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## Effect of Fortification with Sage Loaded Liposome on the Chemical, Physical, Microbiological Properties and Cytotoxicity of Yoghurt

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**Abstract**

The using of plant extract in dairy field is a recent trend to achieve health benefits. Sage (*Salvia officinalis L*) is a medicinal plant contained a large number of bioactive and biological compounds. The phenolic compounds, chemical composition and physical properties of sage phenolic extract (SPE) loaded in liposomal system were firstly investigated, and then it was used to fortify yoghurt (5, 10, 15 and 20 %) to produce functional dairy product. Obtained data revealed that the encapsulation efficiency of SPE in liposome was 83.7%, the particle size ranged between 168.30 to 273 nm and Zeta potential was +35.4 mV. SPE exhibited an inhibition activity against all tested pathogenic bacteria and the antibacterial activity of SPE was increased significantly after encapsulation in liposome. The highest antibacterial activity was noted against *S. aurous* and *B. cereus.* On another vision; protein, total solids and ash contents of fresh yoghurt increased with increasing the concentration of SPE liposome but decreased after 15 days of storage. The pH values and diacetyl contents of liposome fortified yoghurt decreased compared to control throughout storage. The acetaldehyde content of control significantly decreased during cold storage while it increased with increasing concentration of SPE liposome in yoghurt. The viscosity of fresh yoghurt increased with the increase of added liposome but decreased after 15 days of storage. Fortification of yoghurt samples with SPE liposome significantly enhanced the viability of *L. rhaminosus* along the storage period. The high selectivity index values of SPE suggest their safety and cytotoxic selectivity effect on cancer cell.

*Keywords:* Sage; Liposome; Encapsulation efficiency; Yoghurt; Antibacterial activity; Cytotoxic effect.

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## Introduction

Polyphenols are secondary metabolites of plants. These compounds exhibit positive effects on human health. Due to their antioxidant; antimicrobial, anti-inflammatory, anti-osteoporotic and hepato-protective activity against some chronic diseases such as cancer, type-II diabetes, cardiovascular disease or non-alcoholic fatty liver diseases [1]. Sage (*Salvia officinalis L*) is an important medicinal plant, revealed a great number of bioactive compounds and a variety of biological activities. Various studies have shown antioxidant properties, antibacterial, antifungal and anti-inflammatory activity of sage leaves hydroalcoholic and aqueous extracts [2]. However, they are highly unstable prone to degradation by light, oxygen or enzymes. In addition, they may lose their activity by interaction with other food components such as proteins and carbohydrates. So; it might be preserving their properties and protected their activity. Encapsulation is an effective way to protect polyphenols from adverse in conditions, mask their odor and color or inhibit the interaction with other food constituents [3]. As an encapsulation technique, liposome can allow successful delivery of phenolic compounds. Liposome are enclosed spherical vesicles organized by one or several concentric phospholipidic bilayers with an internal aqueous phase which make them a suitable carrier for both water and oil soluble functional compounds. However, the liposome structure is flexible fragile and to overcome this disadvantage their surfaces can be coated with a polymer to ensure their stability. Chitosan, pectin or combined chitosan/pectin coatings can be used as protective coatings for liposome in the acidic milieu of the stomach and triggered release systems in the colon [4].

In another side, yoghurt is one of the important foods to provide the human digestive system with beneficial bacteria strains [5]. Also, it can be employed as a vehicle for bioactive compounds particularly antioxidants in order to improve antioxidant capacity and phenolic in food formulation.

In the present study; sage phenolic extract (SPE) was encapsulated in liposome; and the obtained liposome were characterized. The sage loaded liposome were used to fortify yoghurt then, the chemical and physical properties of obtained yoghurt were followed storage of 15 days at 4°C. Assessment the cytotoxicity of SPE, encapsulated SPE and the fortified yoghurt was carried out on different cancer cell lines.

## Materials and Methods

Dried leaves of sage (moisture 2.87%, ash 6.03%, fat 10.1%, protein 5.84%, fiber 13.65% and total carbohydrate 61.51%) were obtained from a local market in Cairo, Egypt. The dried leaves were powdered using a mortar (total solids 97.13%).

Soy lecithin (69.3% phosphatidyl choline, 9.8% phosphatidyl ethanolamine, and 2.1% lyso phosphatidylcholine) was provided by Lipoid AG (Ludwigshafen, Germany).

Sodium acetate and glacial acetic acid were purchased from Carl Roth GmbH& Co. KG (Karlsruhe, Germany).

Folin-Ciocalteu reagent, chitosan, Gallic acid and 1,1-diphenyl-2-pycrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (St. Louis, USA).

Imported low heat full cream milk powder with 23.6% protein, 40% lactose, 28.2% fat, and 8.2% ash were obtained from the local market.

Strains of *Streptococcus thermophilus*, *Lactobacillus delbuerkii ssp. bulgaricus*, *Lactobacillus casei* were obtained from stock cultures from the Dairy Microbiology Lab., National Research Centre, Dokki, Cairo, Egypt, and propagated in sterilized reconstituted skimmed milk (10% w/v) before use.

