Original Article

New Food Preservation candidate Zataria multiflora endrimer Synthesis and Antimicrobial Assessment

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ABSTRACT

It is widely acknowledged for its antibacterial and antioxidant properties. Zataria multiflora, also designated as ZTM, is a distinguished botanical species. Therefore, it represents an excellent alternative to products containing synthetic preservatives. Therefore, it can be concluded that this is the optimal choice. The objective of this experiment was to ascertain the efficacy of a novel nanoformulation, designated dendrimer-ZTM, in achieving the desired outcome. The composition of the nanoformulation is a pegylated anionic linear globular G2 dendrimer. The objective of this experiment was to ascertain whether the nanoformulation was capable of preventing Escherichia coli from penetrating the mayonnaise. Once the dendrimer had been synthesized, an extract from Z. multiflora was added to it. In the final stage of the process, a range of techniques, including zeta potential analysis, atomic force microscopy (AFM), and Fourier transform infrared (FTIR) spectroscopy, were employed to provide a comprehensive and accurate description of the material. The results of the experiment demonstrated that the nanoformulation exhibited intriguing protective effects. This result was observed at the conclusion of the experiment. The findings of the investigation revealed that the minimal inhibitory and fatal value was 1500 µg/mL. Furthermore, even at the lowest concentrations tested (0.1 and 0.01 per hour), the nanoformulation demonstrated the capacity to markedly diminish the levels of E. coli present in bacterial cultures. The efficacy of the nanoformulation was demonstrated by its successful performance. During the inspection of the nanoformulation, its identification was initially determined. The antioxidant and antibacterial properties of dendrimer-ZTM indicate that it has the potential to be an effective natural dietary supplement, as evidenced by our findings. This conclusion was reached as a result of a comprehensive analysis of the data collected.

Keywords: *Zataria Multiflora*, Dendrimer, Nano-Formulation, Antioxidant, Antibacterial.

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1. Introduction

In recent times, there has been an increasing demand for food products of superior quality that are readily accessible. However, the proliferation of microorganisms and the occurrence of chemical and oxidative changes frequently result in the deterioration of food quality. Despite the common use of chemical preservatives and antibacterial agents to address these issues, concerns persist regarding their potential adverse effects on human health. The use of these synthetic preservatives has been associated with the formation of hazardous and carcinogenic compounds, giving rise to considerable concern among global public health experts (1). Mayonnaise, a long-standing and popular condiment, is highly susceptible to microbial growth due to its nutrient-dense composition. Manufacturers have historically utilized synthetic compounds with antibacterial properties, such as benzoate, to ensure the safety and quality of their products. Nevertheless, concerns have been raised regarding the potential adverse effects of this material on human health. Therefore, it is recommended that it be used in very small quantities (2). In light of the pivotal role mayonnaise plays in the dietary habits of the Iranian population, it is of paramount importance to implement measures to prevent the proliferation of bacteria that may result in foodborne illnesses. The appeal of natural preservatives as a substitute for synthetic ones has increased considerably. Scientists have identified a number of plant-derived chemicals that demonstrate a diverse range of antibacterial and antioxidant properties. An exemplar of such a plant is Zataria multiflora, a plant frequently utilized in Iran, Afghanistan, and Pakistan. This herb has been demonstrated to possess a number of therapeutic effects, including disinfectant, antiviral, antibacterial, and antifungal attributes. The extract of this substance contains phenolic components, including carvacrol and thymol, which confer upon it robust antioxidant and antibacterial properties. Consequently, it can be employed as a preservative to prevent spoilage (3–5). Nevertheless, the limited capacity to dissolve and the modifications in quality within the food environment have impeded the extensive deployment of natural preservatives. Researchers are actively engaged in investigating methods to enhance the efficacy of these natural preservatives and overcome the aforementioned obstacles (6). Nanotechnology is currently capable of addressing a multitude of challenges. Nanoparticles are composed of biodegradable constituents. Dendrimers, a class of chemical compounds, have recently emerged as a subject of interest in this domain due to their distinctive structure and architecture (7). The surface of these nanoparticles is decorated with a variety of functional groups, which endow them with remarkable physical, chemical, and biological properties. Moreover, they possess a remarkable capacity for transporting substances, exhibit minimal toxicity, and do not significantly stimulate the immune system. Dendrimers, including polyamidine and polypropylene imine, have been extensively synthesized for a multitude of applications (7). Nevertheless, the presence of surface functional groups containing several positive charges gives rise to concerns regarding toxicity. To address this issue, two primary approaches have been proposed: surface engineering and the integration of biodegradable components during the manufacturing phase. The tri-block citric acid-polyethylene glycol-citric acid dendrimer exhibits both biocompatibility and biodegradability. It has the capacity to accommodate substantial amounts of chemicals, stabilize various compounds, enhance solubility, and mitigate the aforementioned issue of toxicity. The objective of this study is to examine the potential of these particles to absorb Z. multiflora and utilize its antibacterial and antioxidant properties in food products, as this specific application has not been previously investigated.

