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SYNTHESIS CHARACTERIZATION AND ITS ANTIOXIDANT AND ANTICANCER ACTIVITIES ON CHITOSAN-CURCUMIN NANOPARTICLES

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ABSTRACT

Chitosan is a agent that are used in the manufacturing of drugs for the drug dissolution. Chitosan exhibits an intrinsic antioxidant activity and anticancer activity. Curcumin is low in toxicity and acts as a anticancer agent. The aim of the paper is to extract chitosan nanoparticles and loaded with curcumin to undergo antioxidant activity and anticancer activity for liver cells. Chitosan is extracted from crab shells of Metacarcinus magister and curcumin extraction is carried out at different microwave operating powers.CLCNPs are characterized by UV analysis, FTIR spectroscopy, SEM analysis and XRD studies. Antioxidant activity is analysed by FRAP and DPPH assay. Anticancer activity is analysed against the Hep-G2 cell line by MTT assay. The work represents that curcumin loaded chitosan nanoparticles has more effective inhibition against cancer cells than chitosan nanoparticles for liver cancer cells. Thus curcumin loaded chitosan nanoparticles acts as a agent of drug delivery for treatment of liver cancer.

Keywords: Liver cancer, CLCNPs, Hep-G2 , MTT Assay , FRAP and DPPH Assay.

1. INTRODUCTION

In the recent days, researchers are focused on an effective drug delivery entity where the drug is encapsulated and furthermore utilized to pronounce the drug at the targeted site. If the formulation is of nanosize, it saves the loaded drug from degradation by improper pH and increases its half-life. Cancer is part of the vast majority of grave deadly diseases in today's earth because of its noteworthy complicated nature. Chemotherapy is a the vast majority of regular treatment utilized to pronounce anticancer drugs suitably to patients for quenching uncontrolled proliferation of cancerous cells. Resmi et.al., (2020). But these agents cannot reveal the wanted result in the vast majority of of the cases because of several drawbacks for instance grave side effects, low water solubility and brief circulation time.Curcumin, a polyphenol is known for its antioxidative and anti-inflammatory properties. Curcumin conducts its part as an antioxidant by scavenging the free radicals alive to in the peroxidation reaction. The anti-inflammatory property of curcumin has been explored to makewell inflammatory-mediated conditions as an example cancer, diabetes and atherosclerosis. Polymeric nanoparticles, for instance chitosan nanoparticles are predominantly explored to make better the bioavailability and absorption, to save early, hasty degradation of drugs because of lofty reactions and to temperance the rate of drug roll out in the system. Chitosan is an N-acetylated derivative of chitin, which is a naturally occurring polysaccharide exhibit in the shells of marine crustaceans. It's a linear polyamine composed of β-[1– 4]-linked D-glucosamine and N-acetyl-D-glucosamine subunits and can be cross-linked with anions, because of the presence of free amine groups. Chitosan nanoparticles are mainly utilized for controlled and improved drug delivery. In this study, curcumin-loaded chitosan nanoparticles were synthesized, characterized and to enquire the presence of enhancement in the antioxidant activity and anticancer activity.

2. MATERIALS AND METHODOLOGY

2.1. CHEMICALS AND LABORATORY EQUIPMENTS

Acetate of sodium $C_2H_3NaO_2$, Acetic acid glacial CH_3COOH, Hydrochloric acid, Sulfuric acid H_2SO_4, Potassium bromide KBr, Ethanol C_2H_6O, Sodium hydroxide NaOH, N-acetyl glucosamine $C_8H_{15}NO_6$, Sonicator,UV-vis spectrophotometer,Autoclave, Centrifuge, weighing balance, pH meter, cold room, Hot water bath, etc.

2.2. COLLECTION OF EXPERIMENTAL SAMPLE

Shells of Metacarcinus magister were collected from the local fish markets of Coimbatore. Figure (1) shows a schematic representation of the production of chitosan from crab shells. The shells were washed thoroughly with tap water and then with distilled water to remove impurities and pulverised into powder. Chitosan was extracted in accordance to the method described Samrot et.al., (2018) with some modifications.

