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Synthesis, Characterization, Antioxidant and Cytotoxic Studies of Embelin Loaded N,O-CMC Nanoparticles

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ABSTRACT

Embelin is a long alkyl chain substituted hydroxy benzoquinone and naturally isolated from Embelia ribes. Embelin was reported for a widespread range of pharmacological properties that including anticancer activity. However, the potency of embelin as an anticancer molecule is inadequate due to its high lipophilicity. N,O-Carboxymethyl Chitosan (N,O-CMC) are elegance biopolymers which are toxic-free, biodegradable and biocompatible. This is seemly appropriate for numerous biological utilization, such as gene therapy, drug administration and tissue engineering. Hence, in the present study, we aimed to synthesize and characterize embelin loaded N,O-CMC nanoparticles and study its antioxidant and cytotoxic properties. The embelin N,O-CMC nanoparticles were prepared by loading embelin into N,O-CMC nanoparticles and characterized by FT-IR, DLS, SEM, Zeta potential and XRD measurements. Antioxidant and cytotoxic studies were conducted for the synthesized embelin loaded N,O-CMC nanoparticles using DPPH and MTT assay methods, respectively. The synthesized embelin loaded N,O-CMC nanoparticles with particle size ranges from 650-850 nm. The charge of embelin loaded N,O-CMC nanoparticles were confirmed by Zeta potential measurement and the value was found to be −47.8 mV. In the DPPH method, embelin loaded N,O-CMC nanoparticles showed IC_{50} value between 125-250 μg/ml. In MTT assay, embelin loaded N,O-CMC nanoparticles exhibited a significant reduction in the growth of osteosarcoma MG-63 cells. These findings demonstrating that the embelin loaded N,O-CMC nanoparticles were efficient nanocarrier for delivering embelin to cancer cells.

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INTRODUCTION

Cancer is one of the major health problems in all over the world. Currently used and commercially available anticancer drugs have some limitations such that non-specific biodistribution, deficiency of water solubility, low bioavailability and varied therapeutic indices. Nano-therapeutics can be opted as a solution to overcome these limitations. Chitosan is a toxic-free, biocompatible and biodegradable polymer that is being extensively explored for pharmaceutical purposes which includ-

ing drug administration (Anitha *et al.*, 2011; Rejinold *et al.*, 2011a,b), gene delivery (Jayakumar *et al.*, 2010), wound covering (Kumar *et al.*, 2010) and tissue engineering (Wang *et al.*, 2005). N,O-CMC has been synthesized by using chitosan, sodium hydroxide, isopropanol and chloroacetic acid with a temperature of 50-70°C. It has numerous attractive physical and biological properties such as moisture retention, water-solubility, biocompatibility and gel-formation, all of these together make it as a promising biomaterial (De-Abreu and Campana-Filho, 2009).

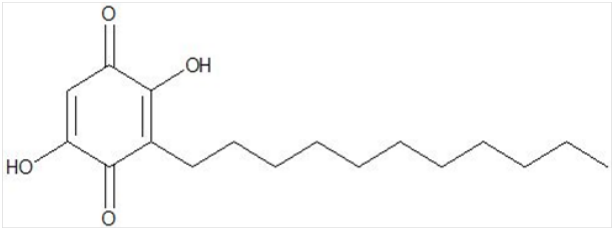

Figure 1: Structure of embelin

Pharmaceutical companies are progressively concerned in products derived from plants which achieve multiple natural purposes, safe and cheap when related with a synthetically derived agent. Embelin is a hydrophobic benzoquinone obtained from Embeliaribes. It has been reported to possess potential biological effects including antifertility, anti-implantation, antitumour, antioxidant, analgesic, anti-inflammatory, hepatoprotective, wound healing, antibacterial, antidiabetic and anticonvulsant properties (Mahendran *et al.*, 2011a,b,c). 125-Embelin also proven that it can be used for the treatment of inflammatory bowel syndrome and neurodegenerative disorders. Additionally, there are numerous articles reported its antitumor potential and capability to enrich TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, moderate NF-κB signaling cascade for increasing tumour cell apoptosis and suppress STAT3 as well as Akt/mTOR/S6K1 signaling cascades (Li *et al.*, 2019).

Regardless of these excellent characteristics, the main problem with embelin is it has poor solubility in water, hence greatly impacting its bioavailability and clinical efficacy. The oral bioavailability of embelin was very low and reported as 30.2 ± 11.9% (Li *et al.*, 2019). Different studies of embelin suggested that the poor bioavailability of embelin is caused majorly by low absorption, rapid metabolism and instantaneous elimination of embelin itself (Pathan and Bhandari, 2011).

Earlier studies in our laboratory, we have prepared embelin silver nanoparticles and tested for its *in-vitro* cytotoxicity study to enhance the aqueous solubility and bioavailability of this naturally isolated hydroxyl benzoquinone compound which is embelin (Othman and Sekar, 2019). However, the water solubility has not been attained as we expected when embelin was converted into embelin silver nanoparticles. Hence, in the present study we aimed to synthesize, characterize and antioxidant studies of embelin loaded N,O-CMC nanoparticles. Along with that, we also evaluated its cytotoxic effect against osteosarcoma MG-63 cells and 3T3 cells.

