Nanoliposome-encapsulated Phenolic Rich Fraction From *Alcea Rosea* as a Dietary Phytobiotic in Mice Challenged by *Escherichia Coli*

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Abstract

Background: This research was performed to evaluate the antibacterial and health-promoting potentials of the nanoliposome-encapsulated phenolic rich fraction (PRF) from *Alcea rosea* leaves as a dietary phytobiotic in mice challenged by enteropathogenic *Escherichia coli* (*E. coli*; O157: H7).

Results: The overall results demonstrated that the nanoliposome-loaded PRF contained gallic acid, salicylic acid, pyrogallol, cinnamic acid, catechin, naringin, ferulic acid. The *E. coli* challenge in mice impaired the weight gain, food intake, liver enzymes, lipid peroxidation, morphometric characteristics of the ileum, up-regulated the inflammatory genes (COX2, iNOS), down-regulated the antioxidant-related genes (SOD and GPx) and increased the population of *E. coli* in the ileum. The dietary inclusion of nonencapsulated PRF and nanoliposome-encapsulated PRF at the concentration of 10 mg TPC/kg BW/day improved these parameters however the nanoliposome-encapsulated PRF appeared to be more effective as compared to nonencapsulated PRF in improving the health parameters in mice.

Conclusion: Consequently, the nanoliposome-encapsulated PRF could play a critical role as a promising phytobiotic against *E. coli* infection in mice.

Background

Various antibiotics such as β -lactams, chloramphenicol, tetracyclines, aminoglycosides, macrolides, glycopeptides, quinolones, streptogramins, oxazolidinones, lipopeptides and mutilins have been developed to cure infectious diseases [1]. Infectious diseases are increasingly an important public health issue. They are among the major causes of death worldwide. The increase is mainly attributed to the development of antibiotic-resistant strains such as *Pseudomonas aeruginosa*, *Escherichia coli* (*E. coli*), *Proteus vulgaris*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Salmonella typhi* and many more [2, 3]. The impact of antibiotic resistance such as the rapid increase in the cost of drugs and increase in morbidity and mortality [4, 5] has spearheaded the search for new alternative sources of antimicrobial agents that are effective, cheap, accessible and possess few or no undesirable side effects such as bioactive constituents of plants [6–8].

Several studies have been confirmed the natural products possess higher chemical novelty than chemically synthesized products and thus this fact has led to the search of active compounds in plants including flavonoid and phenolic compounds. These compounds have been reported to be potentially significant in therapeutic applications as antimicrobial agents. They offer major opportunities for finding novel low molecular weight molecules that are active against microbes [9, 10].

Alcea rosea L. (*A. rosea*) belongs to the family of *Malvaceae* and is a famous and common name as Hollyhock. It is an ornamental plant that is widely grown in the eastern Mediterranean to central Asia [11]. Most of the subspecies have been reported mainly found in Iran and Turkey [12]. Different aerial parts of plants including seed, root, leave and flower has been applied in traditional medicine like irritated stomach, throat pain, fever, kidney pain, and antimicrobial and anti-inflammatory agent [12, 13]. Most of

these biological properties in this plant related to the natural bioactive compound especially phenolic compounds. Phenolic compounds consider as abundant and interesting micronutrients due to its outstanding pharmaceutical properties such as natural antioxidant and antimicrobial potential [14, 15]. Despite their wide range of biological potentials of phenolic compounds, the low water solubility and poor absorption are the main problems that appear to remain difficult to be employed clinically. The encapsulation using liposomal technology as carriers aimed at enhancing the bioavailability of pharmaceuticals and nutraceuticals benefits of polyphenols [16].