2. Materials and Methods

The chemical compounds citric acid and polyethyleneglycol diacid (molecular weight 400) were procured from Merck (Darmstadt, Germany) for use in the experiment. The investigation was conducted using chemical reagents and solvents from Sigma Aldrich (MO, USA) in analytical grade, which did not require further purification. The E. coli O157:H7 bacterium strain (NCTC: 12900) was procured from the microbiological collection of the Laboratory of Food and Aquaculture at the Islamic Azad University, Shahr-e-Qods Branch, Tehran, Iran.

2.1 Extraction Method

The Z. multiflora extract was obtained from the above-ground portions of the Z. multiflora plant through the process of hydro-distillation, utilizing a Clevenger apparatus. Subsequently, the extract was subjected to gas chromatography-mass spectrometry analysis to identify its specific chemical compounds.

2.2 Synthesis of the Dendrimer

The initial stage of the experiment involved the introduction of a quantity of 2 moles of N',N'-dicyclohexyl carbodiimide (DCC) into a solution containing 10 mL of polyethylene glycol. Subsequently, the mixture was stirred for a period of 15 minutes. Subsequently, a quantity of 3 moles of citric acid was introduced into the solution, followed by an hour of stirring at ambient temperature. Subsequently, 5 mL of the DCC solution was introduced, and the mixture was agitated for an additional 15 minutes. Subsequently, two moles of citric acid were introduced into the solution, which was then agitated for a period of 24 hours at ambient temperature. The process was terminated by the introduction of water, and the resulting second-generation dendrimer was purified using filter paper. To remove any remaining dimethyl sulfoxide (DMSO) and unreacted substances, the dendrimer underwent dialysis for 24 hours (10). In order to incorporate the extract into the dendrimer, a solution containing 1% (w/v) of the extract and Tween 80 at a concentration of 2% (v/w EO) was introduced into the 2 mL solution mentioned earlier. The mixture was agitated continuously throughout the night. The final nano-formulation was separated from the solution by centrifugation and subsequently washed with doubly

distilled water to remove any residual extract. To facilitate additional analysis, the final product was diluted in water and stored at 4°C. The loading efficiency was evaluated through the use of absorption spectroscopy at a wavelength of 230 nm.

2.3 Characterization

A variety of characterization techniques were employed to analyze the products at each stage of the process in this investigation. The aforementioned techniques included atomic force microscopy (ICON, BRUKER, USA), Fourier transform infrared spectroscopy (AVATAR, Thermo, USA), dynamic light scattering (DLS), and zeta potential (ZEN3600, MALVERN, UK).

2.4 Antioxidant Activity Assay

In summary, a solution comprising 0.2 mg of beta-carotene in 10 mL of chloroform (yielding a total volume of 1 mL) was augmented with 25µL of linoleic acid and 200mg of Tween 40. Following the removal of the chloroform through distillation, 100 mL of distilled water saturated with oxygen was added while maintaining vigorous stirring. Subsequently, a volume of 2.5mL of the aforementioned reagent was introduced into each test tube, and its antioxidant properties were assessed under a variety of conditions. Butylated hydroxytoluene was utilized as a positive control. The absorbance was ultimately determined by absorption spectroscopy at a wavelength of 470 nm, and the outcomes were computed using the formula outlined by Becerril et al. (11).

Eq. (1) $I(\%) = \frac{Asample - Acontrol}{Acontrol(0) - Acontrol} \times 100$

Where

$A_{control}$: Absorption of the control

A_{sample}: Absorption of the sample

$A_{control\,(0)}$: Absorption of the control at t=0

2.5. Antibacterial activity

In summary, a solution containing 0.2 mg of the nano-formulation was tested for antimicrobial properties using the disc covering method with brain heart infusion (BHI) agar. Each disc contained 20 µL of the sample and served as a positive reference (12). The minimal inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) against both bacteria were determined using the broth microdilution assay in a 96-well plate, in accordance with guidelines from the Clinical & Laboratory Standards Institute (CLSI) (13).

2.6 Software and Statistical Analysis

A one-way analysis of variance (ANOVA) was conducted to perform the requisite statistical analysis. Imaging analysis was conducted using Image J and SPIP (Scanning Probe Image Processor Software). All experiments were repeated five times, and P-values less than 0.05 were considered significant. A Box-Behnken experimental design with three levels (low, medium, high) represented as −1, 0, and +1, respectively, was utilized to create a model and analyze the responses influenced by three independent factors (Table 1).

