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Piperidone Analogue of Curcumin-loaded HPβ-cyclodextrin liposomes as delivery system for human cancer therapy

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Abstract : Cancer is a broad term used to describe different types of tumors affecting various parts of the human body. The resistance to multiple therapeutic agents, toxicity to healthy tissues, and lack of effective therapies obligate scientists to keep looking for new agents. Curcumin (diferuloylmethane) is a natural product. It has been reported that curcumin has anti-inflammatory, anti-diabetic, and anti-cancer effects. However, clinical use of curcumin is limited due to its poor aqueous solubility. Recently, a curcumin analogue, 5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4-piperidone of curcumin (PAC), was synthesized to overcome this limitation. This compound showed significantly higher anticancer activity on breast cancer cell lines and colon cancer cell lines as compared to native curcumin. However, aqueous solubility of this new chemical compound limited its use and application.

Liposomes were found to be the most promising system to use to overcome aqueous solubility and delivery limitations. Liposomes are a self-assembly of phospholipid molecules. They are biodegradable, biocompatible, and nontoxic carrier systems. These features make liposomes an ideal carrier system for anticancer agents. In this study liposomes were utilized to overcome PAC limitations. This project was designed to develop liposomal delivery system for PAC for cancer treatment and evaluate the anti-cancer properties of this system. Liposomal PAC formulae were prepared by the film hydration method and were optimized by adding hydroxypropyl-beta-cyclodextrin (HPβCD) and characterized in term of particle size, entrapment efficiency, release profile and cytotoxic activity. Liposomes with an average size below 150 nm and zwitterionic charge were obtained. Indeed, no major differences were seen in particle size and surface charge. However, HPβCD inclusion gave satisfied incorporation capacity reaching 68.1%. In addition, HPβCD inclusion in the liposomes resulted in increased *in vitro* release rate compared to conventional liposomes. On colon cancer cells, Annexin V/PI- Flow Cytometery cytotoxicity results revealed that the PAC-loaded HPβ-cyclodextrin liposomes trigger apoptosis by 75% in response (10 μM), whereas it was only 43% in response to the same concentration of PAC conventional liposomes, which confirmed their potential anti-cancer activity. On breast cancer cells, Annexin V/PI- Flow Cytometery cytotoxicity results showed that while PAC conventional liposomes have only minor cytotoxic effect (22-25%), PAC-loaded HPβCD liposomes induced 53% and 70% apoptosis in response to 5 μM and 10 μM, respectively. The cytotoxicity of PAC-loaded HPβ-cyclodextrin liposomes was more pronounced than PAC conventional liposomes in both cells, suggesting the benefits of using HPβ-cyclodextrin. Therefore, PAC-loaded HPβ-cyclodextrin liposomes indicate significant potential as delivery vehicles for the treatment of cancers.

***Keywords**: liposomes, colon cancer, Hydroxypropyl-beta-cyclodextrin, curcumin analogs, Piperidone analogue of curcumin.*

Introduction

Curcumin is well-known in China and India. Curcumin is the active component of turmeric extract and has anticancer properties. However, its poor aqueous solubility, low gastrointestinal absorption, high-rate metabolism, low bioavailability, and low stability in neutral pH conditions limit its clinical use. Two main strategies were pursued to solve the problems related to using curcumin for clinical applications. First, curcumin analogues were synthesized, which keep the nontoxic features of the molecule, while improving its pharmacological and pharmacokinetics properties. Second, drug delivery systems were developed, which enable protection, targeting and stability of the molecules.

5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4piperidone (PAC) was one of these curcumin analogues, that showed promising anticancer effects [1-3] (Figure 1). Although PAC is 27-fold more soluble than curcumin in water, water's solubility is still considered poor and cannot be formulated in traditional dosage form [1]. Liposomes are one of the most important novel drug delivery systems. Their importance lies in Their composition, which makes them able to carry lipophilic and hydrophilic drugs [4]. Liposomes can be formulated in different dosage forms, such as suspension, aerosol, gel and cream. Also, liposomes can be administered *in vivo* topically or parenterally [5, 6]. Entrapment of anticancer drugs into liposomes resulted in an increased circulation lifetime and protection from drug metabolic degradation. Moreover, liposomes are capable of targeting tumors by leaving the blood and accessing the tumor because of their size [7]. Many different liposome formulations of various anticancer agents have been shown to be less toxic and more effective than the free drug such as platinum compounds (cisplatin and oxaplatin), anthracyclines (doxorubicin and daunorubicin), paclitaxel, camptothecin derivatives, antimetabolites (methotrexate, cytarabine), and Vinca alkaloids (vincristine, vinblastine and vinorelbine) [8].

Cyclodextrin (CD) is a hydrophobic water-soluble oligosaccharide produced enzymatically from starch. According to the number and size of glucose units, CDs are classified as α-CD, β-CD, and ɤ-CD, having six, seven and eight glucose units, respectively. CDs contain hydrophobic cavities in which they can accommodate hydrophobic drugs without any effect on them, while CDs' outer surface permits aqueous solubility [9]. In order to increase CDs' water solubility, some chemical modifications have been applied. These modifications resulted in CDs with different properties and toxicity profiles. Hydroxypropyl β-CD (HPβCD), sulfobutylether β-CD (SBEβCD) and methylated βCD(MβCD) are examples of modified CDs. CDs can enhance the solubility, stability and even bioavailability of drug molecules by inclusion complex formation[10, 11]. Hydroxypropyl betacyclodextrins are modified β-CDs having a higher aqueous solubility (above 60%), easy complex formation and a proven safe profile, especially for parenteral uses [12]. Complexation CD-drug into liposomes combines the advantages of both CDs (such as increasing the solubility of drugs) and liposomes (such as targeting of drugs) into a single system and thus overcomes the problems associated with each system [13]. By forming water-soluble complexes, CDs would allow

insoluble drugs to accommodate in the aqueous phase of vesicles and thus potentially increase drug-to-lipid mass ratio levels (enhance drug-loading capacity), increase the range of insoluble drugs amenable for encapsulation, allow drug targeting, and reduce drug toxicity [14, 15]. On the other hand, problems associated with intravenous administration of CD complexes, such as their rapid removal into urine and toxicity to kidneys, especially after chronic use, can be circumvented by their entrapment in the aqueous phase of liposomes [16, 17].

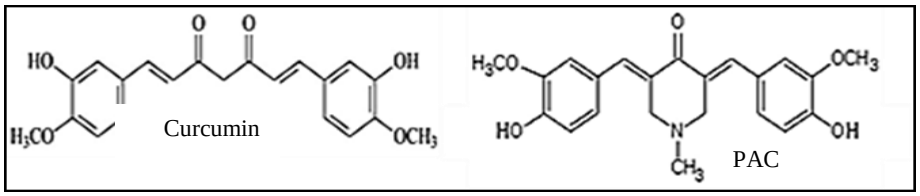


Figure 1. Chemical structure of curcumin and the novel analouge of PAC.

Materials and methods

1. Materials

Materials were purchased from the following sources: Dulbecco's modified eagle's medium (DMEM), dipyridamole, RPMI 1640 and Hydroxypropyl-βcyclodextrin (HPβCD) (Sigma-Aldrich, St. Louis, MO, USA); Lα-phosphatidylcholine (Avanti Polar Lipids, Alabama, USA) ;penicillin/streptomycin (Thermo Fisher Scientific, Auckland, NZ); fetal bovine serum (Moregate Biotech, Hamilton, NZ); cholesterol (Alpha Chemika, Mumbai, India); chloroform (VMR-Prolabo Milan, Italy); methanol (Restek, Bellefonte, PA USA); acetonitrile (Sigma-Aldrich, Lyon, France). All organic solvents were analytical grade reagents. Cancer cell lines were purchased from the American Type Culture Collection (ATCC), Manassas, USA.

