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Green Synthesis of SP-SeNPs as Promising Antioxidant Agent and Pancreatic Lipase Inhibitor

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The purpose of this study was to evaluate pancreatic lipase inhibitory activity and antioxidant effect of *Spirulina platensis* containing selenium nanoemulsion nanoparticles (SP-SeNPs). The SP-SeNPs were prepared and characterized to find mean particle size and zeta potential to evaluate the possible lipoprotein lipase and pancreatic lipase inhibitory as well as antioxidant and free radical scavenging activities. Different antioxidant tests were employed, namely, reducing power, chelating activity on Fe^{2+}, free radical-scavenging and total antioxidant activities. The obtained results showed that the shapes of SP-SeNPs were spherical and the mean particle size was 39.86 ± 0.14 nm and negative zeta potential was +33.14. The SP-SeNP at 60 ppm and epicatechin (100 ppm) produce the same ratio of inhibition 18%. However, lipoprotein lipase activity was increased to less than 20 and 27 % at 80 and 100 ppm SP-SeNPs. Also, the present results indicate that the SP-SeNPs at concentration of 80 and 100 ppm showed inhibitory effects more than epicatechin (100 ppm) against pancreatic lipase activity. However, the reducing power of SP-SeNPs and butylated hydroxytoluene (BHT), butylated hydroxy anisole (BHA) and α-tocopherol decreased in the order of BHA> SP-SeNPs >BHT > α-tocopherol. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. However, the chelating activity of SP-SeNPs of 1.50 mg/mL was nearly equal to EDTA at 0.037 mg/mL (43.67%) for an incubation time of 60 min. This indicates that the chelation property of the samples on Fe^{2+} ions may afford protection against oxidative damage. The SP-SeNPs of 6 mg/mL had the highest radical scavenging activity when compared with 0.6 mg/mL Trolox. The antioxidant activity of SP-SeNPs and both standards decreased in the order of SP-SeNPs > α-tocopherol > Trolox > BHA > BHT. The results suggested that the inhibitory activity of SP-SeNPs is closely related to the tertiary structural change in lipoprotein lipase and pancreatic lipase. Also, the antioxidant activity of SP-SeNPs could be due to presence of carotenoids, ω-3 or ω-6 polyunsaturated fatty acid, γ-linolenic acid, sulpholipides, glycolipids, potassium, zinc and selenium, vitamin A, vitamin E and a variety of minerals.

Keywords: *Spirulina platensis* nanoparticles, Selenium nanoemulsion, Pancreatic lipase, Lipoprotein lipase, Chelating activity.

INTRODUCTION

Spirulina is a blue-green microalgae have a spiral cellular structure and has an extraordinary capacity to survive under conditions that are too harsh for other algae [1,2]. Two species of Spirulina that are most commonly used in nutritional supplements are *Spirulina platensis* is the most common species of Spirulina family that large amounts of protein, ω-3, ω-6, γ-linolenic acid (GLA), sulfolipids, vitamins and minerals [2]. It is, therefore, a potential therapeutic agent for treating oxidative stress-induced diseases [3].

The adipocyte hormone, leptin, has a central role in the regulation of food intake, energy expenditure and body fat stores [4,5]. Circulating leptin concentrations are well correlated with adipose stores in humans [6-8] and animals [8-10]. However, leptin production is also acutely regulated by nutritional status. For example, circulating leptin decreases after fasting [9,11-13] or energy restriction [14,15] and increases after refeeding or overfeeding [12,16]. *In vitro* studies have shown that insulin increases leptin expression and secretion in isolated rodent [17-19] and human [20,21] adipocytes.

Recently, algae are considered to be a good source for anti-obesity agents [22]. Jung *et al.* [23] reported that the edible brown alga *Ecklonia stolonifera* and its constituent fucosterol decreased lipid accumulation in 3T3-L1 pre-adipocytes by inhibiting the expression of adipocyte marker proteins peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα). Choi *et al.* [24]

indicated that a major phlorotannin dieckol from *Ecklonia cava* decreased body weight gain (38%) in high-fat diet-fed mice by activating AMP-activated protein kinase (AMPK) signaling.

