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RESEARCH ARTICLE

SYNTHESIS, CHARACTERIZATION AND HEMOCOMPATIBILITY EVALUATION OF CURCUMIN ENCAPSULATED CHITOSAN NANOPARTICLES FOR ORAL DELIVERY

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Abstract

Curcumin has a diverse therapeutic potential in health and disease. However, the poor aqueous solubility and low bioavailability of Curcumin have limited its potential when administrated orally. The aim of this study was to assess the potential of nanoparticles to improve the pharmacokinetics of Curcumin, with a primary goal of enhancing its bioavailability. Chitosan nanoparticles containing Curcumin (CCN1, CCN2 and CCN3) formulations were synthesized by ionic gelation of chitosan with tripolyphosphate anions (TPP) resulting in particles size smaller than 224 nm. The encapsulation efficiency of nanoformulations was over 73%. The nanoformulations exhibited slow and sustained *in vitro* release over 53% of Curcumin from the Curcumin encapsulated chitosan nanoparticles after 12 hours in PBS at pH 7.4 when freeze-dried particles were used. The nanoformulations were stable, hemocompatible and produced no hemolysis.

INTRODUCTION

The turmeric (*Curcuma longa* Linn.) is a great medicinal plant extensively used in Ayurveda, Unani and Siddha for curing both infectious and non-infectious diseases including a variety of cancer. The extract from the turmeric rhizomes is composed of mainly three phenolic compounds called curcuminoids such as Curcumin or Curcumin-I, Curcumin-II (demethoxycurcumin) and Curcumin-III (bisdemethoxycurcumin) (Masuda, T. *et. al.*,(1992), Sreejayan, S. *et. al.*,(1994) and Unnikrishnan, M.K. *et. al.*,(1995)). Curcuminoids are potential therapeutic molecules being used for health promotion and disease prevention (Diana, Guzman-Villanueva. *et. al.*,(2013)). Generally, Curcumin as principle nutraceutical molecules along with others has been given a warming welcome in the food and pharmaceuticals industries due to their broad spectrum biological activities (Sabita, N. Saldanha. *et. al.*, (2012)).

Besides unique nutraceutical properties of these lipophilic molecules, pharmacokinetics and pharmacodynamics qualify them to slip through the biological membranes and barriers to interact physically with diverse range of molecular targets. Several research investigations have focused on the safety and efficacy of Curcumin included transcription factors, growth factors and their receptors, cytokines, enzymes, and genes at the molecular level exhibiting drug and gene interaction. In addition, therapeutic functions of Curcumin showed anti-inflammatory effect in acute, sub-acute and chronic levels *in vivo* and *in vitro* models (Arora, R.B. *et. al.*,(1972)). It has been documented from clinical studies that administering Curcumin even up to 1600mg/kg/day for 4 weeks in phase-I trials in male volunteers did not produce any serious side effects. Further, phase-II trials conducted in patients with rheumatoid arthirties and osteoarthritis were proved to be safe (Gupta, S.C. *et. al.*,(2013)).

Unfortunately due to limited aqueous solubility, the Curcumin molecules cannot be readily absorbed from the gut region imparting very low concentration in serum and poor bioavailability to exhibit therapeutic activity at the targeted tissues (Dulbecco, P. *et. al.*,(2013)). In recent years, investigators are shifting their attention towards the use of drug-loaded nanoparticles for targeted delivery applications (Kumaresh, S. Soppimatha. *et. al.*,(2001) and Mohan Raj, V.J. *et. al.*,(2013)). Today, due to technological innovations nontoxic, biocompatible, inexpensive and biodegradable nanoparticles with various colloidal dimensions are being developed to enhance the penetration ability, reduce the frequency of doses, toxicity and to improve the therapeutic efficacy (Anwunobi1, A.P. *et. al.*,(2011), Letizia, Da Sacco. *et. al.*,(2010), Paul, D.R. *et. al.*,(2008) and Sunil, A. Agnihotri. *et. al.*,(2004)). The purpose of this study was to synthesize Curcumin encapsulated nanoparticles (CCN) to improve the solubility and stability of the Curcumin in gastrointestinal (GI) conditions by evaluating their particle size, encapsulation efficiency, drug release and haemocompatibility aspects.

