Encapsulation of Green Tea Extract in Nanoliposomes and Evaluation of its Antibacterial, Antioxidant and Prebiotic Properties

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Received: 10 May 2015 **Accepted:** 12 Sep. 2015

Abstract

Background: The application of natural compounds including green tea extract (GTE) in food and pharmaceutical industries is limited. Encapsulation in nanoliposomes could be used as a delivery system to protect these compounds during processing and storage.

Objective: In this study encapsulation of green tea extract in nanoliposomes and evaluation of its antibacterial, antioxidant and prebiotic properties were evaluated.

Methods: GTE was encapsulated in liposomes by thin film layer method and reached to nanoscale with sonication. The prebiotic activity of 1% nanoliposomal GTE was evaluated on the growth of *lactobacillus casei* and *bifidobacterium lactis* in MRS broth medium. Furthermore, the antioxidant activity of nanoliposomal GTE was estimated by DPPH assay. The antibacterial activity of nanoliposomal GTE against *Bacillus cereus* (ATCC11778), *Salmonella typhimurium* 138 phage type 2, *E. coli* O_{157}:H_7 and *Listeria monocytogenes* (ATCC19118) was determined using well diffusion technique.

Results: The mean diameter of nanoliposomes was about 44.7± 1.9 nm and had 0.203 ±0.014 polydispersity index. Entrapment efficiency of nanoliposomal GTE under the optimum conditions was 97%. Antibacterial activity of GTE was significantly increased after encapsulation in nanoliposomes. The strongest antibacterial activity of nanoliposomal GTE was seen against *listeria monocytogenes* with an inhibition zone of 16.2 mm while *E. coli* was the most resistance strain with an inhibition zone of 14 mm. Furthermore, the antioxidant activity of GTE was significantly increased after nanoliposome encapsulation since the IC_{50} value of nanoliposomal GTE was decreased to 1.78 µg ml^{-1}. Moreover, addition of 1 % nanoliposomal GTE enhanced the growth rate of *Lactobacillus casei* and *Bifidobacterium lactis* to a significant extent.

Conclusion: Nanoencapsulation effectively enhanced beneficial properties of GTE.

Keywords: Antibacterial activity, Antioxidant activity, Green tea extract, Nanoliposome encapsulation, Prebiotic properties

Introduction

Nowadays, increasing consumers demand for natural compounds has led to the growing use of these substances in food industries as antimicrobial, antioxidant and preservative agents. Furthermore, some of these resources exhibit prebiotic activity which defined as"non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [1]. Green tea is one of the most favorable natural compounds that obtained from the young shoots of tea plant *Camellia sinensis* belongs to the family *Theaceae*. Green tea contains high quantity of catechins that are chemically defined as flavan-3-ols [2]. The major polyphenol in green tea is catechins and among them (-)-Epigallocatechin-3-gallate (EGCG) is the main and biologically most-active compound [3]. Recently, green tea has been acquired great attention in pharmacological and food industries due to some beneficial effects including antimicrobial, antioxidant and prebiotic activities and promoting human health [4, 5]. There are contradictory reports on the antibacterial activity of GTE against bacterial pathogens. Hara and Ishigami (1989) showed *Salmonella typhimurium* and *Campylobacter jejuni* resistance to GTE, while some others reported sensitivity of *S. typhimurium* to GTE [6]. Furthermore, different researchers have proved that GTE exhibited a growth stimulatory effect on the beneficial bacteria [7, 8]. Cabrera et al. has attributed prebiotic role of GTE to the presence of many essential dietary compounds and trace elements. Moreover, the antioxidant activity of GTE has been well documented [9,11]. This effect is due to the polyphenol content which has potent free radical scavenger activity through donating hydrogen atoms, interrupting chain oxidation reaction and accepting free radical.