Liposomes consist of one or more spherical lipid bilayers encapsulating bioactive molecules. They can entrap both lipophilic and hydrophilic molecules in their unique individual structure [16]. In such a way that lipophilic molecules are inserted into the phospholipid bilayer membrane, and hydrophilic molecules are encapsulated into the aqueous center of the liposome. In other words, the nanoliposomes' internal compartment is filled with a polar liquid media such as a buffer or water containing dissolved hydrophilic compounds [17]. The nano-scaled liposomes called nanoliposomes as the targeted-drug delivery systems are the most applicable nano-carriers in the pharmaceutical industry. They have successfully improved the therapeutics' efficiency in a wide range of biomedical applications. Nanoliposomes facilitate the cellular uptake of their contents and efficiently stabilize them [18, 19]. This research was performed to synthesis the nanoliposome-encapsulated phenolic rich fraction from *A. rosea* and to evaluate its antibacterial and health-promoting activities in mice challenged by enteropathogenic *E. coli* (O157: H7).

Materials And Methods

Plant material and reagents

The fresh leaves of *A. rosea* were purchased from the herbal medicine market of Mashhad, Iran. The soybean lecithin (purity of 99%) was purchased from Sigma Aldrich (Germany). The *E. coli* (O157: H7) as a food-borne pathogen was obtained from the microbial culture collection of Islamic Azad University of Mashhad, Iran. For the gene expression analysis the RNeasy Mini kit (Qiagen, Hilden, Germany), cDNA synthesis Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany), and SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) were used. The DNA extraction kit used in this study was QIAamp DNA Stool Mini Kit from Qiagen GmbH, Hilden, Germany. The other reagents not mentioned here were from Merck (Germany).

Fractionation and total phenolic determination

In the first step, the fresh leaves were cleaned by sterile distilled water, dried in shadow for two weeks in room temperature. The dried leaves finely ground (powder form) using a grinder mill. Then, the 100g of the dried powder was extracted with 900 mL aqueous methanol (80% (v/v)) and 100 mL of 6 M HCl using the reflux method for 2 hours [31]. Finally, the extract was filtered and the filtrate was evaporated at the temperature of 60°C by rotary evaporator (Buchi, Flawil, Switzerland). In the second step, the dried aqueous-methanolic extract was fractionated using separating funnel and different solvents including

hexane, chloroform, ethyl acetate, n-butanol, and water-based on the Oskoueian et al [32]. Upon fractionation, the supernatant was filtered and concentrated using a vacuumed rotary evaporator. The total phenolic compounds (TPC) evaluation of each fraction was carried out by adding 0.1 ml of the extract, 2.5 ml of Folin-Ciocalteu reagent (1:10 v/v), and 2 ml of 7.5 % sodium carbonate into a test tube covered with aluminum foil. The test tubes were vortexed and the absorbance was measured at 765 nm [32]. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight. The fraction containing the highest phenolic content is named as a phenolic rich fraction (PRF).

Nanoliposomes preparation

The four grams of lecithin were agitated for 2 h by 196 g of hot water (80 °C) using stirrer at 300 rpm. Then, the PRF after dissolving in ethanol was added to the mixture and stirred for 2h to reach the final concentration of 2000 ppm. Finally, the solution was bath-sonicated at 80% power (Sonorex RK100, Germany) during 4 to 6 minutes and the obtained nanoliposomes-loaded PRF from *A. rosea* was prepared and used for further characterization.

Characterization of nanoliposomes

At the beginning, the nanoliposomes-loaded PRF was diluted by water (1:20) to decrease the aggregation and inhibit the noise scattering. The dynamic light scattering (DLS) method was performed to determine the average size of particles and their stability (zeta potential). The measurements were analyzed three times by a Malvern Zetasizer Nano ZS (Malvern, UK). Moreover, Field Emission Scanning Electron Microscopy (FESEM) was applied to verify the shape and nanoliposomes size dimensions. The total phenolic content of nanoliposomes was determined as described earlier in the fractionation section [32].

Phenolic profiling of nanoliposomes

The Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) analysis was carried out to investigate the types of phenolic compounds present in the nanoliposomes. Briefly, the solvents comprising of deionized water (solvent A) and acetonitrile (solvent B), the pH of solvent A was adjusted to 2.5 using concentrated trifluoroacetic acid. The column was equilibrated by 85 % solvent A and 15 % solvent B for 15 min before injection. Then, the ratio of solvent B was increased to 85 % after 50 min. After 5 min (at the 55th minute of running the experiment), the ratio of solvent B was reduced to 15 %. This ratio was maintained for 60 min for the next analysis with a flow rate of 1 ml/min. An analytical column (Intersil ODS-3 5um 4.6×150 mm GI Science Inc. USA) was used for the detection of phenolic at 280 nm. The phenolic standards used in this study were gallic acid, syringic acid, vanillic acid, salicylic acid, caffeic acid, pyrogallol, catechin, cinnamic acid, ellagic acid, naringin, chrysin, and ferulic acid.

