Investigation of chitosan, its depolymerized products, and nanoformulation as novel anticonvulsants

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Objectives
Chitosan is a natural biopolymer that possesses various biological activities. The aim of the current study was to evaluate the potentiality of chitosan and its enzymatically depolymerized products as anticonvulsants.
Materials and methods
In the current study, chitosan enzymatic depolymerization was carried out using *Bacillus cereus* chitosanase followed by fractionation of the produced chitooligosaccharides. Phase I anticonvulsant activity of chitosan as well as its enzymatically depolymerized products was evaluated using pentylenetetrazole-induced seizures, maximal electric shock, and neurotoxicity tests. In phase II, median effective dose, median toxic dose, and protective index were determined. In addition, γ-aminobutyric acid brain level and acute toxicity were evaluated.
Results and conclusion
The results indicated that the fraction with the lower degree of acetylation and longer chains of glucosamine (COS_H) possessed rapid onset of action with the highest protection (75%) at 0.5 h and long-acting effect for 4 h. In addition, the median effective dose of COS_H was 12.7-fold more potent than the reference ethosuximide, whereas in the maximal electric shock test, COS_H showed lower potency than phenytoin. The median toxic dose was 1.4-fold and 7.9-fold higher than ethosuximide and phenytoin, respectively. The protective index was 18.1-fold and 3.98-fold higher than ethosuximide and phenytoin, respectively, with a significant increase in γ-aminobutyric acid neurotransmitter brain level. In an attempt to prolong the anticonvulsant effect of COS_H, a nano-formulation was carried out in which the particle size was estimated as 188.7±0.26 nm. After that, an equivalent dose of a combined treatment of COS_H and the nanoformula (each 15 mg/kg) was evaluated in which a prolonged effect was achieved up to 24 h.

Keywords:
anticonvulsant, chitosan, depolymerized products, nanoformulation

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Introduction

Chitosan is a natural biopolymer commercially prepared by the deacetylation of chitin, a widely distributed natural polysaccharide that exists in crustacean shells, cell walls of fungi, insects cuticle, yeasts, and other invertebrates [1]. Chitosan is a linear chain of β-(1-4)-linked D-glucosamine units with less than 20% of N-acetyl-D-glucosamine. Accordingly, three different types of reactive functional groups (amino/acetamido groups at C-2 as well as primary and secondary hydroxyl groups at C-3 and C-6 positions) participate in its various biological activities including anti-inflammatory, and immune-modulatory [2], antitumor [3], and antimicrobial [4]. Although chitosan has been widely applied in the biomedical sector [5], the main drawbacks for extending its application are its high viscosity and low water solubility. Therefore, the partial depolymerization of chitosan to produce water-soluble chitooligosaccharides (COS) has been proposed to overcome the aforementioned drawbacks [6].

COS are water-soluble homo-oligomer or hetero-oligomers of D-glucosamine and N-acetyl-D-glucosamine with an average molecular weight of less than 3900 Da [7]. They have been estimated to possess various biological activities, including prebiotic, antioxidant [8], antitumor [9], neuroprotective [10], antibacterial [11], antifungal [12,13], immuno-modulatory [14], hepatoprotective [15], and hypolipidemic effects [16]. Furthermore, acid hydrolysis was the most common traditional method applied for the preparation of COS, but in the last few years, enzymatic hydrolysis using chitosanases has been addressed as an eco-friendly method with high yield of the resultant product [6].

Epilepsy can be defined as an abnormal electrical activity that takes place inside the entire brain or in one of its focal locus and can lead to seizures [17], which has been globally estimated as the third most common neurological disorder [18]. One of the main challenges in epilepsy treatment is mainly related to the drug resistance developed by approximately one-third of the patients, especially at the later stage of treatment [19]. The drug resistance development may cause a greater risk of brain damage, which consequently will increase the mortality rates. Another challenge in epilepsy treatment is associated with the difficulty of the drug to localize inside the brain at therapeutic concentrations owing to the blood–brain barrier, which allows only the lipophilic drug with low molecular weight to reach the brain [20]. The need to screen for novel antiepileptic drugs together with employing advanced delivery systems became inevitable to overcome the drug resistance and to enhance the ability of the drug to cross the blood–brain barrier for effective treatment.

Nanotechnology as the delivery system is considered one of the main approaches to enhance the bioavailability of antiepileptic drugs [21]. The antiepileptic drugs can be either coated or encapsulated within particles in nanoscale size. Although there are different types of nanoparticles (NP), polymeric NPs are considered the most popular and suitable system to deliver antiepileptic drugs owing to the ability to control the particle size and the ability of polymeric NPs to enhance the in-vivo stability of the drug in the biological fluid and extend its release over a prolonged time [22–24].