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Eq. (2) $Y = \beta_0 + \beta_1 A\ \beta_2 B + \beta_3 C + \beta_1\beta_1 (A\times A) + \beta_2\beta_2 (B\times B) + \beta_3\beta_3 (C\times C) + \beta_1\beta_2 (A\times B) + \beta_1\beta_3 (A\times C) + \beta_2\beta_3 (B\times C)$

3. Results

3.1 Extract Analysis

The quantitative and qualitative analysis of Z. multiflora using gas chromatography-mass spectrometry enabled the identification of a number of compounds present in the plant's volatile oil, including their respective percentages. The most prominent compounds identified were thymol (22.23%), carvacrol (46.52%), p-cymene (2.13%), and γ-terpinene (6.84%). It should be noted that discrepancies in the reported values may be attributed to a number of factors, including weather and geographical conditions (14).

3.2 Dendrimer Synthesis and Loading

Fourier transform infrared (FTIR) analysis was conducted to identify molecular interactions and functional groups (Figure 3). Peaks related to –OH, -CH, -C=O, -CH, and –C-Oester were observed at 3372 cm-1, 2970 cm-1, 1643 cm-1, 1427 cm-1, and 1046 cm-1, respectively. Additionally, peaks at 1919 cm-1, 1567 cm-1, and 1211 cm-1, which relate to -CH aromatic, –C=C, and –C-O aromatic, were identified, confirming the presence of oil in the dendrimer. The size of the nanoparticles was assessed using atomic force microscopy (AFM) before and after loading with the Z. multiflora extract (Figures 4 & 5). The dendrimers exhibited a nearly spherical shape with a diameter of 5-15 nm, with a mean size of 13 ± 4.22 nm. However, following the loading of the extract, the particle size range increased to 33-15 nm, with an average size of 22±5.09 nm. The enlargement of the image and the modification of the spherical morphology of the dendrimer substantiated the

successful loading process. The results of the DLS analysis indicated that the particle size of dendrimer-ZTM was approximately 33 nm larger than that of the dendrimer alone. Furthermore, the zeta potential analysis proved to be a valuable indicator of the changes in dendrimer loading, demonstrating that the nanoformulation possessed a neutral charge (Figure 6).

3.3. Antioxidant activity

The objective of this study was to investigate the impact of three variables (temperature, amount of dendrimer-ZTM, and incubation period) on antioxidant activity with the goal of achieving maximum yield. In this experiment, the rapid color loss of beta-carotene-linoleic acid in the absence of antioxidants is caused by the oxidation of beta-carotene and linoleic acid, which results in the formation of free radicals. The formation of free radicals in linoleic acid is caused by the removal of a hydrogen atom by highly unsaturated beta-carotene molecules, which results in the oxidation and decomposition of beta-carotene. The loss of orange coloration can be quantified using a spectrophotometer. The third experiment yielded the fewest responses, while the second experiment produced the most. In order to account for the nonlinearity of the relationship between the response and the main variables, a model was constructed which included three parameters, interactions between the main parameters, and the second power of each main parameter. An analysis of variance (ANOVA) was conducted using a second-order mathematical model. The results of the analysis of variance (ANOVA) on the modelling are presented in Table 3 and Equation 2. A P-value of 0.05 during the modeling process indicates the significance of the effect of each term. The P-value of 0.002 and the R-sq value of 97.55 indicate that the model is an accurate representation of the data and that the interactions between the variables B×B, C×C, A×B, and A×C do not significantly impact the outcome. A contour map was employed to investigate the influence of the parameters on antioxidant activity. The graphs illustrate the influence of two parameters on the response while maintaining a constant value for the third parameter. The color in the upper left corner of the graph is interpreted for each contour line. The figures illustrate a slight change in color, with a transition in hue from blue to red representing the highest reaction level. The graphs demonstrate that elevating the parameter values enhances the antioxidant activity, with this increase being pronounced until the midpoint of the graph and then gradually diminishing. Based on these findings and the subsequent target tuning, it was determined that the values of 5, 6, and 7 exhibited the highest antioxidant activity.

3.4. Antibacterial Activity

Figure 8 illustrates the inhibitory activity of dendrimers and the nano-formulation against bacterial growth. The dendrimer demonstrated inhibitory activity against the growth of approximately 20% of E. coli and 23% of Staphylococcus, respectively. Furthermore, the inhibitory analysis demonstrated that the zone of inhibition diameter of the nano-formulation against E. coli was ±8.11, which was markedly larger than that of the empty extract. Table 4 presents the nano-formulation's minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC). Figures 9 and 10 illustrate the bactericidal effect of the non-formulated sample in comparison to the control group in a food sample of mayonnaise. The findings suggest that an extended storage period is associated with a notable reduction in bacterial growth.