*Bacillus cereus* (ATCC133018), *Salmonella typhimurium* 9027, *Staph aurius* (ATCC 25175) obtained from the stock cultures of the Agricultural Research Centre in Giza., *E. coli* O157:H7 (ATCC 6933) and *Listeria monocytogenes V7* and *Yersenia enterocolitica subsp. enterocolitica* ATCC9610TM were obtained from Liofil chem S.r.l. Italy. *Lactobacillus rhamnosus* Tistr 541 and *Lactobacillus reuteri* B-14171 were obtained from Cairo Microbiology Resources Center (Cairo-Mircen, Faculty of Agriculture Ain Shams University).

Colorectal adenocarcinoma (Caco-2 cell line), prostatic carcinoma cell line (PC-3), lung carcinoma (A-549 cell line) and BJ-1 "A telomerase-immortalized normal foreskin fibroblast cell line was obtained from Karolinska Center, Department of Oncology and Pathology, Karolinska Institute and Hospital, Stockholm, Sweden.

**Methods**

*Preparation of sage phenolic extract (SPE)*

Twenty grams of sage leaves powder was soaked in 120 ml of ethanol (80%) for 24 h at 4 °C, filtered using filter paper (Whatamn No.1) and then evaporated using rotary evaporator. The residual sage phenolic extract was freeze-dried using Lab conco, USA, at -52 EC for 48 h at a pressure below 0.1 MPa, ground to fine powder and kept at -18°C until used. Freeze-dried SPE contained 0.58±0.01mg catechin/g of phenolics, 0.044±0.001mg Rutin/g of flavonoids and exhibited antioxidant capacity of 1.08±0.004 mmol Trolox /g in term of DPPH assay.

*Determination of antioxidant capacity*

The DPPH radical-scavenging activity was determined using the method proposed by Brand-Williams et al. [6]. An aliquot (100 µL) of the sample solution was mixed with 2.9mL of 1,1-diphenyl-2-pycrylhydrazyl (DPPH) in methanol. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517nm by a UV-visible spectrophotometer. The results were corrected for dilution and expressed as mmol trolox equivalent/g.

*Determination of phenolic content*

The method described by Naczk and Shahidi [7] was used for the determination of total phenolic compounds content of SPE extract. Briefly, 0.5-mL of the extract was diluted with 8 mL distilled water, 0.5 mL Folin-Ciocalteu's phenol reagent and 1 mL saturated sodium carbonate solution was added. The mixture was vortexed for 15 Sec. left to stand at room temperature for 30 min, and its absorbance was measured at 725 nm using Spectrophotometer (model 2010, Cecil Instra. Ltd., Cambridge, London). The results are expressed as mg catechin equivalents per g of the extract.

*Determination of flavonoid content*

Aliquot (0.5ml) of the sample was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance this mixture was measured at 415 nm. The flavonoid content was calculated using a standard calibration of Rutin solution and expressed as mg of Rutin equivalent (RE) per g of sample [8].

*Identification of phenolic and flavonoid compounds in SPE by HPLC*

The phenolic and flavonoids components of SPE were separated using HPLC Agilent 1260 series system. The separation was carried out using a C18 column (4.6 mm × 250 mm i.d., 5 µm). The mobile phase consisted of water: 0.02% tri-floro-acetic acid in acetonitrile (80:20) at a flow rate 1 ml/min. and the separated components were monitored at 280 nm. The

injection volume was 10 µl for each of the sample solutions. The column temperature was maintained at 35°C.

*Preparation of dispersion liposomal systems with SPE*

The methods described by El-Messery et al. [9] were used for encapsulation of SPE in liposome. SPE (0.2% w/v) was added to 2% lecithin solution in acetate buffer (0.1M pH 3.5). The prepared liposome were added to chitosan solution (0.8%, w/v) in acetate buffer (pH = 3.5 ± 0.1; 0.1 mol/L) of the ratio of 1:1 (w/w) and stirred over night at room temperature. By this way, negatively charged liposome were coated with positively charged chitosan layer.

*Encapsulation Efficiency (EE)*

The dialysis tube diffusion technique was used to determine Encapsulation Efficiency [10]. Aliquot (5 ml) of the liposome suspension was placed in the dialysis tube, closed tightly and dialyzed against the acetate buffer (0.1 M pH 3.5) at 37ºC under continues stirring. Samples were taken from the dialysate at successive intervals and assayed, for the release of total phenolic compounds.

*Measurements of particle size distribution and zeta (ζ) potential*

Average particle size (PS), and size distribution (particle dispersity index; PDI) of liposome preparations were measured by dynamic light scattering (Zetasizer var. 704 instruments (Malvern Instruments, Malvern, UK) The sample was suspended and diluted with ultrapure MQ water before measurement it's light scattering for a laser beam (633 nm) at an angle of 173 at 25˚C over time intervals [11].