Nanoparticles can reduce side effects in patients by targeting the area of disease directly and removing the need of circulation throughout the body [25,26]. *In vivo* tests have been conducted with *Spirulina platensis* and reported for example, its significantly reduced the body weight, fatty liver, amount of white adipose tissue and levels of triglyceride and glucose in obese mice fed a high-fat diet [27,28]. But there are no reports of the effect of *Spirulina platensis* on adipocyte glucose utilization and leptin production in primary cultures of isolated adipocytes. As an extension of our interested research program to evaluate the medicinal importance of new synthesized drugs and natural products [29-33]. A facile route is reported to evaluate the therapeutic potential of *Spirulina platensis*-selenium nanoemulsion (SP-SeNPs) on leptin secretion from cultured rat adipocytes.

EXPERIMENTAL

Spirulina platensis water extract powder (100%) was purchased from Zazzee Natruals, USA. All other chemicals used in this study were of the analytical grade.

Green synthesis of *Spirulina platensis*-selenium nanoemulsion (SP-SeNps): A 20 mM ascorbic acid (Vc) solution was freshly prepared by dissolving 35.2 mg Vc powder in 10 mL of Milli-Q water. *Spirulina platensis* water extract (SPWE) was dissolved in deionized water and diluted in deionized water (90 mL) in a conical flask as follows: Dissolved selenious acid (H_2SeO_3, 0.013 g, 0.01 mmol) in 10 mL deionized water was added to the solution, with continuous stirring and heating at 60 ºC for 10 h; forming *in situ* after which 200 L of 40 mM ascorbic acid was added as a catalyst; the ruby red SeNPs were suspended and characterized by transmission electron microscopy (TEM).

***Spirulina platensis in situ* selenium nanoparticles (SP-SeNPs) characterization:** The crystal-line characteristics and grain dimensions of SP-SeNPs were determined by the X-ray diffraction pattern at 25-28 ºC with nickel (Ni) (D8 Advance X-ray diffractometer) filtered using Cu*K*α (λ = 1.54184 Å) radiation as X-rayed source. Scanning electron microscope and field transmission microscope at 15 Kv and 200 Kv have investigated the morphology and size of the SP-SeNPs.

Lipoprotein lipase (LPL) inhibition reaction: The ability of the SP-SeNPs to inhibit LPL was measured using the modified method previously reported by Schotz *et al.* [34]. An activator consisted of Apo C-II from human plasma and diluted to 1 µg/mL with 0.002 M *Tris* HCl (pH 8.0) was prepared. In the preparation of substrate, 0.6 mL triolein, 24 mL apo C-II, 3.6 mL of 1% BSA solution, 3.6 mL of 1% Triton X-100 and 28.8 mL of 0.2 *Tris* HCl buffer (pH 8.0) were mixed. The mixture was then sonicated in ice for 3 min. Enzyme LPL from bovine milk was prepared by diluting with 0.02 M *Tris* HCl (pH 8.0) to a concentration of 25 units/mL. The LPL activity was then determined using a method reported by Chung & Scanu [35]. Briefly, 0.5 mL of previously diluted LPL was added to 0.5 mL of SP-SeNPs serial concentrations (10, 20, 40, 60, 80 and 100 ppm) as well as epicatechin (100 ppm) as a standard drug in test tubes followed by pre-incubation at 4 ºC for 30 min. Then, 1 mL of substrate emulsion was added to the mixture of enzyme and extract, followed by incubation in water bath at 37 ºC to initiate hydrolysis. The reaction was stopped with the addition of 1 mL of 1 M NaCl. Control samples were consisted of mixture of enzyme and substrate only. The liberated free fatty acids (FFA) were titrated with 0.01 M NaOH until pH 9.4 using autotitrator (Metrohom, 785 DMP Titrino). The amount of liberated free fatty acid (FFA) was reflected by the amount of base required by the incubation mixture which is equivalent to LPL activity. Control sample was equivalent to 100% enzyme activity. The experiment was repeated thrice for each sample extract and percent inhibition was calculated.

Pancreatic lipase inhibition assay: Porcine pancreatic lipase (PPL, type II) activity was measured using *p*-nitrophenyl butyrate (*p*-NPB) as a substrate. The method used for measuring the pancreatic lipase activity was modified as described by Kim *et al.* [36] and Zheng *et al.* [37]. PPL stock solutions (1 mg/mL) were prepared in a 0.1 mM potassium phosphate buffer (pH 6.0) and the solutions were stored at –20 ºC. To determine the lipase inhibitory activity, SP-SeNPs final concentrations (5, 10, 20, 30, 60, 80 and 100 µg/mL) or Orlistat (at same concentrations) as a standard drug were pre-incubated with PPL (1000 ppm) for 1 h in a potassium phosphate buffer (0.1 mM, pH 7.2, 0.1% Tween 80) at 30 ºC before assaying the PPL activity. The reaction was then started by adding 0.1 µL NPB as a substrate, all in a final volume of 100 µL. After incubation at 30 ºC for 5 min, the amount of *p*-nitrophenol released in the reaction was measured at 405 nm using a UV-visible spectrophotometer (BioTek Synergy HT, Winooski, USA). The activity of the negative control was also examined with and without an inhibitor. The inhibitory activity (I) was calculated according to the following formula:

