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Synthesis and Characterization of Mangiferin Loaded N,O-CMC Nanoparticles and its Cytotoxic Effect on Osteosarcoma MG-63 Cells

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ABSTRACT

Mangiferin is a xanthone glycoside, naturally isolated from *Mangifera indica*. Mangiferin has been reported for a wide range of pharmacological activities and its anticancer potential is very well known. However, the mangiferin anti-cancer potency is inadequate due to its poor water solubility. N,O-Carboxymethyl Chitosan (N,O-CMC) is a smart biopolymer, in which its biocompatible, biodegradable and non-toxic making it ideal for abundant biological applications include the delivery of lipid soluble drugs. Also useful to improve and replace biological tissues and gene therapy. Hence, this study attempts to synthesize and characterize mangiferin-N,O-CMC nanoparticles and evaluate its antioxidant and cytotoxic properties. The mangiferin-N,O-CMC nanoparticles were prepared by loading mangiferin into N,O-CMC nanoparticles and characterized by FT-IR, DLS, SEM, Zeta potential and XRD measurements. *In-vitro* antioxidant was carried out by the DPPH method. The synthesized mangiferin-N,O-CMC nanoparticles with particle size ranges from 200±10 nm. The charge of N,O-CMC nanoparticles were confirmed by Zeta potential and found to be −45.8 mV. In the DPPH method, mangiferin-N,O-CMC nanoparticles showed IC_{50} value between 7.8-15.6 μg/ml. In MTT assay, mangiferin-N,O-CMC nanoparticles exhibited a significant reduction in the growth of osteosarcoma MG-63 cells and there is no toxic effect against normal 3T3 cells. These findings designated that the synthesized mangiferin-N,O-CMC nanoparticles were very efficient nanocarrier in delivering the mangiferin to cancer cells.

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INTRODUCTION

Mangiferin is a xanthone glycoside, mainly obtained from *Mangifera indica* and reported to possess many pharmacological effects that including antioxidant, analgesic, anti-inflammatory, antidiabetic, neuroprotective, hepatoprotective, cardioprotective and anticancer studies (Mahendran *et al.*, 2014; Sekar, 2015). Over the past few decades, mangiferin has been comprehensively studied concerning the anticancer properties. There are few evidences strongly supported that mangiferin has been used to prevent the growth and development of cancer cells by mod-

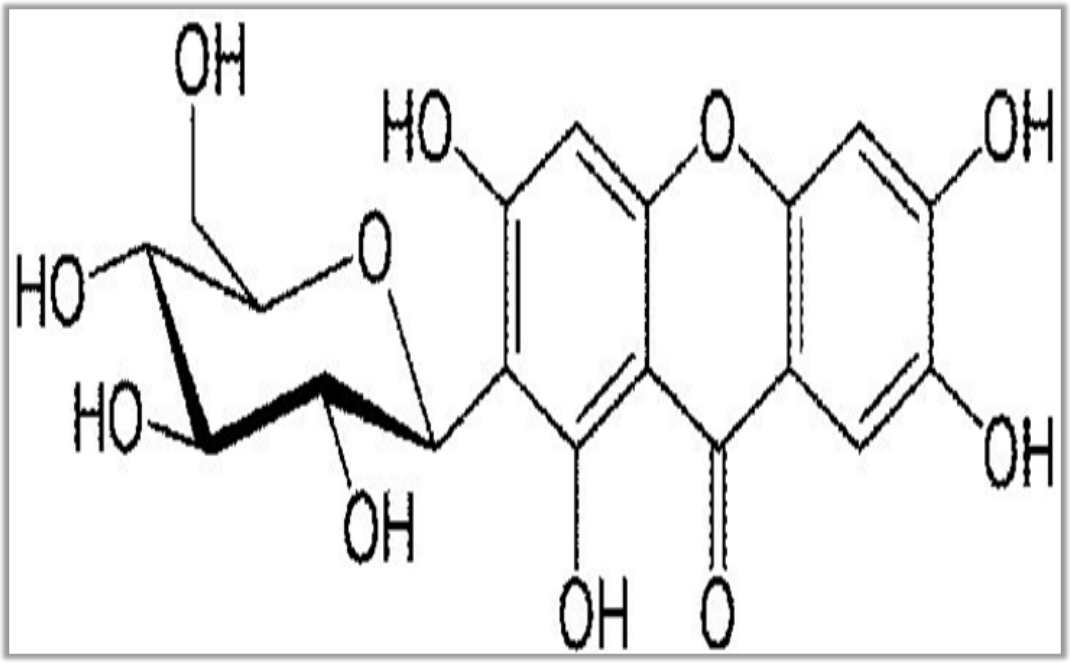

Figure 1: Structure of mangiferin

ulation of many molecular pathways. The literature of mangiferin constantly showed that it has synergistic effects with chemotherapeutic agents include the etoposide, oxaliplatin and doxorubicin (Gold-Smith *et al.*, 2016). Perhaps the most noticeable anti-proliferative effect of mangiferin on tumour cells has been detected in animal experimental models, where the tumour volume was reduced similarly with a standard drug cisplatin. Mangiferin also exhibits other promising features including low toxicity with wide oral safety margin, as pointed out by (Gold-Smith *et al.*, 2016).

Nevertheless, mangiferin has low solubility in water which confines its clinical efficacy and bioavailability. (Hou *et al.*, 2012), also reported that the absorption range of mangiferin was improved when large dose was administration. Also mentioned that the mangiferin pharmacokinetics profile was nonlinear in human, the major reason for poor bioavailability of this naturally isolated mangiferin is mainly because of its low water solubility and absorption. In this regard, (Othman and Sekar, 2019) attempted to increase its aqueous solubility and bioavailability by converting mangiferin into silver nanoparticles and studied for its in-vitro cytotoxicity study against cancer cells. However, the water solubility has not been achieved as expected when mangiferin was converted into silver nanoparticles.