MATERIALS AND METHODS

Chitosan (MW of 30 kDa) was a gift sample from Laxmish I P, Cochin Central Marine Fisheries, Cochin, India. Curcumin from Amruta Herbals Pvt. Ltd., Indore. Pentasodium tripolyphosphate (TPP) was purchased from Sigma (St. Louis, MO, USA), Acetic acid and Tween 80 were purchased from Hi-media Chemical Co. (Mumbai, India). Double distilled water was used throughout the study. All other reagents were of analytical grade unless otherwise stated.

Preparation of Curcumin encapsulated chitosan nanoparticles by ionic gelation method

Curcumin encapsulated nanoparticles were prepared by ionic gelation method as reported by Calvo. *et. al.*,(1997) with slight modification. The nanoparticles were easily prepared upon addition of TPP to a chitosan solution containing Curcumin and mixing by using magnetic stirrer. Encapsulating polymer solution was prepared by dissolving chitosan in acetic aqueous solution to achieve 1.0, 2.0 and 3.0 (mg/ml) different formulations. Drug solution was prepared separately by dissolving Curcumin (2 mg) in ethanol (500 µl) and added with 2% (w/v) Tween 80. The chitosan and drug solutions were mixed together using magnetic stirrer (25°C, 30 min) and 0.25% (w/v) TPP was added drop-wise while stirring. During the ionic interaction of chitosan (Jessica, D. Schiffman. *et. al.*,(2007)) with TPP establishes an equilibrium leading to the reduction of the aqueous solubility of chitosan. In the process of doing so, it traps the Curcumin into the core to encapsulate resulting in nanoformulations. The resultant nanoparticles suspension was centrifuged (10,000 rpm, 30 min), the particles were washed thrice with distilled water and freeze dried. While preparing nanoparticles, it is critical to regulate the ratio between chitosan and TPP in order to control the uniform size of the nanoparticles in the reacting mixture. The procedure was repeated for all the three formulations CCN1, CCN2 and CCN3 prepared (Amir, D. *et. al.*,(2008), Nasiri1, M. *et. al.*,(2012), Paresh, N. Patel. *et. al.*,(2011), Rabindra, K. Nanda. *et. al.*,(2012) and Yan, Wu. *et. al.*,(2005)).

Particle size distribution

The size of the nanoparticles was determined by photon correlation spectroscopy (Zetasizer 4000, Malvern Instruments Ltd., Malvern, UK). It is routinely used method to determine the mean hydrodynamic diameter and the particle size distribution to study the polydispersity index (PDI = 22/Γ2) of the nanoparticles. The dynamic light-scattering measurements were done with a wavelength of 532 nm at 25°C with an angle detection of 90º.

Evaluation of Curcumin encapsulation efficiency (%)

The amount of Curcumin encapsulated during the formulations was calculated by the difference between the total amount of Curcumin added initially into the preparation medium and the amount of Curcumin remained in the supernatant after centrifugation. The Curcumin present in the supernatant was determined spectrophotometrically by measuring the absorbance at 426.7 nm using UV–VIS spectrophotometer (Shimadzu 1650, Kyoto, Japan) (Ravikumara, N.R. *et. al.*,(2009)).

***In vitro* release study of Curcumin**

The *in vitro* release (Rabindra, K. Nanda. *et. al.*,(2012)) of Curcumin from Curcumin encapsulated chitosan nanoparticles were carried out by dialysis method. The nanoparticles were redispersed in freshly prepared in PBS (5ml, pH 7.4) and dialyzed using membrane with 12 kDa cut-off pore dimensions. During dialysis, the bag was placed in a jar containing PBS (150 ml) and incubated at 37°C in a shaking water bath (50 rpm). The amount of Curcumin released from the Curcumin encapsulated chitosan nanoparticles was measured by sampling out 1 ml each

time at predetermined time intervals (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 36, and 48 hour). From an aliquot, the amount of Curcumin released was determined spectrophotometrically at 426.7 nm. A standard calibration curve was drawn using Curcumin as reference standard (Ravikumara, N.R. *et. al.,* (2009) and Vyjayanthimala. *et. al.,*(2013)).

Stability studies

Curcumin encapsulated chitosan nanoparticles were evaluated to assess their stability at three different temperature levels as described by Vyjayanthimala. *et. al.,*(2013). The three different nanoparticles were incubated at 4^{o}C, room temperature and 45^{o}C for a period of 1 month. The samples were collected at every week and measured the amount of Curcumin release using UV-Spectrophotometer at 426.7 nm.

