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Enhancing the Antibacterial and Anticancer Activity of Ciprofloxacin by Encapsulating into Shellac-Chitosan Nano Particles (CNPs)

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ABSTRACT

This study demonstrates a preparation of a nanocarrier for drug delivery system which based on combining two natural materials shellac (SH) and chitosan (CH). The chitosan shellac NPs (SH-CH NPs) was loaded with ciprofloxacin drug to enhance its anticancer and antibacterial activity. The shellac – chitosan NPs (SH-CH NPs) size was measured using DLS and its size was58.8nm ± 3.4, while the morphology of the nano compound was identified using FESEM, TEM techniques. We diagnosed SH-CH NP synthesis by FTIR, HNMR and DSC techniques. The results of MTT test showed a high inhibition rate of 55.45% for A375 melanoma cells, and a low inhibition rate for normal cells (HFF) range (8.51%-32.93%) when incubated with Cipro-SH-CH NPs. Also, the results reinforced the possibility of using ciprofloxacin loaded on chitosan – shellac NPs as a promising nanocarrier with low cytotoxicity, and high activity as an antibacterial with a diameter of 40 mm with both type of positively and negatively charged bacteria.

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INTRODUCTION

As a result of their availability and degradability, enhanced encapsulation, release control, and therapeutic efficiency of nano-encapsulated medicines, as well as decreased toxicity, natural sources of nanoparticles have been utilized as a drug delivery medium. Kraisit. P*et al*. [1] Used ionic cross-linking technique to link chitosan with shellac to encapsulate vaccine serum albumin, a type of a protein. The particle size ranged from 100 to 300 nm. The chemical structure and surface area of nanostructures, as well as their size on the nanoscale (1-100 Nm), give them distinct characteristics properties. Chitosan is a natural biopolymer with cationic properties derived from the deacetylation of chitin [poly— (1-4)-N-acetyl-dglucosamine], a crustacean extract.[2][3] The benefits of employing chitosan as nanocarrier include biocompatibility, biodegradability, low toxicity, excellent mucoadhesion, and membrane permeability enhancement, by allowing previously closed epithelial cell connections to open. It dissolves in an acidic solution with a pH of less than 6.4–6.5.[4][5]Furthermore, the protonation of fundamental a lower pH, amino groups leads

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for crosslinking with positive charged glutamic acid. Shellac (SH) is a natural biopolymer with an anion charge which exist in China, Thailand and India, a result of the lac bug, Laccifer lacca, produces a lot of SH. Resinous secretion can occur processed to produce shellac, which is utilized in paints, food, and pharmaceutical industries to a lesser extent. Above pH 7.0, SH begins to dissolve (pKa is 6.9–7.5), which may be advantageous when utilized in bodily fluid pH (pH 7.4).[6] Fig. 1 shows the primary shellac structure, which is made up of single esters and poly esters with hydroxyl and carboxyl groups. Shellac in alkaline liquids has the capacity to deprotonate[7].This causes numerous electrolyte complexes to form with the glutamic chitosan salt of GCH. Both chitosan and glutamic acid were used as salts in this study. Because of its great stability and solubility in water, Shellac has been used in the form of ammonium salt, while chitosan glutamate (GCH) is soluble in the digestive system across a wide pH range. The reflux condenser process produced GCH from the chitosan base[8].The use of nanoscale particles such as nano shellac particles or nano chitosan nanoparticles has helped to develop and improve the efficiency of many treatments by controlling the period and rate of drug delivery and reducing repeated doses and side effects. Shellac was used few times as nanocarrier for delivering berberine, vancomycin, and chlorhexidine. [9][10][11][12] . Nano chitosan production and amphotericin loading were accomplished using the phase separation technique. A new solvent was also developed, and the effectiveness of nanodrugs was tested in vitro and in vivo (pathology).[13] in this research we combined between two natural materials (shellac and chitosan) to manufacture a natural nanocarrier to be loaded with ciprofloxacin drug.(Fig. 2)

Ciprofloxacin is a quinolin-4 (1H)-one having carboxylic acid, cyclopropyl, fluoro, and piperazin-1-yl substituents at positions 1, 3, 6, and 7in its structure, respectively.[14][15] It's an anti-infective, a topoisomerase IV inhibitor, antibacterial medicine, an inhibitor of DNA synthesis, an EC 5.99.1.3 [DNA topoisomerase (ATP-hydrolyzing)] inhibitor, an antibiotic agent, and an environmental toxin. It's a fluoroquinolone antibiotic, a quinolone antibiotic ,a quinolone, N-aryl piperazine, a quinolone monocarboxylic acid, and an amino quinoline, amongst others. [10][16] Ciprofloxacin is a second-generation fluoroquinolone antibiotic that is much utilized to treat mild to severe infections of the urinary and pulmonary systems caused by organisms that are sensitive. A limited number of instances of lupus have been linked to ciprofloxacin. The goal of this research is to improve the release efficiency and

