The Pharma Innovation Journal 2018; 7(11): 156-161

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(11): 156-161

www.thepharmajournal.com
Received: 15-09-2018
Accepted: 20-10-2018

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Pharmaceutical evaluation of enzymatically produced chitooligosaccharides

G Arunkumar and K Bhanumathi

Abstract
Chitooligosaccharides (COS) are the depolymerized products of chitosan polymer. COS has valuable medicinal properties and potent than chitosan. In this study, pharmaceutical potential of enzymatically produced COS was analyzed using Total Antioxidant assay, Protein denaturation inhibition assay, Membrane stabilization assay and Nitric oxide (NO) scavenging assay. COS have shown maximum TAA activity of 168.70 ± 0.45 mg/g equivalents of ascorbic acid at concentration of 1000 µg/ml. Protein denaturation inhibition assay of COS revealed 89.97 ± 0.51% activity at 1000 µg/ml concentration and the IC50 value was found to be 175.44 µg/ml. Membrane stabilization assay confirmed the cell membrane protecting ability of COS with 74.03 ± 2.95 % activity at 1000 µg/ml concentration and the EC50 was found to be 560.45 µg/ml. COS have shown highest NO scavenging activity of 63.73 ± 0.57 % at 500 µg/ml with IC50 value of 277.35 µg/ml. Hence, enzymatically produced COS could be used as drug of choice to treat certain protein and cell membrane related diseases and disorders.

Keywords: COS, enzyme, protein denaturation, membrane stabilization, Nitric oxide

Introduction
Free radicals are the main cause for the damage of DNA and proteins. Reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical and superoxide anion are generated as a result of normal metabolism of oxygen, which are capable of oxidizing biomolecules such as lipids, proteins, carbohydrates and DNA that leads to oxidative stress. However, cellular antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and thioredoxins will neutralize ROS. Excessive generation of ROS cannot be quenched by the limited amount of cellular defense system. The excess generation of ROS results in aging, cancer, inflammation, rheumatoid arthritis etc. However, COS of different molecular weight have the property of neutralizing the ROS and helps to combat against oxidative stress and prevent cellular injury.

COS were found to inhibit and kill many pathogenic microorganism. The mechanism of its antibacterial effect of COS might be due to cell lysis, cytoplasmic membrane destruction and chelation of trace metal cations. The antimicrobial effect is due to the presence of cationic amines in COS, which can interact with negatively charged residues of lipids, proteins and carbohydrates located on cell surface (Chen C *et al*., 1998) [3]. Interestingly, antibacterial effect of COS also depends on molecular weight, especially COS greater than 10 Kda is required to exhibit antibacterial property.

COS also found to be effective against tumor growth, metastasis and angiogenesis. Cationic nature of COS and molecular weight found to be critical for antitumor effect (Muzarelli R.A.A, 1977) [15]. Hexameric COS possess excellent inhibitory activity against angiogenesis (Xiong C *et al*., 2009) [31]. However, N-acetylated COS was found to be more potent than fully deacetylated COS in angiogenesis prevention (Wang Z *et al*., 2007) [28]. COS cause induction of lymphocyte factor, T cell proliferation and acquired immunity to achieve its antitumor effect. Antiinflammatory effect of COS is due to the presence of free amino groups. The structure of COS resembles the units of proteoglycans present in connective tissue and cartilage. COS and its monomer glucosamine replenished the damaged and inflamed cartilage by getting absorbed in to it (Olivier *et al*., 2004) [17]. Moreover, it has no side effects like other steroidal anti-inflammatory drugs. COS can acts as biocompatible and nontoxic drug of choice for the betterment of arthritic patients (Olivier & Jean-Yves, 2007) [18]. COS also prevents blood pressure, as it comprises Angiotensin converting enzyme (ACE) inhibitory activity (Xia, 2003) [30]. COS of low degree of deacetylation has highest ACE inhibitory activity (Park *et al*., 2003) [21].

Structural modification of COS, leads to increase in ACE inhibition and reduction of blood pressure (Ngo *et al*., 2008) [20].