The biological activities of chitosan have been attributed to the activity of its hydrolyzed products generated in the target tissues [25]. In addition, chitosan has been successfully used in the preparation of nanodelivering systems for various antiepileptic drugs [17,26]. This study aimed to investigate the possible anticonvulsant effect of chitosan and its depolymerized derivative (COS) as well as COS loaded into polylactic co glycolic acid nanoparticles (PLGA-NPs) as well as determination of the possible mechanism of action.

Materials and methods

Preparation of chitooligosaccharide

In the current study, COS was prepared by the enzymatic hydrolysis of medium-molecular-weight chitosan (Sigma-Aldrich, Saint Louis, MI, USA) using chitosanase prepared in our laboratory.

Chitosanase preparation

The used chitosanase in the current study was prepared according to Ismail [27], in which shrimp byproducts were fermented under solid-state fermentation using *Bacillus cereus* SSW1 (accession number MK533796). In brief, 5 g of microwave pretreated shrimp byproduct moistened with 10 ml of salt solution composed of KCl, 3%; K_2HPO_4, 0.15%; $MgSO_4$, 0.015%; and $FeSO_4.7H_2O$, 0.01% were cultivated by the bacterial strain and then incubated for 24 h at 37°C. At the end of the incubation period, extraction of the fermented substrate was carried out using distilled water followed by enzyme purification by fractional precipitation using ethanol. The fraction obtained at concentration of 50% was further used in the hydrolysis of chitosan. The activity of the enzyme was estimated using dinitrosalicylic acid method, and 1 U of the enzyme was defined as the amount of enzyme capable for the release of 1 μmol of D-glucosamine per minute under the assay conditions.

Hydrolytic conditions

The hydrolysis was performed in 250-ml screw-caped bottles in which 100 ml of 1% chitosan solution of pH 5 prepared as described by Uchida and Ohtakara [28] was hydrolyzed by the addition of the enzyme in the ratio of 0.28 U/mg chitosan in a final volume of 150 ml and then incubated at 50°C for 2 h. At the end of the hydrolysis period, denaturation of the added enzyme was performed by boiling of the reaction mixture for 10 min followed by centrifugation for 10 min at 5500 rpm (4°C) and then air drying of the clear supernatant [27].

The resulting solution of COS mixture (COS_T) was further separated to low (COS_L) and high (COS_H) fractions extracted from silica gel 60 thin-layer chromatography plates (Merck, Darmstadt, Germany) using propanol: water: ammonia (7 : 2 : 1 v/v) as the mobile phase [29].

Characterization of chitooligosaccharide

The structural differences of the resulted fractions were estimated by determining the total content of reducing sugars of each fraction using the Nelson-Somogyi method [30,31] and Fourier transform infrared spectroscopy (FTIR-8300; Shimadzu, Kyoto, Japan).

The degree of acetylation for both fractions was calculated as described by Kasaai [32], on the basis of FTIR analysis data according to equations 1 and 2.

$$A = -\text{Log } T \quad \text{Eq. (1)}$$

$$DA\ (\%) = (A_{1640}/A_{3430}) \times (100/1.33) \quad \text{Eq. (2)}$$

where T is the transmittance, A is the absorbance, and DA is the degree of acetylation.

Nanoformulation

Preparation

COS_H was loaded into PLGA nanoparticles (COS_H-PLGA-NPs) by a modified double emulsion/evaporation method [23]. In brief, 1 ml of aqueous solution of 10 µg/ml of COS_H was added to 50 µg of PLGA dissolved into 2.5 ml of dichloromethane followed by emulsification by a probe-sonicator for 1 min at 40% voltage efficiency. The produced emulsion was then added drowsily to a 10 ml aqueous solution containing 2.5% polyvinyl alcohol (PVA) and emulsified by a probe-sonicator for four successive cycles at 40% voltage efficiency (1 min/cycle). The formed emulsion was allowed to stir for 1 h at 1200 rpm at room temperature until the dichloromethane was completely evaporated. The produced NPs were separated by centrifugation at 10000 rpm for 40 min followed by washing with distilled water and stored at −20°C.

Entrapment efficiency

The entrapment efficiency was indirectly measured by taking certain volume from the supernatant after separation of the NPs, and the unentrapped amount of COS_H was determined by the Nelson-Somogyi method. The percentage of the entrapment efficiency was calculated using the following formula:

$$\text{Entrapment efficiency \%} = \frac{(\text{Total amount of } COS_H \text{ used} - \text{amount of } COS_H \text{ unentrapped})}{\text{Total amount of } COS_H \text{ used}} \times 100$$

The effective diameter and zeta-potential

Zetasizer (Malvern, Cambridge, UK) was used to measure the effective diameter, size distribution, and zeta-potential of the produced NPs. The NPs were initially diluted with distilled water, and both the effective diameter and polydispersity index were determined using the Dynamic Light Scattering mode at 25°C, whereas the zeta potential (mV) was measured by the laser Doppler velocimetry.

