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ORIGINAL ARTICLE

Fabrication of chitosan-bis (4-formyl-2 methoxy phenyl carbonate) Schiff base nanoparticles and evaluation of their antioxidant and anticancer properties

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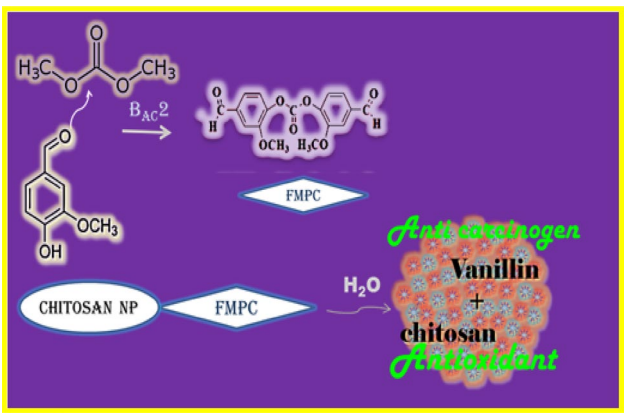
Abstract

The present study details on the mechanism of synthesis of bis (4-formyl-2 methoxy phenyl carbonate), using two green reagents dimethyl carbonate and vanillin for application as therapeutic agent. The synthesized FMPC was identified from the ^{13}C nuclear magnetic resonance spectra. The novel modified Schiff base nanoparticles resulted from the crosslinking of FMPC with chitosan were confirmed by cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance spectroscopy. The incorporation of the FMPC was identified from the amorphous X-ray diffraction patterns of C-FMPC-Nps. The thermal stability of the formed nanoparticles was predicted using thermogravimetric analysis. The morphology of the nanoparticles as observed from HRTEM was found to be smooth and spherical in nature. Both FMPC and C-FMPC-Nps showed significant radical scavenging potential and anticancer property. The carbonate ester backbone and the moiety present in chitosan-FMPC-nanoparticles, underwent hydrolysis at the targeted cancer causing microenvironment to release vanillin and chitosan and enhance the anticancer activity. Both FMPC and C-FMPC-Nps exhibits a dose dependent cytotoxicity towards the different cell lines and it was tested with a commercial drug for application studies.

Graphical abstract

Effective synthesis of FMPC, successful incorporation onto chitosan nanoparticles for the formation of C-FMPC-Nps. The formed Schiff base compound proves to have enhanced antioxidant and anticancer efficacy.



Keywords FMPC · DMC · Vanillin · Chitosan · Anticancer · Oxaliplatin

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Abbreviations

FMPC	Bis (4-formyl-2 methoxy phenyl carbonate)
DMC	Dimethyl carbonate
^{13}C-NMR	^{13}C nuclear magnetic resonance
Nps	Nanoparticles

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C	Chitosan
CP/MAS	^{13}C-NMR cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance
XRD	X-ray diffraction
TGA	Thermogravimetric analysis
HRTEM	High resolution transmission electron microscopy
TPP	Sodium tripolyphosphate
ATR	Attenuated total reflectance
D_2O	Deuterium oxide
EI-MS	Electron ionization mass spectrometry
ZrO	Zirconium oxide
KHZ	Kilohertz
TMS	Tetramethyl silane
TEA	Triethyl amine
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
DPPH	(1,1-Diphenyl-2-picryl hydrazyl)
MEM	Minimal essential medium
FBS	Fetal bovine serum

Introduction

Chitosan is a linear poly-amino saccharide obtained by *N*-deacetylation of chitin, the second most abundant natural bio-polymer [1]. While, chitin occurs naturally in cell walls of fungus and crustacean shells, chitosan is a fully biodegradable and biocompatible natural polymer, and can be used as an adhesive and as an antibacterial and antifungal agent. Chitosan offers many advantageous properties, including hydrophilicity [2], non toxicity [3], biodegradability [4] and biocompatibility [5] and it has been well documented for the preparation of value added products such as food additives, cosmetics and drugs [6].

Chitosan, due to its biocompatible properties, has been extensively studied for making it as prospective drug carrier. Chitosan can be modified accordingly to its uses as the elemental composition of the chitosan polymer is carbon (44.11%), hydrogen (6.84%) and nitrogen (7.97%) with an average molecular weight of ~ 5.3×10^5 Daltons [7]. Even though search to form chitosan as micro and nano system for biomedical application is common, majority of the attempts are for preparation and synthesis of chitosan nanoparticles [8]. Researchers have suggested using chitosan to coat nanoparticles made of other materials, in order to reduce their impact on the body and increase their bioavailability. Nano sizing of chitosan and its derivatives were found to have high saturation solubility and dissolution rate due to large surface area and small diffusion layer thickness compared to larger particles [9]. Polysaccharide nanoparticles with ultratiny volume can penetrate through the cells and tissue gap easily and enhance the availability of the drug at the target site [10].

Recently, *N*-(2-hydroxyl) propyl-3-trimethylammonium chloride [11], *N*-trimethyl chitosan [12] and glucomannan-chitosan [13] nanoparticles were fabricated for the delivery of proteins by ionic gelation method. Among wide variety of crosslinked chitosan nanoparticles, vanillin crosslinked chitosan nanoparticles and microparticles have received considerable attention as vanillin is regarded as a safe material and exert potent antioxidant activity, antibacterial and anti-inflammatory activity [14]. There is a great need to address the challenge of preparing stable vanillin and it can be achieved by crosslinking it with chitosan to form chitosan particles with prolonged functionality that finds application in food and drug administration [15]. It has recently been reported, that vanillin cross linked chitosan microspheres was explored as bioactive microcarrier of drugs such as the control release of resveratrol [16]. In addition, chitosan vanillin nanoparticles were fabricated, that can act as a promising vehicle for the delivery of 5-flurouracil against HT-29 cells [17]. Novel polymeric prodrug of vanillin termed poly(vanillin oxalate) nanoparticles, have been developed with great potential that finds application as novel antioxidant therapeutics and drug delivery systems [18].

In this current framework, efforts were made to synthesize FMPC, using green reagents vanillin and DMC. Herein, the carbonate linkages present in FMPC which links the vanillin moieties together would provide enhanced stability to FMPC. The objective of the present study is to develop a novel, facile route to fabricate C-FMPC-Nps, which would act as a potential biomaterial in therapeutics. The incorporated FMPC will undergo hydrolysis at the site of carbonate linkages producing hydroxyl groups. This would act as a radical scavenger at the targeted site making the C-FMPC-Nps a better antioxidant and anticancer agent.

Materials and methods

Instrumentation

FTIR spectra of the samples were analyzed using Alpha Bruker spectrophotometer in attenuated total reflectance (ATR) mode in the range of 4000–400 cm^{-1}.

^{1}H-NMR and ^{13}C-NMR spectra of the samples were recorded on a Bruker D8 Avance 500 spectrometer at 25 °C using Deuterium oxide (D_2O) as solvent.

Mass spectrum was recorded on an electron ionization mass spectrometry (EI-MS) JEOL JMS-AX 500. Solid state NMR spectroscopy was conducted using a Bruker Avance 600 spectrometer (14.10 T). Solid powder samples were packed in a zirconium oxide (ZrO) ceramic cylindrical rotor with 3.2 mm diameter and spun at 4.5 kHz. The chemical

shift of the ^{13}C nuclei was estimated using tetramethyl silane (TMS), with $\delta = 0$ ppm as external reference. Contact time of cross-polarization was 2.5 ms with accumulation of 1400–4000 scans.

Elemental analysis was performed on a Heraeus CHN-O-Rapid analyser.

XRD patterns were done with a Bruker D8 Advance XRD machine using Cu-Ka ($\lambda = 1.54$ Å) source. The layer spacing of the samples was calculated according to the Bragg's equation. The diffraction angles 2θ were set between 2° and 75° incremented with a scanning angle of 1° min^{-1}.

Thermal stability was examined by the TGA of TA Perkin Elmer instrument at a heating rate of 20 K min^{-1} in the temperature range between 30 and 700 °C under nitrogen atmosphere.

For high resolution transmission electron microscopy (HRTEM) studies of nanoparticles, 5 mL of the sample was coated on a carbon-coated copper HRTEM grid, which was subsequently air dried and was analysed by HRTEM (JEM 2100, Jeol, Peabody, MA, USA) operating at a high voltage of 200 keV.

The bioassay of the nanoparticles was done using Shimadzu UV-3101 spectrophotometer.

Chemicals

Analytical grade dimethyl carbonate (DMC), 4-hydoxy-3-methoxy benzaldehyde (vanillin) and triethyl amine (TEA) were purchased from Sigma Aldrich. Chitosan of low molecular weight (85% deacetylation) with viscosity > 200 cps, sodium tripolyphosphate (TPP) with 85% purity and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] with 98% purity, drug oxaliplatin obtained from local drug store and DPPH (1,1-diphenyl-2-picryl hydrazyl) were also obtained from Sigma Aldrich. All other reagents were of analytical grades and were used without any further purification. Milli-Q grade (resistivity of 18.2 MU cm^{-1}) water was used in all the experiments.

In vitro bio assays

Antioxidant activity by DPPH method

The free radical scavenging activity was measured by DPPH assay as described below.

(a) The determination of the antioxidant activity of the FMPC, was done by a slight modification of the literature protocol [19]. 2 mL of 100 µM FMPC prepared in distilled ethanol was added to ethanol solution of 0.5 mL of 100 µM DPPH in test tube. Ascorbic acid (100 µM) was used as a standard antioxidant. The test tubes were kept in dark room for 20 min at ambient temperature and measured for absorbance at 517 nm.