MATERIALS AND METHODS

Isolation of Embelin from Embeliaribes

Embelin was isolated from *Embelia ribes* and purified based on our earlier published protocol (Othman and Sekar, 2019) The purified embelin was stored at room temperature until further use. The chemical structure of embelin was shown in Figure 1.

Synthesis of N,O-CMC from Chitosan

Mixed 2 g of Chitosan and 20 ml of isopropyl alcohol and made it into a form of a slurry. Then 10 ml of 5 M NaOH solution was added drop-wise into the slurry and the reaction mixture was continuously stirred for 3 h at 60°C by adding mono chloroaceticacid in dropwise slowly at consistent intermissions. The obtained solution was filtered and the residue has been washed using enough quantity of methanol and made it to be dried completely in a hot air oven at 37°C for 24 h. The characterization was done after the sample was dried and used for the preparation of nanoparticles (Anitha *et al.*, 2012).

Synthesis of N,O-CMC Nanoparticles

10 ml of 0.05 % N,O-CMC solution was prepared using distilled water and added 0.2 ml 0.5% TPP solution under continuous and constant stirring for 30 min. The obtained nanoparticles were purified by centrifugation process at 20000 rpm for 45 min and lyophilized. The characterization of lyophilized N,O-CMC nanoparticles, was done for further experiment (Anitha *et al.*, 2009).

Synthesis of Embelin-Loaded N,O-CMC Nanoparticles

100 mg of N,O-CMC was dissolved in 200 ml of Millipore water and kept it under continuous stirring. Then, 20 mg/ml solution of embelin was prepared using ethanol and added drop-wise with frequent intervals into N,O-CMC solution under continuous stirring. 0.5% of the TPP solution was added into the resultant solution of polymer and continuously stirred for 1 h, resulting in the formation of embelin loaded N,O-CMC nanoparticles (Anitha *et al.*, 2012).

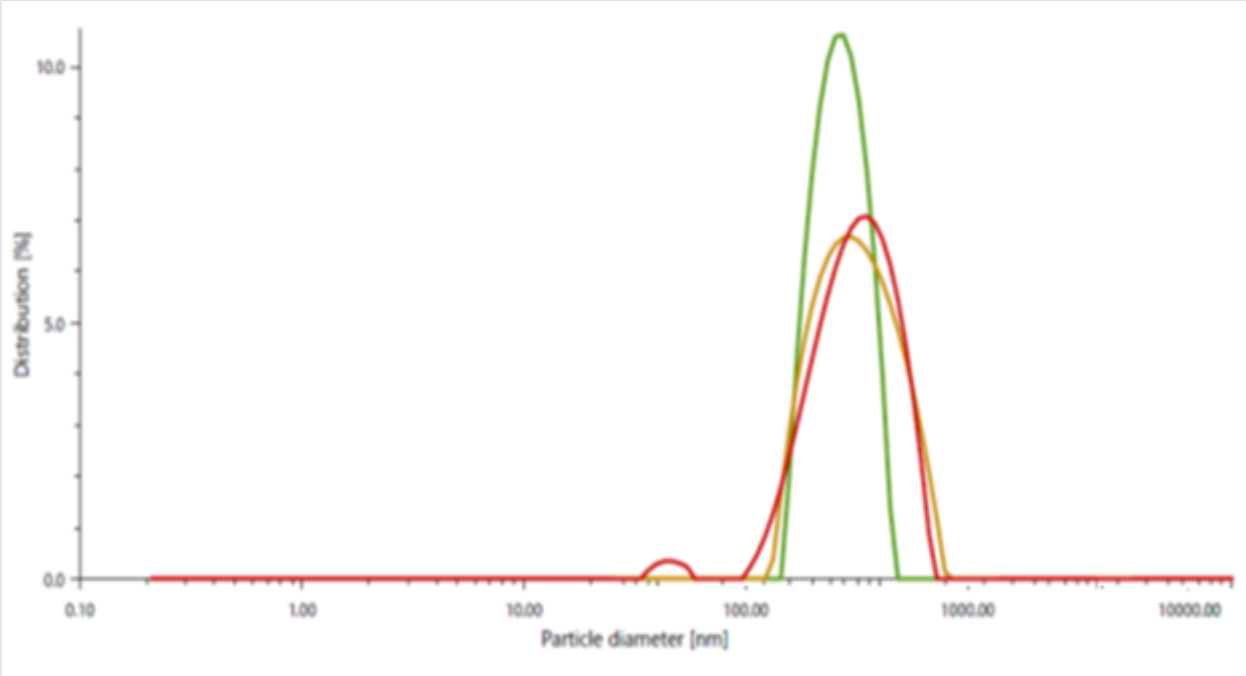

Figure 2: Particle size distribution of embelin loaded N,O-CMC nanoparticles by DLS