$$\text{Inhibitory activity (\%)} = 100 - \frac{(B-b)}{(A-a)} \times 100$$

where A is the activity without inhibitor; a is the negative control without inhibitor; B is the activity with inhibitor; and b is the negative control with inhibitor. DMSO was used as negative control and its activity was also examined.

Antioxidant activity

Determination of reducing power: The reducing power of SP-SeNPs was measured according to the method of Oyaizu [38]. Various concentrations of SP-SeNPs (20-140 µg) in 1 mL of distilled water were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 7.6) and 2.5 mL potassium ferricyanide (1%, w/v) and then the mixture was incubated at 50 ºC for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper-layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%, w/v) and the absorbance was measured at 700 nm. α-Tocopherol, BHA and BHT were used as standard antioxidants. Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of chelating activity on Fe^{2+}: The chelating activity of SP-SeNPs on ferrous ions (Fe^{2+}) were measured according to the method of Decker & Welch [39]. Aliquots of 1 mL of different concentrations (0.25, 0.50, 1.0, 1.25 and 1.5 mg/mL) of samples were mixed with 3.7 mL of deionized water. The mixture was incubated with $FeCl_2$ (2 mM, 0.1 mL) for 5, 10, 30 and 60 min. After incubation the reaction was initiated by addition of ferrozine (5 mM and 0.2 mL) for 10 min at room temperature and then the absorbance was measured at 562 nm in a spectrophotometer. A lower absorbance indicates a higher chelating power. The chelating activity of SP-SeNPs on Fe^{2+} were compared with that of EDTA at a level of 0.037 mg/mL. Chelating activity was calculated using the following formula:

$$\text{Chelating activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Control test was performed without addition of SP-SeNPs

Determination of free radical-scavenging activity: The free radical scavenging activity of SP-SeNPs were measured with 1,1-diphenyl-2-picrylhydrazil ($DPPH^{\bullet}$) using the slightly modified methods of Brand-William *et al.* [40] and Takashira & Ohtake [41]. Briefly, 6×10^{-5} mol/L $DPPH^{\bullet}$ solution in ethanol was prepared and 3.9 mL of this solution was added to 0.1mL of SP-SeNPs (2- 6 mg/mL) and Trolox solution (0.02- 0.06 mg/mL). The mixture was shaken vigorously and the decrease in absorbance at 517 nm was measured at 5, 10, 30 and 60 min. Water (0.1 mL) in place of SP-SeNPs were used as control. The percent inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of SP-SeNPs sample.

Determination of total antioxidant activity: The antioxidant activity was determined according to the thiocyanate method of Osawa & Namiki [42] with slight modifications. For stock solution, 10 mg of SP-SeNPs was dissolved in 10 mL water. Then the solution of SP-SeNPs or standards samples (tocopherol, Trolox, BHA and BHT) [100 mg/L] in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.6) was added to 2.5 mL of linoleic acid emulsion. Fifty mL linoleic acid emulsion contained Tween-20 (175 µg), linoleic acid (155 µL) and potassium phosphate buffer (0.04 M, pH 7.6). On the other hand, 5.0 mL of control containing 2.5 mL of linoleic acid emulsion and 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.6). Each solution was then incubated at 37 ºC in a glass flask in the dark. At 24 h intervals during incubation, 0.1 mL of this incubation solution was added to 4.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after 0.1 mL of 0.02 M $FeCl_2$ in 3.5% (w/v) HCl was added to the reaction mixture, the absorbance of the red colour was measured at 500 nm in a spectrophotometer. The solutions without added SP-SeNPs or standards were used as the control. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of SP-SeNPs or standards.

Statistical analysis: All data were statistically evaluated with SPSS/13 software. Hypothesis testing methods included one way analysis of variance (ANOVA). *P*-values of less than 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Transmission electron microscopy (TEM) analysis showed that the size of SP-SeNPs was around 39.86 ± 0.14 nm with negative zeta potential of +33.14 (Fig. 1).