Hemocompatibility study of Curcumin encapsulated chitosan nanoparticles

Fresh blood from a healthy volunteer was collected in a blood collection tube containing anticoagulant (EDTA, 0.5 mg). The blood was centrifuged (2000 rpm, 20 min) at room temperature using REMI 24C centrifuge. The concentrated leukocyte band (a Buffy-coat) and a small portion of the plasma was removed. Later, the concentrated RBCs in the packed cells were separately collected and washed thrice with normal saline (0.9% NaCl). The RBC cells and saline were taken in 1:1 ratio and centrifuged (2000 rpm, 10 min). The supernatant was discarded and washings were repeated thrice. Washed RBCs were further diluted to a 50% hematocrit by adding normal saline. Hemolysis experiments were followed in accordance with a method used previously in our laboratory with slight modifications.

100 µl cell suspensions taken into a clean dry test tube was added with an appropriate negative control in normotonic condition. The positive control was prepared with 100 µl cell suspensions by diluting with double distilled deionized water (3 ml) and the RBCs lysis was compared. 100 µl of Curcumin encapsulated chitosan nanoparticles formulations (CCN1, CCN2 and CCN3), where drug-free chitosan nanoparticles formulation (chitosan-alone) served as blank nanoparticles. Then, the individual category nanoparticles were further added with100 µl of RBC suspension made to 3 ml by adding normal saline. The experiment was carried out in the triplicate. All the samples were incubated at 37^{o}C for 1 hour in a water bath (ILE instrument, Bangalore). The reaction was terminated using 50 µl of gluteraldehyde (2.5%). The samples were then centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 426.7 nm using UV-VIS spectrophotometer (Optizen 2120UV Plus, Mecasys co., Ltd, Korea.) (Ravikumara, N.R. *et. al.,* (2009))

$$\text{Hemolysis}\,(\%) = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the positive control}} \times 100$$

RESULTS AND DISCUSSION

Curcumin is practically insoluble in water. Because of this reason, Curcumin encapsulated chitosan nanoparticles were prepared by ionic gelation method using TPP ions (Table.1). The data from Fig.1 on size variability suggested the ratio between TPP and chitosan is critical and controls the size and the size distribution of the nanoparticles (Yaowalak, Boonsongrit. *et. al.,*(2006)).The particle size of Curcumin encapsulated chitosan nanoparticles formulations (CCN1, CCN2 and CCN3) was evaluated by Zeta Sizer is presented in Table.2. The size of the nanoparticles ranged between 173 nm and 224.2 nm, where the maximum size of nanoparticles was observed in CCN3 (224.2 nm) as compared to other formulations and the least size was seen in CCN1 (173.0 nm). The size of the nanoparticles varied with the ratio between Curcumin and chitosan concentration. It is evident from the earlier researchers that the particle size is dependent on the chitosan concentration, the minimum size corresponding to the lowest chitosan concentration (Calvo. *et. al.,* (1997)).

The particles distribution index (PDI) values of Curcumin encapsulated chitosan nanoparticles (CCN1, CCN2 and CCN3) are shown in Table.2. The PDI values ranged between 0.395 and 0.523, where CCN1 exhibited the higher value than that of CCN2 (0.440) and the PDI values decreased as the ratio between Curcumin and chitosan increased. Table.2 shows the results of encapsulation efficiency and loading capacity of the Curcumin encapsulated chitosan nanoparticles. The encapsulation efficiency was maximum with the lower Curcumin concentration (CCN1) and minimum with the higher Curcumin concentration (CCN3). The encapsulation efficiency ranged between 61.22 to 74.44%. Conversely the loading capacity of nanoparticles increased as the concentration of the Curcumin increased. The increase of Curcumin concentration leads to a decrease of encapsulation efficiency and an enhancement of loading capacity, possibly due to effect of the chain length of chitosan as longer chains of high

