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Inhibition of HIV-1 infection with curcumin conjugated PEG-citrate dendrimer; a new nano formulation

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Abstract

Background Nano-drug delivery systems have become a promising approach to overcoming problems such as low solubility and cellular uptake of drugs. Along with various delivery devices, dendrimers are widely used through their unique features. PEG-citrate dendrimers are biocompatible and nontoxic, with the ability to improve drug solubility. Curcumin, a naturally occurring polyphenol, has multiple beneficial properties, such as antiviral activities. However, its optimum potential has been significantly hampered due to its poor water solubility, which leads to reduced bioavailability. So, the present study attempted to address this issue and investigate its antiviral effects against HIV-1.

Method The G2 PEG-citrate dendrimer was synthesized. Then, curcumin was conjugated to it directly. FTIR, HNMR, DLS, and LCMS characterized the structure of products. The conjugate displayed an intense yellow color. In addition, increased aqueous solubility and cell permeability of curcumin were achieved based on flow cytometry results. So, it could be a suitable vehicle for improving the therapeutic applications of curcumin. Moreover, cell toxicity was assessed using XTT method. Ultimately, the SCR HIV system provided an opportunity to evaluate the level of HIV-1 inhibition by the curcumin-dendrimer conjugate using a p24 HIV ELISA kit.

Results The results demonstrated a 50% up to 90% inhibition of HIV proliferation at 12 µm and 60 µm, respectively. Inhibition of HIV-1 at concentrations much lower than CC50 (300 µM) indicates a high potential of curcumin-dendrimer conjugate against this virus.

Conclusion Thereby, curcumin-dendrimer conjugate proves to be a promising tool to use in HIV-1 therapy.

Keywords PEG-citrate dendrimer, Drug delivery, Curcumin, HIV-1, Antiviral, Herbal drug

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Introduction

Nanomedicine has been significantly refined over the last few decades, particularly in drug delivery [1, 2]. Using nanoscale delivery vehicles makes it possible to achieve improved solubility and circulating half-life of poorly water-soluble drugs. They also increase specificity, reduce side effects, improve bioavailability, and diminish drug metabolism. Moreover, intracellular delivery of macromolecules and targeted delivery of drugs to specific cells or tissues can be achieved [2–5]. The application of nanodelivery systems in the clinical treatment and prevention of various diseases has the greatest potential due to these specific characteristics [2, 3].

Since viral diseases have generally been the main cause of illness, researchers have worked continuously to discover new antiviral agents. AIDS is one of the significant viral diseases. Despite ongoing improvements in HIV prevention and treatment, the HIV/AIDS pandemic continues to be a major problem for the majority of developing countries [6], with over 38.4 million living with HIV/AIDS as of 2021 [7].

Highly active antiretroviral therapy (HAART), the current HIV/AIDS therapy strategy, involves administering patients three or more antiviral medications concurrently. Despite significant advancements in HIV/AIDS treatment, several problems persist. The evolution of viral resistance, life-long administration, poor patient adherence, and high prices, among other factors, impede broad use [8, 9]. In addition, the side effects of drugs due to their toxicities are also a concern. Increased rates of heart disease, diabetes, liver disease, cancer, and accelerated ageing were monitored in patients taking HAART experience [10]. Therefore, there is an enormous need to investigate novel methods for creating nontoxic, lower-dosage therapeutics [2]. To conquer the drawbacks of the existing drugs, such as their undesirable side effects, traditional herbal therapy, and natural remedies are gradually gaining acceptance in the modern world, and they provide new possibilities for future medicines for a wide range of diseases [11–14].

Curcumin is a yellow hydrophobic polyphenol derived from the rhizome of turmeric (Curcumin longa). It has a broad range of biological and pharmacological activity, including anti-inflammation, antioxidant, antiproliferative, antifungal, antiviral, antibacterial, and anti-cancer activity, with low or no inherent toxicity [15–19]. However, the poor bioavailability of curcumin due to low absorption in the small intestine, poor solubility, rapid degradation, and swift systemic elimination have been highlighted as critical issues in the therapeutic potential of this useful natural drug [20].

Several methods based on nano vehicles have been recently investigated to conquer these restrictions. Curcumin is encapsulated in liposomes, micelles, solid lipid microparticles, Chitosan, dendrosome, and dendrimers or complexes with phospholipids and cyclodextrin [21–26]. Likewise, nanoformulations such as lipid nanoparticles, nanoemulsions, polymer nanoparticles, and nanocrystal dispersions have improved curcumin's solubility, stability, and bioavailability [27–37].

Among various nanocarriers, dendrimers are excellent pharmacological transporters because of unique features such as well-defined chemical structure, rounded shape, uniform size distribution, and controlled surface functionalities. Dendrimers improve the solubility and bioavailability of weakly water-soluble medicines. Additionally, they may extend the period that pharmaceuticals remain in the body, increase the stability of bioactives, and protect them from the biological environment [38].

Small therapeutic molecules can be entrapped within the dendritic architecture and safely transported to target places. Furthermore, drugs can interact with the surface of dendrimers with electrostatic or covalent interactions. These systems can enhance the solubility and bioavailability of the drug and act as modulators for targeting drug delivery systems [39, 40].

To be used for drug delivery, dendrimers must be nontoxic, non-immunogenic, and biodegradable [41]. However, the inherent toxicity of dendrimers prevents their use in biological systems. One strategy to eliminate or reduce the toxicity of dendrimers is designing dendrimers composed of biocompatible building blocks by operating biodegradable core and branching units or utilising metabolic pathway intermediates [38].

Recently, various biodegradable dendrimers have been developed with monomers of natural metabolites from varied metabolic pathways, including citric acid dendrimers, phosphate dendrimers, and lysine dendrimers [38].

Citric acid–polyethylene glycol-based dendrimers (PEG-citrate dendrimers) are particularly interesting since they have citric acids as peripheral groups with a negative charge, which are not harmful to biological membranes. Furthermore, they have polyethylene glycol (PEG) in the core, which is biodegradable. In addition, these dendrimers have shown regulated release of mefenamic acid, diclofenac, and 5-amino salicylic acid [42, 43]. Namazi and Adeli synthesised PEG-citrate dendrimers and entrapped small drug molecules in the above dendrimers. These hydrophobic drugs and molecules were becoming soluble in aqueous solutions. Besides, they were stable at room temperature for a long time and did not release drugs. Also, they have shown a controlled release of guest molecules [44]. In another study performed by Haririan and colleagues, two conjugates of cisplatin (G1+Pt and G2+Pt) with these biocompatible dendrimers were prepared, and their potential cytotoxic effects were examined using an MTT assay. They

described that these conjugates have high potency and minimum hemolysis. So, they are suitable candidates against cancerous cell lines as efficient and novel antitumor agents [45]. Another study performed a series of in vitro structure-associated cell toxicity evaluations on G1 (generation 1) and G2 (generation 2) PEG-citrate dendrimers. They examined the in vitro cytotoxicity of dendrimers using methods such as crystal violet staining, MTT, and LDH tests. Based on their results, these dendrimers do not have any harmful effects up to the concentration of 0.05 mg/ml. They described the potential of these structures to be employed in various areas of nanomedicine would be highly significant [46]. Because of the benefits of PEG-citrate dendrimers, they are selected in this study. Here we introduced a facile and efficient procedure with green chemistry to synthesise these applicable and biodegradable dendrimers.

On the other hand, the use of curcumin in cancer treatment has been studied a lot, but few studies have been done regarding its antiviral properties. Therefore, considering the need that existed in this field as well as the high potential of curcumin as an antiviral drug, in this study, we conjugated curcumin to G2 PEG-citrate dendrimer to improve its solubility and cell uptake. Finally, its effect on human immunodeficiency virus (HIV-1) was investigated.

Materials and methods

Materials

Polyethylene glycol (PEG) 600, Citric acid, dicyclohexylcarbodiimide (DCC), Dimethyl Sulfoxide (DMSO), Methanol, Chloroform, Iodine, Toluene, Calcium Chloride anhydrous, Acetone, Curcumin and TLC Silica gel 60 were purchased from Merck Co. and Dialysis membrane (100–500 Da and 500–1000 Da) was obtained from Spectrum Company.

