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ORIGINAL RESEARCH PAPER

Antibacterial properties of biologically formed chitosan nanoparticles using aqueous leaf extract of *Ocimum basilicum*

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ABSTRACT

Objective(s): Chitosan nanoparticles (CNPs) were prepared based on the ionic gelation of chitoan with anionic compounds of *Ocimum basilicum* leaf extract.

Materials and Methods: After addition of *Ocimum basilicum* leaf extract to chitosan solution, the physicochemical properties of the nanoparticles were determined by Field Emission Scanning Electron microscope (FESEM), Fourier Transform Infrared (FTIR) analysis, X-ray diffraction (XRD) Pattern, and Dynamic Light Scattering (DLS). The antibacterial activity of CNPs was evaluated by agar disc diffusion method.

Results: The synthesized nanoparticles were found to be nearly spherical shape with size in the range of 135-729 nm. FTIR analysis revealed the presence of polyphenolic; proteins and alkaloids compounds act as effective agents for converting chitosan to CNPs. Moreover, the synthesized nanoparticles showed potent antibacterial activity against Gram positive and Gram negative bacteria.

Conclusion: These results reveal that natural sources of materials such as plants could be used for preparation of CNPs instead of use of chemical substances.

Keywords: *Biosynthesis, Chitosan nanoparticles, Leaf extract, Ocimum basilicum*

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INTRODUCTION

Chitosan is a poly-cation natural de-acetylated polymer, which is formed of N-acetyl D- glucosamine and D-glucosamine groups. There is chitosan with different molecular weights and it has been interesting properties therefore can have many applications in industry and particularly drug delivery [1,2]. Chitosan particles could be produced in the forms of microcapsules, microspheres and nanoparticles [1]. Chitosan through its amino groups will participate in various chemical reactions [3]. Natural polymers have many advantages compared to synthetic polymers, especially since these polymers are non-toxic, biodegradable and bio-compatible [4,5]. Nanoparticles (NPs) are small sized (1-100 nm) compounds that are able to function as whole units. These compounds are becoming widespread for their use in consumer products and medical applications with potential for utilization as therapeutic compounds, transfection vectors, anti-microbial agents and fluorescent labels [6]. Biogenic nano-scale metal particles using leaf extract is gaining predominant importance [7]. Since, bionanotechnology is an interesting and emerging technical tool for the synthesis of ecofriendly and reliable methodology of nanoscale materials using biological source [8]. Currently most of the application of CNPs are as reducing agent metal nanoparticles [9] drug delivery system [10], gene therapy [11], removal of heavy metals [12], antibacterial activity [13], food packaging [14] and etc. Array of

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conventional methods have been employed in synthesis of nanoparticles [3,15], but these conventional methods are bound with various limitations such as expensive, generation of hazardous toxic chemicals etc., which has upsurge the researches to develop safe, ecofriendly alternative approaches in synthesis of nanoparticles among which biological systems have been focused and exploited as a preferred green principle process for synthesis of nanoparticles. Undoubtly, biological systems have a unique ability for production of precise shape and controlled structures [16]. Among variety of methods developed to prepare CNPs, ionic gelation technique has attracted considerable attention due to this process is non-toxic, organic solvent free, convenient and controllable [17]. Ionic gelation technique is based on the electrostatic interaction between the positively charged primary amino groups of chitosan and the negatively charged groups of polyanion, such as sodium tripolyphosphate (TPP) [18-20]. In the present work, we report a new method to prepare CNPs based on ionic gelation technique by using negatively charged groups of leaf extract of *O. basilicum* instead of any other synthetic negatively charged materials. The genus *Ocimum* comes under lamiaceae family and is found in many part of the world like tropical and subtropical regions of Asia, Africa and Central and South America. It is source of essential oils and aroma compounds, a culinary herb and an attractive, fragrant ornamental plant [21]. Also *O. basilicum* extract has an antibacterial, antifungal and antioxidant properties [22,23]. Phytochemical screening of the methanol extracts of *Ocimum* showed the presence phenolic content, glycosides, anthraquinones, terpenoids, tannins, lignin and saponin as chemical constituents [21]. Probably these bio-molecules of leaf plant extract can act as biosynthesizing agents for CNPs. It seems that this system has some interesting features: (a) The nanoparticles are obtained under biological system, very mild conditions without the need of high temperature, surfactant and some other special experimental technology (b) The nanoparticles have small particle size and positive surface charges, which may improve their stability in the presence of biological actions [24] and is favorable for some drugs due to the interaction with negatively charged biological membranes and site – specific targeting *in vivo* [25,26]. The biomolecules of the plant extract are the possible compounds in the biosynthesis of nanoparticles [27]. However, aromatic and medicinal plants contain many biologically active compounds [28]. On the other hand, to the best of our knowledge, there is no report of CNPs biosynthesis by utilizing of aqueous leaf extracts of *O. basilicum*. So, this study aims at biosynthesis of CNPs using *O. basilicum* leaf extracts and investigation of their antibacterial effects for the first time.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *O. basilicum* purchased from the company of Pakanbazr (Isfahan, Iran), were surface sterilized by immersion in 70% ethanol for 2 minutes and then 5% sodium hypochlorite for 5 minutes, followed by three times rinsing in sterile water after each step. The seeds were next soaked in a dilute solution of benomyl and transferred to plastic pots containing peat moss (Klasmann-Deilman, potgrond H) under equal greenhouse condition at Imam Khomeini International University (Qazvin, Iran) in February 2015. At late vegetative stage, leaves of the plants were harvested. The samples were immediately frozen in liquid nitrogen and stored frozen at – 80 °C until use.