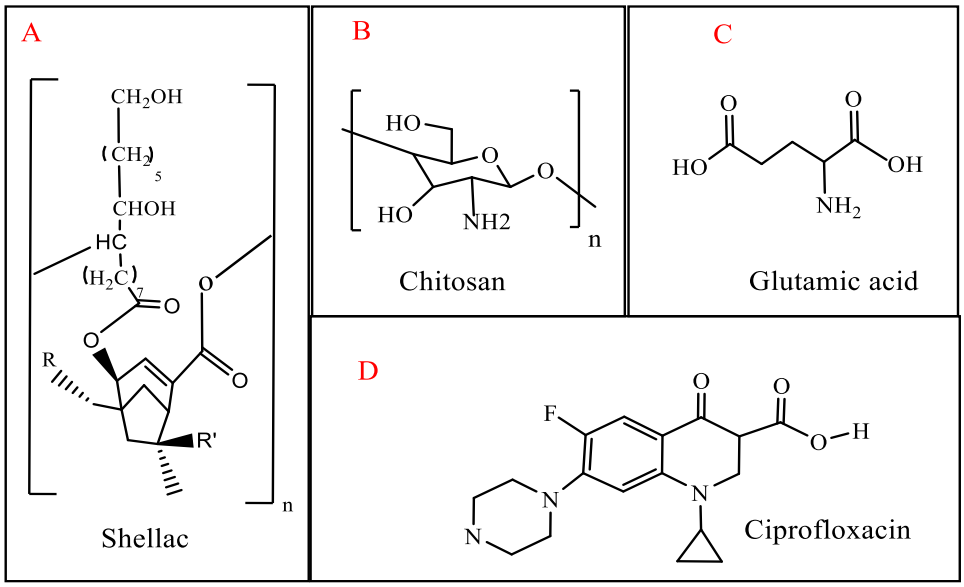

Fig. 1. The chemical structure of; A: Shellac, B: chitosan, C: Glutamic acid, and D: Ciprofloxacin drug

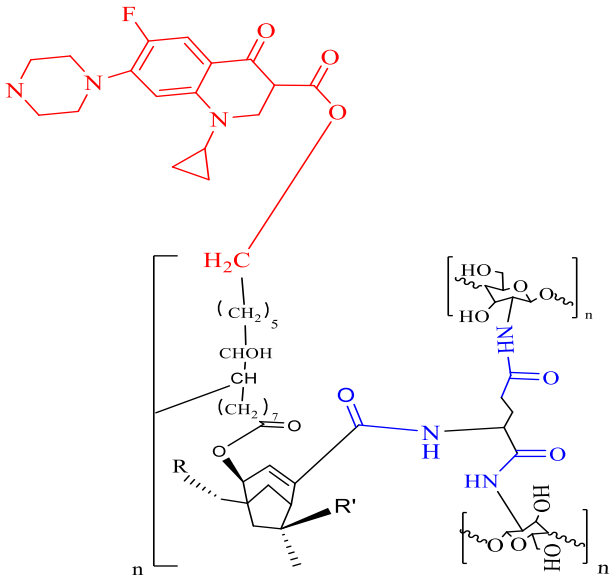

Fig. 2. A schematic structure of ciprofloxacin loaded Shellac- Chitosan NPs

biological activity of antibacterial and anticancer ciprofloxacin drug [17].

MATERIALS AND METHODS

Materials

The alkaline solution of shellac ammonium was obtained kindly from prof. Vasselin Paunov who in turn received it also as a gift from (Stroever Schellack Bremen, Germany). At pH > 7, shellac was used as an ammonium salt in a soluble form (25 wt.%). Sigma-Aldrich was the company which provided ciprofloxacin drug (100%). Nanochitosan Shaanxi Sang herb Bio-Teching (95.5%). Glutamic acid was obtained from Fluka (Switzerland), while DMSO solvent was purchased from Gainlan Chemical Company, UK. The cell lines for MTT testing were obtained from Pastor Institute in Tehran, Iran. While the bacteria were grown and tested in biology department, College of Science, University of Babylon. Deionized water was used in all experiments.

Preparation of the Ciprofloxacin loaded Shellac-Chitosan (Cipro- SH-CHNPs)

The preparation method followed in this research was depenent on the method that used by Kraisit. P*et al*[1] with some modifications. First step, glutamic chitosan linkage was prepared as followed, (0.5 gm) of glutamic acid was dissolved in 0.81 ml of 0.01 M diluted hydrochloric acid, then (0.25 gm dissolved in 2.0 mL DMSO) of nanochitosan was added. Themixture was placed in a round flask (100 ml) and refluxed for about an hour at a temperature of 60-65°C.

Secondly, shellac – glutamic chitosan prepared by mixing (0.25gm) glutamic chitosan which dissolved in 2.0 ml DMSO with (0.25gm) of ammonium shellac. The mixture was placed in a round flask of (100ml) for refluxing about 1 hour at 60-65°C. A precipitate (SH-CH) was then filtered and washed with absolute ethanol. The last step which is loading the drug ciprofloxacin (Cipro), (0.25gm) of ciprofloxacin was placed in a round flask of (100ml) with an excess of thionel chloride $SOCl_2$ and stirred. An equivalent amount of SH-CH (0.25gm) was added. That mixture refluxed for about an hour at 60-65°C. Cipro-SH-CH precipitate is then filtered and washed with absolute ethanol (Fig. 3).