Chitosan, a well-known amino polysaccharide with its biodegradability, biocompatibility and low cost. The interesting characteristics of chitosan have made it be widely used in pharmaceuticals, agriculture, medicine, food and biotechnology fields. Although chitosan is insoluble in water,its water solubility could be improved when it was converted into carboxymethyl chitosan (Yang *et al.*, 2017). N,O-CMC has been prepared using chitosan, isopropanol, chloroacetic acid and sodium hydroxide by maintaining the temperature at 50-70 °C. It has abundant attractive physical and biological properties such as biocompatibility, moisture retention, aqueous solubility and gel-formation. All of these properties together make it that N,O-CMC as a promising biomaterial (De-Abreu and Campana-Filho, 2009). Hence, the present study aims to synthesize, characterize mangiferin loaded N,O-CMC nanoparticles and subsequently evaluating its effect against osteosarcoma MG63 cells. Along with that we also evaluated its antioxidant activity by DPPH method.

MATERIALS AND METHODS

Isolation of Mangiferin from *Mangifera indica*

Mangiferin was isolated from *Mangifera indica* and purified based on our earlier published protocol (Othman and Sekar, 2019). The chemical structure of mangiferin is shown in Figure 1.

Preparation of N,O-CMC from Chitosan

2 g of Chitosan was mixed with 20 ml of isopropyl alcohol and prepared into the form of a slurry. This was followed by a dropwise addition of 10 ml of 5M NaOH solution. The reaction mixture was then stirred continuously for 3 h at 60^{o}C by adding

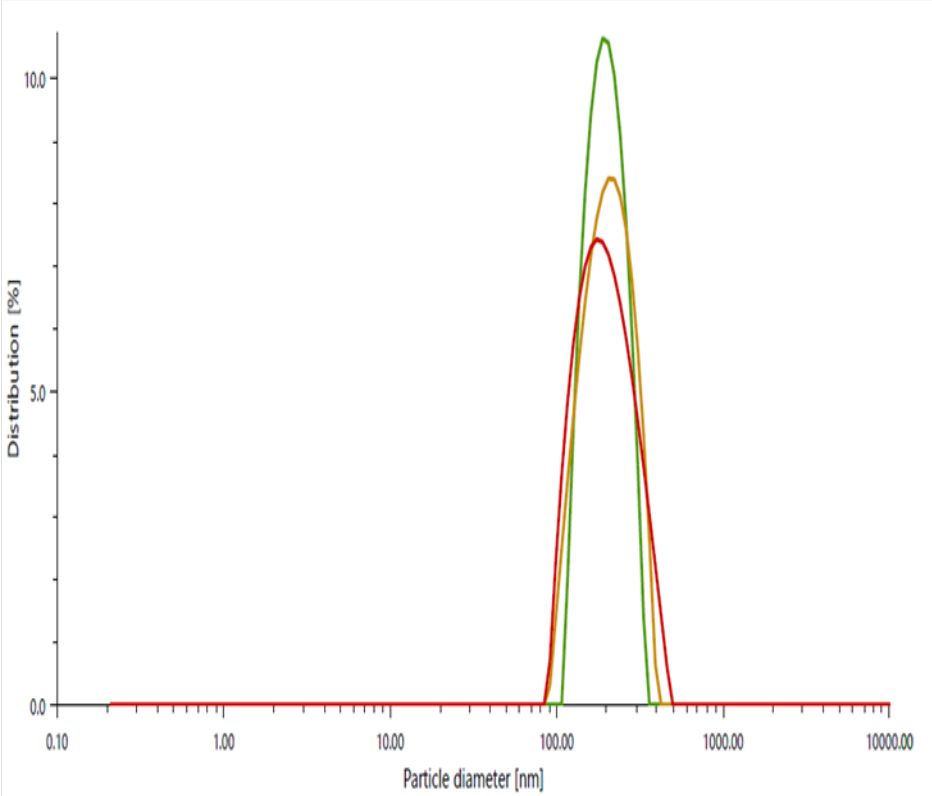

Figure 2: Particle size distribution of mangiferin loaded N,O-CMC nanoparticles by DLS

