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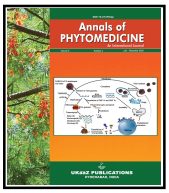

Original article: Open access

Synthesis and characterization of chitosan nanoparticles loaded with 6-gingerol isolated from *Zingiber officinale* Rosc.

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Abstract

6-gingerol is known to possess various pharmacological activities such as anticancer, obesity, anti-inflammatory, antioxidant, antibacterial, antiangiogenic as well as gastroprotective activities. Dried rhizomes (1000 gm) of *Zingiber officinale* Rosc. was subjected to soxhlet extraction to obtain 200 gm of thick paste. It is mixed with silica gel to make fine powder of 230 gm. This fine powder is subjected to column chromatography. All fractions of n-hexane and ethyl acetate in the ratio of 6:4 and 5:5 showed the same Rf value (0.93) on thin layer chromatography (TLC), so they are mixed and evaporated to collect 6-gingerol. High performance liquid chromatography (HPLC) of 6-gingerol showed a retention time of 9.339 min. Structure of 6-gingerol ($C_{17}H_{26}O_4$) was elucidated by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and mass spectroscopy (MS). Plane chitosan nanoparticles (PCNPs) and 6-gingerol loaded chitosan nanoparticles (6-GCNPs) were prepared by using ion gelation method. Synthesized nanoparticles were characterized by fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), particle size analyzer, polydispersity index (PI), and zeta-potential (ZP). FTIR analysis showed the presence of various functional groups. The results of SEM and ZP analysis showed that 6-GCNPs have nanoparticle size (196 nm) and stability (–14.1). Our study confirms the easy method for the preparation of 6-gingerol loaded chitosan nanoparticles and put forth the way to evaluate their biological activities.

1. Introduction

Nanotechnology is becoming boon to mankind because of its wide application in different fields since few decades. Feynman (1960) coated nanotechnology as “There’s Plenty of Room at the Bottom”. Researchers all over the world explored and achieved many revolutionary successes in this field, and were able to manufacture nanoparticles of various sizes and shapes. In brief, nanoparticle (NPs) is a candidate having dimensions approximately 100 nm (Laurent *et al*., 2008). Contingent upon the general shape, these materials can be 0D, 1D, 2D or 3D (Tiwari *et al*., 2012). The significance of these materials acknowledged when researchers found that size can impact the physiochemical properties of a substance, for example, the optical properties. Silver, gold, platinum and palladium NPs of size 20 nm have colors like black, wine red, yellowish gray and dark black, respectively. It is an important to note that color and properties of NPs changes with their size and shape. This property makes them to be utilized in bioimaging techniques (Dreaden *et al.,* 2012). Shin *et al.* (2016) reported that NPs are not simple molecules but they are composed of 3 different layers, *viz*., a central portion of NPs called as core, surface layer (modified by addition of different molecules) and a shell layer. Because of all these outstanding properties, they are widely employed in various fields, *viz*., drug delivery (Imad Uddin *et al.,* 2019), chemical and biological sensing (Barrak *et al*., 2016), gas sensing (Rawal and Kaur, 2013; Mansha *et al.,* 2016; Ullah *et al.,* 2017), CO_2 capturing (Ramacharyulu *et al.,* 2015; Ganesh *et al.,* 2017) and as diagnostic tool in many diseases (Shaalan *et al*., 2016).

Major limitation in terms of drug treatment of many life threatening diseases is their serious side effects (Ferlay *et al*., 2013). Patients usually prefer to the alternative safe methods of treatment. Noteworthy, plants are immeasurable source of medicine and used by mankind since ancient times. Recognizable portion of clinically used drugs are of plant origin (Sekiwa *et al*., 2000). *Z. officinale* belongs to family Zingiberaceae, commonly called as adrak in hindi language, not only used as spice but also used as a medicine in different parts of the world (Gaikwad *et al*., 2017). Main constituents are 6-gingerol, 8-gingerol and 10-gingerol, 6-shogaols, and paradols. These one or more active constituents are involved in various biological activities like antidiabetic, anticancer, anti-inflammatory, and antimicrobial (Mao *et al*., 2019). Sunghun *et al*. (2015) used HPLC method for content analysis of 6-gingerol, an active constituent of *Z. officinale* rhizome. Kumara *et al*. (2017) reported the anticancer potential of 6-gingerol. As a boon to mankind, nanotechnology is used to improve the site specific drug delivery and pharmacokinetic profile of many commonly used drugs. Thus, current study is designed to isolate 6-gingerol from ginger rhizome extract and to synthesize 6-gingerol loaded chitosan nanoparticles.

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2. Materials and Methods

2.1 Chemicals and reagents

Chitosan was purchased from Vihan Life Sciences, Ahmednagar, India. Sodium tripolyphosphate (STTP), sodium hydroxide, ethanol, glacial acetic acid, n-hexane, ethyl acetate and all other chemicals are of laboratory grade and purchased from SD Fine Chemicals Limited, Mumbai, India. Silica gel (70-230 mesh size) was purchased from Merck Specialities, Pvt. Ltd., Hyderabad, India. The double distilled grade water was used in the experiment.

