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RESEARCH ARTICLE

Synthesis characterisation and neuroprotectivity of *Silybum marianum* extract loaded chitosan nanoparticles

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ABSTRACT

Aim: *Silybum marianum* extract (SME) possesses neuroprotective potency through its high antioxidant content. We attempted to increase the effectiveness of SME by encapsulating them in chitosan. Neuroprotective potency of SME and SME-loaded chitosan nanoparticles (SME-CNPs) were shown in SH-SY5Y cell line against H_2O_2-induced oxidative stress.

Methods: We produced CNPs and SME-CNPs by ionic gelation method and properly determined their physical characteristics. Encapsulation efficiency, loading capacity, and *in vitro* release tests were performed for SME-CNPs. The neurotoxicity and neuroprotective efficiency in SH-SY5Y cell line against H_2O_2 was also investigated.

Results: The size of SME-CNPs was 168.2 ± 11.12 nm with zeta potential 10.6 ± 1.0 mV. The encapsulation efficiency and loading capacity were successfully achieved at 96.6% and 1.89% respectively. SME and SME-CNPs improved cell viability higher than 80%, and SME-CNPs exhibited significant neuroprotective effects against H_2O_2 damage.

Conclusions: It was concluded that SME and SME-CNPs highly prevent damage caused by H_2O_2 and reduce cell damage *in vitro* by their neuroprotective effects.

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KEYWORDS
Alzheimer's disease; chitosan nanoparticles; encapsulation; ionic gelation; neuroprotective effect; oxidative damage; *Silybum marianum* extract

Introduction

Silybum marianum, commonly called milk thistle, belongs to the Asteraceae family and contains valuable biological active components such as silibinin and a complex of flavonolignan and phytosterols known as silymarin, silychristin, dihydrosilybin, silydianin, and so on (Fernandes *et al.* 2018, Wang *et al.* 2020b). According to high-quality publications related to the pharmacological effects of *S. marianum*, its antimicrobial, anticancer, hepato-protective, cardiovascular-protective, skin-protective, and neuroprotective potentials are highlighted (Wang *et al.* 2020b). *In vitro* and *in vivo* studies have shown that *S. marianum* and its active ingredients have neuroprotective effects own to its anti-inflammatory and antioxidant properties thus, it is emphasised as a remarkable plant to be used for neurodegenerative diseases such as Alzheimer's Disease (AD) (Kumar *et al.* 2015, Filippopoulou *et al.* 2017, Nazir *et al.* 2018). AD is a complex neurodegenerative disease and progresses depending on age. Therapeutic approaches are being developed but an effective therapeutic method has not been developed yet. Current applications for AD treatment are on researching new approaches, developing new formulations, and repositioning active ingredients via new technologies such as in silico methods (Akhtar *et al.* 2021). Since the bioavailability of drugs in the brain is poor, drug delivery systems drugs are being improved to increase the bioavailability and efficacy of drugs and facilitate their delivery to the brain (Cortés *et al.* 2020, Nguyen *et al.* 2021). Drug delivery systems have attracted great attention and their use in treatments against various diseases such as cancer and neurodegenerative diseases have great potential in recent years. The data obtained from several studies reveal that nanoparticle-based drug delivery systems decrease the side effects caused by the pharmaceutical formula and increase the drug bioavailability (Jang *et al.* 2014). Also, the effectiveness of the nanotechnology-based drug delivery changes according to their fabrication methods. Chitosan is a very attractive polymer for drug delivery systems for use in medicine and pharmaceuticals in terms of its properties such as biocompatiblity, biodegradablity,

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and strong antibacterial effect (Narayanan *et al.* 2014). In addition to these known features, it has been reported that chitosan exhibits neuroprotective properties too. Therefore, it is hoped that *Silybum marianum* extract-loaded chitosan nanoparticles can exhibit a stronger neuroprotective effect and thus this nanodrug can be used against neurological damage (Budama-Kilinc et al. 2017, Chen *et al.* 2018, Ragusa *et al.* 2018, Wang *et al.* 2020a, Karavelioglu and Cakir-Koc, 2021).