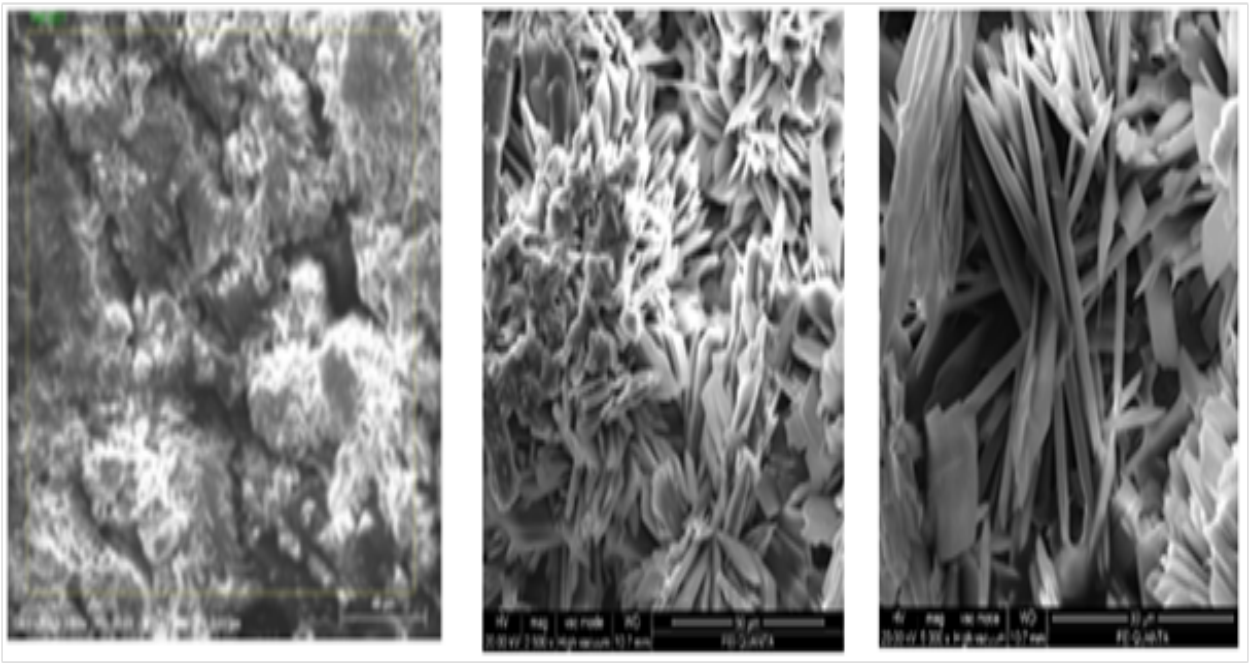

Figure 3: SEM Images of mangiferin loaded N,O-CMC nanoparticles in different magnifications scale

Table 1: *In-vitro* antioxidant activity of mangiferin-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	0.127	75.67%	0.128	76.90%	0.135	74.76%	75.78±1.07%	
500	0.129	75.29%	0.139	74.91%	0.142	73.46%	74.55±0.97%	
250	0.166	68.20%	0.186	66.43%	0.176	67.10%	67.24±0.89%	
125	0.176	66.28%	0.199	64.08%	0.188	64.86%	65.07±1.11%	
62.5	0.194	62.84%	0.207	62.64%	0.210	60.75%	62.08±1.15%	
31.2	0.223	57.28%	0.233	57.94%	0.240	55.14%	56.79±1.46%	
15.6	0.232	55.56%	0.250	54.49%	0.265	50.47%	53.51±2.68%	7.8-15.6
7.8	0.308	41.00%	0.301	45.67%	0.323	39.63%	42.10±3.17%	μg/ml

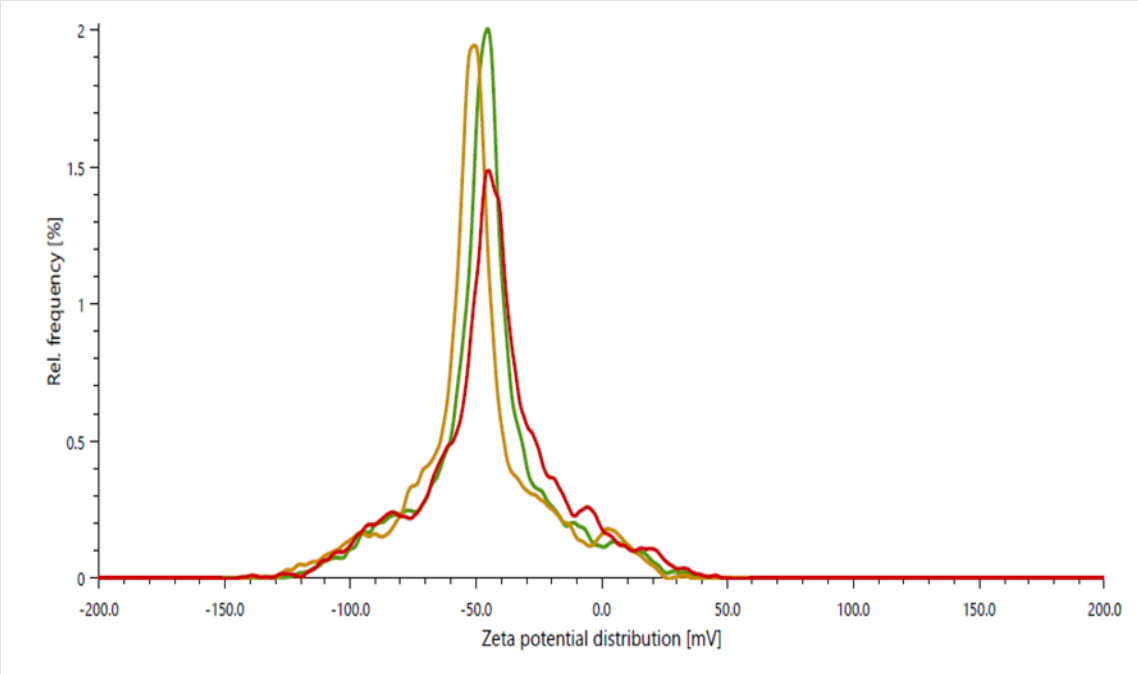

Figure 4: Zeta Potential distribution of mangiferin loaded N,O-CMC nanoparticles

monochloroacetic acid in dropwise slowly at consistent intermissions. After that, the solution was filtered and the residue was washed using enough quantity of methanol and dried in a hot air oven at 37°C for 24 h. The dried sample was characterized and used for nanoparticle synthesis (Anitha *et al.*, 2012).

Preparation of N,O-CMC Nanoparticles

10 ml of 0.05 % N,O-CMC solution was prepared first using distilled water, then 0.2 ml 0.5% TPP solution was added under continuous stirring for 30 min. Then, the resultant nanoparticles were centrifuged at 20000 rpm for 45 min and lyophilized. The lyophilized N,O-CMC nanoparticles were used for characterization and further studies (Anitha *et al.*, 2009).

