Investigation of Physicochemical, Antioxidant, and Antimicrobial Properties of Thymol and Resveratrol Encapsulated with Zein-Caseinate Nanoparticle

FatemehSadati Khadar[1], Elham Mahdian[1*], EsmaeilAtaye Salehi[1], Reza Karazhian[2]

[1]Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran

[2]Industrial Microbial Biotechnoogy Department, Institute of Industrial Biotechnology, ACECR, Mashhad, Iran

Corresponding author: Elham Mahdian[*]

Email:elhammahdian@iauq.ac.ir

Abstract

Thymol and resveratrol are organic, phenolic compounds with wide-ranging potential applications in the food industry, mainly use as an anti-oxidation and antibacterial additives. However, their weak stability, low water solubility, and bioavailability have severely limited their industrial applications. Herein, zein-casein nanoparticles loaded with thymol and resveratrol have been successfully prepared using the liquid-liquid dispersion method to empower their stability, solubility, antioxidant, and antimicrobial properties. As a result, formulation of 75% thymol with 25% resveratrol mixing ratio 1:20 (75T25R20) was the most stable nanoparticle with zeta potential of -19.54 compared to only thymol or resveratrol nanocapsules (100T0R20 and 0T100R20) with zeta potential of -1.22 and -4.12 respectively. In addition, scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical activity increased to 58.77% in 75T25R10 nanocapsule compared to the antioxidant activity of nanocapsule of only thymol or resveratrol with DPPH of 20.33% and 13.67% respectively. The synergic effect of the thymol and resveratrol also improved the antimicrobial activity. For example, minimum inhibitory concentration (MIC) against e.coli bacteria in culture medium, drops from 300-350 for sole thymol/resveratrol nanocampule (100T0R10, 0T100R10) to 100 for 75T25R10. Finally, scanning electron microscopy (SEM), and Fourier transforms infrared spectroscopy (FT-IR) have been used for the physical characterization of the resultant nanoparticles (NPs). It is worth mentioning that formed zein-casein nanoparticles loaded with thymol and resveratrol have good redispersibility and physical stability stored at -20 ^{0}C. Therefore, this investigation introduces an excellent organic candidate for delivering hydrophobic active compounds, which is of significance in the cosmetics and especially food industries.

*Keyword*s: liquid-liquid dispersion method, antioxidant, caseinate, nanoparticles, phenolic compounds

1. Introduction:

Natural antioxidants are used more frequently in foods today due to their health benefits. Thymol and resveratrol are natural polyphenols with high antioxidant properties. However, adding this antioxidant directly to food is difficult due to its insolubility in organic solvents, low bioavailability, and oxidation sensitivity. Researchers today are increasingly using bioactive compounds like polyphenols and natural antioxidants since polyphenols are antibacterial compounds able to absorb free radicals[1]. Since polyphenolic compounds have these properties, they can prevent cancer, inflammation, and cardiovascular diseases. Nevertheless, the low stability, large molecular size, oxygen sensitivity, light, heat, and bitter taste cannot be directly used for enriching and preserving food [2].

Thymol (2-isopropyl-5-methylphenol) is a natural monoterpenoid phenol derivative of p-Cymene, isomeric with carvacrol, found in the oil of thyme, and extracted from *Thymus vulgaris* (common thyme), *ajwain,* and various other plants as a white crystalline substance with a pleasant aromatic odor and strong antiseptic properties[3,4]. Monoterpene phenols, such as thymol, break apart bacterial cell membranes when combined with unsaturated fatty acids, increasing cellular permeability[5]. In addition, the antioxidant activity of thymol related to its phenolic structure plays a vital role in reducing and neutralizing free radicals and peroxides, whose antioxidant activity is helpful for protection against inflammation in addition to the antibacterial and antifungal properties of thymol[6]. The hydrophobic and volatile characteristics of thymol make its use in food challenging.

Plants produce resveratrol (3,5,4′-trihydroxy-trans-stilbene) as a phytoalexin when injured or under attack by pathogens, such as bacteria or fungi [7,8]. Grapes and their products are the primary sources of this substance in the human diet. The presence of the hydroxyl group on the aromatic ring in the composition of resveratrol causes antimicrobial activity. In addition, resveratrol has been proven to have antibacterial, antifungal, anti-inflammatory, anticancer, and antioxidant properties. However, the most common problem with resveratrol as a commercial drug, supplement, and functional food product is its low aqueous solubility, short half-life, high photosensitivity, rapid metabolism, and low oxidation stability [9].

Encapsulation is a promising method to overcome the problems of instability of natural phenolic compounds, increasing solubility in the lipid membrane of cells, accessibility, and controlling the release of compounds over time. Nutraceuticals and bioactive compounds are transferred to target parts through nanoencapsulation, which encapsulates less than one micron-sized carrier. Various biopolymer and lipid nanocapsules are used in food systems [10,11]. Proteins, polysaccharides, or their complexes are used to produce biopolymeric nanocarriers. Biopolymeric nanocapsules increase the bioavailability and solubility of hydrophobic compounds, protect these compounds against harmful environmental factors (such as oxygen and light), control their release in the food and body, and prevent inappropriate taste and color [12,13]. The complex between protein and polysaccharide biopolymers has superior stability and usability compared to either biopolymer alone. The most recent phenolic nanostructures, their preparation methods, size, and their efficacy are listed in the table 1.

Table 1. Synthase phenolic nanoparticles

Preparation method	Particles Size (nm)	Encapsulation efficiency	Application	Reference
Liquid-liquid dispersion	<100	17-46.3	Increase antioxidant activity	[14]
Electrospinning	207-275.5	-	Increase fish fillet shelf life	[15]
Liquid-liquid dispersion	155.5 - 225.4	-	Increase antioxidant activity	[16]

Zein, which makes up roughly 45 to 50 percent of corn's protein content, is frequently employed as a secure, biodegradable, and biocompatible biopolymer. Zein is a member of the prolamin family, which also includes hydrophobic amino acids like leucine, alanine, and proline. Zein has also been used by the pharmaceutical industry to coat capsules, regulate release, and mask the taste and smell of medications [17]. Zein and casein complexes have been used in various studies [18–24]. Moreover, thymol and resveratrol have been encapsulated by various methods to increase stability [23,25–29].