One of the major problems which greatly limit application of natural compounds in food preparation and pharmaceutical industries is low efficiency resulting from sensitivity to adverse environmental conditions and decrease of stability and bioavailability during processing and storage [12]. Therefore, search for a new method in order to selectively protect natural compounds during processing and storage is important. For this purpose, various methods have been suggested including encapsulating main compounds, such as nanoparticles [13], coprecipitation [14], coacervation of core micelles [15], microparticles [16] and liposomes [12, 17, 18].

Encapsulation in nanoliposomes, which are microscopic vesicles consisting of membrane-like phospholipid bilayers surrounding an aqueous medium [19], could be used as delivery systems to protect reactive or sensitive compounds. Conventional liposomes contain lecithin and cholesterol, which have hydrophilic and lipophilic groups to form a lipid bilayer that encapsulates an aqueous phase. The common technologies for the preparation of liposome are thin film dispersion, reverse evaporation, pH gradient method, high pressure homogenization, emulsification, freeze-drying, centrifugation, pressure extrusion and melting technologies. The objectives of this study were to evaluate

the effect of nanoliposome encapsulation, using thin film method, on the antimicrobial and antioxidant activities of GTE. Besides, the potential of encapsulated extract, to act as a prebiotic to enhance the growth of beneficial bacteria including *Bifidobacterium lactis* and *Lacobacillus casei* in vitro condition, was assayed as well.

Materials and methods

Preparations of GTE

Green tea leaves were collected from Gilan, Iran. Green tea aqueous extract solution was prepared by mixing ground tea leaves in distilled water (100 °C, 1: 10 w/v), brewing for 10 min with stirring, and removing solid matter by filteration. The extract solution was dried at 60°C and stored at 4 °C until used [20].

Preparations of nanoliposomal GTE

To prepare nanoliposomal GTE adequate amount of lecithin, cholesterol and DSPE-mPEG 2000 (1, 2-Distearoyl-phosphatidyl ethanol amine- methyl- polyethylene glycol conjugate- 2000 (Na+ salt)) (Nanocs, USA) was dissolved in 5 mL of ethanol (Carlo erba, France). Then, the solution was put in a round-bottomed flask and the solvent was evaporated off using a rotary evaporator (IKA, Germany) at 50 °C. The resulted film was further dried with the vacuum pump (2.5 CFM Vacuum Pump, Camarillo, CA, USA). The dried lipid film was hydrated with 10 ml of deionized water containing GTE. By shaking for 30 min at 30 °C (mesonix sonicator, Melville, New York, USA), The emulsion was dispersed to form liposomes [21, 22], then treated with probe ultrasound sonicator (mesonix sonicator, Melville, New York, USA) for 30 min and quickly sealed and stored in darkness at 4°C.

Physicochemical characterization and morphology of nanoliposomal GTE

Physicochemical characterization of nanoliposomal GTE, including determination of entrapment efficiency, particle size, size distribution, and zeta potential, were determined as follows: Liposome encapsulation efficiency was determined using the dialysis technique for separating the non-entrapped extract from liposomes [23, 24, 25]. Briefly, 3 mL of extract-contained liposomal dispersion was dropped into a cellulose acetate dialysis bag (Spectra/ Por, MW cut-off 12,000, Spectrum, Canada) immersed in 800 mL of deionized water and magnetically stirred at 30 rpm (Model TK22, Kartell Italy). Samples, taken at time intervals from the receiver solution, were replaced with equal volumes of fresh solvent. GTE was spectrometrically assayed at 270 nm (UV-1601 Shimadzu, Markham, Ontario, Canada). The experiment was stopped when constant extract concentration values were obtained in subsequent withdrawals from the receiver phase (taking into account the progressive dilution of the medium). The percent of encapsulation efficiency (EE %) was then calculated according to the following equation:

$$EE\% = \frac{\text{Total GTE} - \text{diffused GTE}}{\text{Total GTE}} \times 100$$

