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RESEARCH ARTICLE

Synthesis and characterization of chitosan nanoparticles of *Achillea millefolium* L. and their activities

[version 1; peer review: 2 approved with reservations]

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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

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Approval Status ? ?

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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[1]. 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[2]. 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[3–5]. Different biological activities of CSNPs have been reported, such as antimicrobial, antioxidant, anticancer[6], drug delivery, tissue engineering, carbon nanotube, food preservative, and purification of water[7]. CSNPs successfully used in drug delivery for treatment of various diseases, including ocular drug delivery[8], per-oral drug delivery[9], nasal drug delivery[10], pulmonary drug delivery[11], mucosal drug delivery[12], gene delivery[13], buccal drug delivery[14], vaccine delivery[15], vaginal drug delivery[16], and cancer therapy[17] 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[18,19]. 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 urolithiasis.

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. 5g 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 10 ml chitosan solution by magnetic stirring prior to adding the 5 ml 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[20–22]:

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 10^8 CFU/ml for final use, which is equal to 0.5 McFarland standards, as previously performed in the literature[23–25] to obtain a similar concentration of each bacterium. Each reading was taken thrice.

Antibacterial screening of AMCSNPs was done using well-diffusion method[31]. 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. Crystallization 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.

% Inhibition = {(Abs. Control- Abs. Sample)/ Abs. Control} * 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).

% Inhibition = {(Slope Control- Slope Sample)/ Slope Control} * 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).

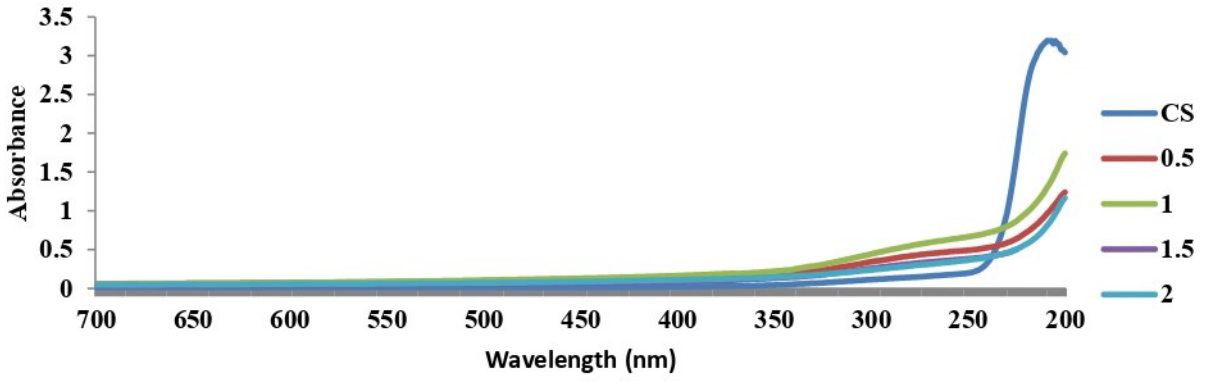


Figure 1. UV-Vis spectrum of chitosan (CS) and chitosan nanoparticles prepared with different concentrations of sodium tripolyphosphate (0.5%, 1%, 1.5% and 2%).

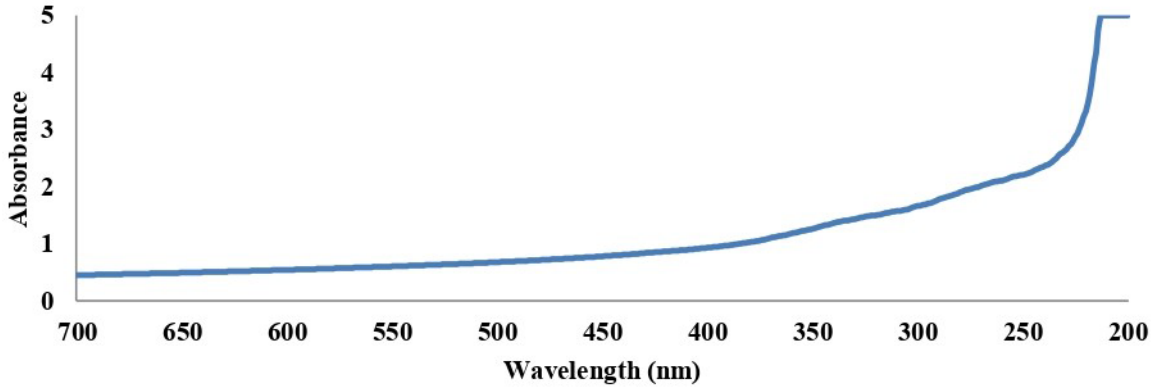


Figure 2. UV absorption spectrum of *Achillea millefolium* chitosan nanoparticles (0.1% chitosan, 1% sodium tripolyphosphate and 20% *A. millefolium*).

