test tubes were used to determine the MIC for ethanolic *A. fasciculifolius* gum extract. From 1 mg/ml to 625 mg/ml, 5 µl of the prepared bacterial suspension was added to each ethanolic extract concentration. The tubes were incubated at 37°C for 24 hours. After 24 hours, the tubes were examined for turbidity due to the growth of inoculated bacteria. To estimate the MIC, the first concentration in which microbial growth did not occur and was not seen was recorded. All the cultures that showed no visible growth were cultured on media and incubated at 37 °C for 24 h. The MBC was taken as the concentration that kills all bacterial cells cultured and prevents the growth of any bacterial colony on media (3).

2.5. Preparation of meatball samples and microbial counting

The meat and fat were ground with 1.5% salt using a meat grinder with a blade diameter of 3 mm. For 1000 g of beef, 200 g of fat, 15 g of salt, and 50 g of bread were mixed and ground again (11). Then, they were heat-treated with 80°C water vapor for two hours until the center temperature reached 72°C. Approximately 10^{10} CFU/g *Cl. perfringens* (activated according to the manufacturer's instructions) were inoculated separately into meatballs. Ethanolic *A. fasciculifolius* extract was injected into the meatball samples at concentrations of 0 to 8000 ppm, and the samples were stored at 4 to 12°C and examined for 1 to 96 hours. The proposed tests of Design-Expert software with the Central Composite method were designed (file version: 12.0.3.0).

2.6. Microbial count

Clostridium perfringens bacteria count in meatballs Sulfite polymyxin sulfadiazine agar was the medium for the growth of *Cl. perfringens*. The plates were incubated for 37 h at 37°C, and the colony counter was used for counting (12).

2.7. Data analysis

The phenolic compounds analysis: All experiments were performed in triplicate. Average results were reported as the mean and standard error, and SPSS (Version 18.0, SPSS Inc., Chicago, USA) statistical software, one-way ANOVA followed by Duncan's multiple range test, were used. Optimization and modeling of the microbial population of *Cl. perfringens* with the extract concentration, time, and storage temperature were evaluated by Design-Expert software for RSM design and the Central Composite method. Analysis of variance was performed at a 95% confidence level.

3. Results

3.1. Modeling of samples

Table 1 presents the critical variables employed in the response surface methodology (RSM) modeling for optimizing the growth dynamics of Clostridium perfringens in meatballs. Three key factors were investigated: Factor A: Concentration of A. fasciculifolius extract, ranging from 0 to 8000 ppm, allowing for assessment of the extract's antimicrobial efficacy across various concentrations; Factor B: Storage time, spanning from 12 to 96 hours, enabling evaluation of both short-term and extended storage effects on microbial growth; and Factor C: Storage temperature, varying between 4°C and 12°C, covering typical refrigeration temperatures and slightly elevated temperatures that might occur during storage or handling. These factors were chosen to comprehensively analyze the impact of extract concentration,

time, and temperature on *C. perfringens* growth in meatballs. The RSM approach allows for the examination of potential interactions between these variables and their combined effects on microbial proliferation. This range covers typical refrigeration temperatures and slightly elevated conditions, allowing for assessing temperature abuse scenarios. The table meticulously details each factor's minimum and maximum values alongside their coded representations (-1 to +1) used in the experimental design. This coding facilitates standardized comparison across factors with different units and magnitudes. The comprehensive range of each factor ensures a thorough exploration of the experimental space, enabling robust modeling and optimization of the bacterial growth dynamics under various conditions.

Table 1. The input factors for modeling and optimization.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High
A	Cons. Extract	ppm	Numeric	0.0000	8000.00	-1 ↔ 0.00	+1 ↔ 8000.00
B	Time	hr.	Numeric	12.00	96.00	-1 ↔ 12.00	+1 ↔ 96.00
C	Temperature	degree centigrade	Numeric	4.00	12.00	-1 ↔ 4.00	+1 ↔ 12.00

3.2. Phenolic profile of A. fasciculifolius gum extract

The phytochemical analysis of the *A. fasciculifolius* ethanolic extract revealed substantial quantities of bioactive compounds. Specifically, the total phenol content was determined to be 24.39 ± 1.2 mg GAE/g (milligrams of gallic acid equivalents per gram of extract), indicating a high concentration of phenolic compounds. Additionally, the extract exhibited a notable total flavonoid content of 15.12 ± 0.8 mg RUE/g (milligrams of rutin equivalents per gram of extract). These measurements, utilizing standardized equivalents for quantification, clearly indicate the extract's rich phytochemical profile. The relatively high values for phenolic and flavonoid contents suggest that the *A. fasciculifolius* extract is a potent source of bioactive compounds, which may contribute to its potential therapeutic and antimicrobial properties. Table 2 offers a more detailed breakdown of the specific phenolic compounds detected by GC analysis. Notably, hesperidin emerged as the most abundant compound, constituting 17.615% of the total phenolic content.