Animal trial

The 40 white male Balb/c mice (20-25 g) were obtained from the Razi vaccine and serum research institute of Mashhad. The mice were kept in the individual cages at 58%±10% humidity and 23°C±1°C

with 12-hour light/dark periods for 7 days to be adapted with lab condition. We divided the mice into four groups of ten. With free access to the standard pellet diet (Javaneh Khorasan, Mashhad, Iran) and tap water. The experimental treatments were as follow:

T1: normal diet

T2: normal diet +infected by *E. coli* (O157: H7) on day 21

T3: Normal diet enriched by nonencapsulated PRF (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21

T4: Normal diet enriched by nanoliposome-encapsulated PRF (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21

All mice received experimental treatments for 4 weeks and the oral infection through gavage needle (10^8 CFU of *Escherichia coli* O157: H7) was conducted for once on day 21. Animals were monitored daily for general health and the amount of food eaten. At the end of the experiment (day 28), the mice were euthanized with pentobarbital-HCL (50 mg/kg, i.p.) and sacrificed. The blood, liver, and ileum samples were collected immediately and used for liver enzyme analysis, lipid peroxidation assay, gene expression analysis, and morphometric evaluation of ileum, respectively. The mice were weighed three times at the beginning, middle, and at the end of the experiment. All animal experiments were conducted according to the ethical principles approved by the Islamic Azad University of Mashhad with the code of ethics IR.IAU.MSHD.REC.1399.016.

Liver enzymes and lipid peroxidation assay

The main liver enzymes in the serum including alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) were determined using blood auto-analyzer (Hitachi 902, Japan). The lipid peroxidation in the liver tissue was determined as described earlier by Shafaei et al. [33]. Briefly, the liver tissue was homogenized and 200 μ l of lysate were mixed by distilled water (300 μ l), BHT (35 μ l), sodium dodecyl sulfate (165 μ l) and thiobarbituric acid (2 ml) respectively. Then, after heating (90°C for 60 min) the cooled solution was mixed by 2 mL of n-butanol and centrifuged at 2000 $\times$ g for 5 min. Finally, the absorbance of the n-butanol part was read at 532 nm and the results were expressed as percentage malondialdehyde (MDA) changes relative to the control.

Histopathology and morphometric analyses

At the end of the *in vivo* experiment, the mice were sacrificed and the liver, kidney, and ileum were separated and washed using the physiological serum. Then, they were fixed in buffered formalin (10% formalin in 0.1M sodium phosphate buffer, pH7). Finally, they were paraffinized, sliced, and stained according to the hematoxylin/eosin protocol [33]. The histopathology slides were observed under a light microscope using a magnification of 20X. The morpho-structural characteristics of ileum including villus height, villus width, crypt depth, and goblet cell count were determined [34].

Gene expression analysis

To investigate the response of ileum tissue to different treatments, the expression of major inflammation biomarker genes such as cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS) and antioxidant-related genes including superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined. The mice’s ileum tissues which were freshly frozen in the liquid nitrogen, crushed and prepared for RNA extraction by an RNeasy Mini kit (Qiagen, Hilden, Germany) following the recommended protocols. Then, cDNA was synthesized using a Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Next, the sets of primer sequences for the key genes and a housekeeping (Beta-actin) gene were applied in the experiment as shown in Table 1. The SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used in a comparative Real-time PCR (Roche Diagnostics). The targeted genes were amplified as follows: 95°C for 5 min (1X) for initial denaturation, followed by 35 cycles of 95°C for 30s, primer annealing at 60 and 58 for 30s for the inflammatory genes and antioxidant genes, respectively and extension of 72°C for 30s. The expressions of genes were normalized to beta-actin as a reference gene and then normalized to the expression of respective genes in the control group [35]. The characteristics of the primer used in this study are presented in Table 7.