2.2 Extraction and isolation of 6-gingerol

Fresh ginger rhizome (3 kg) was purchased from a local market, washed thoroughly to remove the dust particles, and then it is shade dried for 1 week at a temperature of 37°C. This is authenticated at Botanical Survey of India, Hyderabad. Herbarium with a reference number PRIP/PCOG/19-20/001 is stored in the Department of Pharmacognosy, Pulla Reddy Institute of Pharmacy, Hyderabad. Dried ginger rhizome (≈1000gm) was pulverized into small pieces and divided into suitable portions. Each portion was subjected to soxhlet extraction for 48 h by using a sufficient amount of ethanol as a solvent at a temperature of 45-50°C. Obtained extract fractions were mixed and after passing through filter paper reduced to a thick paste by keeping in a hot air oven at a temperature of 45°C. The percentage yield was calculated by using the following formula:

% Yield= $(W1/W2)100$. Whereas, W1 is the weight of ginger thick paste obtained after drying in a hot air oven and W2 is the weight of dried pulverized ginger. 200 gm of ginger thick paste was triturated with 25-30 gm of silica gel to make a fine powder of 230 gm. A column of dimensions 99.5 cm length X 3.4 cm diameter was used and the bottom of the column was packed with a cotton plug to avoid leakage of silica gel. A slurry of silica gel (70-230 mesh size) made with a sufficient amount of n-hexane was added to the column. By keeping an adequate amount of cotton plug on the top ginger powder (230 gm) was added. Sufficient space was left on top to load the required amount of eluent. Care was taken to avoid drying out of the column throughout the purification process.

After 24 h, the column was defatted by using 500 ml of pure n-hexane. Later on, 500 ml of three different ratios (9:1, 8:2, and 7:3) of n-hexane and ethyl acetate are prepared. Each of these ratios is divided in to two fractions of 250 ml each and separately passed through the column. As no spot was obtained with each fraction, purification was forwarded to the next solvent ratio, *i.e.*, 6:4. 2000 ml (divided into 8 fractions of 250 ml each) of 6:4 ratio of n-hexane and ethyl acetate was passed through the column. Each aliquot collected is subjected to TLC by using merck TLC plates (2 cm X 5 cm) coated with silica gel 60 G F254 (stationary phase). Solvent ratio used to collect aliquot is also used as mobile phase. Iodine chamber is used for visualisation of spots. All these fractions were mixed as they showed the same Rf value on TLC. Next 3000 ml (divided into 12 fractions of 250 ml each) of 5:5 ratio of n-hexane and ethyl acetate was passed through the column. All these 12 fractions were mixed as they showed the same Rf value on TLC. Out of these 20 fractions, 8 of 6:4 ratio and 12 of 5:5 ratios were mixed as they have the same Rf value of 0.93 and were evaporated to collect 6-gingerol.

2.3 HPLC, ^{1}H NMR and mass spectroscopic analysis of 6-gingerol

HPLC analysis was performed with empower software version-2, runtime of 20 min by using 0.01M KH acetonitrile as mobile phase A at a flow rate of 1 ml/min, and injection volume of 10.00 µl. Acetonitrile is used to prepare 5 mg/ml of 6-gingerol. Column used in HPLC (Waters – separation module 2695) is made up of intersil ODS-3V with dimensions of 5 mm X 250 mm X 4.6 mm. To evaluate the number and nature of protons present in 6-gingerol (dissolved in DMSO), ^{1}H NMR (300 MHz) is performed by using bruker avance 300, rheinstetten, Germany. Later on to evaluate the fragmentation pattern and molecular weight of 6-gingerol mass spectroscopy is performed by using synapt mass spectrometer from waters corporation, United Kingdom.

2.4 Preparation and characterization of PCNPs and 6-GCNPs

25 ml of low molecular weight chitosan solution (0.5 mg/ml) was prepared by dissolving 12.5 mg of chitosan in 25 ml of 1% acetic acid and pH was adjusted to 4 by adding 1M NaOH solution. Similarly, 25 ml of STPP solution (0.7 mg/ml) was prepared by dissolving 17.5 mg of sodium tripolyphosphate (STTP) in 25 ml of distilled water and pH was adjusted to 2 using 1M HCl. Four different formulations; A, B, C, and D of PCNPs were prepared by adding 600 µl of chitosan solution to 150,300,450 and 600 µl of STPP solution, respectively. 6-GCNPs were prepared in two steps: In the first step, range of gingerol-STTP solutions were prepared by adding 225 µl (formulation E), 450 µl (formulation F), and 900 µl (formulation G) of gingerol to 600 µl of above prepared STTP solution and then in second step, 600 µl of chitosan was added to above prepared gingerol-STTP solutions to make gingerol loaded CNPs. Both of these nanoparticles are prepared by slight modification of iconic gelation method reported by Othman *et al.* (2018). Flow chart explaining extraction and isolation 6-gingerol is described in Figure 1.