Table 2. Comparison of phenolic compounds identified in ethanolic extracts.

Compound	Amount	Percentage (%)
Hesperidin	17.615±0.004	20.76%
p-Coumaric acid	11.953±0.005	14.08%
Quinic acid	8.781±0.009	10.34%
Rosmarinic acid	8.333±0.006	9.82%
Chrysin	7.202±0.015	8.48%
Caffeic acid	6.026±0.005	7.10%
Aconitic acid	4.333±0.009	5.10%
Salicylic acid	3.834±0.007	4.52%
Vanillin	3.212±0.006	3.78%
Rutin	2.665±0.110	3.14%
Gallic acid	2.428±0.005	2.86%
Hyperoside	2.428±0.020	2.86%
Protocatechuic acid	2.122±0.002	2.50%
Quercetin	2.354±0.577	2.77%
Chlorogenic acid	1.857±0.020	2.19%
Malic acid	1.380±0.01	1.63%
Tannic acid	1.311±0.001	1.54%
Luteolin	0.00±0.00	0%
Kaempferol	0.00±0.00	0%
Apigenin	0.00±0.00	0%
Total	**84.834**	**100%**

Other major compounds included p-coumaric acid (11.953%), quinic acid (8.781%), and rosmarinic acid (8.333%). Interestingly, some common flavonoids such as luteolin, kaempferol, and apigenin were not detected. In total, 20 different phenolic compounds were identified and quantified. The high hesperidin content is noteworthy due to its well-documented antioxidant and anti-inflammatory properties. This diverse phenolic profile likely contributes significantly to the extract's overall bioactive effects, underscoring the potential of *A. fasciculifolius* as a source of natural bioactive compounds.

3.3. Investigation of MIC and MBC

The MIC, the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism, was found to be 78 mg/ml for *C. perfringens*. The MBC, defined as the lowest concentration of an antimicrobial agent required to kill a particular bacterium, was determined to be 156 mg/ml. These results indicate that the *A. fasciculifolius* gum extract exhibits significant antimicrobial activity against *C. perfringens*, with bacteriostatic effects observed at lower concentrations and bactericidal effects at higher concentrations.

3.4. Modeling bacterial growth dynamics in meatballs

Table 3 outlines the experimental design and results for modeling the growth dynamics of *C. perfringens* in meatballs treated with *A. fasciculifolius* gum extract. The table presents 20 experimental runs, systematically varying three factors: extract concentration (0-8000 ppm), storage time (12-96 hours), and storage temperature (4-12°C). The response variable is the log count of *C. perfringens*. Notable observations include lower bacterial counts at higher extract concentrations, shorter storage times, and lower temperatures. For instance, run 6 (8000 ppm extract, 12 hours, 12°C) resulted in a low bacterial count of 3.5 log CFU, while run 7 (0 ppm extract, 96 hours, 12°C) showed the highest count of 10.5 log CFU.

3.5. Growth dynamics of Clostridium perfringens

The growth of *C. perfringens* and *Pseudomonas aeruginosa* was analyzed using a quadratic model, with bacterial counts transformed to a logarithmic scale. An analysis of variance (ANOVA) was conducted to assess the significance of independent variables and their interactions. Multivariate

correlation analysis yielded coefficients for the final equation. Variables not significantly affecting the response were excluded to refine the model. The model's performance was evaluated based on lack of fit values, R^2 and adjusted R^2 coefficients, and the model's p-value. Table 4 presents a comprehensive ANOVA for the quadratic model describing *C. perfringens* growth dynamics. The model demonstrates high significance ($p<0.0001$) with an R^2 value of 0.9840, indicating excellent fit. All linear terms (A, B, C) and some interaction terms (AB, AC) are significant ($p<0.05$). Extract concentration (A) exhibits the most substantial effect (F-value = 461.88), followed by storage time (B) and temperature (C). The significant quadratic term A^2 suggests a non-linear relationship between extract concentration and bacterial growth. The non-significant lack of fit (p=0.2496) further validates the model's adequacy. High adjusted R^2 (0.9696) and predicted R^2 (0.9095) values, coupled with an adequate precision of 29.6061, demonstrate the model's robustness and predictive power for *C. perfringens* growth under various conditions in the presence of *A. fasciculifolius* gum extract.

Table 3. Experimental tests and results.