Pharmacological studies

Experimental animals

Male albino mice weighing 20–25 g were obtained from the Animal House Colony of National Research Center, Giza, Egypt. The mice were housed in polypropylene cages under standardized conditions: temperature (23±2°C) light (12 h light/dark cycle), and humidity (55±5%). All mice were allowed free access to water and standard chow as well as being acclimatized to laboratory conditions before the start of the experimental studies. The studies involving animals were conducted according to the guidelines of the ethical committee of National Research Center for experimental animal use, with ethics approval number: 14412012022.

Phase I study

Chitosan and its depolymerized products (COS_T, COS_L, and COS_H) (30 mg/kg) as well as a mixture of COS_H (15 mg/kg) and COS_H-PLGA-NPs (15 mg/kg) were injected intraperitoneally, and then after the assigned time intervals (0.5, 4, and either 8 and/or 24 h), the following tests were carried out.

Subcutaneous pentylenetetrazole-induced seizures test

In this test, seizures were induced via subcutaneous injection of pentylenetetrazole (scPTZ) (85 mg/kg) in the loose fold of the skin located on the back of the mouse neck. It has been reported that this dose induces a clonic seizure that persists for at least 5 s in 97% of the tested mice [33]. The compounds under investigation were injected, and then after the designated time interval, PTZ was subcutaneously injected followed by 30 min of observation for the appearance of seizures. The absence of threshold convulsion, which is defined as one episode of clonic convulsions that persist for at least 5 s, indicated the ability of the compounds under investigation to protect against scPTZ-induced seizures [34].

Maximal electric shock test

Mice were injected with chitosan and its depolymerized products (COS_T, COS_L and COS_H) (30 mg/kg) as well as a mixture of COS_H (15 mg/kg) and COS_H-PLGA-NPs (15 mg/kg), and then after the designated time interval, electroconvulsions were induced in mice via delivering an electric current of fixed intensity (25 mA) and stimulus duration (0.2 s) through an ear-clip electrode Rodent Shocker generator (constant current stimulator Type 221; Hugo Sachs Elektronik, Germany). Prevention of the tonic hind limb extension indicated the ability of the tested compounds to inhibit the spread of MES-induced seizures [35].

Neurotoxicity

Rotarod test was used to detect minimal neurological deficits indicating neurotoxicity. Mice were trained to maintain equilibrium on a 1-inch-diameter revolving knurled plastic rod for 1 min at a speed of 10 rpm in

each of three trials using a rotarod device (UGO Basile, 47600, Varese, Italy). Only mice that achieved this criterion were included in this experiment. The test compounds were injected, and then after the designated time interval, neurotoxicity was examined by the inability of mice to maintain equilibrium for at least 1 min on the rotating rod [36].

Phase II study anticonvulsant evaluation

The median effective dose (ED_{50}) is the dose of the compound that protects 50% of mice against scPTZ and MES-induced seizures. To determine the ED_{50} of COS_H, various doses were administered to different mice groups until at least three points were achieved in the range of 16–83% seizure protection [37]. The neurotoxicity was expressed as median toxic dose (TD_{50}). The protective index (PI) value was calculated as the ratio of TD_{50} to ED_{50} ($PI=TD_{50}/ED_{50}$) [38].

Determination of γ-aminobutyric acid

The γ-aminobutyric acid (GABA) was estimated in whole brain tissue homogenates using enzyme-linked immunosorbent assay (Life Span Bio Sciences Inc., LifeSpan BioSciences, Inc., Seattle, WA, USA). COS_H (30 mg/kg, intraperitoneal) or bromazepam (30 mg/kg, intraperitoneal) used as a reference standard was injected in different groups of mice. After 2 h, mice from control, COS_H, and reference standard groups were sacrificed, and brains were extracted, weighed, and frozen instantly in −80°C freezer [38].

Acute toxicity test

The tested compounds at different doses up to 500 mg/kg were injected to adult male albino mice (n=6) via intraperitoneal route. Mice were observed for any gross behavioral changes and deaths, delayed effects, and toxic symptoms for 14 days [39,40].