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2.3. EXTRACTION OF CHITOSAN

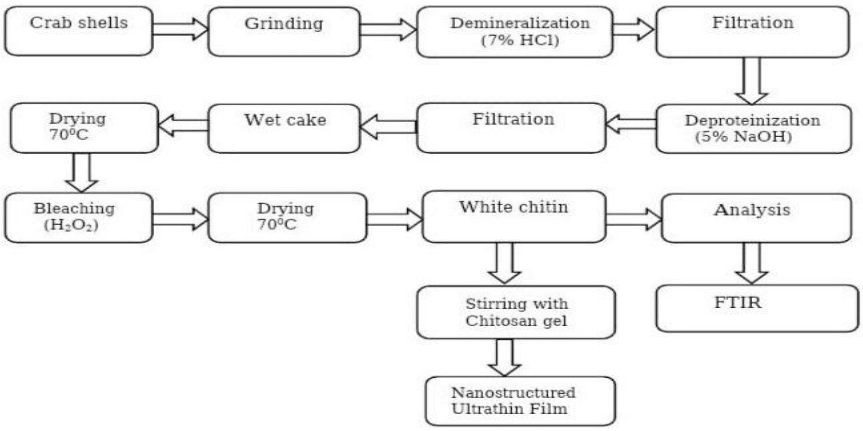

Figure (1). Schematic representation of the production of chitosan from crab shell.

2.3.1. DEMINERALIZATION

In Demineralization process, crab shell powder was added slowly to 7% HCl with continuous stirring to avoid effervescence and heated at 60℃ for 2 hours to remove carbonate and phosphate content from the crab shell powder

2.3.2. DEPROTEINIZATION

In Deproteinisation step, the acid hydrolyzed sample was treated with 5% W/V sodium hydroxide to reduce nitrogen content of protein, followed by washing to remove any traces of chemical and soluble impurities. The filtered sample was then dried in an oven at 70℃ for 3 hours. The dried demineralised, deproteinized and deodorized white sample was obtained. In bleaching step, the dried sample washed with hydrogen peroxide to reduce pigment of chitosan, followed by drying and storing under air tight condition.

2.3.3. CHITOSAN PRODUCTION

Deacetylation of chitin was achieved by reacting chitin with 12.5 M NaOH at a solid/liquid ratio of 1:15 (g/mL). The reaction mixture was cooled down and kept frozen at −83 ◦C in an ultra-freezer for 24 h afterwards, the temperature of the mixture was raised to 115 ◦C, and the reaction proceeded with agitation at 250 rpm for 4 or 6 h. The resulting chitosan was filtrated, washed with distilled water until neutral pH .

Figure (2). Crab shell of *Metacarcinus magister*

Figure (3). Crab shell powder

Figure (4). Chitosan

2.4. EXTRACTION OF CURCUMIN

2.4.1. MICROWAVE-ASSISTED EXTRACTION OF CURCUMIN

For microwave-assisted extraction of curcumin, 0.5 g of turmeric powder was weighed and dissolved in 10 ml acetone and put in a microwave chamber (domestic microwave). Acetone which was used as an extraction solvent has a good dissipation factor which can be heated up to a high extent and dissipate the microwave energy. Extraction was carried out at different microwave operating powers varied between 100-450 W and different irradiation times of 0.5-3 min. The samples were subjected to microwave irradiation in an intermittent way of irradiation-cooling irradiation for an extraction time of up to 3 min because longer irradiation time and higher power caused boiling of solvent. After that, the solvent was separated through a 0.45 µm filter and evaporated under a vacuum.

2.5. PREPARATION OF CHITOSAN NANOPARTICLES

Chitosan nanoparticles were synthesised by an ionic gelation. The range of the two compounds (positively charged chitosan and negatively charged sodium tripolyphosphate) in the different ratios was based on pilot experiments, in which only one type of phenomena was observed: an almost clear solution, an opalescent suspension and no aggregates. The opalescent suspension was of interest to us in the formation of nanoparticles . Different concentrations of chitosan (1-5 mg) was added to 1 % acetic acid (v/v) and mixed well using magnetic stirrer and adding 1 % TPP (w/v) drop by drop under magnetic stirring. Then the solution was centrifuged at 10,000 rpm for 10 minutes to remove residual TPP and the particles were freeze dried.