*Transmission of electron microscope*

Twenty microliters of diluted samples were placed on a film-coated 200-mesh copper specimen grid for 10 min and the excess fluid was removed using filter paper. The grid was then stained with one drop of 3% phosphor-tungstic acid and allowed to dry for 3 min. The coated grid was dried and examined under the TEM microscope (JEM-2100 Electron Microscope. The samples were observed by operating at 160 kV**.**

*Inclusion of SPE liposome into yoghurt formulation*

Full cream milk powder was reconstituted in distilled water (16% w/v). SPE loaded liposome solution was used to replace 5 ($T_1$), 10 ($T_2$), 15 ($T_3$) and 20 ($T_4$) % of the water used for reconstitution of full cream milk powder. All treatments were heated to 90˚C for 10 min and cooled to 42˚C. 2% Starter cultures (*S. thermophilus* and *Lb. delbuerkii ssp. bulgaricus* (1:1)) and 2% probiotic bacteria *(L. rhamnosus*) were added and poured into cups (100 ml) and incubated at 42ºC until complete coagulation. All yoghurt cups were stored in a refrigerator (6±ºC) and analyzed when fresh and after 3, 7 and 15 days during storage for 15 days.

*Physicochemical analysis of functional yoghurt*

Total solids (T.S), fat, total nitrogen (T.N), ash content and titratable acidity (T.A) were determined according to AOAC [12]. The pH values were measured using a digital laboratory Jenway 3510 pH meter, UK. Bibby Scientific LTD. Stone, Stafford shire, ST 15 OSA. Diacetyl and acetaldehyde contents were determined according to Less and Jago [13].

*Apparent viscosity of functional yoghurt*

Yoghurt samples were gently stirred 5 times clockwise direction with a plastic spoon prior to viscosity measurements. Apparent viscosity was measured at 7ºC using a Brookfield digital viscometer (Middleboro, MA 02346, U.S.A). The sample was subjected to shear rates ranging from 3 to 100 $S^{-4}$ for upward curve. Viscosity measurements were

expressed as centipoise (cP.s) and were performed in triplicate [14].

**Antibacterial activity assay**

The antibacterial activity of SPE and SPE- loaded liposome were evaluated by the well diffusion technique according to NCCLS [15]. The test was conducted against six common foodborne pathogenic bacteria namely: *Bacillus cereus* (ATCC133018), *Salmonella typhimurium* 9027, *Staph aurius* (ATCC 25175), *E. coli* O157:H7 (ATCC 6933), *Listeria monocytogenes V7* and *Yersenia enterocolitica subsp. enterocolitica* ATCC9610TM. Each microorganism was cultured in brain heart infusion broth then spread onto surface of nutrient agar plates with wells (8 mm in diameter) full of 70 µl of SPE and SPE loaded liposome. After incubation for 24 h at 37°C, all plates were examined for the diameter of the inhibition zone. The experiment was repeated two times, and the results (mm diameter of inhibition zone) were expressed as average values.

*Evaluation of Prebiotic activity*

The prebiotic activities of SPE and SPE loaded liposome were investigated on the growth of *Lactobacillus casei*, *Lactobacillus rhamnosus* Tistr 541 and *Lactobacillus reuteri* B-14171 previously 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 6000 g for 10 min at 4°C (Sorvall, model RC-5C, rotor GS3, 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 of SPE and SPE loaded liposome and incubated at 37°C for 48 h. The bacterial growth was monitored at 6, 12, 24 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. Bacterial count was calculated through enumerating the colony numbers on the plates [16].

*Microbiological analysis of yoghurt*

All yoghurt samples (control and treatments) were microbiologically examined after 0, 3, 7 and 15 days of cold storage period. Total aerobic colony count (TACC), mold and yeast, and coliform bacteria were enumerated using the selective media and the conventional methods according to FDA [17]. *Streptococcus thermophilus* was enumerated on M17 agar after aerobic incubation at 37°C for 48h. *Lactobacillus bulgaricus and Lactobacillus rhamnosus* were enumerated using modified MRS agar supplemented with 0.05% L-cysteine-HCl and the plates were incubated at 37°C for 48 h [16].

*Cancer cell viability assay (CCVA)*

The CCVA was assayed according to Batran et al. [18], the cells were seeded at concentration of $10x10^3$ cells per well in case of PC3, $20x10^3$ cells/well in case of A-549 and Caco-2 cell lines and 40 $x10^3$ cells/well in a fresh complete growth medium in case of BJ-1 using 96-well microtiter plastic plates at 37 ºC for 24 hours under 5% $CO_2$, in a water jacketed carbon dioxide incubator. After 48 hours' incubation, the medium was aspirated and then MTT salt were added to each well and incubated for further four hours at 37ºC under 5% $CO_2$. To stop the reaction and dissolving the formed crystals, 10% sodium dodecyl sulphate (SDS) solution was added to each well and incubated overnight at 37ºC. The absorbance was measured using a microplate multi-well reader at 595nm and a reference wavelength of 690 nm. Cell viability was assessed according to the mitochondrial-dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan.