Results and discussion

Chitooligosaccharide preparation

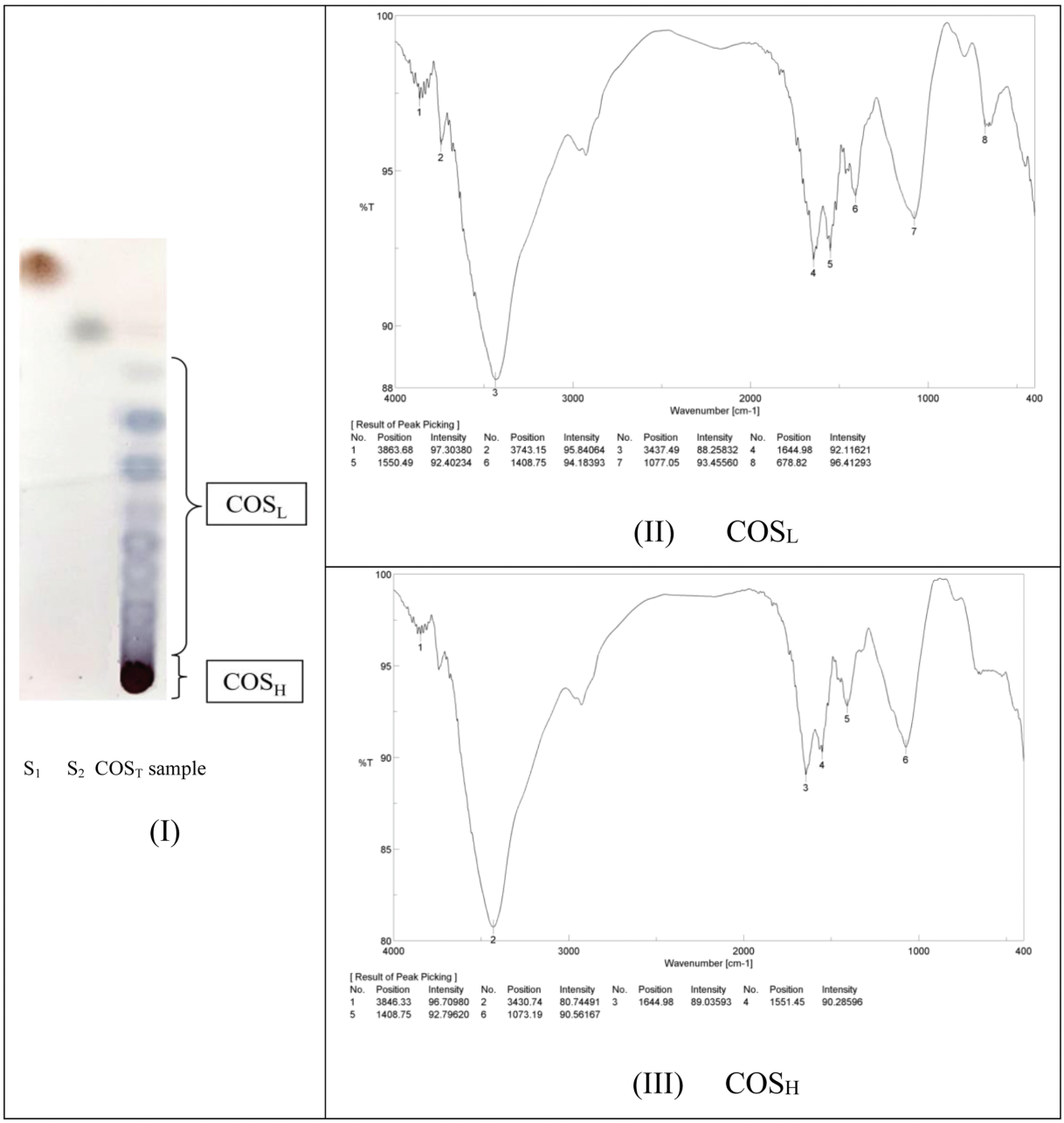
Enzymatic hydrolysis of chitosan using chitosanases was very expressive, which can be explained by their efficiency in the hydrolysis of chitosan under easy controllable mild conditions with the release of high yield of the resulted product [12,41–44]. In the current study, the hydrolysis of chitosan was carried out using *B. cereus* SSW1 chitosanase and then the produced mixture was separated into two fractions (Fig. 1). The molecular weight of the produced mixture (COS_T) was previously estimated at about 2000 Da with an average content of reducing sugars of 91.74 mg glucosamine/g and degree of acetylation of 40.7% [8]. In the current study, the total content of reducing sugars in each fraction was determined. The result indicated that the amount of reducing sugars in COS_L fraction (130±2.93 mg glucosamine/g) was three fold higher than that present in the COS_H fraction (41.61 ±1.46 mg glucosamine/g). Li *et al.* [45] indicated that the increase in the total content of reducing sugars in chitosan hydrolyzate was directly related to the production of reduced chains of glucosamine units.