Table 1. Coding factor for each parameter.

	Coded Value		
Parameter	-1	0	+1
Concentration (A)	1	1.5	2
Temperature (B)	30	35	40
Time (C)	1	2	3

Table 2. Experimental design table

Std-Order	Run-Order	A	B	C	Response (%)
6	1	1	0	-1	88.3
8	2	1	0	1	90.68
1	3	-1	-1	0	74.56
14	4	0	0	0	86.69
15	5	0	0	0	86.18
10	6	0	1	-1	86.42
11	7	0	-1	1	86.33
5	8	-1	0	-1	76.19
7	9	-1	0	1	84.53
4	10	1	1	0	88.79
3	11	-1	1	0	84.33
13	12	0	0	0	86.64
9	13	0	-1	-1	78.75
2	14	1	-1	0	83.12
12	15	0	1	1	89.24

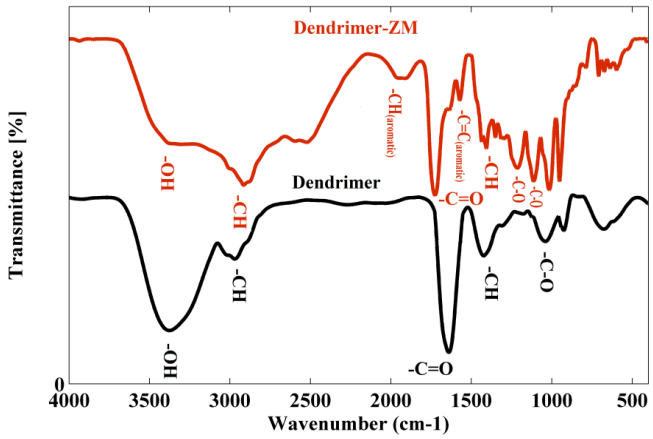

Figure 1. FTIR spectrum of dendrimer (black) and nano-formulation (red).

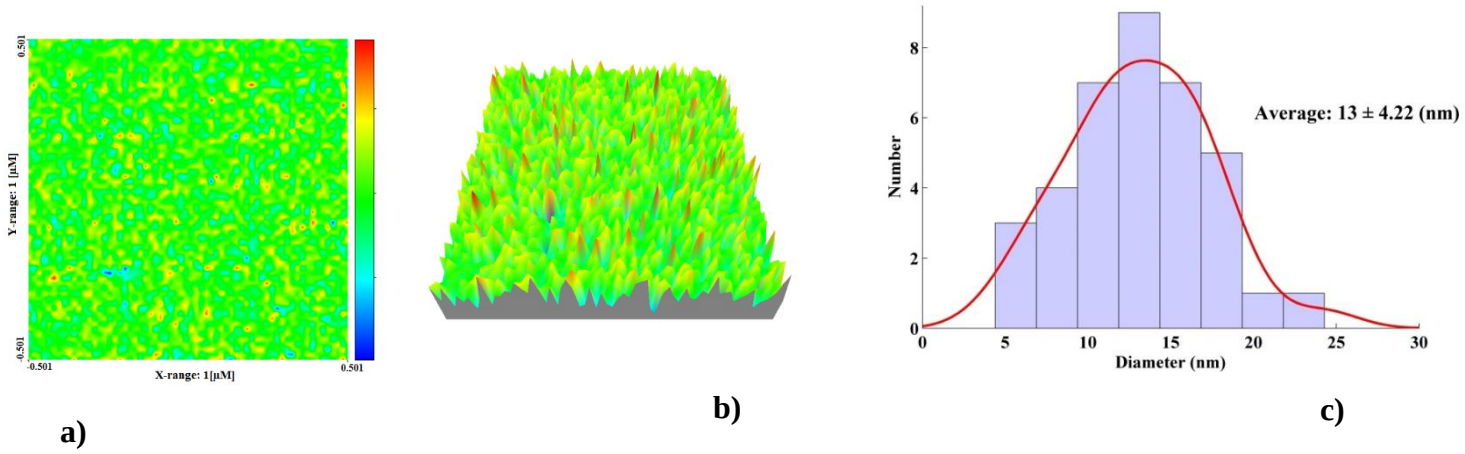

Figure 2. a) 2d, b) 3d, and c) size distribution plots obtained from AFM analysis of dendrimer