2. PAC syntheses

The curcumin analogue, PAC 5-Bis (4-hydroxy-3-methoxybenzylidene)-N-methyl-4piperidone, was synthesized using a previously outlined method[3]. Structures of the starting reagents N-methylpiperidone (1.13 g, 10 mmol), vanillin (3.04 g, 20 mmol), and 5N hydrochloric acid and final compound PAC are shown in Figure 2.

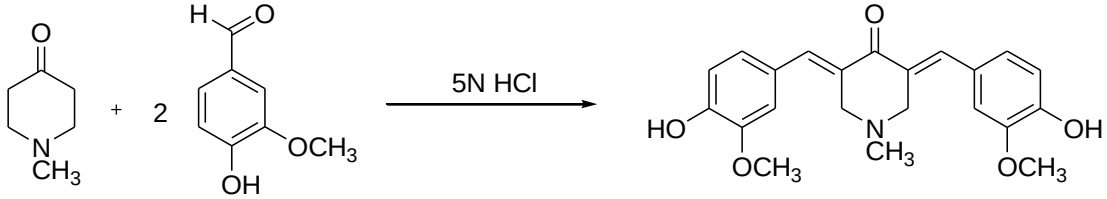


Figure 2: Chemical synthesis of PAC.

1.1. Fourier transforms infrared spectroscopy for PAC (FT-IR)

PAC was confirmed by Perkin Elmer FT-IR Spectrum BX apparatus (Perkin Elmer, USA). The PAC sample was prepared by conventional the potassium bromide (KBr) disc method (2 mg sample in 98 mg KBr) and examined in the transmission mode. A KBr disc was prepared at a pressure of 10 ton. The spectrum was scanned at a resolution of 2 cm^{-2}, over a frequency range of 4000—400 cm^{-1}. The different wavenumbers of the spectrum were compared to the literature to determine the different functional groups in PAC.

1.2. Nuclear magnetic resonance for PAC (NMR)

The molecular structure of PAC was confirmed by using a Bruker NMR spectrometer (Bruker, Germany) operating at 500 MHz for 1H and at 125.76 MHz for ^{13}C. TMS was used as an internal standard and chemical shift values were recorded in ppm on δ scale. The NMR measurements were run in deuterated dimethylsulfoxide (DMSO-d6).

3. Solubility study

The maximum solubility of PAC in Hydroxypropyl-βcyclodextrin (HPβCD) was determined. An excess amount of PAC was added to 3 ml of different concentrations of HPβCD solutions. The mixtures were then incubated at 25°C with gentle stirring for 72 h. Finally, the solutions were centrifuged at 6000 rpm for 10 min to precipitate the undissolved PAC. The supernatant was filtered through a membrane filter (0.45 μm). After the appropriate dilution with acetonitrile, the PAC concentration in the supernatant solution was measured by HPLC in triplicate and the final concentration of each sample was expressed as a mean ± SD. The maximum solubility of PAC was calculated according to the calibration curve.

4. HPLC assay for PAC

We developed a simpler, accurate and rapid reverse phase liquid chromatographic isocratic RP-HPLC analytical method utilizing UV detection. The HPLC system consisted of a Waters 1525 binary pump separation module (Waters, USA), fitted with C18 column (300 × 4.6 mm). The autosampler injection system (Waters 2707) used was a 10 μL sample loop. A Millipore Swinnex type filter (pore size = 0.45 μm) was used, obtained from Millipore (Bangalore, India). We also used a Waters HPLC system equipped with a Waters 484 variable UV absorbance detector and a Waters 2707 plus autosampler. A Waters 515 solvent delivery system was used to operate the gradient flow through a (4.6 × 150 mm), 3 μm spherical particles column. The mobile phase consisted of acetonitrile-5% acetic acid (50:50, v/v) at a flow rate of 1 ml/min and the run time was 7.0 min. A Waters 2489 UV/Visible detector set at a wavelength of 392 nm was used for detection. Degassing was achieved by filtration through a 0.45 μm Millipore membrane filter and sonication for 10 min. The injection volume was 10 μl and detection was at 392 nm. The HPLC system was operated at ambient temperature. Data were collected with a Breeze Chromatography Manager Data

Collection System. A daily standard calibration curve (6 standards ranging from 100 to 10000 ng/ml) was performed to determine the unknown PAC concentration in the formulations. (Under publication)

5. Preparation of conventional PAC liposomes

Conventional PAC liposomes (CD-free liposomes) were prepared by the thin-film evaporation method. In brief, different Lα-phosphatidylcholine to cholesterol ratios (w/w)ranging from 10:1 to 4:1 were tested before settling on a fixed ratio of 10:5 based on tests to determine optimal encapsulation of PAC by liposomes as indicated in Table 1. PAC (0.015 g), Lα-phosphatidylcholine (0.08 g) and cholesterol (0.02 g) were dissolved in 10 ml of a chloroform and methanol mixture (1:2 v/v). The solution was evaporated by using a rotary evaporator (Büshi, Switzerland) under 50–100 kg/cm² nitrogen flow for 1 hour to obtain a dry thin lipid film. The lipid film was then hydrated 10 in ml 1% sorbitol solution and resuspended by hand shaking at 25 °C for 15 minutes. The hydrated solution was sonicated for 60 minutes in the presence of ice. The PAC-liposome was purified by ultracentrifuge (Beckman, Germany) at 20,000 rpm for 10 minutes for three times to remove free-PAC and impurities and kept at 4 °C prior to use. The empty (PAC-free) liposomes were used as a control to study the effect of Lα-phosphatidylcholine and cholesterol on all cell lines.

6. Entrapment of HPβCD-PAC complex into liposomes

HPβCD-PAC loaded liposomes were prepared by using the protocol described for conventional curcumin liposomes with a small variation in the contents of the starting solution. In conventional PAC liposomes were made by PAC incorporated into the dried phospholipid film before hydration. For the HPβCD-PAC system, the starting solution additionally contained the HPβCD-PAC complex in which PAC and HPβCD were mixed in the ratio 1:1 (w/w) and were added to the Lα-phosphatidylcholine to cholesterol mixture. Then, the procedure continued as described above.

Formulation name	Compsition	Molar ratio (PC:CH)	Molar ratio (PAC: HPβCD)
LIPOPAC	**PC:CH**	**10:5**	
LIPOPAC-HPβCD	**(PC:CH): HPβCD**	**10:5**	**1:1**

Table 1. Composition of prepared liposomal formulations.

7. Physicochemical characterization

7.1. Zeta potential and size measurements

Hydrodynamic diameter, mean particle size, polydispersity index (PDI) and zeta potential of liposomal formulations were determined by the Photon Correlation Spectroscopy (PCS) machine and through electrophoretic mobility titration (Zetasizer Nano Series ZS4700, Malvern Instruments, UK). All liposome formulations were diluted with 1 ml of filtered distilled water. Hydrodynamic diameter (based

on volume measurement), PDI and zeta potential were obtained from the average of three measurements at 25 °C. The refractive index of liposome and water were set at 1.42 and 1.33, respectively. The potentials were calculated by using the Smoluchowski approximation.