(b) Antioxidant property of C-FMPC-Nps suspension was determined, by slight modification [20]. 0.2–2.2 mg/mL of C-FMPC-Nps were mixed with 0.5 mL of 100 µM DPPH which was subjected to stirring for 2 h under dark condition at ambient temperature and measured for absorbance at 517 nm. Chitosan was used to compare the antioxidant activity of the prepared nanoparticles.

All the determinations were performed in triplicate and the activity was measured using the following equation.

$$\text{DPPH radical scavenging activity}\,(\%) = \frac{A_c - A_s}{A_c} \times 100\%$$

A_c is the absorbance of control (all reagents except standard/sample), A_s is the absorbance of standard/sample.

Evaluation of cytotoxicity against cell lines (cell culture preparation and experimental design)

Human cancer cell line MCF-7 and A 2780 used in this study was procured from National Centre for Cell Science, Pune. HT-29 (ATCC HTB-38) cell lines were procured from American Type Culture Collection (Rockville, MD, USA). MCF-7 and A 2780 cells were grown in Minimal essential medium (MEM) supplemented with 4.5 g/L glucose, 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37 °C in 5% CO_2 incubator. HT-29 cells were grown on E-MEM Minimum Essential Medium (MEM) with Earle's salt and nonessential amino acids, supplemented with 5% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, and 2 mM L-glutamate. The MTT assay developed by Mosman [21] was modified and used to determine the inhibitory effects of test compounds on cell growth in vitro. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5×10^3 cells/well in growth medium and cultured at 37 °C in 5% CO_2 to adhere. After 48 h incubation, the supernatant was discarded and the cells were pre-treated with growth medium and were subsequently mixed with different concentrations of test compounds (12.5, 25, 50, 100, 200 µg/mL) to achieve a final volume of 100 µL and then cultured for 48 h. All experiments were carried in triplicates.

The compound was prepared as 1.0 mg/mL concentration stock solutions in DMSO. Culture medium and solvent were used as controls. Each well then received 5 µL of fresh MTT (5 mg/mL in PBS) followed by incubation for 2 h at 37 °C. The supernatant growth medium was removed from the wells and replaced with 100 µL of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance

(OD) of the culture plate was read at a wavelength of 570 nm on an ELISA reader, Anthos 2020 spectrophotometer. Percentage inhibition of compounds against the cell lines were calculated using the following formula [22].

$$\% \text{ cell survival } = \frac{A_t - A_b}{A_c - A_b} \times 100$$

where A_t is the absorbance of test, A_b is the absorbance of the blank (media), A_c is the absorbance of control (cells)

$$\% \text{ cell inhibition } = 100 - \% \text{ cell survival}$$

IC_{50} determination

The drug concentration that reduced the viability of cells by 50% (IC_{50}) was determined by plotting triplicate data points determined by plotting a graph of log (concentration of compound) versus % cell inhibition.

Synthesis of FMPC

8.4 mL of 0.1 M DMC was allowed to react with 30.4 g of 0.2 M vanillin in the presence of 13.9 mL of triethyl amine base and refluxed at 86–88 °C for about 48 h. The crude compound obtained was subjected to purification using dry diethyl ether to get a final brown coloured product FMPC with 40% yield. FTIR: The peaks obtained ($_{max}/cm^{-1}$) are as follows: 1668 (ester and aldehyde C=O str), 1590 (C=C aromatic str), 1087 (ester C–O sym str), 1039 (ether C–O str), 878 and 798 (phenyl C–H out-of-plane bends). ^{1}H-NMR (500 MHz, D_2O): δ_H ppm 9.4 (2H, s, Ar–*CHO*, H-g), 7.4 (2H, d, J = 2 Hz, –O–C–C=*CH*–, H-e), 7.3 (2H, s, O–C–*CH*=C–CHO, H-c), 6.7 (2H, d, J = 8 Hz, –O–C–*CH*=CH–, H-f), 3.7 (6H, s, –OCH_3, Ar-OCH_3, H-h). ^{13}C-NMR (500 MHz, D_2O): δχ ppm 193 (Ar–*C*HO), 171 (–O–*C*=O–O–(Ar)), 148 ((–O–*C*–Ar) and (CH_3O–*C*(Ar)), 126 (CHO–*C*(Ar)), 116 (–O–CH=*C*H(Ar)) and (–O–CH=CH–*C*H(Ar)), 103 (CH_3O–C=*C*H(Ar)), 56 (Ar-OCH_3).

Preparation of chitosan nanoparticles

Initially, with 1 g of chitosan prepared in 100 mL of 1% aqueous acetic acid, 0.1 g of TPP in 100 mL of water was gently dripped and kept at room temperature for 10 min to get an opalescent suspension. The obtained suspension was subjected to centrifugation at 10,000 rpm for 30 min at 4 °C. The formed nanoparticles were again re-suspended in distilled water, sonicated, centrifuged and freeze dried.

Fabrication of C-FMPC-Nps

100 mg of chitosan nanoparticles dispersed in methanol was taken in a 100 mL round bottom flask. Excess amount of FMPC was added to the same and refluxed for 48 h at 60 °C with continuous stirring. FMPC crosslinked chitosan nanoparticles were collected, after washing the particles with methanol several times by centrifugation at 10,000 rpm for 20 min. The C-FMPC-Nps were finally dried in a vacuum oven at 40 °C for 12 h. FTIR: ν_{max}/cm^{-1} 1646 (–CH=N– str), 1600 (C=C aromatic str), 1374 (sym angular def of CH_3), 1254 (ester C=O assy str), 1152 (anti sym C–O–C), 887 (sym str of C–O–C). ^{13}C-CP MAS (150.32 MHz,): δ_c ppm 171 [O–(*C*=O)–O/**(Ci)**], 167 [–H*C*=N–/**(Cg)**], 148 [O–*C*=C– and CH_3O–*C*–Ar/**(Ca and Cb)**], 126 [–N=CH–*C*–(Ar)/**(Cd)**], 116 [–C=*C*H–*C*H–(Ar)/**(Ce and Cf)**], 103 [CH_3O–C=*C*H–C–(Ar)/**(C1 and Cc)**], 82 **(C4)**, 74 **(C5 and C3)**, 56 [*C*H_3O–(Ar)/**(Ch, C6 and C2)**], 22 (*C*H_3CO– of chitosan).

Results and discussion

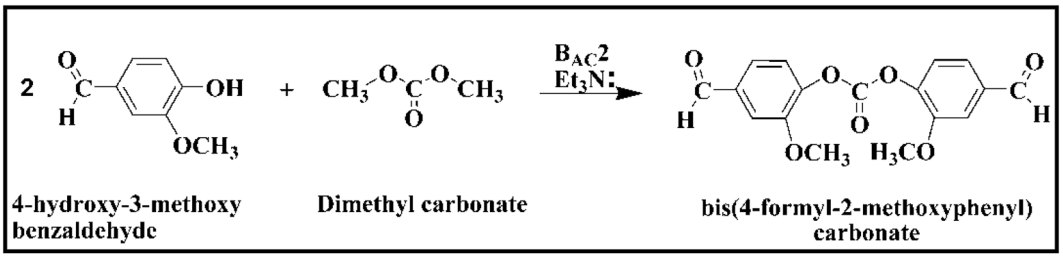
Mechanism of FMPC synthesis and its reaction with chitosan for effective Cytotoxicity

Studies were carried out to check the detailed mechanism undergone on the formation of C-FMPC-Nps. If the backbone of the compound is stable then the anticancer activity was enhanced. For this initial steps were carefully optimized for effective synthesis of FMPC and incorporating into chitosan nanoparticles. On study it was found that the DMC hits the hydroxyl group of vanillin and carboxy methylation [23] reaction takes place through $B_{AC}2$ mechanism (Scheme 1). This resulted in the formation of FMPC that was carried out at 88 °C and methanol was formed as byproduct [24]. Next step is the formation of C-FMPC-Nps which was done through condensation reaction of aldehyde functionality of vanillin with the free amine groups on chitosan. This resulted in formation of Schiff base chitosan exhibiting the presence of azomethine (–CH=N–) linkage in C-FMPC-Nps (Scheme 2) [25].