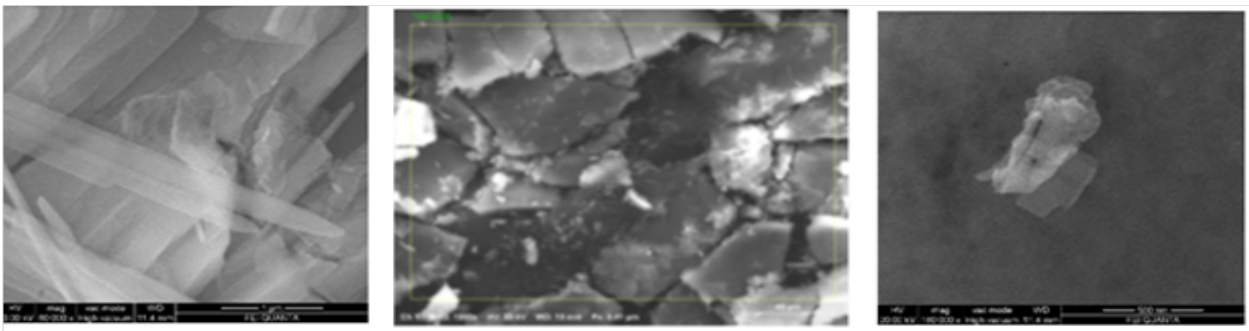

Figure 3: SEM images of embelin loaded N,O-CMC nanoparticles in different magnifications scale