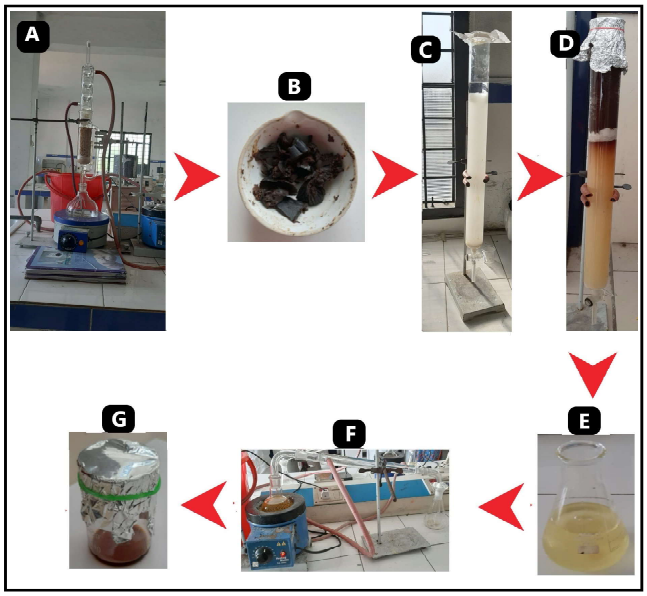

Figure 1: Extraction and isolation of 6-gingerol from ginger rhizome. A: Soxhlet extraction of ginger by using ethanol; B: Dried ethanolic extract; C: Preparation of column; D: Column loaded with ginger extract powder; E: Isolated fraction containing 6-gingerol; F: Evaporation of solvent to collect 6-gingerol; G: 6-gingerol collected after evaporation of solvent.

Functional groups present in these nanoparticles were determined by FTIR analysis (Shimadzu-8400S). FTIR spectrometer was operated with working range of 4000 to 500 cm, no. of scans were 10, resolution was 4 cm^{-1} and apodization was done by square triangle method. Polydispersity index, particle size and stability of solution (zeta potential) were determined by using Horiba – SZ-100 Zeta Sizer. For measurement of particle size and PI instrument was operated with scattering angle of 90°, temp of holder was 25°C, viscosity of dispersion medium was 2.035 mPa.s, representation of result by scattering light intensity method, and count rate was 342 kCPS. Whereas, for measurement of zeta potential and electrophoretic mobility mean (EMM), zeta sizer was operated with temp of holder at 25°C, viscosity of dispersion medium was 0.893 mPa.s, conductivity was 0.221 mS/cm, and electrode voltage was 3.3 V. Morphological features of PCNPs and 6-GCNPs were determined by using scanning electron microscope (SEM) (Hitachi-S3700N) operated at magnification of 2K to 8K, Acceleration voltage of 30K V, emission current of ≈ 100000 nA, and working distance of 9.1 to 9.2 mm.

2.5 Statistical analysis

All the data of seven different formulations of particle size, and polydispersity index are analyzed by pareto analysis in microsoft excel -2010.

3. Results

3.1 HPLC, ^{1}H NMR and mass spectroscopic analysis of 6-gingerol

The percentage yield of ethanolic ginger extract was found to be 20 percent. All 20 fractions of 6:4 and 5:5 ratios of n-hexane and ethyl acetate showed a single visible spot on TLC plates with the same Rf value of 0.93 representing the presence of 6-gingerol. In HPLC analysis, major peak with a retention time of 9.339 min corresponding to % area of the peak about 18.114 percent indicates 6-gingerol (Figure 2). Further structure of 6-gingerol ($C_{17}H_{26}O_4$) was elucidated by ^{1}H NMR and MS. ^{1}H NMR (300MHZ, DMSO) chromatogram of 6-gingerol showed following peaks: δ: 0.83-*0.860 (3H, t,* j=6.6Hz, H-9), 1.00-1.45 (8H, m, H-5, H-6, H-7, H-8), 1.91-1.99 (1H, d, j=24Hz, H-4), 2.45-2.67 (2H, s, H-2, H-3), 3.28-3.45 (2H, m, J=51Hz, H-1), 3.737 (3H, s, -OCH3), 3.995-4.066 (1H, m, H-4), 6.641 (1H, dd, J=6Hz, 2Hz, H-6'), 6.738 (1H,s, H-2'), 6.782 (1H, d, J=8Hz, H-5') (Figure 3). Further molecular weight 6-gingerol was found to be ≈ 293.01 by using mass spectroscopy. Peak with m/z value of 293.1 represents 6-gingerol. An abundant product ion of m/z value of 137.1 represents breakage between C1 and C2 and the remaining part of the molecule showed a peak with m/z value of 151.1. Another abundant product ion of m/z value of 223.1 represents breakage between C4 and C5. In addition to these there are 3 less abundant ion peaks of m/z value of 239.1, 209.2, and 127 (Figure 4).