molecular weight chitosan can entrap greater amount of Curcumin when gelated with TPP as observed in the previous study (Yan, Wu. *et. al.*,(2005)). The failure to increase encapsulation efficiency proportionate with increase in Curcumin concentration may be due to shorter chains low molecular weight chitosan used in the present study.
The *in vitro* release data for Curcumin loaded chitosan nanoparticles (CCN1, CCN2 and CCN3) are shown in Table.3 and Fig. 2. The release pattern demonstrated slow and sustained gradual release of Curcumin at each point of time from nanoparticles at slightly basic pH 7.4. However between 53.24% and 62.66% release was observed after 6 hour. The results are in close agreement with recent studies which exhibited slow and sustained release of drug from drug-loaded chitosan nanoparticles. Sustained drug release from the nanoparticles is an important event, as it will increase the drug bioavailability and prolong the therapeutic effect. Although, the burst release of Curcumin lasted for 30 min from the nanoparticles that was < 20% and the dug was gradually released on an incremental basis attaining a range between 65.71% and 78.22% release in 12 hours. There was no initial rapid release of the Curcumin from Curcumin loaded chitosan nanoparticles (CCN1, CCN2 and CCN3) formulations. The results thus demonstrate that the concentration of Curcumin to chitosan polymer (ratio) in the Curcumin loaded chitosan nanoparticles influenced these physicochemical characteristics and the release profile of the nanoparticles. Upon storage, the nanoparticles exhibited good stability for a month at the different temperature conditions (Table.4). The Curcumin encapsulated nanoformulations showed a slight degradation at the elevated (45^{o}C) temperature in comparison with the CCN2 and CCN3 nanoformulations at 4^{o}C and room temperature (Fig.3). The haemocompatibility study is of prime concern for cellular assays. We optimized all nanoformulations concentration of blank and Curcumin encapsulated chitosan nanoparticles on red blood cells which exhibited no toxicity with concentration ranging between 500 - 1500 µg/mL as shown in Fig.4.

Table 1. Preparation of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles

Sl. No	Formulations code	Curcumin (mg)	Chitosan (mg)	0.25% TPP solution (ml)	Curcumin : Chitosan Ratio
1	CCN1	2	4	3	1:2
2	CCN2	2	8	3	1:4
3	CCN3	2	12	3	1:6

Table 2. Particle size and encapsulation efficiency (%) of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles.

Sl. No	Formulation code	Curcumin : Chitosan ratio	Particle Size (nm)	PDI	Encapsulation efficiency (%)
1	CCN1	1:2	173.0	0.523	73.44
2	CCN2	1:4	177.5	0.440	64.55
3	CCN3	1:6	224.2	0.395	61.22

Table 3. *In vitro* release of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles. at pH 7.4 (37 °C)

Time Interval (Hours)	Curcumin encapsulated chitosan nanoparticles		
	CCN1 (%)	CCN2 (%)	CCN3 (%)
30min	20.01	21.25	19.99
1	26.06	24.56	25.44
2	30.04	26.88	28.66

3	34.08	32.11	31.56
4	44.52	36.44	38.97
5	52.55	47.34	45.23
6	62.66	58.42	53.24
8	71.11	66.98	61.64
12	78.22	69.13	65.71

Table 4. Stability study of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles.

Sl. No	Time in Week	CCN 1 Formulation			CCN 2 Formulation			CCN 3 Formulation		
		4°C	Room Temp	45°C	4°C	Room Temp	45°C	4°C	Room Temp	45°C
1	0	100	100	100	100	100	100	100	100	100
2	1	99.27	99.01	94.64	98.01	97.89	95.65	97.02	94.99	95.03
3	2	98.11	98.16	92.01	96.55	97.01	94.90	97.67	96.68	95.98
4	3	97.12	97.79	91.44	97.99	98.66	97.10	96.42	97.88	96.12
5	4	96.68	96.04	90.42	96.04	95.67	89.11	94.78	96.21	92.33