*Statistical analyses*

All data were expressed as the mean of triplicate determinations. Statistical analysis was carried out using SPSS for Windows, Version Rel. 10.0.5., Inc. $p<0.05$ were considered to be significant.

**Results and Discussion**

*Phenolic profile of SPE*

The phenolic profile (mg/g) of SPE obtained after ethanol/water extraction is shown in **Fig. 1**, while concentration of the identified compounds is given in **Table 1** fourteen compounds were detected. Eight of which were phenolic acid derivatives and six flavonoids.

**Table 1. Phenolic profile (mg/g) of SPE**

| Phenolic compounds (PC) | Concentration of PC (mg/g) |
|---|---|
| Gallic acid | 4.58 |
| Chlorogenic acid | 3.84 |
| Catechin | 2.26 |
| Coffeic acid | 1.12 |
| Syringic acid | 0.08 |
| Rutin | 0.00 |
| Ellagic acid | 0.16 |
| Coumaric acid | 2.41 |
| Vanillin | 1.69 |
| Ferulic acid | 0.57 |
| Naringenin | 0.32 |
| Propyl Gallate | 0.012 |
| Querectin | 0.76 |
| Cinnamic acid | 0.094 |

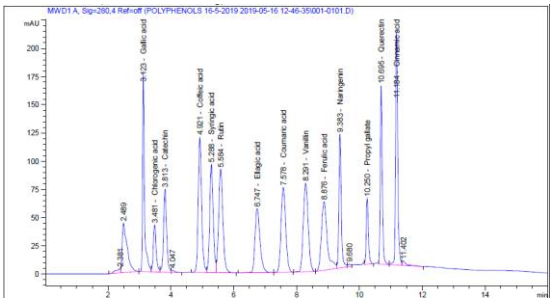

**Fig.1. Phenolic profile (mg/g) of SPE**

*Characterizations of SPE loaded liposome*

By definition, encapsulation efficiency is the amount of core material encapsulated inside the particles. Encapsulation efficiency of SPE loaded liposome was found to be 83.7%. In other words, about 83% of the added SPE were entrapped within the liposome and less than 17% has been remained un-encapsulated. It is generally accepted that the encapsulation efficiency of the active substances within liposomal structure can be affected by the size and/or specific surface areas of the liposome [19]. Previous studies which reported high yields (83–95%) of nanoliposome [9] to agreement with the present results by El-Said et al. [10]. **Fig. 2** shows the morphology of SPE loaded liposome. Zeta potential of SPE loaded liposome was +35.4mV since negatively charged of SPE enhanced the positively charge of liposome. The particle size of SPE loaded liposome ranged between 168.30 to 273 nm.

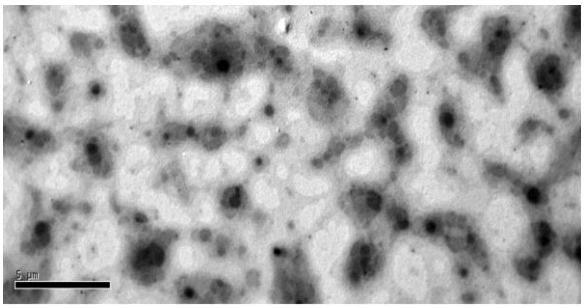

**Fig. 2. Micrograph of SPE loaded liposome**

*Chemical composition of functional yoghurt*

Data presented in **Table 2** showed that the mean of moisture content decreased slightly by increasing concentration of SPE loaded liposome ($T_1>T_2>T_3>T_4$) also during storage compare to control samples. The mean values of total solids slightly increased by increasing concentration of SPE liposome ($T_4>T_3>T_2>T_1$) also during the storage period. This may be due to including lecithin as an emulsifier in liposome preparation that could increase total solids [20]. Protein and ash contents increased significantly with increasing concentration of SPE loaded liposome at fresh while the values significantly decreased after 15 days of storage. The pH values of yoghurt from

different treatments decreased in descending order ($T_4>T_3>T_2>T_1$) compared to control in fresh and after 15 days of storage, which was probably due to the production of lactic acid by starter culture and probiotic bacteria [21]. Generally, the functional yoghurt with SPE loaded liposome exhibited lower pH and higher acidity after 15 days of storage than the control yoghurt, the results are in the line with Zhong et al. [21]. The pH and acidity results reflect the growth and activity of yoghurt starter and probiotic bacteria as confirmed by microbiological evaluation (Table 6). Development of acidity during storage was more pronounced to control yoghurt compared to yoghurt fortified with SPE loaded liposome. This suggest that the added liposome affected the growth and acidity of the used starter and probiotic organisms [24].

Diacetyl values increased by increasing percentage of added loaded SPE loaded liposome in yoghurt compared to control. These results may be due to the enhanced activity of starter and probiotic bacteria [22]. After 15 days of cold storage; diacetyl recorded highest value in all treatment compared to control.