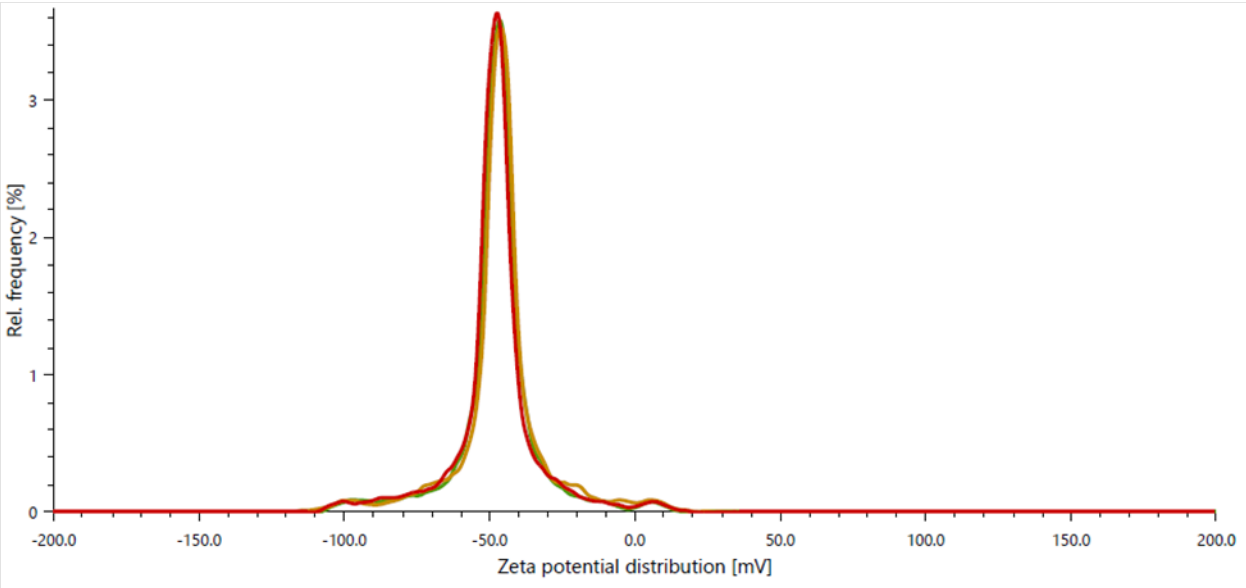

Figure 4: Zeta potential distribution of embelin loaded N,O-CMC nanoparticles

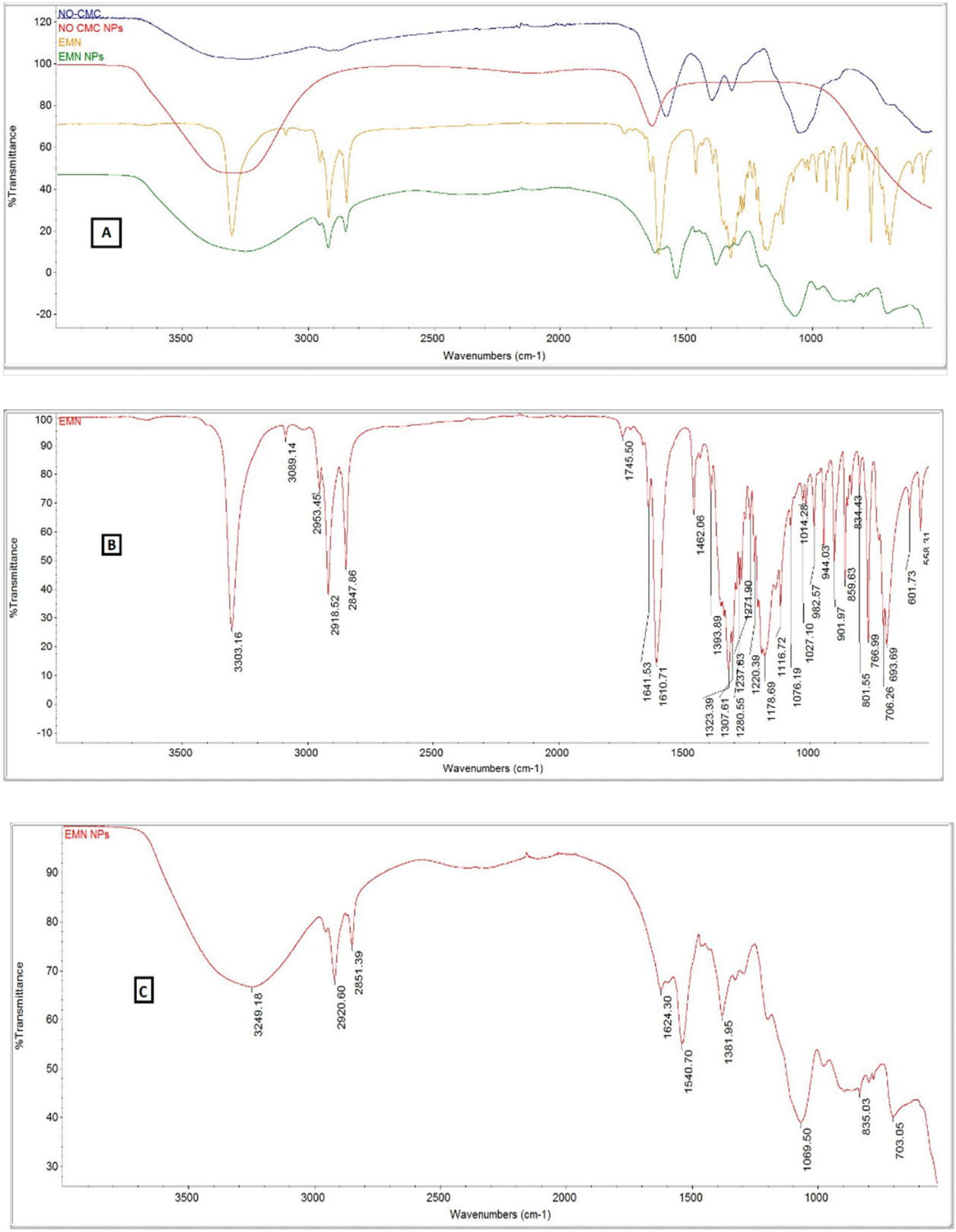

Figure 5: A) Comparison of FT-IR spectrums of N,O-CMC, N,O-CMC nanoparticles, embelin and embelin loaded N,O-CMC nanoparticles B) FT-IR spectrum of Embelin C) FT-IR spectrum of Embelin loaded N,O-CMC nanoparticles