Table 7. The primer sets characteristics used in this study.

Gene	Forward (5′ →3′)	Reverse (5′ →3′)	References
COX2	caagcagtggcaaaggcctcca	ggcacttgcatgatggtggct	[36]
iNOS	caccttggaagttcacccagt	accactcgtacttgggatgc	[37]
SOD	gagacctgggcaatgtgact	gtttactgcgcaatcccaat	[35]
GPx	caagttttgatgccctggt	tcggacgtacttgagggaat	[35]
β-actin	cctgaaccctaaggccaacc	cagctgtggtggtgaagctg	[33]

E. coli population analysis

The real-time PCR (LightCycler 96 instrument, Roche, Basel, Switzerland) was used to determine the fold changes of *E. coli* (O157: H7) population in the ileum digesta. Since the major sites of microbial fermentation and propagation and colonization of enteropathogens in the monogastric is the ileum, that is why in this study the population of *E. coli* was analyzed only in the ileum section. The real-time PCR condition was 95°C for 5 min (1X) for initial denaturation, followed by 35 cycles of 95°C for 30s, primer annealing at 60, and 55 for 25s for the *E. coli* and total bacteria, respectively and extension of 72°C for 20s. The primer characteristics are shown in Table 8. The DNA from ileum digesta was extracted using QIAamp DNA Stool, extraction kit (QIAGEN, Germany). The SYBR GREEN Master Mix (BIOFACT, Korea) was used in this study. The previously published primers were used for quantitative real-time PCR assay. The real-time PCR data were analyzed using $\Delta\Delta C_t$ method to determine the fold changes in the *E. coli* bacteria population and the data were expressed as fold changes of *E. coli* relative to the total bacteria [38, 39].

Table 8. The list of the primers used for ileum microbial population analysis

Bacteria	Forward (5' →3')	Reverse (5' →3')	References
<i>E. coli</i> (O157:H7)	ttaccagcgataccaagagc	caacatgaccgatgacaagg	[39]
Total bacteria	cggcaacgagcgcaaccc	ccattgtagcacg tgtgtagcc	[40]

Statistical Analysis

The data obtained were subjected to the general linear models (GLM) procedure of SAS [(9.1) (2002-2003) (SAS Institute Inc., Cary, NC, USA)] in a completely randomized design (CRD) and the means were compared with Duncan's Multiple Range Test. The difference was considered significant when the P-value was < 0.05.

Results And Discussion

Fractionation and total phenolic determination

The fractionation with different polarity solvents resulted in the extraction of most of the phenolic compounds from leaves of *A. rosea* in different quantities which the highest phenolic compounds detected in ethyl acetate fraction 26.1 ± 4.36 followed by n-butanol (16.7 ± 3.29) > water (12.6 ± 3.94) > chloroform (8.3 ± 2.76) > hexane (7.5 ± 2.63) mg GAE/g DW of extract, respectively. The ethyl acetate fraction appeared to be the fraction with the highest content of phenolic compounds hence named as a phenolic rich fraction (PRF) and was used for further experiments. The earlier studies observed the highest phenolic concentration in the ethyl acetate fraction when different polarity solvents were applied for the extraction of phenolic compounds [20, 21].

Physicochemical characteristics of PRF-loaded nanoliposome

The characteristics of nanoliposomes-loaded PRF are shown in Table 1. The particle size is an important factor in the stability and bioavailability of nanoliposomes. The particle size was 254.3 ± 7.91 nm that displayed the nanometre size of the particles. As indicated in Table 3, the PDI values were reported 0.31 ± 0.08 indicating the homogenous dispersion [22]. The zeta potential values as the marker of the stability behavior of a colloid showed moderate stability of colloidal dispersion with the value of -34.04 ± 0.6 mV based on the classification of Kumar et al. [23]. The FESEM image presented in Figure 1 displayed the spherical shape of nanoliposomes. The nanoliposome contained total phenolic compounds of 1.9 ± 0.12 mg GAE / g DW.