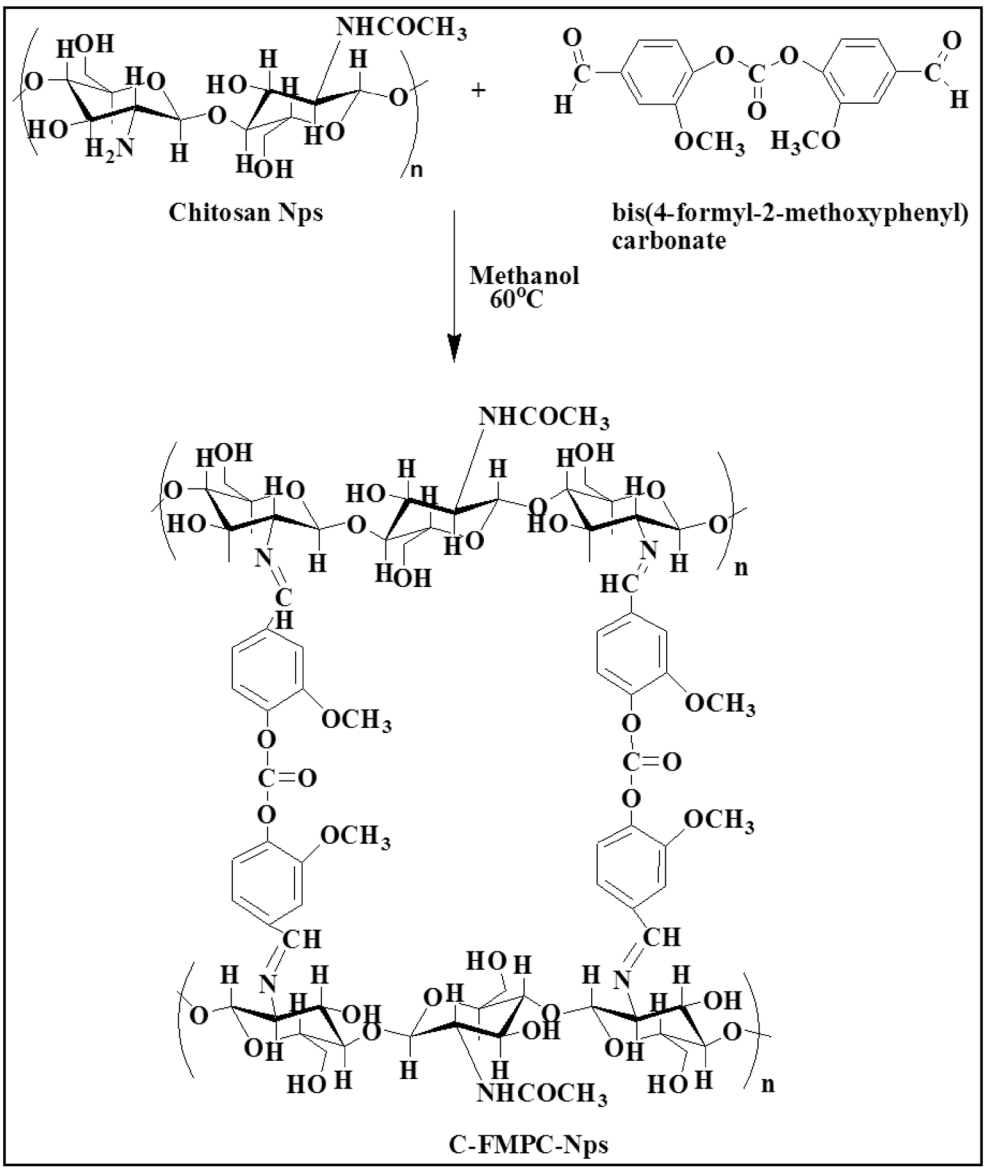
The successful formation of schiff base was confirmed by elemental analysis (Table 1). From the results it could be inferred that the C-FMPC-Nps showed an increase in nitrogen percentage but a decrease in carbon and hydrogen percentage compared to chitosan. This behaviour would lead to a conclusion, that FMPC has been successfully incorporated onto chitosan backbone. In addition, the decrease in the C/N ratio is an indicative of the successful crosslinking of chitosan with the pendent FMPC to form Schiff base [26, 27].

Spectral studies for the formation of (FTIR, NMR and Mass) of FMPC and C-FMPC-Nps

Various studies were performed to analyze and predict the formation of C-FMPC-Nps through FTIR, NMR and Mass spectroscopy.



Scheme 1 Synthetic route for the preparation of FMPC



Scheme 2 Schematic structure of newly synthesized C-FMPC-Nps Schiff base

Table 1 Elemental analysis of C-FMPC-Nps

Element %				
Sample	C	N	H	C/N
Chitosan	40.43	6.57	7.17	5.63
C-FMPC-Nps	33.63	5.63	7.31	4.60

FTIR confirms the presence of (C–O) phenolic stretch at 1299 cm^{-1} present in vanillin (Fig. 1), was found to be absent in the synthesized FMPC which is a clear indication of the successful formation of FMPC. In the formed C-FMPC-Nps (Fig. 2b), the peak at 1668 cm^{-1} corresponds to free aldehyde group of vanillin (Fig. 2a) which was found to be absent and a new peak appeared at 1646 cm^{-1} that indicates the formation of schiff base [28]. The absence of peak at 1559 cm^{-1}, in C-FMPC-Nps corresponding to the amide II band of chitosan, was a clear evidence for the covalent attachment of FMPC with chitosan. In addition, the identification of peaks at 1600 cm^{-1} and 1152 cm^{-1} are due to the presence of C=C aromatic stretching vibration of vanillin and C–O–C anti symmetric bridging vibration of chitosan. This confirms the successful incorporation of FMPC with chitosan [29].

The ^{1}H-NMR spectrum of FMPC (Fig. 3), exhibits the same characteristic resonances of vanillin [30]. Here, the formation of FMPC was confirmed by the reappearance of proton signal which corresponds to aldehyde functionality of vanillin at 9.4 ppm [31]. Therefore, it proves that vanillin –OH is involved in reaction with the carbonyl group of DMC through $B_{AC}2$ mechanism.

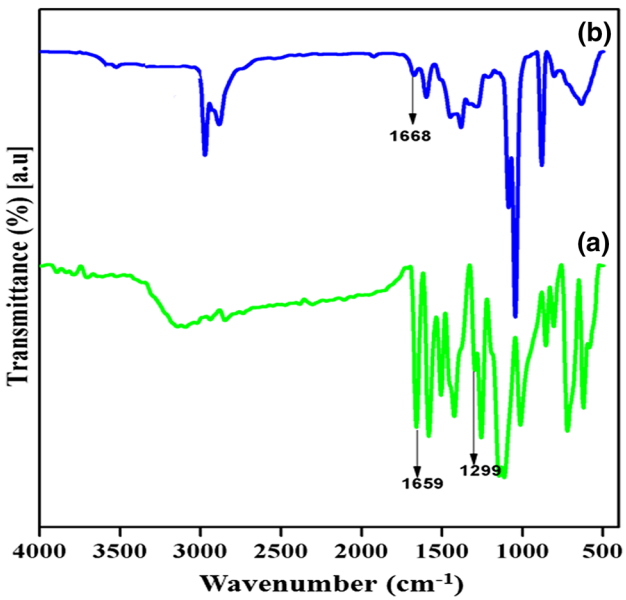


Fig. 1 FTIR of (**a**) vanillin and (**b**) FMPC

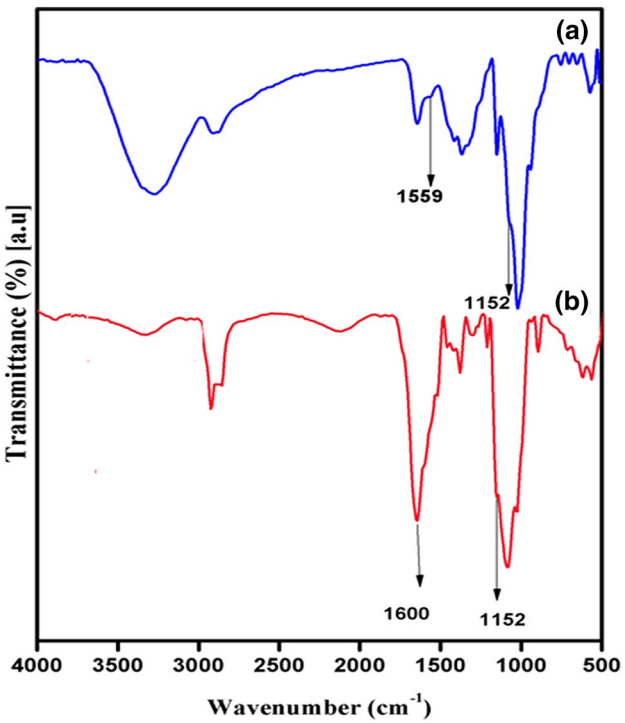


Fig. 2 FTIR of (**a**) chitosan and (**b**) C-FMPC-Nps

Further confirmation was done through ^{13}C-NMR spectrum (Fig. 4). The characteristic resonance at 193 ppm indicates the presence of free (–C=O) group in FMPC [32]. In addition to the signals found in FMPC, the most predominant resonances at 171 and 148 ppm corresponds to (–*C*=O–) of ester and (–O–*C*–(Ar)) of carbonate linkage that exhibited the formation of FMPC with a stable carbonate backbone [33].

The EI mass spectra (Fig. 5) of the synthesized FMPC showed a molecular ion peak at (m/z) 328.18 that was in good agreement with the calculated theoretical values.

In the CP/MAS ^{13}C-NMR spectrum of C-FMPC-Nps (Fig. 6), the peak of aldehyde carbon at 195 ppm [34] disappeared and a new signal at 167 ppm appeared. This has been attributed to the formation of schiff base chitosan, resulted from the condensation reaction between chitosan and FMPC [35].