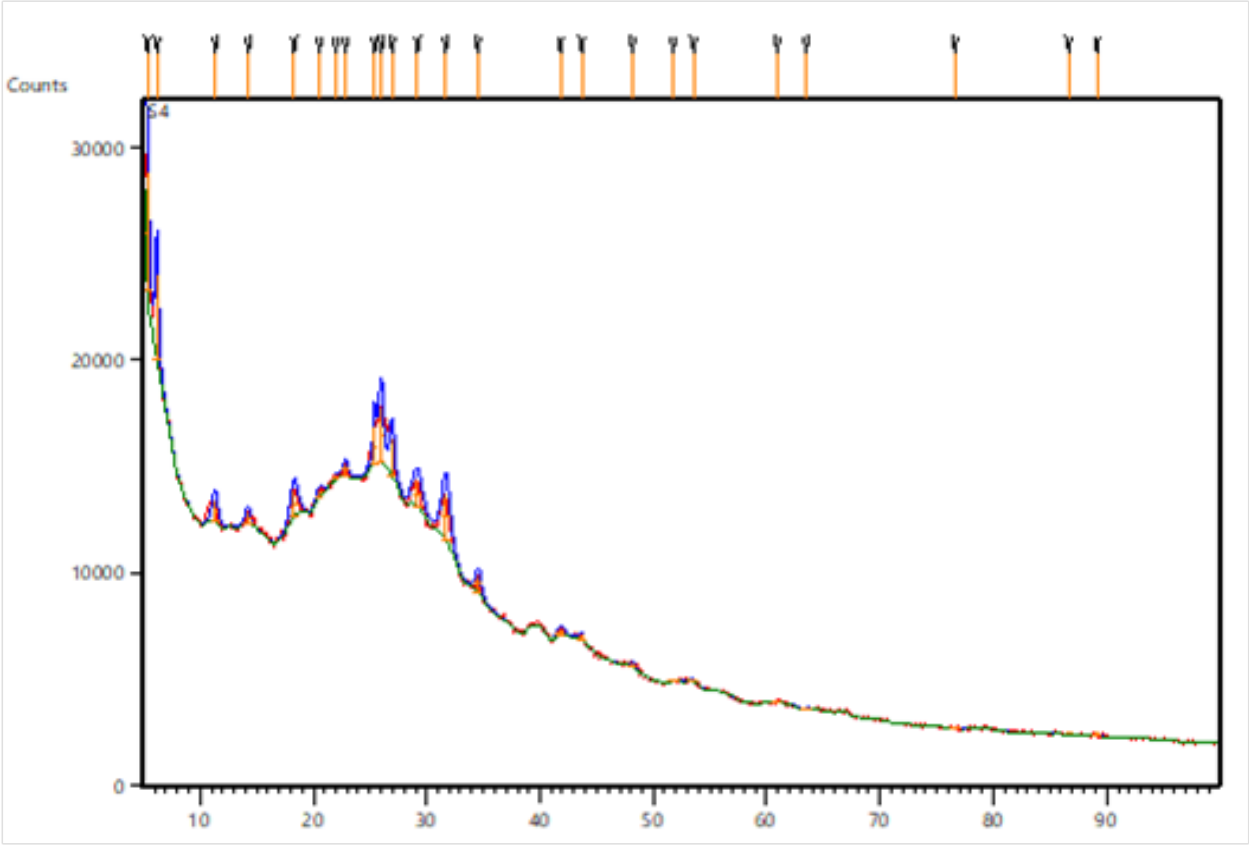

Figure 6: XRD pattern of embelin loaded N,O-CMC nanoparticles

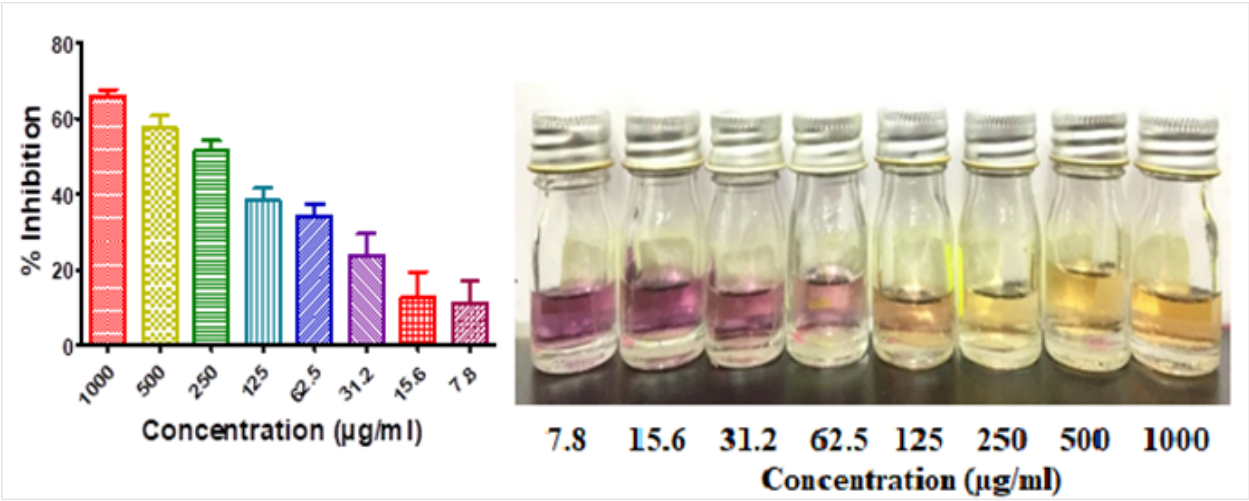

Figure 7: *In-vitro* antioxidant activity of embelin loaded N,O-CMC nanoparticles by DPPH method

Characterization of Synthesized Embelin Loaded N,O-CMC Nanoparticles

The prospective interaction between embelin and N,O-CMC nanoparticles were identified using Fourier Transform Infrared Spectroscopy (FT-IR). X-ray diffraction (XRD) analysis was used to explore the inherent attributes of embelin which is present in embelin loaded N,O-CMC nanoparticles. The size distribution of N,O-CMC nanoparticles were analyzed by Dynamic Light Scattering (DLS). Surface morphology and size of the obtained nanoparticles were further visualized by Scanning Electron Microscopy (SEM). The determination of the surface charge of the nanoparticles were done by zeta potential measurements.