Acetaldehyde values of control sample decreased significantly during cold storage while it increased with increasing the added percentage of SPE loaded liposome in yoghurt but decreased during storage. This may be due to the variable ability lactic acid bacteria to lowest acetaldehyde to ethanol. The same was reported by Abd El-Aziz et al. [23] who recorded a positive relation between acidity and diacetyl contents.

**Table 2. Chemical composition of yoghurt supplemented with SPE liposome**

| Treatments | Storage Days | Total solids % | Fat% | Protein % | Ash% | pH | Acidity % | Diacetyl (µm/100 g) | Acetaldehyde (µm/100 g) |
|---|---|---|---|---|---|---|---|---|---|
| Control | Fresh | 14.21$^{e}$ ±0.01 | 4.00$^{a}$ ±0.10 | 3.50$^{b}$ ±0.20 | 0.56$^{c}$ ±0.03 | 4.69$^{d}$ ±0.03 | 0.95$^{a}$ ±0.04 | 33.60$^{e}$ ±1.63 | 13.28$^{e}$ ±0.05 |
| $T_1$ | | 14.44$^{d}$ ±0.13 | 4.00$^{a}$ ±0.30 | 3.55$^{b}$ ±0.25 | 0.67$^{b}$ ±0.03 | 4.72$^{d}$ ± 0.01 | 0.91$^{ab}$ ±0.14 | 114.40$^{d}$ ±3.10 | 14.78$^{d}$ ±0.22 |
| $T_2$ | | 14.58$^{c}$ ±0.03 | 4.00$^{a}$ ±0.20 | 3.58$^{b}$ ±0.04 | 0.75$^{b}$ ±0.09 | 4.83$^{c}$ ±0.02 | 0.89$^{bc}$ ±0.01 | 162.40$^{c}$ ±0.53 | 19.78$^{c}$ ±0.22 |
| $T_3$ | | 14.71$^{b}$ ±0.31 | 4.00$^{a}$ ±0.10 | 3.90$^{b}$ ±0.40 | 0.87$^{a}$ ±0.03 | 4.88$^{b}$ ±0.01 | 0.85$^{cd}$ ±0.03 | 194.00$^{b}$ ±2.0 | 22.32$^{b}$ ±0.68 |
| $T_4$ | | 14.90$^{a}$ ±0.15 | 4.00$^{a}$ ±0.30 | 4.09$^{ab}$ ±0.09 | 0.94$^{a}$ ±0.05 | 4.99$^{a}$ ±0.01 | 0.82$^{d}$ ±0.01 | 209.20$^{a}$ ±1.08 | 24.36$^{a}$ ± 1.36 |
| Control | 15 | 15.06$^{c}$ ±0.12 | 4.20$^{a}$ ±0.30 | 3.46$^{c}$ ±0.04 | 0.51$^{d}$ ±0.04 | 4.34$^{c}$ ±0.04 | 1.77$^{a}$ ±0.06 | 79.20$^{e}$ ±0.80 | 9.56$^{c}$ ± 0.19 |
| $T_1$ | | 15.35$^{bc}$ ±0.24 | 4.20$^{a}$ ±0.36 | 3.52$^{c}$ ±0.08 | 0.64$^{c}$ ±0.06 | 4.38$^{c}$ ±0.04 | 1.74$^{a}$ ±0.03 | 147.20$^{d}$ ±0.80 | 12.40$^{b}$ ± 0.4 |
| $T_2$ | | 15.40$^{ab}$ ±0.17 | 4.20$^{a}$ ±0.17 | 3.54$^{c}$ ±0.06 | 0.73$^{b}$ ±0.03 | 4.54$^{b}$ ±0.12 | 1.72$^{a}$ ±0.04 | 250.40$^{c}$ ±6.95 | 12.48$^{b}$ ± 0.31 |
| $T_3$ | | 15.84$^{a}$ ±0.11 | 4.20$^{a}$ ±0.26 | 3.78$^{b}$ ±0.04 | 0.85$^{a}$ ±0.03 | 4.67$^{a}$ ±0.03 | 1.47$^{b}$ ±0.02 | 320.40$^{b}$ ±3.40 | 18.60$^{a}$ ±0.02 |
| $T_4$ | | 15.87$^{a}$ ±0.21 | 4.20$^{a}$ ±0.17 | 3.98$^{a}$ ±0.02 | 0.91$^{a}$ ±0.04 | 4.69$^{a}$ ±0.05 | 1.41$^{b}$ ±0.11 | 396.80$^{a}$ ±0.80 | 19.01$^{a}$ ± 0.16 |

Control: yoghurt without sage phenols extract (SPE) liposome, T1: yoghurt with 5% SPE liposome, T2: yoghurt with 10% SPE liposome, T3: yoghurt with 15% SPE liposome, T4: yoghurt with 20% SPE Liposome. The means with the different capital (A, B, C…) superscript letters within the same column indicate significant (P≤0.05) differences between treatment