Extraction and biosynthesis of CNPs

All chemicals were purchased from Merck or Aldrich. Low molecular weight chitosan was dissolved at 2% (w/v) with 0.5% (v/v) acetic acid and then raised to pH 5 with 1N NaOH under magnetic stirring for 24 hours and brought to volume in a 200 ml volumetric flask. Frozen collected leaves were used for the preparation of *O. basilicum* leaf extract. 10 gram of finely cut leaves was taken and boiled in 100 ml of distilled water for 5 minutes. After cooling, the obtained extract was filtered through Whatman No. 1 filter paper and filtered extract was stored at 4 °C. CNPs formed spontaneously upon addition of 10 ml of the *O. basilicum* leaf extract to 40 ml of chitosan solution (1mg/ml) under magnetic stirring at 60 °C and 110 rpm to obtain an opalescent solution.

Characterizations

CNPs were characterized by following measurements. Particles size distributions of CNPs were determined using Dynamic Light Scattering (DLS, Zetaplus, and Brookhaven). X-ray powder diffraction patterns of CNPs were obtained by a GNR, apd-2000

diffractometer. FTIR spectra of CNPs were taken with potassium bromide pellets on a Bruker Tensor 27 spectrometer. Field Emission Scanning Electron Microscopy (FESEM, HITACHI, S-4160) was used for visualization of CNPs.

Assays for antibacterial activity

The antibacterial activity of biosynthesized CNPs have tested against two different types of microorganisms by using disc diffusion method. For the determination of anti-bacterial activity of CNPs, the Gram-negative *Escherichia coli*, and Gram-positive *Bacillus vallismortis* were used. Luria-Bertani media was prepared and poured into sterilized petriplates and then plates were spreaded with of the bacteria separately. Then sterile discs were kept and the samples (with an equal volume) were added to the disc and the plates were incubated at 37 °C overnight. Then zone of inhibition was measured.

RESULTS AND DISCUSSION

Biosynthesis and characterisation of CNPs

At the early stage of the experiment, there was no or little amount of CNPs in the solution, thus the system showed the property as a clear solution. Gradually the amount of CNPs in the solution increased, and the system changed initially from a clear solution to an opalescent emulsion, indicating the formation of CNPs. Fig 1 displays the FESEM images of the biosynthesized nanoparticles. Nearly spherical nanoparticles are seen that appear to be well separated and stable over the steps of the preparation process. This result is comparable with the results of many previous researchers [13,29,30]. FTIR spectra of pure chitosan, reaction solution of chitosan with leaf broth of *O. basilicum* including CNPs, and pure leaf broth of *O. basilicum* are shown in Fig 2. The peak observed at 3438.10, 2925.34, respectively in the reaction solution of chitosan with leaf broth of *O. basilicum*. The band at 3448.61 Cm^{-1} corresponds to the combined peak of the NH_2 and OH groups stretching vibration in chitosan. For reaction solution, the intensities of amide bands at 1560.77 Cm^{-1}, which can be observed clearly in pure chitosan, decrease dramatically, and one new absorption band at 1629.68 Cm^{-1}, which can be assigned to the absorption peak of the $NH3^{+}$ absorption of chitosan is observed.