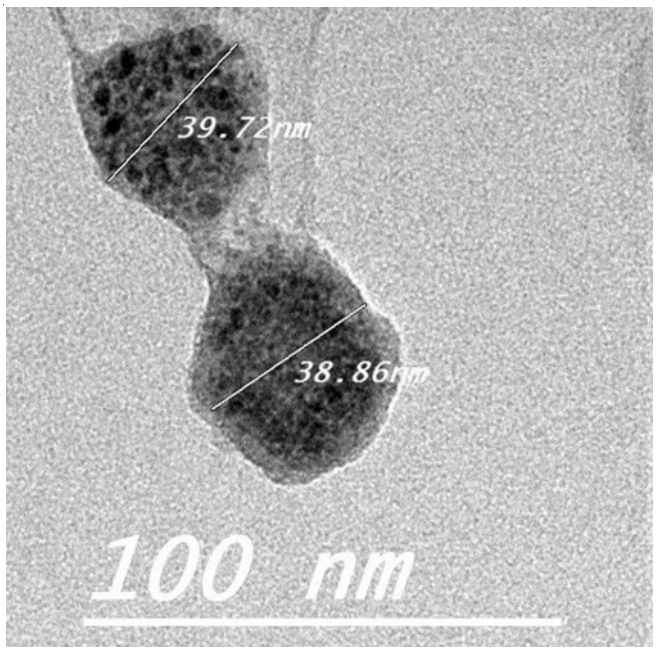

Fig. 1. TEM image of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE)

Pancreatic lipase is the key enzyme for dietary fat digestion [43] and inhibition of the enzyme could be an effective way to alter fat absorption. In fact, Orlistat, pancreatic lipase inhibitor and sibutramine, an appetite suppressor is the only two anti-obesity medications currently approved [44]. However, because Orlistat can result in undesirable side effects, such as fecal incontinence, flatulence and steatorrhea, its use may be limited [45]. Therefore, it may be worthwhile to search the natural substances that show potent inhibitory activity against pancreatic lipase and have fewer side effects.

Fig. 2 shows the inhibitory activity of lipoprotein lipase by SP-SeNPs serial concentrations (10, 20, 40, 60, 80 and 100 ppm) as well as epicatechin (100 ppm). The SP-SeNPs at 60 ppm and epicatechin (100 ppm) produce the same ratio of inhibition 18%. However, lipoprotein lipase activity was increased to less than 20 and 27 % at 80 and 100 ppm SP-SeNPs. The SP-SeNPs inhibitory activity due to presence of various extents and this phenomenon was explained by previous reports on natural lipoprotein lipase-inhibitory compounds in plants,

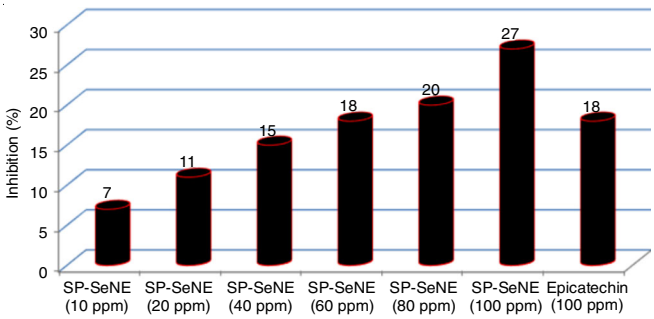

Fig. 2. Inhibitory effects (%) of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE) serial concentrations (10, 20, 40, 60, 80 and 100 ppm) as well as epicatechin (100 ppm) on lipoprotein lipase (0.1 mg/mL). All experiments were repeated three times with triplicate readings

such as polyphenols, saponins and terpenes [46]. These effects could be due to presence of carotenoids, ω-3 or ω-6 polyunsaturated fatty acid, γ-linolenic acid, sulpholipides, glycolipids, potassium, zinc and selenium, vitamin A, vitamin E and a variety of minerals in SP-SeNPs [47].

Fig. 3 shows inhibitory effects of *Spirulina platensis*-selenium nanoemulsion (SP-SeNPs) and orlistat (5, 10, 20, 30, 60, 80 and 100 µg/mL) on pancreatic lipase (1000 ppm). Based on a comparison of the absorbance at 700 nm, the inhibitory effects of SP-SeNPs at a concentration of 70 µg/mL is equal half value of inhibitory effects on pancreatic lipase when compared to orlistat at the same concentration.