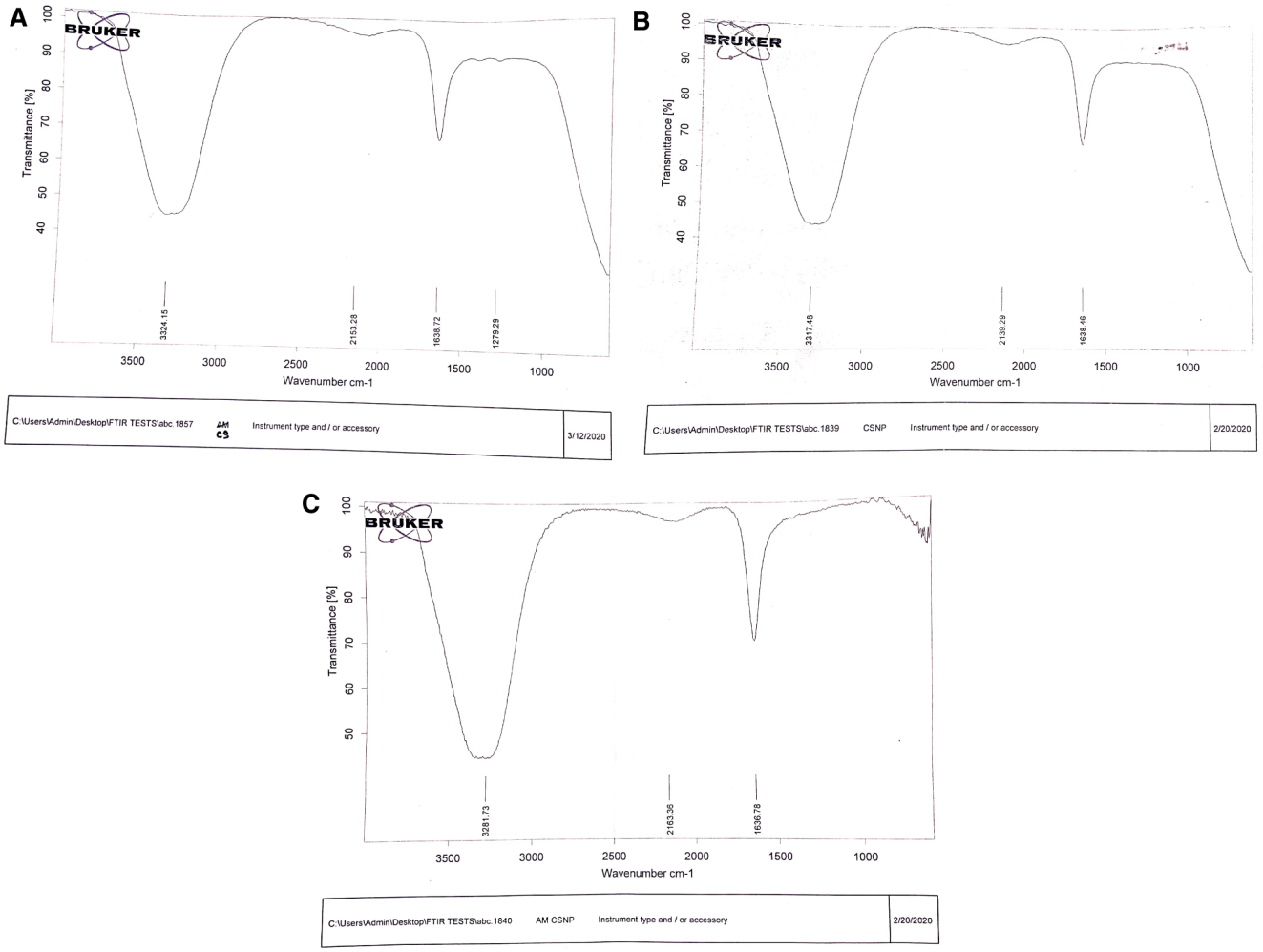


Figure 3. Fourier Transform-Infrared graph of chitosan (**A**), chitosan nanoparticles, (**B**) and *Achillea millefolium* chitosan nanoparticles (**C**).

