Synthesis and characterization of chitosan nanoparticles of *Achillea millefolium* L. and their activities [version 1; peer review: awaiting peer review]

Dolly Kain, Suresh Kumar

Medicinal Plant Research Laboratory, Department of Botany, Ramjas College, University of Delhi, Delhi, 110007, India

**Abstract**

**Background:** *Achillea millefolium* L. is an herbal aromatic plant of family Asteraceae reported to have various medicinal activities in the literature. The current study evaluated the potential of chitosan nanoparticles of *A. millefolium* as an effective strategy for targeted treatment of bacterial diseases and urolithiasis.

**Methods:** *A. millefolium* was collected from Poonch, Jammu and Kashmir, and its inflorescence extracted in water by maceration. Chitosan nanoparticles of *A. millefolium* (AMCSNPs) were prepared by ionic gelation method using 0.1% chitosan, different concentrations of the cross-linking agent sodium tripolyphosphate (STPP; 0.5%, 1%, 1.5%, 2%) and different concentrations of *A. millefolium* extract (0.5%, 1%, 1.5%, 2%). Characterization of AMCSNPs was done using UV-Vis spectroscopy, Fourier transform-infrared (FT-IR) spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy (TEM). Antibacterial screening of AMCSNPs was performed by well-diffusion method. Antiurolithiatic screening of AMCSNPs was done by nucleation and aggregation assay.

**Results:** The best chitosan nanoparticles of *A. millefolium* (AMCSNPs) were obtained with 0.1% chitosan, 1% STPP and 20% *A. millefolium*. These AMCSNPs showed maximum zone of inhibition of 30±0.5 mm using the well-diffusion method against both *Bacillus subtilis* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) and maximum antiurolithiatic activity with 68% inhibition shown at aggregation stage.

**Conclusions:** The current study suggests that AMCSNPs are an excellent strategy for targeted drug delivery for treatment of bacterial diseases and urolithiasis.

**Keywords**

*Achillea millefolium* L., AMCSNPs, Drug delivery, Targeted treatment, Antibacterial, Antiurolithiatic
Corresponding author: Suresh Kumar (suresh.kumar@ramjas.du.ac.in)

Author roles: Kain D: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Kumar S: Conceptualization, Formal Analysis, Resources, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: The University Grant Commission, University of Delhi provided financial support.

Copyright: © 2020 Kain D and Kumar S. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Kain D and Kumar S. Synthesis and characterization of chitosan nanoparticles of Achillea millefolium L. and their activities [version 1; peer review: awaiting peer review] F1000Research 2020, 9:1297
https://doi.org/10.12688/f1000research.26446.1

Introduction
Natural biopolymers are attractive products of living organisms as they serve a number of different applications for human health due to their biodegradability, such as vaccine delivery, drug development, and food preservatives. Chitosan (CS) is a natural biopolymer and a derivative of chitin. It is obtained from different sources of chitin and differs on the basis of its degree of deacetylation. In the last few years, CS nanoparticles (CSNPs) have drawn much attention due to their biodegradability, biocompatibility, quantum size effects, large surface to volume ratios, and their simple and inexpensive production. Different biological activities of CSNPs have been reported, such as antimicrobial, antioxidant, anticancer, drug delivery, tissue engineering, carbon nanotube, food preservative, and purification of water. CSNPs successfully used in drug delivery for treatment of various diseases, including ocular drug delivery, per-oral drug delivery, nasal drug delivery, pulmonary drug delivery, mucosal drug delivery, gene delivery, buccal drug delivery, vaccine delivery, vaginal drug delivery, and cancer therapy have been reported. Achillea millefolium is a perennial herbal aromatic plant belonging to the family Asteraceae with characteristically finely divided leaves and inflorescence in corymbose cluster. It has been reported to have different medicinal activities, including antibacterial and diuretic. The current study was designed to evaluate the potential of CSNPs of A. millefolium (AMCSNPs) as an effective alternative of targeted drug delivery and treatment of various diseases, including bacterial infections specifically uratiition.

Methods
Collection, identification, and extraction of A. millefolium. A. millefolium was collected from Pathanteer Village, Mendhar Tehsil, Poonch District (Jammu and Kashmir, India; GPS coordinates 33° 39’ 40” N – 74° 11’ 11” E) and identified by Raw Materials Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR), Pusa with reference IDNISCAIR/RHMD/consult/2018/3293-94. 15g powder of inflorescence of A. millefolium was extracted in 50 ml of water by maceration at 90°C using water bath.