Physicochemical characterization of Cipro-Loaded Shellac-Chitosan NPs

FTIR and 1HNMR Studies

(FTIR) Fourier Transform Infrared analysis was carried out by means of KBr technique using Shimadzu 8400 spectrophotometer (400-4000 cm-1), (Japan). Likewise, nuclear magnetic resonance (1HNMR) analysis was carried out by dissolving the loaded drug in DMSO solvent using Varian Inova 500 MHz, (USA) spectrometer instrument. These

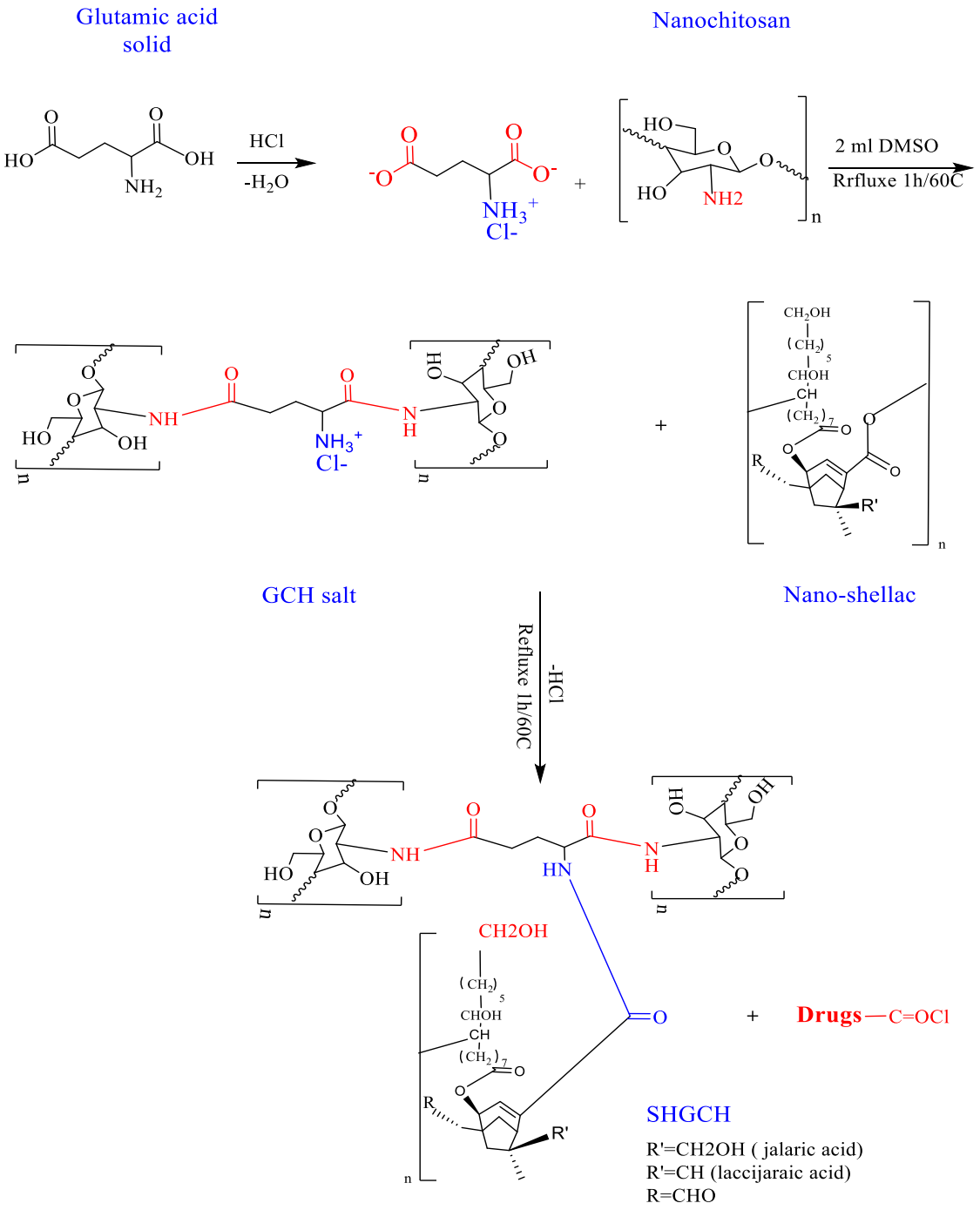

Fig. 3. A Schematic represents the preparation steps of Cipro loaded SH-CH NPs

analyses were used to describe the formation of Cipro-loaded on shellac- chitosan NPs, and to validate the cross-linking between the anionic molecules of Shellac and the cationic molecules of chitosan[18].

Cipro-SH-CH NPs Size Measurements

The average nanosize of Cipro-SH-CH NPs was characterized in terms of their size and morphological examination using the light scattering technique (DLS), TEM analysis using

Zeiss equipment, Germany, EM10C, 100Kv, as well as Field Emission Scanning Electron Microscope (FESEM –Tescan, Mira3) in their suspension form at pH 4.