The aim of this study is to establish *in vitro* AD model by oxidative damage and toxic effect of H_2O_2 on SH-SY5Y cell line, and to investigate the neuroprotective effects of *S. marianum* extract (SME) and the SME loaded chitosan nanoparticles (SME-CNPs) in this *in vitro* model.

Materials and methods

Chemicals and procedures

S. marianum liquid extract was obtained from Immunat Herbal Pharmaceuticals (Mugla, Turkey). Chitosan (50.000–190.000 kDa, low molecular weight), sodium tripolyphosphate (TPP), tween 80, and all the chemicals used in nanoparticle synthesis were purchased from Merck (Darmstadt, Germany). All the chemicals and solvents were analytical grade. For cell culture studies, SH-SY5Y human neuroblastoma cell was obtained from American Type Culture Collection (ATCC). Foetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12), Trypsin-EDTA, and Trypan Blue were purchased from Gibco® (MT, USA), antibiotic solution of penicillin-streptomycin from PAN Biotech. 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), phosphate-buffered saline (PBS), phenazine methosulfate (PMS), H_2O_2, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and DAPI were purchased from Merck, and Santa Cruz Biotechnology® (Texas, USA), respectively.

In this study, it is very important to encapsulate the SME with chitosan. For this purpose, the optimal SME concentration on SH-SY5Y cell line was determined. Initially, cell viability assays were performed for both SME and chitosan, then SME-loaded chitosan nanoparticles (SME-CNPs) were synthesised and characterised. On the other hand, *in vitro* AD model was generated by the induction of H_2O_2 and finally, the neuroprotective effect of SME-CNPs was evaluated. All the experimental sets in this study were triplicated.

Cell culture

SH-SY5Y cell line is a human neuroblastoma cell type that is often used as an *in vitro* model of neuronal diseases (Xie *et al.* 2010, Kovalevich and Langford, 2013, Xicoy *et al.* 2017). The cells were maintained in DMEM/F-12 supplemented with 10% FBS (v/v) and 0.5% penicillin/streptomycin (v/v). Cultures of SH-SY5Y were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Determination of the cytotoxic effects of SME on SH-SY5Y cell line was performed with XTT method (Karavelioglu and Cakir-Koc, 2021). Cells were seeded at a density of $1x10^4$ cells/well into 96-well plates and incubated for 24 h. Then, the cells were treated with SME with a concentration range between 2.5 and 20 μg/mL for 24 and 48 h and afterward XTT assay was performed. Cell viability was measured by a microplate reader (Fluoroskan Ascent, Thermo Labsystems, Helsinki, Finland) at 570 nm wavelength.

To examine the neuroprotective effect of SME on 1 mM H_2O_2-induced SH-SY5Y cell line, cells were seeded at a density of $1x10^4$ cells/well into 96-well plates, at 37 °C in a humidified 5% CO_2 atmosphere. Non-toxic concentrations of SME (at a range of 2.5 to 20 μg/mL) were added into wells and cultured for 24 h in an incubator. Subsequently, an XTT assay was performed to evaluate cell viability and proliferation for the optimum application.

Antioxidant capacity

In the body, antioxidants have a protective role against to destructive effects of free radicals damage such as H_2O_2-induction (Halliwell, 1996, Marques *et al.* 2014, Rubio *et al.* 2016). Therefore, the CUPRAC method was used to examine the total antioxidant capacity of SME (Rubio *et al.* 2016). Briefly, solutions of copper (II) chloride ($CuCl_2$), ammonium acetate (NH_4Ac), and neocuproine (Nc) were prepared. Different concentrations of SME and trolox compound were used as standard and added into 1 ml of $CuCl_2$, NH_4Ac, and Nc buffer solutions respectively at certain concentrations. The absorbance values were measured at 450 nm wavelength, and the graphs of extracts corresponding to trolox calibration curve were created (Apak *et al.* 2008).

