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Biomedical applications of ferulic acid encapsulated electrospun nanofibers

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ABSTRACT

Ferulic acid is a ubiquitous phytochemical that holds enormous therapeutic potential but has not gained much consideration in biomedical sector due to its less bioavailability, poor aqueous solubility and physiochemical instability. In present investigation, the shortcomings associated with agro-waste derived ferulic acid were addressed by encapsulating it in electrospun nanofibrous matrix of poly (D,L-lactide-co-glycolide)/polyethylene oxide. Fluorescent microscopic analysis revealed that ferulic acid predominantly resides in the core of PLGA/PEO nanofibers. The average diameters of the PLGA/PEO and ferulic acid encapsulated PLGA/PEO nanofibers were recorded as 125 ± 65.5 nm and 150 ± 79.0 nm, respectively. The physiochemical properties of fabricated nanofibers are elucidated by IR, DSC and NMR studies. Free radical scavenging activity of fabricated nanofibers were estimated using di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay confirmed the cytotoxicity of ferulic acid encapsulated nanofibers against hepatocellular carcinoma (HepG2) cells. These ferulic acid encapsulated nanofibers could be potentially explored for therapeutic usage in biomedical sector.

1. Introduction

Recently, there has been an emerging research interest is noticed in renaissance of naturally occurring phenolic compounds as a potential therapeutic agents against broad range of infirmities including cancer, diabetes, aging, cardiovascular and neurodegenerative disease [1–3]. Among the naturally occurring polyphenolic plant constituent, ferulic acid possess a wide range of biomedical effects including antioxidant, anti-inflammatory, antimicrobial, antiallergic, anticarcinogenic, antithrombotic, antiviral, hepatoprotective, and vasodilatory actions [4–6]. Ferulic acid has also been approved globally as food additive to prevent the peroxidation of lipids as it affectively scavenges the superoxide anion radical [7,8]. However, despite the broad range of bioactivities, the therapeutic applications of ferulic acid are limited due to its poor bioavailability, low water solubility and physiochemical instability in human body fluids [9,10]. The conjugation of such bioactive molecules with biodegradable and biocompatible polymeric matrix, improves the water solubility of drug molecules due to the presence of interactions between drug and polymer matrix. Polymeric matrix provides an environment to the drug in which dispersed drug molecules can reside and experience the intermediate mobility and solubility as compare to pure components [11]. This in turn results into advanced pharmacokinetics properties as well as reduced side effects of drug [12,13]. A number of approaches have been introduced to formulate such systems which consists of biodegradable polymers along with antioxidant compound in order to improve the safety, therapeutic efficacy, stability and reduce toxicity by delivering the drug in a sustained manner for a prolong period [10,14]. In this investigation, we used electrospinning approach to encapsulate agro-waste derived ferulic acid in PLGA/PEO polymeric nanofibrous matrix to combat the shortcomings associated with it. These ferulic acid encapsulated nanofibers were then evaluated for its antioxidant and cytotoxic potential against HepG2 cells.

2. Materials and methods

2.1. Materials

Ferulic acid was isolated from *Parthenium hysterophorus* plant which was locally obtained and its identification was authenticated by Forest Research Institute (FRI), Dehradun, India. The polymers, PLGA (Mw∼45,000) and PEO (Mw∼900,000) were

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purchased from Sigma–Aldrich (St. Louis, MO, US). Dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell culture-grade dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay kit, acridine orange (AO), ethidium bromide (EtBr), Dulbecco's Modified Eagle Medium (DMEM) and all analytical grade chemicals were procured from Himedia (India). Human hepatoma cell line (HepG2) was obtained from National Center for Cell Science (NCCS), India.

2.2. Extraction and characterization of ferulic acid

Plant sample (whole plant) was dried under sunlight and kept in oven at 40 °C for 12 h. The dried samples were grounded to powder for the extraction of ferulic acid as described earlier [15,16]. Briefly, 2.0 g powder of plant sample was taken in 250 mL round bottom flask containing 60 mL NaOH (2 M). To prevent the oxidation of ferulic acid during alkali treatment, 0.001 g $NaHSO_3$ was added and kept within rotary shaker at 25 °C, 180 rpm for 24 h. Sample was then centrifuged at 12,000 rpm for 10 min and the obtained supernatant was acidified with by 2 M HCl solution ($pH \leq 2$). Acidified sample was treated three times with 60 mL ethyl acetate and concentrated for the extraction of ferulic acid. The concentrated extract was dissolved in equal volume of acetonitrile/water for further analysis [17]. Ferulic acid bands from TLC plate was scraped and dissolved in 2.0 mL acetonitrile. The samples ($n = 3$) were quantitative analyzed using HPLC (Knauer, Germany) with C_{18} column having mobile phase consisting of acetonitrile/water (80:20) and 0.1% acetic acid and absorbance was taken at 320 nm.

2.3. Preparation of electrospun nanofibers

Polymers (PLGA:PEO:1:1) at an absolute concentration of 2 wt% were dissolved in DCM/DMF (4:1, v/v) solvent to prepare PLGA/PEO nanofibers. A blended solution containing ferulic acid and PLGA/PEO polymers was prepared by dissolving ferulic acid (2 mg/mL) in the above prepared 2 wt% PLGA/PEO polymeric solution. PLGA/PEO and ferulic acid containing PLGA/PEO solutions were then separately loaded into a 5.0 mL syringe which is attached to a metallic needle (21 G). Electrospinning process was performed at a fixed applied voltage (18 kV) and solution flow rate (0.5 mL/h). The fabricated nanofibers were collected on aluminum foil collector, placed at 12 cm horizontal distance from the metallic needle tip. The collected nanofibers were placed in desiccators for 24 h to get rid of any residual solvent.