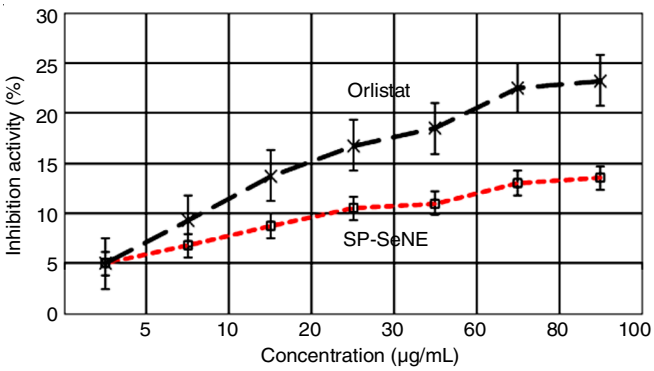

Fig. 3. Inhibitory effects (%) of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE) and Orlistat (5,10, 20, 30, 60, 80 and 100 µg/mL) on pancreatic lipase (1000 ppm). All experiments were repeated three times with triplicate readings

Fig. 4 shows the reducing power of *Spirulina platensis*-selenium nanoemulsion (SP-SeNPs), α-tocopherol, BHA and BHT. The reducing power of SP-SeNPs increased with the increasing concentration. Based on a comparison of the absorbance at 700 nm, the reducing power of SP-SeNPs at a concentration of 110 µg/mL was higher than BHT and α-tocopherol as well as less than BHT at the same concentration. This indicates that SP-SeNPs was electron donors and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction. Also, 110 µg/mL SP-SeNPs is the best concentration which exhibits the most reducing power. The reducing power of SP-SeNPs and BHA, BHT and α-tocopherol decreased in the order of BHA > SP-SeNPs > BHT > α-tocopherol.

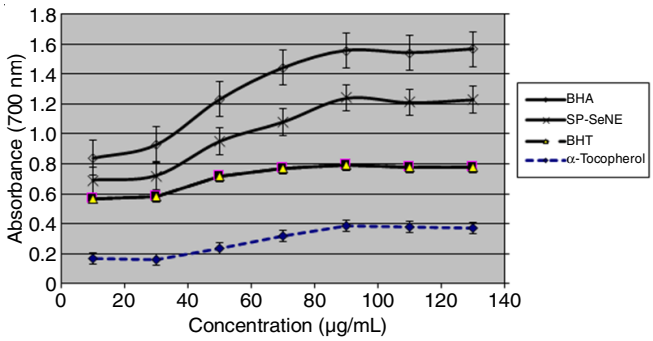

Fig. 4. Reducing power of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE), BHA, BHT and α-tocopherol

Fig. 5 shows the chelating effect of SP-SeNPs. All samples at 1.25 and 1.50 mg/mL concentration showed more than 30 % chelating effect on ferrous ions at an incubation time of 120 min. Also, the chelating activity of SP-SeNPs at a concentration of 1.0, 1.25 and 1.50 mg/mL are the same at the zero time. In addition, the maximum chelating activity of SP-SeNPs at a concentration at 1.50 mg/mL. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. However, the chelating activity of SP-SeNPs of 1.50 mg/mL was nearly equal to EDTA at 0.037 mg/mL (43.67%) for an incubation time of 60 min. This indicates that the chelation property of the samples on Fe^{2+} ions may afford protection against oxidative damage.

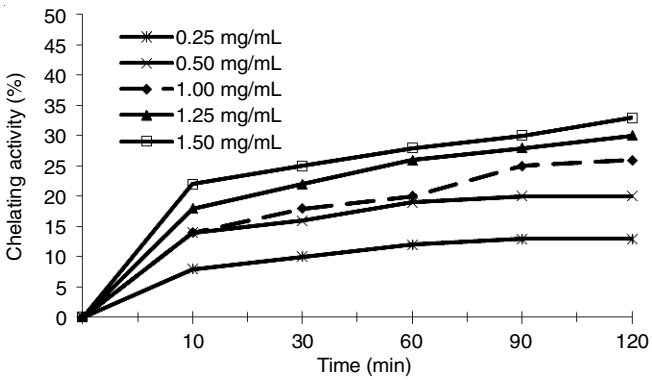

Fig. 5. Chelating effects of different concentrations of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE) on Fe^{2+} ions at different incubation times with $FeCl_2$

The DPPH• radical scavenging effects of SP-SeNPs are presented in Fig. 6 and showed appreciable free radical scavenging activities. The free radical scavenging activity of SP-SeNPs was compared to Trolox, as a synthetic antioxidant. SP-SeNPs of 6 mg/mL had the highest radical scavenging activity when compared with 0.6 mg/mL Trolox.