Calorimetry Using Differential Scanning Analysis

Thermograms of Cipro-loaded shellac–chitosan NPs were based on a DSC (DSC 7, Perkin-Elmer, USA). 2–4 mg of material was precisely weighed and packed into a solid metal pan. The test was carried out with a nitrogen gas purge between 25 and 300°C at a rate of 10°C/min.[2]

Release Rate Study of Cipro-SH-CH NPs

The releasing drug rate was calculated by taking 0.01gram dissolved in 10.0 ml of 1:1 DMSO:H_2O of the drug loaded on shellac-chitosan NPs and dissolved in two pH medium. An acidic solution (pH 2) and basic solution (pH 7.2). the drug was put in a dialysis bag with a pore diameter size of 2.5 nm and then was submerged in a 50 ml buffer solution (at pH 2&7.2). A magnetic stirrer was used to gently move the bag at 100 rpm. The absorbance of the released drug was measured in acid and basic solution at 37°C at each 2 hours for 12 hours. The amount of the released drug was measured using the equation below[19]:

$$\%\, In\ vitro\ Cipro - SHGCH\ release = \frac{M_{released}}{M_{total}} \times 100$$

where $M_{released}$ represents the amount of Cipro-SH-CH NPs freed from the nanoshellac-chitosan particles at the time t while M_{total} represents the entire amount of Cipro drug loaded on SH-CH NPs.

The Antibacterial Action of Cipro-SH-CH NPs

The antibacterial activity of Cipro-SH-CH NPs was studied on two types of bacteria, *Escherichia coli* and *Staphylococcus Aurrous*. These microorganisms were chosen because of their relevance to [19]technique was used to calculate the inhibitory impact of chemicals produced on these bacteria[20], and it involves the following steps:

1. making of numerous holes in bacteria-planted dishes.
2. (0.1mL) (25mg/1ml) of certain compounds extracted from bacteria-inoculated cultivars.
3. Incubate the dishes for 24 hours at 37C° in an incubator.
4. The inhibition zone was determined.

Anticancer Examination of the Drugs Loaded on the Prepared Cipro-SH-CH NPs

Reagent preparation: MTT is soluble in ethanol (20 mg/ml),water (10 mg/ml), buffer salt solution, and culture medium (5 mg/ml). In the laboratory, dissolve 5 mg/ml of powder in PBS and gently vortex, then filter and store at-20°C for testing.

MTT Method test

Pouring 10,000 cells from various A375andnormal cell line HFF into 96-well plate under various concentrations of Ciprofloxacin loaded on shellac-chitosan NPs and incubate for 24 hours at 37 °C in an incubator with 5% CO_2. In addition to the drug-treated cells, we cultured the cells without the drug and without the cells as negative and positive controls, respectively, in

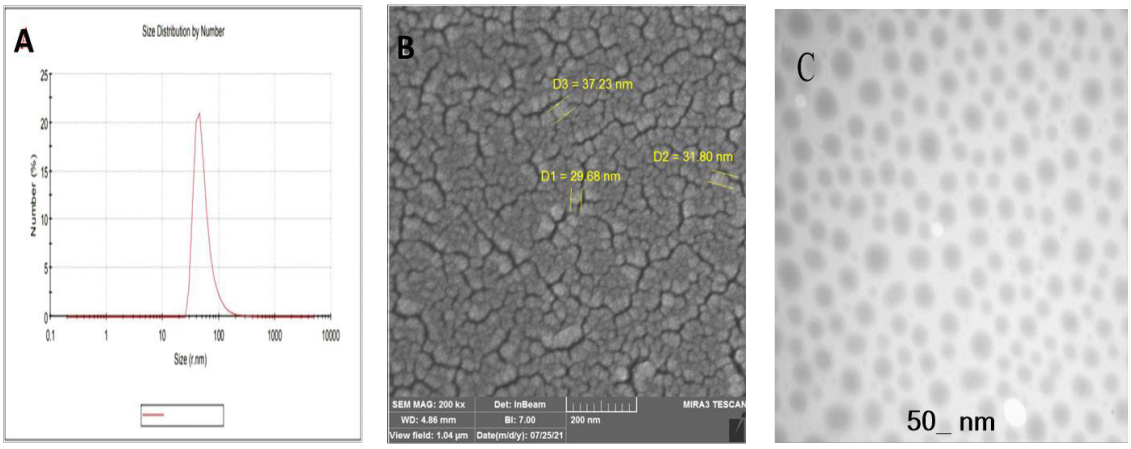

Fig. 4. (A) The average particle size distribution of Cipro loaded shellac-chitosan NPs using DLS instrument(B) The FESEM image of shellac – chitosan nanoparticles prepared by mixing 0.25% of glutamic-chitosan with 0.25% shellac (C) TEM image of Cipro-loaded SH-CH nanoparticles (prepared by mixing SH-CH 0.25 wt.% with Cipro 0.25 wt.%).

addition, cells with free drug. After 24 hours, 10 µl of MTT solution was added to 100 µl of cells culture supernatant in a sampler and gently shake until smooth using a shaker. In an incubator (CO_2 5% and 37 °C), the plates were left for 5 h. Then, after emptying the midst, fill each well with 100µl of DMSO and wait for the formazan crystals to dissolve and create a pink-purple solution. After that, a Dana 3200 microplate reader was used to measure the light absorption of the samples at 570 nm. Prism software version 8.2 was used to calculate the IC_{50} value. [21].