Table 1. Physical and phytochemical characteristics of nanoliposome-loaded by *A. rosea* phenolic rich fraction

Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Total phenolics (mg GAE / g DW)
254.3±7.91	0.31±0.08	-34.04±0.6	1.9±0.12

Phenolic compounds analysis

The profiling of phenolic compounds presented in the nanoliposomes is shown in Table 2. The obtained results illustrated various types of phenolic compounds in the nanoliposomes whereas the cinnamic acid and gallic acid are the main and abundant phenolic compounds with the respective values of 805±8.2 and 728.2±3.1 µg/g DW.

Table 2. Phenolic compounds presented in the nanoliposome-loaded by *A. rosea* phenolic rich fraction

Phenolic compounds contents (µg/g DW)						
GA	SA	PY	CA	CT	NA	FE
728.2±3.1	577±6.4	411±6.5	805±8.2	236±5.7	342±3.3	221±6.2

GA: gallic acid; SA: salicylic acid; PY: pyrogallol; CA: cinnamic acid; CT: catechin; NA: naringin; FE: ferulic acid. The analyses were performed in triplicates

Animal trial

The average daily weight gain and feed intake are shown in Table 3. The results revealed that the *E. coli* challenge (T2) impaired the daily weight gain and feed intake significantly ($p<0.05$) as compared to the group of un-challenged mice (T1). Moreover, the dietary addition of phytogetic compounds in the forms of nonencapsulated PRF and nanoliposome-encapsulated PRF in the groups of *E. coli*-challenged mice (T3 & T4) improved these parameters significantly ($p<0.05$). The results confirmed that dietary incorporation of phytogetic compounds in the form of nanoliposome-encapsulated PRF was more effective than in the form of nonencapsulated PRF in improving the daily weight gain and feed intake. The observed improvement in the daily weight gain and food intake at the presence of dietary phytogetic supplements could be associated to the antioxidant and antibacterial activity of bioactive compounds detected in the PRF such as gallic acid, salicylic acid, pyrogallol, cinnamic acid, catechin, naringin, ferulic acid.

Table 3. The averages of mice body weight changes and feed intake during experiment receiving different treatments

Average	T1	T2	T3	T4	SEM
Average daily weight gain	0.23 ^a	0.16 ^{cd}	0.18 ^c	0.21 ^{ab}	0.06
Average daily feed intake	3.5 ^a	2.6 ^c	2.9 ^{bc}	3.1 ^b	0.12

T1: normal diet; T2: normal diet +infected by *E. coli* (O157:H7) on day 21; T3: Normal diet enriched by nonencapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157:H7) on day 21; T4: Normal diet enriched by nanoliposome-encapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157:H7) on day 21

Different letters in the same raw indicated significant difference (p<0.05)

The analysis were performed in triplicates

Liver enzymes and lipid peroxidation analysis

The results of liver enzymes analysis and lipid peroxidation in the liver tissue are presented in Table 4. Similar to the observation made in Table 3, the liver enzymes and lipid peroxidation increased significantly (p<0.05) upon *E. coli* challenge when compared to the un-challenged group of mice. Further, the inclusion of nonencapsulated PRF and nanoliposome-encapsulated PRF alleviated the liver enzymes (SGOT, SGPT, ALP) and lipid peroxidation in the liver tissue significantly (p<0.05). In line with the results observed in daily weight gain and food intake, providing PRF in the form of nanoliposome-encapsulated PRF was more effective in modulation of liver enzymes and lipid peroxidation as compared to the nonencapsulated PRF. In summary, these results postulated that enteropathogenic *E. coli* induced oxidative stress in the liver as indicated by lipid peroxidation and resulted in liver malfunction as shown by the increase in the liver enzymes. The antioxidant compounds detected in the PRF such as gallic acid, salicylic acid, pyrogallol, cinnamic acid, catechin, naringin, ferulic acid could alleviate the oxidative stress caused by *E. coli* and enhanced the function of the liver. Consistent with the results observed in this study early reports indicated the hepatoprotective activity of phenolic compounds against enteropathogens endotoxins [24].