In-vitro Antioxidant Activity of Embelin Loaded N,O-CMC Nanoparticles by DPPH method

The *in-vitro* antioxidant study of embelin loaded N,O-CMC nanoparticles were carried out by the DPPH method using the standard procedure men-

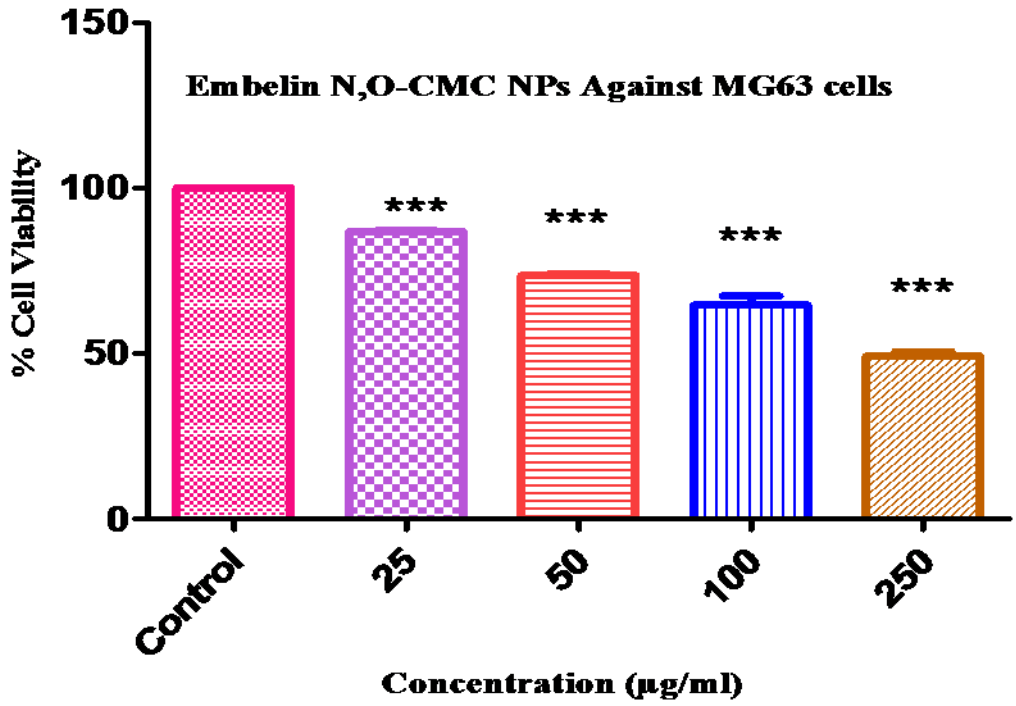

Figure 8: Effect of embelin loaded N,O-CMC nanoparticles against Osteosarcoma MG63 Cells [*P<0.001, when compared to control(n=3), Turkey-Kramer].**

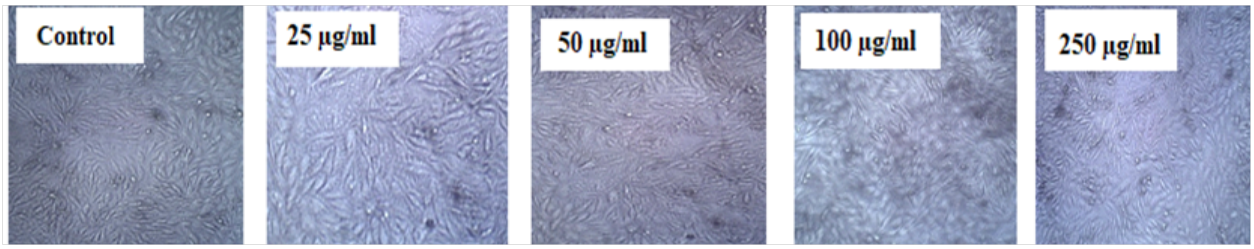

Figure 9: Morphological changes induced in Osteosarcoma MG-63 Cells upon treated with embelin loaded N,O-CMC nanoparticles in different concentrations

Table 1: *In-vitro* antioxidant activity of embelin loaded N,O-CMC nanoparticles by DPPH Method

Concentration (μg/ml)	Absorbance value at 490 nm in DPPH method						% Inhibition (Mean±SD)	IC_{50}
	1^{st} Trial	% Inhibition	2^{nd} Trial	% Inhibition	3^{rd} Trial	% Inhibition		
Control	0.522	–	0.554	–	0.535	–	–	
1000	0.186	64.37%	0.189	65.88%	0.173	67.66%	65.97±1.65%	
500	0.209	59.96%	0.255	53.97%	0.221	58.69%	57.54±3.16%	
250	0.245	53.07%	0.288	48.01%	0.252	52.90%	51.33±2.88%	125-
125	0.342	34.48%	0.331	40.25%	0.321	40.00%	38.24±3.26%	250
62.5	0.363	30.46%	0.353	36.28%	0.345	35.51%	34.08±3.16%	μg/ml
31.2	0.429	17.82%	0.424	28.47%	0.399	25.42%	23.90±5.49%	
15.6	0.492	5.75%	0.481	13.18%	0.432	19.25%	12.73±6.76%	
7.8	0.496	4.98%	0.487	12.09%	0.447	16.45%	11.17±5.80%	