In addition, the functional groups and the chemical bonds of each fraction were estimated by FTIR analysis, and the results are shown in Fig. 1. The FTIR spectrum for both fractions manifested the characteristic bands of chitosan; the broad band appeared at about 3430 cm^{-1} corresponding to the stretching vibration of O–H merged with that of N–H; the bands at about 1640 and 1550 cm^{-1} corresponding to the stretching of C=O amide I and the bending of N–H amide II, respectively; at 1408 cm^{-1} corresponding to CH_2 bending; and at about 1075 cm^{-1} corresponding to the stretching of C–O. On the basis of FTIR analysis, the degree of acetylation was calculated as 49.43 and 40.83% for COS_L and COS_H, respectively.

Formulation and characterization of chitooligosaccharide (high-molecular-weight fraction)-loaded nanoparticles

NPs loaded with COS_H were fabricated by the double emulsion/evaporation method. The entrapment efficiency was calculated indirectly and found to be 71.1±1.8%. The size of the produced NPs was analyzed by a zeta sizer and found to be 188.7±0.26 nm with narrow size distribution (0.14±0.007), whereas the particle surface charge (zeta potential) was −1.63 ±0.108 mV (Fig. 2). Hence, COS in general are water soluble. The double emulsion/evaporation method had been employed previously to encapsulate COS into PLGA NPs [8]. The entrapment efficiency obtained in the current study was comparable to the one obtained previously when the total COS or inulin was encapsulated into PLGA NPs by the double emulsion/evaporation method [8,46]. The COS_H was fabricated into NPs with size similar to the one obtained by Haggag *et al.* [47], which were employed to enhance the anticonvulsant activity of Zaleplon. Particles with size around 200 nm were found to transport across BBB with greater extent [48], which indicates the applicability of the formed NPs in the current study to deliver the COS across BBB [49].

Figure 1



Thin-layer chromatography plate of (I) COS_T sample in which S_1 and S_2 are standards of N-acetyl glucosamine and glucosamine, respectively, in addition to FTIR analysis of (II) COS_L and (III) COS_H fractions. COS, chitooligosaccharide; FTIR, Fourier transform infrared spectroscopy.