Determination of ROS and apoptosis

To determine the effect of H_2O_2 and SME on apoptosis, the 4′,6-diamidino-2-phenylindole (DAPI) staining method was performed. SME-treated SH-SY5Y cells

were exposed to 1 mM H_2O_2 for 30 min. Subsequently, the medium in the wells was replaced with DAPI solution and incubated at 37 °C for 15 min in the dark. Apoptotic cells were observed with a fluorescence microscope (Leica, DM3000) (Pajaniradje *et al.* 2014). Cells appearing in bright blue colour indicate the presence and density of apoptotic cells.

The ROS production was evaluated by the DCFH-DA assay to determine the effect of H_2O_2 and SME. SME-treated SH-SY5Y cells were exposed to 1 mM H_2O_2 for 30 min. After that, the medium in the wells was replaced with DCFH-DA solution and incubated at 37 °C for 30 min and staining was performed. The cells were observed using fluorescence microscopy in the dark (Wang *et al.* 2009).

Synthesis of nanoparticles

The ionic gelation technique is widely used for the synthesis of chitosan nanoparticles which has been improved through a number of methodological modifications (Desai, 2016). Chitosan nanoparticles (CNPs) and SME-loaded chitosan nanoparticles (SME-CNPs) were synthesised by ionic gelation method and for this purpose chitosan in a concentration of 0.1% (w/v) was dissolved in 1% (w/v) acetic acid solution, and then stirred overnight at 1000 rpm. After that tween 80 and SME (20 μg/mL) were added into 5 ml of chitosan solution and again stirred for 1.5 h at 1000 rpm. SME was not added for blank CNPs. TPP solution (0.1% w/v) as a crosslinking reducing agent was mixed in distilled water at 1000 rpm for 1 h. 2 ml TPP was added into the chitosan solution and stirred for 1 h at 1500 rpm. Finally, the mixture was centrifuged at 4100 rpm for 30 min. Thus, pellets were discarded and the lyophilised supernatant was used for further characterisation studies (Karavelioglu and Cakir-Koc, 2021).

Characterisation of nanoparticles

The size, polydispersity index (PDI), and zeta potential values of the nanoparticles were analysed by the Zetasizer Nano ZS (Malvern Instruments, UK) instrument. The particle size and PDI values were measured in the ZEN0040 cuvette using the dynamic light scattering (DLS) technique. The zeta potential values were measured in the DTS1070 cuvette and by the electrophoretic light scattering (ELS) technique (Ragusa *et al.* 2018).

Encapsulation of SME into chitosan was evaluated by FTIR analysis. Chitosan polymer, TPP, tween 80, SME, and lyophilised CNPs and SME-CNPs were used for FTIR analysis. The wavelength and absorbance values of the samples were measured and the characteristic peaks were evaluated depending on the chemical bond and functional groups.

The shape and surface morphologies of lyophilised CNP and SME-CNPs were examined using Scanning Electron Microscopy (SEM) (Zeiss EVO® LS 10). Briefly, lyophilised nanoparticles were placed on carbon tapes on grids and subsequently coated by gold-palladium. SEM images were taken at an acceleration voltage of 7 kV.

Encapsulation efficiency, loading capacity, and in vitro release test

One of the important issues of this study was the encapsulation efficiency (EE) and loading capacity (LC) of SME-CNPs. To examine that different dilutions of SME were prepared and a calibration curve and equation were created by taking absorbance measurements in the UV spectroscopy. Absorbance values of SME-CNPs were periodically taken in the UV spectroscopy. The SME amount was found by substituting the obtained data in the graph equation. After that, in order to calculate the EE and LC, the calculated formulas were used as our previous study (Yeşilkır-Baydar, 2021) inspired from Ragusa *et al* (2018) and Sato *et al.* (2020) (Ragusa *et al.* 2018, Sato *et al.* 2020).