The study was designed to investigate the interaction between resveratrol and thymol as representative phenolic compounds, and make a conclusion about their combined effect, synergistic or antagonistic, on the antioxidant properties of the samples. To accomplish this objective, we measured the antioxidant activity of samples by employing the following two methods: ferric reducing antioxidant power (FRAP), scavenging of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Then antimicrobial activity of the nanoparticles were measured in the culture and milk medium. In the next step, the kinetic release of nanoparticles were tested and finally, the structure of nanoparticles were confirmed by FT-IR and Scanning electron microscopy (SEM) analyses.

2. Materials and Methods

2.1. Materials

TSB and Tryptic Soy Agar (TSA) microbial culture media (Merck, Germany) with purity levels of more than 95 and 97%, respectively. They used in the study to obtain thymol and resveratrol. The solvents used in this study were obtained from Sigma-Aldrich (USA), and lyophilized Escherichia coli (O 157:H7) and Listeria monocytogenes (19118 TTAC) bacteria were obtained from the microbial collection of Shahid Beheshti University (Iran). All other chemicals were at analytical grade and purchased from chemical suppliers.

2.2. Preparation method

The liquid-liquid dispersion method was used to prepare zein-caseinate nanoparticles [30–32]. Based on this method, 2g of zein desolved in 100 ml of aqueous ethanol solution (80% by volume) until obtaining a concentration of 20 mg/ml. Then, thymol and resveratrol were added (in a ratio of 1:1) to the zein solution with different ratios of the core-to-wall (Table 2). A magnetic stirrer was used to stir slowly for 12 hours. The stock solution (mixing of two solutions) was added to deionized water (containing 2 mg/ml of sodium caseinate) at a ratio of 10:1 drop by drop under constant stirring with the stirrer at a speed of 750 rpm for 30 minutes. Then, stirring was completed using an ultra-turrax (IkaT10 basic, Germany) at 19000 rpm.

Table 2. The treatments used in this research

Treatments	Core/wall	Resveratrol (%)	Thymol (%)
100T0R10 (sole thymol)	1:10	0	100
75T25R10	1:10	25	75
50T50R10	1:10	50	50
25T75R10	1:10	75	25
0T100R10	1:10	100	0
100T0R20	1:20	0	100
75T25R20	1:20	25	75
50T50R20	1:20	50	50
25T75R20	1:20	75	25
0T100R20 (sole resveratrol)	1:20	100	0

Then, ethanol was evaporated using a rotary evaporator (Buchi, Switzerland) under a vacuum at 30°C. A centrifuge (Thermo, Japan) was used at 4000 rpm for 10 min to separate possible particles from the agglomeration of nanoparticles. The final dispersion was freeze-dried, and the resulting nanoparticle powder was packed and kept at -20°C for the tests.

2.3 Encapsulation efficiency (EE)

Freshly prepared samples containing thymol and resveratrol were centrifuged at 4°C at 10000 rpm for 30 min. The encapsulation efficiency values of thymol and resveratrol were determined by finding the amount of these compounds in the total disperse (C_W) and supernatant (C_S) using the device HLPC (Equation 1) [33].

$$\text{Encapsulation efficiency } (\%) = \frac{C_W - C_S}{C_W} \times 100 \qquad (1)$$

2.4 Determination of particle size, polydispersity index (PDI), and zeta potential

The encapsulated particles size were measured 30 min after production using Dynamic Light Scattering (DSC). The refractive index was 1.33, and the dielectric constant was 78.5 at 25°C at a scattering angle of 173 degrees [34].

The zeta potential is a reliable indicator of the stability of colloid dispersion under storage conditions. Zeta Sizer (Malvern, England) device was used to determine this quantity, which is based on the amount of electrophoretic displacement of surface electric charge particles. In this test, the refractive index was 1.33, and the dielectric constant was 78.5 at 25°C [35,36].

2.5 Determination of antioxidant activity

2.5.1 DPPH radical scavenging

The free radical-scavenging activity of the extracts was determined with 2'-diphenyl-1-picrylhydrazyl radical (DPPH). Different concentrations of each extract were added at an equal volume to the methanol solution of DPPH (100μM). The samples were kept at room temperature in the darkness [11]. The absorbance of each sample was measured at 517nmby a spectrophotometer after 30min (Biochrom, England).

2.5.2 Ferric reducing antioxidant power (FRAP)

The ability of extracts to reduce iron (III) was evaluated using the method of Huange et al. [37]. Extracts (2.5ml) were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$; 10 g/L) and incubated for 30 min at 50°C. Then, 2.5 ml of trichloroacetic acid (100 g/L) was added to the solution and centrifuged for 10 min. Finally, 2.5 ml of supernatant was combined with 2.5 ml of distilled water and 0.5ml of $FeCl_3$ (1g/L). The absorbance of samples was measured at 700 nm, and higher absorbance values indicated higher reducing power.

2.6 Antimicrobial properties

A microbroth dilution test was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in the TSB culture medium [38]. A specific concentration of nanoparticles carrying thymol-resveratrol was prepared in ethanol. In the next step, TSB culture medium was used to prepare different concentrations of the antibacterial solution, starting with a stock solution. Then, the solution and culture medium contained 10^8 *Escherichia coli* CFU/ml and *Listeria monocytogenes* were added to special tubes with a volume of 240 µl. A negative control was prepared using 240 µL TSB, and a positive control was prepared by substituting an antimicrobial treatment with TSB, and their absorbance was determined by spectrophotometric method at zero time and 24h after incubation at a wavelength of 630 nm.