Methods

Synthesis of PEG-citrate dendrimer G1 (generation 1) and G2 (generation 2)

This procedure used 3.7 mmol poly (ethylene glycol) (PEG) (2 ml). First, 3.7*2 mmol DCC was dissolved in 15 ml anhydrous DMSO and added to a PEG flask. Next, the mixture was stirred for about 15 min at room temperature. After that, 3.7*2 mmol citric acid was added and stirred for one more hour. The reaction was stopped by adding 20 ml ddH2O (double distilled water) and was filtered by filter paper. Then the clear solution was transferred to a dialysis bag (cut off 100–500Da, SPECTRUM) and dialysed against ddH2O for 17 h to separate the unreacted material. Eventually, the product was removed from the dialysis bag and dried in a Freeze dryer.

For the synthesis of generation 2 (G2), a solution of G1 (0.5 g, 0.52 * 10−3 mmol) was dissolved in anhydrous DMSO, and then the equivalent of (0.52×6 mmol) DCC was added. After that, the container was closed immediately and stirred for 15 min. Then, (0.52×6 mmol) citric acid was added and stirred again for one hour. Next, the reaction was stopped by adding 20 ml ddH2O and filtered with filter paper. Afterward, the clear solution was collected and conducted into dialysis bags (cut off 500-1000Da, SPECTRUM). Finally, the above procedures were repeated to purify G2 PEG-citrate dendrimer.

Synthesis of curcumin-dendrimer conjugate

- Procedure A: use of anhydrous Toluene and Methanol as solvents, CaCl2 as the catalyst, and 110˚C.

In this process, a solution of G2 PEG-citrate dendrimer (0.0415.

g, 0.019 mmol) in 10 ml methanol was used. Curcumin was dissolved in 10 ml toluene (0.13 g, 0.35 mmol) and was added to the G2 PEG-citrate dendrimer solution along with CaCl2 (0.002 g, 0.018 mmol) (as a catalyst). The reaction was performed in a round-bottom flask with reflux and stirred at 110˚C for 72 h. After that, solvents were removed by using a rotary evaporator. Then, the reaction was stopped by adding ddH2O, and the product was filtered with a 0.45-micron filter to remove non-conjugated curcumin. Ultimately, the curcumin-dendrimer conjugate was dried in a Freeze dryer.

- Procedure B: Synthesis with DMF as a solvent and DCC.

In this procedure, terminal groups of G2 PEG-citrate dendrimer (200 mg in anhydrous DMF and sulfuric acid) were activated with 373 mg DCC. After 15 min, 667 mg of curcumin was added and stirred for 72 h. Next, the reaction was ended by adding 20 ml ddH2O. It was filtered through filter paper to remove precipitated free curcumin, and di-cyclohexyl urea (DCU) formed, followed by gel filtration chromatography (Sephadex G-75 M) to eliminate the solvent. Eventually, the curcumin-dendrimer conjugate was dried in a Freeze dryer.

- Procedure C: Combination of procedures A and B and develop a novel method with higher efficiency.

Here, the combination of procedures A and B were used. First of all, a solution of G2 PEG-citrate dendrimer ((200 mg, 0.1 mmol) in 10 ml toluene) with 2.22 mg anhydrous CaCl2 and 280.55 mg (0.1 *18 mmol) EDC was prepared. Thereupon, curcumin (667 mg, 0.1 *18 mmol) dissolved in 5 ml methanol was added, and the resultant reaction mixture was refluxed and stirred using

a magnetic bead at 110˚C for 72 h. Subsequently, solvents were removed with a rotary evaporator, and curcumin-dendrimer conjugate was dissolved in water by adding 30 ml ddH2O and shaking. Precipitated EDC and Free curcumin were removed with filtering by filter paper, and a 0.45-micron filter was used to remove non-conjugated curcumin. Lastly, the produced curcumin-dendrimer conjugate was obtained as a solid by freeze-drying.

Characterisation methods

Purification of the products was confirmed by thin-layer chromatography (TLC) using TLC Silica gel 60 aluminum sheets (Merck) and different ratios of chloroform/methanol as solvents.

Instrumental measurements FT-IR spectra for G2 PEG-citrate dendrimers and curcumin-dendrimer conjugate were obtained utilising an FT-IR spectrometer (Shimadzu 4300, Japan) using the KBr disk method. H NMR spectra were recorded with (Bruker spectra spin 400 MHz, Leipzig, Germany) using DMSO-d6 as a solvent. LC-MS spectra were carried out in an LC-MS triple quad Agilent 6410 system.

Zeta potential and particle size distribution The particle size and distribution of prepared G2 PEG-citrate dendrimers and curcumin-dendrimer conjugate were measured by Malvern Nano-ZS zeta potential and particle size analyser (manufactured by Malvern in England). To avoid clumping before measurement, the dried powder samples were suspended in water and softly sonicated. The obtained homogenous suspension's mean diameter, size distribution, and surface potential charge were then calculated.

Preparation of the curcumin calibration curve in DMSO

Free curcumin in DMSO was used to generate a standard concentration curve. A range of 0.001 to 0.01 mg/ml of curcumin in DMSO was prepared, and the solutions' absorbance was measured at 429 nm using an ultraviolet-visible (UV–Vis) spectrophotometer. Curcumin concentration in the conjugate was calculated using the absorption intensity of the conjugate in the standard calibration curve.

Evaluation of increasing the water solubility

The improved solubility of the curcumin-dendrimer conjugate was confirmed using a UV-Vis spectrophotometer: 3.7 mg (0.01 mmol) of free curcumin and 59.2 mg (0.01 mmol) of curcumin-dendrimer conjugate were separately dissolved in 1 ml of ddH2O. After that, both samples were centrifuged at 13,000 RPM for 2 min to eliminate any undissolved amounts of curcumin. The supernatant liquids were diluted (10 µl in 990 µl ddH2O) to achieve 100µM solutions, and their UV-Vis absorbance was recorded.

In vitro cell uptake studies by fluorescence microscopy

For a comparative visualisation of curcumin and curcumin-dendrimer conjugate uptake, Hela cells were seeded at a seeding density of 10,000 cells/well in 200 µl medium in 96-well plates. The cells were incubated for 24 h at 37˚C for attachment. The attached cells were then treated with a constant concentration (100 µM) of free curcumin (dissolved in DMSO) and curcumin-dendrimer conjugate (dissolved in culture medium) for 4 h at 37˚C in a cell culture incubator (Hera Cell, Thermo Scientific, Waltham, MA). The final concentration of DMSO in the culture medium was less than 0.1%. After incubation, the cell monolayers were rinsed three times with 1 ml PBS (0.01 M, pH 7.4). Fresh PBS (0.01 M, pH 7.4) was added to the wells, and the fluorescence images of curcumin were captured using a Nikon Eclipse TE300 fluorescence microscope with a Nikon F601 camera (Nikon, Japan).

Uptake kinetics of curcumin-dendrimer conjugate versus free curcumin by flow cytometry

To determine the uptake kinetics of curcumin-dendrimer conjugate and free curcumin, HeLa Cells were treated with the same amount of curcumin-dendrimer conjugate and free curcumin for increasing periods of time. Hela cells were seeded at a seeding density of 10,000 cells/well in 96-well plates and allowed to grow overnight. Then, the cells were treated with 100 µM curcumin-dendrimer conjugate and free curcumin for 0 to 7 h. The cells were detached and transferred to FACS (Fluorescence-activated cell sorting) tubes, and intra-cellular curcumin fluorescence was analysed by flow cytometry. Each measurement required at least 10,000 events. The relative fluorescence intensity per cell count was calculated by comparing the peak observed in each sample to a non-treated sample as a control.