7.2. Encapsulation efficiency of liposomal PAC

The indirect method was used to determine drug entrapment efficiency percent (EE %). After preparing the fresh liposome formulations, free PAC was separated from liposome by ultra-centrifugation at 40,000 rpm for 60 min at 4 °C using a Beckman ultracentrifuge (Le 90, Beckman, CA, USA) using a TI-100X rotor. The free PAC in the supernatant was determined by high-performance liquid chromatography (HPLC) (WaterE600 system, MA, USA). The samples were filtered using a Phenex RC 0.45 mm membrane filter (Millipore,Germany) and injected in triplicate into the HPLC. PAC concentration was determined using the regression equation obtained from the calibration curve. The % entrapment efficiency (EE %) was calculated by the following applying equation:

$$EE\ (\%) = \frac{\text{Total amount of drug - amount of the free drug}}{\text{Total amount of drug}} \text{x } 100$$

7.3. In vitro release study

The *in vitro* drug release from liposomal formulations was carried out using dialysis technique. An open-ended tube was used. A dialysis membrane (Membrane Filtration Products, TX, USA) was soaked overnight in 20 ml receptor medium (PBS buffer pH 7.4 and 5%Tween 80) and mounted tightly on one end of the tube. Two ml of liposomal suspension (containing 60 µg of PAC) was placed on this tube. The dialysis tube was suspended in a 100 ml beaker containing 40 ml of 5% Tween 80 in PBS (pH 7.4) to maintain a sink condition. The test was performed using a shaker water bath (VS-8480, Vision Scientific, Daejeon, VS8480; South Korea), which accurately controlled the bath under a shaking condition of 50 rpm and 37 °. The samples were withdrawn at predetermined time intervals (0.25, 0.5, 1, 2, 3, 4, 6, and 8 hours) and replaced by 5% Tween 80 in PBS (pH 7.4) solution. The drug content in the withdrawn samples was estimated by HPLC and the cumulative percentage of drug release was calculated and plotted against time.

8. In vitro evaluation of the anticancer activity of encapsulated PAC

8.1 Quantification of liposomal PAC for in vitro cytotoxicity test

For *in vitro* quantification of PAC in liposomes, the liposomal formulations were diluted with ethanol in a ratio 1:10 and vortexed. 100 µl from the diluted sample was added to HPLC vials. The linear equation obtained from the calibration curve was used to determine PAC concentration in liposomal formulations.

8.2 Cell culture

LoVo (human colon cancer cell line, ATCC-CCL-229 ^{TM}) cells were cultured in DMEM, while MDA-MB-231 (human breast cancer cell line, ATCC-HTB-26^{TM}) cells were cultured in RPMI 1640. The media were supplemented with 10% FBS and 1% antibiotic/antimycotic. All cells were incubated at 37 ^{0}C in a humidified incubator with 5% CO_2 and cultures were regularly examined using an inverted microscope. Cells were passaged when the tissue culture plate became confluent by sucking the old medium, washing cells with 1x PBS, and cells were detached by trypsin for 1-3 minutes incubation at 37 ^{0}C. After that, trypsin was neutralized by addition of serum containing the medium. Following proper homogenizing of the suspended cells, the cells were centrifuged to discard trypsin. Then, the cell pellet was re-suspended into fresh medium and distributed in new culture plates. Cell cultures were performed in 10 ml cell culture dishes.

8.3 Cytotoxicity assessment by flow cytometry

Cell death was assessed by the Vybrant® Apoptosis Assay Kit #2 and Flow Cytometry. This was performed at the Flow Cytometry Core Facility at the King Faisal Specialist Hospital and Research Center (Riyadh, KSA). After PAC/LIPOPAC treatment, cells were harvested by trypsinization, centrifuged and re-suspended in 1 ml PBS. Subsequently, cells were stained by Alexa Fluor 488 annexin V/propidium iodide (PI) and analyzed by the flowcytometer. The percentage of cells was determined by FACSCalibur apparatus and CellQuest Pro software (Becton Dickinson, Biosciences, N J, USA).

9. Results

9.1. Spectroscopy for PAC results

9.1.1. Fourier transform infrared spectroscopy (FT-IR)

The spectrum obtained for FT-IR analysis showed a major broad peak at 3318 cm-¹ indicating hydroxyl group for PAC. Peak at 1744 cm-¹ attributed to ν(C=Cring) aromatic ring stretching vibration. The peak at 1670 cm-1 corresponds to ν(C=C). The peak at 1593 cm-1 corresponds to ν(C=O) in plane bending aromatic stretching vibration (Figure 3).

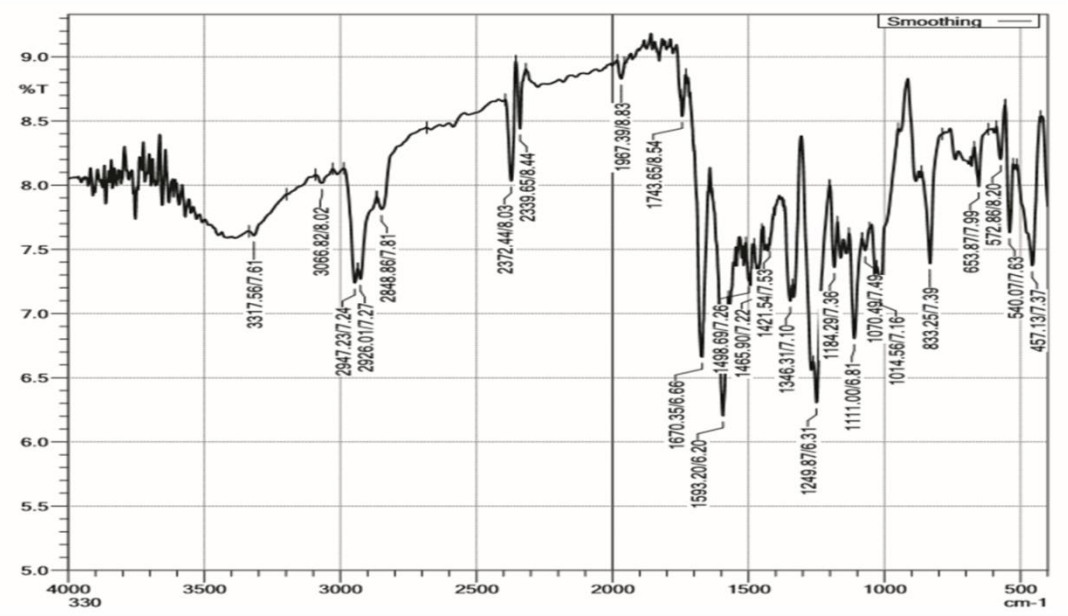


Figure 3. FT-IR spectrum of PAC.

9.1.2. Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR spectrum in Figure 4 of PAC contains the singlet at 3.83 ppm due to the two methoxy groups and the singlet at 9.75 ppm due to the protons of the two hydroxyl groups, respectively, which reflects its symmetric structure. The other singlets at 6.88 ppm, 6.96 ppm, 7.5 ppm and 7.08 ppm are assigned to the aromatic protons. The spectrum is also characterized by a singlet at 2.41 ppm which is assigned to the proton of the N-CH3 group.

The ^{1}H NMR spectrum can be summarized as:

^{1}H-NMR (DMSO-d_6): δ 2.41 (s, 3H, N-CH_3), 3.72 (s, 4H, 2 x CH_2), 3.83 (s, 6H, 2 x OCH_3), 6.88 (d, J = 7.5 Hz, 2H, Ar-H), 6.96 (d, J= 8.0 Hz, 2H, Ar-H), 7.08 (s, 2H, Ar-H), 7.54 (s, 2H, 2 x Ar-CH=C), 9.75 (br. s. 2H, 2 x OH)