2.4. Spectroscopic and microscopic analysis

Infrared absorption of isolated ferulic acid, PLGA/PEO and ferulic acid encapsulated PLGA/PEO nanofibers were examined by infrared (IR) spectrometer (Thermo Nicolet Nexus 6700, US) while nuclear magnetic resonance (NMR) experiments were performed using 500 MHz high resolution NMR spectrometer (Avance 500 Bruker Biospin Intl. AG, Switzerland). The phase behavior of samples (10–12 mg) was observed by heating them at 10 °C/min from 25 to 500 °C under the nitrogen atmosphere using a differential scanning calorimeter (DSC; EXSTAR TG/DTA 6300). Morphology of fabricated nanofibers was observed using field emission scanning electron microscopy (FESEM; Quanta 200F Model, FEI, Netherland) at an accelerated voltage of 15 kV. Samples for FESEM were prepared by cutting them into 1 × 1 cm pieces and sputter coated with gold for 1 min using a Biotech SC005 sputter coater. The diameter of nanofibers was measured from FESEM

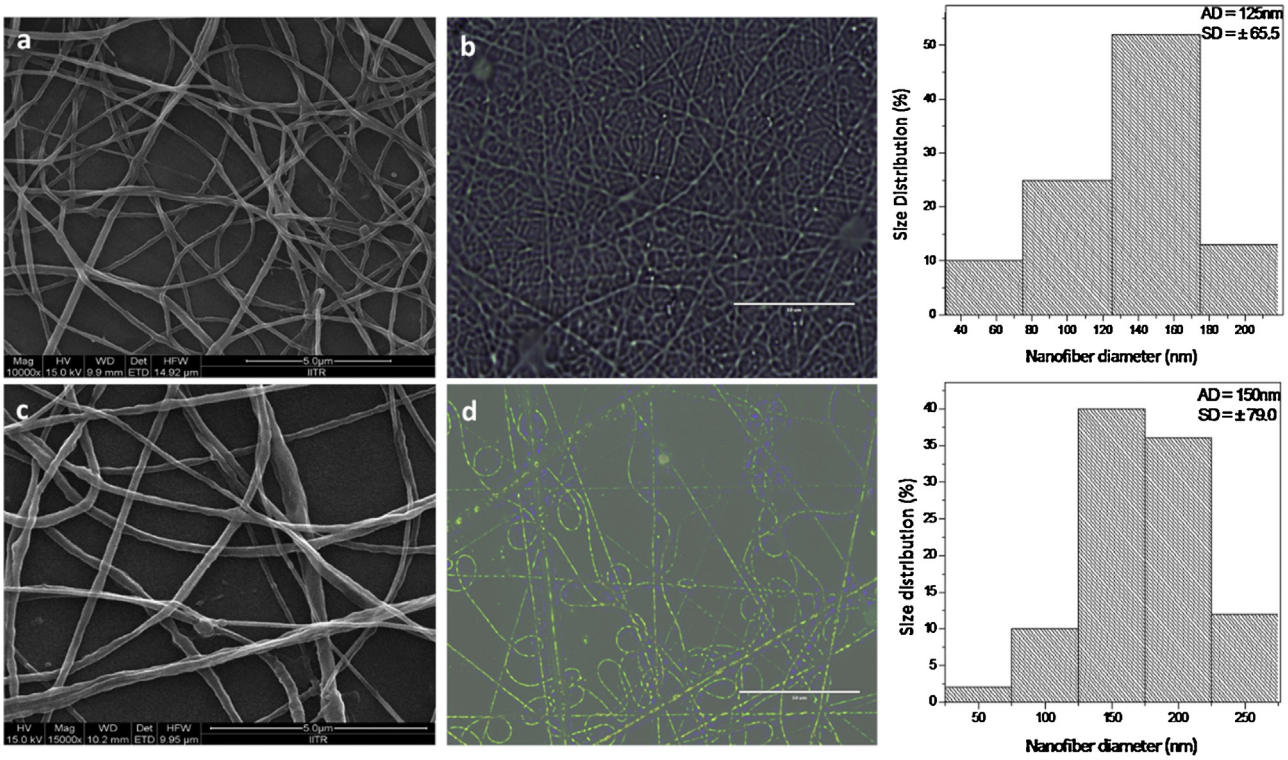

Fig. 1. FESEM and fluorescent micrographs of PLGA/PEO (a and b) and ferulic acid (FA) encapsulated PLGA/PEO (c and d) nanofibers with corresponding diameter distribution histograms.