Cytotoxicity assay

Using a cell proliferation XTT kit (Roche Diagnostics, Germany), the cellular toxicity of substances in HeLa cells was evaluated. Cells were cultured in triplicate in 96-well plates (10000 cells/well) containing fresh phenol-red free RPMI medium in the presence or absence of various concentrations of free curcumin, G2 PEG-Citrate dendrimer and curcumin-dendrimer conjugate. After incubation at 37 °C with 5% CO^2 for 24 h, 50 µL of prepared XTT mixture was added to each well. The cells were incubated for an additional 4 h at 37 °C to allow the production of XTT formazan. An ELISA plate reader (BioTek ELx800) was used to measure absorbance at a test wavelength of 450 nm and a reference wavelength of

630 nm. The concentration that reduces the proliferation of 50% of cells was CC50.

Inhibition of HIV p24 Core antigen production (HIV replication)

In this study, single-cycle replicable (SCR) HIV virions were used. SCR virions are replicable in no more than one cycle [47]. Vesicular stomatitis virus glycoprotein (VSVG)-SCR virions can infect and replicate in HeLa cells by assembling inactive virions. Single-cycle replicable (SCR) HIV virions were produced as previously described [47, 48]. HeLa cells (10000) were seeded in each well of 96 plates containing 100 µl of the complete medium in the presence or absence of various concentrations of free curcumin, G2 PEG-citrate dendrimer, and curcumin-dendrimer conjugate, which were filtered by 0/2 micron Millipore filters before use. Nevirapine (a HI-1/2 RT inhibitor) was used as a positive control. Each well was infected with 200 ng of p24 SCR HIV virions. After incubation at 37 °C with 5% CO^2 for 48 h, cells were washed two times with pre-warmed 5% FBS-supplemented RPMI. According to the manufacturer's instructions, the complete medium (100 µl) was applied to each well, and the p24 antigen (Ag) assay was carried out on the supernatants using a quantitative p24 ELISA method (HIV p24 ELISA, Biomerieux, France). The IC50 of the curcumin-dendrimer conjugate was calculated, and the selectivity index (SI) was evaluated as the ratio of CC50 to IC50.

Statistical analysis

The average standard deviation of three separate experiments was used to show the data. T-Student test and ANOVA were used for statistical analysis. P values less than 0.05 were considered statistically significant.

Result

Synthesis of G2 PEG-citrate dendrimer

According to the stoichiometric relationship between the density and molecular weight of materials, the required amount of each material was determined, and generation2 (G2) of PEG-Citrate dendrimer was prepared. The inner core of the dendrimer is polyethylene glycol (PEG); in G2, six citric acids were attached to both sides and finally, a water-soluble G2 PEG-citrate dendrimer was synthesised.

Drug conjugation

Three methods were investigated for the synthesis of curcumin dendrimer conjugate, and finally, method C, mentioned in part 2-2-2, was chosen due to its higher efficiency. Figure 1 shows curcumin-dendrimer conjugate

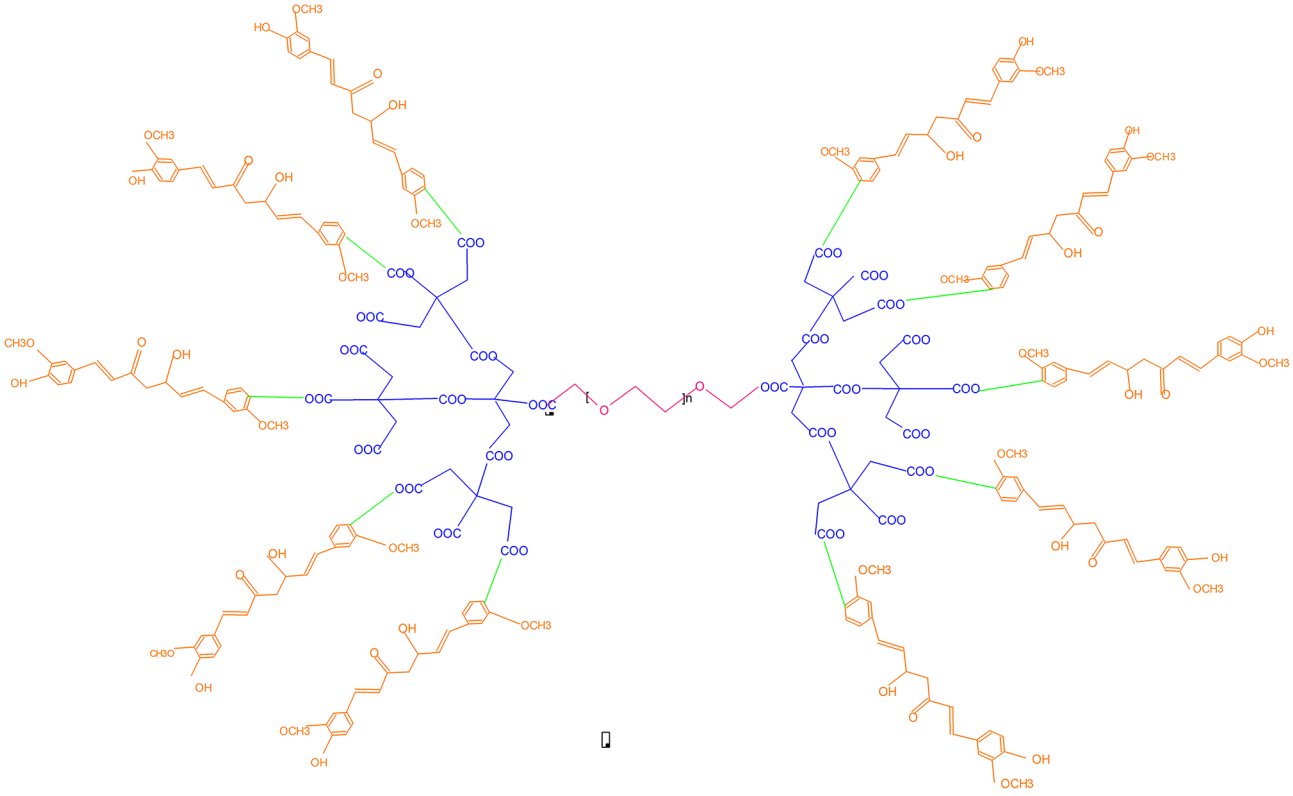

Fig. 1 Schematic figure of curcumin-dendrimer conjugate. PEG in the core of dendrimer is shown with dark pink color, peripheral groups (citric acids) are shown with blue color, ester bonds are shown with green lines and curcumin molecules are shown with orange color

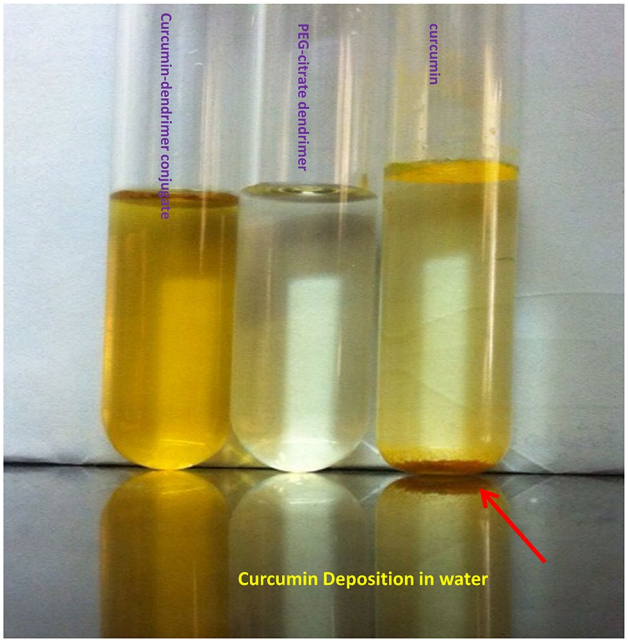

Fig. 2 Comparison of curcumin-dendrimer conjugate and free curcumin solubility in water

by forming an ester linkage between dendrimer and curcumin. Hydroxyl curcumin groups were directly conjugated to carboxylic groups of citric acid in G2 PEG-citrate dendrimer in a mixed solvent system using EDC as a coupling agent. The conjugate displayed an intense yellow colour (Fig. 2) with an average curcumin content of 4.7 µg in 50 µg of the curcumin-dendrimer conjugate.