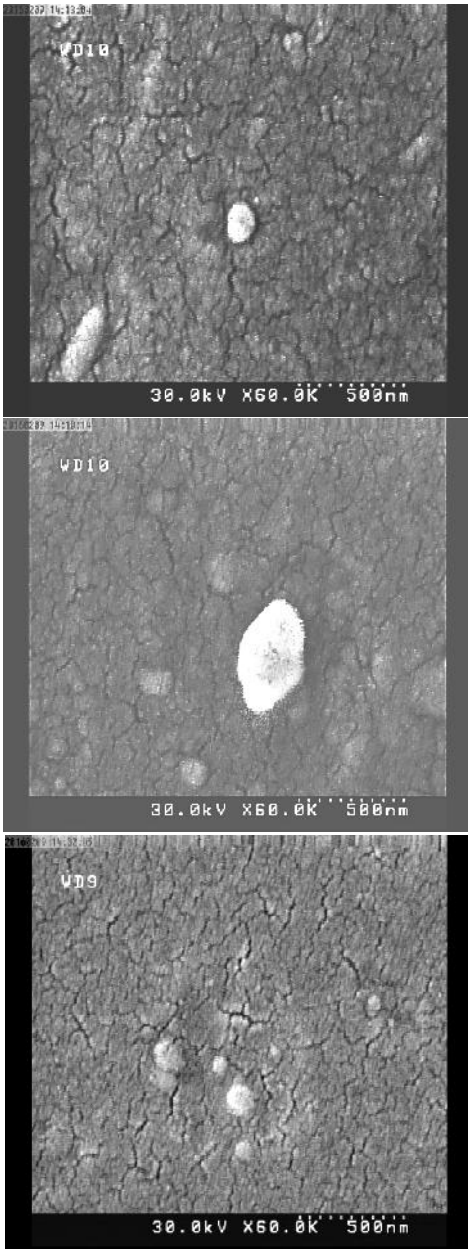


Fig. 1. FESEM images of prepared CNPs

The absorption peak at 1414.08 Cm^{-1} in reaction solution could be assigned to symmetric stretching vibrations of - COO anion groups. This result indicates that carboxylic groups of the leaf broth compounds are dissociated into - COO groups which complex with protonated amino groups of chitosan through electrostatic interaction to form CNPs. The peak at 3384.07 Cm^{-1} indicates alcohol and phenolic

OH groups along with the peak of 1605.78 Cm^{-1} which represents $CONH_2$ group in leaf broth. These peaks decrease dramatically in reaction solution. Aqueous extract of leaves of *O. basilicum* contain different compounds such as limonene, camphene, myrtenol, linalool, flavonoids, tannins, proteins and triterpenoids [22,31]. These chemical constituents are mainly responsible for various biological activities. It is postulated that anion groups of leaf broth such as OH (alcoholic and polyphenolic compounds) and COO^- (aminoacids residues, free carboxylate groups in proteins, carboxylic acids, alkaloids) interact with the ammonium groups of chitosan, which series to enhance both the inter and intra molecular interaction in CNPs.Fig 3 shows the XRD pattern of chitosan (Fig 3a) and the biosynthesized CNPs (Fig 3b). Chitosan gives two characteristic peaks at 2*è* = 10° (weak diffraction peak) and 20 ° (strong diffraction peak), indicating the high degree of crystallinity of chitosan [32]. However, there are no comparable peaks in the diffractograms of the CNPs. The broad peaks are attributed to the increased amorphous nature of the CNPs.