The particle size and polydispersity index of nanoliposomal GTE were determined by DLS (Dynamic laser light scattering) (Brookhaven Instruments Corporation, Holtsville, NY, USA). The zeta potential of the liposomes were measured according to fang 2005 by a laser scattering method using Zeta sizer Nano- ZS (Malvern Instruments, England, UK) equipped with a helium-neon

laser with a λ of 630 nm. Liposomal suspensions were diluted 100- fold with double-distilled water before the measurement of both particle size and zeta potential. The determination was repeated three times for each sample. The morphology of the nanoliposomal GTE was observed by TEM (Transmission Electron Microscopy). One drop of nanoliposomal GTE solution was 10- fold diluted with deionized buffer, dripped to the copper mesh surface, allowed to air dry for 5 min. The samples were then negatively stained with 1% phosphotungstic acid and observed by a high-voltage transmission electron microscope (Zeiss - EM10C - 80 KV, Germany).

Antibacterial activity assay

The antibacterial activity of free and nanoliposomal GTE was evaluated by the well diffusion technique according to [26]. The test was conducted against four common foodborne pathogenic bacteria: *Bacillus cereus* (ATCC11778), *Salmonella typhimurium* 138 phage type 2, *E. coli* O_{157}:H_7 and *Listeria monocytogenes* (ATCC19118). Briefly, each microorganism, cultured in brain heart infusion broth, was diluted to approximately 10^6 colony-forming unit/ml in sterile saline solution. The nutrient agar plates were surface inoculated with 0.1 ml of diluted cultures. The wells (8 mm in diameter) were cut from the agar and inoculated with 70 µl of 1% free and nanoliposomal GTE solutions. After incubation for 24 h at 37°C, all plates were examined for any zone of growth inhibition around the wells. The experiment was repeated two times, and the results (mm of zone of inhibition) were expressed as average values [27].

Total phenolic contents (TPC)

Determination of phenolic compounds in GTE solution, before and after liposomal encapsulation was accomplished as suggested by Barros et al. [2007]. In order to estimate total phenolics, 1 ml of extracts (5 mg mL^{-1}) was combined with 1 ml Folin and Ciocalteu's phenol reagent (Merck, Germany). Later, on 1 ml saturated Na_2Co_3 (7.5%, w/v) solution (Merck, Germany) was added to the mixture after 3 min and total volume of mixture was adjusted to 10 mL with distilled water. This reaction mixture was then kept in dark for 90 min and then absorbance was read at 725 nm. Standard curve was calculated by using Gallic acid.

Evaluation of antioxidant activity

The hydrogen atom or electron donation abilities of the GTE solution, before and after liposomal encapsulation, were measured from the bleaching of the purple-colored methanol solution of DPPH (2, 2-diphenyl-1-picryl hydrazyl). This spectrophotometric assay was done using the stable radical DPPH as a reagent according to the method of Burits and Bucar [2000]. Briefly, 50 µL of the free and nanoliposomal GTE (various concentrations) were added to 5 ml of the DPPH solution (0.004% methanol solution). After 30 min incubation at room temperature, the absorbance was read against pure methanol at 517 nm. The radical-scavenging activities of the samples were calculated as percentages of inhibition according to the following equation: $I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$; Where A_{blank} is the absorbance of the control (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compounds. Extract concentration providing 50% inhibition (IC50) was calculated from the plot of inhibition percentages against extract concentration using PHARM/ PCS-version 4.

Evaluation of prebiotic activity

The prebiotic activity of 1% free and nanoliposomal GTE was investigated on the growth of *Lactobacillus casei* (Lc-01) and *Bifidobacterium lactis* (Bb-12). These strains were obtained from the CHR-Hansen (Horsholm, Denmark) and activated by inoculating in de Man- Rogasa-Sharpe (MRS) broth and incubating at 37°C for 24 h. The probiotic biomass in late-log phase was harvested by centrifugation at 6000g for 10 min at 4 ° C (Sorvall, model RC-5C, rotor GS-3, Newtown, CT), and washed twice in sterile saline solution. Then 10^3 CFU ml^{-1} of bacterial suspension was inoculated into 20 ml of fresh MRS broth containing 1% of free and nanoliposomal GTE and incubated at 37°C for 48 h. The bacterial growth was monitored at 0, 6, 12, 24, 36 and 48 h by preparing 10- fold serial dilutions and spreading 100 µl aliquot on the surface of MRS agar plates. The plates were incubated at 37°C for 48 h under anaerobic and aerobic condition, for *B. lactis* and *L. casei*, respectively. Bacterial count was calculated through enumerating the colony numbers on the plates.