2.6. PREPARATION OF CURCUMIN LOADED CHITOSAN NANOPARTICLES

An aqueous solution of 9 ml of sodium tripolyphosphate pentabasic (TPP) (1mg/ml, pH 5) was added dropwise to 17.5 ml in chitosan solution (1mg/ml) with and without curcumin (4mg/ml). The chitosan solution was prepared by dissolving in a 1% acetic acid and the pH was adjusted to 5.0 with 0.1 M NaOH. CCN was prepared by mixing the desired volume of curcumin solution with TPP (0.133% w/v in pure water) at different ratios dropwise at 600 rpm using a magnetic stirrer before adding it to the chitosan solution. It was observed that the formation of nanoparticles without any aggregation, even after the particles were left overnight on magnetic stirring. After overnight stirring the Curcumin loaded chitosan nanoparticles (CLCNPs) were collected by centrifugation at 8000 rpm at 4 °C for 30 min . Finally, the obtained solution was stirred for 45 min until it was evenly dispersed. It was degassed via ultrasonication for 10 min. The pellet was washed three times with 10% aqueous ethanol. Then transferred into a new polystyrene tube at − 80 ◦C for 72 h and lyophilized. This formulation was selected for further studies and characterization as well as for entrapment of curcumin. The pellets were then resuspended in 10% aqueous ethanol and then the particles were freeze dried.

2.7. CHARACTERIZATION OF CURCUMIN LOADED CHITOSAN NANOPARTICLES

2.7.1. UV ABSORPTION STUDIES

The Absorbance studies of curcumin loaded chitosan nanoparticles were performed in a UV 1700 spectrophotometer using a 10 mm path length quartz cuvette. The spectra were measured in the 200–700 mm wavelength range. The quantity of the curcumin stock solution was increased from 1% to 10%, and to achieve a final concentration of 100%. The absorption spectra were recorded after each concentration.

2.7.2. SEM OBSERVATION

Scanning electron microscopy (SEM) was used for the study of the morphology of the surfaces of nanoparticles. A drop of the nanoparticles suspension was placed onto the aluminium stubs located on the surface of the sample stub and dried. Subsequently, the stub was coated with a platinum layer by the Auto Fine Platinum Coater before imaging. The morphological structure and the diameter of the fibers were analyzed by SEM with an acceleration voltage of 10 kV. To render the electrically conductive samples, they were gold sputter-coated under an argon atmosphere before the experiment.

2.7.3. FTIR SPECTRAL STUDY

The FT-IR spectra of curcumin-loaded chitosan nanoparticles were analysed by (FTIR 4600) spectrophotometer to investigate the possible entrapment of curcumin with the polymer matrix. The CLCNPs were crushed with KBr to get the pellets by applying a pressure of 600 kg/cm2. FTIR spectra of the above sample were recorded at 4mm/sec at a resolution of 2cm-1 in the wavenumber range of 650-4000 cm-1 and the characteristic peak of the above sample was measured.

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2.7.4. XRD STUDY

The X-ray Diffraction (XRD) was used to confirm the nature of crystal structure of the formed curcumin loaded chitosan nanoparticles. To observe the physical nature of curcumin-loaded CPNs, X-ray diffraction (XRD) analysis of the samples were done by (X'Pert-Pro) X-ray Diffractometer using 30 milliamps and 45 kV current with a monochromatic copper anode radiation (λ = 1.54060Å). The scan rate was 0.02° 2θ/s, in the scan range from 2θ = 4 to 60°.

2.8. ANTIOXIDANT ACTIVITY

2.8.1. DPPH ASSAY

Radical scavenging activity was determined according to (Braca et al., 2001) .The stock solution was prepared by dissolving 4 mg DPPH (2, 2, diphenyl-1-picryl hydrazyl) with 100 mL methanol and stored in dark at 20°C until required. Then control 500 µL of DPPH and 500 µL of ethanol in a test tube. Different concentrations (10 µL, 20 µL, 30 µL, 40 µL, 50 µL) of CLCNPs added in test tubes and makeup up to 500 µL by adding ethanol. Finally, add DPPH 500 µL to all test tubes. The reaction mixture was shaken well and incubated in the dark for 30 minutes at room temperature. The absorbance was measured at 517 nm where lower absorbance indicated higher free radical scavenging activity.