Preparation of Mangiferin-Loaded N,O-CMC Nanoparticles

200 mg of N,O-CMC was dissolved in 400 ml Millipore water and kept it under continuous stirring. Then, 40 mg/ml solution of mangiferin was prepared using ethanol and added drop wise with frequent intervals into N,O-CMC solution under continuous stirring. 0.75% of the TPP solution was then added into the resulting polymer solution. After stirring continuously for 2 h, the mangiferin loaded N,O-CMC nanoparticles were formed (Anitha *et al.*, 2012).

Characterization of Synthesized N,O-CMC

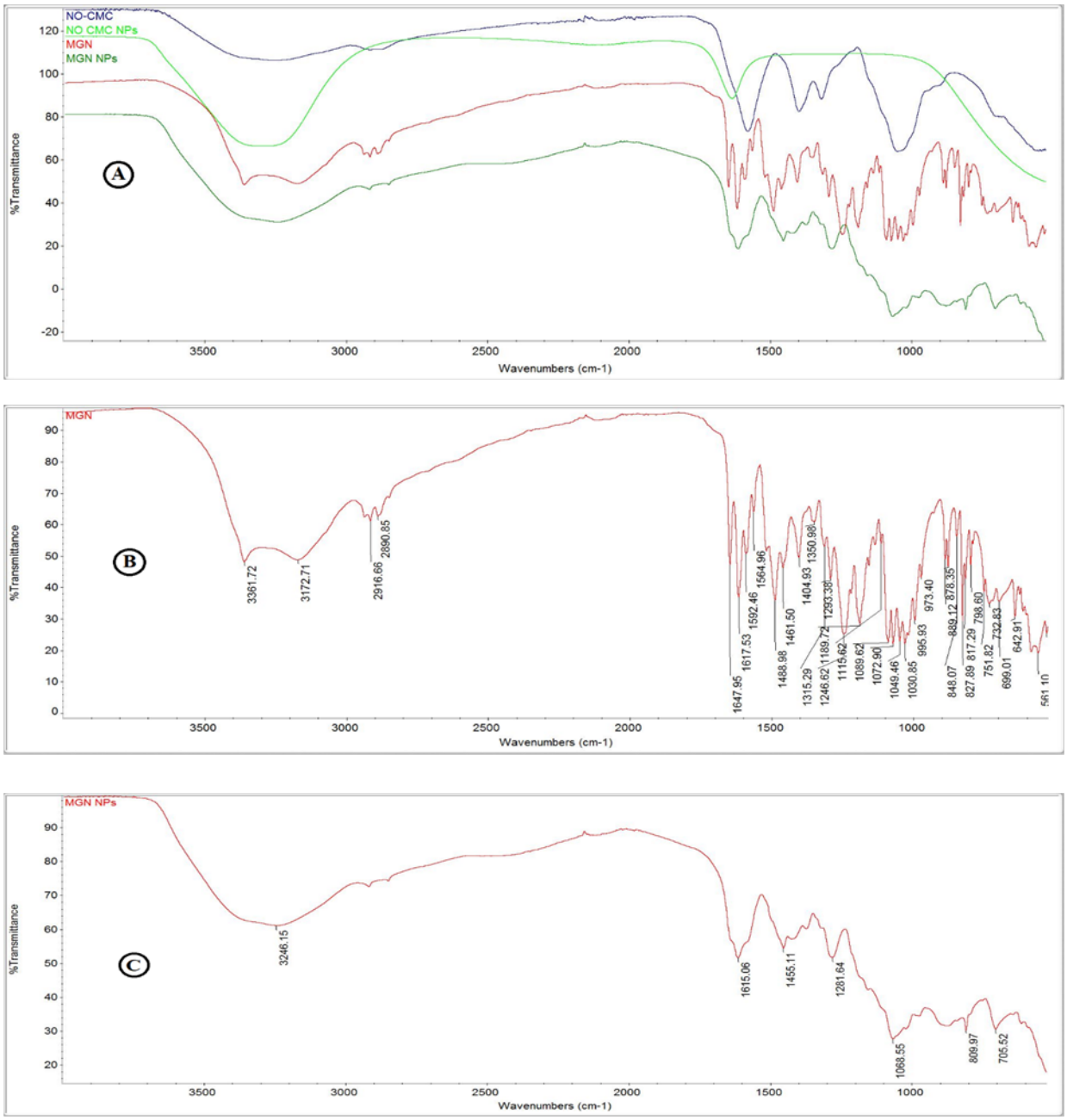

Figure 5: A) Comparison of FT-IR spectrums of N,O-CMC, N,O-CMC nanoparticles, mangiferin and mangiferin loaded N,O-CMC nanoparticles B) FT-IR spectrum of mangiferin C) FT-IR spectrum of mangiferin loaded N,O-CMC nanoparticles

Nanoparticle

The potential interaction between mangiferin and N,O-CMC nanoparticles was identified using Fourier Transform Infrared Spectroscopy (FT-IR), whereas X-ray Diffraction (XRD) analysis was done to understand the physical nature of mangiferin that is present in mangiferin-N,O-CMC nanoparticles. The size distribution of N,O-CMC nanoparticles was analyzed by Dynamic Light Scattering (DLS). The surface morphology and the surface charge of nanoparticles were further determined by Scanning Electron Microscopy (SEM) and Zeta Potential measurements, respectively.