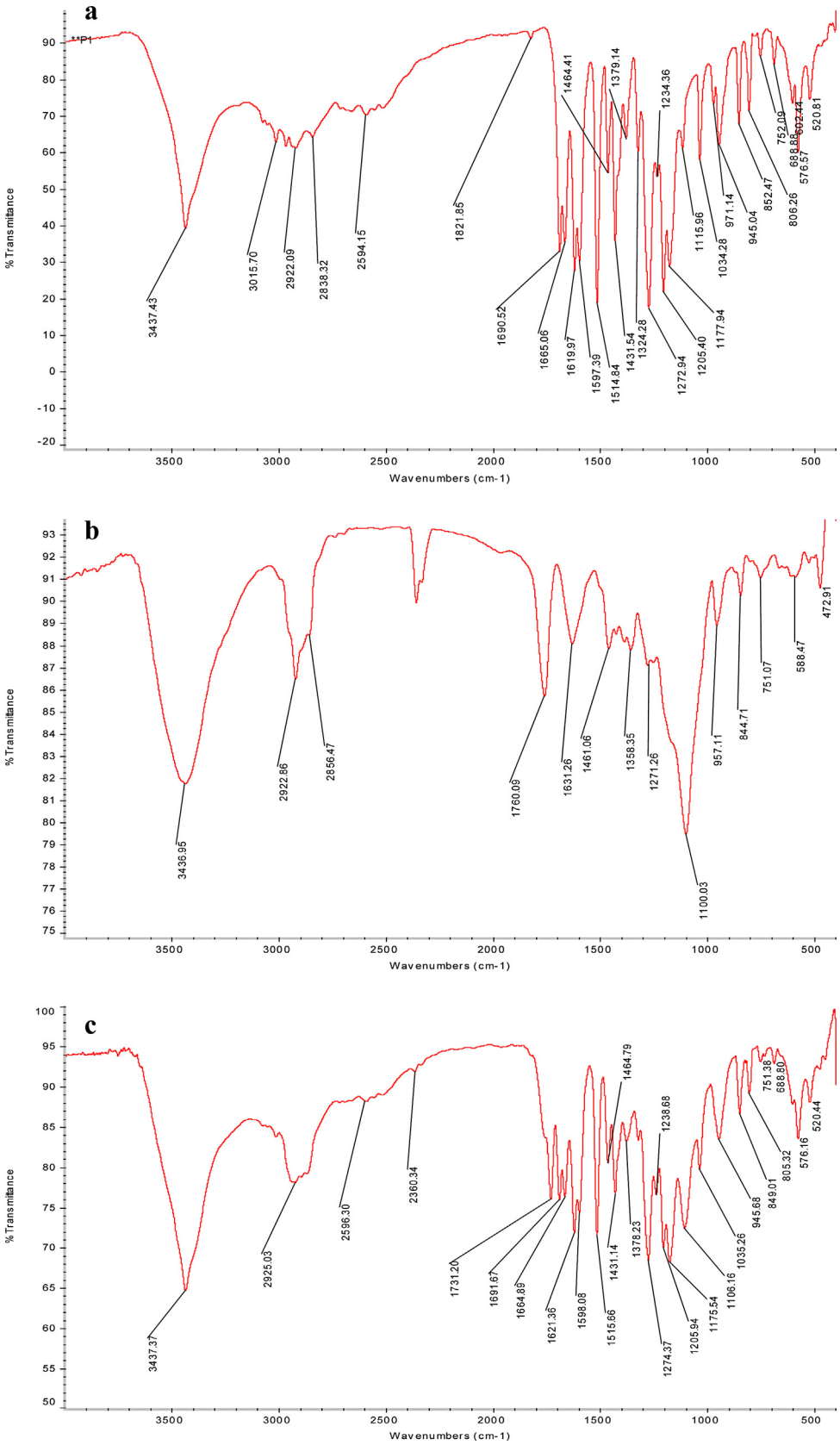

Fig. 2. IR spectra and of isolated (a) ferulic acid (b) PLGA/PEO and (c) ferulic acid encapsulated PLGA/PEO nanofibers.

images using visualization software Image J. The morphology and the ferulic acid encapsulation in the PLGA/PEO nanofibers were also illustrated by fluorescent microscopy. For sample preparation, nanofibers were collected onto a glass cover slip for 2 min and observed immediately under the microscope (Evosfl, AMG groups, USA).