Statistical analysis

The data collected in this study were expressed as mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA). Multiple comparisons were performed by Tukey's test. Statistical significance was set at p <0.05. All analyses wereperformed using SPSS Version 16.0.1 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Characterization and morphology of nanoliposomal GTE

Characterization and morphology of nanoliposomal GTE including particle size, polydispersity index, zeta potential and entrapment efficiency were assessed. Using TEM and DLS measurements, the morphology and size distributions for the nanoliposomal GTE were obtained. The liposome was vesicles with rounded shape (Figure 1a), and the majority were separately dispersed (Figure 1b). The mean diameter of nanoliposomes was about 44.7 ± 1.9 nm and had 0.203 ± 0.014 polydispersity index. Zeta potential value for the nanoliposomal GTE was found to be -12.6 mv. Entrapment efficiency of nanoliposomal GTE under the optimum conditions was 97 %.

Antibacterial activity

The antibacterial activity of 1% free and nanoliposomal GTE against four pathogenic bacteria was investigated and the results are shown in Table 1. The free GTE exhibited inhibitory effect against all tested bacteria. *Salmonella typhimurium* showed greater inhibition zone (12.3 mm) comparing to the other strains while *B. cereus* was the least sensitive strain. Antibacterial activity of GTE was significantly increased after encapsulation in nanoliposomes. The strongest antibacterial activity of nanoliposomal GTE was seen against *Listeria monocytogenes* with an inhibition zone of 16.2 mm while *E. coli* was the most resistance strain with an inhibition zone of 14 mm.

Total phenolic content

In this work, the total phenol content of free and nanoliposomal GTE was analyzed. As shown in Table 2, the total phenol content in free and nanoliposomal GTE was 29.65± 1.5 and 88.86± 3.84 mg gallic acid g^{-1} extract, respectively.