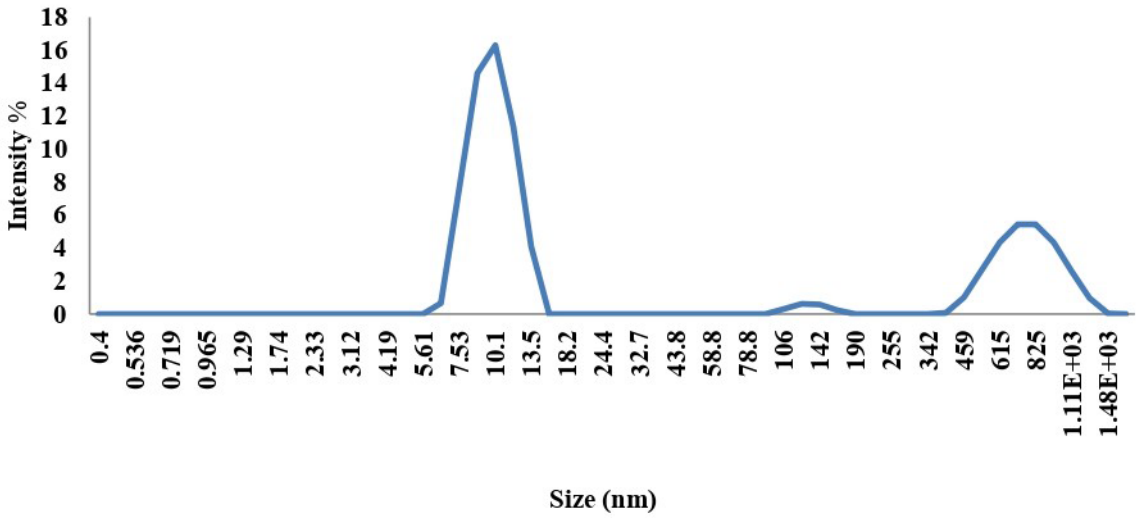


Figure 4. Particle size distribution of *Achillea millefolium* chitosan nanoparticles, as revealed by Dynamic Light Scattering.

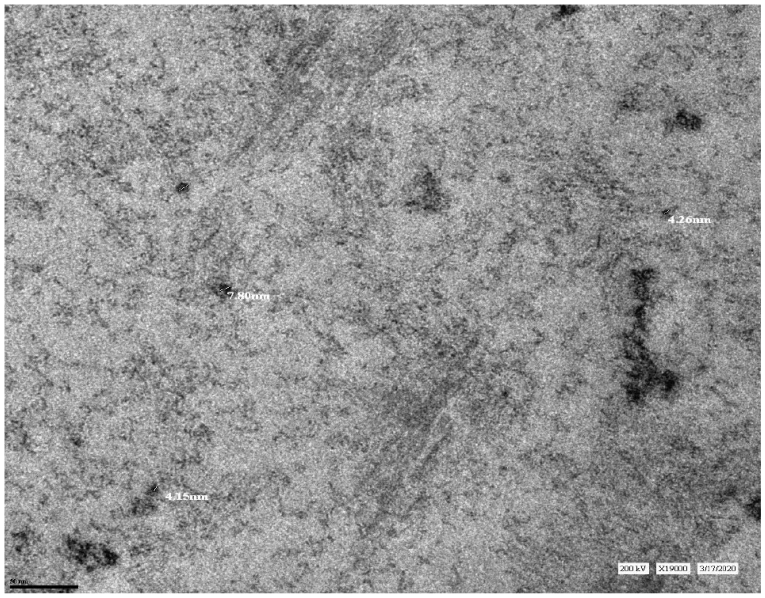


Figure 5. Transmission Electron Microscopy showing *Achillea millefolium* chitosan nanoparticles.

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).

Antiurolithiatic 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 55.132% 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 AMCSPs, respectively, (Figure 7).

Discussion

For characterization of nanoparticles, different techniques used in the literature including UV- Vis spectroscopy, FTIR spectroscopy, DLS and TEM[29,30]. 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.*[31]. A shift of