Std	Run	Factor 1 A: Cons. Extract ppm	Factor 2 B: Time	Factor 3 C: Temperature	Response 2 *Cl. perfringens*
16	1	4000	54	8	5
20	2	4000	54	8	5.6
19	3	4000	54	8	5.3
15	4	4000	54	8	5.1
12	5	4000	96	8	5.6
13	6	4000	54	4	4.8
5	7	0	12	12	7.2
6	8	8000	12	12	3.5
10	9	8000	54	8	4.2
3	10	0	96	4	8.9
9	11	0	54	8	9.1
17	12	4000	54	8	5.1
11	13	4000	12	8	4.8
7	14	0	96	12	10.5
2	15	8000	12	4	3.5
1	16	0	12	4	6.3
8	17	8000	96	12	4.3
18	18	4000	54	8	4.8
4	19	8000	96	4	4.1
14	20	4000	54	12	5.1

Table 4. Results of analysis of variance of dynamic growth of *Clostridium perfringens* bacteria.

Source	Sum of Squares	Df.	Mean Square	F-value	p-value
Model: Quadratic	66.92	9	7.44	68.45	< 0.0001
A-Cons. Extract	50.18	1	50.18	461.88	< 0.0001
B-Time	6.56	1	6.56	60.40	< 0.0001
C-Temperature	0.9000	1	0.9000	8.28	0.0164
AB	2.53	1	2.53	23.30	0.0007
AC	0.6613	1	0.6613	6.09	0.0333
BC	0.1013	1	0.1013	0.9320	0.3571
A^2	4.88	1	4.88	44.90	< 0.0001
B^2	0.0384	1	0.0384	0.3536	0.5653
C^2	0.3728	1	0.3728	3.43	0.0937
Residual	1.09	10	0.1086		
Lack of Fit	0.7113	5	0.1423	1.90	0.2496
Pure Error	0.3750	5	0.0750		
Cor Total	68.01	19			
Std. Dev.	0.329		R^2	0.9840	
Mean	5.46		Adjusted R^2	0.9696	
CV %	5.84		Predicted R^2	0.9095	
			Adeq. Precision	29.6061	

3.6. Factors influencing C. perfringens growth in meatballs

The study investigated the combined effects of *A. fasciculifolius* gum ethanolic extract concentration, storage time, and storage temperature on *Clostridium perfringens* growth in meatball samples. Results (Fig. 1) revealed a significant inverse relationship between extract concentration and bacterial growth ($p\leq0.05$). Higher extract concentrations, particularly at 6000 ppm and above, showed a marked inhibitory effect on *C. perfringens* proliferation, suggesting a potential threshold for optimal antimicrobial activity. Storage duration positively correlated with bacterial growth, with peak counts observed after 96 hours, highlighting the critical role of storage time in food safety. Storage temperature significantly

influenced bacterial proliferation ($p \leq 0.05$), with lower temperatures suppressing growth. Minimum growth occurred at 4°C, emphasizing the importance of proper refrigeration in pathogen control. A quadratic equation was developed to model these relationships, incorporating interaction terms and a quadratic term, indicating complex, non-linear interactions between variables. After removing ineffective variables, the final model was expressed as:

$$Log\ (C.\ perfringens\ count) = 4.47 - 0.009A + 0.034B + 0.47C - 0.00005AB - 0.0001AC + 8.32A^2$$

where, A=Extract concentration (ppm), B=Storage time (hours), C=Storage temperature (°C).

The model demonstrated high adequacy based on fitted coefficients, providing a reliable tool for predicting *C. perfringens* growth under various conditions. This comprehensive analysis offers valuable insights for developing effective strategies to enhance food safety and extend the shelf life of meat products.

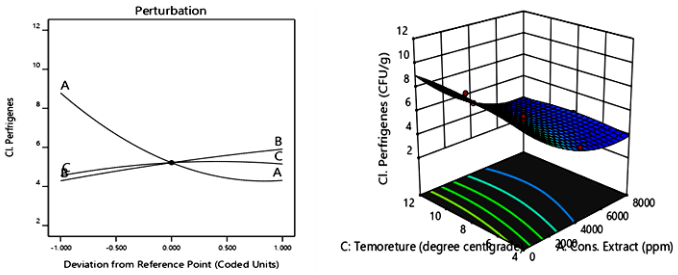

Fig. 1. Effect of ethanolic extract concentration of *A. fasciculifolius* gum (A), storage time (B), and storage temperature (C) on the growth of *Clostridium perfringens.*