Crude extract of the plant was evaporated using rotary evaporator (Khera KI-102), which resulted in the semi solid form of extract. This was then weighed and dissolved in a known amount of solvent for making a stock concentration of the plant extract. Different concentrations were made by serial dilution.

Bacterial strains and chemicals
Strains of Bacillus subtilis (MTCC 441) and Pseudomonas aeruginosa (MTCC 1688) were procured from MTCC Chandigarh. All the chemicals used (chitosan, acetic acid, sodium tripolyphosphate (STPP), Tween-80, calcium chloride, sodium oxalate, tris buffer and NaCl) were of good quality and purchased from Fisher Scientific International, Inc.

Synthesis of chitosan nanoparticles of A. millefolium
CSNPs were prepared by ionic gelation method. 10ml 0.1% chitosan solution was made in 1% acetic acid with different percentages of the cross-linking agent STPP (0.5%, 1%, 1.5% and 2%). 5ml of STPP was added drop wise to the chitosan-acetic acid solution, which was magnetically stirred at room temperature. An opalescent color was observed, and stirring was continued for 60 min.

To obtain AMCSNPs, variable concentrations of plant extract (5%, 10%, 15% and 20%) were added to the 10ml chitosan solution by magnetic stirring prior to adding the 5ml STPP drop wise. This solution was stirred for a further 2 h followed by centrifugation at 10000g for 10 min and then the AMCSNPs were washed three times with distilled water. The pH of the nanoparticles was maintained at 4.8, and 1–2 drops of 1% Tween-80 was used to prevent agglomeration.

Percentage encapsulation efficiency of each concentration of extract was determined using the following formula:

Encapsulation efficiency (%) = (total amount – free amount/total amount) *100.

Characterization of chitosan nanoparticles of A. millefolium
UV-Vis spectroscopy using SPUV-1000 spectrophotometer attached to Mwave professional software 2.0 (or any software used to obtain the UV-Vis absorption spectra) and spectrum between 200 nm-700 nm was obtained for determining the main absorbing region. FTIR (Fourier Transform-Infrared) Spectroscopy using spectrometer (Brukers) in the range of 1000 cm⁻¹-3500 cm⁻¹ to identify the peaks of main functional groups, DLS (Dynamic Light Scattering) in the range between 0 nm to 1000 nm using zetasizer Nano ZS90 (Malvern Instruments Ltd., UK) at room temperature for particle size distribution and TEM (Transmission electron microscopy) at an accelerating voltage of 200 kV using Tecnai G2 30U-twin kV Ultra-twin microscope to study the morphology.

Antibacterial screening of chitosan nanoparticles of A. millefolium
Primary culture of bacteria was obtained from lyophilized culture by inoculating in LB broth, which was incubated in an incubator shaker at 120 rpm and 37°C for 12–16 h. Pure colonies of each bacterium were obtained from primary culture by streak plate method using LB agar plates, which were inoculated in LB broth, incubated in an incubator shaker at 120 rpm 37°C for 12–16 h. Absorption of bacterial culture was adjusted to 0.1±0.02 at 600nm using SP-UV1000 spectrophotometer to reach the concentration of 10⁶ CFU/ml for final use, which is equal to 0.5 McFarland standards, as previously performed in the literature to obtain a similar concentration of each bacterium. Each reading was taken thrice.

Antibacterial screening of AMCSNPs was done using well-diffusion method. 1.5% LB agar plates were used and a 5mm cork-borer made four wells in each plate. 20 µl of B. subtilis and P. aeruginosa culture was added to the plates and spread using a glass spreader. 100 µl of AMCSNPs was poured in the wells. Plates were sealed with parafilm, incubated at 28°C for 12–16 h and the zone of inhibition (ZOI) recorded.
Antiurolithiatic screening of chitosan nanoparticles of A. millefolium

Nucleation and aggregation assays were used to determine the antiurolithiatic potential of AMCSNPs.