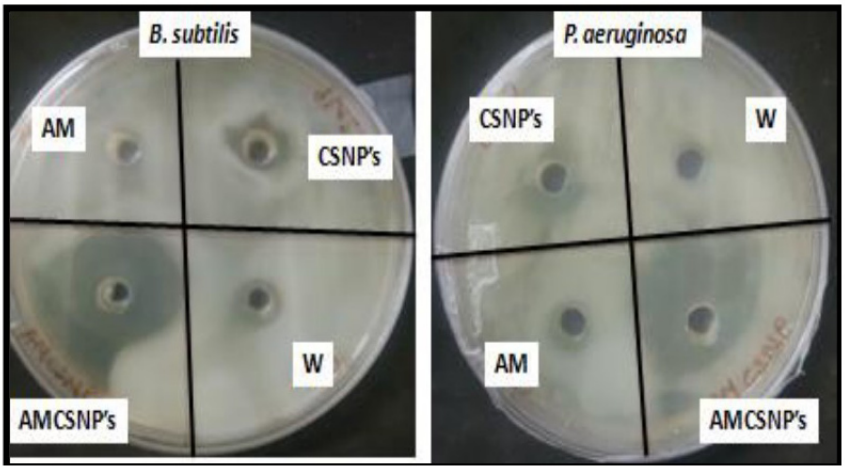


Figure 6. Antibacterial activity of *Achillea millefolium* chitosan nanoparticles. CSNP's= Chitosan nanoparticles, AM= *A. millefolium* and W= water.

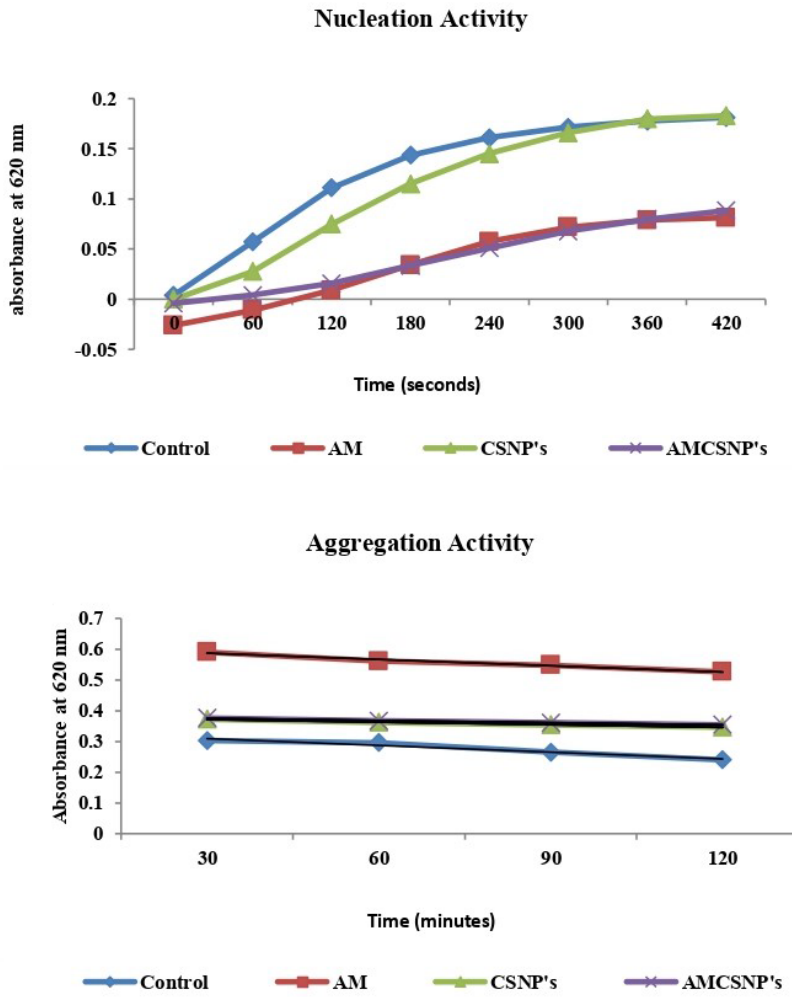


Figure 7. Antiurolithiatic activity of *Achillea millefolium* chitosan nanoparticles (AMCSNP's) as shown by a nucleation assay (**A**) and aggregation assay (**B**). AM= *A. millefolium*, CSNP's= chitosan nanoparticles.

FTIR peaks from 3317.48, 2139.29 and 1638.46 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.*[21].

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[36–38]. Antibacterial screening of *Ocimum basilicum* CSNPs against *E. coli* and *Bacillus vallismortis* 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 phytopathogens of tomato *Xanthomonas* and *Erwinia* strains by Oh *et al.*[29]. Gallic acid-chitosan conjugates have been reported to inhibit the formation of calcium oxalate crystals by Queiroz *et al.*[40]. Antiurolithiatic activity of *Aerva lanata* chitosan nanoparticles at 0.8 µg/ml concentration through nucleation assay have been reported by Chandirika *et al.*[41] and *Tridax procumbens* by Chandirika *et al.*[42]. In our study, AMCSNPs showed excellent antibacterial activity against both *B. subtilis* and *P. aeruginosa*, and significant antiurolithiatic activity at aggregation stage of urolithiasis.