XRD of C-FMPC-Nps

Figure 7 indicates the XRD profile of pristine chitosan, vanillin and C-FMPC-Nps respectively. The high degree of crystallinity of chitosan (Fig. 7a) is indicated by the presence of sharp diffraction peak at $2\theta = 20.3°$ and a weak diffraction peak at $2\theta = 11.6°$ [36]. The presence of a sharp diffraction peak of vanillin at $2\theta = 13.3°$ (Fig. 7b), appeared as semi crystalline peak at $2\theta = 13.7°$ in

Fig. 3 ^{1}H-NMR of FMPC

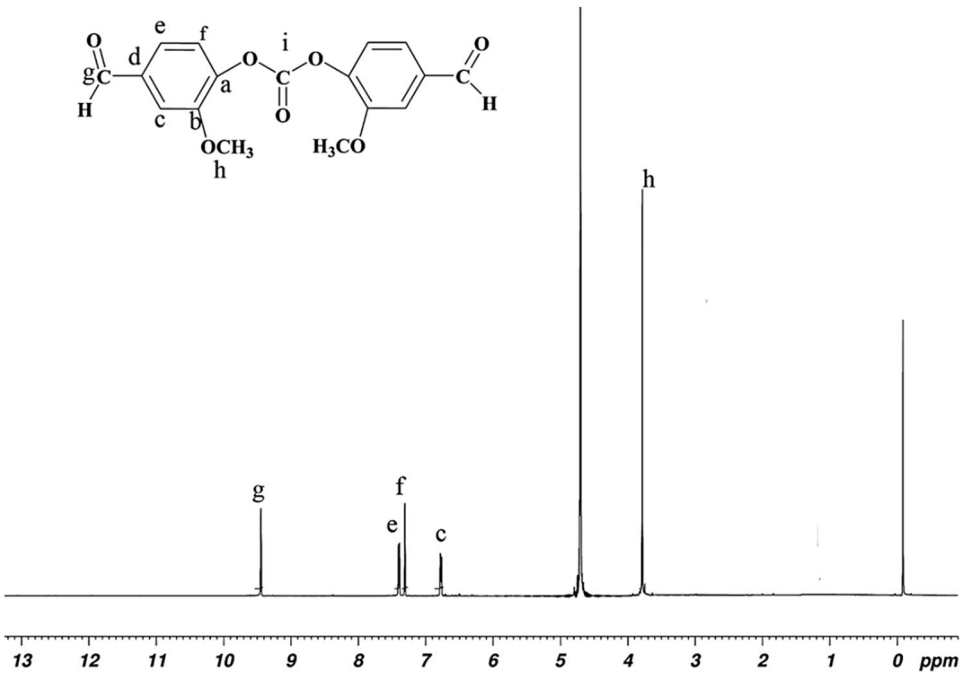
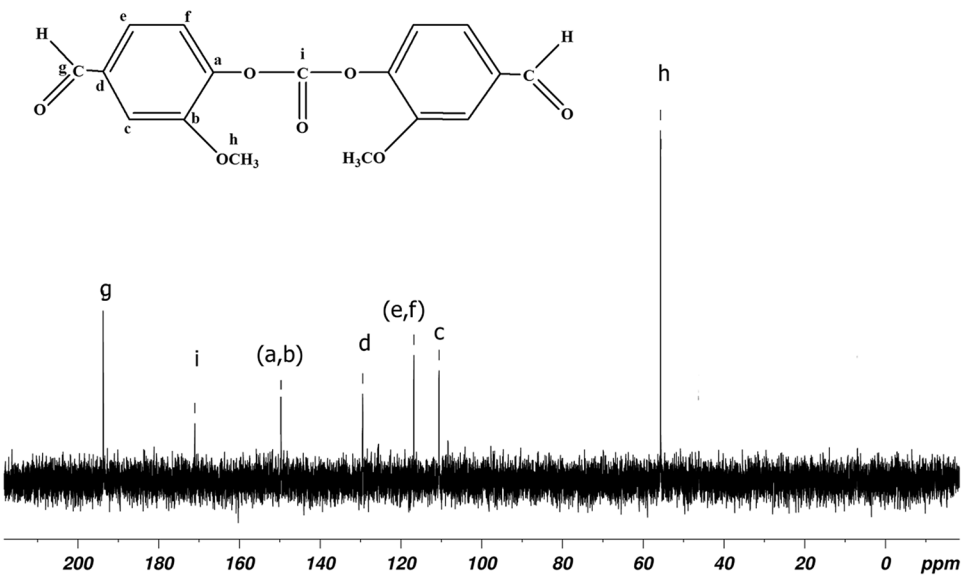


Fig. 4 ^{13}C-NMR of FMPC



C-FMPC-Nps (Fig. 7c) [37]. Herein, for C-FMPC-Nps the observed peak at $2\theta = 13.7°$ with weak reflection exhibits the existence of interdigited hydrogen bond between chitosan and vanillin unit present in FMPC [38]. In addition, C-FMPC-Nps exhibits a broad peak at $2\theta = 20.6°$ with decrease in magnitude that is an indicative of molecular miscibility between the chitosan, TPP and FMPC. Thus, the successful formation of C-FMPC-NPs has been confirmed by the XRD patterns.

TGA of C-FMPC-Nps

The thermal stability of chitosan, vanillin and the prepared C-FMPC-Nps was studied from TGA results and are indicated in Fig. 8. For chitosan, vanillin and C-FMPC-Nps, the initial weight loss around 50–150 °C is due to the moisture vaporization and bound water of hydration. Chitosan (T_{max} at 298 °C) (Fig. 8a) was an indicative of disruption of strong inter and intra molecular hydrogen bonds, due to the

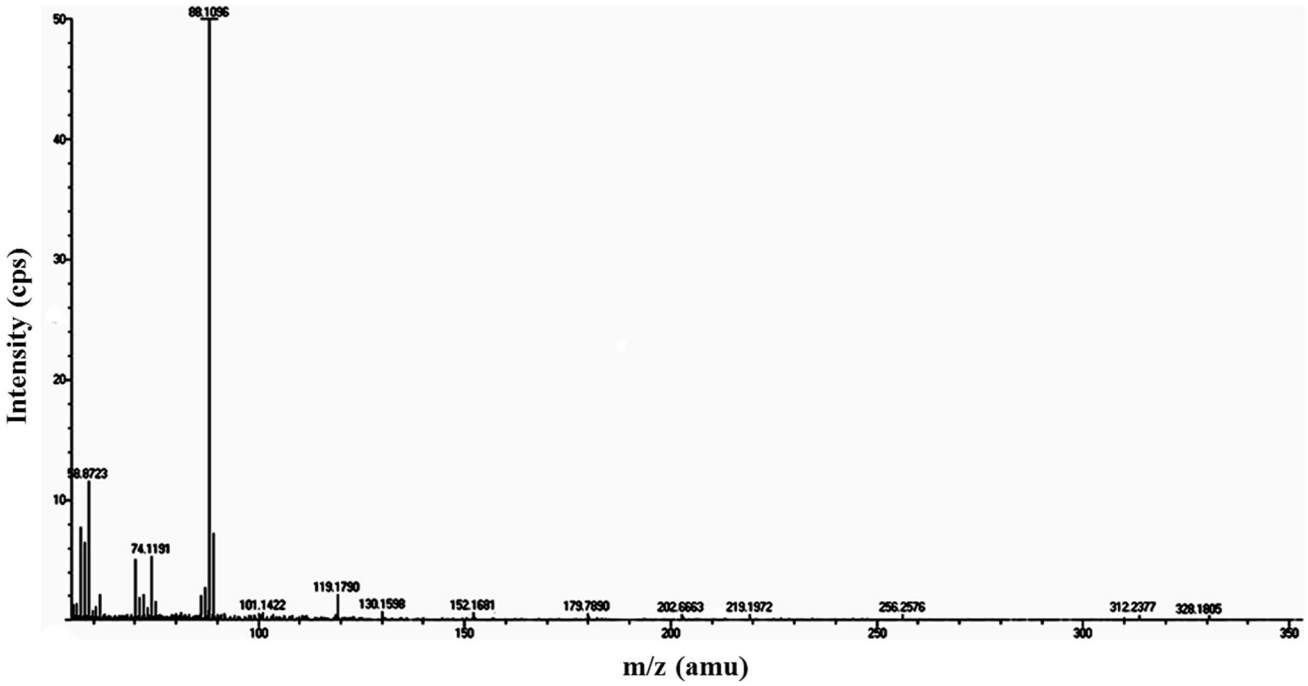


Fig. 5 Mass spectra of FMPC

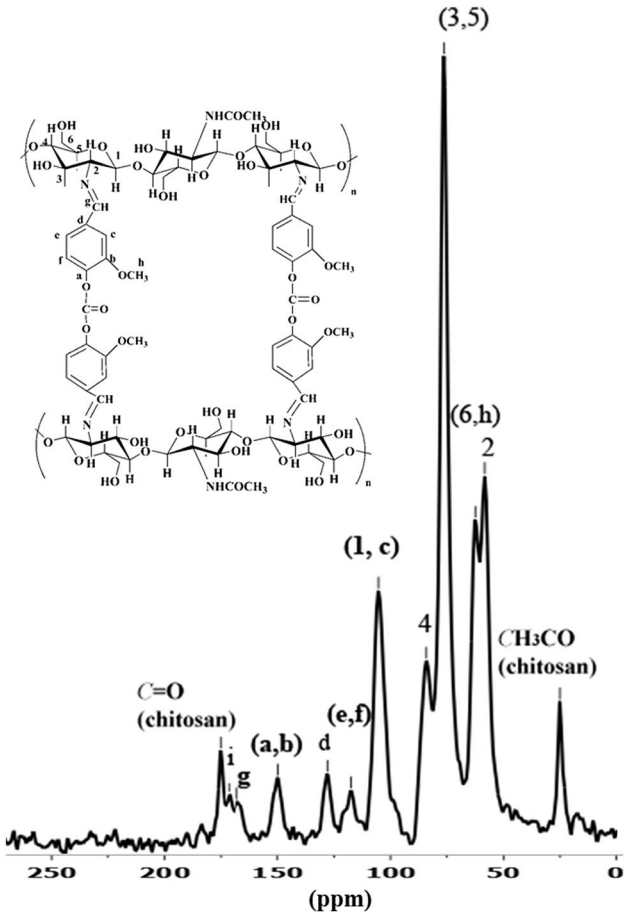


Fig. 6 (CP/MAS) ^{13}C-NMR of FMPC

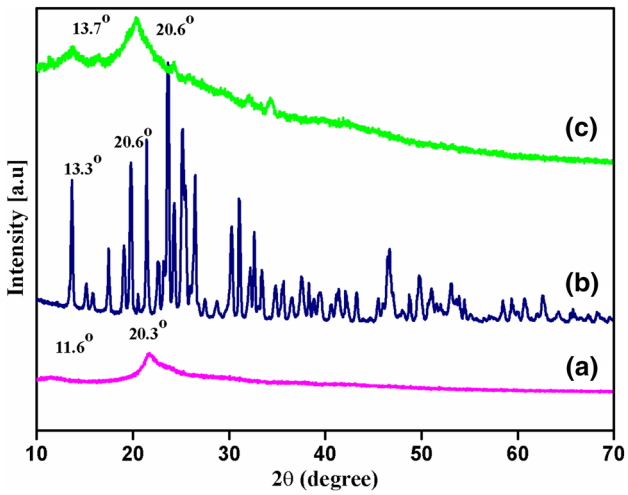


Fig. 7 XRD of (**a**) chitosan, (**b**) vanillin and (**c**) C-FMPC-Nps

depolymerisation and thermal decomposition of the acetylated and deacetylated units of polymeric chitosan [39]. For vanillin (Fig. 8b) T_{max} value has been observed at 230 °C that corresponds to the degradation or thermal evaporation of vanillin [40].