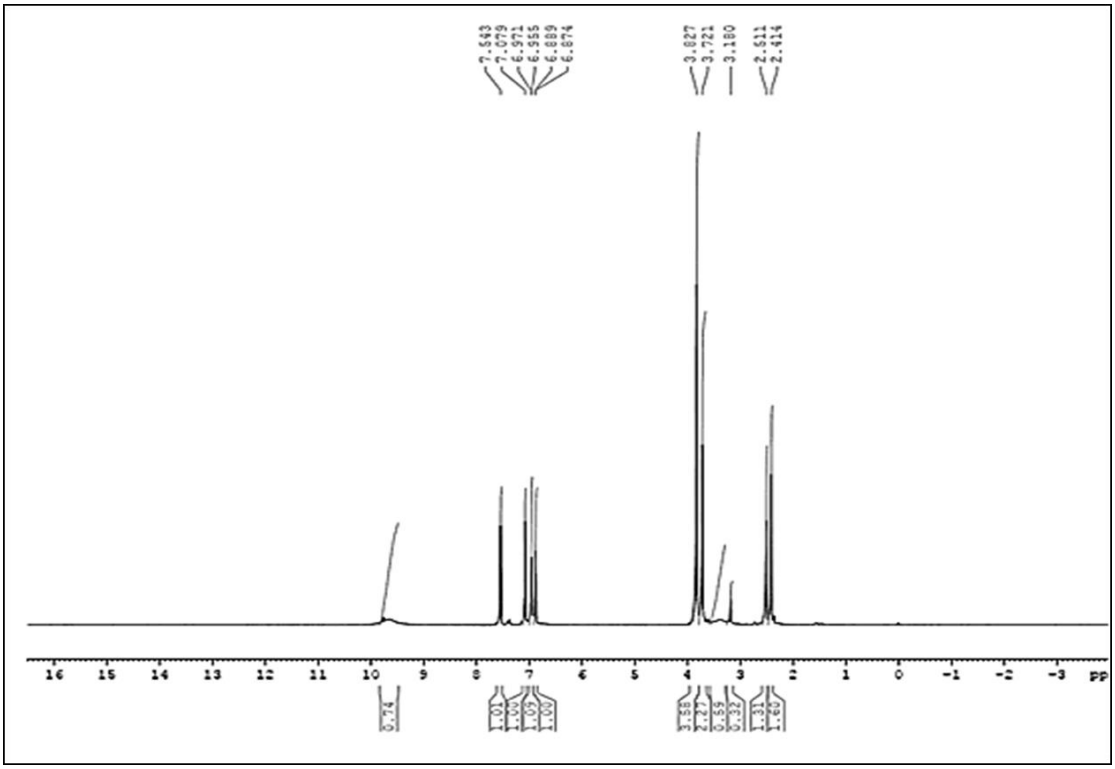


Figure 2. ^{1}H NMR spectrum of PAC.

^{13}C NMR spectrum of PAC in Figure 5 exhibited 12 singles from 21 carbons. The two carbonyl carbons signal appeared at 186.5 ppm. The signal at 56.1 ppm was assigned to (-CH=CH-). The signal at 57.1 ppm was due to carbons of methoxy groups. The signals of the carbons of the aromatic ring appeared at 115.5, 116.2, 124.7, 126.7, 131.5 ppm. The highest field signal at 45.9 ppm was assigned to a carbon of the N-CH_3 group.

The ^{13}C NMR spectrum can be summarized as:

^{13}C-NMR (DMSO-d_6): δ 45.9 (N-CH_3), 56.1 (2 x CH_2), 57.1 (2 x OCH_3), 115.5, 116.2, 124.7, 126.7 (Ar-CH and Ar-C), 131.5 (2 x Ar-CH=*C*), 135.4, 148.0, 148.7 (2 x Ar-*C*H=C, 4 x Ar-C), 186.5 (O=C)

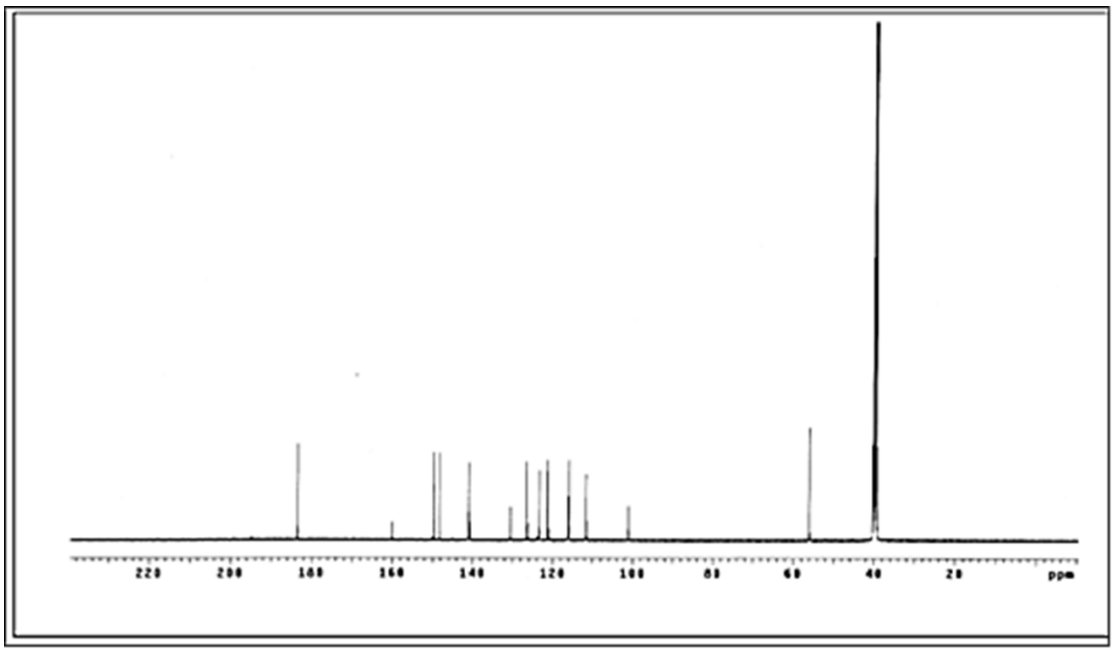


Figure 3. ^{13}C NMR spectrum of PAC.

9.1.3. HPLC assay

The proposed method was optimized to develop a suitable analytical method for the analysis of PAC. Figure 6, shows the chromatogram A, for the blank, mobile phase, and chromatogram B represents PAC with the average retention times of 5.8 ± 0.92 with no interfering peaks. This is an indication of the specificity of the HPLC assay method.

A. B.

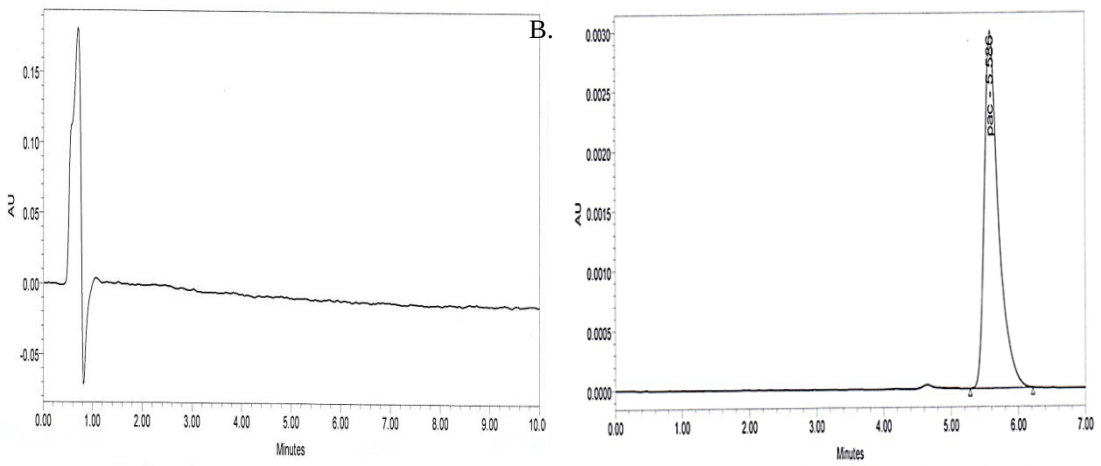


Figure 6. HPLC chromatograms of mobile phase (chromatogram A) and HPLC chromatograms of mobile phase containing 1000ng/ml PAC and 100 µg/ml (chromatogram B).

9.2. Solubility results

PAC showed very low (363 ng/mL) aqueous solubility (Figure 7). The addition of HPβCD has enhanced PAC aqueous solubility. The aqueous solubility of PAC increased by increasing the HPβCD concentration and maximum solubilization was achieved in 100 µM HPβCD solutions.