*Phenolic content (PC) of yoghurt*

**Table 3** shows that the addition of SPE loaded liposome to milk prior to yoghurt manufacture increased significantly ($p < 0.05$) the PC content of yoghurt. During storage, the PC content in yoghurt increased steadily with SPE loaded liposome during storage to reach at least 3 times its content than control

**Table 3. Phenolic compounds content (mg catechin equivalent/g) in yoghurt samples during storage period.**

| Treatments* | Storage period (day) | | |
|---|---|---|---|
| | Fresh | 7 | 15 |
| **Control** | 0.360±0.042 | 0.305±0.005 | 0.268±0.009 |
| **$T_1$** | 0.520±0.014 | 0.514±0.004 | 0.506±0.005 |
| **$T_2$** | 0.730±0.025 | 0.713±0.012 | 0.694±0.005 |
| **$T_3$** | 0.944±0.013 | 0.916±0.007 | 0.899±0.004 |
| **$T_4$** | 1.04±0.002 | 0.927±0.007 | 0.918±0.004 |

*See footnote table (2)

at the end of storage period. Similar results were reported in previous studies [9,10]. During storage, PC content of treated yoghurt ($T_1$, $T_2$ and $T_3$) were decreased but still high than control sample. This can be explained by the binding of polyphenols to milk proteins.

*Viscosity of yoghurt*

**Fig. 3, 4** show that the apparent viscosity of yoghurt increased dramatically with increased addition of SPE loaded liposome compared to the control. The significant increase in apparent viscosity of fresh samples may be due to absorption of water by lecithin used to the preparation of liposome which would increase the viscosity [20], also, the increase of the total solids in yoghurt with added liposome **(Table 1)** may also be responsible for the increased viscosity. After 15 days of storage, the viscosity of control yoghurt and yoghurt with added liposome decreased which may be due to protein degradation. Several researchers reported that yoghurt is a thixotropic fluid, so viscosity measurements showed the thixotropic characteristics of yoghurt samples which showed a reduction of viscosity in time, in accordance with the data presented in the literature as confirmed by Dabija et al. [24].

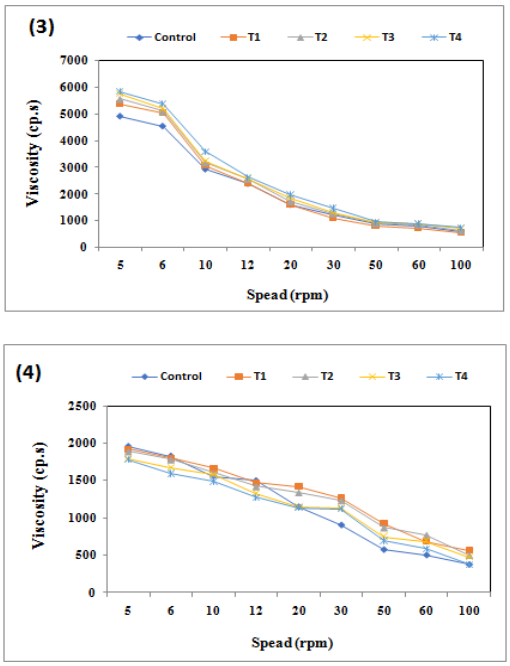


**Fig. 3,4 Effect of SPE loaded liposome on the viscosity of yoghurt samples fresh (3) and after 15 days (4) of cold storage.**

*The antibacterial activity of SPE and SPE loaded liposome*

The antibacterial activity of SPE and SPE loaded liposome against six pathogenic bacteria are shown in **Table. 4.** SPE inhibited variably all tested bacteria where the highest inhibition zone was for *S. aurous* (16.0 mm) while *Salmonella typhimurium* was the

**Table 4. Antimicrobial activity of SPE and its liposome**

| Treatments | Diameter of inhibition zone (mm) | | | | | |
|---|---|---|---|---|---|---|
| | ***E. coli O157:H7*** | ***Yersinia enterocolitica ATCC 9610*** | ***Salmonella ATCC 9027*** | ***Listeria monocytogenesV7*** | ***Staph. aurous ATCC 25175*** | ***Bacillus cereus ATCC 33018*** |
| SPE | $5.3^{Bd}$ | $5.0^{Bd}$ | $4.3^{Be}$ | $8.5^{Bc}$ | $16.0^{Ba}$ | $9.2^{Bb}$ |
| SPE liposome | $15.5^{Ae}$ | $20^{Ab}$ | $16.1^{Ad}$ | $19.1^{Aac}$ | $27.1^{Aa}$ | $21.3^{Ab}$ |

The means with the different capital (A, B, C…) superscript letters within the same column indicate significant (P≤0.05) differences between treatments. Means with the different small (a, b, c,) superscript letters within the same row are significantly (P≤0.05) different between treatment

least sensitive strain. Antibacterial activity of SPE was significantly increased after encapsulation and the strongest antibacterial activity was seen against *S. aurous* and *B. cereus* with inhibition zones of 27.1 and 21.3 mm respectively. This may be due to the interaction between SPE liposome and bacterial cells through various ways including inter membrane transfer, contact release, absorption, fusion and phagocytosis [25].