Table 4. The results of liver enzymes analysis and lipid peroxidation in the liver tissue

Parameters	T1	T2	T3	T4	SEM
SGOT (IU/L)	129.8 ^d	173.1 ^a	157.2 ^b	142.1 ^c	4.67
SGPT (IU/L)	106.1 ^d	169.4 ^a	136.4 ^b	124.6 ^c	5.63
ALP (IU/L)	147.3 ^d	215.6 ^a	171.4 ^b	158.9 ^c	7.42
MDA* (%)	100 ^d	173 ^a	156 ^b	142 ^c	4.49

T1: normal diet; T2: normal diet +infected by *E. coli* (O157:H7) on day 21; T3: Normal diet enriched by nonencapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157:H7) on day 21; T4: Normal diet enriched by nanoliposome-encapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157:H7) on day 21

Different letters in the same raw indicated significant difference (p<0.05)

*Expressed as malondialdehyde changes relative to the control group (T1)

The analysis were performed in triplicates

Histopathology and morphometric analyses

The histopathological characteristics of mice liver, kidney and ileum upon treatment by pathogenic bacteria, nonencapsulated PRF, and nanoliposome-encapsulated PRF are presented in Figure 2. The results showed normal architecture of the liver, kidney, and ileum in the control group (T1). The administration of *E. coli* (O157: H7) in the days of 21 did not induce any prominent histomorphological changes in the liver and kidney tissues. Furthermore, it was obvious from the results that the treatment of mice challenged by *E. coli* (O157: H7) bacteria using nonencapsulated PRF and nanoliposome-encapsulated PRF did not affect the histomorphology of the liver and kidney.

The morphometric analysis of ileum including villus height, villus width, and crypt depth and the number of goblet cells in treated mice are reported in Table 5. The mice challenged by *E. coli* showed a significant (p<0.05) decrease in the villus height, villus width, and the numbers of goblet cells. The *E. coli* infection increased the crypt depth significantly (p<0.05). The dietary inclusion of 10 mg TPC/kg BW/day from nonencapsulated PRF and nanoliposome-encapsulated PRF significantly (p<0.05) improved the villus height and width, crypt depth together with the number of goblet cells. These results were consistent with the early studies who reported the role of plant bioactive compounds in improving the morphostructure of ileum in enteropathogens infected or uninfected rabbit [25] pig [26] rat [27] and broiler chickens [28].

Table 5. Morphometric analysis of ileum upon different treatments

Parameters	T1	T2	T3	T4	SEM
Villus Height (µm)	438.2 ^a	327.6 ^d	396.2 ^c	414.8 ^b	6.41
Villus Width (µm)	115.1 ^a	89.6 ^d	94.2 ^c	100.7 ^b	4.23
Crypt Depth (µm)	140.3 ^d	192.8 ^a	182.1 ^b	169.7 ^c	4.66
Mean number of Goblet cells	5.5 ^a	3.7 ^c	4.3 ^b	4.4 ^b	0.47

T1: normal diet; T2: normal diet +infected by *E. coli* (O157: H7) on day 21; T3: Normal diet enriched by a nonencapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21; T4: Normal diet enriched by nanoliposome-encapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157:H7) on day 21

Different letters in the same raw indicated significant difference (p<0.05)

The analysis were performed in triplicates

Gene expression analysis

The expression of COX2 and iNOS as major biomarkers of inflammation and SOD and GPx genes as biomarkers of antioxidant activity in the ileum are shown in Table 6. The infection caused by *E. coil* (T2) significantly (p<0.05) up-regulated the expression of COX2 and iNOS and down-regulated the expression of SOD and GPx as compared to uninfected mice (T1). The dietary addition of 10 mg TPC/kg BW/day from nonencapsulated PRF and nanoliposome-encapsulated PRF could significantly (p<0.05) suppressed the COX2 and iNOS as inflammatory markers and enhanced the expression of SOD and GPx genes as an indicator of cellular antioxidant redox potential. The regulation of inflammatory- and antioxidant-related genes is attributed to the anti-inflammatory and antioxidant activities of bioactive compounds detected in the PRF such as gallic acid, salicylic acid, pyrogallol, cinnamic acid, catechin, naringin, ferulic acid [29].