In-vitro antioxidant activity by DPPH method

A 100 μl of mangiferin-N,O-CMC nanoparticle solution in different concentrations were added to 2 ml of 100 μM DPPH solution which was prepared earlier by using methanol. The reaction mixture was incubated at 37^{o}C for 20 min and the absorbance was determined at 490 nm using UV-visible spectrophotometer (Othman and Sekar, 2019). The percentage inhibition was calculated as follows,

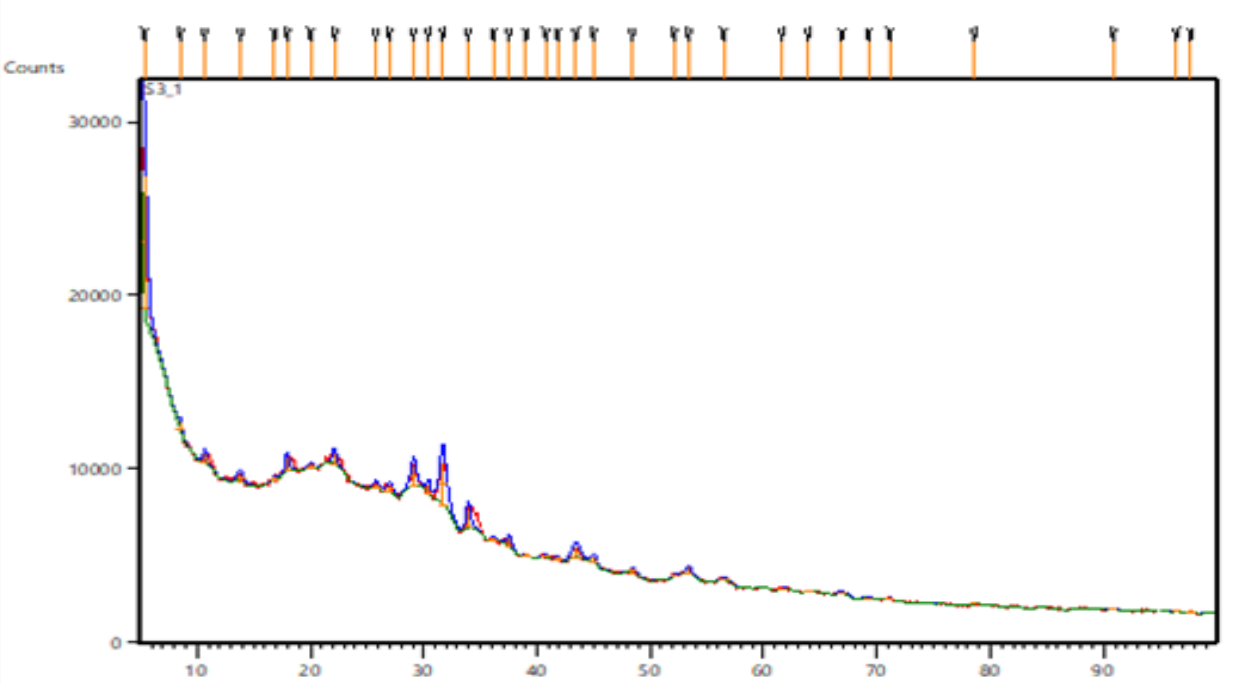

Figure 6: XRD Pattern of mangiferin loaded N,O-CMC nanoparticles

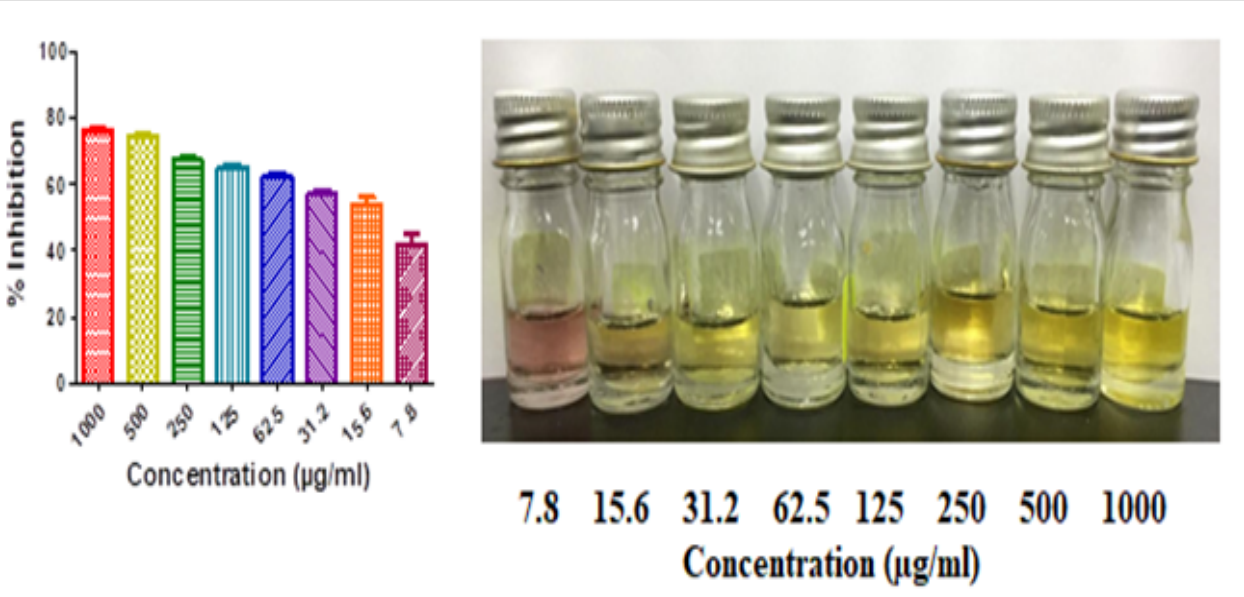

Figure 7: *In-vitro* antioxidant activity of mangiferin-N,O-CMC nanoparticles by DPPH method

Percentage inhibition =

$$\left[\left(\frac{Abs\ Control\ -Abs\ Sample}{Abs\ control}\right)\times 100\right]$$

***In-vitro* cytotoxic activity by MTT assay**

The *in-vitro* cytotoxic study of mangiferinloaded 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. Note that, control values were set at 100% viable and the respective concentrations were calculated and expressed as a percentage of the control.