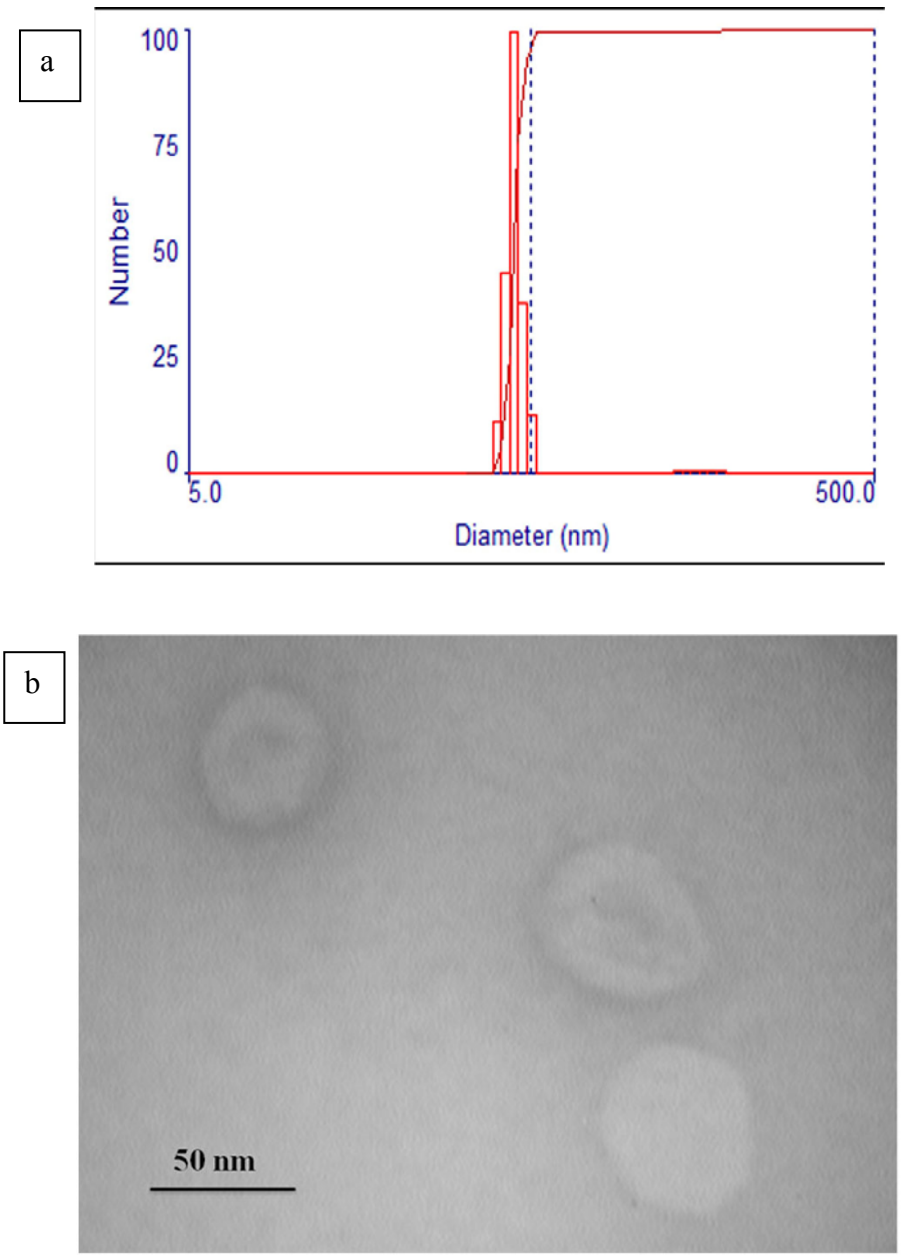

Figure 1- (a) Particle size distribution by intensity and (b) transmission electron micrograph of nanoliposomal GTE

Table 1- Antibacterial activities (mm of zone inhibition) of free and nanoliposomal green tea extract (GTE)

Bacterial strains	Inhibition zone (mm)	
	Free GTE	Nanoliposomal GTE
Listeria monocytogen	10.92 ± 0.05	16.2 ± 0.01
Bacillus cereus	10.61 ± 0.02	15.6 ± 0.03
Salmonella typhimurium	12.3 ± 1.3	15.7 ± 0.5
E. coli O157: H7	12.1 ± 0.03	14 ± 0.9

Antioxidant activity

The antioxidant capacity of free and nanoliposomal GTE was evaluated as DPPH scavenger activity and expressed in term of IC_{50} values. The result is presented in Table 2. The free GTE showed IC_{50} of 12.49 ± 0.6 µg ml^{-1}. The antioxidant activity of GTE was significantly increased after nanoliposome encapsulation since the IC_{50} value of nanoliposomal GTE was decreased to 1.78 ± 0.3 µg/ml.

Prebiotic activity

The results of *L. casei* counts in culture media containing 1 % of free and nanoliposomal GTE are shown in Figure 2. In the control group, no significant changes were found in bacterial count during the first six-hour. Afterward, the growth significantly enhanced, since at 24 h bacterial population reached to full growth equal to 9.3 ± 0.16 log CFU ml^{-1} and remained constant during 48h. The growth of *L. casei* in medium containing free GTE generally showed similar trend to that in control. Although the free GTE increased bacterial count compared to the control group, this difference is not of practical importance. Addition of 1 % nanoliposomal GTE enhanced the growth rate of *L. casei* to a significant extent in 6h and 12 h, since the bacterial number was 0.5 and 0.6 log higher than that of control group, respectively. The results of *B. lactis* counts in culture media containing 1 % of free and nanoliposomal GTE are shown in Figure 3. Addition of 1% free GTE significantly increased growth rate of *B. lactis* in all the interval times compared to the control group. In the groups containing nanoliposomal GTE, the growth curve was similar to the free GTE supplemented group but with higher counts. The maximum differences in *B. lactis* count between control and nanoliposomal GTE supplemented groups were 1.1 log experienced at hour 18.