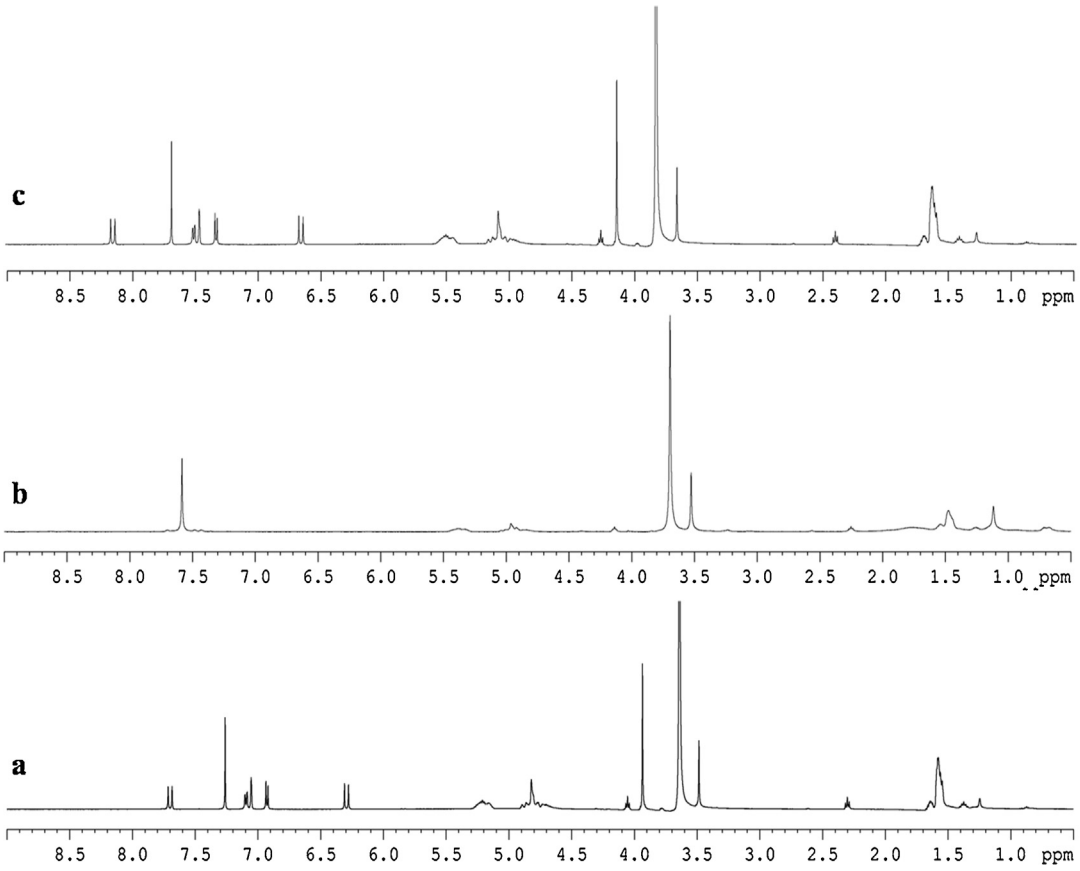

Fig. 3. ^{1}H NMR spectra of isolated (a) ferulic acid (b) PLGA/PEO and (c) ferulic acid encapsulated PLGA/PEO nanofibers.

2.5. Free radical scavenging activity

The antioxidant activity of ferulic acid before and after encapsulation was measured using DPPH radical-scavenging assay as reported earlier [14]. Briefly, 0.2 mL solution containing ferulic acid encapsulated PLGA/PEO nanofibers (10 mg in 5 mL DCM/DMF; $n = 3$) was added in 2.8 mL of 1×10^{-4} M methanol solution of DPPH radical and allowed to stand in dark at room temperature for 60 min. Similarly, the equivalent amount of ferulic acid (2 mg/mL) in PLGA/PEO solution before electrospinning was used as control and assayed for comparative study. The absorbance was recorded at 517 nm.

2.6. In vitro *cytotoxic activity against HepG2 cells*

I*n vitro* cell viability of HepG2 cells on ferulic acid encapsulated PLGA/PEO nanofibers was estimated using MTT assay [18]. Briefly, the sterilized electrospun samples ($n = 3$) were kept in 24 well microtiter plates and DMEM medium supplemented with 10% fetal bovine serum (FBS) was added to each well. Diluted suspension of HepG2 cells (1×10^{5} cells/mL) was then added to each well and plates were incubated in humidified atmosphere containing 5% CO_2 at 37 °C in a CO_2 incubator. The HepG2 cells cultured in presence of isolated ferulic acid only were used as positive control. Cells were harvested after 24 h and washed carefully with PBS (pH 7.4) to remove unattached cells. FESEM was used to observe the cell morphologies on each sample. For FESEM imaging, the adhered cells on samples were secured by keeping them in 2.5% glutaraldehyde/PBS solution at 4 °C for 4 h. Consequently, the samples were desiccated stepwise with different graded ethanol solutions (25%, 50%, 75%, 90% and 100%) and coated with gold.

2.7. Cytotoxic analysis using *fluorescent microscopy*

HepG2 cells (1×10^{5} cells/mL) cultured in presence of different formulations (as described above) were stained with acridine orange (AO) and ethidium bromide (EtBr) (100 μg/mL stock solution) for 10 min in order to determine the cell death type. The stained cells seeded on fabricated nanofibrous samples were than observed under the fluorescence microscope (Evosfl, AMG groups, USA).

Table 1
Change in chemical shifts (ppm) in ^{1}H NMR of isolated ferulic acid and ferulic acid encapsulated PLGA/PEO nanofibers.

Type of protons	Isolated ferulic acid	Ferulic acid encapsulated PLGA/PEO nanofibers	Change in chemical shift (ppm)
$-OCH_3$	3.6 ppm	3.8 ppm	+0.2
Aromatic	6.3–7.7 ppm	6.8–8.3 ppm	+0.5–0.6
Phenolic	9.4 ppm	9.8 ppm	+0.4
Carboxylic	11.92 ppm	12.05 ppm	+0.13