Antimicrobial studies revealed the effectiveness of COS against different bacteria like *Escherichia coli, Staphylococcus aureus, S. lactis, Bacillus subtilis* and fungi such as *Rhodotorula bacarum, Saccharomyces cerevisiae, Mucor circinelloides, Rhizopus apiculatus, P. charlesii, Aspergillus niger* (Wang *et al*., 2007) [29]. COS produced using *Chaetomium globosum* KM651986 fungal chitosanase found to be effective against *S. aureus, B. subtilis, Pseudomonas aeruginosa, E. coli* and *Candida albicans* (Shehata A. N & El Aty A.A.A, 2015) [25]. COS exerted antitumor property by enhancing the activity of natural killer lymphocytes in sarcoma180 bearing mice (Maeda & Kimura, 2004) [10]. COS induce apoptotic effect on hepatocellular carcinoma via Bcl-2 associated X protein (Xu *et al*., 2008) [32]. Antiangiogenic effect of completely deacetylated COS dimers and hexamers was evaluated (Xiong *et al*., 2009) [31]. *In vitro* and *in vivo* studies revealed *N*-acetylated COS inhibit angiogenesis more effectively than deacetylated COS (Wang *et al*., 2007) [28]. It also induce DNA fragmentation mediated apoptosis of Ehrlich ascites tumor cells (Harish Prashanth K.V & Tharanathan R.N, 2005) [8]. *In vitro* antitumor potential of novel COS obtained using *Capsicum annuum,* was evaluated in HEP-G2 hepatocellular carcinoma cell line, MCF -7 breast carcinoma cell line and HCT-116 colon carcinoma cell line (Sanaa *et al*., 2012) [23].

COS proved to have anti-inflammatory activity by enhancing the migration of peritoneal macrophages into inflammatory areas in mouse (Moon J *et al*., 2007) [13]. It suppresses LPS mediated production of TNFα, IL-6 in RAW264.7 cells (Yoon H *et al*., 2007) [33]. Matrix metalloproteinases (MMPs) are the endopeptidases that degrades extracellular matrix components. COS with molecular weight less than 5Kda exhibited highest inhibitory effect on MMP-2 by chelating Zn^{2+}, which is a cofactor of MMP-2 (Gorzelanny *et al*., 2007) [7]. Inhibitory effect of N-acetylated (NA) COS (1-3 Kda and < 1Kda) against DNA and protein oxidation was evaluated (Chen A *et al*., 2003) [2]. Mouse macrophages treated with COS resulted in increased level of intracellular glutathione and radical scavenging activity. NA-COS ranges between 1-3 Kda was more efficient than NA-COS<1Kda in preventing protein oxidation and intracellular free radical production in live cells (Ngo D *et al*., 2009) [16].

COS have excellent wound healing property exerted by improvement of cell repair (Mori T *et al*., 1997) [14]. Both N-acetylated and deacetylated COS in hexameric form were enhance the release of IL-8, a chemoattractant of polymorphonuclear cells from fibroblasts in rats. It also has wound break strength and collagenase activity (Minagawa T *et al*., 2007) [12]. Collagenase produced by inflammatory cells and fibroblasts cells, which induce wound healing (Clark R.A.F & Denver M.D, 1985) [4]. COS used to deliver therapeutically important genes, as it forms stable complexes with plasmid DNA (Koping Hoggard *et al*., 2001) [9]. Interestingly, COS – DNA complex remained stable at pH 6, while it disassociates above pH 7, makes it very useful in gene delivery (Strand S.P *et al*., 2005) [26].

2. Materials and Methods

2.1Production of Chitooligosaccharides (COS)

Mixture of 1ml chitosan and 1ml bacterial chitosanase enzyme was incubated at 50˚ C for 3h. COS were used for further experiments.

2.2 Evaluation of Total Antioxidant Activity (TAA) by Phosphomolybdenum assay

TAA was estimated by phosphomolybdenum assay (Prieto *et al*., 1999) [22]. Enzymatically produced COS of different concentration ranging from 200µg/ml to 1000 µg/ml were taken in individual test tubes and made upto 1 ml using distilled water and 2 ml of Molybdate reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tubes were incubated at 95°C for 90min. After incubation, the tubes were cooled to room temperature for 20-30 min and the absorbance of the reaction mixture was measured at 695nm. All experiments were performed in triplcates and the results were expressed as mean ± SD. Ascorbic acid was used as the positive reference standard.

2.3 Protein denaturation inhibition assay (Tanford, 1968) [27]

The reaction mixture (0.5ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.1 ml of COS at different concentration. The samples were taken in a test tube and incubated at 37ºC for 30 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660nm for control test 0.05 ml distilled water was used instead of COS. The percentage inhibition of protein denaturation was calculated by the following formula. All experiments were performed in triplcates and the results were expressed as mean ± SD.