0.01 M PBS solution (pH 7.4) was prepared and used as the release medium, in order to obtain *in vitro* release profiles of SME-CNPs. SME-CNPs were added to 20 ml of PBS solution and the solution was continuously stirred at 350 rpm and 37 °C. Their absorbance was measured in a UV spectrophotometer (Shimadzu, Japan) from the 0^{th} hour and at certain intervals. When the absorbance change was ended, the test was stopped and an *in vitro* release profile was obtained by creating a graph from these absorbance values (Ragusa *et al.* 2018).

Neuroprotective effect of nanoparticles

The neuroprotective effect of fabricated CNPs and SME-CNPs was determined by the XTT method. SH-SY5Y cells were seeded into 96-well plates and two groups of treatment were prepared. One of them was treated with CNPs and the other was treated with SME-CNPs at the concentrations between 2.5 and 20 μg/mL. After a 24-h incubation, groups were exposed to 1 mM H_2O_2 for 30 min to induce neurotoxicity and, cell viability was determined by XTT method.

Statistical analysis

Statistical analysis of the obtained data was performed using The GraphPad statistical software. By comparing samples and control means, mean values were considered statistically significant at $p < 0.05$, and data were presented as mean ± standard deviation.

Results and discussion

H_2O_2–induction of SH-SY5Y and evaluation of neuroprotective effect of SME

In vitro modelling of neurodegenerative diseases is possible with the treatment of oxidative agents such as H_2O_2. In this study, the neuroblastoma cells (SH-SY5Y) were induced with H_2O_2 and 1 mM of H_2O_2 caused significant oxidative damage. Also, the determination of the cytotoxic concentrations of SME was critical to examine its neuroprotective effect. For this purpose, concentrations of SME up to 20 μg/mL were applied to SH-SY5Y cells and were evaluated as non-toxic. Thence, it was determined that the SME has a neuroproliferative effect on SH-SY5Y cells (data not shown).

Oxidative damage which is one of the pathogenesis of neuronal diseases, especially for Alzheimer's Disease was evaluated by treating SH-SY5Y cells with SME and that induced with H_2O_2 as mentioned above. Obtained results showed that the increasing concentrations of the SME increased cell viability (Figure 1). In the experiment with 24 h pre-treatment, 5 and 10 μg/mL SME failed to prevent neurotoxicity, whereas 15 and 20 μg/mL SME preserved cell viability up to 79% and 84% against H_2O_2, respectively. In the experiment with 48 h pre-treatment, despite 5 and 10 μg/mL SME started to show the effect on cell viability, it did not have any significant effect. However, 15 and 20 μg/mL SME preserved cell viability up to 80% and 90%, respectively.

In vitro antioxidant capacity of SME on SHSY-5Y

The antioxidant capacity of SME was determined using the CUPRAC method compared to trolox compound, which has antioxidant properties. Trolox was used as a standard to obtain the calibration curve. According to the results, the R2 value was found to be 0.9989 and the line equation was $y = 0.0008\ x + 0.2359$. The obtained absorbance values of SME at certain concentrations were substituted for the "y" value in the equation, and the "x" values corresponding to trolox equivalents were found and shown on the calibration curve (Figure 2). It was evaluated that the antioxidant capacity of SME at 20 μg/mL concentration was higher than the other concentration values, and it corresponded to the antioxidant effect of 1641 μM trolox compound.