To determine the antimicrobial activity of 2% fat milk with encapsulated nanoparticles, 4.5 ml of product were mixed with 5.5 ml of milk and vortexed at laboratory temperature for 30 min. A specific concentration of nanoparticles carrying thymol-resveratrol was prepared in ethanol. Then, 10^8 CFU/ml of *Escherichia coli* and *Listeria monocytogenes* bacteria were cultured, diluted with 0.1 peptones after 24h of incubation at 21°C, and added to the TSA culture medium using the diffusion method in agar. , and survivors were enumerated after plating on TSA and incubating at 32 °C (for L. monocytogenes) or 37 °C (for E. coli O157:H7) for 24 h. The detection limit was 1.0 log CFU/mL [38]. The antimicrobial properties of these substances in milk were determined using the bacteria destruction and inhibition zone.

2.7 Solubility and stability measurement

To determine the solubility of the products, Jiang and Zhu applied various methods [39]. To do this, a specific quantity of prepared samples was first stirred into the media (milk) and centrifuged for 10 minutes. After drying the supernatant at 105°C, the solubility of the samples was determined using Equation 2.

$$\text{Solubility (\%)} = \frac{W_2 - W_1}{W_0} \times 100 \qquad (2)$$

Where W_0 indicates the weight of the prepared samples, W_1 shows the dried weight of the supernatant of the media (milk) without nanoparticles, and W_2 donates the dried weight of the food containing the samples.

To check the stability of the samples, the redispersibility time of the samples of dried nanoparticles in milli-Q water was determined, and the agglomeration or sedimentation was visually checked [40].

2.8 Release of thymol and resveratrol

A total of 1ml of sample (5mg per ml of double-ionized water) was added to dialysis bags (3.5 KD, diameter 22 mm, thickness 0.9 ml). Then, the dialysis bag was immersed in 85ml of double-distilled water. In the next stage, 1ml of the uncoated sample (25mg of sample to 4 ml of double distilled water) was added to the dialysis bag of 3.5 kDa. Finally, the dialysis bag was immersed in 120 ml of double distilled water. The water temperature was 25°C, and the stirring speed was 100 rpm. The sampling was conducted in 14 steps between time intervals of 0 to

8 hours. Each time, 3 ml of the solution was sampled to determine the amount of released essential oil, and the same amount of double distilled water was added to the medium. The amount of essential oils of thymol and resveratrol was measured by reading the absorbance at 276 and 306 nm, respectively [41].

2.9 Fourier transforms infrared spectroscopy (FTIR)

The interactions between the compounds were investigated through the Fourier transform infrared spectrometer (Shimadzu, Japan). The wave number of the device was set between 400 and 4000 cm^{-1}, and the resolution was $1cm^{-1}$ and it was analyzed through a Fourier transform infrared spectrometer equipped with diamond ATR cells [42].

2.10 Scanning electron microscopy (SEM)

A scanning electron microscope was used to examine the structure of the samples (Oxford, England). First, freeze-dried samples (Christ α 1-4, Germany) are glued to the conductive carbon base with silica glue, and then the bases are covered in gold and platinum coating/spraying machine [43].

2.11 Statistical analysis

This research was conducted in a completely randomized design with three replications to investigate the effect of the independent variable. The results were analyzed using SAS 9.4 statistical software, and the average data were compared using Duncan's multi-range test at the statistical level of 5%.

3 Results and Discussion

3.1 Encapsulation efficiency

The results showed that the formulation type significantly affects the encapsulation efficiency of thymol (p<0.05). According to Duncan's test method, 100% thymol encapsulation was the most efficient, and the core ratio of 1:20 for their treatment had the highest thymol encapsulation efficiency. When the core-to-wall ratio was 1:20, thymol encapsulation efficiency decreased to 75% with increasing thymol concentration in the formulation, and thymol encapsulation efficiency increased with increasing core-to-wall ratio, except for the treatment containing 100% thymol (Table 3).

Table 3. Encapsulation efficiency of treatments

| Treatments	Encapsulation efficiency (%)	
	Thymol	Resveratrol
100T0R10	36.52±0.28[d]	0.0±0.0[i]
75T25R10	39.48±0.53[c]	83.26±1.0[c]
50T50R10	31.45±0.39[e]	30.87±0.29[e]
25T75R10	40.33±0.51[b]	39.57±0.50[e]
0T100R10	0.0±0.0[i]	16.40±0.2[i]
100T0R20	97.14±2.50[a]	0.0±0. 0[i]
75T25R20	16.84±0.3[h]	64.08±1.50[d]
50T50R20	26.52±0.26[g]	89.67±1.0[b]
25T75R20	30.72±0.22[f]	97.00±0.22[a]
0T100R20	0.0±0.0[i]	18.73±0.50[g]

The effectiveness of liquid-liquid dispersion method was often shown by the high encapsulation efficiency of the treatments. Additionally, the treatments with increased thymol concentrations showed improved efficacy. This increase in effectiveness may be due to the increased likelihood of thymol being physically trapped due to its higher concentration. The least amount of chemical bonds is feasible between surfactants (ionized hydroxyl groups) and thymol in order to impact the surface charges and the attraction between particles (zeta potential). Physical trapping occurs between and inside the thymol molecules of the caseinate and zein molecules [44].

In addition, the formulation type significantly affected the resveratrol capsulation efficiency (p <0.05). Table 3 indicates that the highest encapsulation efficiency of resveratrol was found in 25T75R20 with 97%.The encapsulation efficiency of resveratrol rose from 25 to 75% with increasing concentrations of resveratrol in the formulation. Following the increase in the core-to-wall ratio, the encapsulation efficiency of resveratrol decreased, except for the treatment containing 75% thymol. At 100% concentration of resveratrol, the protein-protein interactions are weakened, leading to the entry and loss of more extract and protein into the clear liquid and participating in particle formation.