The scavenging activity against DPPH was calculated using the following formula:

$$\text{Scavenging activity} = \frac{\text{A517 (sample)} - \text{A517 (control)}}{\text{A517 (control)}} \times 100$$

Where, A517 (control) was the absorbance of the control (DPPH solution without the sample), A517 (sample) was the absorbance of DPPH solution in the presence of the sample (extract/standard).

2.8.2. FRAP ASSAY

The antioxidant capacity of samples was estimated according to the procedure described by Benzie and Strain (1996) and as modified by Pulido et al. (2000) . FRAP reagent (900 µl), prepared freshly and incubated at 37 ºC, was mixed with 90 µl of distilled water and 30 µl of the test sample, or acetone (for the reagent blank). The test samples and reagent blank were incubated at 37 ºC for 30 minutes in a water bath. The FRAP reagent contained 2.5 ml of 20mmol/l TPTZ solution in 40 mmol/l HCl plus 2.5 ml of 20 mmol/l FeCl3.6H2O and 25 ml of 0.3 mol/l acetate buffer, pH 3.6 (Benzie and Strain, 1996). At the end of the incubation period, the absorbance readings were recorded immediately at 593 nm using a spectrophotometer. The known Fe (II) concentration ranging between 100 and 2000 µmol/l (FeSO4.7H2O) was used for the preparation of the calibration curve. The parameter Equivalent Concentration (EC1) was defined as the concentration of antioxidant has a ferric- TPTZ reducing ability equivalent to that of 1 mmol/l FeSO4.7H2O. EC1 was calculated as the concentration of antioxidants giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1 mmol/l concentration of Fe (II) solution determined using the corresponding regression equation.

Percentage of inhibition = A (control) -A (test) / Acontrol X 100

2.9. IN-VITRO ANTICANCER ACTIVITY

2.9.1. CELL LINE

The human Liver Cancer-Hep G2was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 370C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

2.9.2. CELL TREATMENT PROCEDURE

The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x105 cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 370C, 5% CO2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and an aliquot of the sample solution was diluted to twice the

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desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 370C, 5% CO2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

2.9.3. MTT ASSAY

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore,the amount of formazan produced is directly proportional to the number of viable cells.

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 370C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader.

The percentage cell viability was then calculated with respect to control as follows

% Cell viability = [A] Test / [A]control x 100

The % cell inhibition was determined using the following formula.

% Cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log concentration and IC50 was determined using GraphPad Prism software (70, 71).

3. RESULTS AND DISCUSSION

3.1. UV ANALYSIS OF CLCNPs

We found an absorption peak at 420 nm with a 10 % of curcumin entangled in chitosan nanoparticles. After incorporating two distinct concentrations of curcumin, 1% and 10%, onto chitosan, the absorption peaks are shifted to 300, 320, 340, and 400 nm, respectively, by increasing the concentration of curcumin to 100%. When the curcumin content is reduced to 1%, the absorption peak shifts to around 300 nm. A characteristic peak is noticed in the range of 300 nm to 400 nm for this peak. The creation of intermolecular hydrogen bonds between curcumin and chitosan nanoparticles (CLCsNPs) may be responsible for the absorbance peak of curcumin- loaded chitosan nanoparticles (CLCsNPs). As evidenced by this result (Figure 5), curcumin was obviously loaded onto chitosan nanoparticles.

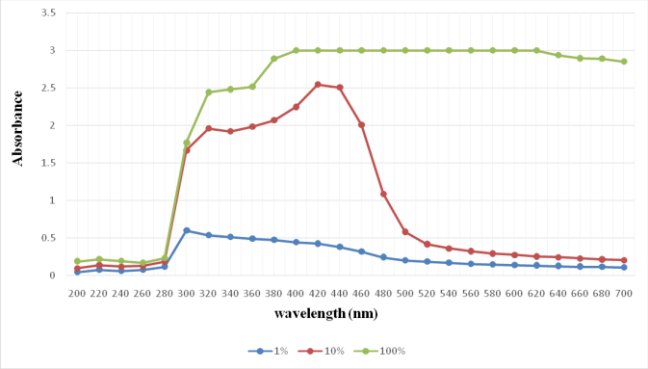

Figure (5). UV Profile of Curcumin loaded chitosan nanoparticles

3.2. SEM ANALYSIS OF CLCNPs

The morphology was confirmed by SEM. The SEM image of curcumin-loaded chitosan nanoparticles (Figure 6) shows spherical particles with a size range of 200 nanometers. Nanoparticles should be large enough to avoid rapid leakage into blood capillaries but small enough to avoid capture by macrophages stuck in the reticuloendothelial system when delivering drugs via nanoparticles .