It is evidenced from Fig. 8c, for C-FMPC-Nps (T_{max} = 269) the thermal stability, was comparatively higher than vanillin. This could be due to the incorporation of stable carbonate backbone present in FMPC moiety. But, rather the decrease in the stability of C-FMPC-Nps compared

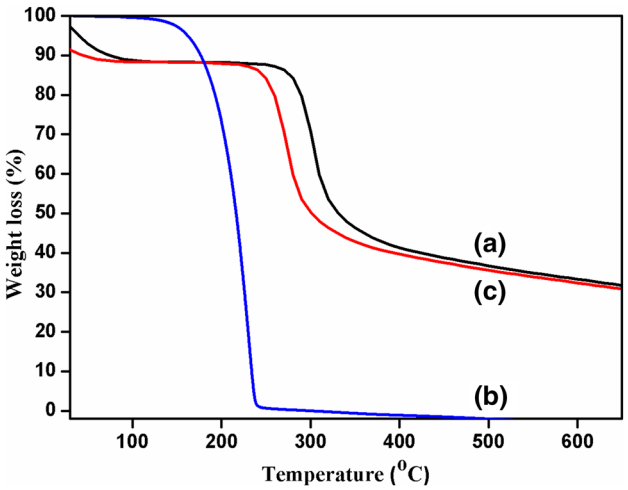


Fig. 8 TGA of (**a**) chitosan, (**b**) vanillin and (**c**) C-FMPC-Nps

to chitosan was because of the introduction of the FMPC moiety in chitosan. FMPC decreases the number of amine groups on chitosan by obstructing the chain packing of chitosan resulting in the decreased stability of C-FMPC-Nps [41].

TEM observation of C-FMPC-Nps

The visualization of the particles as performed by TEM analysis indicates the size and morphology of the formed C-FMPC-Nps (Fig. 9). TEM image revealed that the size of the nanoparticles were around 25–30 nm in diameter with smooth surface and spherical morphology. The electrostatic repulsion, hydrophobic interaction and hydrogen bonding exists between different functionalities present in C-FMPC-Nps that results in transparent colloidal aggregates,as clearly observed from TEM analysis [42].

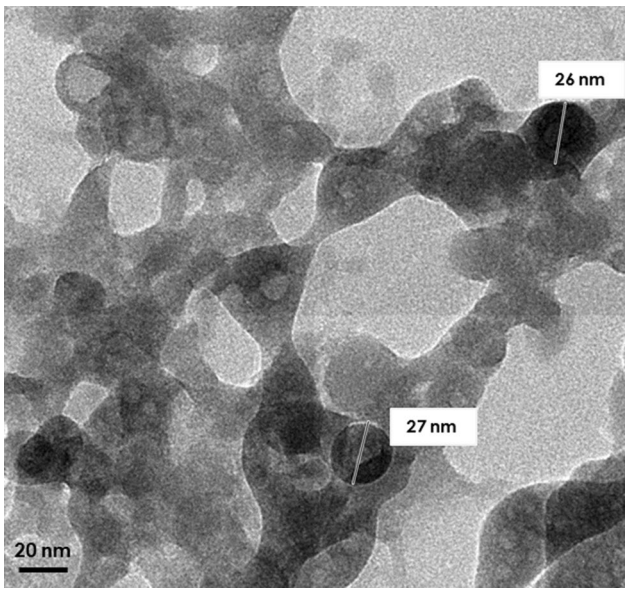


Fig. 9 HRTEM image of C-FMPC-Nps

DPPH radical scavenging activity of FMPC and C-FMPC-Nps

DPPH is a relatively stable nitrogen centred free radical. It has the ability to react with electron/hydrogen atom donating reducing agents and converting it into a nonradical 1,1-diphenyl-2-picryl hydrazine (yellow) diamagnetic molecule which can be measured spectrophotometrically [43].

The IC_{50} values of the newly synthesized FMPC and standard ascorbic acid to scavenge the DPPH radicals were found to be 48.7% and 89.6% respectively. FMPC was found to be almost equally potent as compared to ascorbic acid by DPPH assay. The functional groups present in the targeted compound are highly responsible for the free radical scavenging activity. It could be understood from the structure that the antioxidant activity of the FMPC might be attributed to the electron donating nature of the substituent $–OCH_3$ [44]. The presence of $–OCH_3$ on FMPC increases both the electron density and electron mobility in the core structure [45] and stabilizes the generated radical during oxidation [46] thus, exhibiting predominant DPPH radical scavenging activity.

Figure 10 represents the antioxidant activity of the C-FMPC-Np. The IC_{50} values of the chitosan and C-FMPC-Nps were found to be 1.859 and 0.9739 mg/mL respectively. The antioxidant activity of C-FMPC-Nps indicates that the nanoparticles act as a potent antioxidant in scavenging DPPH free radicals.

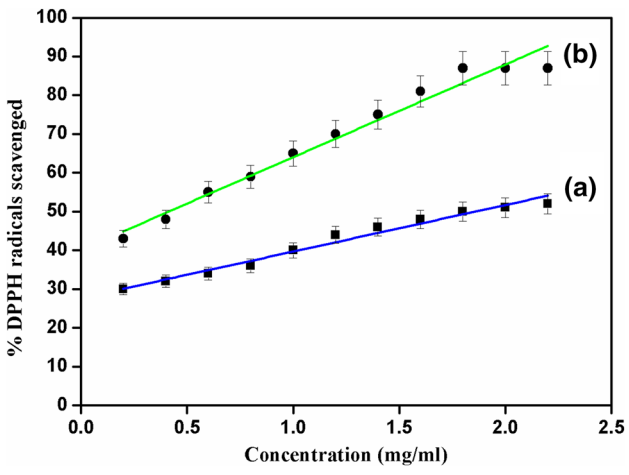


Fig. 10 DPPH radical scavenging property (**a**) chitosan (**b**) C-FMPC-Nps. Each value represents the mean ± SD (n = 3)

The antioxidant property is far higher than the synthesized FMPC. The reason behind this high activity might be due to the synergistic effect of both FMPC and chitosan [47]. Notably, the electron donating $–OCH_3$ present in vanillin moiety, the hydroxyl groups present at C3 and C6 position of chitosan and the presence of (–C=N–) linkage enhanced the radical scavenging activity of the C-FMPC-Nps. Wherein, the high electron density over nitrogen present in the schiff base (–C=N–), overlaps with the neighbouring (–O˙) radical electrons formed at the C3 carbon of chitosan would stabilize the free radical formed [48]. Further, the increased electron fluidity of the prepared nanoparticles upon formation of the schiff base [49] would resulted in the enhanced antioxidant property of the schiff base C-FMPC-Nps [50].

Inhibitory effect of FMPC and C-FMPC-Nps against MCF-7, HT-29 and A 2780 cell line

As the antioxidant result of FMPC is encouraging, further screening of FMPC for anticancer activity against MCF-7, HT-29 and A 2780 cell lines were undertaken. The in vitro cytotoxic activity was accessed and graphically represented for Chitosan and FMPC in Fig. 11 and C-FMPC-Np's in Fig. 12. The IC_{50} value was determined to be 0.501 µg/mL, 23.098 µg/mL and 25.189 µg/mL respectively for MCF-7 cell line and all other cell line values are tabulated. (Table 2). From the result it could be inferred that the electron withdrawing $–OCH_3$ group is highly responsible for the positive effect against stress related H_2O_2 scavenging anticancer activity of the synthesized compound [51].