Recently Cantor *et al*. [26] reported that after coating the liposome with Eudragit E-100 (cationic polymer) antibacterial activity increased approximately 12.5 times and these vehicles would protect the entrapped peptides from degradation by bacterial proteases.

*Growth of yoghurt starter and probiotics in media*

The *LAB* count in culture media containing SPE and SPE loaded liposome are shown in **Table 5.** In control group, no significant changes were found in bacterial count during the first six hours. Afterward, the growth significantly enhanced, up to 24 h whereas the count reached a maximum and remained constant up to 48h. Addition of both SPE and SPE loaded liposome improved the growth of probiotic *Lactobacilli*, Behradmanesh *et al*. [27] found growth of *L. casei*, *L. rhaminosus* and *L. reuteri* in medium containing SPE generally showed similar trend in control. The addition of SPE loaded liposome enhanced significantly the growth rate of *L. rhaminosus* after 24h and 48 h, since the bacterial number was 1.0 and 0.95 log higher than control, respectively. This may be improved ingredient bioavailability and enhanced the antioxidant activity [28]. Also, addition of SPE significantly increased growth rate of *L. casei* and *L. reuteri* in all interval times compared to the control group and these results agree with Shori and Albloushi [29] who found the SPE to enhance the growth of the *Lactobacillus plantarum* in yoghurt.

*Microbiological quality of yoghurt*

Data presented in **Table 6** show that, the counts of *Lb. delbrueckii ssp. bulgaricus* and *Str. thermophilus* were higher in control sample than yoghurt treated at 1st day of storage then yoghurt cultures were slightly decreased for all samples during storage. Similar findings were obtained by El Batawy [30] who recorded decreases in the growth of yoghurt cultures during cold storage period. Also, the current study revealed a gradual decrease in the viability of probiotic strains (*L. rhaminosus*) in yoghurt during the cold storage period that could be due to the sensitively of this bacterial strain to acid development that was in consistent [31]. On the other hand, fortification of yoghurt with SPE loaded liposome enhanced significantly the viability of *L. rhaminosus* probiotic strain along the storage period that was in line with Bisar et al. [31]. In general, the food industry has targeted bacterial populations over $10^6$ probiotics/g at the time of consumption of strain added to food [32]. Moreover, yeast and mold could be observed and counted after 7 days of the storage in control and after 14 days in treated yoghurt samples ($T_2$ and $T_3$) and this in line with El Batawy et al. [30], while yoghurt samples were free from coliform bacteria.

**Table 5. Effect of SPE and SPE loaded liposome on growth of Lactic acid bacteria (log cfu/g)**

| Treatments | *L. casei* | | | | *L. rhamnosus* | | | | *L. reuteri* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6h | 12h | 24h | 48h | 6h | 12h | 24h | 48h | 6h | 12h | 24h | 48h |
| Control | $8.1^{B}$ | $8.6^{B}$ | $8.8^{C}$ | $8.9^{C}$ | $8.0^{B}$ | $8.2^{C}$ | $8.9^{C}$ | $9.0^{B}$ | $8.18^{A}$ | $8.32^{A}$ | $8.61^{B}$ | $8.71^{A}$ |
| SPE | $8.35^{A}$ | $8.85^{A}$ | $9.1^{B}$ | $9.3^{B}$ | $8.6^{A}$ | $8.8^{A}$ | $9.1^{B}$ | $9.2^{B}$ | $8.1^{A}$ | $8.3^{A}$ | $8.7^{A}$ | $8.76^{A}$ |
| SPE loaded liposome | $7.45^{C}$ | $8.5^{B}$ | $9.4^{A}$ | $9.7^{A}$ | $7.7^{C}$ | $8.6^{B}$ | $9.9^{A}$ | $9.95^{A}$ | $7.71^{C}$ | $8.0^{B}$ | $8.8^{A}$ | $8.85^{A}$ |

The means with the different capital (A, B, C…) superscript letters within the same column indicate significant ($P \leq 0.05$) differences between treatments