Encapsulation efficiency generally refers to the wall material's potential to retain or trap the core material inside the particles. Encapsulation efficiency is also related to the shelf life of the material enclosed inside the wall. A successful encapsulation produces a powder with minimal surface material on the powder particles and maximum preservation of the core material [45]. Many factors influence encapsulation efficiency, including the wall and core material characteristics, the core-to-wall material ratio, the produced emulsion characteristics, process conditions, moisture content, particle size, feed viscosity, and the total solids. One of the important factors in boosting encapsulation effectiveness is the ideal choice of the core-to-wall material ratio [45]. Total solids, core-to-wall material ratio, and wall material type all have a substantial impact on encapsulation efficiency. The inability of the wall material to build a solid wall structure and a thick layer around the core may be the cause of the reduction in efficiency. Efficiency can also be increased by adding more total solid matter [46].

3.2 Particle size, PDI, and zeta potential

The PDI assesses the size distribution of nanoparticles. Better uniformity is indicated by a lower PDI value. All of the nanoparticles studied had PDI values between 0.14 and 0.85 (. 1), showing the uniformity of the size distribution[30]. In addition, zeta potential measurements are shown that the zeta potentials of all samples are negative though thymol and resveratrol content varied, and that all zeta potentials were between -0.3 and -19.5 mV. This result suggested that sufficient electrostatic repulsion existed between the nanoparticles, resulting in the good stability of the dispersions of NPs. The treatment of 25T75R10 with 0.855 resulted in the highest quantity of PDI.
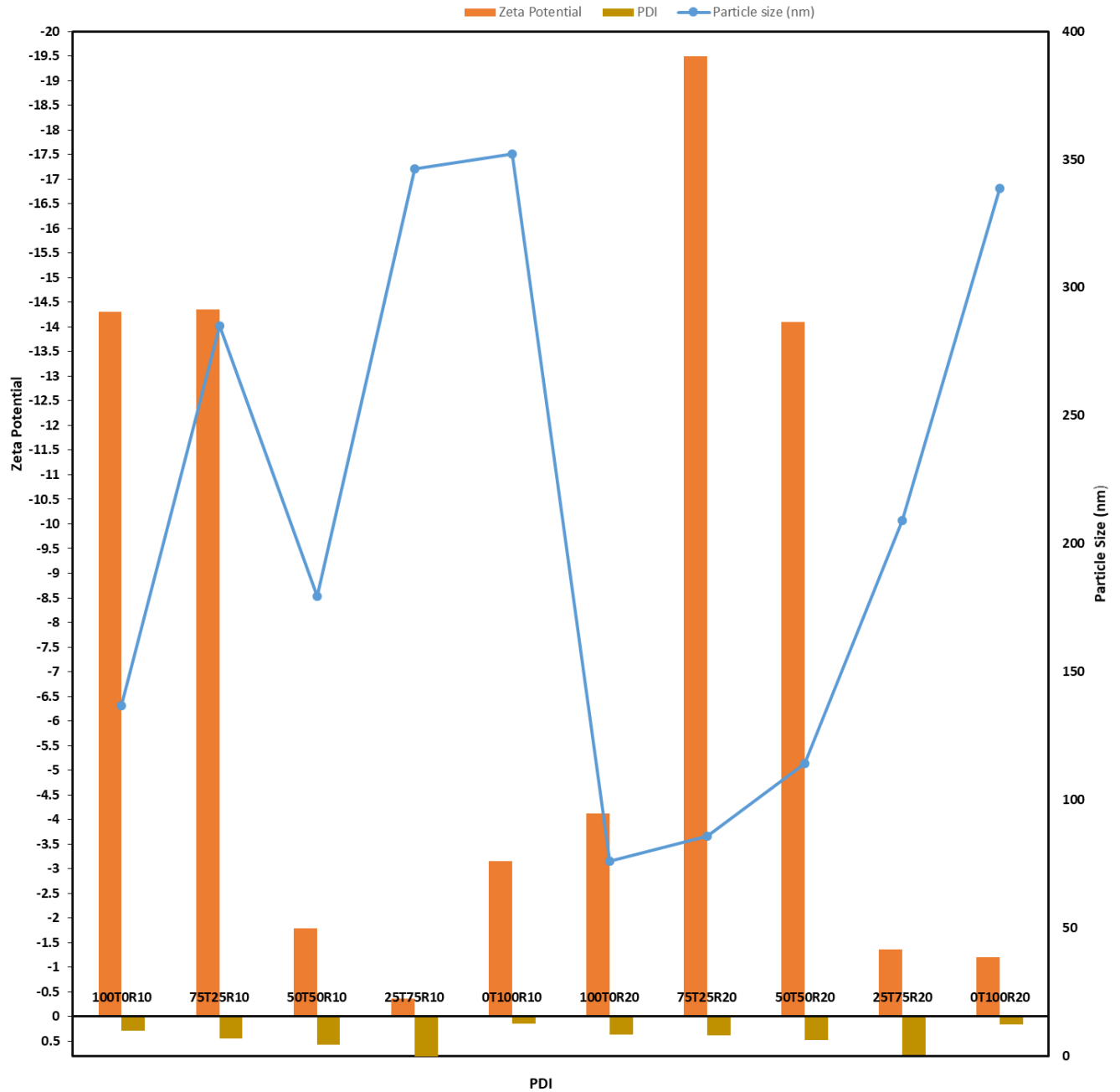
1. Prticle Size, PDI, and Zeta Potential measurment of thymol-resveratrol formed nanocapsules

Samples' particle size increased with an increase in the core-to-wall ratio. According to the results, formulations containing more thymol had the smallest particle size (<100nm), while formulations containing a large amount of resveratrol had the most significant size (>300nm). As a result, formulations with high level of resveratrol produce unstable suspension that contain accumulated particles that lead to the formation of larger particles and a cloudy suspension due to sedimentation in the suspension. This aggregation may be due to the fact that resveratrol nanoparticle surfaces are not saturated with zein and caseinate [47]. In addition, the large particle size in the core-to-wall ratio of 1:10 can be related to the high concentration of the core, increasing the viscosity of the outer phase and accumulating many droplets. In addition, PDI index of less than 0.5 suggests that nanoparticles are distributed uniformly, and a value of more than 0.5 implies aggregated particles. Lower concentrations of thymol and

resveratrol caused PDI levels to rise, while larger 1:10 ratios caused PDI values to rise. The therapy with 100% resveratrol had the lowest quantity of PDI, indicating reduced particle accumulation

The zeta potential of all production treatments was in the negative range, the lowest of which was related to the treatment containing 25% thymol and 75% resveratrol with a core-to-wall ratio of 1:10.The highest was related to the treatment containing 75% thymol and 25% resveratrol with a core-to-wall ratio of 1:20.