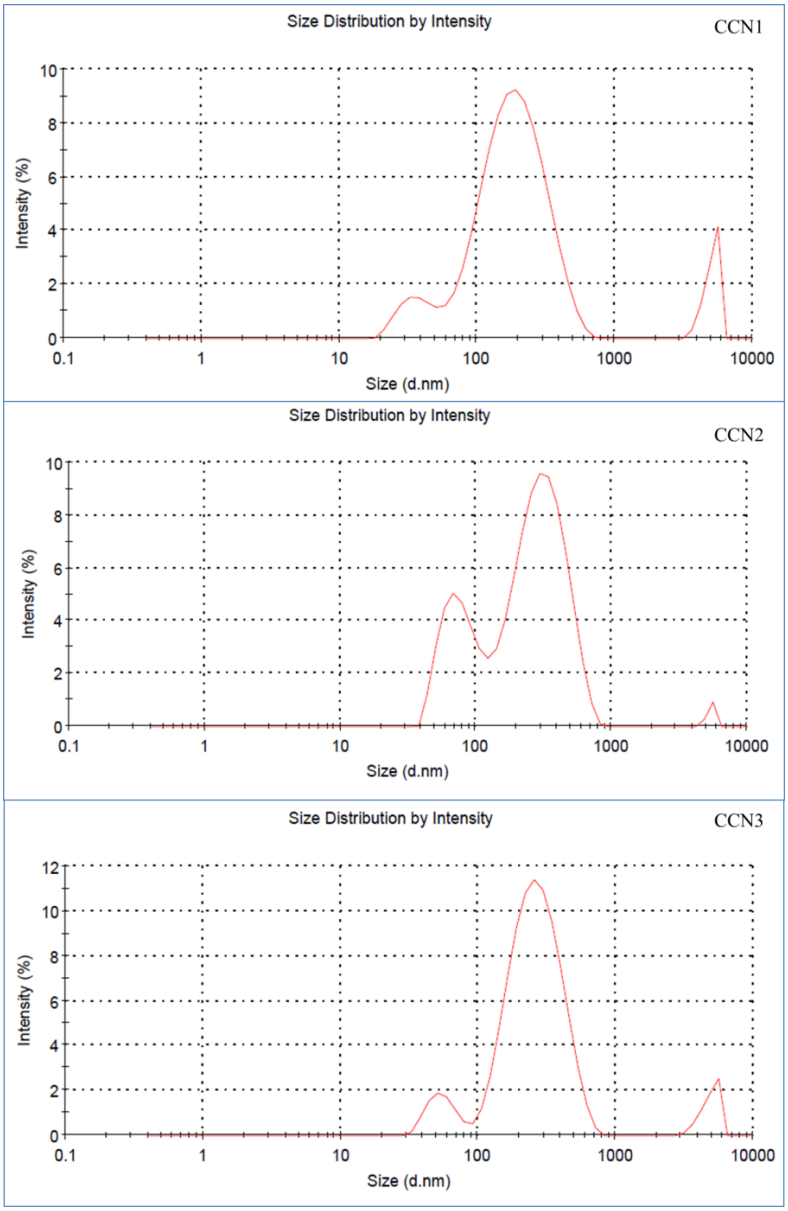

Fig 1. Size variability of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles.

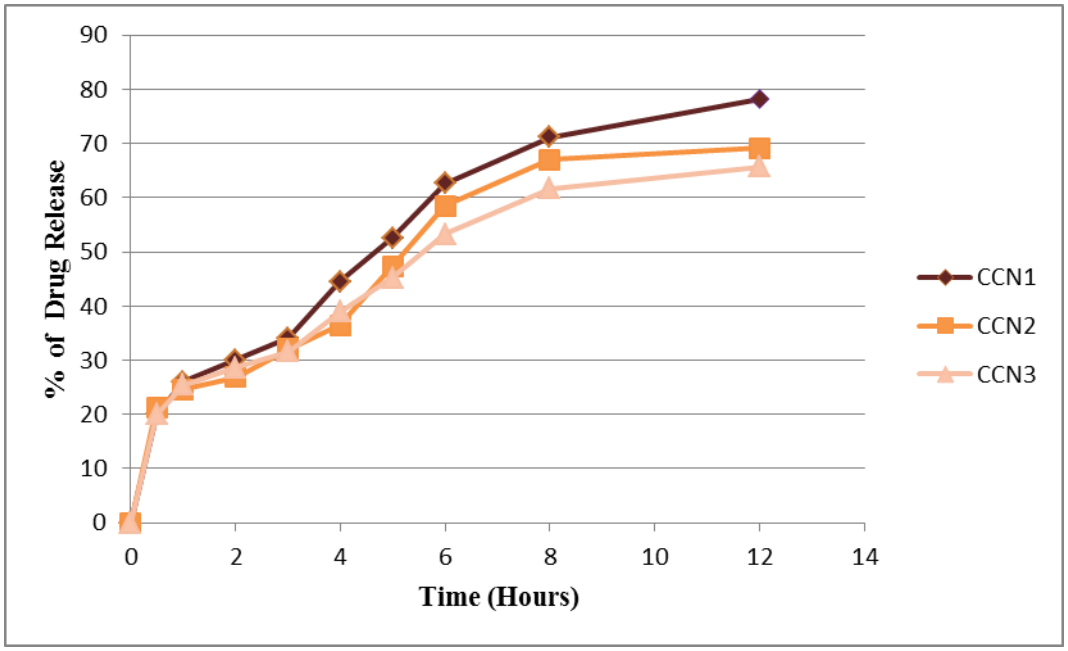

Fig 2.*In vitro* release of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles.