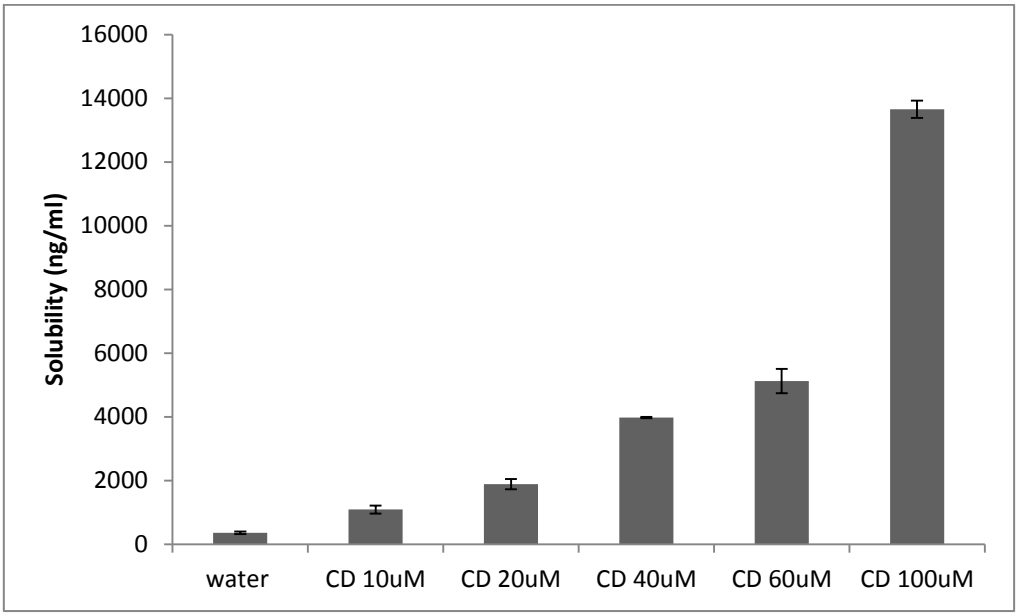
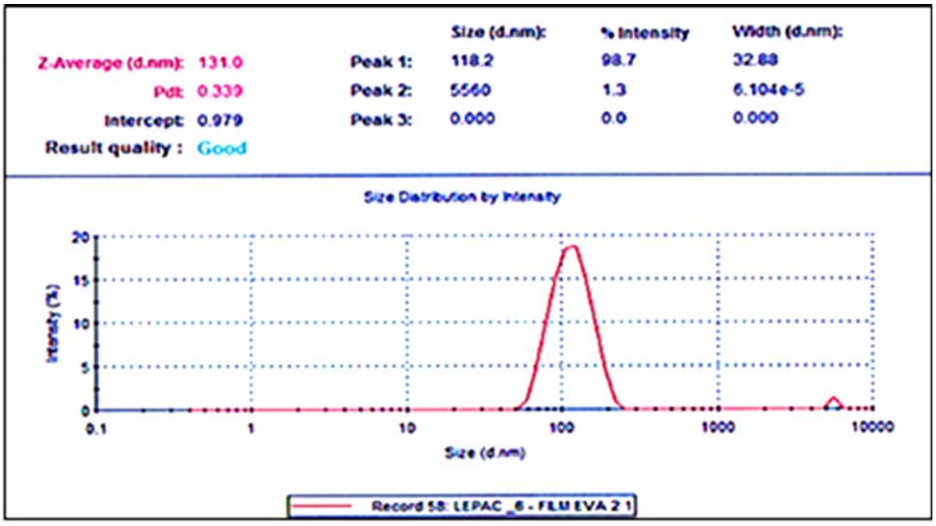


Figure 7. PAC solubility in different HPBCD concentrations (n = 3±SD).

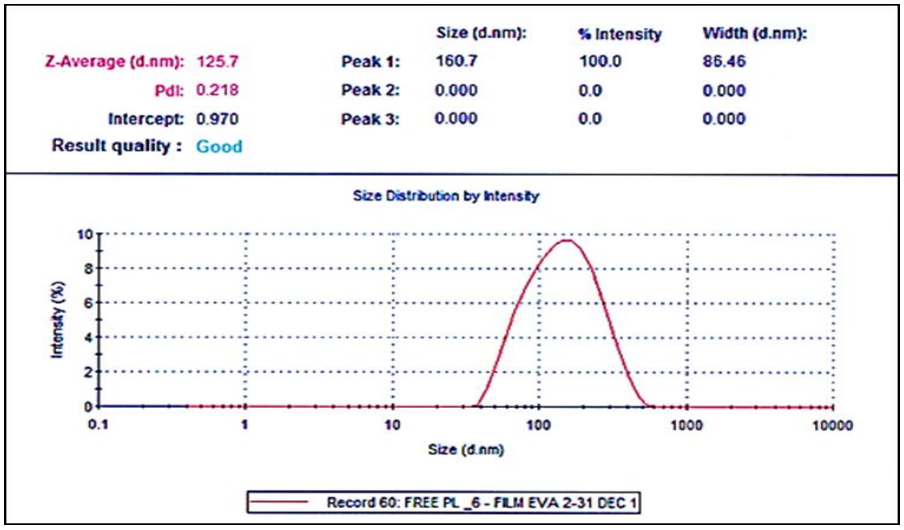
9.3. Physicochemical characterization of the liposome

9.3.1. Size measurements and zeta potential

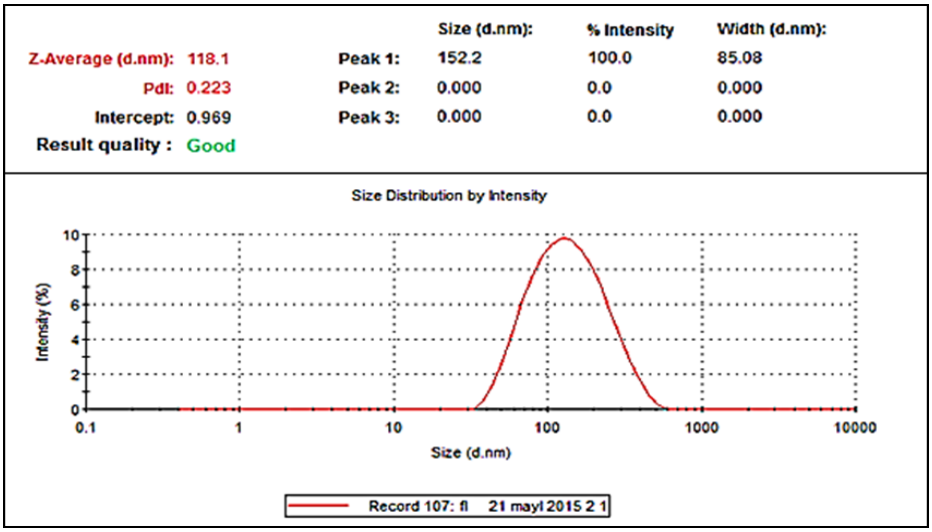
Representative DLS results of PAC liposomal are seen in Figures 8. In this study, the effect of HPβCD incorporation into the liposomal formulation was evaluated by formulating PAC liposomes with/without HPβCD. The free liposomes in which no drug was added showed a mean particle size of 124.7 nm and PDI of 0.273 (Figure 8C). The mean particle size of PAC-loaded HPβCD liposomes and PAC conventional liposomes were 138.4 and 106.9 nm, respectively, and PDI values were 0.373 and 0.224, respectively, as seen in Figure 8A and Figure 8B. Lower PDI in both formulations indicates the liposomes populations were homogeneous in size (PDI < 0.3 indicates good homogeneity) [18]. It could be inferred from the results that there was no significant impact of the HPβCD incorporation into liposomes on the size or distribution of particles. The small variations between the size of liposomal formulations could be attributed to the handling variation between one preparation and another. The zeta potential values for liposomal formulations are shown in Table 2. The zeta potential remained in the range of low negative values with little differences among all formulations (-2.76 mV to-3.84 mV), which is according to what was expected due to PAC liposomes prepared from neutral lipids (phosphotidylecholine) which possess nonzero z-potentials over a wide range of ionic strengths. This is due to the fact that zwitterionic head group of neutral lipids absorbs anions or cations, leading to slightly negative or positive z-potentials, while cholesterol has no charge [19].