4. Discussion

The ethanolic extract of *A. fasciculifolius* demonstrated significant total phenol and flavonoid content, aligning with similar research findings. Hadzri et al. (13) showed that extraction of *Phyllanthus niruri* by Soxhlet using methanol as solvent had the highest yield, followed by ethanol, ethyl acetate, and hexane. This trend in solvent efficacy is consistent with our findings and highlights the importance of solvent selection in phytochemical extraction. Do et al. (14) reported that the amounts of phenolic compounds in total aqueous extract were significantly lower than in other solvents, with extraction efficiency decreasing as the water content in the solvent increased, except for the methanol system. This phenomenon may be attributed to the increased extraction of non-polar compounds, such as carbohydrates and terpenes, in aqueous extracts. Additionally, the formation of complexes between certain phenolic compounds and methanol, acetone, or ethanol could contribute to this trend. These findings underscore the complex interplay between solvent polarity and phytochemical extraction efficiency. Our study identified distinct phenolic compound profiles across different extracts: p-coumaric acid predominated in methanolic extract, hesperidin in ethanolic extract, and salicylic acid in aqueous extract. This variability in phenolic profiles emphasizes the importance of solvent selection in targeting specific bioactive compounds. Similarly, Lekmine et al. (15) identified 17 phenolic compounds in the ethanolic extract of *Astragalus gombiformis* aerial parts with crasiliol. silymarin, quercetin, and kaempferol being the most abundant. This diversity in phenolic profiles across Astragalus species suggests potential for varied biological activities. Wang et al. (16) on *Astragalus membranaceus* root extract revealed a rich profile of isoflavonoids and saponins, which exhibited significant antioxidant and anti-inflammatory properties. This finding supports the potential health benefits of Astragalus species and warrants further investigation into the bioactivities of *A. fasciculifolius* extracts. The antimicrobial activity of medicinal plants is typically associated with phenolic compounds containing hydroxyl (-OH) groups. These groups can interact with microbial enzymes, inhibiting their metabolism (16). Additionally, phenolic compounds may bind to phospholipids in cell membranes, reducing selective permeability and disrupting cellular functions. This mechanism leads to the loss of cellular components, impaired energy metabolism, and disrupted nutrient absorption, electron transfer, and genetic material synthesis (17). Our study found that *Clostridium perfringens* was more sensitive to *A. fasciculifolius* gum extract than *Pseudomonas aeruginosa*, exhibiting lower minimum inhibitory and lethal concentrations. This differential sensitivity can be attributed to structural differences between Gram-positive and Gram-negative bacteria. The absence of an outer membrane in Gram-positive bacteria allows easier penetration of phenolic compounds. In contrast, the outer membrane of Gram-negative bacteria acts as a barrier against excess fatty acids (18). A recent study by Chen et al. (19) on Astragalus polysaccharides demonstrated their ability to modulate gut microbiota composition and enhance the production of short-chain fatty acids, suggesting potential prebiotic effects. This finding opens new avenues for exploring the broader impacts of Astragalus extracts on microbial ecosystems beyond direct antimicrobial activity. Khan et al. (20) investigated the antimicrobial properties of a methanolic extract of *Astragalus eremophilus*, reporting the highest inhibitory halos against *Salmonella* Typhimurium, *Enterococcus faecalis*, *Klebsiella pneumoniae,* and *Staphylococcus aureus*, with a minimum inhibitory concentration of 7.5 mg/mL. They attributed these antimicrobial properties to phenolic compounds, tannins, saponins, and flavonoids. This aligns with our findings and reinforces the potential of Astragalus species as sources of natural antimicrobial agents. Furthermore, a comprehensive review by Ghabeshi et al. (21) highlighted the diverse pharmacological activities of Astragalus species, including immunomodulatory, anti-inflammatory, and antioxidant effects. This broader perspective on the biological activities of Astragalus extracts suggests that the antimicrobial properties observed in our study may be part of a more complex array of beneficial effects. In conclusion, our study, supported by recent literature, demonstrates the significant antimicrobial potential of *A. fasciculifolius* extracts. The variability in phenolic profiles across different extraction methods and the

differential sensitivity of microbial strains highlight the importance of optimizing extraction techniques and considering targeted applications. Future research should focus on isolating and characterizing specific bioactive compounds, exploring their mechanisms of action, and investigating potential synergistic effects among different phytochemicals present in *A. fasciculifolius* extracts.

5. Conclusion

In this investigation, researchers explored the potential biostatic properties of Anazrut (*Astragalus fasciculifolius*), a plant native to Iran, West Asia, and South Asia. The focus was on its traditional use in Iranian medicine. Three solvents—methanol, ethanol, and water—were employed to analyze the phenolic compounds in Anazrut gum extract. Gas-mass chromatography revealed that the highest phenolic compound content was observed in the methanol-extracted extract, with P-coumaric acid compounds being the most abundant. The study also assessed the antimicrobial activity of Anazrut gum extract against *Clostridium perfringens*. The MIC was 78 mg/ml, while the MBC was 156 mg/ml. These concentrations indicate that Clostridium perfringens is sensitive to Anazrut gum extract. In summary, Anazrut gum extract shows promise as a potential antimicrobial agent against Clostridium perfringens. Further research is warranted to explore its applications fully.

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