Observation of intracellular ROS level and apoptotic cells

DCFH-DA staining was performed to obtain the intracellular ROS levels of SH-SY5Y cells which were initially treated with SME and then induced with H_2O_2. Staining with DCFH-DA is based on the principle of emitted fluorescent light that is a result of its oxidation to dichlorofluorescein (DCF) by ROS. The effect of H_2O_2 on ROS formation was examined under a microscope using DCFH-DA staining and it was observed that H_2O_2 caused an increase in the intracellular ROS level as expected. In order to examine the inhibition of H_2O_2-induced ROS formation of SME, the cells

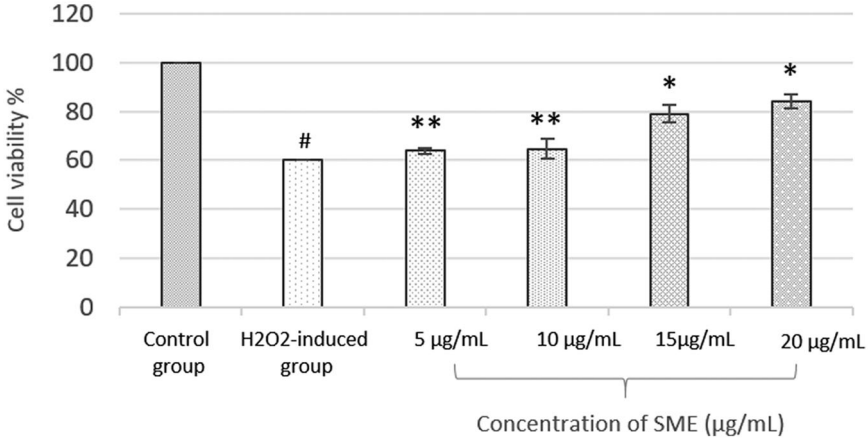

Figure 1. Neuroprotective effect of different SME concentrations on the cell viability against H_2O_2 damage on SH-SY5Y cells. The results were indicated as #$p < 0.001$ compared with the control and *$p < 0.001$ and **$p < 0.01$ compared with the H_2O_2 group.

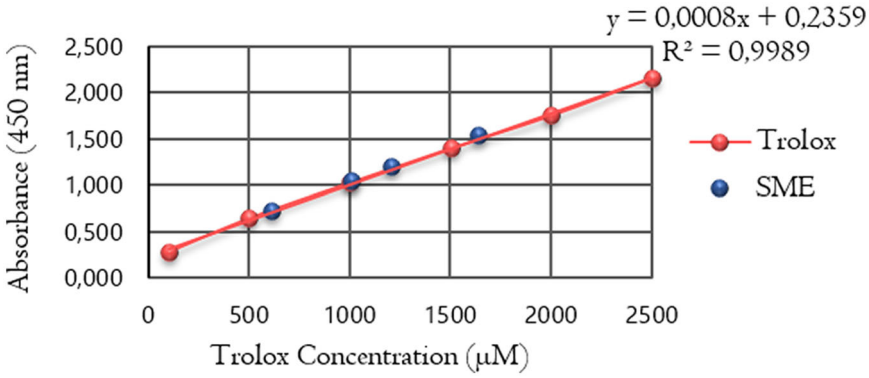

Figure 2. The calibration curve of antioxidant activity of Trolox-SME. The 20 μg/mL SME, which has the highest antioxidant capacity, was identified to correspond to the antioxidant effect of 1641 μM trolox compound.

Table 1. Size, Zeta potency and distribution results of CNPs and SME-CNPs (*[a]n = 5, SD: Standard deviation for 5 determinations.).*

Nanoparticles	Size (nm) (mean ± SD[a])	Zeta Potency (mV) (mean ± SD[a])	Polidispersity (mean ± SD[a])
Blank chitosan nanoparticles (CNPs)	154.7 ± 10.05	18.6 ± 1.4	0.135 ± 0.009
S. marinum extract loaded chitosan nanoparticles (SME-CNPs)	168.2 ± 11.12	10.6 ± 1.0	0.209 ± 0.012

treated with SME were exposed to H_2O_2 for 30 min, then DCFH-DA staining was performed, and their fluorescent images were examined under a microscope. It was qualitatively observed that increasing concentrations of SME reduced the H_2O_2-induced intracellular ROS level and the ROS level of 20 μg/mL SME had similarity to control cells (data not shown).