The zeta potentials of all treatments were in the range of -0.3 to -0.19mV, which indicates solid electrostatic stabilization. However, the highest amount of zeta potential was related to the treatment containing 25% thymol and 75% resveratrol with a core-to-wall ratio of 1:10. In general, the zeta potential in the mentioned range confirms that thymol and resveratrol have been loaded into the nanoparticles. In addition, the core-to-wall ratio was an essential factor in the deformation and stability of nanoparticles, which was consistent with the findings of Zhang et al.[40].

3.3 Antioxidant activity

Table 4 shows that 75T25R10 with 58.77% had the highest ability to inhibit DPPH free radicals, followed by 25T75R10. The increase in the wall, except for the treatment with 75% thymol, led to a decrease in the ability to inhibit DPPH free radicals

Table 4.Antioxidant activity for nanoparticles containing different core/wall ratios

Treatments	DPPH (%)	FRAP m mol Fe / 100 g sample
100T0R10	20.33 ± 0.33^g	0.107 ± 0.003^h
75T25R10	58.77 ± 1.50^a	0.323 ± 0.002^a
50T50R10	20.67 ± 0.20^g	0.323 ± 0.002^a
25T75R10	38.17 ± 0.30^d	0.217 ± 0.001^d
0T100R10	$\mathbf{13.67\pm0.51^i}$	0.05 ± 0.001^j
100T0R20	32.67 ± 0.10^f	0.176 ± 0.001^f
75T25R20	34.67 ± 0.30^e	0.192 ± 0.002^e
50T50R20	40.00 ± 0.20^c	0.257 ± 0.002^c
25T75R20	54.67 ± 0.50^b	0.298 ± 0.003^b
0T100R20	$\mathbf{17.83\pm0.10^h}$	0.087 ± 0.001^i

*The data are the average of three replicates ± standard deviation, and similar lowercase letters in each column indicate a lack of significance at the 5% level.

The results also indicated that resveratrol inhibited DPPH free radicals by 75% when a 1:20 core-to-wall ratio was used in the formulation of treatments. Thymol is a phenolic acetyl compound with vigorous antioxidant activity. Treatments containing higher amounts of thymol showed higher DPPH than resveratrol.

The results also confirmed the synergistic effect of resveratrol and thymol. However, the amount of DPPH was higher than other studied synergistic compounds, indicating the perfect combination of these two antioxidant compounds. The mutual interaction of thymol with resveratrol leads to a synergistic or antagonistic effect based on their antioxidant capacity. The antioxidant activity of mixtures of phenolic compounds cannot be determined from data obtained for individual compounds due to their unpredictable interactions.

Like the ability to inhibit DPPH free radicals, the treatment containing 75% thymol and 25% resveratrol with a ratio of 1:10 cores had the highest iron ion reduction power, followed the treatment containing 25% thymol and 75% resveratrol with a core-to-wall ratio of 1:20. The increase in the wall, except for the treatment with 75% thymol, led to a decrease in the reducing power of ferric ions. Treatment formulations using 1:20 core-to-wall ratios increased iron ion reduction power by 75% when resveratrol was added to the formulation (table 4).

The reducing power method measures antioxidant activity by reducing iron, which indicates the antioxidant properties of hydrophilic compounds and confirms the data from the DPPH test to a large extent. The reducing power method showed the antioxidant activity of all treatments at different concentrations more clearly.

3.4 Antimicrobial properties of nanoparticles

Table 5 shows the inhibitory activity of the prepared treatments. The highest MIC in all media (culture and milk) was related to the 0T100R10 treatment. Therefore, Listeria bacteria in the TSB culture medium were the most sensitive bacteria to the prepared treatments. Treatment 75T25R10 has the strongest treatment with the lowest MIC with 100, 50, 200, and 100 of *e.coli*, *listeria monocytogenes* in culture, and milk respectively. In most treatments, the bacteria in milk were more resistant to these nanoparticles than the culture medium. MIC is the lowest antibacterial concentration that inhibits a microorganism's visible growth after one day of incubation. MICs can be detected on solid, agar, or diluted liquid media after pure culture isolation.

Table 5. Effect of the type of formulation on the MIC (μg/ml)

Treatments	MIC			
	Culture medium		Milk	
	e.coli	*Listeria monocytogenes*	*e.coli*	*Listeria monocytogenes*
100T0R10	**300**bB	250aC	400aA	300aB
75T25R10	100fB	50dC	200dA	100dB
50T50R10	250cB	250aB	300bA	300aA
25T75R10	200dB	150bC	250cA	200bB
0T100R10	350aB	250aD	400aA	300aC
100T0R20	**200**dB	150bC	300bA	200bB
75T25R20	200dB	150bC	300bA	200bB
50T50R20	150eB	100cC	300bA	150cB
25T75R20	150eB	100cC	250cA	150cB
0T100R20	300bB	250aC	400aA	300aB

*The data are the average of three replicates ± standard deviation, and similar lowercase letters in each column indicate a lack of significance at the 5% level.