Synthesized FMPC with carbonate ester backbone structure has high response towards H_2O_2. Thus under the

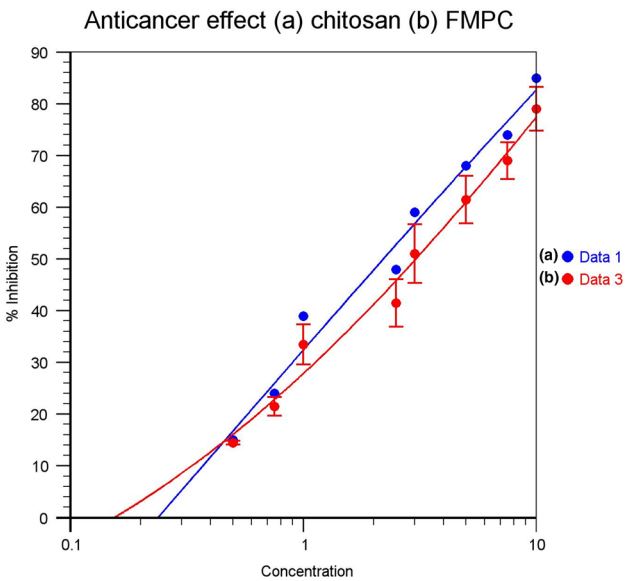


Fig. 11 Anticancer effect of synthesized (**a**) chitosan and (**b**) FMPC

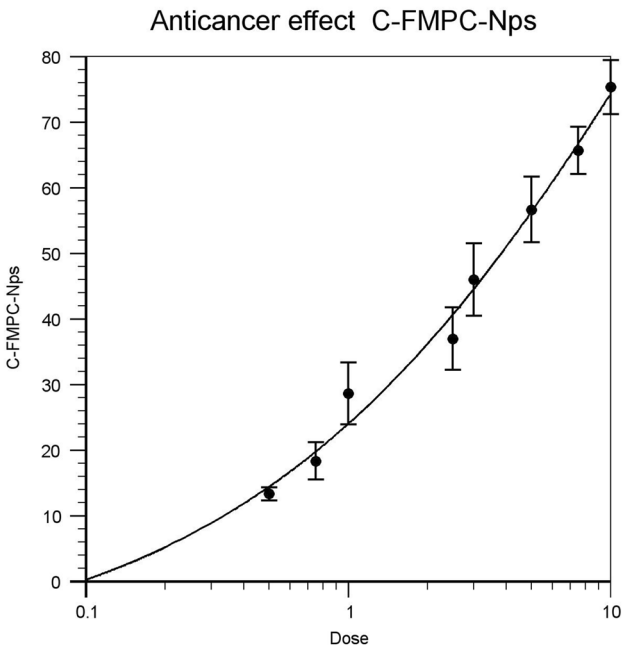


Fig. 12 Anticancer effect of C-FMPC-Nps

cancer induced acidic environment the carbonate back bone might be cleaved [52] to release vanillin, which suppress the generation of ROS, such as H_2O_2 and exerts, excellent activity against oxidative stress [53]. Thus FMPC exhibits dose dependent cytostatic/cytolysis behaviour [54] towards all cell lines. The high levels of H_2O_2 found in cancer cell line induce the hydrolytic cleavage of carbonate ester bonds present in FMPC [55]. Thus the release of vanillin from FMPC would result in the anticancer activity of the synthesized FMPC. The percentage inhibition of C-FMPC-Nps on MCF-7 cancer cell line is indicated and the activity of the nanoparticles was compared with chitosan. The inhibitory effect of C-FMPC-Nps was higher on comparison with chitosan (Fig. 13). The increase in the anticancer activity of C-FMPC-Nps arises from the schiff base formation between vanillin and chitosan. Here, the reactive oxygen species produced at the targeted site were quenched by the localized electron dense nitrogen atom present in the schiff base. In addition to the aforementioned property, the hydrolytic cleavage of the carbonate backbone [56] and the

Table 2 IC50 values of the FMPC and C-FMPC-Nps against MCF-7, HT-29 and A 2780 cell line

	Anticancer activity cell lines IC50 (µg/mL) ± S.D		
	MCF-7	HT-29	A 2780
Chitosan	0.501 ± 1.34	12.45 ± 1.71	13.67 ± 3.12
FMPC	35.39 ± 1.16	65.21 ± 2.43	47.28 ± 1.87
C-FMPC-Nps	25.189 ± 2.05	59.32 ± 1.18	67.55 ± 2.21

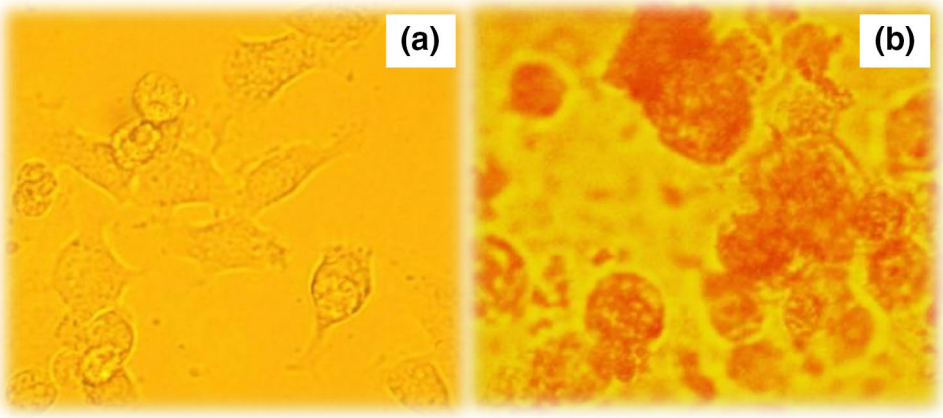


Fig. 13 Images of (**a**) control and (**b**) FMPC treated MCF-7 cells

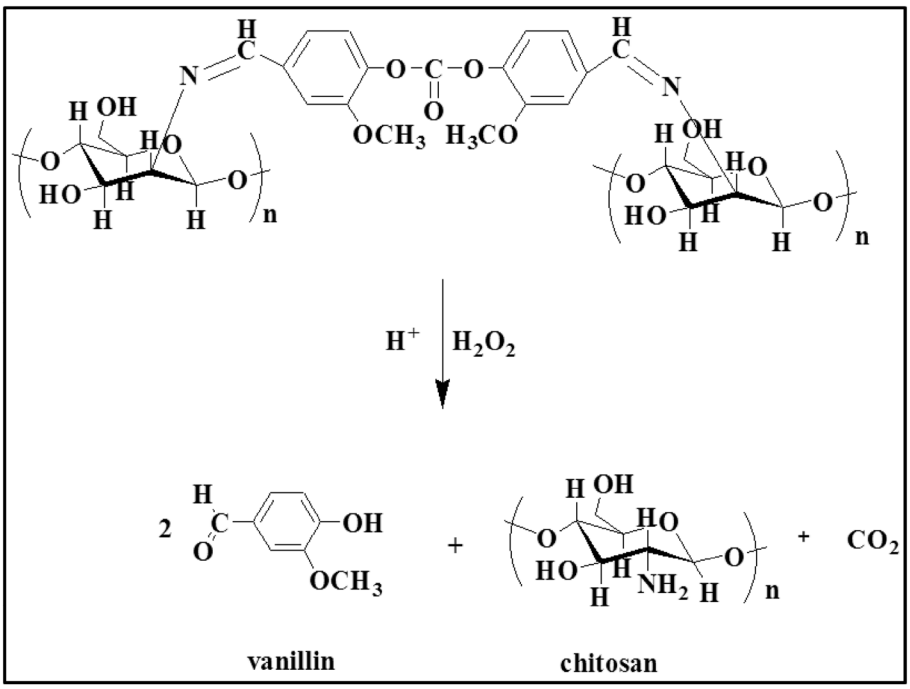
C=N linkage [57], would release vanillin and chitosan from C-FMPC-Nps.

The synergistic effect of both chitosan and vanillin moieties released at the targeted carcinogenic site would enhanced the anticancer activity thus curtailing the growth of cancer cells (Scheme 3). The inhibitory effect led us to speculate, that there is a steady and linear increase in the cytotoxic effect with increase in concentration of the prepared nanoparticles [58].

Cytotoxic response of different cell lines to the commercial drug oxaliplatin

One of the goals of oncology is to predict the response of patients with cancer to chemotherapeutic agents. One method is by employing drug response assays in vitro. The MTT assay is one of the methods used to predict the drug response in malignancies [59]. Incorporation of cell culture studies offers good possibility to assess the drug in controlled conditions. The cytotoxic response of different cell lines is evaluated using high-throughput MTT assay. It measures the cell respiration and the amount of formazan produced is proportional to the number of living cells present in culture. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the drug. MTT was used to assess the cell viability as a function of redox potential. Actively respiring cells convert the water soluble yellow tetrazolium MTT to an insoluble purple formazan.

Scheme 3 Hydrolytic cleavage of C-FMPC-Nps



It has been claimed that, the MTT formazan gives rise to extracellular deposits of needle-shaped crystals by exocytosis. These crystals were dissolved in DMSO. The resulting purple solution was spectrophotometrically measured and the percentage of viability was calculated. The results (Fig. 14) exposed that with increasing time period from 12 to 72 h, the vero cells adhere and proliferate on C-FMPC-Nps facilitating cell attachment [18]. The significant increase in the optical density (OD) value of C-NMIC-Nps suggested a good development of cells within. The images of the viable cells in vero cell lines treated using control and C-FMPC-NPs after 72 h were shown in Fig. 15. The test performed on vero cell lines, showed the cell viability of 93.5% at a maximum concentration of 0.5 mg/mL after 72 h of incubation. This suggested that the prepared C-FMPC-Nps was found to be less cytotoxic in vero cell lines.