The XRD of the CNPs is characteristic of an amorphous polymer which is compatible with some of previous studies [13,23]. The results of Dynamic

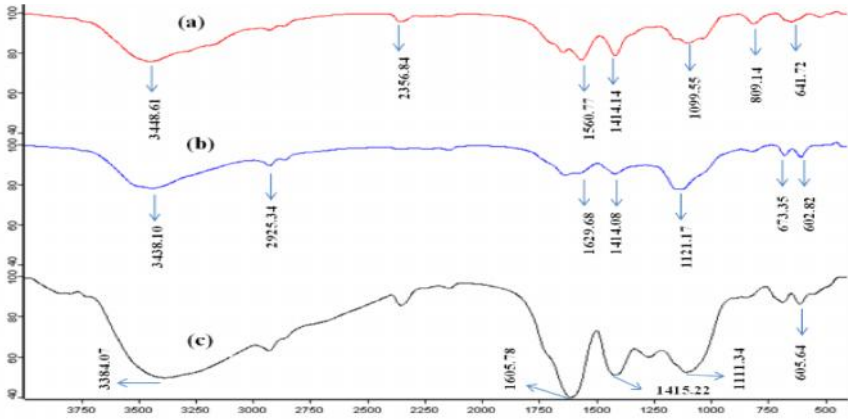


Fig. 2. FTIR spectra of (a) chitosan (b) CNPs, and (c) *O. basilicum* leaf extract

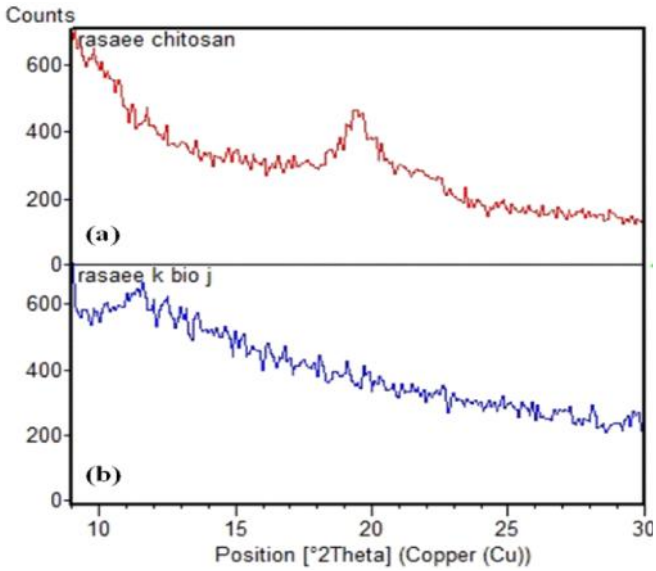


Fig. 3. XRD patterns: (a) Chitosan and (b) Biosynthesized CNPs

Light Scattering (Fig 4) show that the majority of the biosynthesized CNPs have a size between 135 and 729 nm. considering that phytochemicals have a main role in formation of CNPs [33], it can be said that whatever be smaller the size of this phytochemicals existing in the leaf extract, as a result will be smaller obtained particle size.

Antibacterial activity of CNPs

Antibacterial activity test of CNPs and chitosan powder solution (with a concentration of 1 mg/ml) was performed on two bacteria including E. coli and B. vallismortis using disc diffusion method (Fig 5). The created inhibition zone by the disc containing CNPs was measured after 24 h. According to the obtained results based on the size inhibitory zone around the discs, it was found that Gram-negative bacteria (E. coli) more be sensitive to CNPs compared to Gram-positive bacteria (B. vallismortis).

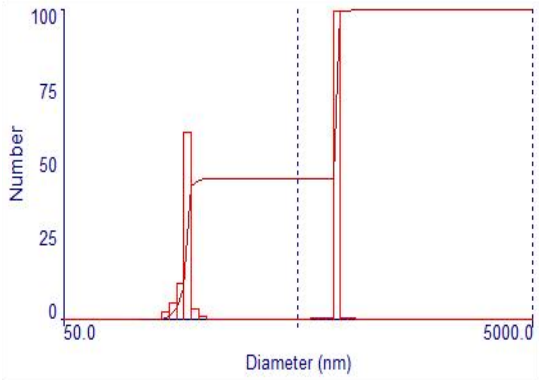


Fig. 4. Size of chitosan nanoparticles

It was also found that the inhibition zone size created by the disc containing chitosan powder solution around the bacteria is lower compared to the disc containing biosynthesized CNPs. To description antibacterial activity of CNPs can be mentioned to several mechanisms. The first mechanism is including linkage and chelated necessary metal elements for the enzymes and proteins activity, and finally inhibit the growth of bacteria [34]. The second mechanism is involves combining chitosan with non-ionic groups on the surface of bacterial cells [35,36].