Statistical Analysis

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

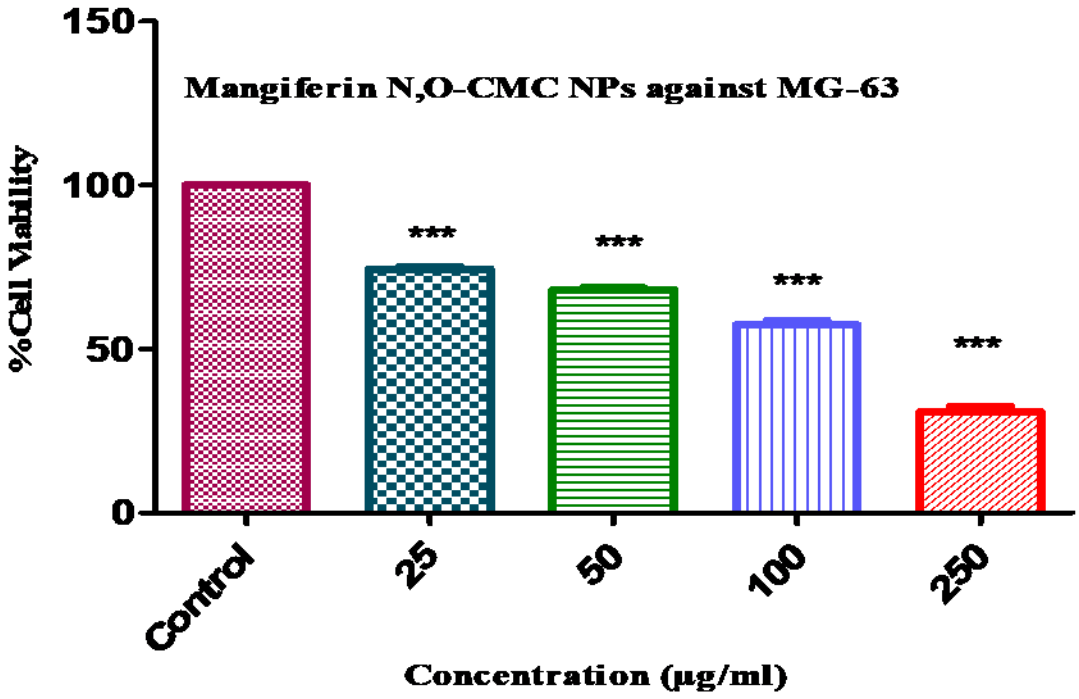

Figure 8: Effect of mangiferin loaded N,O-CMC nanoparticles against Osteosarcoma MG63 Cells. [*P<0.001, when compared to control(n=3), Turkey-Kramer]**

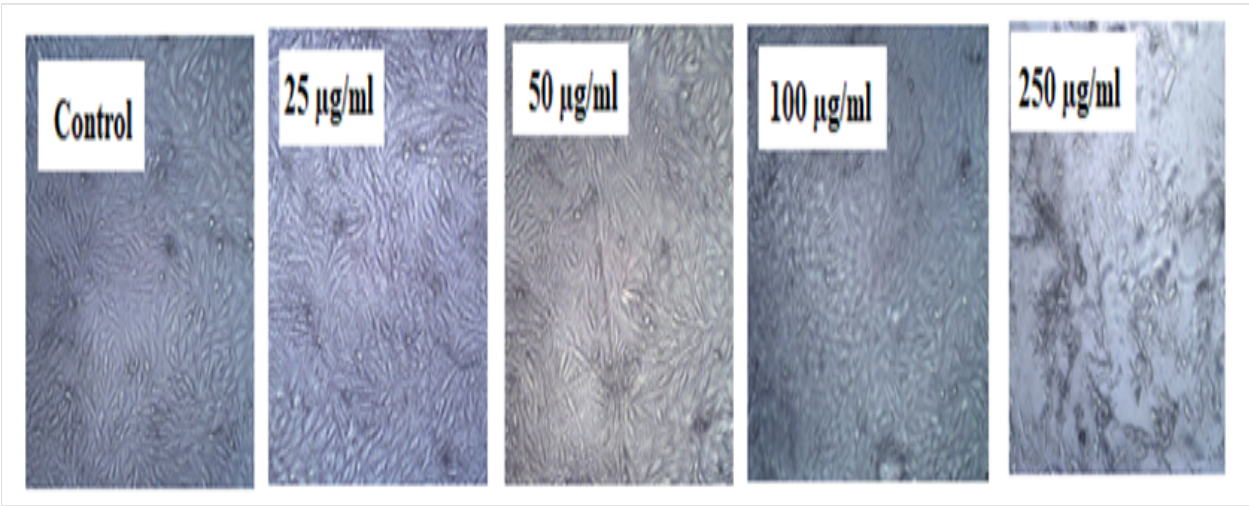

Figure 9: Morphological changes induced in Osteosarcoma MG-63 Cells upon treated with mangiferin loaded N,O-CMC nanoparticles in different concentrations