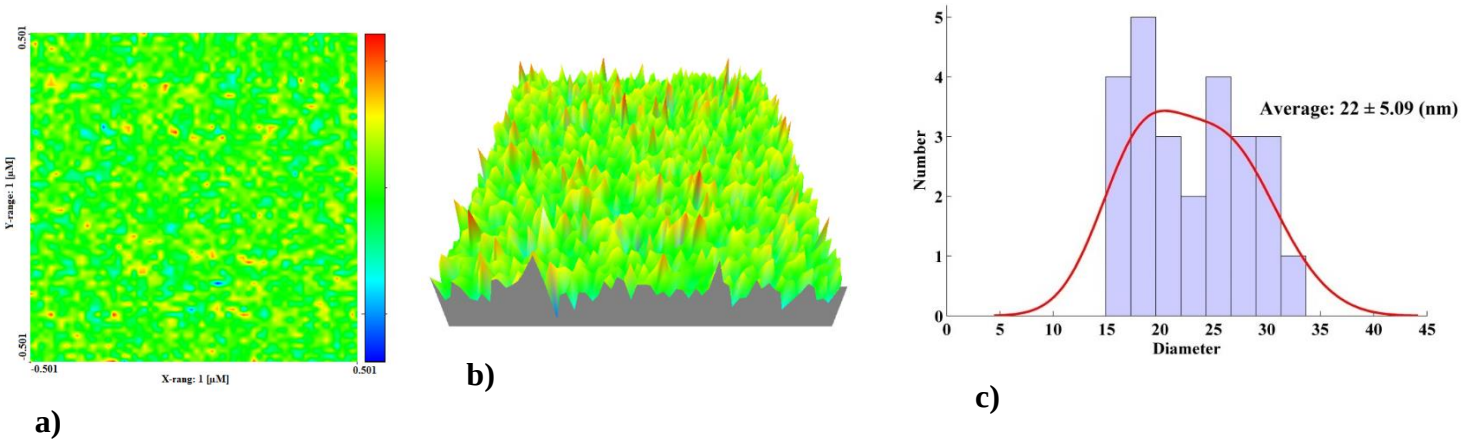

Figure 3. a) 2d, b) 3d, and c) size distribution plots obtain from AFM analysis of Nano formulation

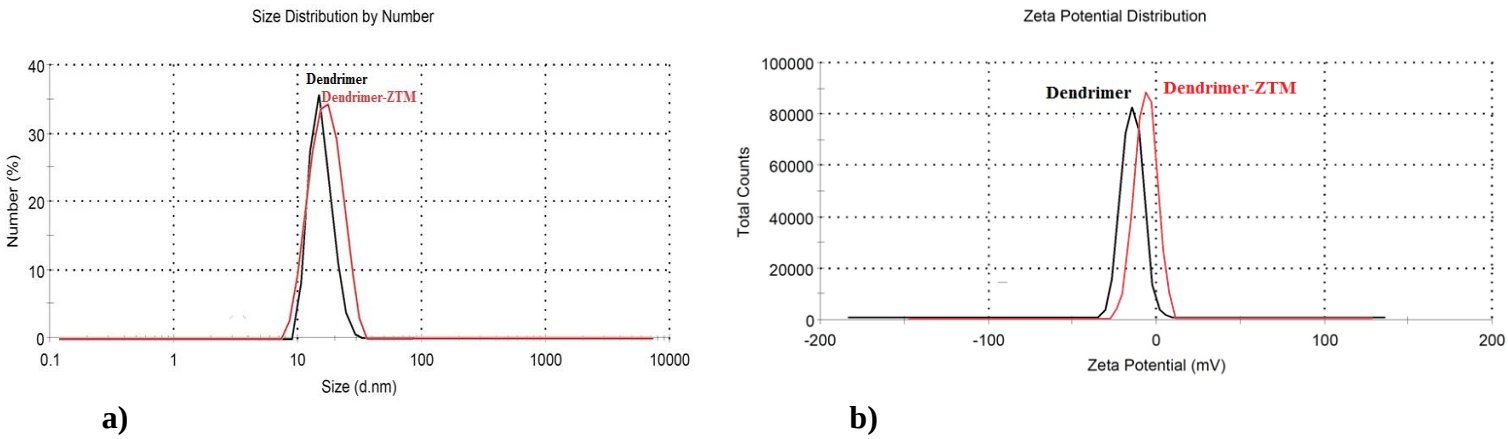

Figure 4. a) DLS and b) Zeta potential of the dendrimer (black) and nano-formulation (red)

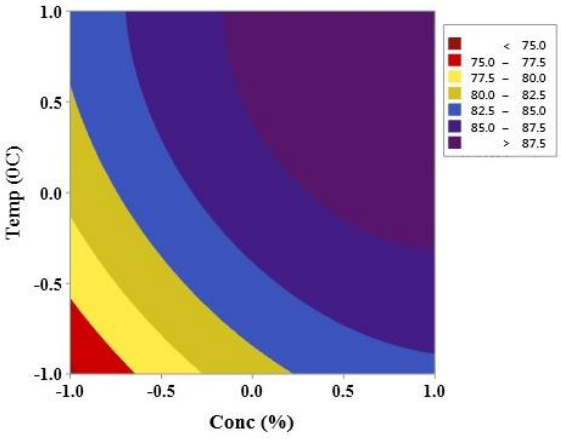

Figure 5. Contour plot of concentration-temperature interaction.