**Table 6. Microbiological analysis of yoghurt fortified with SPE liposome during storage**

| Treatments* | Counts (log cfu/ml) Storage period (day) | | | |
|---|---|---|---|---|
| | **Fresh** | 3 | 7 | 15 |
| | | ***Lb. delbrueckii ssp. bulgaricus*** | | |
| Control | $7.90^{A}$ | $7.0^{C}$ | $6.81^{B}$ | $6.18^{D}$ |
| **T1** | $7.11^{E}$ | $6.98^{C}$ | $6.51^{C}$ | $6.01^{E}$ |
| **T2** | $7.2^{D}$ | $7.1^{B}$ | $6.81^{B}$ | $6.5^{C}$ |
| **T3** | $7.28^{C}$ | $7.11^{B}$ | $6.85^{B}$ | $6.62^{B}$ |
| **T4** | $7.31^{B}$ | $7.21^{A}$ | $7.1^{A}$ | $6.78^{A}$ |
| | | ***Str. thermophilus*** | | |
| Control | $8.1^{A}$ | $7.77^{A}$ | $7.12^{D}$ | $6.23^{C}$ |
| **T1** | $7.8^{B}$ | $7.71^{AB}$ | $7.30^{C}$ | $6.80^{B}$ |
| **T2** | $7.81^{B}$ | $7.75^{B}$ | $7.41^{B}$ | $6.91^{A}$ |
| **T3** | $7.95^{A}$ | $7.80^{AB}$ | $7.51^{A}$ | $6.95^{A}$ |
| **T4** | $8.1^{A}$ | $7.95^{A}$ | $7.60^{A}$ | $7.00^{A}$ |
| | | ***L. rhamnosus*** | | |
| Control | | | | |
| **T1** | $7.18^{C}$ | $7.65^{B}$ | $7.40^{D}$ | $6.55^{D}$ |
| **T2** | $7.25^{C}$ | $7.86^{A}$ | $7.55^{C}$ | $6.78^{C}$ |
| **T3** | $7.38^{B}$ | $7.98^{A}$ | $7.71^{B}$ | $6.88^{B}$ |
| **T4** | $7.61^{A}$ | $7.56^{C}$ | $7.80^{A}$ | $6.98^{A}$ |
| | | **Total bacteria count** | | |
| Control | $8.18^{C}$ | $8.2^{C}$ | $8.0^{C}$ | $7.95^{C}$ |
| **T1** | $8.12^{C}$ | $8.23^{C}$ | $8.25^{B}$ | $8.10^{B}$ |
| **T2** | $7.95^{D}$ | $8.11^{D}$ | $8.30^{B}$ | $8.11^{B}$ |
| **T3** | $8.65^{A}$ | $8.71^{A}$ | $8.61^{A}$ | $8.21^{AB}$ |
| **T4** | $8.5^{B}$ | $8.65^{B}$ | $8.51^{A}$ | $8.30^{A}$ |
| | | **Yeast and mold** | | |
| Control | ND | ND | $2.3^{A}$ | $3.1^{A}$ |
| **T1** | ND | ND | $2.0^{B}$ | $2.5^{B}$ |
| **T2** | ND | ND | $2.0^{B}$ | $2.0^{C}$ |
| **T3** | ND | ND | ND | $2.0^{C}$ |
| **T4** | ND | ND | ND | ND |

*****See footnote table (2). The means with the different capital (A, B, C…) superscript letters within the same column indicates significant (P≤0.05) differences between treatments**.**

*Cytotoxic activity effect of SPE, SPE liposome and yoghurt fortified with SPE liposome using different types of cell lines*

According to the results in **Table 7 and 8**, SPE possessed high cytotoxic activity over colon and prostate cancer cells. Also, SPE liposome assayed for their cytotoxic potentiality, the results imply the activity of the SPE with moderate activity on colon cancer cells and weak activity on prostate cells, while SPE liposome alone were examined its effect on cancer cells as well, to confirm the responsibility of the powder of their anticancer activity. SPE was further assayed in a dose-dependent manner at 4 different concentrations ranged from from100-12.5 ug/ml to calculate IC 50 and SI. SPE liposome showed IC50 23.1±0.3 on CaCo2 with SI 2.1, while SPE had IC50 5.5±0.5 on PC3 with SI value 8.7. The high selectivity index values of SPE suggest their safety

**Table 7. *In vitro* cytotoxicity of samples, against human colon tumor (CaCo2), lung tumor (A-549) cell lines and human prostate tumor (PC3) at concentration and human normal skin fibroblast 100ug/ml***

| Treatments | caco2 | A-549 | Pc3 | BJ-1 |
|---|---|---|---|---|
| SPE | 96.9 | 12.1 | 88.8 | 66.5 |
| SPE liposome | 60.8 | ND | 27.9 | 7.1 |
| liposome (without SPE) | 3.7 | ND | 11.7 | 0 |

**Table 8.** ***In vitro*** **cytotoxicity percent of samples, against human colon tumor (CaCo2) and human prostate tumor (PC3) at concentration 100ug/ml***

| Treatments* | PC3 | caco2 |
|---|---|---|
| Control | 71.5 | 47.5 |
| $T_1$ | 53.9 | 44.2 |
| $T_2$ | 82.3 | 49.0 |
| $T_3$ | 53.1 | 45.4 |
| $T_4$ | 42.1 | 47.4 |

*See footnote table (2).

and selectivity of the cytotoxic effect on cancer cell rather than normal cells.

## Conclusion

It could be concluded that liposomal systems in dispersion were employed for the delivery of sage phenolics via yoghurt. It was observed that stability of Sage phenolic extract (SPE) in yoghurt samples showed better results even during storage period of 15 days. To suggest the form of liposomal system to be used in liquid foods, physicochemical properties of such foods including pH, zeta potential etc. will be crucial.

## Conflicts of interest

No conflict of interest was reported by the authors.

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None

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