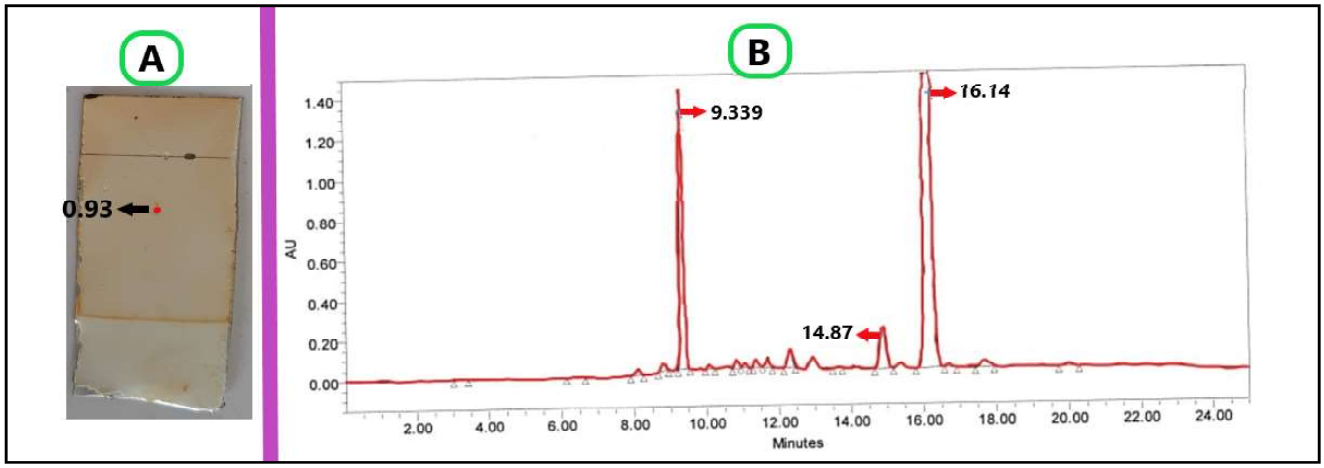

Figure 2: Thin layer chromatography showing Rf value of 0.93, corresponds to 6-gingerol. High performance liquid chromatogram of 6-gingerol, the retention time of 6-gingerol is found to be 9.339.

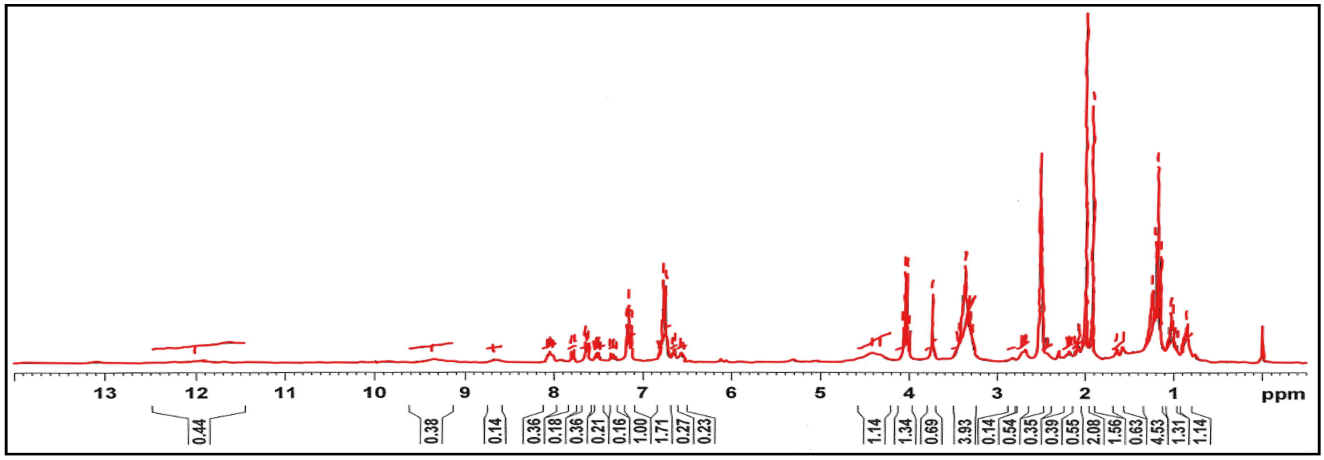

Figure 3: Proton nuclear magnetic resonance spectra of 6-gingerol.

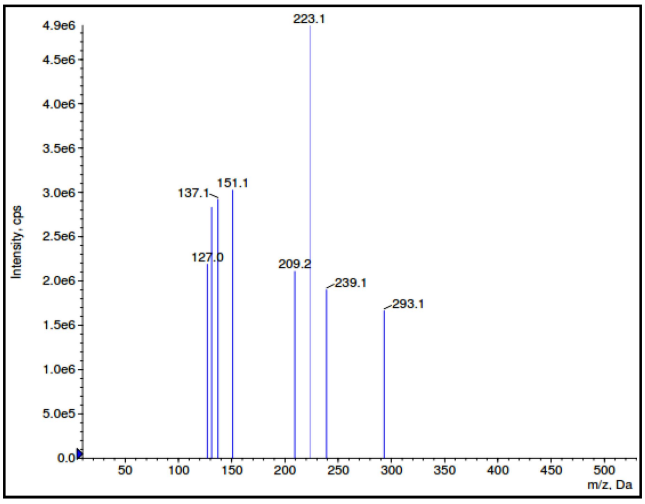

Figure 4: Mass spectra of 6-gingerol, m/z value of 293.1 represents 6-gingerol.