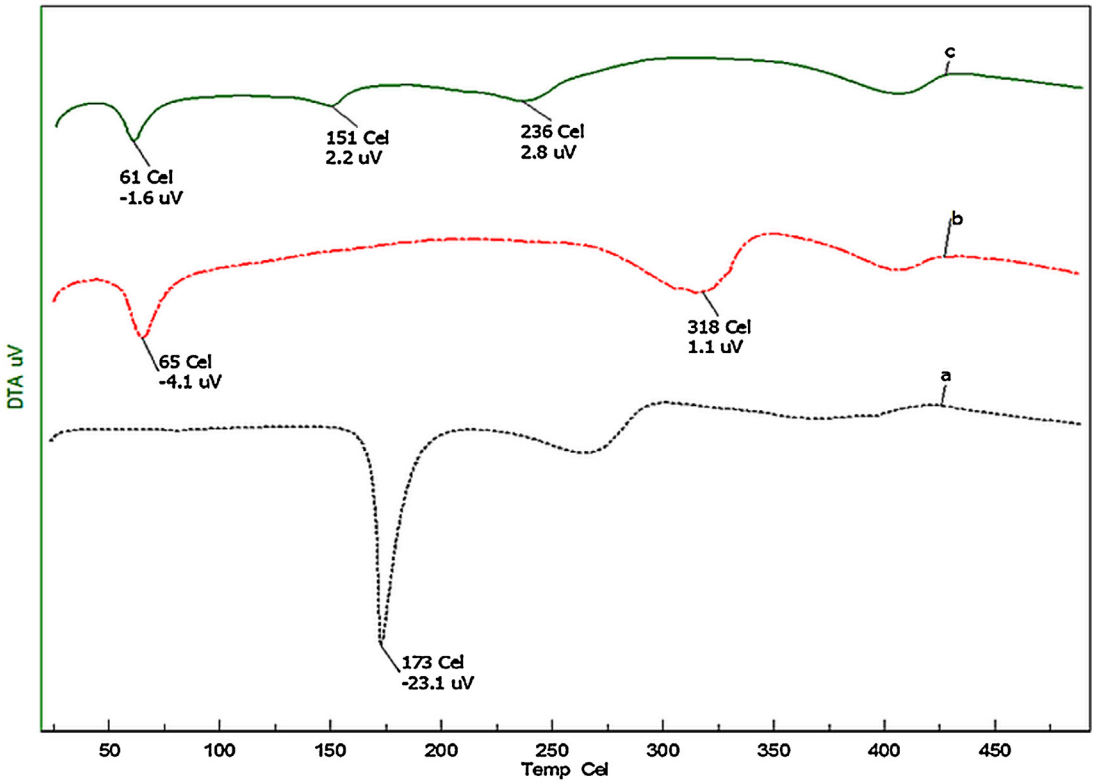

Fig. 4. DSC thermograms of isolated ferulic acid (FA), PLGA/PEO and ferulic acid encapsulated PLGA/PEO nanofibers (PLGA/PEO-FA).

2.8. Statistical analysis

The experiments were performed in triplicate ($n = 3$) and the results are expressed as mean values with ±standard deviation (SD). Statistical analysis was performed using one-way ANOVA test. Statistical significance values of $p < 0.05$ were considered.

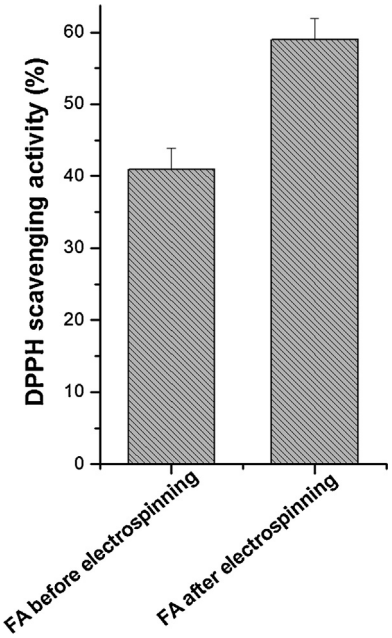

Fig. 5. Antioxidant activity of ferulic acid (FA) before and after encapsulation (electrospinning).

3. Results and discussion

3.1. Extraction and characterization of isolated ferulic acid

Isolation and quantification of ferulic acid from *P. hysterophorus* sample were carried out using alkali treatment and chromatographic techniques, respectively. The TLC chromatogram of plant sample indicated the presence of ferulic acid when sprayed with 10% ferric chloride solution and compared with standard ferulic acid (Supplementary Fig. 1). HPLC assessment of TLC spots for ferulic acid (retention time: 29.07 min) was performed for further purification (Supplementary Fig. 2). The approximate content of ferulic acid in whole plant was recorded to be 145–160 mg/100gram (45–50 mg, 64–70 mg and 32–35 mg in root, stem and leaves, respectively).

3.2. Morphology of electrospun nanofibers

Key parameters (polymer concentration, ferulic acid concentration, applied voltage, solution flow rate and tip to collector distance) that affect the fiber morphology were optimized for successful preparation of the ferulic acid encapsulated nanofibers as described earlier [19,20]. FESEM analysis depicted the average diameter of nanofibers as 125 ± 65.5 nm and 150 ± 79.0 nm for ferulic acid-free PLGA/PEO and ferulic acid encapsulated PLGA/PEO nanofibers, respectively (Fig. 1a and b). The surface of nanofibers appeared smooth and free of ferulic acid crystal on the fibrous surface. Data depicts that ferulic acid homogenously dispersed in the nanofibers with slight enhancement in the fiber diameter as shown in their corresponding diameter distribution histograms (Fig. 1). The encapsulation of ferulic acid within the nanofibers was further assessed using auto-fluorescence properties of ferulic acid (Fig. 1b and d). The intersectional fluorescent images represent