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

2. 4 Membrane Stabilization assay (Oyedepo and Femurewa, 1995) [19]

Preparation of Red Blood Cells (RBCs) Suspension

Fresh whole human blood (10 ml) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline.

Heat Induced Hemolysis

The 2ml of reaction mixture consisted of 1ml of COS of various concentration and 1ml of 10% RBC suspension. Control test tube consisted of saline instead of drug. Diclofenac sodium was taken as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56ºC for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500rpm for 5 min and the absorbance of the supernatants was taken at 560nm. The experiment was performed in triplicates. % membrane stabilization activity was calculated by the formula

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

2.5 Nitric Oxide (NO) scavenging assay

NO scavenging activity can be estimated by the use of Griess IlIosvoy reaction (Garrat, 1964) [6]. The compound sodium nitroprusside decomposed in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite). The quantitative estimation of nitrate and nitrite can be determined using Griess reagent (1% sulfanilamide, 2% H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For this experiment, the sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations (200-1000 µg/ml) of 0.1 ml COS and incubated at 30ºC for 2 hours. The distilled water was taken as control. After incubation, 0.5 ml of Griess reagent was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 540nm. Inhibition of nitrite formation by COS and the standard antioxidant ascorbic acid were calculated with respect to the control. All experiments were performed in triplicates and the results were expressed as mean ± SD.

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

3. Results

Enzymatically produced COS was tested for various bioactivities viz. TAA, protein denaturation inhibition activity, NO scavenging activity and Membrane stabilization activity. The activity observed in different assays represented and confirmed the pharmaceutical potential of COS.

3.1 TAA of COS

The antioxidant activity of COS was determined by TAA assay. Different concentration of COS (200 – 1000 µg/ml) have shown wide range of TAA activity (Table 1). The TAA activity of COS shown minimum of 12.43 ± 0.40 and maximum of 168.70 ± 0.45 mg/g equivalents of ascorbic acid at concentration of 200 and 1000 µg/ml respectively (Fig.1).

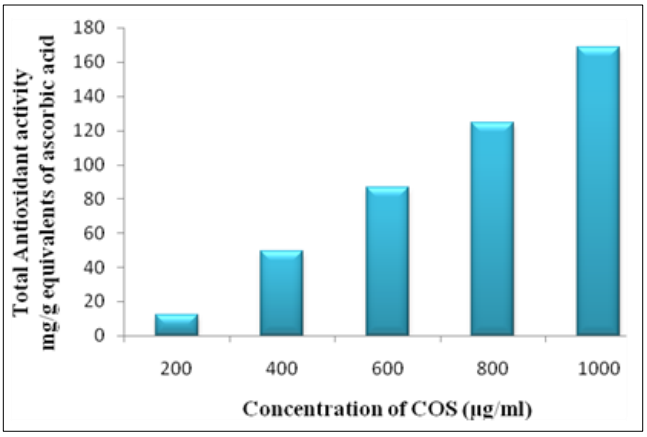

Fig 1: TAA of COS

3.2 Protein denaturation inhibition activity of COS

Protein denaturation inhibition activity revealed the ability of COS to inhibit the protein denaturation. The protein denaturation inhibition activity exhibited by varying concentration of COS was provided in the (Table 1). COS have shown 56.66 ±0.40 % and 89.97 ± 0.51% protein denaturation inhibition activity at concentration of 200 and 1000 µg/ml respectively. Interestingly, COS have shown protein denaturation activity, greater than salicylic acid at lower concentration of 200 and 400 µg/ml. However, at higher concentration from 600 µg/ml to 1000 µg/ml, COS have shown protein denaturation activity lesser than salicylic acid (Fig. 2). This confirmed that COS could be better drug of choice to combat protein denaturation related diseases and disorders. The Effective concentration 50 (EC50) value was found to be 175.44 µg/ml (Table 1).