3.2 Particle size distribution, polydispersity index, zeta potential, and electrophoretic mobility mean assessment of PCNPs and 6-GCNPs

The particle size of formulation A was found to be 1867 nm, and it was decreased as the STTP volume was increased. Formulation D with the highest volume of STTP showed the particle size of 861.3 nm. Polydispersity index also called a heterogeneity index decreased as the size of nanoparticles was decreased. The PI of formulation A, B, C, and D were found to be 1.060, 0.780, 0.769, and 0.522, respectively. Whereas ZP was decreased with the increase in the volume of STTP, the lowest ZP of around –16.1 was measured with formulation D. Electrophoretic mobility mean was also decreased with a decrease in the size of nanoparticles. Formulation A with the highest particle size showed EMM of –0.000089 cm^2/Vs, and formulation D with the lowest particle size showed EMM of –0.000124 cm^2/Vs. In 6-GCNPs particle size was increased as the volume of 6-gingerol added was increased. Formulation E with the lowest volume of 6-gingerol showed the

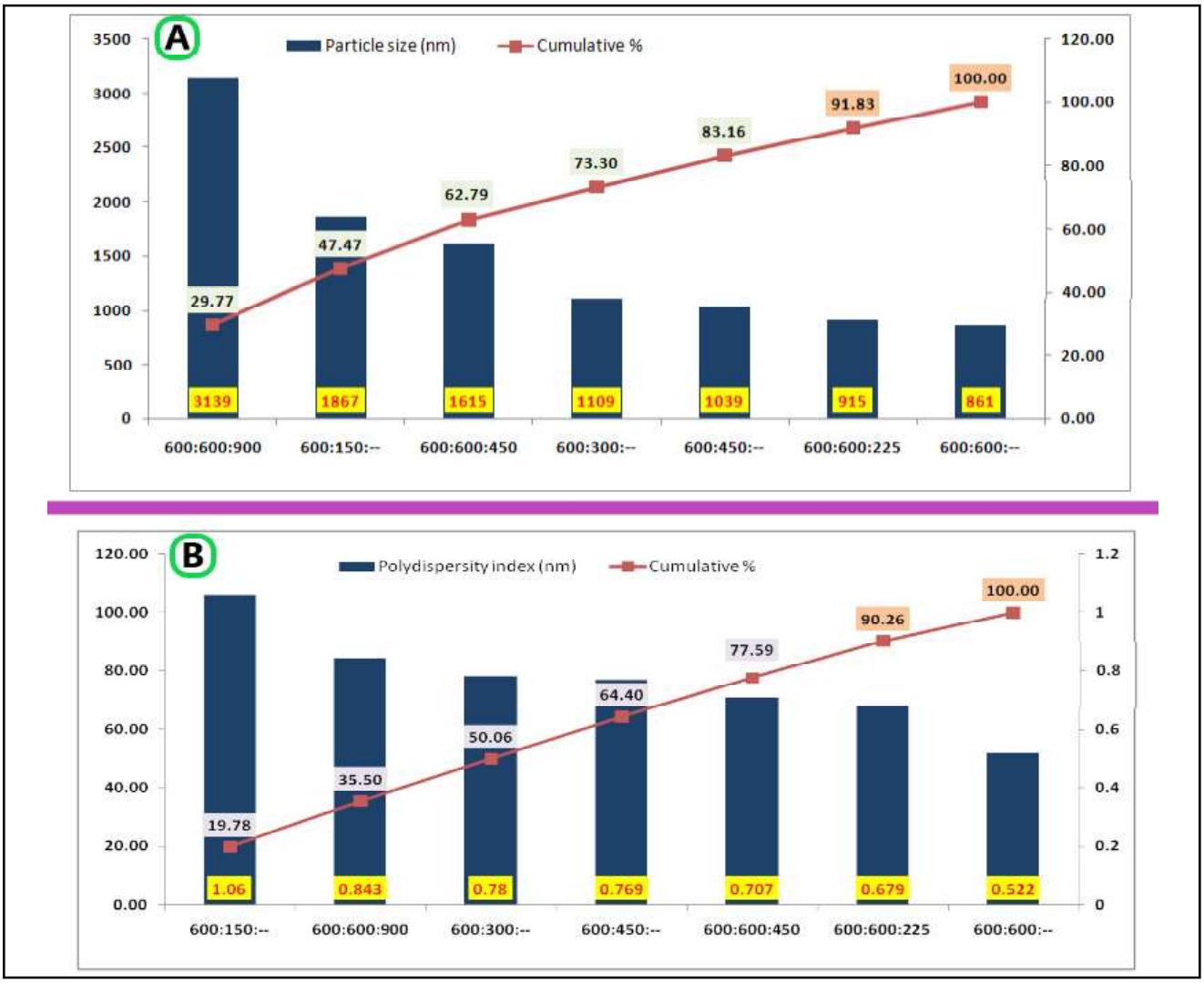

Figure 5: Pareto analysis of particle size (A) and polydispersity index (B) of all seven formulations, formulations D (600:600) and E (600:600:225) are selected as they are above 80 percent.