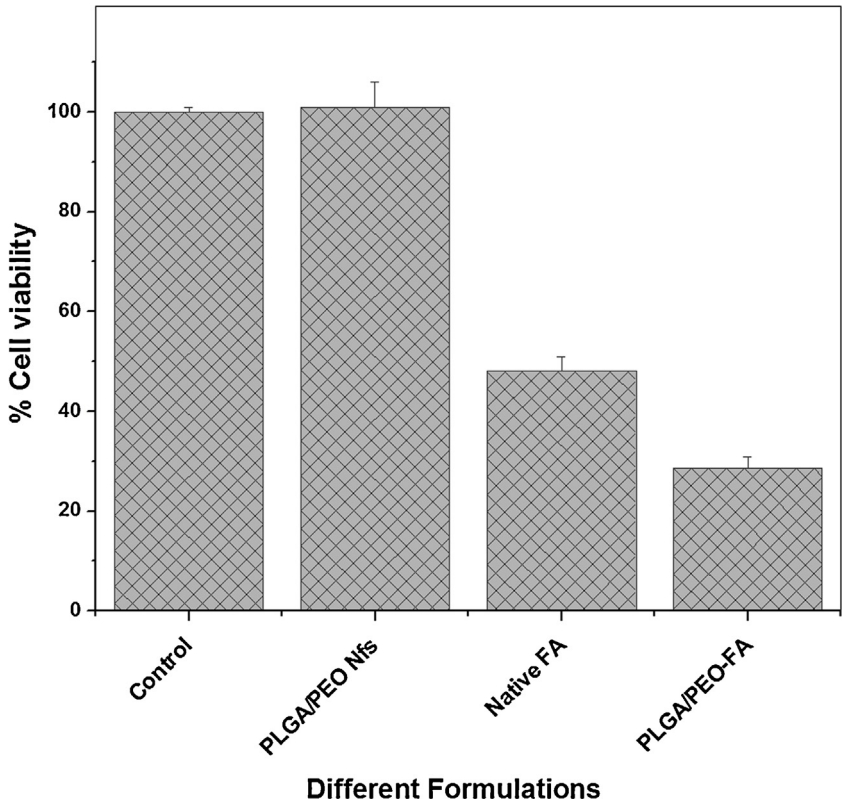

Fig. 6. Colorimetric MTT viability assay against HepG2 cells treated with different formulations coverslips (control), PLGA/PEO nanofibers, native ferulic acid and ferulic acid encapsulated PLGA/PEO nanofibers after 24 h at A570 nm. Error bars represent mean ± standard deviation for three independent experiments ($n = 3$). Nfs = nanofibers.

that ferulic acid predominantly reside in the core region of encapsulated PLGA/PEO nanofibers.

3.3. Spectroscopic and thermal analysis

The successful incorporation as well as the presence of the secondary interactions between ferulic acid and nanofibers has been confirmed by IR (Fig. 2) and NMR spectroscopy (Fig. 2B, 500 MHz, solvent: $CDCl_3$). The presence of secondary interactions reflected the compatibility among the drug molecules and carrier nanofibrous matrix. The IR spectrum of isolated ferulic acid showed the characteristic bands at 3437.43 cm^{-1} for —OH group, 3015.70 cm^{-1} for —CH group, 1690.52 cm^{-1} for —C=O group, 1665.06 cm^{-1} for alkene group, 1619.97–1431.54 cm^{-1} for C=C group, 1272.94–1234.36 cm^{-1} for C—O—C group and from 900 to 650 cm^{-1} for —C—H group. The IR data obtained for ferulic acid was found to be in agreement with the earlier reports [21,22]. The IR spectrum for PLGA/PEO nanofibers shown peaks at 3436 cm^{-1} (—OH group), 2922–2856 cm^{-1} (—CH vibrations), 2360 cm^{-1} (C≡C group), 1760–1630 cm^{-1} (—C=O group) and 1271–1100 cm^{-1} (C—O group). The IR spectrum for ferulic acid encapsulated PLGA/PEO nanofibers contained all the peaks for PLGA/PEO fibers as well as for ferulic acid. In addition, the spectrum of ferulic acid encapsulated nanofibers also showed the shifting of absorption peaks (1640–1820 cm^{-1}) to lower wavenumber which possibly could be due to the presence of weak interactions such as hydrogen bonding between the PLGA/PEO carbonyl groups and ferulic acid hydroxyl groups. The shifting of peaks and presence of the ferulic acid peaks in the IR spectra of the composite nanofibers conjointly suggested the interaction between the ferulic acid and polymeric matrix which ensures a stabilized environment for the composite structure and provides a high degree of compatibility between the components of nanofibers.

The stability studies were performed using NMR to examine the stability of ferulic acid after encapsulation due to its peculiar instability [23]. ^1H NMR spectrums of isolated ferulic acid, PLGA/PEO nanofibers and ferulic acid encapsulated PLGA/PEO nanofibers displayed no considerable difference for the peaks of ferulic acid (Fig. 3) thereby indicating the integrity of ferulic acid molecules and the retention of its activity even after electrospinning process. In addition, small peaks (ranges from 6.3–7.7 ppm) for ferulic acid in the NMR spectrum of ferulic acid encapsulated PLGA/PEO nanofibers have been found to be slightly shifted towards 6.8 ppm to 8.3 ppm and the methoxy group has been shifted from 3.6 ppm to 3.8 ppm after encapsulation.

The variation in chemical shifts variation which occurred after encapsulation of isolated ferulic in PLGA/PEO nanofibers has been listed in Table 1. This shifting could be attributed to the presence of weak interaction bonds such as hydrogen bonds between the ferulic acid and polymeric matrix.

The phase transition behavior and physical stability of isolated ferulic acid, ferulic acid free and ferulic acid encapsulated nanofibers were evaluated by DSC analysis (Fig. 4). DSC thermograms of isolated ferulic acid exhibited a single sharp endothermic peak at melting point of 173 °C whereas thermograms of PLGA/PEO nanofibers showed first endothermic peak due to glass transition temperature (T_g) at 65 °C and a second endothermic peak at 318 °C due to the melting of crystalline phase of polymeric matrix [24–26]. In case of ferulic acid encapsulated nanofibers melting temperature point was shifted to the lower values compared to isolated ferulic acid (173–151 °C). This shifting could be attributed to the improved orientation in the molecular chains of encapsulated ferulic acid and large surface area to volume ratio of

nanofibers. The decrease in the melting point could also be observed due to the presence of amorphous ferulic acid in the PLGA/PEO nanofibers instead of crystalline state of isolated ferulic acid. This also suggested that that drug molecules are uniformly dispersed in the nanofibers.