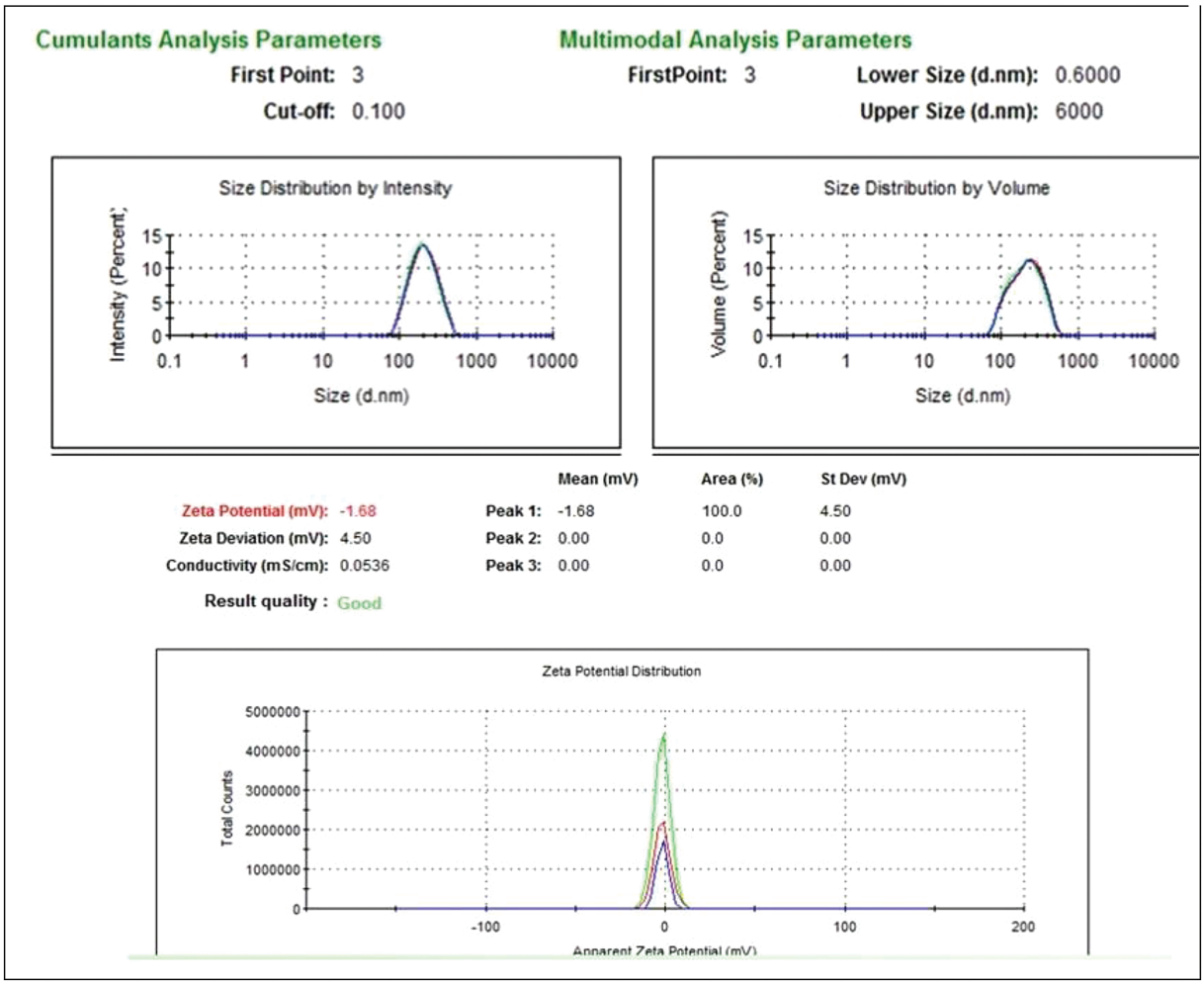
Pharmacology

Anticonvulsant activity

Phase I anticonvulsant evaluation: scPTZ and MES tests are considered the 'gold standard' screen tests for phase I screening of anticonvulsants conferring to the Antiepileptic Drug Development (ADD) program of the National Institute of Neurological Disorders and Stroke (NINDS), Epilepsy section standard procedure [50,51]. In the current study, the anticonvulsant activity of the parent compound chitosan and its depolymerized products, namely, COS_T, COS_L, and COS_H (30 mg/kg) were evaluated in scPTZ and MES seizure tests as well as neurotoxicity tests at different time intervals (Table 1).

The scPTZ test is considered a useful screen test for the identification of compounds that elevate seizure threshold and prediction for the therapeutic efficacy against generalized myoclonic seizures [52,53]. In scPTZ test, both COS_T and COS_H exhibited rapid onset of action with highest protection (75%) against scPTS-induced seizures compared with chitosan (0%) and COS_L (50%) at 0.5 h. The seizure protection of COS_H (75%) was further extended providing a long-

Figure 2



Zeta potential analysis of COS_H loaded into PLGA nanoparticles. COS, chitooligosaccharide.

Table 1 Subcutaneous pentylenetetrazole-induced seizures test (phase I anticonvulsant evaluation)

Groups	Percentage protection (%)			
	0.5 h	4 h	8 h	24 h
Chitosan (30 mg/kg)	0	25	–	–
COS_T (30 mg/kg)	75	50	–	–
COS_L (30 mg/kg)	50	50	–	–
COS_H (30 mg/kg)	75	75	25	–
COS_H (15 mg/kg)+COS_H-PLGA-NPs(15 mg/kg)	50	75	50	50
Ethosuximide (120 mg/kg)	50	25	–	–

COS, chitooligosaccharide; PLGA, polylactic co glycolic acid; NP, nanoparticle.

acting effect at 4 h, whereas treatment with COS_T (50%) demonstrated decrease in the percentage protection against scPTZ-induced seizures revealing its short-acting effect. Meanwhile, COS_L showed similar protection from seizures at 4 h compared with 0.5 h (50%), whereas chitosan protection was slightly enhanced at 4 h to reach 25%. Interestingly, at 0.5 h, COS_T and COS_H at dose of 30 mg/kg showed higher percentage protection than the reference drug ethosuximide (120 mg/kg), whereas COS_L (30 mg/kg) demonstrated a similar effect. Moreover, the effect of ethosuximide at 4 h decreased reaching 25%, whereas COS_L, COS_T, and COS_H showed percentage protection against PTZ-induced seizures ranging between 50 and 75% (Table 1). In general, the degree of polymerization and the degree of acetylation and the amino content are the main variables that influence the biological activities of

COS [6]. In the current study, the high protection effect of COS_T and COS_H might be attributed to their low degree of acetylation and consequently high amino content in addition to their constituent longer chains of glucosamine compared with COS_L. Moreover, the enhanced effect of chitosan after 4 hours might be attributed to its *in vivo* generated hydrolyzed products as reported by Aam *et al.* [25].