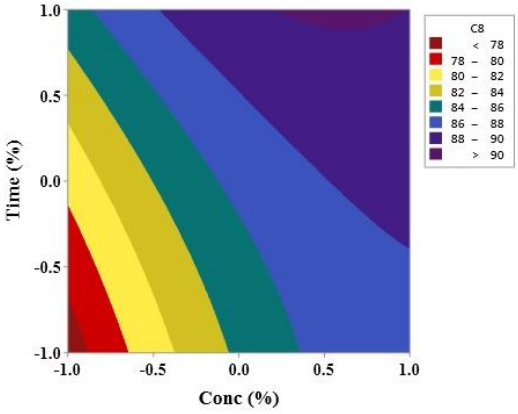

Figure 6. Contour and concentration-time interaction.

Table 3. Results obtained from the ANOVA

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	86.503	0.661	130.85	0.000	
A	3.910	0.405	9.66	0.000	1.00
B	3.252	0.405	8.03	0.000	1.00
C	2.640	0.405	6.52	0.001	1.00
A×A	-2.032	0.596	-3.41	0.019	1.01
B×B	-1.772	0.596	-2.97	0.031	1.01
C×C	0.453	0.596	0.76	0.481	1.01
A×B	-1.025	0.573	-1.79	0.133	1.00
A×C	-1.490	0.573	-2.60	0.048	1.00
B*C	-1.190	0.573	-2.08	0.092	1.00

Y= 86.503 + 3.910 A + 3.252 B + 2.640 C - 2.032 (A×A) - 1.772 (B×B) + 0.453 (C×C) - 1.025 (A×B)- 1.490 (A×C) - 1.190 (B×C) (Eq. 3)

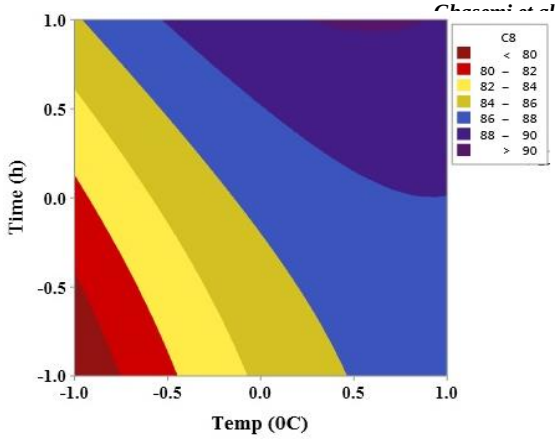

Figure 7. Contour plot of time-temperature interaction.

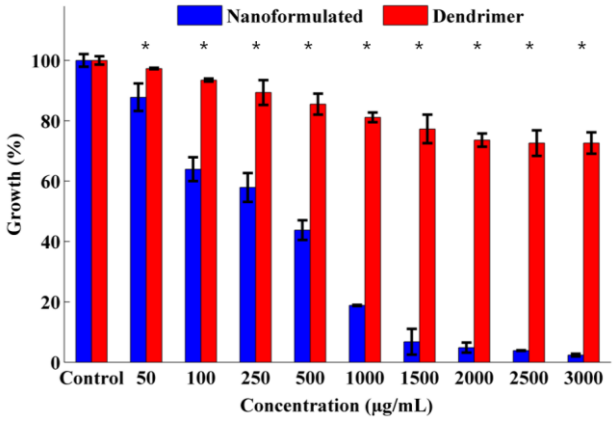

Figure 8. Inhibitory growth of the nano-formulation (blue) and dendrimer (red) against *E. coli* O157:H7. The sign (*) indicates a significant difference.

Table 4. MIC and MBC results.