Table 2- Total phenolic content (mg galic acid/ g of extract) and DPPH antioxidant activity (IC_{50} value in µg/ml) of free green tea extract (GTE) and nanoliposomal GTE

Sample	Total phenolic content (mg g^{-1})	IC_{50} (µg ml^{-1})
Free GTE	29.65 ± 1.5[a]	12.49 ± 0.6*[a]
Nanoliposomal GTE	88.86 ± 3.84[b]	1.78 ± 0.3[b]

*Results are the average of three replications ± standard deviation.
Different letters within columns represent significant differences (p<0.05)

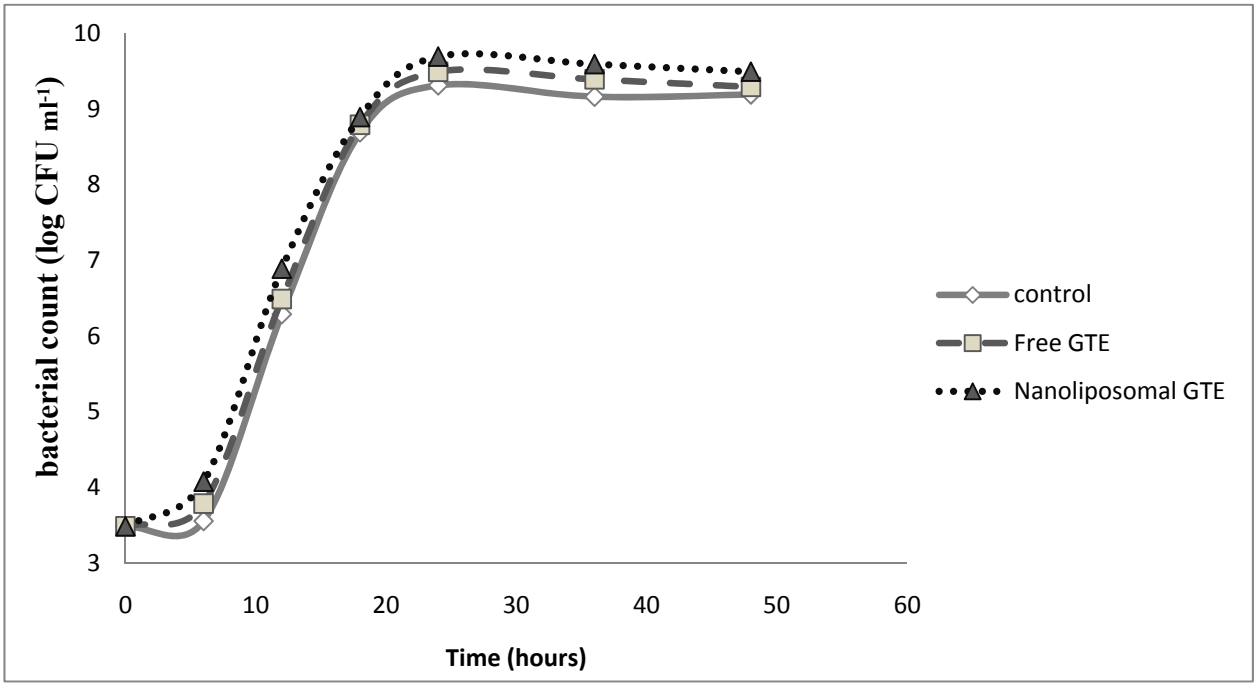

Figure 2- Effect of free green tea extract (GTE) and nanoliposomal GTE on the growth of *Lactobacillus Casei* during 48 h