RESULTS AND DISCUSSION

Characterization of Ciprofloxacin Loaded Shellac-Chitosan NPs

The two natural biopolymers (shellac and chitosan) were prepare as nanocarrier for ciprofloxacin drug which they were combined by the ionic crosslinking technique. The particle size of the colloid was 58.8 nm ± 3.4, size range, Fig. 4. TEM was used in order to confirm the morphological characteristic of the Cipro-loaded shellac-chitosan nanoparticles, as shown in Fig. 3. The spherical shape of the nanoparticles was detected. This outcome could be prove that the nanoparticle was formed by using shellac and chitosan for the encapsulation of ciprofloxacin. The FT-IR spectra of SH, Cipro, CG, Cipro-loaded shellac-chitosan NPs and physical mixture are shown in Fig. 5.

Fig. 5 A shows FT-IR spectrum of salt (GC)which illustrate the following bands: NH amide (3736), (3419) NH2 Amine, (3055) = CH Aromatic, (1356) CH Alkane, (1653) C = O Amide, (3265) Phenol OH, (1600) Amine bending NH. As well as, Fig. 5 B demonstrates the spectrum of ^{1}HNMR for salt (GC) using DMSO solvent. It showed a single signal at 3.3ppm for amide NH protons (1H), multiple signal at 2.3ppm for $NH3^+$Gluprotons, and a multiple signal at 2.5ppm for OH protons. For the alcoholicity of chitosan, a single signal at 2.6 ppm belongs to the $HOC=OCH_2$ protons and a single signal at (1.8-1.9) ppm belongs to glutamic $R\text{-}CH_2\text{-}R$ protons. On the other hand, Fig. 5C represents the FT-IR spectrum of the formed polymer SH-CH. It shows the following bands: (3861) O-H alcohol, (3248-3443) N-H amide, (1519) NH Amine, (3061) -CH Aromatic, (2935) CH Alkane, (1649-1660) C = O Amide, (1519) N-H Amide bending, (1068) C-O Alcohol, (3749) O-H free alcohol. While Fig. 5D displays the ^{1}HNMR spectrum of the polymer SH-CH using a DMSO solvent, as it shown that a single signal at 8ppm belonging to the amide NH protons (1H), multiple molarity at 1.9ppm belonging to CH-Alkyl protons and multiple molarity at 2.1-2.3ppm belonging to protons Aryl - CH_3 Shellac has a single signal at 2.5 ppm belonging to the alcoholic OH protons, a single signal at 2.6 ppm, $R\text{-}CH_2\text{-}R$ and a single signal at 3.4 ppm for NH-Aryl chitosan. Fig. 5 E shows the FT-IR spectrum of the Cipro-SH-CH NPs as it can be seen the following bands appeared: (3857) NH Amide, (3136-650) = CH Alkene, (3005-3070) Aromatic CH, (1633) C = O Amide, (1519) CC Aromatic, (340-3377) Amine, NH, (2717-2875-2924). Aromatic CN (1217-1317), Ester C=O (1722), Aromatic -CH, (1421) Halide alkyl CF. while Fig. 5 F displays the ^{1}HNMR spectrum of Cipro-SH-CH nanoparticles using DMSO solvent, as it showed multiple signal at 1.2-1.4 ppm for Alkyl CH protons and multiple signal at 2-2.3 ppm for Glu $C=O\text{-}CH_2$- protons and a single signal at 2 .7 ppm belongs to Cipro-Ar-NH protons, a single signal at 3.5 ppm belongs to alcoholic OH protons, a signal at 3.6-3.8 ppm belongs to $OH\text{-}CH_2$-shellac bound protons, and signal at -4.74.1 ppm belongs to OH-Phenol chitosan protons, and a mono signal at 7.5-8.7 for amide NH. Therefore, these studies proved that the formation of glutamic – chitosan compound, shellac linked with glutamic chitosan NPs, as well as the formation of Cipro-loaded SH-CH nanoparticles.

The DSC analysis was used to validate production of nanoparticles loaded with ciprofloxacin to confirm the stability of ciprofloxacin loaded SH-CH nanoparticles. The diagram depicts a thermal DSC plot of prepared nano-shellac particles loaded with ciprofloxacin. DSC Peak thermal charges for all endothermic materials are indicated (degrees Celsius).The endothermic peaks of all endothermic materials are illustrated in the schematics of DSC by the nanoshellac particles and the Cipro-SH-CH NPs. The absorbent peaks of Cipro-SH-CH loaded on nanoshellac, SH-CH NPs (Onset815.57 °C- End 869.29 °C, Peak=836.23 °C, Peak Height =41.081mW),(Onset 203.48C°–End 240.23°C,Peak=220.06°C,Peak Height =30.0007 mW) were shown in the Fig. 6.