In order to examine the apoptotic effect of H_2O_2 on cells, DAPI staining method was performed. SH-SY5Y cells that had been exposed to only H_2O_2 were stained with DAPI. Observed results showed that the number of apoptotic cells was higher for the cells which were exposed to H_2O_2. Then, as mentioned above, cells that were previously treated with SME were then exposed to H_2O_2 and incubated for 24 and 48 h respectively. After incubation periods, DAPI staining was performed to both groups. Subsequently, samples were examined under a fluorescent microscope. It was observed that increasing concentrations of SME were able to reduce the number of H_2O_2-induced apoptotic cells and 20 μg/mL had a number of apoptotic cells significantly similar to control cells (data not shown).

Characterisation results of nanoparticles

While synthesis of *S. marinum* extract loaded chitosan nanoparticles (SME-CNPs), blank chitosan nanoparticles were also synthesised. Subsequently, nanoparticle characterisation procedures were applied to investigate accurately the effect of SME-CNPs *in vitro*.

The average size calculation of the nanoparticles was mentioned in Table 1 with the results of PDI and Zeta potency. Due to our nanoparticle fabrication procedures, it is revealed that the size of the blank CNPs have a diameter of 154.7 ± 10.05 nm, while SME-CNPs have 168.2 ± 11.12 nm. This is a good and essential range for cellular uptake which is associated with further *in vitro* and *in vivo* studies. The size of synthesised particles is critical and has a specific signification. Generally, nanoparticles which have diameters between 10 and 200 nm are generally preferred to be synthesised because nanoparticles larger than 200 nm are removed from bloodstream rapidly and nanoparticles smaller than 10 nm undergo renal filtration. Nanoparticles' size 10 to 200 nm range are mostly preferred as they can more effectively evade the reticuloendothelial system (RES) and increase the half-life of circulation (de Barros *et al.* 2012). Associated with their increased blood circulation rate, they have an improved targeting effect especially to brain (Lockman *et al.* 2003, Kulkarni and Feng, 2013). On the other hand, Kulkarni *et. al* (2013) reported that cellular uptake of nanoparticles (NPs) of sizes less than 200 nm improves the cellular uptake and can escape from acquaintance by the RES, since *in vivo* investigation of the NPs biodistribution after intravenous administration is critical (Kulkarni and Feng, 2013).

SEM is a versatile, powerful, and useful tool for material characterisation (Müller *et al.* 2020). For this purpose, the distribution of the nanoparticles was analysed by SEM (Figure 3A). It was observed that the