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Figure (6). SEM images of curcumin loaded chitosan nanoparticles.

3.3. FTIR SPECTROSCOPY ANALYSIS FOR CLCNPs

Further, to confirm the loading of drug in the curcumin loaded chitosan nanoparticles formulation, FTIR analysis was conducted. Fig. 7 shows the FT-IR spectra of curcumin loaded chitosan nanoparticles. The IR spectrum of Curcumin demonstrated stretching vibrations due to phenolic hydroxyl groups at 3200–3500 cm-1, a stretching vibration at 1490 cm-1 associated with the aromatic C=C bond and a bending vibration at 1246 cm-1 attributed to the phenolic C-O group. In chitosan, a peak at 1635 cm-1 was observed, and this corresponds to amide I bending vibration. In the CsNPs spectrum, the wave number shifted from 1640 to 870cm-1. The vibrational mode of amide C-O stretching was observed at 1635, cm-1. The spectra were also compared with the standard chitosan and correlations were observed in the spectra are shown in (Figure 7).In the spectra of CLCsNPs, a peak at 686.66 cm-1 was observed, which corresponds to amino deformation, and a change in the peak at 555 cm-1 corresponds to the -keto group of the curcumin. From the FT-IR data, it is confirmed that the NPs were formed due to the interaction between the phospho groups of TPP and the amino groups of chitosan. There was an interaction between the -keto group of curcumin and the amine group of chitosan that resulted in drug loading.

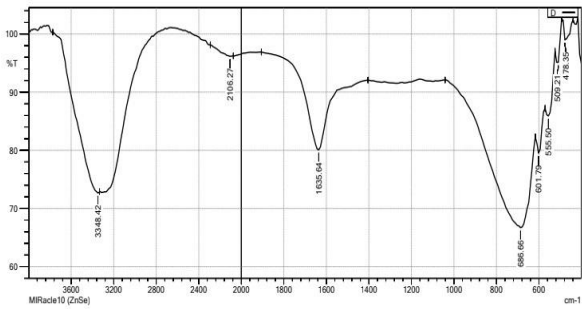

Figure (7). FTIR spectra of curcumin loaded chitosan nanoparticles.

3.4. XRD STUDY ON CLCNPs

Broad XRD patterns of CLCNPs, curcumin and chitosan show several peaks in the 2 range of 15–35°, implying the crystalline nature of the component. The CLCNPs exhibited strong reflections around 22.81°, 26.21°, 27.96°, and 28.66°. The XRD plots for curcumin-loaded CPNs are presented. This result indicates that curcumin encapsulated in NPs is in the solid-state solubilized form in the polymeric matrix. This disordered-crystalline phase of curcumin inside the polymeric matrix helps in the sustained release of the drug from the nanoparticles (Figure 8).

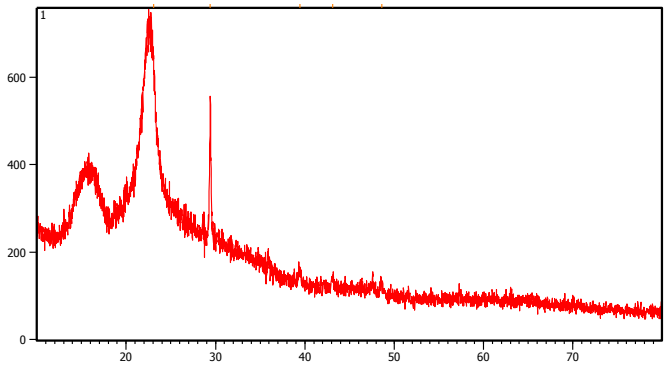

Figure (8). XRD pattern of curcumin loaded chitosan nanoparticles.