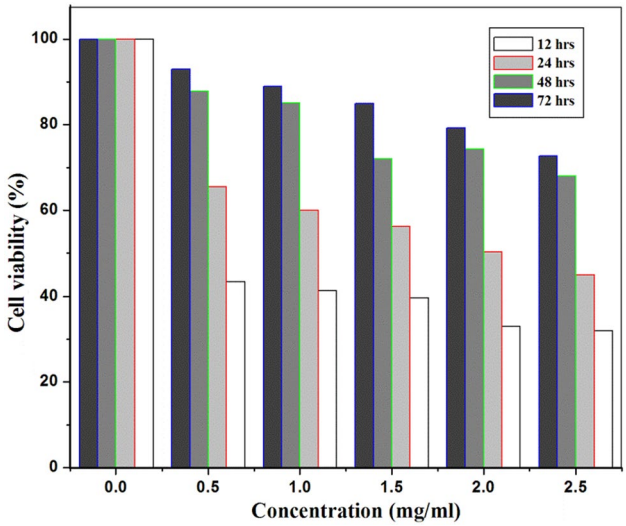


Fig. 14 Cell viability test performed on vero cell lines after cultivating with different concentrations of C-FMPC-NPs

IC_{50} is the concentration of the tested drug able to cause the death of 50% of the cells and can be predictive of the degree of cytotoxic effect. The lower the value, the more cytotoxic is the substance. Figure 16, shows the IC_{50} of drug oxaliplatin against human cancer cell lines. IC_{50} values resulted were 1.65, 0.98 and 0.44 for MCF-7, HT-29 and A 2780 cell lines indicating the cytotoxicity of the drug to that specific cancer cell type. The premise of in vitro drug response testing is that it can provide the knowledge of the relative efficacy of the various agents used in standard therapy before an empiric in vivo trial. Cell-based assays may help in the selection of chemotherapeutic drugs with the greatest likelihood for clinical effectiveness, and in the exclusion of ineffective therapy. This can lead to improved disease management, response, survival and use of financial resources. The IC_{50} was estimated from the curve generated. The lower the IC_{50}, the more cytotoxic the drug is to that specific cancer cell type.

Conclusion

In the present study, synthesis, characterization and mechanism of novel FMPC, C-FMPC and C-FMPC-NPs using DMC has been reported. Successful formation of FMPC and its incorporation in chitosan nanoparticles through cross linking by N-alkylation of chitosan was verified using instrumental analysis. C-FMPC-NPs were proved to be non-toxic and showed high cell viability even when the concentration of the C-FMPC-NPs was 0.5 mg/mL highlighting their potential application in biomedical field. The prepared nanoparticles were found to be more effective than FMPC in the screening of anticancer activity against cell lines. Both FMPC and C-FMPC-Nps exhibits a dose dependent cytotoxicity towards various cell lines. The structure activity relationship revealed that in addition to $–OCH_3$ moiety the –CH=N– imine moiety plays a vital role for the

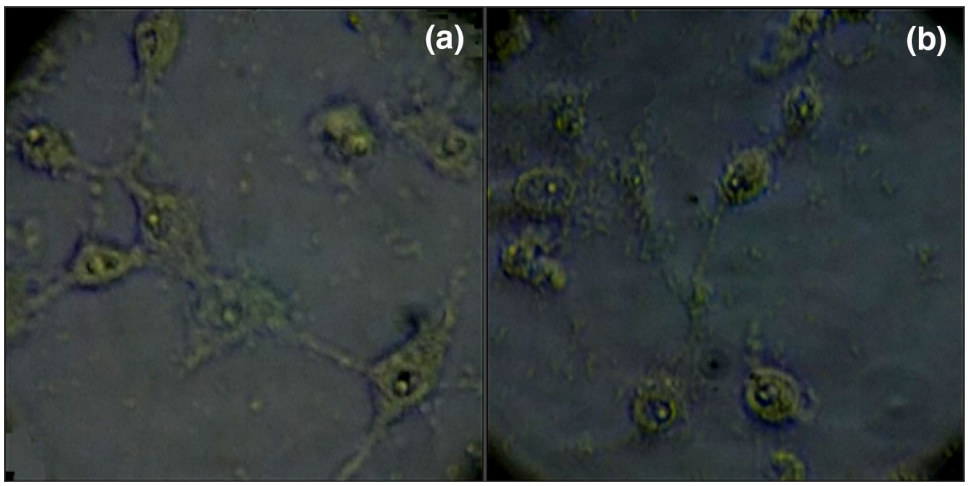
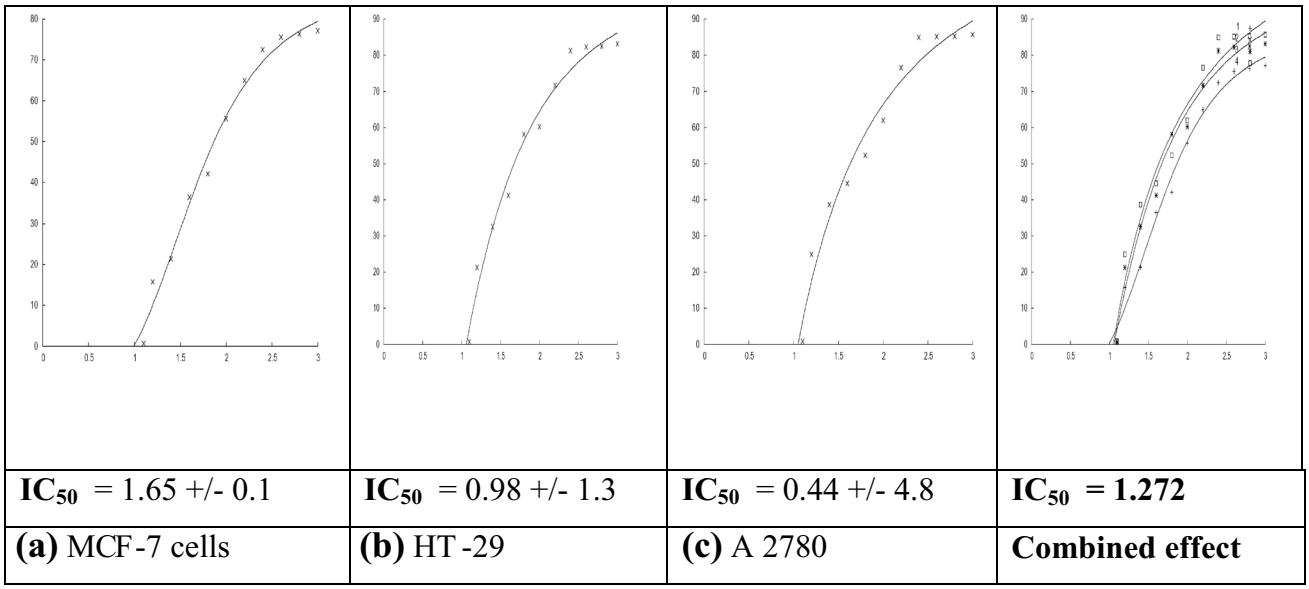


Fig. 15 MTT assay of (**a**) control and (**b**) C-FMPC-Nps



IC_{50} = 1.65 +/- 0.1	IC_{50} = 0.98 +/- 1.3	IC_{50} = 0.44 +/- 4.8	IC_{50} = 1.272
(a) MCF-7 cells	**(b)** HT -29	**(c)** A 2780	**Combined effect**

Fig. 16 IC_{50} of drug oxaliplatin against human cancer cell lines (**a**) MCF-7 cells, (**b**) HT-29, (**c**) A 2780, (**d**) total

enhanced activity of the C-FMPC-Nps to fight against the ROS. Hence it has been concluded that the present work can open new vistas in chitosan base schiff base chemistry and for advanced biological studies.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

References

1. Chethan PD, Vishalakshi B, Sathish L, Ananda K, Poojary B (2013) Preparation of substituted quaternized arylfuran chitosan derivatives and their antimicrobial activity. Int J Biol Macromol 59(2013):158–164
2. Berger J, Reist M, Mayer JM, Felt O, Gurny R (2004) Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm 57(1):35–52
3. Ravi Kumar MNV (2000) A review of chitin and chitosan applications. React Funct Polym 46(1):1–27
4. Yamamoto H, Amaike M (1997) Biodegradation of cross-linked chitosan gels by a microorganism. Macromolecules 30(13):3936–3937
5. Geng X, Oh-Hyeong K, Jang J (2005) Electrospinning of chitosan dissolved in concentrated acetic acid solution. Biomaterials 26(27):5427–5432
6. Pasanphan W, Buettner GR, Chirachanchai S (2010) Chitosan gallate as a novel potential polysaccharide antioxidant: an EPR study. Carbohydr Res 345(1):132–140
7. Zargar V, Asghari M, Dashti A (2015) A review on chitin and chitosan polymers: structure, chemistry, solubility, derivatives, and applications. ChemBioEng Rev 2(3):204–226
8. Liu Z, Jiao Y, Wang Y, Zhou C, Zhang Z (2008) Polysaccharides-based nanoparticles as drug delivery systems. Adv Drug Deliv Rev 60(15):1650–1662
9. Kesisoglou F, Panmai S, Wu Y (2007) Nanosizing-oral formulation development and biopharmaceutical evaluation. Adv Drug Deliv Rev 59(7):631–644
10. Jung T, Kamm W, Breitenbach A, Kaiserling E, Xiao JX, Kissel T (2000) Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? Eur J Pharm Biopharm 50(1):147–160
11. Xu Y, Du Y, Huang R, Gao L (2003) Preparation and modification of N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride nanoparticle as a protein carrier. Biomaterials 24(27):5015–5022
12. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W (2006) Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J. Controll Release 111(1–2):107–116
13. Alonso-Sande M, Cuna M, Remunan-Lopez C, Teijeiro-Osorio D, Alonso-Lebrero JL, Alonso MJ (2006) Formation of new glucomannan-chitosan nanoparticles and study of their ability to associate and deliver proteins. Macromolecules 39(12):4152–4158
14. Divya K, Jisha MS (2018) Chitosan nanoparticles preparation and applications. Env Chem Lett 16(1):101–112
15. Karathanos VT, Mourtzinos I, Yannakopoulou K, Andrikopoulos NK (2007) Study of the solubility, antioxidant activity and structure of inclusion complex of vanillin with b-cyclodextrin. Food Chem 101(2):652–658. https://doi.org/10.1016/j.foodchem.2006.01.053
16. Peng H, Xiong H, Li J, Xie M, Liu Y, Bai C, Chen L (2010) Vanillin cross-linked chitosan microspheres for controlled release of resveratrol. Food Chem 121(1):23–28
17. Li PW, Wang G, Yang ZM, Duan W, Peng Z, Kong LX, Wang QH (2016) Development of drug-loaded chitosan-vanillin nanoparticles and its cytotoxicity against HT-29 cells. Drug Deliv 23(1):30–35
18. Kwon J, Kim J, Park S, Khang G, Kang PM, Lee D (2013) Inflammation-responsive antioxidant nanoparticles based on a polymeric prodrug of vanillin. Biomacromolecules 14(5):1618–1626

19. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT—Food Sci Technol 28(1):25–30
20. Chen F, Shi Z, Neoh KG, Kang ET (2009) Antioxidant and antibacterial activities of eugenol and carvacrol-grafted chitosan nanoparticles. Biotechnol Bioeng 104(1):30–39
21. Mosmann T (1983) Rapid calorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63
22. Sukhramani PS, Patel PM (2013) Biological screening of *Avicennia marina* for anticancer activity. Der Pharm Sin 4(2):125–130
23. Thyriyalakshmi P, Radha KV (2015) Synthesis of dimethyl carbonate (DMC) based biodegradable nitrogen mustard ionic carbonate (NMIC) nanoparticles. RSC Adv 5:10560–10566
24. Aricò F, Chiurato M, Peltier J, Tundo P (2012) Sulfur and nitrogen mustard carbonate analogues. Eur J Org Chem 17:3223–3228
25. Jin X, Wang J, Bai J (2009) Synthesis and antimicrobial activity of the Schiff base from chitosan and citral. Carbohydr Res 344(6):825–829
26. Laus R, Favere VTD (2011) Competitive adsorption of Cu(II) and Cd(II) ions by chitosan crosslinked with epichlorohydrin–triphosphate. Bioresour Technol 102(19):8769–8776
27. Kocak N, Sahin M, Kucukkolbasi S, Erdogan ZO (2012) Synthesis and characterization of novel nano-chitosan Schiff base and use of lead (II) sensor. Int J Biol Macromol 51(5):1159–1166
28. Wang G, Li P, Peng Z, Huang M, Kong L (2011) Formulation of vanillin cross-linked chitosan nanoparticles and its characterization. Adv Mater Res 335–336(2011):474–477
29. Santos JED, Dockal ER, Cavalheir ETG (2005) Synthesis and characterization of Schiff bases from chitosan and salicylaldehyde derivatives. Carbohydr Polym 60(3):277–282
30. Jagadish RS, Divyashree KN, Viswanath P, Srinivas P, Raj B (2012) Preparation of N-vanillyl chitosan and 4-hydroxybenzyl chitosan and their physico-mechanical, optical, barrier, and antimicrobial properties. Carbohydr Polym 87(1):110–116
31. Ghate M, Manohar D, Kulkarni V, Shobhaand R, Kattimani SY (2003) Synthesis of vanillin ethers from 4-(bromomethyl) coumarins as anti-inflammatory agents. Eur J Med Chem 38(3):297–302
32. Stanzione JF III, Sadler JM, La Scala JJ, Reno KH, Wool RP (2012) Vanillin-based resin for use in composite applications. Green Chem 14(8):2346–2352
33. Fache M, Darroman E, Besse V, Auvergne R, Caillol S, Boutevin B (2014) Vanillin, a promising biobased building-block for monomer synthesis. Green Chem 16(4):1987–1998
34. Krishnapriya KR, Kandaswamy M (2010) A new chitosan biopolymer derivative as metal-complexing agent: synthesis, characterization, and metal(II) ion adsorption studies. Carbohydr Res 345(14):2013–2022
35. Amarasekara AS, Razzaq A (2012) Vanillin-based polymers—part II: synthesis of Schiff base polymers of divanillin and their chelation with metal ions. ISRN Polym Sci 2012:1–5
36. Kumar S, Dutta J, Dutta PK (2009) Preparation and characterization of N-heterocyclic chitosan derivative based gels for biomedical applications. Int J Biol Macromol 45(4):330–337
37. Stroescu M, Stoica-Guzun A, Isopencu G, Jinga SI, Parvulescu O, Dobre T, Vasilescu M (2015) Chitosan-vanillin composites with antimicrobial properties. Food Hydrocoll 48:62–71
38. Marin L, Stoica I, Mares M, Dinu V, Simionescu BC, Barboiu M (2013) Antifungal vanillin–imino-chitosan biodynameric films. J Mater Chem B 1(27):3353–3358
39. Singh RK, Kukrety A, Chatterjee AK, Thakre GD, Bahuguna GM, Saran S, Adhikarin DK, Atray N (2014) Use of an acylated chitosan schiff base as an ecofriendly multifunctional biolubricant additive. Ind Eng Chem Res 53(48):18370–18379
40. Kayaci F, Uyar T (2011) Solid inclusion complexes of vanillin with cyclodextrins: their formation, characterization, and high-temperature stability. J Agric Food Chem 59(21):11772–11778
41. Tirkistani FAA (1998) Thermal analysis of some chitosan Schiff bases. Polym Degrad Stab 60(1):67–70
42. Tree-udom T, Wanichwecharungruang SP, Seemork J, Arayachukeat S (2011) Fragrant chitosan nanospheres: controlled release systems with physical and chemical barriers. Carbohydr Polym 86(4):1602–1609
43. Patel Rajesh M, Patel Natvar J (2011) *In vitro* antioxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging methods. J Adv Pharm Educ Res 1:52–68
44. Jagtap RM, Pardeshi SK (2014) Antioxidant activity screening of a series of synthesized 2-aryl thiazolidine-4-carboxylic acids. Der Pharm Lett 6(3):137–145
45. Zhang Y, Feng S, Wu Q, Wang K, Yi X, Wang H, Pan Y (2011) Microwave-assisted synthesis and evaluation of naphthalimides derivatives as free radical scavengers. Med Chem Res 20(6):752–759
46. Mohana KN, Pradeep Kumar CB (2013) Synthesis and antioxidant activity of 2-amino-5-methylthiazol derivatives containing 1,3,4-oxadiazole-2-thiol moiety. ISRN Org Chem 2013:1–8
47. López-Mata MA, Ruiz-Cruz S, Silva-Beltrán NP, Ornelas-Paz JDJ, Ocaño-Higuera VM, Rodríguez-Félix F, Cira-Chávez LA, Del-Toro-Sánchez CL, Shirai K (2015) Physicochemical and antioxidant properties of chitosan films incorporated with cinnamon oil. Int J Polym Sci 2015:1–8
48. Zai-Qun L (2007) How many free radicals can be trapped by (hydroxylphenylimino)methylphenol in the free-radical-induced peroxidation of triolein in micelles? QSAR Comb Sci 26(4):488–495
49. Zhang Y, Zou B, Chen Z, Pan Y, Wang H, Liang H, Yi X (2011) Synthesis and antioxidant activities of novel 4-Schiff base-7-benzyloxy-coumarin derivatives. Bioorg Med Chem Lett 21(22):6811–6815
50. Guo-qing Y, Wen-yue X, Wang H, Sun Y, Hua-Zhang L (2011) Preparation, water solubility and antioxidant activity of branched-chain chitosan derivatives. Carbohydr Polym 83(4):1787–1796
51. Naik N, Vijay Kumar H, Harini ST (2011) Synthesis and antioxidant evaluation of novel indole-3-acetic acid analogues. Eur J Chem 2(3):337–341
52. Park H, Kim S, Kim S, Song Y, Seung D, Hong K, Khang G, Lee D (2010) Antioxidant and anti-inflammatory activities of hydroxybenzyl alcohol releasing biodegradable polyoxalate nanoparticles. Biomacromol 11(8):2103–2108
53. Kim S, Park H, Song Y, Hong D, Kim O, Jo E, Khang G, Lee D (2011) Reduction of oxidative stress by p-hydroxybenzyl alcohol-containing biodegradable polyoxalate nanoparticulate antioxidant. Biomaterials 32(11):3021–3029
54. Ho K, Yazan LS, Ismail N, Ismail M (2009) Apoptosis and cell cycle arrest of human colorectal cancer cell line HT-29 induced by vanillin. Cancer Epidemiol 33(2):155–160
55. Chen W, Han Y, Peng X (2014) Aromatic nitrogen mustard-based prodrugs: activity, selectivity and the mechanism of DNA cross-linking. Chem Eur J 20(24):7410–7418
56. Gillies ER, Fréchet JMJ (2003) A new approach towards acid sensitive copolymer micelles for drug delivery. Chem Commun 14:1640–1641

57. Souza AJD, Topp EM (2004) Release from polymeric prodrugs: linkages and their degradation. J Pharm Sci 93(8):1962–1979
58. Reddy MV, Reddy GCS, Jeong YT (2015) Molybdate sulfuric acid (MSA): an efficient reusable catalyst for the synthesis of tetrahydrobenzo[4, 5]imidazo[2,1-b]quinazolin-1(2H)-ones under solvent-free conditions and evaluation for their in vitro bioassay. RSC Adv 5(15):11423–11432
59. Ashutosh B, Khan I, Vivek KB, Sun CK (2017) MTT assay to evaluate the cytotoxic potential of a drug. Bangladesh J Pharmacol 12:115–118