Structure characterisation

TLC was used to determine the purity of the synthesised compounds, only one spot was observed for each of the materials and products, and no evidence of starting materials was shown. HNMR and FT-IR spectroscopy results indicated the synthesis of G2 PEG-citrate dendrimer and curcumin-dendrimer conjugate.

FTIR results

FTIR spectroscopy was used to ascertain the formation of the G2 PEG-Citrate dendrimer and curcumin-dendrimer conjugate. The FTIR spectra of G2 PEG-citrate dendrimer (orange line), curcumin (violet line), and curcumin-dendrimer conjugate (green line) are represented in Fig. 3. The FTIR spectrum of G2 PEG-citrate dendrimer (orange line) showed a solid C=O stretching absorption at 1720 cm-1, indicating carboxylic acid's appearance and an extensive absorption at 3500 cm-1, which pointed to the hydroxyl of a carboxylic acid's appearance. The peak at 1230 cm-1 corresponds to C–O stretching vibration in the –CO–O– group (ester bond). These results confirmed the formation of the G2 PEG-citrate dendrimer.

The FTIR spectrum of curcumin (violet line spectrum) exhibited an absorption band at 3509 cm-1 attributed to the phenolic O–H stretching vibration. Additionally, sharp absorption bands at 1629 cm-1 (stretching vibrations of the benzene ring of curcumin), 1508 cm-1 (CO and CC vibrations of curcumin), and 1427 cm-1 (olefinic C–H bending vibration) were noticed. In the case of curcumin-dendrimer conjugate (green line), the peak at 3300–3500 cm-1 seems sharper because of the presence of OH (unbound) groups from the curcumin and hydroxyl groups of carboxylic acid from the G2 PEG-citrate dendrimer. The peak observed at 1,736 cm-1 is the characteristic peak of the ester carbonyl group, showing that an ester bond was formed. Also, the band around 1240 cm-1 can be seen due to the C–O stretching frequency of ester linkage in the curcumin-dendrimer conjugate. The signature peak at 1629 cm-1 is found in native curcumin, and curcumin-dendrimer conjugate was due

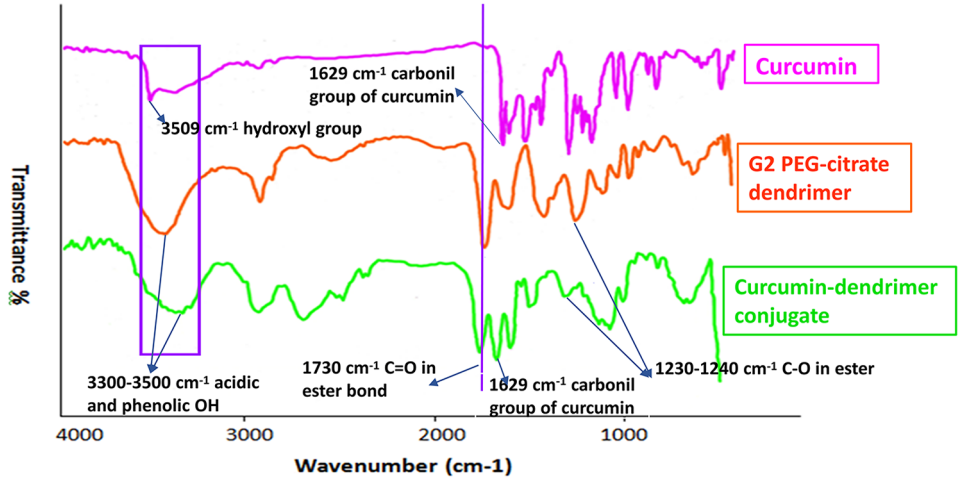

Fig. 3 The infrared spectral features regarding G2 PEG-Citrate dendrimer, curcumin and curcumin-dendrimer conjugate

to stretching vibrations of the curcumin's benzene ring. This marker peak was not found in the G2 PEG-citrate dendrimer, suggesting that the conjugation took place appropriately. Moreover, peaks associated with different groups of curcumin and G2 PEG-citrate dendrimer and the ester linkage peak on FTIR spectra of curcumin-dendrimer conjugate confirmed the conjugation.

HNMR results

The 1 H NMR spectrum of the curcumin-dendrimer conjugate (Fig. 4) shows signals from both G2 PEG-citrate dendrimer and curcumin-dendrimer conjugate. Peaks associated with aromatic protons of curcumin were observed at 7.3–7.7 ppm. A doublet at 6.3– 6.5 ppm also appeared for hydrogen atoms adjacent to the double bond of curcumin. Singlet peaks at 3.82 ppm were attributed to OCH3 groups present in the curcumin moiety in the conjugate. In this figure, signals at 2.7–2.9 and 3.3–3.6 ppm are related to the CH2 groups of citric acid and PEG, respectively.

LC-MS results

The positive ESI tandem mass spectra of G2 PEG-citrate dendrimer and curcumin-dendrimer conjugate are shown in Figs. 5 and 6, respectively. The proposed fragmentation pattern of each compound is also presented at the top of the peaks. PEG can be easily recognised in a spectrum by its equidistant spacing of 44 Da due to its oxyethylene units. As exhibited in Fig. 5, each peak in the range of 564–872 m/z indicated the loss of (CH2CH2O, MW = 44) groups, which corresponded to the oxyethylene units of PEG. Based on the m/z, it is simple to calculate the number of oxyethylene units in each ion (for example, m/z 696.3 has seven oxyethylene units). All of these ions were produced as a result of PEG's dissociation. As shown in Fig. 6, six produced ions of curcumin-dendrimer conjugate with *m/z* of 481 to 789 m/z are for the loss of oxyethylene units of PEG. Further, these fragments have a curcumin unit, and confirm conjugation is done.

Particle size analysis

The size of nanocarriers was determined through the dynamic light scattering (DLS) technique. DLS results indicate that the average G2 PEG-citrate dendrimer and curcumin-dendrimer conjugate diameters were 91 and 101 nm, respectively (Table 1). Furthermore, they had a narrow size distribution.

Concentration of curcumin in the conjugate

The concentration of curcumin in the conjugate was calculated using the conjugate's absorption intensity in the calibration curve.

$y = 0.1214x + 0.0758 => 0.67 = 0.1214x + 0.0758$
$0.1214x = 0.67 - 0.0758 => X = 0.594 / 0.1214 =>$
$X = 4.9$ µg/ml (Concentration of curcumin in 50 µg/ml curcumin-dendrimer conjugate)

Fig. 4 HNMR of curcumin-dendrimer conjugate

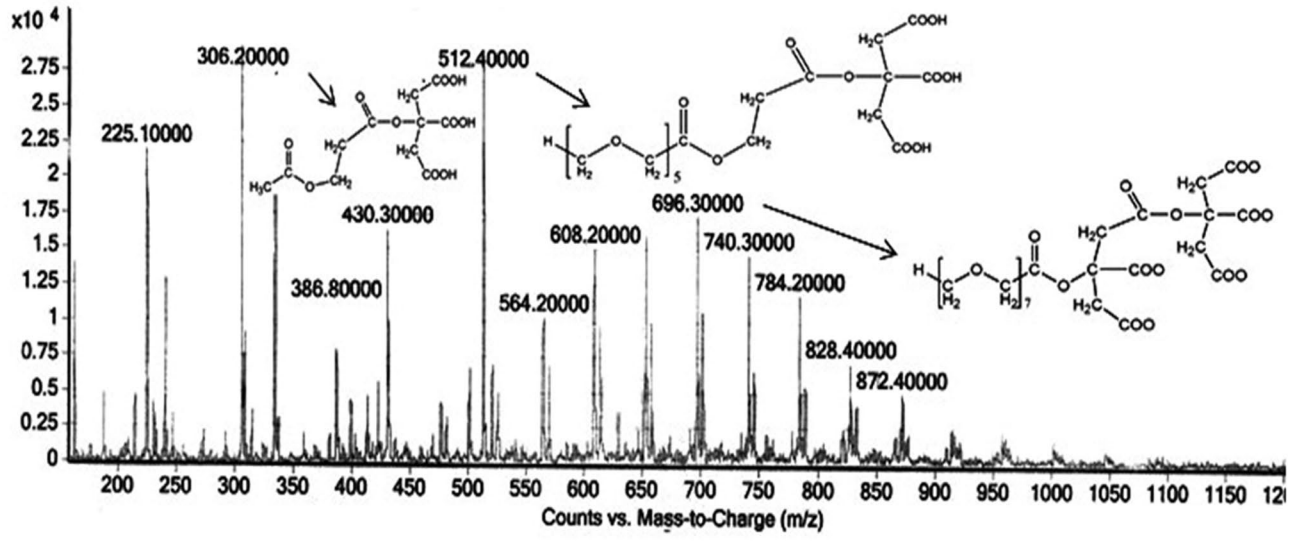

Fig. 5 LC-MS of G2 PEG-citrate dendrimer. Proposed fragmentation pattern of some compounds are presented at the top of the peaks