The preparation of CNPs is based on an ionic gelation interaction between positively charged chitosan and negatively charged compounds [37,38]. Aromatic plants contain chemical substances such as polyphenols, quinines, flavonols/flavonoids, alkaloids, polypeptides or their oxygen-substituted derivatives, also carboxylic acids, carbohydrates, proteins and teriterpenoids. These chemical constituents are mainly responsible for various biological activities [39,40]. These chemical components of plants commonly contain functional

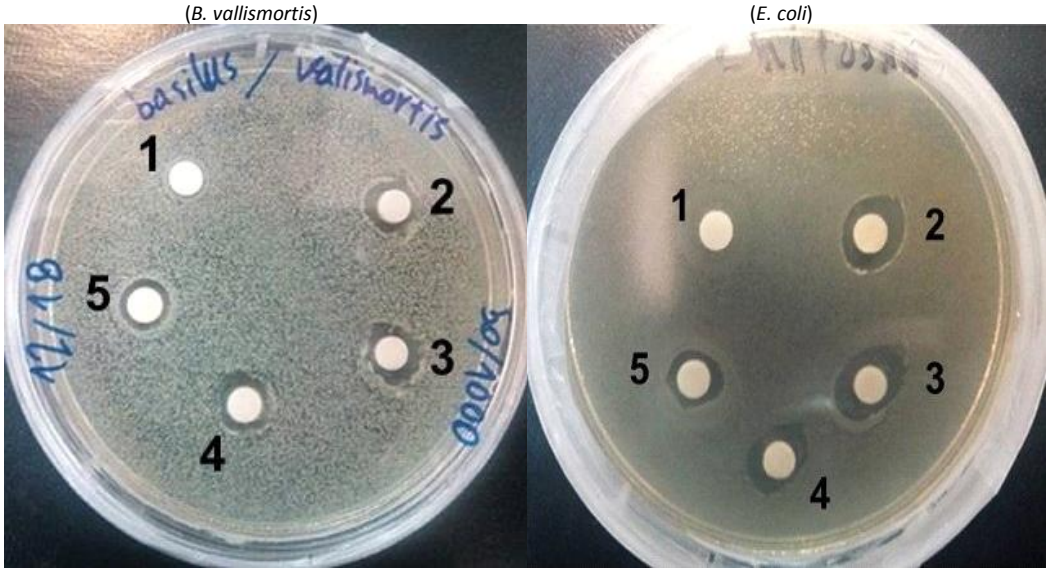
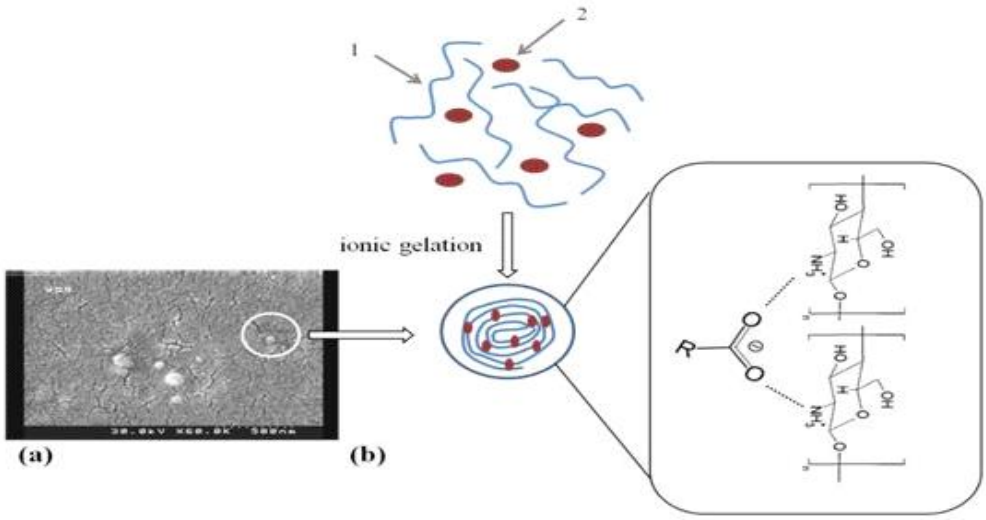