smallest particle size of 915 nm, whereas formulation G with the highest volume of 6-gingerol showed the largest particle size of 3139 nm. The PI of formulations E, F, and G was found to be 0.679, 0.707 and 0.843, respectively. There was an exception with ZP and EMM. Among the different formulations of 6-GCNPs, formulation E showed highest ZP and EMM values of –14.1 and –0.000109 cm^2/Vs, respectively. Thereafter, as the volume of 6-gingerol increased in formulation F and G their ZP and EMM were also increased (Table 1). Pareto analysis of particle size and polydispersity index showed that formulation D and E are more than 80% (Figure 5). Therefore, among all seven, formulation D and E are selected for further characterization.

Table 1: Particle size; polydispersity index, zeta potential, and electrophoretic mobility mean of different formulations

Formulation	Volume of chitosan: STTP: 6-gingerol (µl)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Electrophoretic mobility mean (cm^2/Vs)
A	600:150:—	1867	1.060	–11.6	–0.000089
B	600:300:—	1109	0.780	–12.9	–0.000100
C	600:450:—	1039	0.769	–12.5	–0.000097
D	600:600:—	861	0.522	–16.1	–0.000124
E	600:600:225	915	0.679	–14.1	–0.000109
F	600:600:450	1615	0.707	–24.6	–0.000191
G	600:600:900	3139	0.843	–24.1	–0.000192

3.3 SEM and FTIR analysis of PCNPs and 6-GCNPs

Two best-selected formulations, *i.e.*, D and E are subjected to FTIR analysis and SEM. The average particle sizes were found to be 201.75 nm and 196 nm with PCNPs and 6-GCNPs, respectively (Figure 6). In FTIR analysis PCNPs showed a single broad peak at 3441.12, and 3419.90 cm^{-1} correspondings to STTP cross-linked chitosan nanoparticles, this broad peak is indicating stretching vibrations of the O-H group, an important functional group of chitosan.

Amide-II stretching of the carbonyl group and bending vibration of the NH2 group were indicated by another sharp and intense peak at 1641.48 and 1631.83 cm^{-1}, respectively, the -OH bending, C-N stretching and S=O stretching vibrations are indicated by 1383.01, 1377.22, and 1151.54 cm^{-1}, respectively. Whereas, in 6-GCNPs, a single broad intense peak at 3441.12 and 3417.98 cm^{-1} indicates –OH bond stretching of 6-gingerol, stretching vibrations of aromatic ring was indicated by another sharp peak at 1631.83 cm^{-1}, -OH bending vibrations are indicated by a small and low intense peak at 1383.01 cm^{-1}, $-OCH_3$ stretching vibrations is indicated by 1236.41 cm^{-1} and -CHOH stretching vibrations is indicated by another small peak at 1039.67 cm^{-1} (Figure 7).