Data availability

Underlying data

Figshare: Chitosan nanoparticles of *Achillea millefolium* L., https://doi.org/10.6084/m9.figshare.12936293.v3[43].

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 antiurolithiatic activity AMCSNPs

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

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Dipjyoti Chakraborty
Department of Bioscience and Biotechnology, Banasthali Vidyapith, Banasthali, Rajasthan, India

The authors have used crude extracts of *Achillea millefolium.* The possible constituents of the inflorescence used especially with emphasis on secondary metabolites should be discussed with previous references.

Considering seasonal/low availability of flowers, how do the authors justify using inflorescence? Why not any other plant part?

Justify extraction in water at 90°C, are there any previous reports? What was the duration of extraction?

Why were only *Bacillus subtilis*, and *Pseudomonas aeruginosa* used for the study?

The UV spectrum shows absorbance beyond 1 in the Y-axis (Figure-1). It is generally not recommended to have Abs above 1 as it gives erroneous results and the sample is suitably diluted. The authors may consider.

Since the spectrum shows very low or no absorbance other than the uv region, authors may provide another graph - maybe in the inset showing the spectrum only in the UV region for better signature spectrum.

Figure 2 should also be looked into on similar lines.

The authors have used the "well-diffusion method" for antibacterial screening and thus have not been able to calculate MIC. What was the concentration of extract used for the Zone inhibition study and whether a range of concentration was tried?

Discussion section needs a few lines on the possible constituents of *A. millefolium* extract and its implication on the formation of the nanoparticles and or the bioactivity.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Stress Biology, Secondary Metabolite Biotechnology, Proteomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 31 May 2021

Suresh Kumar

I am thankful to the reviewer for critical analysis of work. The point wise response is as follows:

1. Two reasons for use of Inflorescence, firstly its diuretic uses confirmed by the traditional healers of the collection site. Secondly, crude extract of inflorescence has shown maximum antiurolithiatic activity which was determined in my previous study.

2. The temperature of extraction was used as per literature which gives best result at – 10 to 20 °C than B.P. of solvent used). Duration of extraction was 48 hrs.

3. Both bacteria are reported as causal agent for several human diseases/ infections (urinary tract infections, soft tissue infections, bacteremia, gastrointestinal infections, respiratory system infections) and also it cover the two main categories of gram negative (*P. aeruginosa*) and positive bacteria (*B. subtilis*).

4. The sharp peak at particular wavelength indicates the formation of nanoparticles; further dilution will not cover the entire spectrum and absorption pattern.

5. The antibacterial activity of nanoparticles was determined prepared by 1 mg/ml extract and compared with the same crude extract to determine the enhancement in the activity.

6. GC-MS of crude extract of inflorescence of *A. millefolium* reveals the presence of different bioactive compounds including Lupeol, Stigmasterol, alpha.-amyrin, and gamma.-sitosterol.

Competing Interests: No competing interests were disclosed.

Reviewer Report 12 May 2021

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Alok Arun
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The authors assessed the chitosan nanoparticles isolated from a herbal aromatic plant *Achillea millefolium* (here after referred as AMCSNPs) using ionic gelation method after applying various concentrations of plant extract. Further, the authors measured the antiurolithatic and antibacterial activity using two bacterial species and consequently propose AMCSNPs to be an alternative strategy for targeted delivery.

The research seems to be original and will definitely contribute to broadening the fundamental knowledge about AMCSNPs. However, the authors have potential to make this paper stronger by including few minor details/corrections:

1. Why was only inflorescence used for extract isolation? Providing an explanation can highlight the significant of tissue selection for chitosan nanoparticles isolation.

2. Besides herbarium, did others use any other method to ascertain the identity of plant species used in the study?

3. Authors mention the use of Hess *et al.*, for performing the aggregation assay. There is a brief mention of minor modifications in the protocol. Can they highlight the specific modifications that was used in the protocol?

4. The TEM images are not very clear. Can the authors provide the magnification used for these images?

5. The sentence in discussion, "A shift of FTIR peaks from 3317.48, 2139.29 and 1638.46 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."* is not clear. Can authors rephrase the sentence and provide clarity?

6. The paper will greatly benefit from an image of the plant and the tissue that was used in the study.

7. The study design may have included one more tissue (for example leaf, stem etc) from the plant for comparative purposes.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant Biotechnology, Genomics, Molecular Genetics, Bioinformatics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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