The In-Vitro Cipro-SH-CH NPs Release Evaluation

We concluded that, depending on the drug type ofCipro-SH-CH (weak acid) loaded on the nano-shellac, the release of Cipro-SH-CH NPs in the acidic medium (pH 2) is greater than in the basic

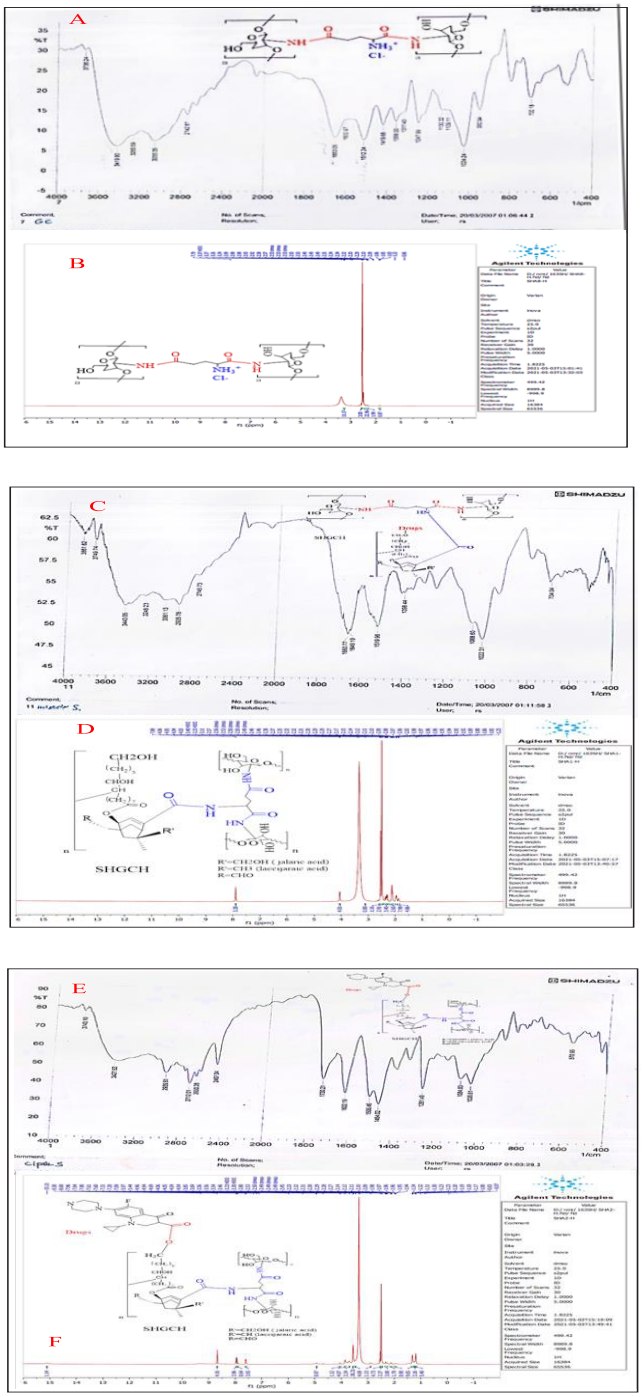

Fig. 5. (A) FT-IR spectrum ofglutamic-chitosan compound (GC). (B)The 1HNMR spectrum of glutamic-chitosan compound (GC).(C) FT-IR spectrum of the SH-CH NPs (D) The ^{1}HNMR spectrum of the polymer SH-CH NPs. (E) FT-IR spectrum of the Cipro-SH-CH NPs.(F) shows the ^{1}HNMR spectrum of Cipro-SH-CH NPs

medium at λmax = 340 nm (Fig. 7). This make us concluded that the speed of drug liberation after the end of the liberation process, which lasts for half a day is faster in acidic medium and reached about 100% of releasing, than in basic medium which the maximum released drug was about

80% after 12 hours. Most drugs are available as weak acids or weak bases. The weak acidic drug is released at a greater rate in the acidic medium (stomach) than in the basal medium (intestines) as a result of the lack of ionization of the drug and the breaking of the ester bond, and the release of

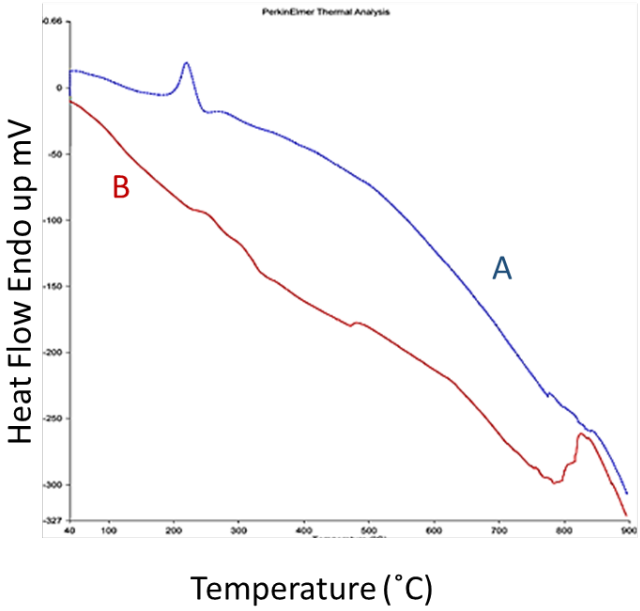

Fig. 6. DSC thermograms of (A) SH-CH NPs and (B) Cipro-loaded shellac–chitosan nanoparticles with CG 0.25 wt.%, SH 0.25 wt.%, and Cipro 0.25 wt.% in a 1:1:1 ratio.