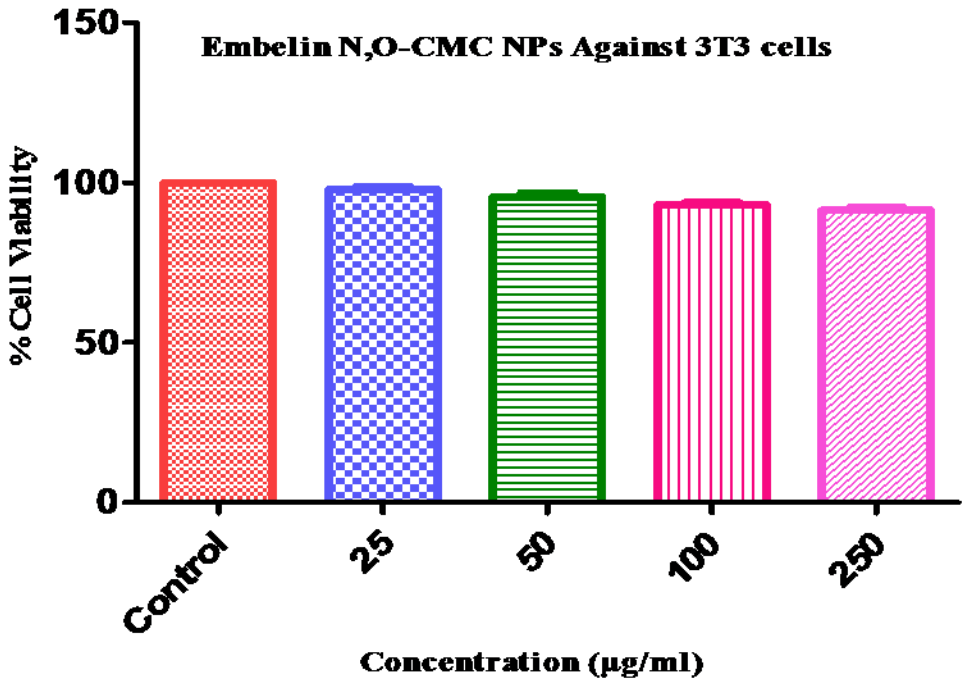

Figure 10: Effect of embelin loaded N,O-CMC nanoparticles against normal 3T3 cells

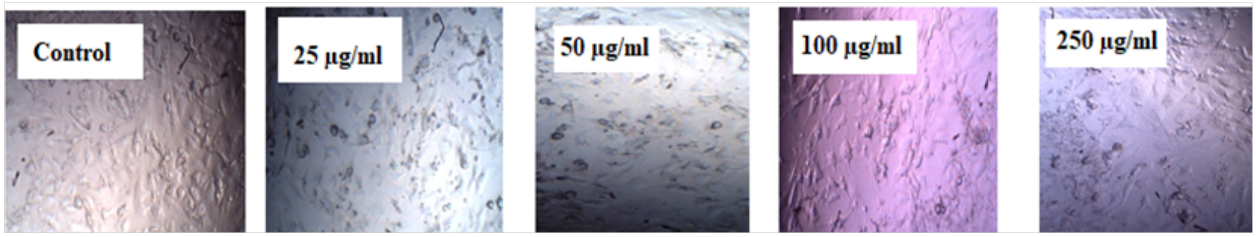

Figure 11: Morphological changes induced in 3T3 cells upon treated with embelin loaded N,O-CMC Nanoparticles in different concentrations

tioned in our earlier published protocol (Othman and Sekar, 2019). The absorbance was measured at 490 nm using a UV-visible spectrophotometer (Othman and Sekar, 2019). The percentage inhibition was calculated using the formula below,

Percentage inhibition = [(Abs Control –Abs Sample) X 100) / (Abs control)]

In-vitro Cytotoxic Activity of Embelin Loaded N,O-CMC Nanoparticles by MTT assay

The *in-vitro* cytotoxic study of embelin loaded N,O-CMC nanoparticles in four different concentrations (25, 50, 100 and 250 μg/ml) were carried out by MTT assay method using the standard procedure mentioned in our earlier published protocol (Othman and Sekar, 2019). The absorbance was measured using a microplate reader at 540 nm. Control values were set at 100% viable and all values were expressed as a percentage of the control and respective concentrations were calculated.

Statistical Analysis

Data were expressed as mean $\pm$ SD of three replicate measurements. The statistical analysis was carried out by one way ANOVA followed by multiple comparison tests of Turkey-Kramer. P values <0.05 were considered as significant.

RESULTS AND DISCUSSION

The size distribution of embelin loaded N,O-CMC nanoparticles was obtained using DLS showed that the nanoparticles lie within a size range of 650-850 nm (Figure 2). The morphology was further confirmed by SEM. The SEM image of embelin loaded N,O-CMC nanoparticles indicates rod shape particles with a size range between 650-850 nm (Figure 3). Zeta potentials were measured for embelin-N,O-CMC nanoparticles and the value was found to

be -47.8 mV (Figure 4).