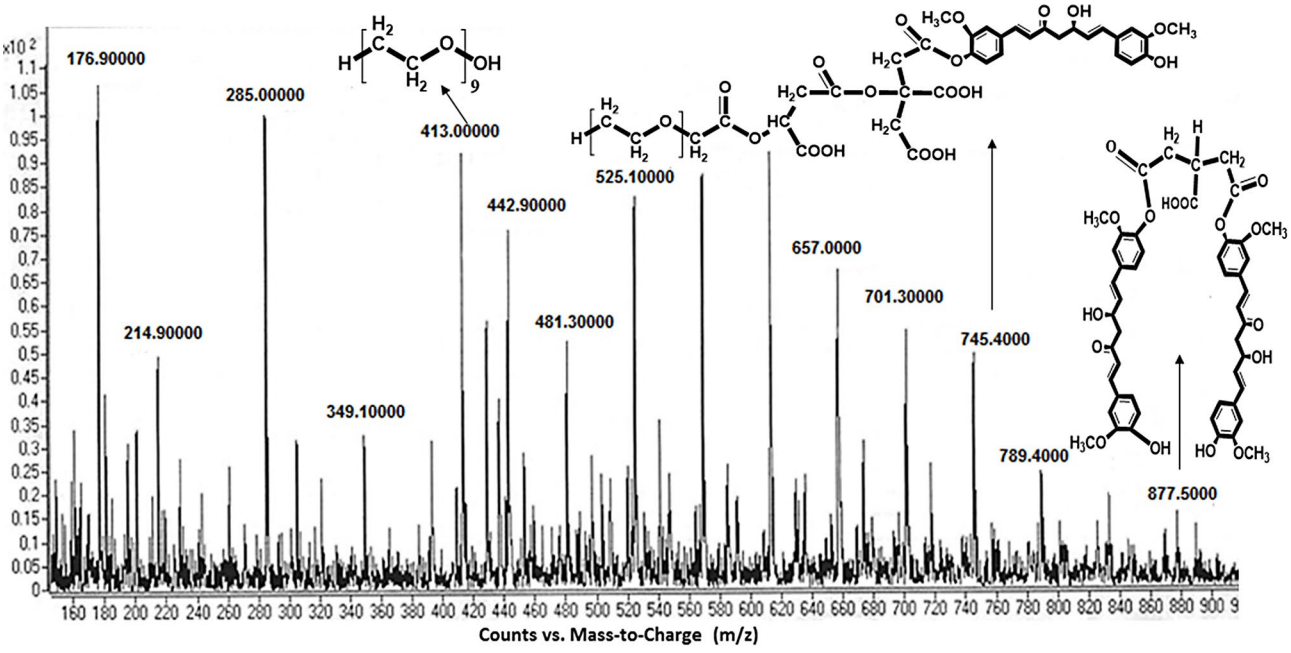

Fig. 6 LC-MS of curcumin-dendrimer conjugate. Proposed fragmentation pattern of some compounds are presented at the top of the peaks

Table 1 Zeta potential and particle size distribution of products

	Size (nm)	(PDI)	ζ-potential (mV)
G2 PEG-Citrat dendrimer	91	0.41 ± 0.2	-3.3 ± 1.3
Curcumin-dendrimer conjugate	101	0.34 ± 0.5	-2.6 ± 0.8

Evaluation of increased water solubility of curcumin

An aqueous solution of a curcumin-dendrimer conjugate produced a precise, well-dispersed formulation with natural curcumin colour (Fig. 2). In contrast, free curcumin is weakly soluble in water with microscopic undissolved flakes of the compound visible in the solution (Fig. 2). Thus, dendrimer conjugation increased the solubility of curcumin in aqueous. To further examine the increased solubility of the curcumin-dendrimer conjugate, absorption spectra of both samples up to a concentration of 100 mM in the range of wavelengths from 200 to 800 nm were measured by a UV-Vis spectrophotometer (Fig. 7). The maximum absorption at a wavelength of 400 nm was obtained. There were approximately zero for free curcumin and G2 PEG-citrate dendrimer, while curcumin-dendrimer conjugate absorbed about 1.1. Once a result, as it was conjugated with dendrimer, a notable improvement in its solubility was observed.

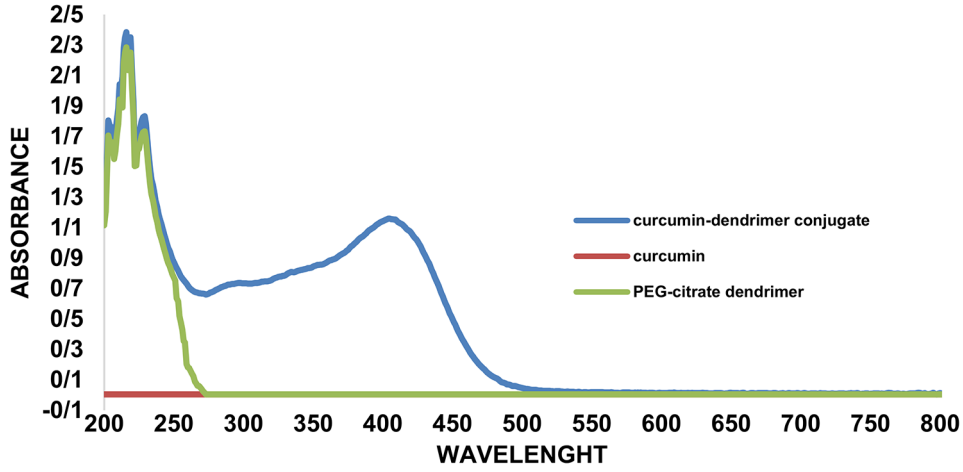

Fig. 7 Solubility comparison of curcumin-dendrimer conjugate and free curcumin by absorption spectrum obtained from a UV-Vis spectrophotometer. The highest absorption value was obtained at 400 nm, which was 0.005 for free curcumin and 1.1 for curcumin-dendrimer conjugate

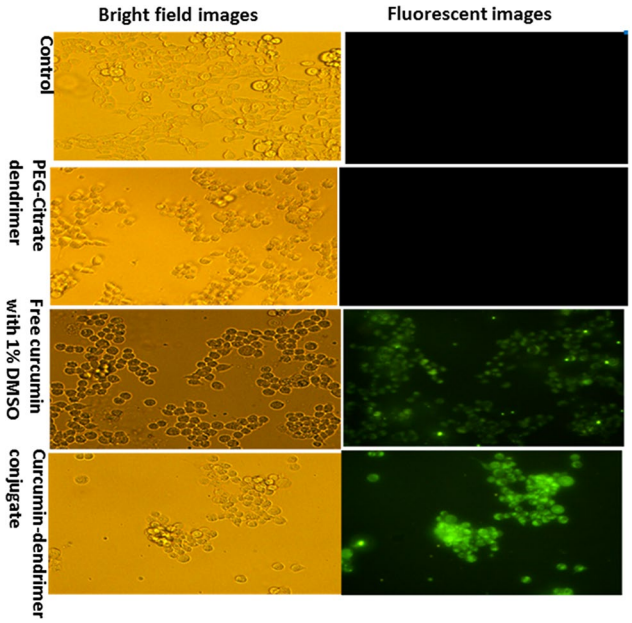

Fig. 8 In vitro cell uptake studies by fluorescence microscopy. Curcumin-dendrimer conjugate entry into Hela cells after 5 h of treatment, as it is clear in the pictures, free curcumin dissolved in 1% DMSO enters the cell in a much smaller amount compared to curcumin-dendrimer conjugate