A: PAC-loaded HPβCD liposomes



B: PAC conventional liposomes



C: free liposomes

Figure 8. DLS plot for the size distribution versus liposomal formulations

Formula	Z average diameter ± σ (nm)	Polydispersity Index (PI) ± σ	Zeta potential± σ (mV)
PAC-loaded HPβCD liposomes	138.4±9.7	0.37±0.02	**-3.6±0.3**
conventional PAC liposomes	106.9 ±11.7	0.22±0.002	**-3.8±0.6**
Free Liposome	124. 7±4.8	0.27±0.01	**-2.8 ±0.01**

Table 2. Particle size, polydispersity index (PI), zeta potential of PAC-loaded HPβCD liposomes, conventional PAC liposomes and free liposomes (σ is standard deviation, (n=3)

9.3.2. Determination of the drug entrapment efficiency (%)

Drug entrapment determination is a method to evaluate efficiency of preparation technique. The indirect method was used to determine drug entrapment efficiency percent (EE%). The percentage of PAC entrapment in liposomes vesicles was 33.5% at PAC conventional liposomes. This EE% values indicate relatively low values of drug to lipid ratio. Another explanation for poor entrapment is the low aqueous solubility of PAC which was not enough for its accommodation inside the aqueous core. Therefore, HPβCD was used to enhance solubility and encapsulation of PAC. This strategy exhibited a significant increase in EE% (68.1%) PAC-loaded HPβCD liposomes.

9.3.3. In vitro Drug Release

In vitro drug release from the PAC liposomal formulations in 5% Tween 80 in PBS buffer of pH 7.4 was performed using the dialysis technique. The calibration curve of PAC was constructed to determine the concentration of the released PAC using the HPLC assay method. The *in vitro* drug release profiles obtained for free PAC, PAC-loaded HPβCD liposomes and PAC conventional liposomes are shown in Figure 9. As seen in these drug release profiles, 83% of free PAC diffused through the membrane within 1 hour. PAC conventional liposomes gave a prolonged drug release profile with only about 36.6% drug release after 8 hours, whereas, PAC-loaded HPβCD liposomes gave 55.35% drug release after the same period of time.

Encapsulation of PAC within liposomes led to a slower release PAC compared to a free PAC, due to the well-known reservoir effect of liposomes. Liposomes' bilayer acts as a barrier and decreases PAC release. The PAC release profile from the liposomes displayed a biphasic release profile. The initial burst was associated with the fast release of drug molecules located in the outer bilayer or free PAC, which is encapsulated in the liposomes. In the second phase, PAC was slowly released corresponding to the diffusion from the inner core into the outer aqueous environment [6]. However, after HPβCD incorporation PAC release was enhanced.

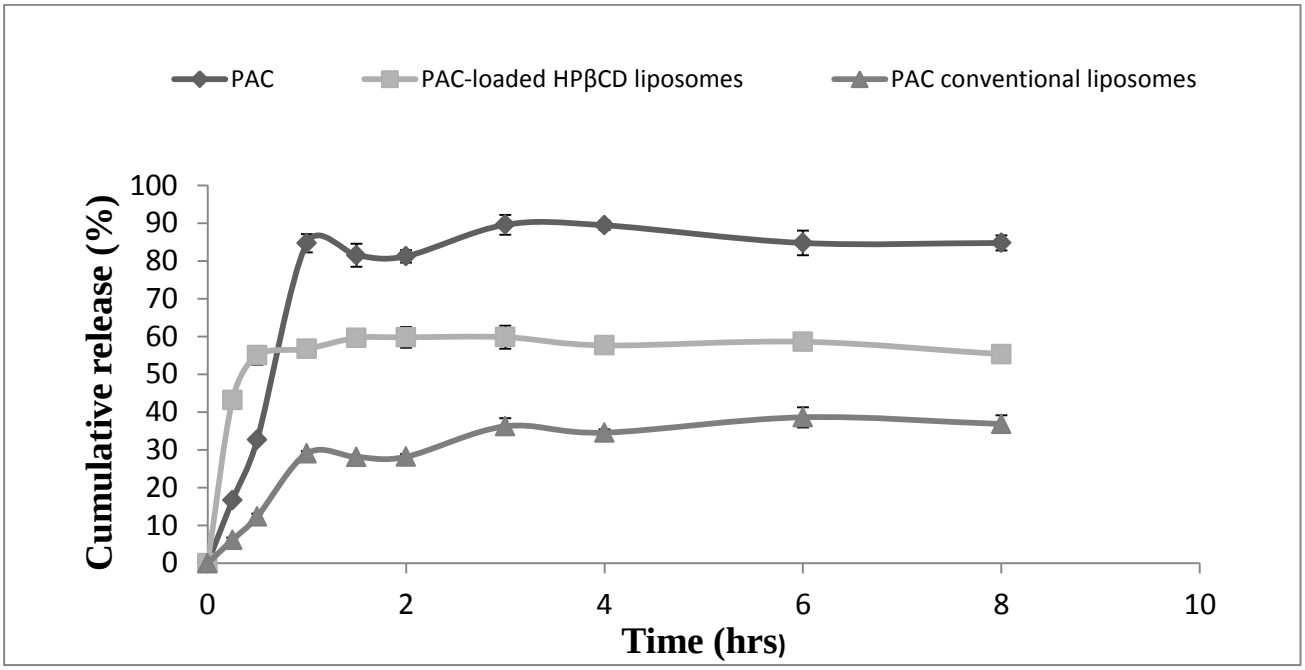


Figure 9. *In vitro* release of free PAC, PAC-loaded HPβCD liposomes and PAC conventional liposomes in 5% Tween PBS buffer pH 7.4 at 37 C

9.4. *In vitro* evaluation of the anticancer activity of encapsulated PAC

9.4.1. PAC liposomal formulations induce apoptosis in colon cancer cells

Liposomal formulations of PAC were made both in the presence and absence of HPβCD, and it has been shown that the presence of HPβCD improves the quality of the formulation. Therefore, it was decided to test the cytotoxic effects of both formulations on the colon cancer cell line (LoVo) known to be sensitive to PAC [2]. Figure 10A shows that PAC-loaded HPβCD liposomes and PAC conventional liposomes triggered mainly apoptosis in LoVo cells. Importantly, the presence of HPβCD enhanced the cytotoxic effect of PAC liposomal formulation (Figure 10B). Indeed, while the proportion of apoptosis reached 75% in response to PAC-loaded HPβCD liposomes (10 μM), it was only 43% in response to the same concentration of PAC conventional liposomes (Figure 10B). However, at higher concentrations, PAC and PAC-loaded HPβCD liposomes had similar cytotoxic effects on LoVo cells (Figure 10B). These results indicate that the formulated PAC is active and the presence of HPβCD enhances this activity but did not improve the pro-apoptotic effect of PAC against colon cancer cells.