Fig. 5. Antibacterial test results for *B. vallismortis* and *E. coli* after 24 h of incubation.1 Distilled water, 2, 3, 4 CNPs and 5 Chitosan



Scheme. 1. Proposed mechanism to form of chitosan nanoparticles by ionotropic gelation: (a) SEM image of the biosynthesized nanoparticle, (b) schematic illustration of the chitosan-anion complex. 1 chitosan and 2 negatively charged functional groups of *O. basilicum* leaf extract such as coo $^-$

groups such as hydroxy (- OH) and carbonyl (- COOH) groups. These groups easily dissociate into hydroxide [- OH] and carboxylate [- COO] anions and a positively charged hydrogen ion (proton). In this study negatively charged functional groups of *O. Basillicum* leaf extract such as [- OH] and [- COO] anions accomplished in the biosynthesis reaction according to the described reaction conditions. Chitosan has fairly long linear structure with rigid conformation. These long molecules in solid state are, mostly, in the form of tightly folded random coils [41]. Chitosan has many hydroxyl group and amine (or amide) groups. These OH and NH_2 groups in the structure of chitosan can activate the electrophilic components of the reaction, such as the carbonyl group, by hydrogen bonding [32]. In acidic medium, amino groups of chitosan are positively charged, resulting in a highly charged polyelectrolyte polysaccharide [30]. Various methods have been developed for the cross-linking of chitosan, which commonly result in gel formation ionic cross-linking reactions with charged ions [42] or molecules [43-46] have also been employed by using ionotropic gelation methods to form hydrogels based on chitosan [30]. Chitosan hydrogels are obtained by ionic gelation, where nanoparticles are formed by means of electrostatic interactions with polyanions [47,48]. In this study, spherical and oval shapes of CNPs were obtained by reacting chitosan with negatively charged functional groups of the leaf extract of *O. basillicum* such as [- OH] and [- COO] anions that the proposed mechanism for the formation of the CNPs is shown in scheme 1. These results are consistent with those of several previous investigations about synthesis of CNPs by using of water-soluble linkages such as phosphates [49], sulfates [50], cyanates [51] and other agents [52,53]. It seems that biocompatible chitosan has been successfully modified by condensation reaction using natural compounds of the plant extract as cross-linking agents to form nanoparticles. Probably in addition to anionic compounds, different types of carboxylic acid such as succinic acid, malic acid and citric acid of the leaf extract have participated in nanoparticle biosynthesis that agrees with previous investigation [30].

The negatively charged surface of the bacterial cell (peptidoglycans) is the target site of the polycation [54]. Because of the high effects of the CNPs on the bacteria, it seems that the biosynthesized CNPs are positively charged [30] therefore, the polycationic CNPs with higher surface charge density interact with the bacteria to a greater degree than chitosan itself. CNPs provide higher affinity with bacteria cells for a quantum-size effect [13]. Because of the larger surface area of the CNPs, nanoparticles could be tightly adsorbed onto the

surface of the bacteria cells so as to disrupt the membrane, which would lead to leakage of intracellular components, thus killing the bacteria cells.

CONCLUSION

We have shown that biocompatible chitosan has been successfully modified by condensation reaction using natural compounds of *O. basilicum* leaf extract as cross-linking agents to form nanoparticles. Opalescent stable colloid systems based on chitosan were fabricated in aqueous medium at room temperature. Biosynthesis of CNPs using green sources like *O. basilicum* is a better alternative to chemical synthesis, science this green synthesis is pollutant free and eco-friendly. The results suggest that *O. basilicum* leaf extract plays an important role in the biosynthesis and stabilization of CNPs. This study also found that the CNPs show antibacterial activity on both Gram positive and Gram negative bacteria and should be explored further for antimicrobial applications. Being of cationic character, chitosan is able to react with polyanions giving rise to polyelectrolyte complexes. Hence chitosan has become a promising natural polymer for the preparation of microspheres/ nanospheres and microcapsules [55]. In addition, since the chitosan microspheres offer highly convenient and flexible systems for different application [15], it is believed that the biosynthesized novel CNPs can be considered for different purposes particularly biomedical application.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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