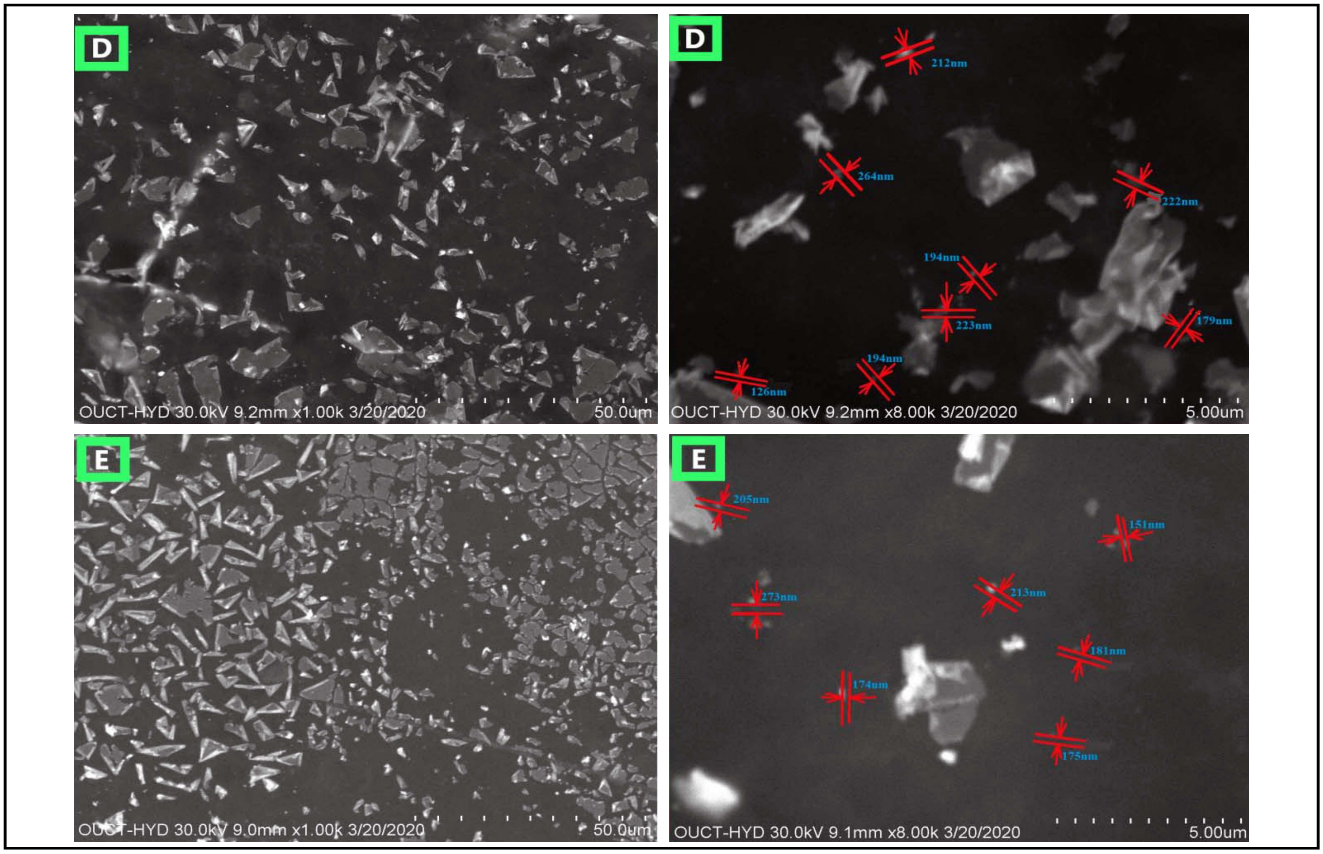

Figure 6: D: SEM images of PCNPs of formulation D at 50 and 5 µm, E: SEM images of 6-GCNPs of formulation E at 50 and 5 µm.