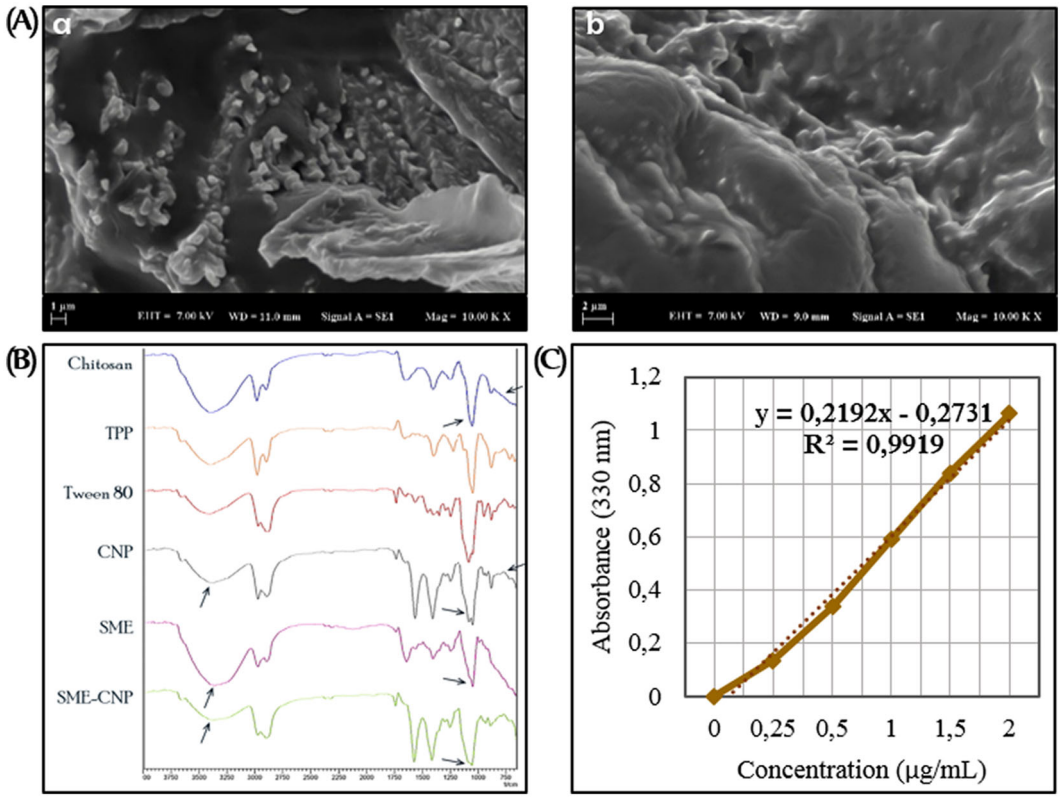

Figure 3. (A). SEM image showing the distribution of CNPs (a) and SME-CNPs (b). (B). FTIR spectrums of chitosan, TPP, tween 80, CNPs, SME, and SME-CNPs. In between 1085 and 1050 cm^{-1} and between 780 and 760 cm^{-1}, new peaks occurred in CNP compared to chitosan. It is observed that the peaks between 1085 and 1050 cm^{-1} blunt in SME-CNP compared to CNP and the peak at 3385 cm^{-1} in CNP shifted to 3355 cm^{-1} in SME-CNP. (C). Calibration curve and equation of SME. From the values, encapsulation efficiency and loading capacity were calculated as 96.6% and 1.89%, respectively.

images obtained in SEM had similar dimensions with the data in DLS dimensional analysis and nanoparticles have spherical shapes at 160 ± 10 nm average diameter (Table 1).

FTIR analysis was evaluated by comparing the specific peaks in the structure of SME, chitosan, TPP, tween 80, and lyophilised nanoparticles in the nanoparticle formulation in which SME-CNPs synthesis was carried out successfully. The spectra were obtained in the range of 4000–750 cm^{-1} as shown in Figure 3(B).

The IR band between 3000 and 2840 cm^{-1} represents the C-H stretching and the peaks between 1650 and 1550 cm^{-1} represent the N-H groups. Comparing chitosan and CNPs, it was observed that the peaks at 2978 and 1649 cm^{-1} in chitosan shifted to 2974 and 1568 cm^{-1} in CNP, and sharper peaks occurred at 1568 and 1408 cm^{-1}. The peaks between 1085 and 1050 cm^{-1} represent C-O stretching and it is seen that the effect of tween 80 causes the formation of a new peak in CNP compared to chitosan. The peaks between 780 and 760 cm^{-1}, specifically in TPP and tween 80 and represent C-H groups, showed their presence in CNP. The peaks between 3450 and 3300 cm^{-1} represent the -OH group. The bands at 3385 cm^{-1} in CNP caused a shift to 3355 cm^{-1} in SME-CNP and that indicates that hydrogen bonding is reduced as a result of the encapsulation of SME. The peaks between 900 and 700 cm^{-1} represent C-H groups; the bands at 804 cm^{-1} in CNP shifted to 785 cm^{-1} in SME-CNP and sharper peaks were formed. Comparing the shifts in some peaks in CNP and SME-CNP, it is indicated that SME was successfully encapsulated into CNPs (de Pinho Neves *et al.* 2014).