3.4. Free radical scavenging activity

DPPH composed of stable free radical molecules with a maximum optical absorption at 517 nm. It can readily undergo scavenging by an antioxidant [27,28]. In this study, the antioxidant activity of isolated ferulic acid and ferulic acid encapsulated PLGA/PEO nanofibers were recorded to be 41% and 59%, respectively (Fig. 5). The data showed a significant ($p \leq 0.05$) difference in the DPPH radical scavenging activity of free ferulic acid and encapsulated ferulic acid which ensures an improved chemical integrity and antioxidant activity of ferulic acid after encapsulation.

3.5. In vitro *cytotoxicity against HepG2 cells*

Hepatocellular carcinoma (HCC) is globally recognized as a major cancer in the world. HCC is commonly preceded due to the hepatocellular damage caused by the generation of reactive oxygen species. Several adjunctive therapies including interferon alpha, tumor necrosis factor (TNF) and anticancer drugs have been used to combat HCC [29,30]. However, in spite of enormous progress, the inherent resistance of cancer cells towards the chemotherapeutic treatments and augmented level of multi-drug resistance protein (MDR) are some of the prime obstacles to treat HCC. Ferulic acid is one of the well-known phytochemical phenolic for its anticancer activity against breast cancer colon cancer, skin cancer, and pulmonary cancer [20,31,32]. The cytotoxicity of ferulic acid encapsulated PLGA/PEO nanofibers toward HepG2 cells are shown in Fig. 6. Data revealed that the polymeric nanofibers without ferulic acid did not display any inhibition in cell growth. Whereas, the inhibition rate of 51.9% and 71.3% ($p < 0.01$) in cell growth were recorded for isolated ferulic acid and ferulic acid encapsulated nanofibers, respectively.

The morphological changes in hepatoma cells were observed in presence of isolated ferulic acid and ferulic acid encapsulated PLGA/PEO nanofibers and shown in Fig. 7. The FESEM images depicted the normal morphology of hepatoma cells on PLGA/PEO nanofibers with good adhesion and proliferation. However, a substantial reduction in cells number and growth was noticed in case of ferulic acid encapsulated nanofibers. To further determine the cell cytotoxicity of fabricated nanofibers towards HepG2 cells, a double staining assay was performed using a mixture of AO and EtBr in 1:1 w/w ratio. The stained HepG2 cells were visualized using fluorescent microscopy. Both the dyes (AO, EtBr) mark DNA and helps in identification of live and dead cells. The intact cells were stained green due to AO whereas the compromised cells (damaged cells) were stained red due to assimilation of EtBr. The intercalation of both the dyes gave orange fluorescence [33]. The cells were found to fluorescence green in case of control and PLGA/PEO nanofibers (Fig. 8), which confirmed the cytocompatibility of fabricated PLGA/PEO nanofibers. However, the cells cultured in presence of isolated ferulic acid and ferulic acid encapsulated nanofibers accumulated EtBr in their cytoplasm and appeared yellow–orange due to co-localization of green and red stains.