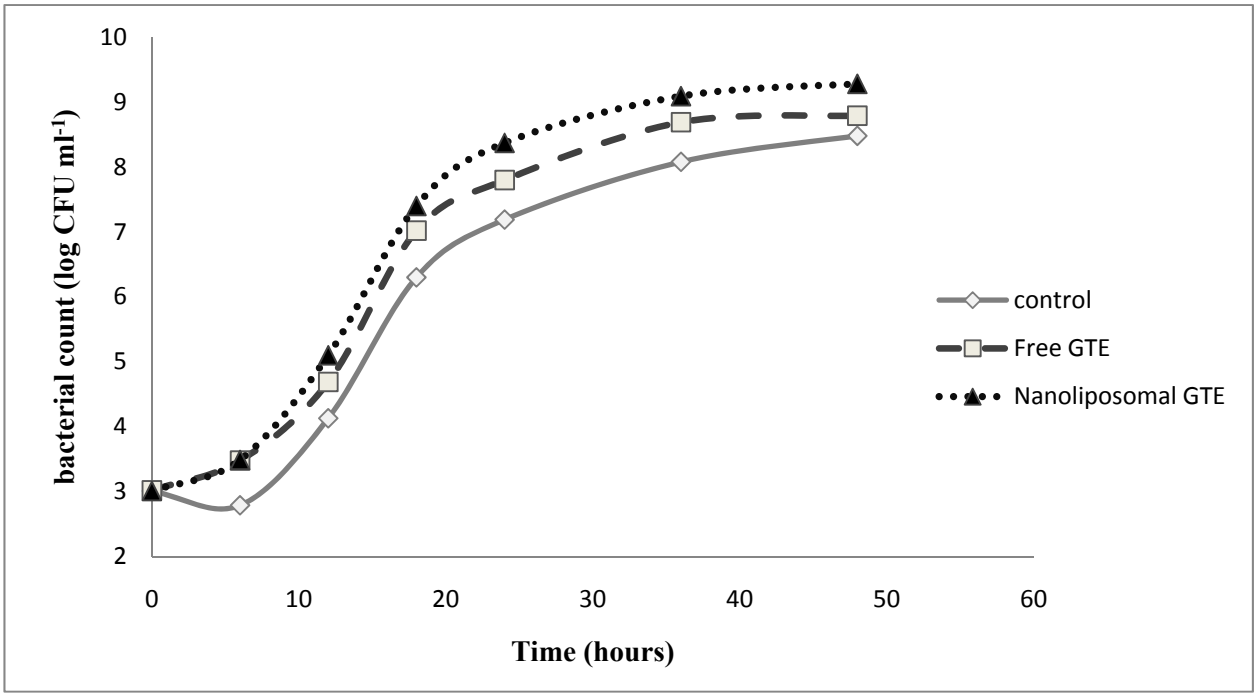

Figure 3- Effect of free green tea extract (GTE) and nanoliposomal GTE on the growth of *Bifidobacterium Lactis* during 48 h

Discussion

Characterization and morphology of nanoliposomal GTE

In the present study characterization and morphology of nanoliposomal GTE including particle size, polydispersity index, zeta potential and entrapment efficiency were assessed. According to the results of DLS, the majority of vesicles were separately dispersed and PI was narrow. In previous studies, nanoliposomes of green tea polyphenol were prepared by different methods including high-pressure homogenizer, thin film ultrasonic dispersion and combination of ethanol injection with dynamic high-pressure microfluidization. Their particle sizes were reported > 100 [18], 160.4 [12] and 66.8 nm [4], respectively. In our study, the particle size of nanoliposomal GTE was 44.7 nm that is 4.5 fold smaller than nonoscale (200 nm). Probably, these differences depend on using mechanical forces that whatever was stronger could more reduce the particle size of liposome. Also, the presence of ethanol in the formulations affects the particle size, since ethanol causes a modification of the net charge of the system and confers it some degree of steric stabilization that may finally lead to a decrease in the mean particle size [28]. Surface charge and thereby the stability of the prepared nanoparticles systems was determined by zeta potential measurement which is an important parameter to reflect the physicochemical and biological stability of nanoparticles in suspension. Zeta potential of the nanoliposomal GTE under the optimum conditions was -12.6 mv, which was higher and lower than this parameter of Zou et al. (-6.16 mv) and Lu et al. (-67.2 mv), respectively. Main compounds of liposomes are cholesterol and phospholipids. The difference of liposome zeta potential is probably attributed to the variations in competence and property of phospholipids. It is documented that the suspension with greater zeta potential is more likely to be stable, which is due to the charged particles repelling each other and thus overcoming the natural tendency to aggregate [28]. Also, Manosroi et al. demonstrated that the leakage rates in negative liposomes were lower than those in positive liposomes. Entrapment efficiency is an important parameter for industrial application of the liposomal system. In general, efficiency depends on wall material, encapsulation formation and interaction between wall material and core material. Entrapment efficiency of nanoliposomal GTE under the optimum conditions was 97 %, which is higher than 78.5 % reported by Zou et al.. Fan et al. found that several factors including appropriate proportions of cholesterol and lipid increase the entrapment efficiency as well as nanoliposome stability.