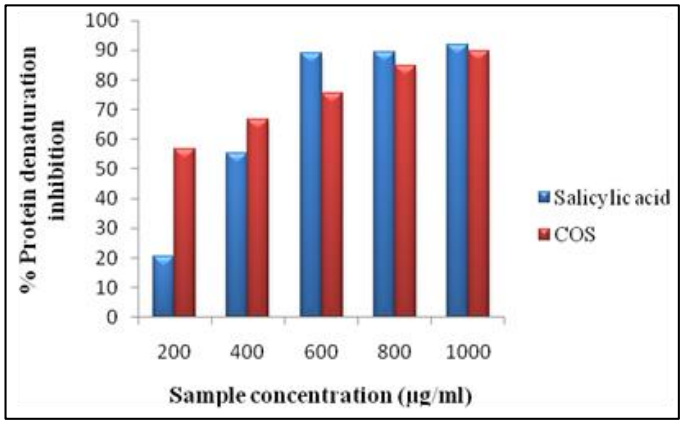

Fig 2: Protein denaturation inhibition activity of COS

3.3 Membrane stabilization activity of COS

Membrane stabilization activity has been done to evaluate the property of COS to protect the cell membrane integrity and viability. COS have shown membrane stabilization activity of 30.23 ± 2.10 % and 74.03 ± 2.95 % at concentration of 200 µg/ml and 1000 µg/ml respectively (Table 1). Different concentration of COS (200 – 1000 µg/ml) have shown membrane stabilization activity higher than the control drug, diclofenac sodium (Fig. 3). The EC50 value was found to be 560.45 µg/ml (Table 1).

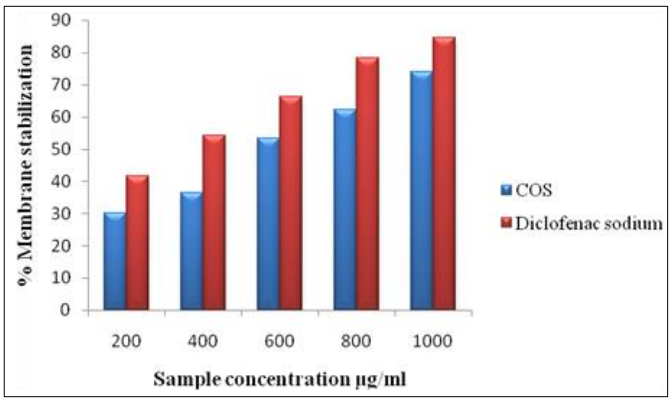

Fig 3: Membrane stabilization activity of COS

3.4 Nitric oxide (NO) scavenging activity of COS

NO scavenging activity has been used to detect the ability of COS to neutralize the NO formed inside the biological system. NO scavenging activity of various concentration of COS (100 – 500 µg/ml) has been evaluated. COS have shown NO scavenging activity of 46.19 ± 0.53 % and 63.73 ± 0.57 % at concentration of 100 and 500 µg/ml respectively (Table 1). COS possessed NO scavenging activity slighty lesser than ascorbic acid at concentrations of 100, 200, 300 µg/ml.

Interestingly, COS have shown NO scavenging activity slightly higher than ascorbic acid at 400 µg/ml. However, at 500 µg/ml concentration, COS have shown equivalent NO scavenging activity, similar to that of ascorbic acid (Fig. 4). The EC50 value was found to be 277.35 µg/ml (Table 1).

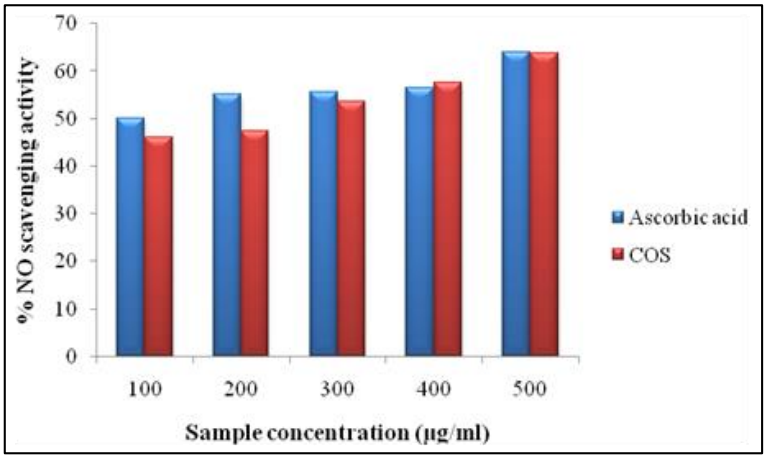

Fig 4: NO scavenging activity of COS

Table 1: Pharmaceutical activity of COS

COS µg/ml	TAA, mg/g equivalents of ascorbic acid	%Protein denaturation inhibition	%Membrane stabilization activity	%NO scavenging activity*
200	12.43 ± 0.40	56.66 ±0.40	30.23 ± 2.10	46.19 ± 0.53
400	49.60 ± 0.87	66.76 ± 0.64	36.32 ± 1.13	47.51 ± 0.98
600	86.40 ± 1.08	75.57 ± 0.68	53.43 ± 0.56	53.51 ± 0.08
800	124.50 ± 0.96	84.77 ± 0.73	62.30 ± 1.00	57.55 ± 0.08
1000	168.70 ± 0.45	89.97 ± 0.51	74.03 ± 2.95	63.73 ± 0.57
EC50 µg/ml	--	175.44	560.45	277.35

*For NO scavenging activity COS (100-500 µg/ml) has been used.