Nucleation assay: The method of Hennequin et al.\(^27\) was used with some minor modifications. Solutions of calcium chloride and sodium oxalate were prepared at a final concentration of 3mmol/l and 0.5mmol/l, respectively, in a buffer containing Tris 0.05mol/l and NaCl 0.15mol/l at pH 5.5. A total of 1.9 ml of calcium chloride solution mixed with 200 µl of AMCSNPs was incubated for 30 minutes in a 37°C water bath. Crystalization was started by adding 1.9 ml of sodium oxalate solution. Equal volume of water has used for a control instead of AMCSNPs. The optical density of the solution was recorded at 620 nm for 420 sec using spectrophotometer SPUV-1000.

\[
\% \text{ Inhibition} = \left( \frac{\text{Abs. Control} - \text{Abs. Sample}}{\text{Abs. Control}} \right) \times 100
\]

Aggregation assay: The method of Hess et al.\(^28\) was used with some minor modifications. 'Seed' CaOx monohydrate (COM) crystals were prepared by mixing calcium chloride and sodium oxalate at 50mmol/l. Both the solutions were equilibrated in a 60°C water bath for 1h and then cooled at 37°C overnight. The crystals were harvested by centrifugation at 10000g and then evaporated at 37°C. COM crystals were used at a final concentration of 0.8 mg/ml, buffered with Tris 0.05 mol/l and NaCl 0.15 mol/l at pH 5.7. A total of 1 ml of AMCSNPs were added in a test tube to 3 ml COM crystal solution and incubated at 37°C. Equal volume of water was used for a control instead of AMCSNPs. Absorption at 620 nm was recorded at different time intervals (30 min, 60 min, 90 min, and 120 min).

\[
\% \text{ Inhibition} = \left( \frac{\text{Slope Control} - \text{Slope Sample}}{\text{Slope Control}} \right) \times 100
\]

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2007. One-way ANOVA was used followed by t-test to determine the significant difference of antibacterial activity between different samples and regression analysis was used to plot graphs of nucleation and aggregation assays.

Results

Synthesis of chitosan nanoparticles of A. millefolium

On addition of STPP to chitosan solution, an opalescent color was observed, which indicates the formation of CSNPs. Different concentrations of STPP (0.5%, 1%, 1.5%, and 2%) were used for nanoparticle preparation and 1.0% STPP was found to be most suitable with sharpest peak shown by UV spectroscopy, indicating the most CSNPs made (Figure 1). Therefore, 1.0% STPP was used to obtain AMCSNPs. Different percentages of A. millefolium water extract (5%, 10%, 15% and 20%) in 0.1% of chitosan solution were used to make AMCSNPs and excellent loading efficiency was observed, i.e. 94%, 94.7%, 94.7%, and 95.2% for 5%, 10%, 15% and 20% A. millefolium respectively. A standard graph for absorbance of A. millefolium extract at 417 nm (maximum absorption verses concentration of extract) was obtained. The amount of loaded extract was determined using the standard graph as a decrease in the absorption values of the supernatant of AMCSNPs indicated the loading of extract of the nanoparticles. Loading efficiency was calculated using the above encapsulation efficiency formula for each concentration. Hence 20% AMCSNPs have been used for further analysis.

Characterization of chitosan nanoparticles of A. millefolium

A broad absorption band between 200 to 300 nm was shown for the UV spectrum of AMCSNPs (Figure 2). FTIR spectra of CS showed peaks at 3324.15, 2153.28, 1638.72 and 1279.29; FTIR spectra of CSNPs showed peaks at 3317.48, 2139.29 and 1638.46; and FTIR spectrum of AMCSNPs showed peaks at 3281.73, 2163.36 and 1636.78 (Figure 3). DLS revealed the size range of nanoparticles with Z average of 118nm having characteristic peaks at 10 nm, 122 nm and 712 nm, and highest intensity was recorded at size 10nm (Figure 4). TEM was used to study the morphology of the nanoparticles, which revealed a spherical shape with smooth surface. TEM also revealed the size of AMCSNPs: <100 nm with smallest size of 4.15 nm (Figure 5).

Figure 1. UV-Vis spectrum of chitosan (CS) and chitosan nanoparticles prepared with different concentrations of sodium tripolyphosphate (0.5%, 1%, 1.5% and 2%).
Figure 2. UV absorption spectrum of *Achillea millefolium* chitosan nanoparticles (0.1% chitosan, 1% sodium tripolyphosphate and 20% *A. millefolium*).