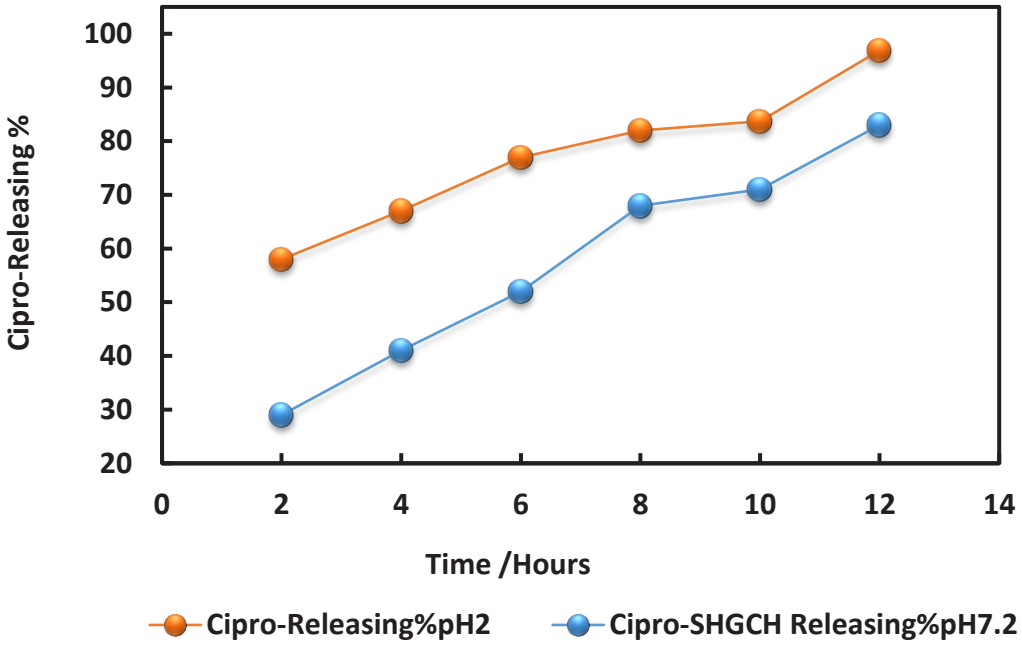

Fig. 7. The drug release amount of Cipro-SH-CH NPs at pH 2 and pH 7.2 at 37 ˚C at 340 nm using uv-vis spectrophotometry

the drug begins gradually.

Biological Potency of Cipro-SH-CH NPs

The biological activity of Cipro-SH-CH NPs was studied versus Gram-positive (*staphylococcus aureus*) and Gram-negative (*E.coli*) bacteria. This study showed that the drug loaded on nano-shellac-chitosan have biological effectiveness to prevent gram-positive and gram-negative bacteria from growing stronger than unloaded drugs, as the results were proven by calculating the radius of inhibition zone to overcome the bacteria which is shown in Table 1 and Fig. 8.

Anticancer Activity of Cipro-SH-CH NPs

The outcomes were revealed through this study is the impact of Cipro-SH-CH NPs on the percentage of cell line inhibition of skin cancer A375. The inhibition ratio tests were about (55.45%, 40.53%, 30.76%,15.41%) at (400, 200, 100, 50) µg/ml of

Table 1. The effect of Cipro-SH-CH NPs, SH-CH NPs,GC, and nonleaded Ciprofloxacin on *Staphylococcus aureus* and *E.coli* bacteria.

Code	sample	*Staphylococcus aureus* radius/mm	*E.coli* radius/mm
A	GC	-	-
B	SH-CH	-	-
C	Ciprofloxacin	20	15
D	Cipro-SH-CH NPs	40	40

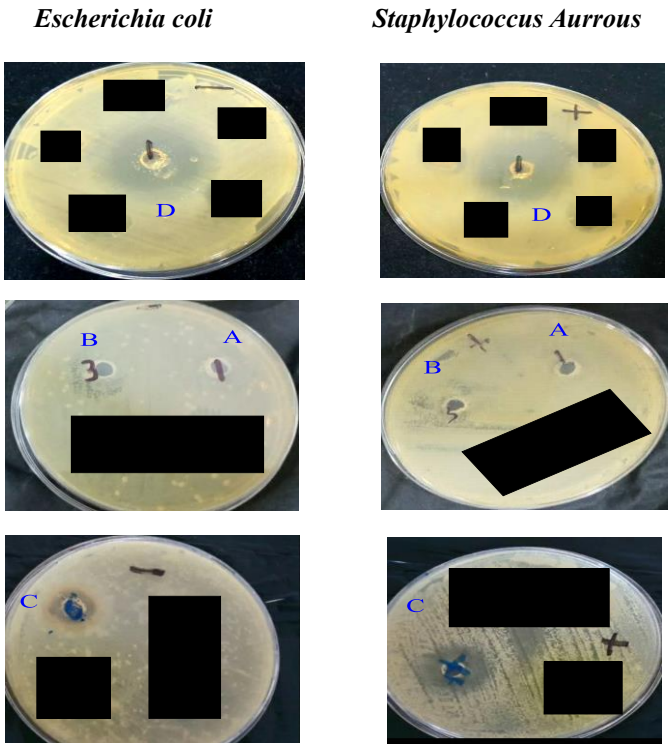

Fig. 8. The effect of (A)GC, (B)SH-CH,(C)Ciprofloxacin, and (D) Cipro-SH-CH NPs on *Staphylococcus Aurrous* and *Escherichia coli* bacteria for 24 hours.