In vitro cell uptake studies by fluorescence microscopy and flow cytometry

Fluorescence microscopy was used to evaluate the cellular uptake of curcumin. The cells were incubated for four h with G2 PEG-citrate dendrimer, curcumin, and curcumin-dendrimer conjugate. Then, cellular uptake was assessed by fluorescence microscopy based on the autofluorescence of curcumin. The microscope images of control HeLa cells without curcumin (Fig. 8, A) and HeLa cells incubated with G2 PEG-citrate dendrimer (Fig. 8, B) showed no fluorescence in the cells. The HeLa cells treated with curcumin-dendrimer conjugate exhibited relatively more green fluorescence (Fig. 8, D) than cells treated with free curcumin (Fig. 8, C). This data suggests that conjugating curcumin to dendrimer enhanced curcumin uptake in the HeLa cells. These observations were further confirmed by flow cytometry.

The flow cytometry histograms from HeLa cells incubated with curcumin and curcumin-dendrimer conjugate are shown in Fig. 9. Non-treated samples were used as a reference value. Part A shows the graphs of curcumin-dendrimer conjugate uptake after treatment of Hela cells at concentrations of 50 and 200 μM for 24 h. As shown in this figure, the fluorescence intensity of cells exposed to higher concentrations of the curcumin-dendrimer conjugate was significantly increased. Likewise, the kinetics of curcumin-dendrimer conjugate and free curcumin in HeLa cell lines were assessed by graphing each sample's fluorescence peak against the incubation period (Fig. 9, B). As a reference, untreated samples were used. The results indicated that the uptake increased with increasing time up to 5 h. free curcumin did not exhibit any appreciable change in fluorescence intensity simultaneously. Dendrimer conjugation appears to enhance curcumin's cellular uptake significantly.

Cytotoxicity studies

Cell cytotoxicity of components for HeLa cells was investigated using the XTT assay. As shown in Fig. 10, there is no significant cytotoxicity in any of the concentrations of the G2 PEG-Citrate dendrimer. It is evident from Fig. 10 that compared to the negative control, almost 85% of cells are viable in all six concentrations of G2 PEG-Citrate dendrimer. These results revealed that the prepared dendrimer is nontoxic to HeLa cells. Free curcumin at the same concentrations did not show any toxicity. Moreover, the same experiments were carried out with curcumin-dendrimer conjugate in the same concentration range.

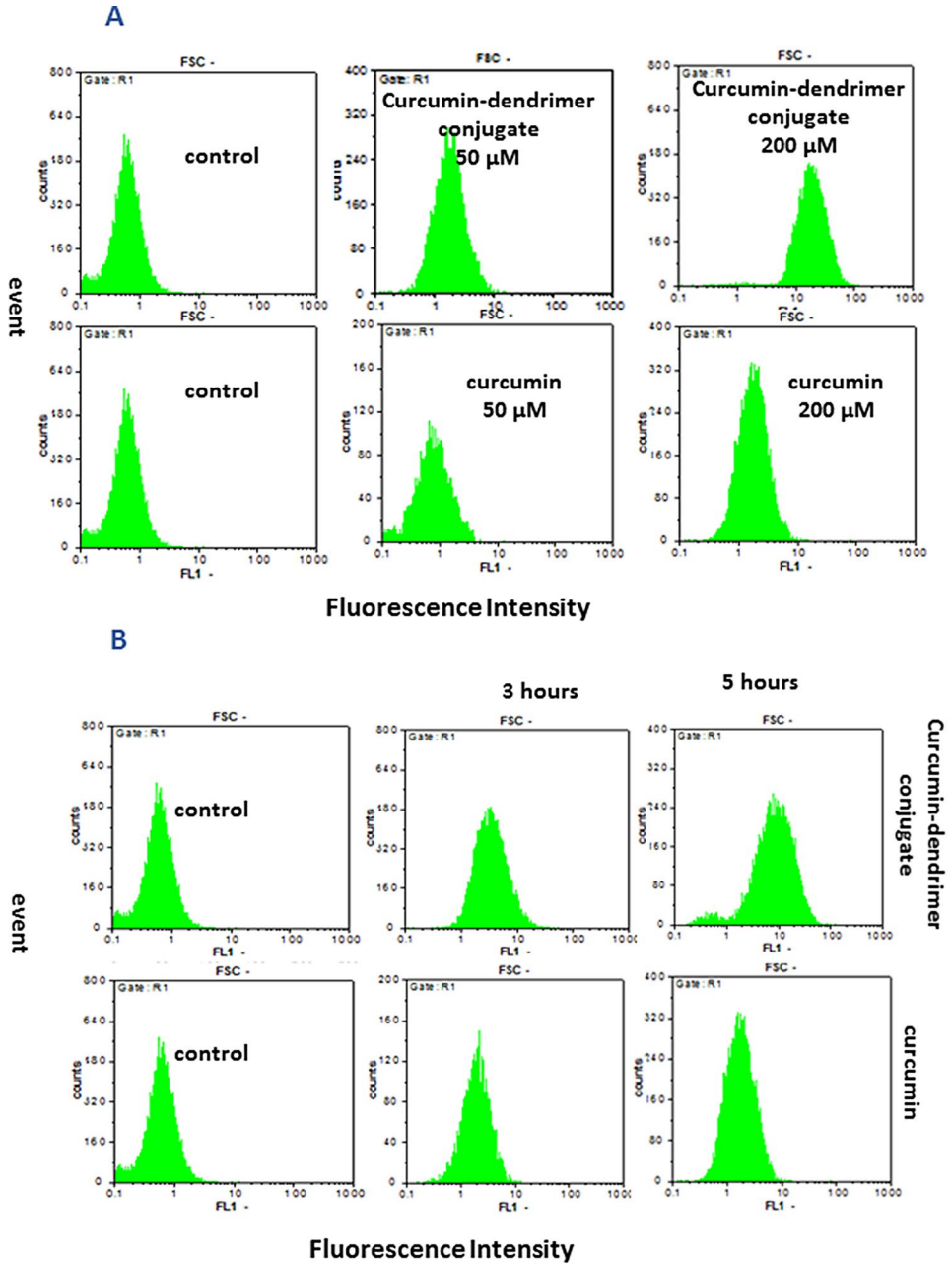

Fig. 9 In vitro cell uptake studies by flow cytometry. A: The graphs of curcumin-dendrimer conjugate uptake after treatment of Hela cells with 50 and 200 µM concentrations for 24 h. B: Plotting each sample's fluorescence peak versus the incubation period (3 &5 h). Untreated samples were used as a reference value

The CC50 values of curcumin-dendrimer conjugate for HeLa cells were observed to be approximately 300 µM.

Inhibition of HIV p24 production (replication assay) by curcumin-dendrimer conjugate

Inhibition of HIV-1 replication with curcumin-dendrimer conjugate was performed using VSVG pseudotyped SCR HIV-1 virions, which can only replicate for one cycle. This experiment demonstrated the ability of curcumin-dendrimer conjugate to inhibit the replication of HIV virions. As shown in Fig. 11, the most functional doses were 12 and 60 µM among different amounts. The results showed that curcumin-dendrimer conjugate inhibited 50% of viral replication at the concentration of 12 µM (IC50) (Fig. 11). At the same time, the cytotoxicity of curcumin-dendrimer conjugate at this concentration was less than 10% (Fig. 10).