The antimicrobial activity of resveratrol is affected by the presence and position of hydroxyl groups on the aromatic ring. Hydrogen bonds can connect hydroxyl groups to enzyme-active sites and disrupt enzyme activity and cell metabolism in bacteria. The growth of gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus cereus,* and *Listeria monocytogenes* was inhibited by phenolic components extracted from *Polygonum cuspidatum* root.

Thymol causes Gram-negative bacteria's outer membrane to separate, releasing lipopolysaccharides while also making the cytoplasmic membrane more permeable. Thymol can also hinder the formation of vegetative bacterial cells and limit the synthesis of toxins by bacteria. Thymol can prevent the synthesis of the toxin generated by Listeria monocytogenes.

The results of the bactericidal activity of the prepared treatments are shown in Table 6. The highest MBC for Escherichia coli (culture medium and milk) was related to the 0T100R10 treatment, but for *Listeria*, there was a correlation between the 100T0R10, 50T50R10, and 0T100R10 treatments. On the other hand, the Listeria bacteria in the culture medium were the most sensitive bacteria to the prepared nanoparticles. The most robust treatment with the lowest MBC was assigned to 75T25R10 with 200, 100, 400, and 200 *e.coli* and *listeria monocytogenes* in culture medium and milk sample respectively. In most treatments, the bacteria in milk were more resistant to these nanoparticles than in the culture medium.

Table 6. Effect of the type of formulation on the MBC (µg/ml)

Treatments	MBC			
	Culture medium		Milk	
	e.coli	*Listeria monocytogenes*	*e.coli*	*Listeria monocytogenes*
100T0R10	600^{bB}	500^{aC}	700^{bA}	600^{aB}
75T25R10	200^{gB}	100^{fC}	400^{eA}	200^{eB}
50T50R10	500^{cB}	500^{aB}	600^{cA}	500^{bB}
25T75R10	400^{dB}	300^{cC}	500^{dA}	400^{cB}
0T100R10	700^{aB}	500^{aC}	800^{aA}	500^{bC}
100T0R20	300^{eC}	200^{dD}	600^{cA}	400^{cB}
75T25R20	300^{eC}	300^{cC}	600^{cA}	400^{cB}
50T50R20	250^{fC}	150^{eD}	600^{cA}	300^{dB}
25T75R20	250^{fC}	200^{dD}	500^{dA}	300^{dB}
0T100R20	400^{dC}	350^{bD}	700^{bA}	600^{aB}

*The data are the average of three replicates ± standard deviation, and similar lowercase letters in each column indicate a lack of significance at the 5% level.

MBC is the lowest concentration of an antimicrobial compound necessary to cause bacterial death, while MIC is the lowest concentration required to prevent the visible growth of bacteria. As the MIC approaches the MBC, the compound becomes more antibacterial. The antimicrobial effect of resveratrol and thymol alone has been investigated in different studies. However, the synergistic effect of these two compounds in nanoparticle form has not been studied. The results of MIC and MBC in the culture medium and milk showed a very high antimicrobial effect of these two compounds in the form of nanoparticles compared many other antimicrobial substances and confirmed this formulation.

3.5 Solubility and stability

The results showed that 100T0R20 has the highest solubility of formed nanoparticles in the milk medium and the lowest solubility was assigned to 0T100R10 with 71.67 and 24 % respectively (table 7). The solubility of the treatments decreased with the increase in the core-to-wall ratio in all treatments. The solubility decreased with the rise of resveratrol when the core-to-wall ratio was 1:20. This reduction in resveratrol solubility can be attributed to the reaction between these compounds (thymol and resveratrol) with the proteins in milk, which reduces their solubility.

Table 7. Solubility of prepared nanoparticles

Treatment	Solubility (%)
100T0R10	59.00±1.00 [d]
75T25R10	35.67±0.50 [g]
50T50R10	49.00±0.80 [e]
25T75R10	27.33±1.20 [i]
0T100R10	24.00±0.90 [j]
100T0R20	71.67±0.20 [a]
75T25R20	66.67±0.33 [b]
50T50R20	62.67±0.44 [c]
25T75R20	42.00±0.60 [f]
0T100R20	31.33±0.77 [h]

*The data are the average of three replicates ± standard deviation, and similar lowercase letters in each column indicate a lack of significance at the 5% level.

After being lyophilized, the redistribution of zein and caseinate nanoparticles loaded with thymol and resveratrol was compared. The outcomes demonstrated that following light shaking, the 100T0R10, 75T25R10, 75T25R20, and 50T50R20 treatments redispersed and created uniform dispersion systems. The dispersion remained practically transparent, and the nanoparticles were entirely deposited at the bottom of the bottle after a gentle shake. The redispersibility observation supports the particle size and zeta potential results. Due to their reduced size and increased zeta potential, these colloidal systems had improved repeatability and stability. After gentle shaking in various treatments, they redispersed in water and created homogenous and milky dispersion systems.