Fig. 7 shows the total antioxidant activity at 100 mg/L of SP-SeNPs in comparison to the same amount of α-tocopherol, Trolox, BHA and BHT. At 100 mg/L concentrations, SP-SeNPs showed higher antioxidant activity than α-tocopherol, Trolox, BHA and BHT on peroxidation of linoleic acid emulsion. The antioxidant activity of SP-SeNPs and both standards decreased in the order of SP-SeNPs > α-tocopherol > Trolox > BHA > BHT.

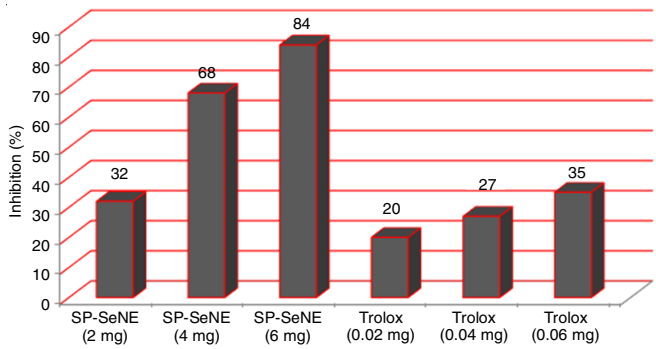

Fig. 6. Scavenging activities of different concentrations of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE) and Trolox against 1,1-diphenyl-2-picryl-hydrazil (DPPH$^{\bullet}$) radical

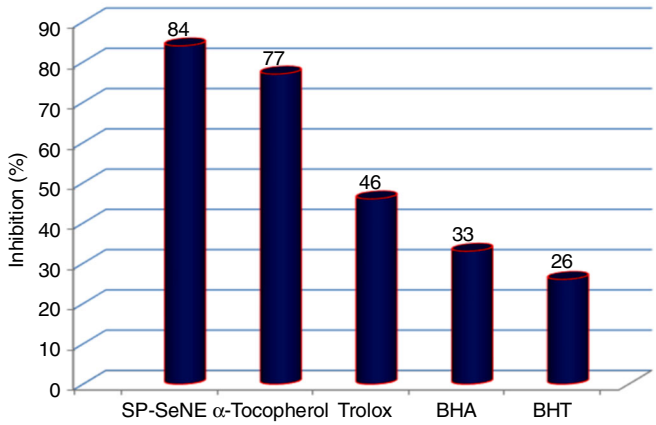

Fig. 7. Total antioxidant activities of *Spirulina platensis*-selenium nanoemulsion (SP-SeNE), α-tocopherol, Trolox, BHA and BHT (100 mg/L concentration) on peroxidation of linoleic acid emulsion

In the reducing power assay, the presence of reductants (antioxidants) in the tested samples resulted in the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The amount of Fe^{2+} complex can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

The SP-SeNPs may serve as a hydrogen donor for α-tocopherol radical, thus regenerating α-tocopherol, a key element of redox balance in biosystems. These effects could be due to presence of carotenoids, ω-3 or ω-6 polyunsaturated fatty acid, γ-linolenic acid, sulpholipides, glycolipids, potassium, zinc and selenium, vitamin A, vitamin E and a variety of minerals in SP-SeNPs [47]. All the mentioned structural conditions may be found in a SP-SeNPs which, in the *in vitro* systems efficiently scavenges hydroxyl radical (OH$^{\bullet}$), superoxide radical (LOO$^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and nitrogen oxide (NO$^{\bullet}$).

Conclusion

The present study showed that the effects of antioxidative activity of *Spirulina platensis* containing selenium nanoemulsion nanoparticles (SP-SeNPs) depend on the presence of carotenoids, ω-3 or ω-6 polyunsaturated fatty acid, γ-linolenic acid, sulpholipides, glycolipids, potassium, zinc, selenium, vitamin A, vitamin E and a variety of minerals. The antioxidant activity of SP-SeNPs and both standards decreased in the order of SP-SeNPs > α-tocopherol > Trolox > BHA > BHT.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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