FT-IR spectrum of N,O-CMC, N,O-CMC nanoparticles, embelin and embelin-N,O-CMC nanoparticles were taken and compared (Figure 5). In the spectrum of N,O-CMC, the peak observed at 3265 cm^{-1} was due to the –OH group, the peak at 1578 cm^{-1} was due to the carboxylic group, and the peak at 1630 $cm^{-1}$was due to the presence of an amino group. After developing nanoparticles, the peak at 1630 cm^{-1} was slightly shifted to 1636 cm^{-1}, confirming that the phosphate groups present in TPP undergoing a cross-linking reaction with the protonated amine groups of N,O-CMC (Anitha *et al.*, 2012). The FT-IR spectrum of embelin showed a peak at 3303 cm^{-1}, attributed to the -OH stretching vibration. Additionally, very sharp absorption bands at 2919 and 2848 cm^{-1} for a long alkyl chain (-CH stretching), 1641 cm^{-1} (α, β- unsaturated C=O) and 1179 (C–O stretching vibration) were observed. Because of the complexation of embelin into an embelin-N,O-CMC nanoparticles, N,O-CMC- related peaks were slightly shifted. When comparing N,O-CMC nanoparticles with embelin-N,O-CMC nanoparticles, a peak shift was observed from 3265 to 3249 cm^{-1} and from 1641 to 1624 cm^{-1}. Furthermore, the peaks in embelin-N,O-CMC nanoparticles exhibited broadening due to the remarkable interaction among the ingredients within the nanoparticles. These data validated that the embelin was present in N,O-CMC nanoparticle matrices.

The XRD of embelin loaded N,O-CMC nanoparticles, was taken to understand its physical nature. The result showed that embelin loaded N,O-CMC nanoparticles did not contain such crystalline peaks, potentially caused by the development of an indefinite any composite during the formation of nanoparticles within the nanoparticle matrix (Figure 6).

In the *in-vitro* antioxidant activity using the DPPH method, embelin-N,O-CMC nanoparticles showed potent antioxidant capability in the tested concentrations (Table 1). The IC_{50} value was found to be between 125-250 μg/ml. There was a colour change from purple to yellow in the DPPH solution indicates that embelin-N,O-CMC nanoparticles having a significant antioxidant activity (Figure 7). This result was consistent with our previous study result, which is on the antioxidant activity of embelin silver nanoparticles in the DPPH method (Othman and Sekar, 2019).

Four different concentrations of embelin-N,O-CMC nanoparticles (25, 50, 100 and 250 μg/ml) were investigated for cytotoxicity study against Human MG-63 (osteosarcoma cells) by MTT assay. Embelin-N,O-CMC nanoparticles exhibited a significant reduction ($P<0.001$) of cancerous cell growth in all the tested concentrations in a dose-dependent manner. Hence, for testing in normal cells (3T3), these concentrations were selected. There was no significant toxicity associated with embelin-N,O-CMC nanoparticles with normal cells indicates the safety of the synthesized nanoparticles (Figures 8, 9, 10 and 11).

Cancer is a serious life-threatening disease in humans. Numerous anticancer medicines are available in the market which are unable to be used extensively in clinical treatment due to their severe harmful outcomes and quick, impactful half-lives *in-vivo*. Nanotechnology combined with therapeutic drugs produces an extended concept in overcoming these issues, concurrently supported by previous studies on anticancer efficacy of nanoparticles loaded with drugs *in-vivo* and *in-vitro* toward various types of cancer (Wang *et al.*, 2016). (Snima *et al.*, 2012) reported that O-CMC-metformin nanoparticles can increase anticancer effects towards pancreatic cancer cells, while the combinatorial anticancer effects of 5-fluorouracil and curcumin-loaded N,O-CMC nanoparticles towards colon cancer cells were demonstrated by (Anitha *et al.*, 2014). Previous findings in our laboratory confirmed that embelin silver nanoparticles showed a significant cytotoxic effect against cancer cells (Othman and Sekar, 2019). In the present study, we successfully synthesized embelin loaded N,O-CMC nanoparticles and characterized by DLS, SEM, FT-IR, Zeta potential and XRD measurements.

In the DPPH method, embelin loaded N,O-CMC nanoparticles showed significant antioxidant activity and the MTT assay results indicated that the synthesized embelin loaded N,O-CMC nanoparticles could inhibit the growth of Osteosarcoma MG-63 cells. These results were very well correlated with our previous study results of embelin silver nanoparticles in DPPH and MTT assay methods (Othman and Sekar, 2019).

CONCLUSIONS

Embelin loaded N,O-CMC nanoparticles were prepared and characterized by FT-IR, DLS, SEM, Zeta potential and XRD. These results showed that embelin was effectively loaded into N,O-CMC nanoparticles with the size ranging at 650-850 nm. This embelin N,O-CMC nanoparticles showed potent antioxidant and cytotoxic properties in DPPH and MTT assay methods, respectively. This preliminary study results indicated that the synthesized embelin N,O-CMC nanoparticles could become a potential medium for transporting hydrophobic drugs such as

embelin comparable to the already reported embelin silver nanoparticles, making it preferable for drug-delivery purposes. However, further studies are recommended to decrease the particle size of embelin-N,O-CMC nanoparticles and tested against other cancer cells to confirm its safety and efficacy.

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