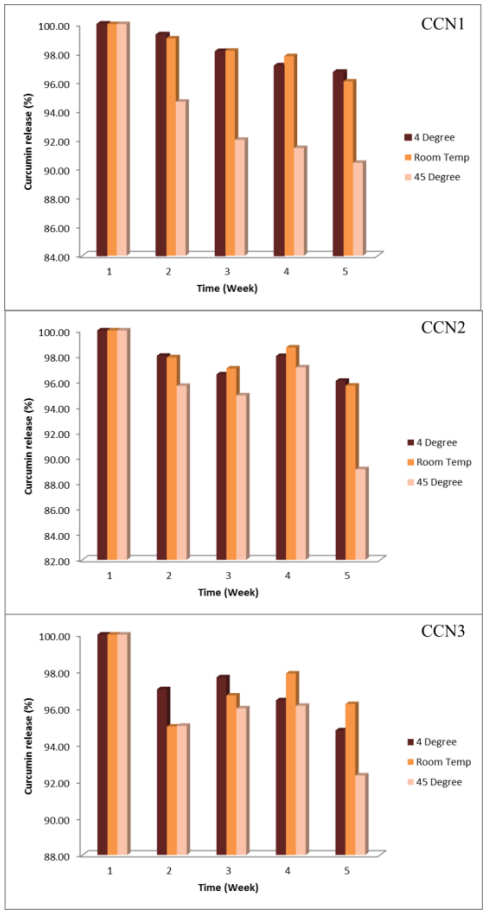

Fig 3. Stability studies of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles.

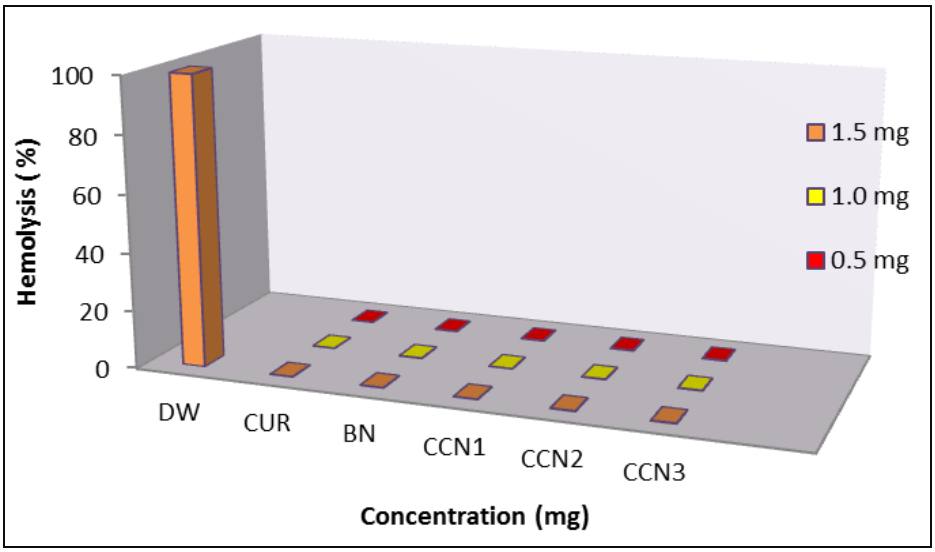

Fig 4. Hemolysis (%) of CCN1, CCN2 and CCN3 Curcumin encapsulated chitosan nanoparticles.

CONCLUSION

The present work demonstrates a simple method in producing Curcumin encapsulated chitosan nanoparticles CCN1, CCN2 and CCN3 can be prepared by cross-linking of chitosan with TPP by ionic gelation process. The formulations were characterized and found to have excellent encapsulation efficiency and better stability. All the formulations exhibited slow and sustained release of Curcumin in 12 hour study duration. The Curcumin encapsulated nanoparticles formulations were proven safe, shown no hemolysis suggesting considering for oral drug delivery applications.

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