Assessment of encapsulation efficiency, loading capacity, and in vitro release profiles

The calibration curve was created to measure the encapsulation efficiency of SME-CNPs. As seen in Figure 3(C), 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 1.5 µg/mL, and 2 µg/mL dilutions of SME were used and the graphic and absorbance value of SME-CNPs were obtained.

Equation of the calibration curve;

$$y = 0.2192x - 0.2731 \quad \text{(Eq. 1)}$$

was found and the R^2 value is calculated as 0.9919.

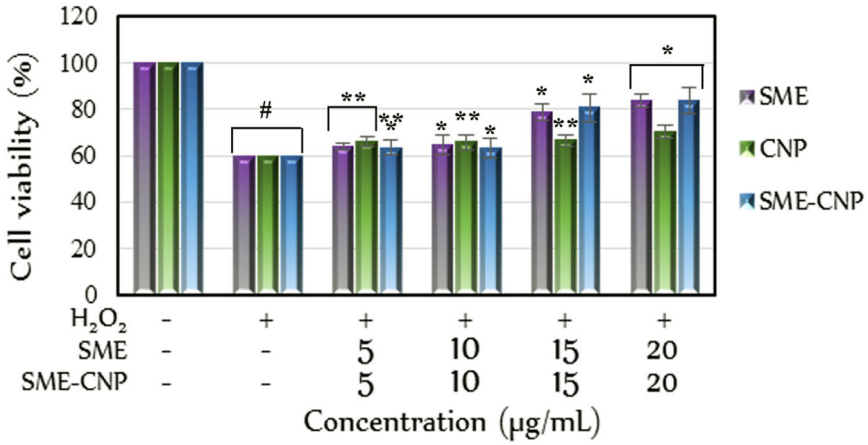

Figure 4. Neuroprotective effect of SME, CNPs, and SME-CNPs on cell viability against H_2O_2-damage on SH-SY5Y cells. SME-CNPs seemed to improve cell viability by up to 84%. The results were indicated as $\#p < 0.001$ compared with the control and $^{*}p < 0.001$, $^{**}p < 0.001$, and $^{***}p < 0.05$ compared with the H_2O_2 group.

The amount of non-encapsulated SME was found as 4.7674 µg from absorbance values. The weight of SME used in encapsulation was calculated as 139.8 µg and the total nanoparticle weight was calculated as 7139.8 µg, and from here, the encapsulation efficiency was calculated as 96.6%.

The absorbance measurements of SME-CNPs in PBS were obtained from the wavelength of 330 nm at 0, 2, 4, 6, 8, and 24th hours, to establish the *in vitro* release profile. At the end of the 24th hour, it was observed that SME-CNPs were able to release approximately 5% (w/w) of the SME. This ratio of release is important for guaranteeing SME released under controlled conditions.

Evaluation of neuroprotective effects

As shown in Figure 4, CNPs exhibited neuroprotective effects and preserve cell viability up to 70%. On the other hand, SME-CNPs improved cell viability up to 84% and exhibited a higher neuroprotective effect than blank CNPs. This indicates that SME enriched the neuroprotective properties of CNPs and reduced H_2O_2 damage.

Conclusion

Herein, we have conclusively demonstrated the neuroprotective effects of SME and SME-CNPs on neurodegenerated cells via H_2O_2 induction that represents the *in vitro* Alzheimer's Disease model. The SME-CNPs displayed desirable loading capacity and encapsulation efficiency and did not exhibit a significant form of toxicity, according to cell viability and proliferation rates. Our results indicate that SME and SME-CNPs are promising candidates for further *in vitro* and *in vivo* evaluation as a potential bioengineered food supplement for the prevention and/or treatment of neurodegenerative diseases, and may help provide a pharmacological product by further studies.

Disclosure of interest

No potential conflict of interest was reported by the author(s).

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