Type	MIC (µg/mL)	MBC (µg/mL)
Escherichia coli O157:H7	1250	2500

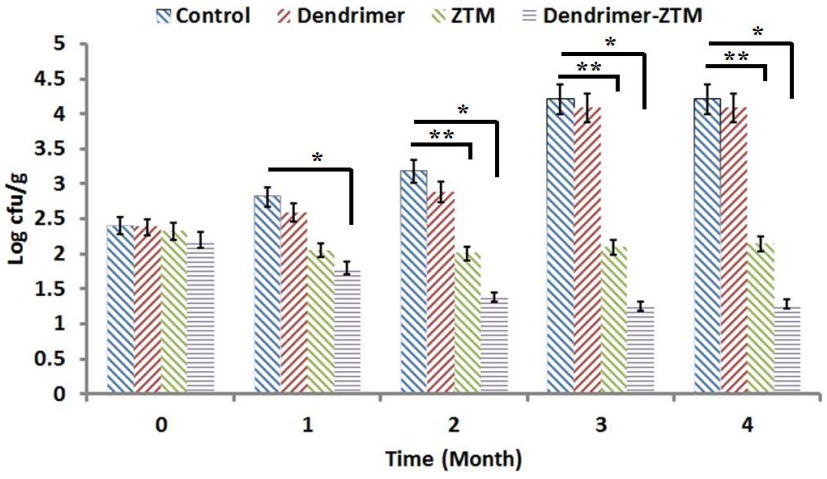

Figure 9. The logarithm of the number of E. coli in mayonnaise, under the influence of different ingredients during 4 months of storage at 4°C.

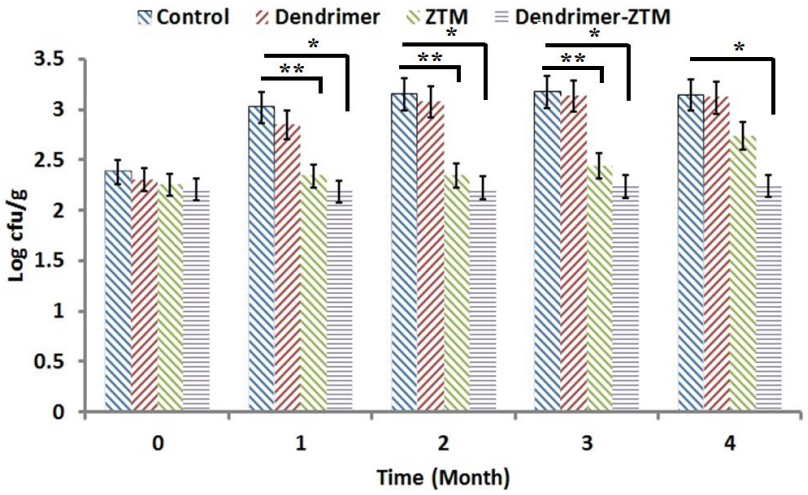

Figure 10. The logarithm of the number of E. coli in mayonnaise, under the influence of different ingredients during 4 months of storage at 15°C.

4. Discussion

A second-generation spherical linear negative dendrimer was prepared by employing a one-pot synthesis utilizing citric acid and polyethylene glycol diacid. This dendrimer was then utilized for the encapsulation of the Z. multiflora extract. Citric acid is a highly utilized substance in the food industry due to its exceptional water solubility, palatable flavor, buffering capabilities, and antibacterial and non-toxic properties (15). Polyethylene glycol is a commonly utilized substance in the food industry due to its compatibility, biodegradability, and solubility (16). The acidic functional groups of polyethylene glycol diacid were activated by interacting with DCC in the synthesis step, thereby facilitating the formation of the desired product. This activation facilitated the conjugation of citric acid via the hydroxyl group, resulting in the synthesis of the first-generation dendrimer (17). The acidic groups of citric acid were activated by DCC and subsequently reacted with the preceding generation of dendrimers, thereby creating the second generation. The process of activating the acid groups and conjugating citric acid is illustrated in Figure 12. The formation of free radicals during the storage and processing of food can lead to spontaneous oxidation, the creation of unwanted chemical compounds, and the development of a rotten flavor in food. The extract of Z. multiflora contains thymol and carvacrol, which have been demonstrated to exhibit potent antioxidant effects (18). A spectrophotometric approach, based on the β-carotene bleaching experiment, was employed to investigate this characteristic. In accordance with the results of this assay, active oxygen species were observed to transform linoleic acid into a peroxyl radical, which subsequently interacted with β-carotene, transforming it into a radical. The alteration of the β-carotene structure results in a reduction in its color intensity. The anti-radical compounds present in the solution compete with β-carotene for reaction with peroxyl radicals, thereby reducing the degradation of β-carotene. (19). The study employed a response surface method based on the Box-Behnken design to analyze the parameters influencing the antioxidant activity of dendrimer-ZTM and achieve the maximum yield. The response surface method is a collection of statistical and mathematical techniques that are employed for the purposes of experiment design, model construction, variable effect analysis, optimal variable value determination, and target response prediction. This strategy offers an alternative to traditional approaches by considering the interactions among various components, as opposed to examining one variable at a time. Classical optimization approaches entail the alteration of individual variables while maintaining other variables within defined parameters. This approach ultimately proven ineffective as it neglects to consider the complex interplay between all variables. The response surface method has addressed this issue by reducing the number of tests, time, cost, and material required (20). Regulatory agencies such as the Food and Drug Administration and the World Health Organization have expressed significant concern regarding foodborne infections. A total of 250+ foodborne illnesses have been identified, with bacteria responsible for 66% of these cases (21). Bacterial food poisoning, which is frequently associated with the consumption of contaminated food containing toxin-producing bacteria, represents a significant foodborne illness. Enterohaemorrhagic strains of Escherichia coli, such as E. coli O157:H7, represent a significant public health concern due to their ability to contaminate rivers and cause a range of severe conditions, including hemorrhagic colitis, hemolytic uremic syndrome, and acute renal failure (22). The antibacterial properties of the dendrimer are attributed to the incorporation of citric acid into its structure, which exhibits effective antibacterial activity (23). The impact of