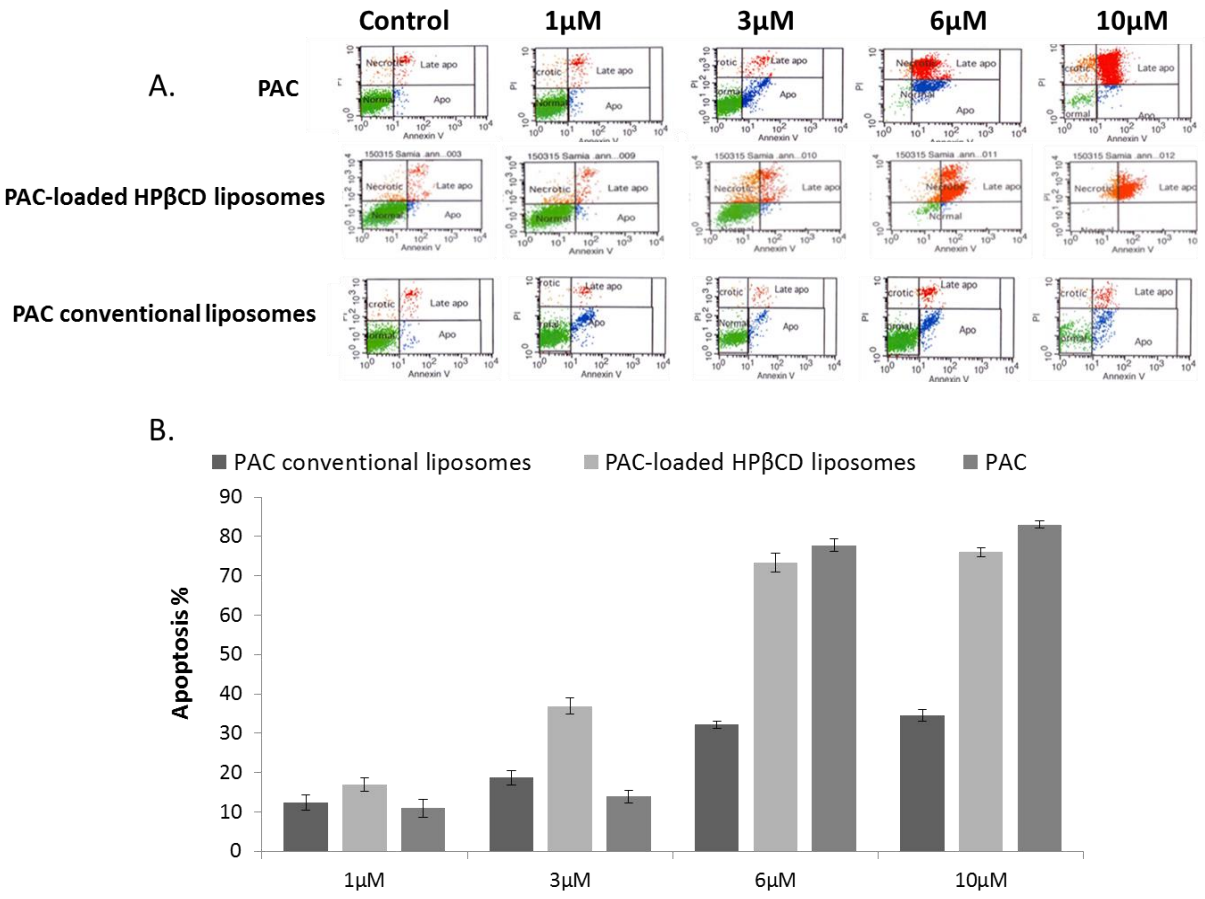


Figure 10. PAC liposomal formulations induce apoptosis in colon cancer cells.

LoVo cells were either sham-treated (DMSO or free liposomes) or challenged with PAC or PAC Liposomal formulations with the indicated concentrations for 72 hours, and then the proportion of apoptosis was analyzed by Annexin V/PI-flow Cytometry. (A) Flow charts. (B) Histogram showing the percentage of induced apoptosis in LoVo cells (early and late apoptosis subtracted from controls [DMSO and free liposomes]. Error bars represent mean +/- SD from three independent experiments conducted in triplicate.

9.4.2. PAC liposomal formulations induce apoptosis in breast cancer cells

It has also been previously shown that PAC triggers apoptosis in breast cancer cells [1]. Therefore, we wanted to test the effect of PAC liposomal formulations against these cells. Figure 11B shows that while PAC conventional liposomes have only minor cytotoxic effect (22-25%), PAC-loaded HPβCD liposomes induced 53% and 70% apoptosis in MDA-MB-231 cells in response to 5 µM and 10 µM, respectively. These effects were similar to those obtained in response to PAC at similar doses (Figure 11B). This indicates that PAC-loaded HPβCD liposomes have stronger pro-apoptotic effect against breast cancer cells than PAC conventional liposomes but did not improve the effect of PAC.

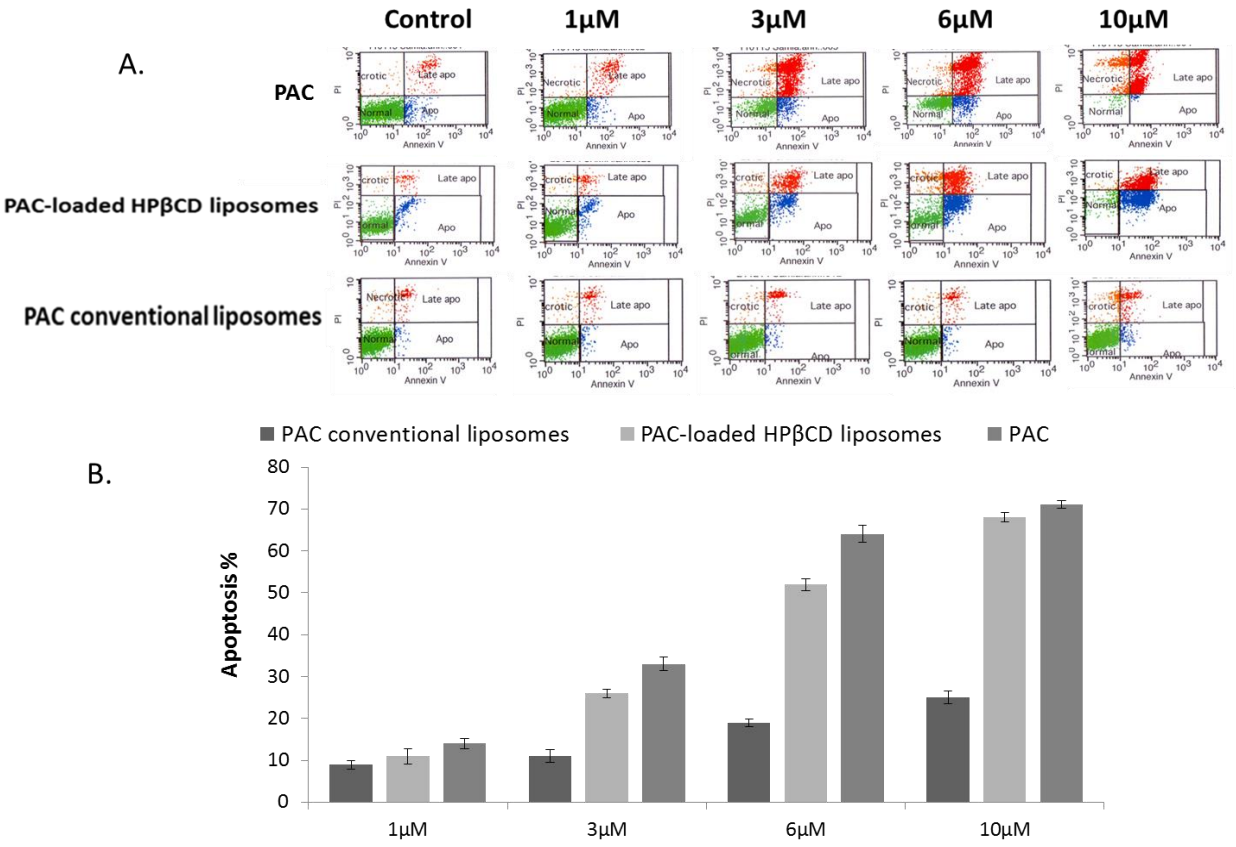


Figure 11. PAC liposomal formulations induce apoptosis in breast cancer cells. MDA-MB-231 breast cancer cells were either sham-treated (DMSO or free liposomes) or challenged with PAC or PAC Liposomal formulations with the indicated concentrations for 72 hours, and then the proportion of apoptosis was analyzed by Annexin V/PI-flow Cytometry. (A) Flow charts. (B) Histogram showing the percentage of induced apoptosis in MDA-MB-231 cells (early and late apoptosis subtracted from controls [DMSO and free liposomes]). Error bars represent mean +/- SD from three independent experiments conducted in triplicate.