Moreover, viral replication of HIV-1 was inhibited up to 90% at the concentration of 60 µM. Results revealed that curcumin-dendrimer conjugate was more functional

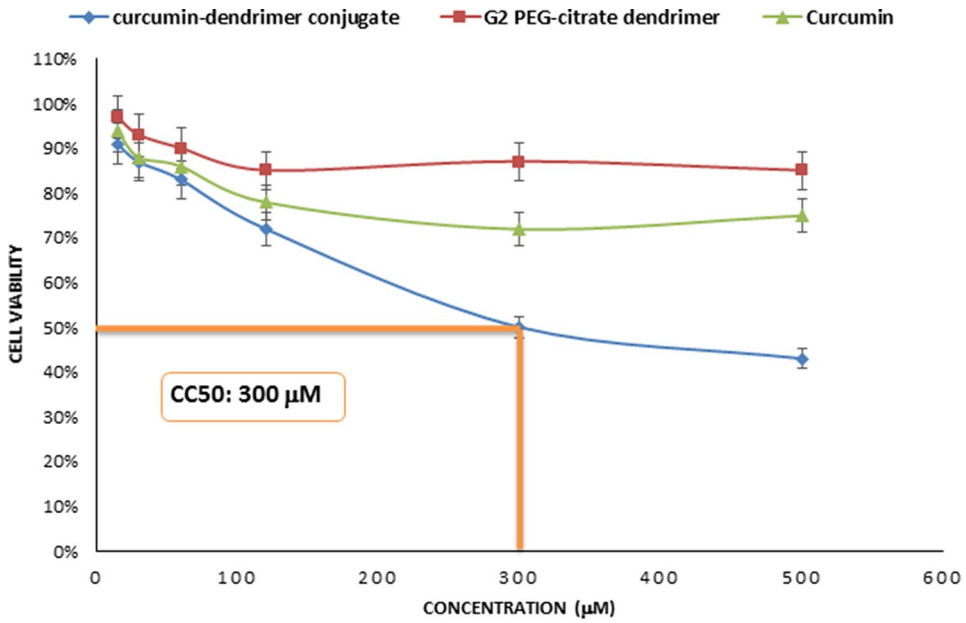

Fig. 10 XTT assay results. The effect of different concentrations of free curcumin, G2 PEG-Citrate dendrimer and curcumin-dendrimer conjugate on the survival of Hela cells using XTT test. The cells were treated with different concentrations of compounds in a period of 24 h

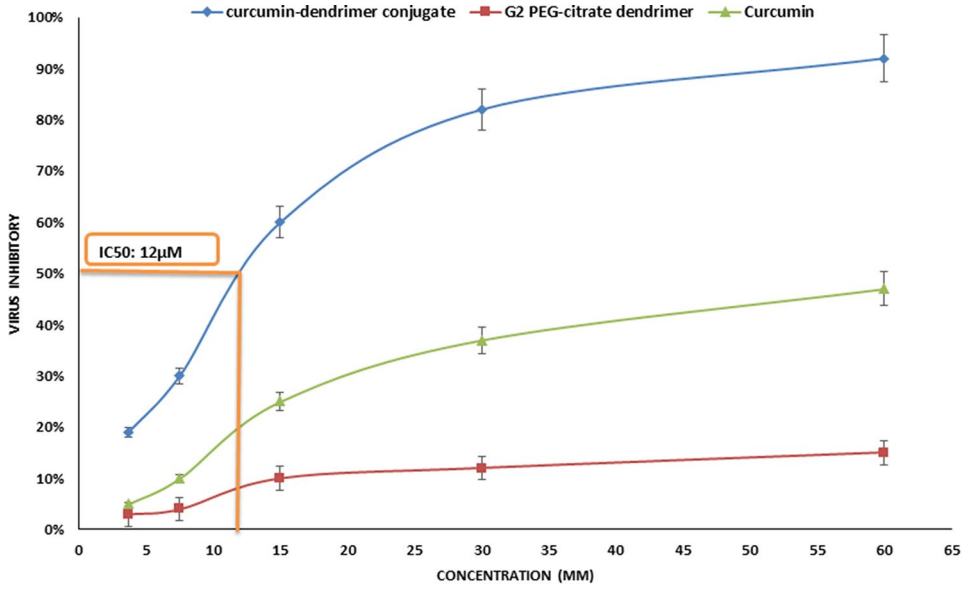

Fig. 11 Curcumin-dendrimer conjugate inhibits HIV-1. Inhibition rate of HIV-1 virus replication by curcumin-dendrimer conjugate, G2 PEG-Citrate dendrimer and free curcumin: IC50 was about 12. ** indicates statistically significant levels of $P \leq 0.05$ compared to the control group (cells not treated with compounds)

in all its doses than free curcumin. In addition, according to the CC50 and IC50 of the curcumin-dendrimer conjugate, the calculated selective index (SI) is 25.

Discussion

One of the essential difficulties in using dendrimers as a drug carrier is their inherent toxicity problems. In most studies, PAMAM (Polyamidoamine) dendrimers were used to deliver and increase the solubility of biological medicines, which can show different degrees of toxicity in cells. The dendrimer utilised in the present study comprises biocompatible monomers, termed biodendrimer. Using biocompatible materials or monomers, including natural metabolites and chemical intermediates found in metabolic pathways, is a suitable strategy to eliminate the cellular cytotoxicity of dendrimers [38, 39]. In the PEG-citrate dendrimer produced in this study, PEG is at the core of the dendrimer, while its surrounding groups are

citric acids (the citric acid cycle intermediates). It also has a negative charge due to the citric acid branches. As a result, it has no interaction with the cell surface and does not damage the cell membrane. The dendrimer is, hence, biodegradable and has minimal toxicity.

XTT test results of this study confirmed the lack of PEG-citrate dendrimer toxicity. They indicated it did not exhibit significant cytotoxicity even at high concentrations (300 mM in Hela cells). Previously, Alavidjeh et al. analysed in vitro cytotoxicity behavior of these dendrimers. According to their results, the dendrimers had no noticeable adverse effects up to a concentration of 0.5 mg/ml [46], which aligns with the results obtained in the present study. Interestingly, under enzymatic conditions inside the cell environment, this dendrimer is also biodegradable. Accordingly, it may be metabolised and eliminated by different biological processes [46, 49]. So, the risk of undesirable toxic accumulations of this material does not exist in cells and organisms.

According to the DLS results, the G2 PEG-citrate dendrimer had a narrow size and low polydispersity. These findings are in line with those reported by Haririan et al. They considered that the G2 PEG-citrate dendrimer had a smaller and narrower size distribution than G1 [45].

Curcumin is known to have incredible biological feature but its instability at physiological pH, water insolubility and poor bioavailability limits its therapeutic efficacy. Numerous studies have been conducted to improve bioavailability, stability and cellular uptake of curcumin. For instance, curcumin loaded glycerol monooleate based nanoparticle (GMO NP) revealed enhanced stability in phosphate buffer saline by protecting encapsulated curcumin against hydrolysis. This nanoparticulate curcumin was more effective than free curcumin against different cancer cell lines due to enhanced cellular uptake [50]. Moreover, curcumin was encapsulated in a nanostructure of monomethoxy poly (ethylene glycol)-oleate (mPEG-OA). Based on the results of fluorescence microscopy and cell-cycle analyses they indicated that the in vitro bioavailability of the curcumin was significantly improved by encapsulating it in the mPEG-OA micelle [51]. Likewise, curcumin loaded folate modified micelle (Cur/PLA-PEG-Fol) was synthesized by phan et al. for targeting cancer therapy. The results demonstrated that folate-modified micelle can act as a potential nanocarrier to improve the solubility and anticancer activity of curcumin [52]. Furthermore, conjugation of curcumin to PLGA polymer have been reported recently. The results showed improvement of curcumin biological activity by increasing its absorption and stability [53]. Additionally, curcumin loaded BTN-PEG-PCL and mono methoxy PEG-PCL (mPEG-PCL) diblock co-polymers series were created. Results indicated that improved cellular uptake and enhanced selective delivery of curcumin to cancer cells were achieved [26]. Also, several kinds of research have been undertaken to enhance the solubility and stability of curcumin through the use of nanocarriers. These methods increased the effectiveness of curcumin by increasing its aqueous solubility. Nevertheless, in most studies, the drug is loaded to the carrier. Therefore, in some cases, the drug is released quickly before reaching the target tissue, so it cannot have the desired effect [54]. On the other hand, the final solution's insolubility is one of the critical issues with drug conjugation to a dendrimer surface [55].