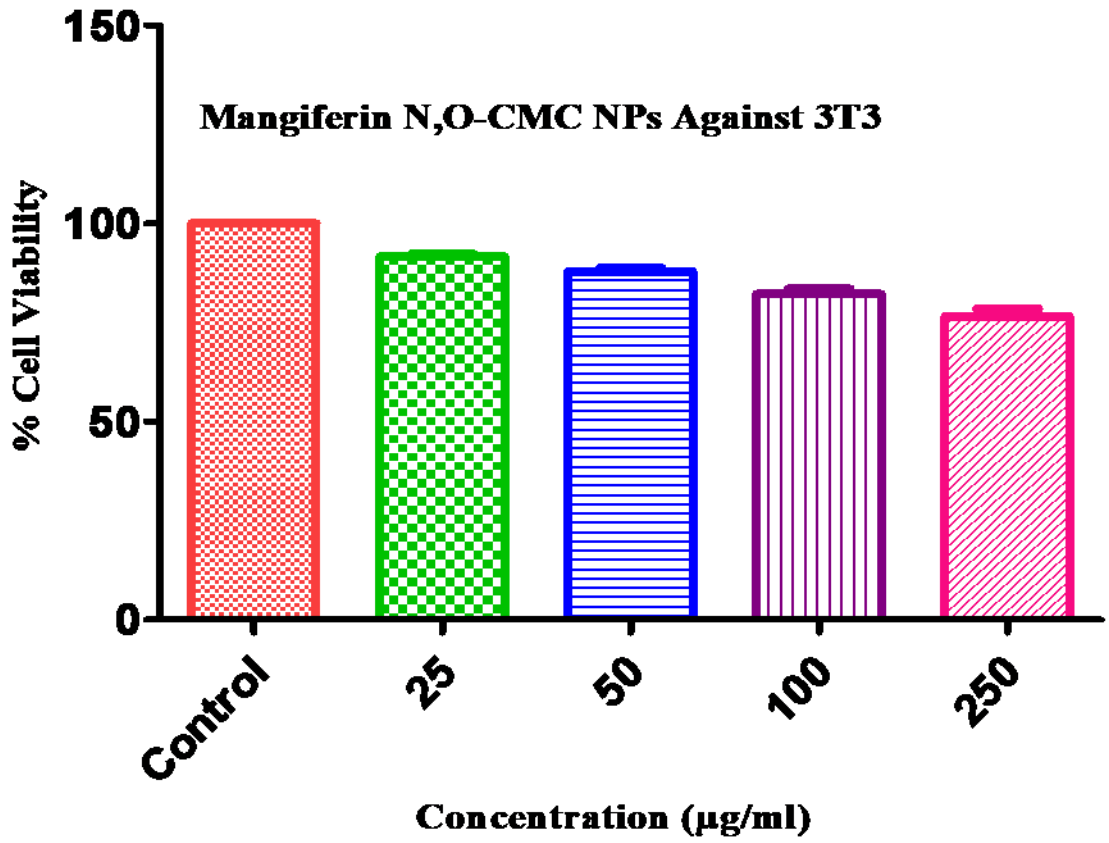

Figure 10: Effect of mangiferin loaded N,O-CMC nanoparticles against normal 3T3 cells

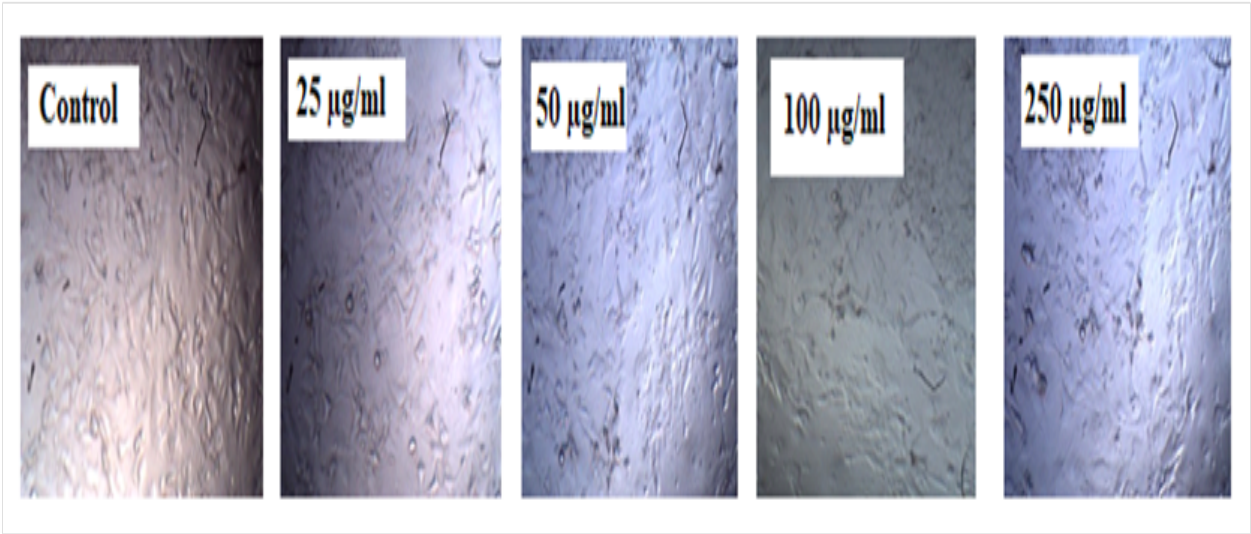

Figure 11: Morphological changes induced in 3T3 cells upon treated with mangiferin loaded N,O-CMC nanoparticles in different concentrations

RESULTS AND DISCUSSION

Characterization of Mangiferin Loaded N,O-CMC Nanoparticles

The size distribution of mangiferin-N,O-CMC nanoparticles obtained DLS. It is observed in Figure 2, that mangiferin-N,O-CMC nanoparticles lied within a size range of 200±10 nm. Based on the SEM investigations as shown in Figure 3, the morphology of mangiferin-N,O-CMC nanoparticles indicated spherical and flower type particles with a size range of 200 nm. Zeta potentials were measured for mangiferin-N,O-CMC nanoparticles and the value was found to be -45.8 mV (Figure 4).