3.6 Release profiles

To check the kinetic release of the prepared NPs, 75T25R10 nanocapsule (75% thymol and 25% resveratrol) was released in the buffer media. Figure 2 shows the release percentage as it changes over time in the buffer environment.
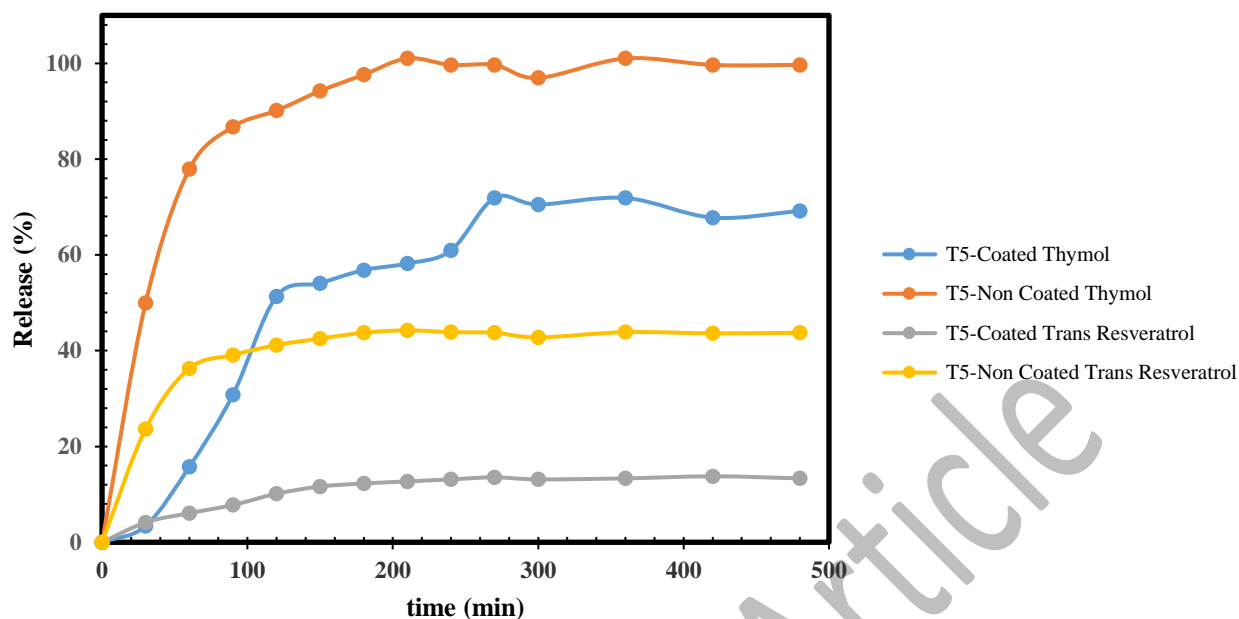
Fig 2. Kinetic release profiles of nanoparticles (sample 75T25R10) in the milli-Q water medium

Resveratrol and thymol were released quickly in the first hour but remained consistent in uncoated samples. Due to the protective nature of the wall material, the samples coated with casein and zein, on the other hand, exhibited a progressive release. The maximum release of resveratrol occurred at 420 minutes, or 42.3% and 13.77%, respectively, in the coated and uncoated samples. The release rate then gradually decreased, reaching its peak for both coated and uncoated thymol at 270 minutes, or 93.5% and 71.88%, respectively. Thymol didn't start to leak slowly until 120 minutes later, whereas resveratrol usually did. The higher release rate and more thymol explosiveness than resveratrol were due to the more significant loading of thymol in the nanoparticle matrix. The percentage of drug release increased with increasing loading. In pervious researches, such as nanoliposome carrying epigallocatechingallate (EG), 4.92% of the uncoated (EG) released within 6h of the bag Dialysis in the buffer medium, while about 6 and 12% of the EG from the nanoliposome enter the buffer medium within 6 and 24h [48]. These results were consistent with those of the present research.

3.7. FT-IR analysis

In this research, FT-IR applied to determine active compounds have been loaded successfully. FT-IR spectrum depicted bellow 1000 cm^{-1}, like 704 cm^{-1}, corresponding to the skeleton vibration of benzene ring trisubstitution; 1080 cm^{-1} assigning to stretching vibration peak of methyl in thymol. ; 29030 cm^{-1} corresponding to methyl group vibration peak; 3380 cm^{-1} belonging to O-H vibration peak. In the case of 75T25R10, they display two absorption peaks at 1660 and 1530 cm^{-1}, which can be attributed to amide I band and amid II band, respectively. A wide absorption peak was observed between 29300 and 3360 cm^{-1} due to the stretching vibration of O-H and N-H [42,49]. 100T0R10 and 0T100R100 on the other hand, do not exhibit the absorption peak between 704 and 1000 cm^{-1}. In contrast, 75T25R10 showed these characteristic absorptions of thymol, indicating that zein nanoparticles were loaded with thymol. The hydrophobic interaction and hydrogen bonds between them may be responsible for their successful combination [42,50]. As reported previously, the hydrophobic interaction between zein and thymol is due to the fact that both are highly hydrophobic molecules [51]. In addition, the hydrogen bond between

them has the potential to widen the absorption peak at 3380 cm^{-1}. In fact, when comparing 75T25R100 to other formulations, there is an expected variation at the aforementioned absorption peaks of them.
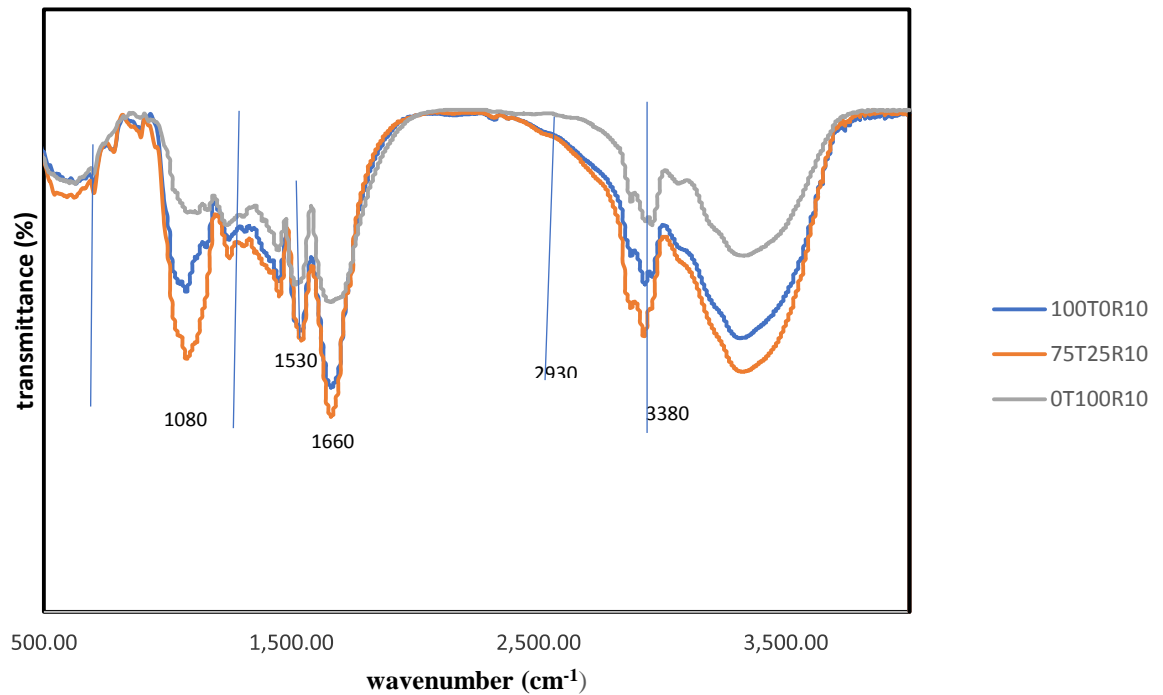