Figure 3. Fourier Transform-Infrared graph of chitosan (A), chitosan nanoparticles, (B) and *Achillea millefolium* chitosan nanoparticles (C).
Antibacterial screening of chitosan nanoparticles of *A. millefolium*
AMCSNPs exhibited excellent antibacterial activity against both Gram-positive *B. subtilis* and Gram-negative *P. aeruginosa*. AMCSNPs showed three-times the increase in antibacterial activity as compared with *A. millefolium* extract only (control); ZOI increased from 10 mm to 30mm against both *B. subtilis* and *P. aeruginosa* with a statistically significant difference between *A. millefolium*, CSNPs and AMCSNPs (Figure 6).

Antiur/o/olithiatic screening of chitosan nanoparticles of *A. millefolium*
AMCSNPs showed significant antiurolithiatic activity with 68% inhibition in the aggregation assay and 51.26% inhibition in the nucleation assay as compared to 55.13% and 9.09% inhibition by *A. millefolium* extract (control). In the nucleation assay, the % inhibition is nearly equal in the case of *A. millefolium* and AMCSNPs, but CSNPs did not show any inhibition. In the aggregation assay there is a significant increase in % inhibition with 9.09%, 63.63% and 68% inhibition by *A. millefolium*, CSNPs and AMCSNPs, respectively, (Figure 7).

**Discussion**
For characterization of nanoparticles, different techniques used in the literature including UV-Vis spectroscopy, FTIR spectroscopy, DLS and TEM. In our study, a UV-Vis absorption band for AMCSNPs of 200-300 nm indicates the presence of a CO group in the CSNPs, as reported by Vaezifer et al. A shift of
Figure 6. Antibacterial activity of *Achillea millefolium* chitosan nanoparticles. CSNP’s= Chitosan nanoparticles, AM= *A. millefolium* and W= water.

**Nucleation Activity**

![Graph showing nucleation activity](image)

**Aggregation Activity**

![Graph showing aggregation activity](image)

Figure 7. Antiurolithiatic activity of *Achillea millefolium* chitosan nanoparticles (AMCSNP’s) as shown by a nucleation assay (A) and aggregation assay (B). AM= *A. millefolium*, CSNP’s= chitosan nanoparticles.
FTIR peaks from 3317.48, 2139.29 and 1636.46 for CSNPs to 3281.73, 2163.36 and 1636.78 for AMCSNPs indicates that the loading of *A. millefolium* into the CSNPs, as reported by Khan et al.32. Our DLS results are comparable to the average size of CSNPs reported in literature, i.e. 189 and 197 nm by Khan et al.32, 216 nm by Agarwal et al.33, and size range of 135–729 nm by Rasaee et al.34 and 6.5-1331.2 nm by Iswanti et al.35. TEM of AMCSNPs in our study revealed a spherical shape with a smooth surface, which was also reported by Da Silva et al.36.

Chitosan is a positively charged macromolecule, which interacts with the negatively charged microbial membrane, and results in the breakage of intracellular components. Chitosan acts as a chelating agent and limits toxin production and microbial growth.37-38. Antimicrobial screening of *Ocimum basilicum* CSNPs against *E. coli* and *Bacillus subtilis* have been reported by Rasaee et al.34, chitosan-tripolyphosphate nanoparticles against *Staphylococcus aureus* and *P. aeruginosa* have been reported by Bangum et al.39, and against pathogen strains of tomato *Xanthomonas and Erwinia* by Oh et al.40. Gallic acid-chitosan conjugates have been reported to inhibit the formation of calcium oxalate crystals by Queiroz et al.41. Anti-urolithiatic activity of *Aerva lanata* chitosan nanoparticles at 0.8 μg/ml concentration through nucleation assay have been reported by Chandirika et al.42 and *Tridax procumbens* by Chandirika et al.43. In our study, AMCSNPs showed excellent antibacterial activity against both *B. subtilis* and *P. aeruginosa*, and significant anti-urolithiatic activity at aggregation stage of urolithiasis.

### Data availability

#### Underlying data


This project contains the following underlying data:

- Output files of chitosan nanoparticles with different concentrations of STPP
- Raw data for Figures 1, 2 and 4
- Unedited and uncropped FT-IR graphs and TEM images of AMCSNPs
- ZOI of antibacterial activity of AMCSNPs
- Absorption values of anti-urolithiatic activity AMCSNPs

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

### Acknowledgements

The authors are thankful to the Principal, Dr. Manoj K. Khanna, Ramjas College, Prof. S.B. Babbar, Head, Prof. K.S. Rao, and Prof. Veena Agrawal, Department of Botany, University of Delhi, Delhi for providing necessary facilities and encouragement during the course of investigation.

### References


The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com