Table 6. Gene expression analysis of the mice received different treatments

Genes	Fold changes				SEM
	T1	T2	T3	T4	
COX2	1.0 ^d	+5.1 ^a	+3.1 ^b	+1.1 ^c	0.06
iNOS	1.0 ^d	+3.1 ^a	+2.2 ^b	+1.3 ^c	0.17
SOD	1.0 ^c	-1.3 ^d	+1.2 ^b	+1.9 ^a	0.09
GPx	1.0 ^c	-1.6 ^d	+0.4 ^b	+1.3 ^a	0.14

T1: normal diet; T2: normal diet +infected by *E. coli* (O157: H7) on day 21; T3: Normal diet enriched by nonencapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21; T4: Normal diet enriched by nanoliposome-encapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21

Different letters in the same column indicated a significant difference (p<0.05)

The analysis was performed in triplicates

***E. coli* population analysis**

Figure 3 illustrated the relative changes in the *E. coli* population upon different treatments. As compared to the uninfected mice, the population of *E. coli* in the ileum of infected mice increased significantly (p<0.05) by 12.6 folds. The dietary supplementation of nonencapsulated PRF and nanoliposome-encapsulated PRF significantly (p<0.05) modulated the population of *E. coli* in the ileum by 7.9 and 4.4 folds, respectively. It seemed that nanoliposome-encapsulated PRF was more potent in inhibiting the population of enteropathogenic *E. coli* in the ileum as compared to the nonencapsulated PRF. Hence, the nanoliposome-encapsulated PRF could play a role as promising phytobiotic against *E. coli* infection in mice. The inhibition of *E. coli* in the ileum by nanoliposome-encapsulated PRF might be due to the antibacterial activity of bioactive phenolics including gallic acid, salicylic acid, pyrogallol, cinnamic acid, catechin, naringin and ferulic acid in the PRF [30].

Conclusion

The dietary inclusion of nonencapsulated PRF and nanoliposome-encapsulated PRF at the concentration of 10 mg TPC/kg BW/day improved the health parameters in the mice challenged by enteropathogenic *E. coli*, however, the nanoliposome-encapsulated PRF appeared to be more effective as compared to nonencapsulated PRF in improving the health parameters. Consequently, the nanoliposome-encapsulated PRF could play a critical role as a promising phytobiotic against *E. coli* infection in mice.

Declarations

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Authors' contributions

NH : Study design, experimental work and writing original draft; EK and EO: analysis, methodology, project administration, supervision, review, and editing of the original draft; All authors read and approved the final manuscript.

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Availability of data and materials

The datasets applied during the current study are available on reasonable request.

Ethics approval and consent to participate

All protocols to use the *invivo* study were reviewed and reported in accordance with ARRIVE guidelines. All animal experiments were conducted according to the ethical principles approved by the Islamic Azad University of Mashhad, IRAN with the code of ethics IR.IAU.MSHD.REC.1399.016.

Consent to Participate:

Not applicable

Consent to Publication:

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The FESEM analysis of nanoliposome-loaded by *A. rosea* phenolic rich fraction

Figure 2

Histopathological analysis of the liver, kidney, and ileum of the mice undergone different treatments. T1: normal diet; T2: normal diet +infected by *E. coli* (O157: H7) on day 21; T3: Normal diet enriched by a nonencapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21; T4: Normal diet enriched by nanoliposome-encapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21

Figure 3

Relative fold changes in the ileum population of *E. coli* (O157: H7). T1: normal diet; T2: normal diet +infected by *E. coli* (O157: H7) on day 21; T3: Normal diet enriched by nonencapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21; T4: Normal diet enriched by nanoliposome-encapsulated phenolic rich fraction (10 mg TPC/kg BW/day) + infected by *E. coli* (O157: H7) on day 21