**Figure3. FT-IR spectra results of samples**

3.8 SEM Results

Morphology of 100T0R10, 0T100R10, and 75T25R10 samples are illustrated in figure 4 to examine the structure of NPs without thymol (0T100R10) and resveratrol (100T0R10) as well as NPs of components mixture (75T25R10). The particle size increased as the amount of thymol added increased. This phenomenon may be explained by the increased amount of hydrophobic thymol incorporated within zein nanoparticles makes the surface of NPs more hydrophobic [52].
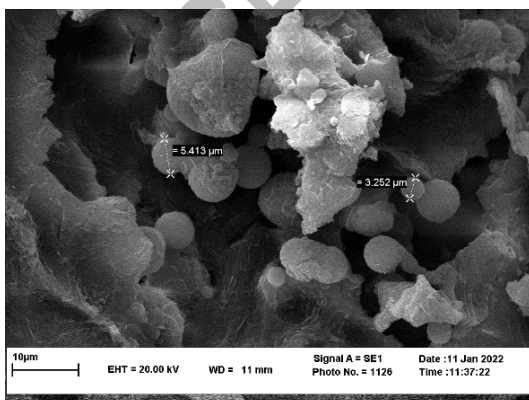
a) 0T100R10

b) 100T0R10
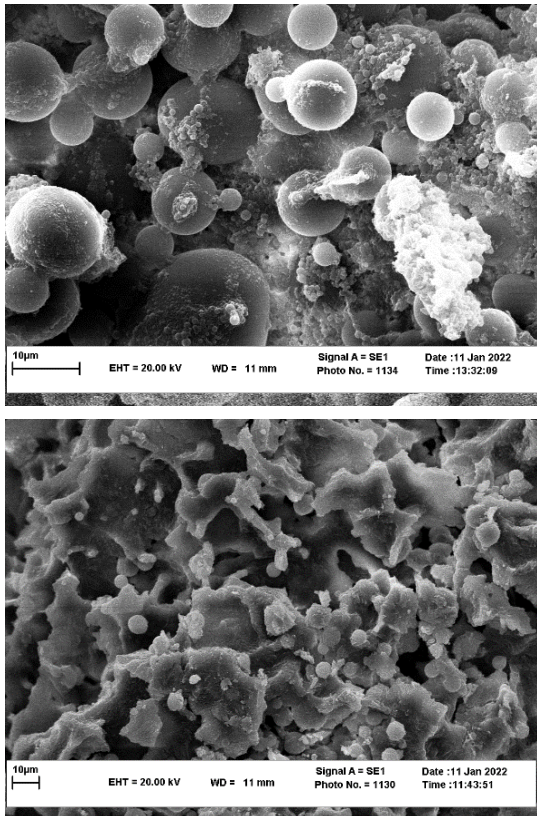
c) 75T25R10

Figure 4. SEM images of a) 0T100R10, b) 100T0R20, c) 75T25R10

On the other hand, by boosting resveratrol levels and lowering thymol levels, the quantity of aggregation and homogeneity were reduced (figure 4C). As a result, 75R25T10 sample showed no surface indentation or, breaking part, and the particles were uniformly spherical. The particle size and homogeneity of zein-casein nanocapsules increased, when thymol and eugenol were added [53]. Coaxial nanofibers with zein walls that included thymol were the subject of an investigation by Liu et al. [19].

4. Conclusion

In this research, "liquid-liquid dispersion" method has been used to prepare the thymol-resveratrol-loaded casein-zein nanoparticles successfully to present a novel nancapsule as an organic additive to enhance antioxidant and anti-bacterial property of dairy products instead of toxin chemical additives. Casein-zein nanoparticles combine with hydrophobic compounds through hydrogen bonding and hydrophobic interaction, showed great improvement of stability, antioxidant, and antibacterial according to a series of characterization methods .

For this purpose, stability, particle size, and polydispersity of the formed nancapsules were measured and found 25T75R20 with the zeta potential of -19.54 as the most stable formula. In addition. Particles size were decreased from 338.65 nm for 100T0R20 to 85.75nm for 75T25R20, due to the thymol and resveratrol interaction .

Furthermore, the as-prepared NPs were quasi-spherical with a narrow size distribution and good stability, as shown by redispersibility and storage experiments. The thymol and resveratrol encapsulation efficiency were 39.48% and 83.26%, respectively, at the optimal preparation condition (75T25R10) .

The antioxidant property of formed nanocapsules were determined by applying DPPH and FRAP methods, also, the antibacterial activity of the prepared nancapsules measured against E. coli and listeria monocytogenes in the

culture and milk mediums. The significant improvement of antioxidant and antibacterial activity were confirmed the synergic effect of thymol and resveratrol compounds .

Hence, the findings from this study suggest that the studied delivery system has the potential to improve functional properties of hydrophobic active compounds for use in the fields of food industry specifically dairy products.

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