Discussion

It has been previously shown that the synthetic compound, PAC, is more potent than its congener, curcumin, in the ability to serve as a cytotoxic agent in human breast cancer cells and human colon cancer cells [1, 2]. It is interesting that PAC does not exhibit cytotoxicity toward normal human breast cells [2]. Therefore, PAC has been seen as a potentially effective anti-cancer agent. However, the effects of this curcumin analog are limited owing to its poor aqueous solubility and its poor pharmacokinetics [2]. To circumvent these limitations, liposomal formulation, LIPOPAC, was synthesized in the hope of preserving the safety profile with enhanced stability and efficiency. CDs in liposomes have been used in recent years as drug delivery vehicles, improving the bioavailability and therapeutic efficacy of many drugs having poor water solubility [20]. Furthermore, liposomal delivery of cyclodextrin-enclosed hydrophobic drugs

has the potential to combine all the delivery advantages that both cyclodextrin and liposomes can offer individually [12, 21].

In our study, we have developed two liposomal systems for PAC delivery: PAC liposomes and PAC/HPβCD inclusion complex liposomes. Both formulations have been assessed in the physicochemical aspects as well as the molecular aspects. From DLS, it is difficult to state clearly that there is a clear distinction in liposome size and zeta-potential between the two liposomal formulations, which means the inclusion of CD does not change the size of the liposomes size or charge significantly. In contrast, PAC/HPβCD inclusion complex liposomes increased the encapsulation ability of liposomes toward PAC. CDs are able to enhance drug loading by forming more soluble complexes that can be incorporated in the aqueous phase of liposomes which provided a relatively larger volume than lipid bilayers [9]. Furthermore, CDs increase drug solubility and availability and preserve the liposomal structural integrity from drug molecules, whereas liposomes prevent drug-CD complexes dissociation due to dilution by the plasma or the renal excretion of CD molecules [12].

The correlation between presence of the CD in liposomes and increased EE% was previously reported of many hydrophobic drugs, such as ketoprofen, betamethasone and riboflavin [22-24]. On the other hand, inclusion of HPβCD in liposomes has a significant effect on accelerating PAC release rate from liposomes [25]. The exact reason behind this behavior is not yet clear. However, alternative mechanisms were proposed to explain this behavior. One study suggested a displacement effect of cholesterol. Because cholesterol has an affinity toward HPβCD, it displaces PAC from the HPβCD cavity and makes a new complexion with HPβCD leaving PAC leak out. In general interaction between HPβCD and cholesterol or PAC depends on their affinity toward HPβCD [6]. In HPβCD-drug complexion, no covalent bonds are formed during formation of the inclusion complex. Therefore, the binding force between HPβCD and PAC molecule is relatively weak and rapid dissociation of PAC/HPβCD inclusion complexes takes place either because of dilution or because of displacement of the included drug. Extraction of cholesterol dose not immediately result in lysis and dissolution of the liposomes. Lipid bilayers losing cholesterol remain intact but become more fluid/or permeable for PAC release [22].

Both liposomal PAC formulations' anticancer activity was examined *in vitro* using breast cancer cells and colon cancer cells. We found that PAC/HPβCD inclusion complexes liposomes have potential anticancer activity similar to free PAC, which confirmed that such a system is capable of encapsulating, PAC within its structure and then delivering it to cancerous cells in active form.

Curcumin-loaded liposomes and curcumin-loaded cyclodextrin liposomes have been extensively tested by several studies. Dhule et al (2012) developed conventional curcumin liposomes and curcumin-loaded HP-ɣ-cyclodextrin liposomes and examined them against two cancer cell lines of osteosarcoma, including KHOS and RFOS. They found while, KHOS cells have a strong sensitivity to both formulations, RFOS cells exhibit resistance to them [26]. Furthermore, Rahman et al (2012) revealed that native and β-

cyclodextrin-enclosed curcumin liposomal formulations were cytotoxic against both lung and colon cancer cells, with the native liposomal formulation was most effective in both cell lines [27]. In Sun et al study (2014), the cytotoxicity of cationic PEG-PEI liposomes loaded with curcumin being more cytotoxic than free curcumin against murine melanoma cells and colorectal adenocarcinoma cells, likely because of its rapid cellular internalization [28]. Tang et al (2007) compared the toxicity and effectiveness of curcumin delivery by DMSO versus liposomes in two aggressive oral cancer cell lines – SCC-1 and CAL-27 –and they have found that the growth inhibition by liposomal curcumin is similar to that achieved with curcumin dissolved in DMSO, and was similar for cells grown for 12 or 36 hours after treatment [29]. Li L et al (2005) encapsulated curcumin in a liposomal delivery system that would allow intravenous administration. They studied the *in vitro* and *in vivo* effects of this compound on proliferation, apoptosis, signaling, and angiogenesis using human pancreatic carcinoma cells. They have found that the activity of liposomal curcumin was equal to that of free curcumin at equimolar concentrations [30].

Overall, we present clear evidence that PAC/HPβCD inclusion complex liposomes could constitute a potent delivery system for PAC. However, further long-term stability and *in vivo* studies could be performed in order to test the liposomal delivery system for clinical use.

Conclusion

The synthesis and establishment of appropriate chemical analysis for PAC were performed to identify and understand its chemical entities. A solubility study was done and HPβCD was found to be capable of increasing PAC aqueous solubility. HPβCD was thus chosen as the method to enhance incorporation capacity in liposomes preparation. Then, liposomes with and without HPβCD were prepared by the film hydration method using PC and cholesterol at a ratio of 10:5 and evaluated. Indeed, no major differences were seen in particle size and surface charge. However, HPβCD inclusion gave satisfied incorporation capacity. In addition, HPβCD inclusion in the liposomes resulted in increased the *in vitro* release rate compared to conventional liposomes, but further investigations are still needed to understand this behavior. Furthermore, in the cytotoxicity studies on colon cancer cells and breast cancer cells, the HPβCD containing liposomes showed potent cytotoxic effect compared to the liposomes without HPβCD.

Future prospects

Based on the results obtained from the current research, HPβCD inclusion in liposomes was found to give the highest EE% for PAC with highest cytotoxicity. However, long-term stability and *in vivo* studies could further be performed in order to test the liposomal delivery system for clinical use.

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List of abbreviations

ATCC	American Type Culture Collection
CD	Cyclodextrin
CAL-27	Oral cancer cell line
C18	C18 bonded silica stationary phase column
DLS	Dynamic light scatter
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
EE%	Entrapment efficiency%
FL	Free liposomes
FT-IR	Fourier transform infra-red Spectroscopy
FBS	Fetal Bovine Serum
HPβCD	Hydroxypropyl-beta-cyclodextrin
KBr	Potassium bromide
KHOS	Carcinoma cell line
LoVo	Human colon cancer cell line
MDA-MB-231	Basal like breast cancer cell line
MβCD	methylated βCD
NMR	Nuclear Magnetic Resonance
nm	Nanometer
PAC	Piperidone analogue of curcumin
PC	Phosphotidylcholine
PBS	Phosphate buffered saline
PDI	Polydispersity index (width of the distribution)
PCS	Photon Correlation Spectroscopy
RP-HPLC	Reverse phase liquid chromatographic
RT	Retention time
RFOS	Carcinoma cell line
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium,
SD	Standard deviation

ATCC	American Type Culture Collection
SCC-1	oral cancer cell line
SBEβCD	sulfobutylether β-CD
UV	Ultraviolet–visible spectroscopy
Z-average	Cumulants mean
ZP	Zeta potential

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