COS_H was further evaluated for its prolonged anticonvulsant effect; however, the protection percentage was inhibited to 25% at the 8-h time point. In an attempt to evaluate the possible maximum anticonvulsant effect by COS_H, a combined treatment of COS_H-PLGA-NPs and COS_H at a dose of 15 mg/kg each to achieve a total dose of 30 mg/kg was tested. Remarkably, the COS_H+COS_H-PLGA-NPs showed an anticonvulsant effect at 0.5 h (50%) that reached its peak at 4 h (75%). A prolonged effect was observed at 8 h (50%) and 24 h (50%), demonstrating that although COS_H was used at a lower dose in this combination, it potentiated the rapid onset of action. Moreover, COS_H-PLGA-NPs played an important role in this treatment combination, as it enhanced the prolonged anticonvulsant effect to reach 8 and 24 h (Table 1). This effect could be referred to the enhanced solubility and the improved penetration through the blood–brain barriers reported for NPs [54] in addition to their efficiency in extending the *in-vivo* response [23].

Phenobarbital was reported to exhibit protection against scPTZ-induced convulsions in 50% or more of mice at doses of 100 and 30 mg/kg at 0.5 and 4 h, respectively. COS_T, COS_L, COS_H, and COS_H+COS_H-PLGA-NPs at a dose of more than three fold lower than phenobarbital demonstrated a similar effect at 0.5 h, whereas at 4 h, a similar effect to phenobarbital (30 mg/kg) was exhibited [34]. Moreover, the effect of COS_T, COS_L, COS_H, and COS_H+COS_H-PLGA-NPs was more potent than ethosuximide, which was reported to exhibit a similar percentage of protection (50% or more) but at doses of 3.33 and 10 fold higher than the tested dose [55].

In the current study, MES test was used as a valid effective model for screening of compounds that block human generalized tonic–clonic seizures. Phenytoin is an active standard in MES test and produces this effect via a mechanism of action that involves blocking of sodium channels [56,57]. Both chitosan and COS_L demonstrated low anticonvulsant effect at 0.5 h, which persisted to 4 h. Treatment with COS_H exhibited a rapid onset of action (0.5 h) with highest protection (75%) in MES test, which is similar to the reference standard phenytoin, whereas COS_T demonstrated 50% protection. Although the protection provided by COS_H decreased to 50% at 4 h and COS_T remained the same (50%), their effect was still long lasting at 4 h, which was half the protection demonstrated by phenytoin (Table 2). Although the combined treatment with COS_H+COS_H-PLGA-NPs showed a low onset of action at 0.5 h, the effect appeared to be long lasting by reaching 50% at 4 h after treatment in MES test. The long-lasting anticonvulsant protective effect was further enhanced at 8 h, reaching its peak effect (75%). The effect decreased at 24 h, but did not fade, to be 25% protection (Table 2). Previous reports showed that phenytoin produces 100% protection at 0.5 h, whereas 75% at 4 h at dose (30 mg/kg, postoperative) in rats [58].

Neurotoxicity of chitosan, COS_T, COS_L, COS_H, and COS_H+COS_H-PLGA-NPs was evaluated using the rotarod test. The test demonstrated that all of the compounds under investigation were devoid from neurotoxicty at the administered dose (Table 3).

Phase II anticonvulsant evaluation: in the present study, COS_H was found to process the highest anticonvulsant activity at 0.5 and 4 h in both scPTZ and MES tests. These promising results lead to phase II evaluation, which includes assessment of ED_{50}, TD_{50}, and PI (Table 4).

Table 2 Maximal electric shock test (phase I anticonvulsant evaluation)

Groups	Percentage protection (%)			
	0.5 h	4 h	8 h	24 h
Chitosan (30 mg/kg)	25	25	–	–
COS_T (30 mg/kg)	50	50	–	–
COS_L (30 mg/kg)	25	25	–	–
COS_H (30 mg/kg)	75	50	0	–
COS_H (15 mg/kg)+COS_H-PLGA-NPs(15 mg/kg)	25	50	75	25
Phenytoin (30 mg/kg)	75	100	–	–

COS, chitooligosaccharide; PLGA, polylactic co glycolic acid; NP, nanoparticle.

Table 3 Neurotoxicity test (phase I anticonvulsant evaluation)

Groups	Neurotoxicity			
	0.5 h	4 h	8 h	24 h
Chitosan (30 mg/kg)	0/6	0/6	–	–
COS_T (30 mg/kg)	0/6	0/6	–	–
COS_L (30 mg/kg)	0/6	0/6	–	–
COS_H (30 mg/kg)	0/6	0/6	0/6	–
COS_H (15 mg/kg)+COS_H-PLGA-NPs(15 mg/kg)	0/6	0/6	0/6	0/6

Data represented indicate the number of mice exhibiting neurotoxcity (falling rotarod)/total number of mice tested in rotarod test. COS, chitooligosaccharide; PLGA, polylactic co glycolic acid; NP, nanoparticle.