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3.5. ANTIOXIDANT ACTIVITY OF CURCUMIN LOADED CHITOSAN NANOPARTICLES

3.5.1. DPPH RADICAL SCAVENGING ACTIVITY

When an antioxidant on the interface with DPPH radical transfers hydrogen or electron atoms to DPPH, the DPPH assay is the radical scavenging of stable 2, 2-diphenyl picryl hydrazyl at room temperature. When DPPH comes in contact with an antioxidant, it gets reduced by donating its free electron. Thus, the greater the degree of decolourization, the greater the scavenging activity of the compound. The reducing capacity of DPPH radicals is calculated by the decrease in their absorbance at 517 nm induced by antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Therefore, DPPH is usually used as a substance to evaluate antioxidant activity. The percentage of DPPH radical scavenging activity of the prepared samples is presented as a tabulation and graph (Table 1) and Figure (09).

Table 1: Percentage of DPPH inhibition in curcumin loaded chitosan nanoparticle.

Sample	Concentration	Abs at	Percentage of inhibition	Standard (Ascorbic
S-1	10 µl	0.289	25. 53 %	15 %
S-2	20 µl	0.265	33.42 %	38 %
S-3	30 µl	0.242	54.92 %	56 %
S-4	40 µl	0.236	69.01 %	73 %
S-5	50 µl	0.167	78.02 %	86 %

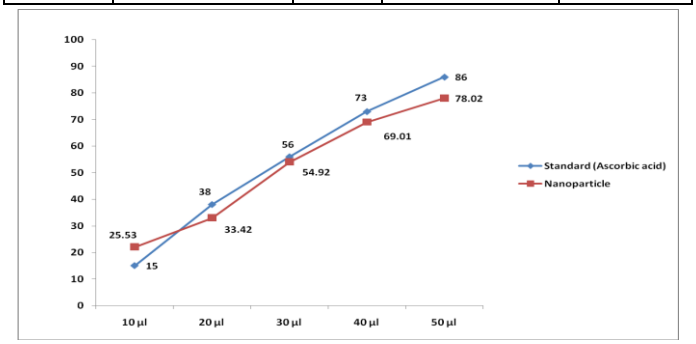

Figure (9). Antioxidant activity of curcumin loaded chitosan nanoparticles.

3.5.2. FERRIC REDUCING ANTIOXIDANT POWER ASSAY

The FRAP reagent was added to a range of Fe2+ solutions of known concentrations to create a standard curve, which allowed the Fe2+ concentration of the samples to be calculated, as well as the "antioxidant capacity of synthesised nanoparticles based on the antioxidant capacity of this material to reduce ferric to ferrous ions". There is a significant increase in the antioxidant activity due to the inclusion of curcumin in the nanoparticles, thereby leading to an overall enhancement in the antioxidant activity. Bhoopathy et.al., (2020). The FRAP values of curcumin-loaded chitosan nanoparticles are shown in Table 2. The graph depicts the progressive decline in peak height (Figure 10).

Table 2: Percentage of FRAP inhibition in curcumin loaded chitosan nanoparticle.

Sample	Concentration	Abs at 593 nm	FRAP Ascorbic acid Standard	Percentage of inhibition Nanoparticles
S-1	10 µl	0.179	33	22.51
S-2	20 µl	0.183	44	32.77
S-3	30 µl	0.186	52	48.00
S-4	40 µl	0.190	71	71.74
S-5	50 µl	0.194	83	84.01

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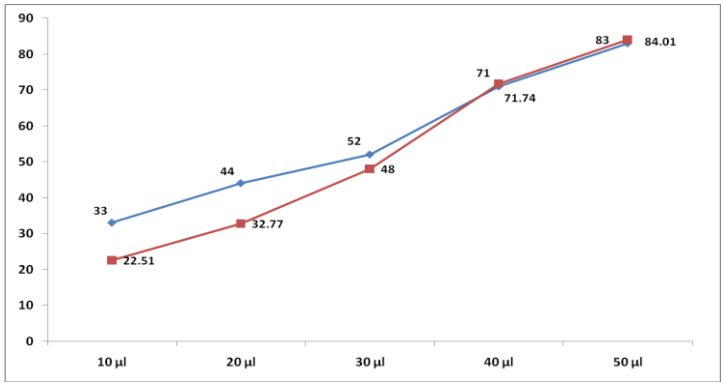

Figure (10). Ferric reducing activity of curcumin loaded chitosan nanoparticles.