4. Discussion

COS known to possess several pharmacological properties like anti-bacterial, antioxidant (Shehata A. N & El Aty A.A.A, 2015) [25], anti-tumor (Maeda & Kimura, 2004) [10], anti-angiogenesis (Xiong *et al*., 2009) [31], anti-inflammatory (Moon J *et al*., 2007) [13] and wound healing property (Mori T *et al*., 1997) [14]. Many research has been done on the anti-tumor effect of COS. The *in vitro* assays viz. TAA, Protein denaturation inhibition, Membrane stabilization and NO scavenging assay were carried out to evaluate the pharmaceutical potential of COS. Firstly, TAA was done to evaluate the antioxidant potential of COS. Antioxidants are considered as the novel molecules, helps to neutralize or prevent the lone pair of electrons from causing lethal or severe damage to vital biomolecules. Hence, antioxidant property of a drug has been considered as the most important and primary need, required by the human body to fight against and to eliminate the disease at the early stage itself. Interestingly, COS produced using chitosanase enzyme have shown TAA of 168.70 ± 0.45 mg/g equivalents of ascorbic acid at concentration of 1000 µg/ml and it can be suggested as supplement to improve human health. Secondly, protein denaturation inhibition activity of COS was evaluated. However, antioxidant properties of COS produced during various time intervals using *Chaetomium globosum* KM651986 has been evaluated and the antioxidant activity was found to be 78% during the short incubation time of five minutes (Shehata A. N & El Aty A.A.A, 2015) [25]. Certain diseases like Alzheimer's, Parkinson's, Huntington's as well as dementia, categorized under protein denaturation diseases (Meredith S. C, 2006) [11]. The pathology of the protein denaturation diseases includes the protein aggregation in the brain and its malfunction. Another critical factor related to protein denaturation is early detection and prevention of protein denaturation, as it's later stage leads to irreversible damage of vital organs like brain and nervous system. Hence, research has been initiated to discover new drug molecules with promising protein denaturation inhibition activity. Interestingly, COS produced using chitosanase, have shown excellent protein denaturation inhibition activity of 89.97 ± 0.51% at concentration of 1000 µg/ml. So, COS has a great hope to prevent these protein denaturation related disease like Alzheimer's, Parkinson's, Huntington's as well as dementia. Thirdly, cell membrane integrity play a pivoted role in regulation of cellular mechanisms and its function. However, alteration in the cell membrane structure leads to several diseases like cancer, alzheimer's, Parkinson and various infectious diseases (Ashrafuzzaman M, & Tuszynski J, 2012) [1]. Membrane stabilization activity of COS was evaluated and found to be 74.03 ± 2.95 % at concentration of 1000 µg/ml. Finally, Nitric Oxide scavenging activity of COS has been clearly demonstrated. Nitric Oxide balance in picogram quantities required for normal physiological function, but found to damage DNA and protein at nanogram quantities. Moreover, excess Nitric Oxide known to cause immunological, cardiovascular, neurodegenerative and mental disorders (Esch T, 2002) [5]. Nitric oxide have also been found to be a pro-inflammatory mediator that proliferated the inflammation process upon overproduction. Inflammation of the gut, lungs and joints are the major parts of the body affected by overproduction of Nitric oxide (Sharma *et al*., 2007) [24]. Interestingly, COS have shown promising inhibitory effect of 63.73 ± 0.57 % Nitric oxide scavenging activity at concentration of 500 µg/ml. Hence, COS can be

used as multifaceted drug of choice to abolish DNA mutation, protein denaturation and cell membrane related diseases like cancer, Alzheimer's, Parkinson's, Huntington's and dementia.

5. Conclusion

Chitooligosaccharides are the derived products obtained from chitosan, with valuable medicinal properties. In this paper, *in vitro* pharmaceutical methods were adopted to evaluate the pharmaceutical potential of chitooligosaccharides. Further, research on pharmacological evaluation of chitooligosaccharides is necessary to reveal the efficacy in animal model.

6. Acknowledgement

The author thanks the Management of Kamaraj College for providing the laboratory facilities to carry out this research successfully.

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