Table 4 Determination of effective dose, toxic dose and protective index of chitooligosaccharide$_H$ in subcutaneous pentylenetetrazole and maximal electric shock tests (phase II anticonvulsant evaluation)

COS_H	scPTZ	MES
ED_{50}	10.3 (5.947–15.42)	19.04 (17.47–20.78)
TD_{50}	>500	>500
PI	>48.54	>26.26

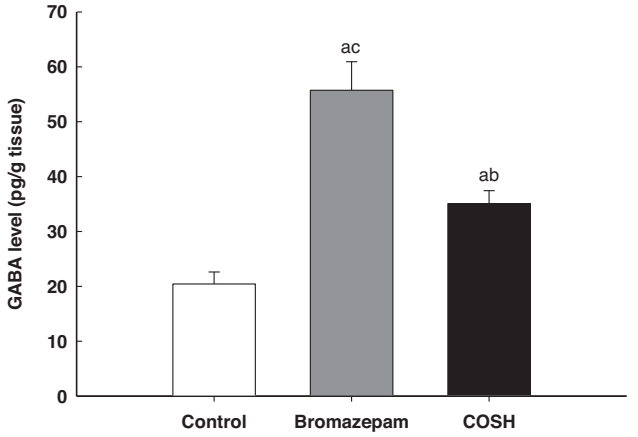
ED_{50} and TD_{50} are expressed in mg/kg. In PTZ test, ethosuximide ED_{50} is 130.55 (98.78–177.87), TD_{50} is 350.1 (268.90–346.20) and PI is 2.68. In MES test, phenytoin ED_{50} is 9.50 (7.24–10.96), TD_{50} is 62.64 (50.52–69.82) and PI is 6.59 [52]. COS, chitooligosaccharide; MES, maximal electric shock; scPTZ, subcutaneous pentylenetetrazole. ED_{50}: median effective dose. TD_{50}: median toxic dose in neurotoxicity test. PI: protective index, PI=TD_{50}/ED_{50}.

In scPTZ, COS_H demonstrated high anticonvulsant activity, where COS_H ED_{50} was 12.7-fold more potent (lower) than ethosuximide. In addition, COS_H exhibited protection against electric stimuli-induced seizures in the MES test, as its ED_{50} was two-fold higher than the reference drug phenytoin, indicating its slight lesser activity than phenytoin. COS_H demonstrated no neurotoxicity in the rotarod test for doses up to 500 mg/kg; thus, the TD_{50} was estimated to be greater that 500 mg/kg. The TD_{50} of COS_H was 1.4 and 7.9-fold higher compared with ethosuximide and phenytoin, respectively. The PI of COS_H calculated as a ration between TD_{50} and ED_{50} and was found to be more than 48.5 and more than 26.26 in scPTZ and MES tests, respectively. These values were 18.1 and 3.98-fold higher than the reference standards ethosuximide and phenytoin, respectively.

Estimation of brain γ-aminobutyric acid

Determination of the mechanism of action of new investigated anticonvulsant compounds is of huge importance for design and development of antiepileptic drug (AED). To determine the possible mechanism of action of COS_H, GABA neurotransmitter level was measured in whole mice brain homogenate. Mice treated with COS_H demonstrated a significant increase in GABA brain level compared with the control value. However, this COS_H-mediated increase in GABA brain was significantly lower than the reference drug bromazepam (Fig. 3).

Figure 3



Effect of COS_H on GABA brain level. Results are presented as mean ±SEM. [a]Significantly different from control at *P* value less than 0.05, [b]significantly different from bromazepam (30 mg/kg) value at *P* value less than 0.05, [c]significantly different from COS_H (30 mg/kg) value at *P* value less than 0.05. COS, chitooligosaccharide; GABA, γ-amino-butyric acid.

Acute toxicity

All mice were devoid of the signs of acute toxicity at doses up to 500 mg/kg.

Conclusion

The biomedical applications of chitosan and its depolymerized products have attracted the research focus. In the current study, the anticonvulsant activity of chitosan and its enzymatically depolymerized products (COS) was evaluated. The results indicated that COS_H fraction possessed the highest anticonvulsant activity in both scPTZ and MES tests via a mechanism involving GABA. Interestingly, an equivalent dose of a combined treatment of both COS_H and the nanoformula (COS_H-PLGA-NPs) exhibited a prolonged anticonvulsant effect up to 24 h. This effect could be attributed to the enhanced solubility and the improved

penetration through the blood–brain barriers resulted in extending the in-vivo effect.

Thus, the findings of the current study may help in resolving huge challenges in epilepsy related to drug resistance via introducing new safe natural treatment alternatives as well as enhancement of the ability of the drug to cross the blood–brain barrier through employing advanced delivery systems.

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