3.6. IN-VITRO ANTICANCER ACTIVITY OF CURCUMIN LOADED CHITOSAN NANOPARTICLES

In vitro anticancer activity against the Hep-G2 cell line at different concentrations was evaluated. The anticancer activities of the CLCNPs nanoparticles were performed with different concentrations, such as 18.75 µg, 37.5 µg, 75 µg, 150 µg, 300 µg. The anticancer activity of CLCNPs nanoparticles against HepG2 increased with the concentration of CLCNPs nanoparticles. The anticancer effect of CLCNPs nanoparticles against HepG-2 cell lines was performed and showed good results. The results show good cytotoxic activity against the cancer cells (Figure 12). The CLCNPs nanoparticles are having good results against Hep-G2 in that 300 µg shows fine results, followed by 75 µg and 150 µg. The lowest cell inhibition action was observed at the concentrations of 18.75 µg and 37.5 µg (Figure 11) & (Table 3).

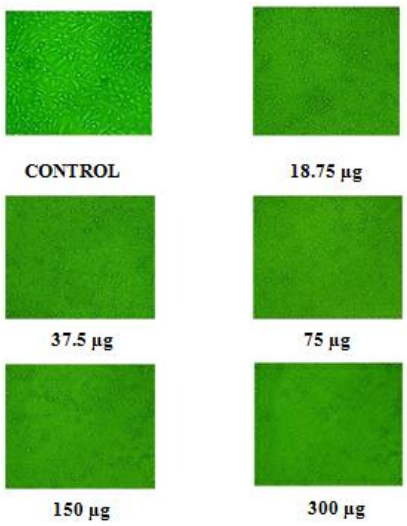

Figure (11): Microscopic images of (HepG2) cell lines after incubation with CLCNPs

Table 3: Percentage of cell inhibition of CLCsNPs.

Conc(µg/ml)	% Cell inhibition	IC 50 174.29	µg/ml
18.75	8.432709		
37.5	19.3356	R^2	0.996
75	31.13288		
150	46.50767		
300	58.34753		

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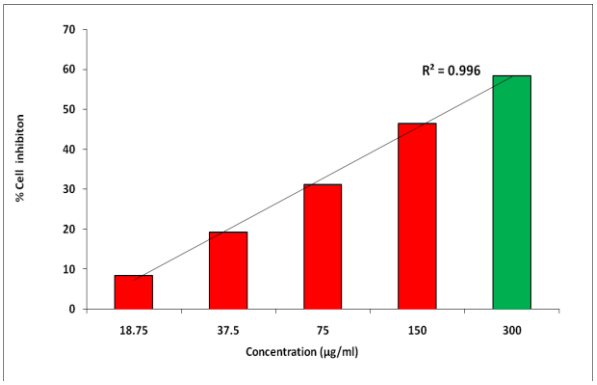

Figure (12):Cell inhibition of CLCNPs against liver cancer (HepG2) cell lines

4. CONCLUSION

Chitosan Nanoparticles Extracted From Crab Shells Have Spherical Shape With Particle Size Range Of 200 Nm. It Has Wide Range Of Antioxidant And Anticancer Activity With Minimal Side Effect. The Studies Revealed That Synthesised Curcumin-Loaded Chitosan Nanoparticles Are Water Soluble As Well As Stable, Bringing Them To The Forefront Of Existing Anticancer Therapeutic Agents. In This Regard, The Entrapment Of Curcumin In Chitosan Nanoparticles Has Opened A New Avenue To Improve The Bioavailability Of Curcumin And Can Make The Drug Responsive For The Treatment Of Cancer. The Anticancer Activity Of Curcumin-Loaded Chitosan Nanoparticles Was Carried Out By Mtt Assay Against Hep-G2 (Liver Cancer Cell) Lines. In This Study, The Increased Anticancer Activity Was Found To Be At An Increased Concentration Of Curcumin-Loaded Chitosan Nanoparticles. This Biosynthesis Approach Was Easy, Large-Scaled And Eco-Friendly. Thus, Chitosan-Based Nano Carriers Could Be Used As A Pertinent Vehicle For The Controlled Delivery Of Curcumin In The Treatment Of Liver Cancer. It May Prove A Promising Carrier Candidate For Cancer Therapeutic Treatment In The Near Future.

DECLARATION OF COMPETING INTEREST

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

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