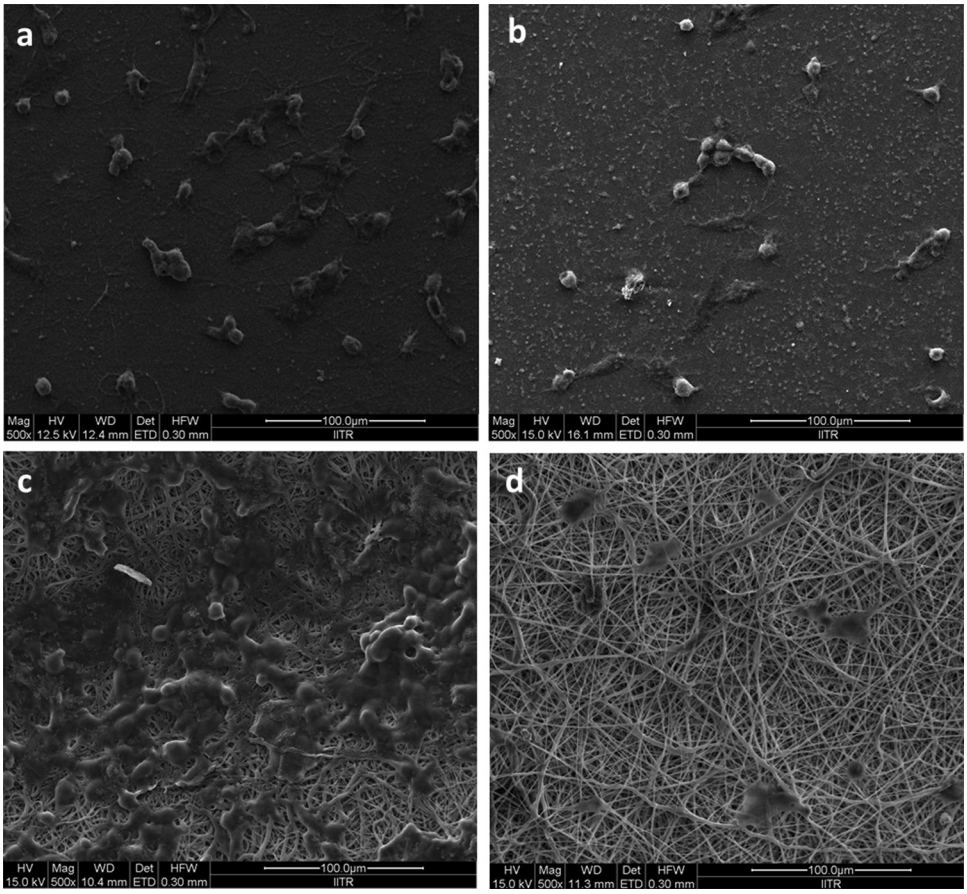

Fig. 7. The effect of (a) control (b) isolated ferulic acid (c) PLGA/PEO nanofibers and (d) ferulicacid encapsulated PLGA/PEO nanofibers on cell morphology of HepG2 cells after 24 h of treatment.

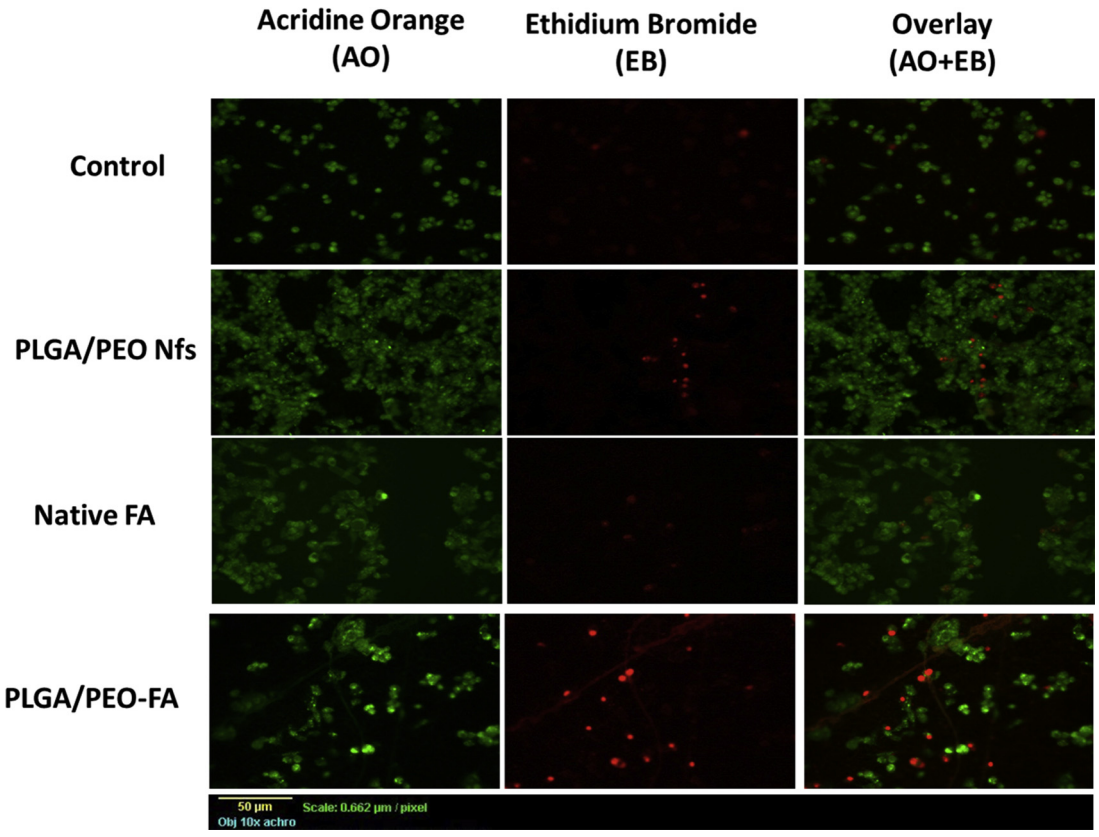

Fig. 8. Fluorescent micrographs of AO:EtBr stained HepG2 cells after 24 h incubation period. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

This observation confirmed the cytotoxic effect of isolated ferulic acid and ferulic acid encapsulated PLGA/PEO nanofibers against HepG2 cell line. Moreover, encapsulated ferulic acid possessed substantial strong cytotoxicity as compared to isolated ferulic acid alone which suggested that polymeric nanofibrous matrix efficiently helps in maintaining its activity for prolong time.

4. Conclusion

In this study, ferulic acid was isolated from *P. hysterophorus* by alkali extraction and its structural characterization was performed using IR and NMR spectroscopy. To enhance the efficiency and stability of isolated ferulic acid, it was successfully encapsulated in the PLGA/PEO electrospun nanofibrous matrix. Microscopic studies revealed the smooth morphology of the fabricated nanofibers with the predominant distribution of ferulic acid in the core region of nanofibers. From the data obtained, we can conclude that the fabricated polymeric nanofibers can act as a protective layer for ferulic acid and can combat its shortcoming such as physiochemical stability and premature degradation. In contrast to isolated ferulic acid, ferulic acid released from PLGA/PEO nanofibers maintained its cytotoxic activity during the whole experiment. The formulated ferulic acid encapsulated nanofibers could be useful in opening up of new therapeutic avenues for liver cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.08.008.

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