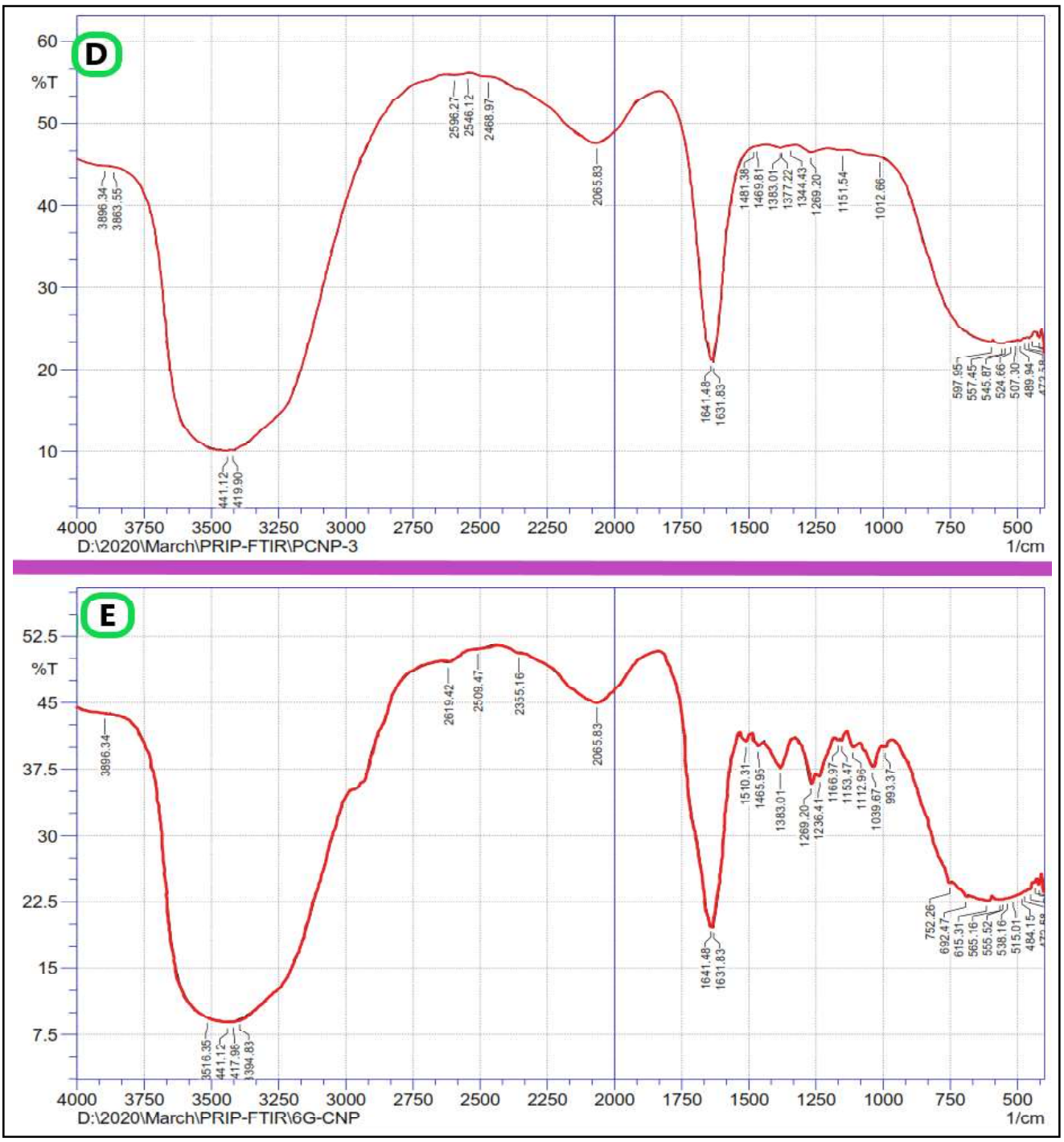

Figure 7: Fourier transform infrared spectroscopy of plain chitosan nanoparticles (D) and 6-gingerol loaded chitosan nanoparticles (E).

4. Discussion

6-gingerol, an active constituent of ginger was isolated by using column chromatography on silica gel of mesh size 70-230. TLC was done for 6-gingerol and the retention factor obtained was 0.93. Our results are very similar to Gaikwad *et al.*(2017) who reported a retention factor of 0.97 for gingerol. This method can serve as a screening step followed by HPLC confirmation. HPLC was performed to identify 6-gingerol based on retention time which was found to be 9.339 min. These results are in accordance with Jazokaite *et al.* (2019) who reported the retention time of 6-gingerol at 10.866 min. By using this method, we can identify, quantify and purify the individual component from the mixture. Later on MS was performed, and the molecular weight of 6-gingerol was found to be 293.1. ^{1}H NMR chromatogram of 6-gingerol showed different peaks and these results are very similar to the values reported by Wu, (2007).

Synthesis of 6-GCNPs was successfully carried out by mixing positively charged chitosan and negatively charged STPP. The formation of nanoparticles due to interaction between chitosan and STPP was also reported by Khan *et al*. (2017). SEM is used to reveal topography and surface morphology of synthesized NPs. Size of 6-GCNPs was found to be 196 nm which falls within the nano range. Similar results were also reported by Bhalekar *et al.,* (2019). Particle size distribution of optimal formulation E was found to be 915 nm. Increase in the size of nanoparticles may be due to encapsulation of 6-gingerol. Thus we can notify as concentration of 6-gingerol increased from 225 µl to 900 µl, particle size increased form 915 nm to 3139 nm. The PI of formulations E, F, and G were found to be 0.679, 0.707 and 0.843, respectively. The PI values are used to describe the distribution of molecular weight. So, as the concentration of 6-gingerol increased from formulation E to F there is increase in the values of PI indicating broader molecular weight of formulation G. However, increase in particle size and high PI are some of the disadvantages of ionic gelation method (Das *et al*., 2019). The ZP, a measure of NPs stability for formulation F was found to be –24.6 mV. This result was in accordance with the findings of Bhalekar *et al.,* (2019) for 6-gingerol solid lipid nanoparticle where ZP was found to be –25.3 mV. This value indicates good stability of NPs. As it is reported that ZP values greater than +25 mV or less than –25 mV have high degrees of stability (Mahobia *et al*., 2016). Electrophoretic mobility is measured by dividing electrophoretic velocity by electric field strength. This may be negative or positive. Negative sign indicates moment of particles against to electrophoretic field. These values may decrease with an increase in the size of particles (Catherine, 2014). Our results are in accordance with this, as EMM decreased with increase in size of NPs in formulations E, F and G. Their negative sign indicates moment against electrophoretic field. Later on among four formulations of PCNPs, formulation D, and among three formulations of 6-GCNPs, formulation E, were selected for further characterization by SEM and FTIR. SEM images showed that NPs are of various shapes and have a PS of 201.75 nm and 196 nm for PCNPs and 6-GCNPs, respectively. Furlani *et al.* (2017) also got the chitosan NPs of size 200 nm. Moreover, in formulation E after adding 6-gingerol, there is no significant increase in size of NPs and both PCNPs and 6-GCNPs got the same particle size.

5. Conclusion

This study presents an easy method to isolate 6-gingerol from ginger rhizome extract by using column chromatography. Different characterization techniques like thin layer chromatography, HPLC, ^{1}H NMR and mass spectroscopy is used to identify isolated compound as 6-gingerol. Moreover, this study also presents an idea to use optimum concentrations of STTP and 6-gingerol for the synthesis of 6-gingerol loaded chitosan nanoparticles. These nanoparticles are characterized by particle size and zeta potential estimations, FTIR and SEM analysis. Researchers through out the world are focusing to use nanotechnology for safe and targeted delivery of different drugs of both synthetic and plant origin. Thus our results supports an idea of using nanotechnology for targeted delivery of 6-gingerol a plant based bioactive molecule. These 6-gingerol loaded chitosan nanoparticles can be further screened for *in vitro* and *in vivo* biological activities.

Conflict of interest

The authors declare that there are no conflicts of interest in the course of conducting the research. All the authors had final decision regarding the manuscript and decision to submit the findings for publication.

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