Antibacterial activity

In this study the free GTE showed antibacterial activity against examined bacterial strains. The antibacterial effect of free GTE has been demonstrated in different studies [6, 8, 29]. Naz et al. and Fan et al. indicated that polyphenols played an important role in protein precipitation and enzyme inhibition of microorganisms. According to the results, antibacterial activity of GTE was significantly increased after encapsulation in nanoliposomes. Liolios et al. explained that use of nanoliposome improves the cellular transport and release of the active component inside the bacterial cell. It may be due to the interaction between nanoliposomes and bacterial cells through various ways including

inter membrane transfer, contact release, absorption, fusion and phagocytosis [4]. However, Zou et al. reported that the antibacterial activity of biological substances reduced by nanoencapsulation processing because of preventing main ingredients incorporated into the nanoliposomes from being released into the media, interacting with proteins, and forming sediments.

Total phenolic content and antioxidant activity

The antioxidant activity of free and nanoliposomal GTE was investigated through DPPH radical scavenging capacity assay. The results of this study showed that both free and nanoliposomal GTE had antioxidant activities. It is reported that the antioxidant activity of GTE is related tocertain phenolic compounds, minerals and vitamins [30]. Moreover, antioxidant activity of GTE was significantly increased after nanoliposome encapsulation. In agreement with our results, Spigno et al. demonstrated that nanoliposome encapsulation technology improved antioxidant efficiency of phenolic compounds against lipid oxidation by increasing extract dispersability in the environment. Furthermore, the results of our study showed that the total phenol content of nanoliposomal GTE was higher than free GTE. This could be the reason for the higher antiradical activity of nanoliposomal GTE compared to free GTE. Accordingly, Luximon-Ramma et al. showed linear correlation between antioxidant activity and phenolic contents of plant, fruits and beverages extracts. However, Yang et al. found that the DPPH radical scavenging activity of vitamin C was not significantly altered by nanoliposome encapsulation.

Prebiotic activity

In this study, both free and nanoliposomal GTE improved the growth of probiotic *L. casei* and *B. lactis*. The prebiotic property of GTE on Bifidobacteria and Lactobacilli has been demonstrated by previous studies [7, 8]. Vodnar and Socaciu found that green tea exerted a stimulating effect on growth of *B. infantis* and *B. breve*, through bifidogenic properties. It is reported that prebiotic effect of GTE is possibly related to the antioxidant and antiradical activities of polyphenol content which resulted in a better environment for the growth and multiplication of probiotic bacteria [20, 31]. Furthermore, Macdonald et al. presented that hydrolytic enzymes of probiotics could convert certain flavonoid glycosides of GTE to their corresponding aglycones. In the present study it was shown that encapsulation of GTE in nanoliposomes improved its growth stimulating effects on tested bacteria. It may be due to increasing ingredient solubility and stability, improving ingredient bioavailability and enhancing antioxidant activity.

Conclusion

The results of this study demonstrated that the nanoliposome encapsulation of GTE improved its beneficial properties including antibacterial and antioxidant activities. Moreover, this is the first report demonstrated modulation of prebiotic properties of biological substances by nanoencapsulation.

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