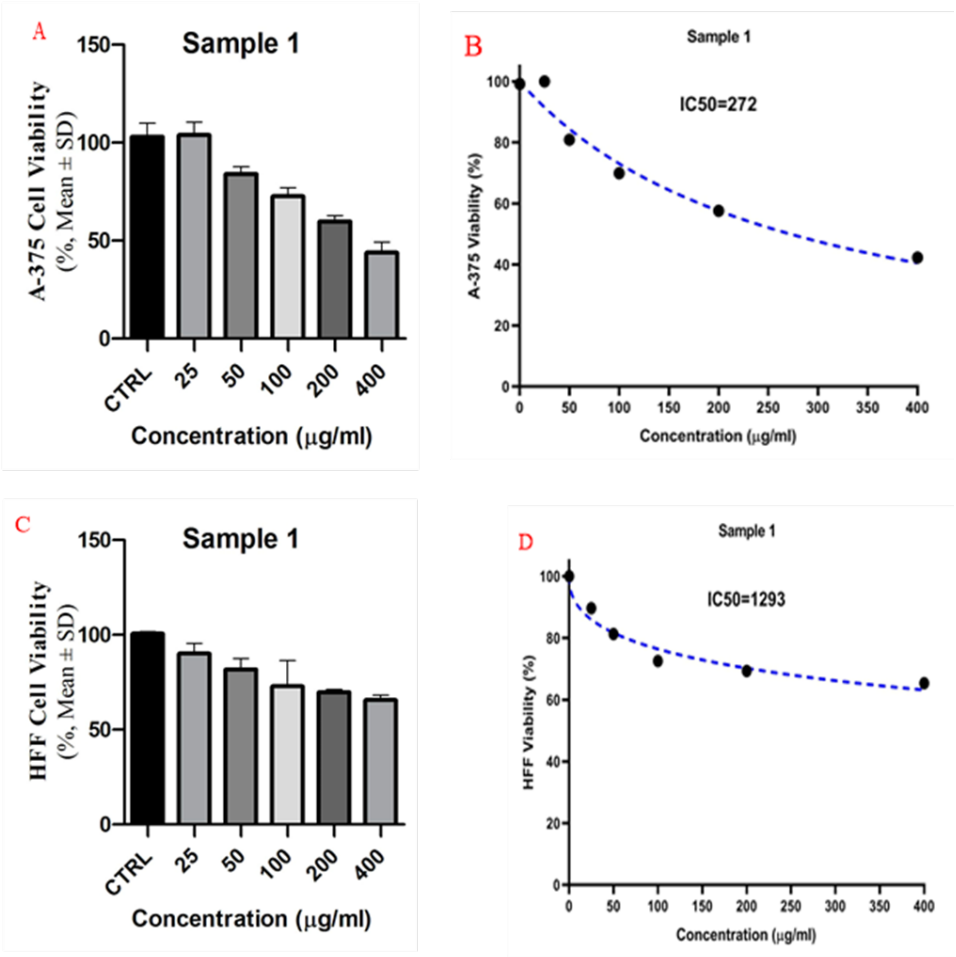

Fig. 9. The effect of Cipro-SH-CH on:(A&B) melanoma cells A375, (C&D) normal cell line HFF using MTT test after 24 hours' incubation at 37°C.

Cipro-SH-CH NPs. When calculating the IC_{50} after Cipro-SH-CH NPs treatment on A375 cancer cells (IC_{50} = 272 µg/ml Cipro-SH-CH NPs)while for the normal cell line using MTT (IC_{50} = 1293 µg/ml HFF). Significant differences appeared ≥ 0.0001, as shown in Fig. 9. As a result, the Cipro-SH-CH NPs test was highly effective against A375 skin cells. While the results of the inhibition ratio test showed a range of (8.51%-32.93%) at concentrations ranging from 25 to400 µg/ml, this demonstrates that the non-toxicity of the prepared nanocarrier and supports the use of Cipro-SH-CH NPs as a pharmaceutical compound.

CONCLUSIONS

This research displays the probability of producing a delivery system consists of shellac and chitosan to be loaded with ciprofloxacin drugs to enhance its antibacterial and anticancer activity. The glutamic chitosan salt was formed and then bonded with shellac and loaded with the antibiotic ciprofloxacin at nano size range. FTIR, HNMR, DSC, FESEM, and TEM were used to identify the formation and the morphology of Cipro-loaded shellac-chitosan NPs. Ciprofloxacin is a powerful anti-bacterial and anti-cancer drug. The efficacy of ciprofloxacin was improved after loading it with shellac-chitosan NPs on melanoma A375 skin cancer cells, as well as its high inhibition rates on bacteria.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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