Other disadvantages of these approaches are batch-to-batch variations in loading contents, poor loading efficiency, and loading contents. Moreover, curcumin was conjugated to (PEG) to create water-soluble curcumin derivatives, but even under neutral circumstances, these compounds were unstable and easily hydrolysed [56]. Due to the presence of PEG in its core, the G2 PEG-Citrate dendrimer created in this study can take a large amount of a drug while remaining soluble. Also, Namazi et al. indicated that the drug/PEG-citrate dendrimer complexes remained stable at room temperature for about ten months, and the drugs were not released. Moreover, their prior studies have shown the ability of PEG-citrate dendrimer in the solubility enhancement of some hydrophobic drugs [44, 45]. So, we attempted to conjugate curcumin to G2 PEG-citrate dendrimer to improve its stability, solubility and hence bioavailability.

In this study, curcumin was conjugated to the surface of the biocompatible PEG-citrate dendrimer with an ester bond to increase its solubility and cell uptake. Therefore, after entering the cell, ester bonds are hydrolysed and lead to the release of curcumin.

The covalent conjugation of a drug molecule onto the dendrimer surface propound a better control over drug release when compared to dendrimer/drug complexes. Dendrimer/drug conjugates are usually stable in vivo due to the covalent linkage between the dendrimer scaffold and the drug [57]. In the present study, to prevent its rapid release in the environment, curcumin was conjugated to the surface of G2 PEG-citrate dendrimer through an ester bond formation at a phenolic hydroxyl group of curcumin that might increase its stability. So after entering the cell, esteric bonds are hydrolysed by cytosolic esterases and lead to the release of curcumin inside the cells [57, 58]. Previously, Namazi et al. have conducted studies related to the drug release and hydrolysis of ester bonds in PEG-citrate dendrimer [59]. Based on their results, the steric bonds of PEG-citrate dendrimer are easily hydrolyzed in basic solutions and lead to release the drug. Moreover, the rate and the amount of the release is as follows pH=10>pH=7.4>pH=1 [59]. Few studies have been performed to conjugate curcumin to dendrimer in which the linker or curcumin derivatives

such as (azide, alkyne, and carboxylic acid) have been used [54, 60, 61]. These methods require multiple steps and materials to synthesise and purify, so it was time-consuming. There was no research based on a direct connection between curcumin and dendrimer.

Shi et al. conjugated curcumin-monocarboxylic acid to PAMAM dendrimer. The curcumin-dendrimer conjugate was a water-soluble and efficient cytotoxic agent for labelling and dissolving amyloid fibrils [60].

Furthermore, Debnath et al. used this monoderivative curcumin-dendrimer conjugate against breast cancer cell lines. Based on in vitro results, curcumin-dendrimer conjugate demonstrated improved cytotoxicity against SKBr3 and BT549 human breast cancer cell lines and triggered apoptosis via caspase-3 activation in vitro [61]. Nevertheless, in the present study, a synthetic methodology was developed, and curcumin was conjugated to dendrimer directly.

Improved solubility of curcumin-dendrimer conjugate compared to curcumin was measured by UV−vis experiment. Unlike other methods in which the hydrophobic compounds are first dissolved in different solvents followed by dilution in water, in this study, direct solubility of curcumin in water was shown and compared with curcumin-dendrimer conjugate solubility. Dolai and colleagues also used this method to compare curcumin and curcumin-galactose conjugate aqueous solubility and noted that this method closely models “real-life” conditions [54].

G2 PEG-citrate dendrimer was extremely hydrophilic and, as expected, significantly increased the water solubility of curcumin. Moreover, the vital aspect of this strategy was that this synthesised curcumin-dendrimer conjugate also acts as a pro-drug. As a result, the ester linkage at the phenolic group on curcumin and dendrimer could be hydrolysed and facilitate the release of curcumin under acidic physiological environments.

Next, the intracellular uptake of curcumin-dendrimer conjugate and native curcumin was revealed by its inherent fluorescence property under flow cytometry and fluorescent microscope. Quantitative measurement of cellular uptake using flow cytometry revealed fourfold higher uptake of the curcumin-dendrimer conjugate than native curcumin. Higher cellular uptake of nanocurcumin has been reported in other studies. Alam et al. entraped curcumin in a dipeptide NP and showed twofold higher uptake of the curcumin-NP compared to free curcumin [62]. Endocytosis pathways, which have been demonstrated to work in the cellular uptake of various nanoparticulate materials, may be responsible for the increased uptake seen here [50, 62, 63]. Moreover, based on flowcytometry studies, a time and concentration dependent increase in cellular uptake of curcumin-dendrimer conjugate was observed. Similar results were also obtained in other researches [36, 50]. Additionally, the results of fluorescence microscope study demonstrated curcumin-dendrimer conjugate was internalized more efficiently and exhibit intense fluorescence in HeLa cells as compared to native curcumin. Therefore, conjugation of curcumin with G2 PEG-citrate dendrimer effectively protected from rapid metabolism and degradation. Considerably, above findings indicated that conjugation of curcumin with G2 PEG-citrate dendrimer improves cellular uptake and stability of curcumin.

Then again, infection with the human immunodeficiency virus (HIV) has been recognised as a significant health problem worldwide. Due to the mentioned potential of curcumin in treating viral diseases, the anti-HIV-1 effects of curcumin-dendrimer conjugate have been studied.

Working with wild types of HIV virions may impose serious infection danger. Using a non-infective HIV strain for research procedures could solve this problem. Earlier studies have been conducted on curcumin inhibition of HIV-1 viruses. Of course, these studies have often been carried out by cloning the HIV genes in a bacterium [64]. Various evidence suggests that the Tat protein secreted by HIV-infected cells is effective in the pathogenesis of AIDS. With this in mind, Barthelemy et al. used Hela cells containing the Lac Z bacterial gene under the control of HIV-1 L. They showed that curcumin at a concentration of 100 nanomolar inhibits Tat proteins, a trans-activator for HIV LTR, up to 80% [65].

Similarly, Balasubramanyam et al. demonstrated that curcumin could inhibit the p53 acetylated by p300. In addition, curcumin can inhibit the secretion of HIV-Tat protein by p300 in vitro [66]. Sui et al. showed that curcumin inhibits the HIV protease enzyme (IC50: 100μM) [67]. While the present study performed with the SCR system that produces a complete virus similar to the natural virus, it was shown that the curcumin-dendrimer conjugate with IC50: 12μM, inhibits the proliferation of HIV, which is much lower than the toxic concentration of the cell and its selected index was 25.

Conclusion

The results of this research indicate that the synthesised curcumin-dendrimer conjugate has an extremely high-water solubility, and its permeability to the cell has increased from 20 to 80%, compared with curcumin. In addition, the curcumin-dendrimer conjugate inhibited the HIV-1 virus proliferation up to 92%. It was also observed that the second-generation PEG-citrate dendrimer alone was not toxic to cells, indicating that this carrier facilitated only the administration of curcumin to the cells.

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Author contributions
Saeideh Ebrahimi: performed most of the experiments, wrote the manuscript and Editing, and performed the formal Analysis of results. Majid Sadeghizadeh performed the data analyses, Conceptualization, editing, and review of the manuscript. Mohammad Reza Aghasadeghi performed the Conceptualization, Investigation, and Resources, and wrote and Reviewed the manuscript. Mehdi Shafiee Ardestani performed the Methodology, and wrote, reviewed, and Edited the manuscript. Shaghayegh AdibAmini performed the Methodology and wrote the Original Draft. Roohollah Vahabpour designed the Methodology, analysis, and Validation. All authors have read and approved the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
NA.

Consent for publication
NA.

Competing interests
The authors declare no competing interests.

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