The FT-IR spectrum of N,O-CMC, N,O-CMC nanoparticles, mangiferin and mangiferin-N,O-CMC nanoparticles were taken and compared in Figure 5. In the spectrum of N,O-CMC, the peak observed at 3265 cm^{-1} was due to –OH group, the peak at 1578 cm^{-1} was due to carboxylic group, and the peak at 1630 cm^{-1} was due to the presence of 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). As for mangiferin, the absorption band at 3362 cm^{-1} was due to the presence of –OH group, the sharp peak at 1648 cm^{-1} was assigned for C=O, the aromatic C=C peak was appeared at 1618 cm^{-1}, 1189 cm^{-1} for C–O and 1073 cm^{-1} for Ar–O–Ar. Due to the complexation of mangiferin into an mangiferin-N,O-CMC nanoparticles, N,O-CMC-related peaks has been shifted. While comparing N,O-CMC nanoparticles and mangiferin-N,O-CMC nanoparticles, a peak shift was observed from 3265 to 3246 cm^{-1} and from 1641 to 1615 cm^{-1}. Furthermore, the peaks present in mangiferin-N,O-CMC nanoparticles exhibited broadening owing to the probable interface among the ingredients within the nanoparticles. These results revealed the presence of mangiferin in N,O-CMC nanoparticle matrices.

The XRD of mangiferin-N,O-CMC nanoparticles was taken to understand its physical nature and the result revealed that mangiferin-N,O-CMC nanoparticles did not comprise with any crystalline type of peaks. This might be probably due to the development of an amorphous complex during the formation of nanoparticle within the nanoparticle matrix as shown in Figure 6.

In the *in-vitro* antioxidant activity using the DPPH method, mangiferin-N,O-CMC nanoparticles showed potent antioxidant activity by increasing the concentrations (Table 1). The IC_{50} value was found to be between 7.8-15.6 μg/ml. As shown in Figure 7, there was a colour change from purple to yellow in the DPPH solution, indicating that mangiferin-N,O-CMC nanoparticles having significant antioxidant activity at lower concentrations. This result was consistent with our previous study results of antioxidant activity of mangiferin in silver nanoparticles in the DPPH method.

In the present study, four different concentrations of mangiferin-N,O-CMC nanoparticles varying from 25 μg/ml to 250 μg/ml were tested for cytotoxicity study against Human MG-63 (osteosarcoma cells) by MTT assay (Figures 8, 9, 10 and 11). Mangiferin-N,O-CMC nanoparticles exhibiteda significant (P<0.001) reduction of cancerous cell growth in all the tested concentrations in a concentration dependent manner. Hence, for testing against normal cells (3T3), these concentrations were selected. There was no significant toxicity related with mangiferin-N,O-CMC nanoparticles with normal cells, thus indicating the safety of the synthesised nanoparticles.

Cancer is well recognized as a serious life-threatening disease of old age. The conventionally used medicines for the treatment of cancer have some boundaries including non-specific targeting and biodistribution, poor bioavailability, lack of aqueous solubility and low therapeutic indices. Because of these reasons, those drugs cannot be used widely in clinical treatment. Mangiferin is a naturally isolated compound from *Mangifera indica* and well-known for its chemotherapeutic properties. However, it is yet been developed as a drug in different dosage forms due to its low bioavailability and lack of water solubility. To solve these problems, the application of nanotechnology in drug manufacturing has been considered as a promising strategy. In addition, the anticancer efficacy of drug nanoparticles *in-vivo* and *in-vitro* toward various types of cancer has been reported in a plethora of studies (Wang *et al.*, 2016).

For instance, the anticancer properties of O-CMC-metformin nanoparticles have been exhibited on pancreatic cancer cells (Snima *et al.*, 2012). On the other hand, (Anitha *et al.*, 2014) , also revealed that the synergistic anticancer activities of 5-fluorouracil and curcumin-loaded N,O-CMC NPs towards colon cancer cells. In the present study, we successfully synthesized mangiferin-loaded N,O-CMC nanoparticles and characterized by DLS, SEM, FT-IR, Zeta potential and XRD measurements. In the DPPH method, mangiferin-loaded N,O-CMC nanoparticles showed significant antioxidant activity at lower concentrations. In MTT assay results indicated that the

synthesized mangiferin-loaded N,O-CMC nanoparticles could inhibit the growth of Osteosarcoma MG-63 cells. These results were in the agreement with the previous study regarding the mangiferin silver nanoparticles in DPPH and MTT assay methods (Othman and Sekar, 2019).

CONCLUSIONS

In this study, mangiferin-N,O-CMC nanoparticles were prepared and characterized by FT-IR, DLS, SEM, Zeta potential and XRD. The results showed that mangiferin was effectively loaded into N,O-CMC nanoparticles with a size range of 200±10 nm. The synthesized mangiferin-N,O-CMC nanoparticles possessed potent antioxidant and cytotoxic properties in DPPH and MTT assay methods, respectively. These pilot study results demonstrated the ability of the mangiferin-N,O-CMC nanoparticles in carrying hydrophobic drugs, thus making it as an alternative promising candidate for drug-delivery applications. However, further studies are warranted to reduce the particle size of mangiferin-N,O-CMC nanoparticles and tested with other type of cancer cells to confirm its safety and efficacy.

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