citric acid on material transport and metabolic pathways is mediated by its influence on pH balance (24). The study demonstrated that the dendrimer's distinctive structural attributes enhance its stability, water solubility, and biocompatibility, with a diameter of less than 50 nm. Further adjustments to the experimental design demonstrated that this particle exhibited robust antioxidant activity under typical conditions. Furthermore, the nano-formulation demonstrated antibacterial activity against bacteria in comparison to the extract. The results suggest that this nano-formulation may prove valuable in the food sector as a natural preservative, assisting in the prevention of bacterial contamination and extending shelf life.

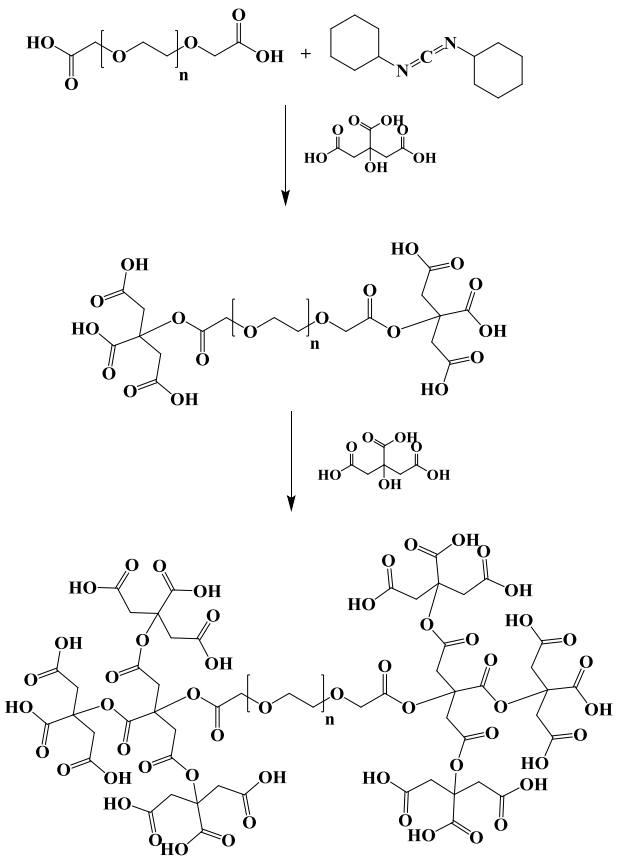

Figure 11. Synthesis of the dendrimer.

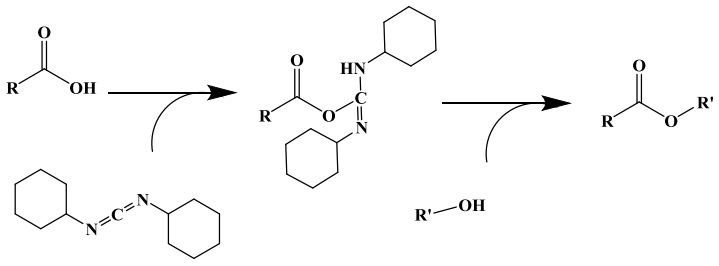

Figure 12. Mechanism of conjugation using DCC.

Abbreviations used

ZTM, *Zataria multiflora*; PH, Power of hydrogen; DLS, Dynamic light scattering; DCC, N', N'-dicyclohexyl carbodiimide; DMSO, (Dimethyl sulfoxide; BHI, Brain heart infusion; CLSI, Clinical & Laboratory Standards Institute; MIC, Minimal inhibitory concentrations; MBC, Minimum bactericidal concentrations; ANOVA, Analysis of variance; AFM, Atomic force microscopy; A_w. Water activity.

Acknowledgment

None.

Authors' Contribution

Experimental Section, Writing Manuscript: R. G.
Statistical Analysis, Edit manuscript: S. M.
Edit manuscript: M. K.

Ethics

The authors certify that all ethical considerations were respected in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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