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3   **A novel pH-sensitive liposomes to trigger delivery of afatinib to cancer cells: Impact**

4   **on lung cancer therapy**

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Abstract

A novel drug delivery systems based on cationic (CL) and pH-sensitive liposomes (PSL) for tyrosine kinase inhibitor afatinib (AFT) were developed to enhance tumor-targetability against NSCLC cells and therapeutic effect. Optimal lipid to drug ratio was selected to prepare AFT-loaded PSL and CL with desirable physiochemical properties based on particle size, drug encapsulation efficiency (EE%), stability and release profiles. Moreover, antitumor activity was performed *in vitro* on human lung cancer cells (H-1975) using a WST-1 assay and Annexin-V apoptosis assay. The mean particle size of the liposomes was less than 100 nm, and EE% was more than 50% with lipid to drug ratio of 1:0.5. Stability data showed that PSL and CL were physically stable for 1 months at 4 and 25 °C. *In vitro* drug release study demonstrated the sustained release of AFT at pH 7.5; while PSL exhibited fast drug release in pH 5.5. This effect revealed that PSL showed pH-sensitive release behaviors. In addition, the *in vitro* cytotoxicity study was employed for AFT-loaded PSL due to optimal characterizations. Thus, *in vitro* anticancer activity revealed that AFT loaded-PSL triggered apoptosis in H-1975 cells. In addition, the inhibitory effect towards H-1975 and HCC-827 was observed, indicating, which indicated high antitumor activity of AFT-loaded PSL. Then, PSL might potentially create practical clinical strategies for better targetability and delivery of AFT for treatment of lung cancer.

Key words: Afatinib, pH-sensitive liposomes, *In vitro* release, Anticancer activity, Lung cancers

1. Introduction

2

42   Cancer is a foremost problem of disease worldwide to human health in recent years.

43   Moreover, lung cancer becomes a serious danger to human health, it is about 1.38 million

44   cancer-associated mortality in males and females in recent decades [1-2]. The incidence of

45   lung cancer has increased significantly in recent years in Kingdom of Saudi Arabia and

46   United Kingdom due to the increased prevalence of cigarette smoking [3-4].

47   Approximately 85% of lung cancer diagnoses are classified as non-small cell lung cancer

48   (NSCLC) and the remaining 15% small cell lung cancer (SCLC) [6]. The unsatisfactory

49   effects after treatment of lung cancer patients using conventional approaches such as

50   surgical resection, radiation, and chemotherapy were perceived [6]. The majority of

51   chemotherapy administration is intravenous, causing pronounced side effects due to their

52   systemic drug distribution. Moreover, the bioavailability of orally administrated anticancer

53   agents is usually compromised by the first-pass metabolism [7]. The cytotoxic effects of

54   chemotherapeutic agents against normal cells, according to dose-response effects, have

55   been recorded, leading to the patient's frail and death [8]. Therefore, the targeted delivery

56   of anticancer drugs has become a focus of scientific research. NSCLC treatment can be

57   improved by targeted delivery of chemotherapeutic agent (s) to suppress the major

58   signaling pathways involved in lung cancer. Then the targeted delivery of anticancer drugs

59   directly into the lungs can increase their accumulation in tumor cells and reduce adverse

60   side effects [9]. To date, numerous epidermal growth factor (EGFR) targeting agents have

61   been approved, including gefitinib, erlotinib, and lapatinib; however, both primary and

62   acquired resistance are significant clinical problems [10]. Afatinib (AFT) is a novel, potent,

63   small-molecule tyrosine kinase inhibitor (TKI), which is now marketed (2013), as a film-

64   coated oral tablet as a dimaleate salt. AFT is an especially effective treatment for nonsmall

65   cell lung cancer (NSCLC) [11]. AFT has ability to bind covalently and irreversibly to the

66   intracellular TK domain, preventing intracellular signaling [12]. By targeting ErbB family

67   receptors, AFT blocks a wide spectrum of cancer-associated ErbB-driven pathways, and

68   thus has broader antitumor activity against receptors with acquired mutations that are

69   resistant to the first generation of TKIs [13]. AFT exposed at low concentration in the

70   tumor cells, which reduced their clinical uses [14]. Therefore, the targeted delivery of AFT

71   has become a focus of scientific research. Nanomedicine is extensively used due to their

72   intrinsic properties such as improved cancer therapy with reduced toxicity. The

73   nanoparticles may be considered as a promising antitumor delivery system for AFT, which

74   may trigger its delivery to the cancer tissues [15]. The liposomes were the interest type of

75   nanomedicine for clinical application in the field of cancer therapy [16]. In addition, they

76   have ability to target tumor tissues via an enhanced permeation and retention (EPR) effect

77   [15]. Liposomes are suitable carriers for pulmonary drug delivery owing to their capacity

78   for targeting to specific cells/tissues [17]. Although drug-loaded liposomes can extend *in*

79   *vivo* circulation and increase chemotherapeutic activity, they may also be limited targeting

80   and fast cleared [18]. It has been reported that pH-sensitive liposomes have received great

81   attention due to their efficient accumulation in the tumor cells [19]. It has been described

82   that tumor tissues exhibited an acidic condition (pH 5.0-6.5) than normal tissues (pH 7.4-

83   7.5) [20]. Therefore, pH-sensitive liposomes were able to deliver the drugs into the tumor

84   cells when the pH value is lower than the normal tissue. Therefore, it may be expected that

85   pH-sensitive liposomes are more efficient for the delivery of AFT than conventional

86   liposomes due to their fusogenic character [21]. pH-sensitive liposomes could increase the

87   intracellular delivery of their content in cancer cells [20-21]. To the best of our knowledge,

88   only one study investigated AFT nanoparticles in term of polymeric micelles as a

89   pulmonary delivery system that improved the therapeutic efficacy in HER2-overexpressed

90   HCT-15-induced tumors [22].

91   In this study, we have prepared three types of liposomes, pH-sensitive liposomes

92   compared with conventional and cationic liposomes containing AFT to study their cancer

93   targeting. An HPLC method was developed and validated for AFT for *in vitro* analysis.

94   The liposomes were characterized in term of particle size distribution, zeta potential and

95   encapsulation efficiency. The surface morphology of the desired liposome was observed

96   by TEM. Moreover, the drug release of AFT was investigated for pH sensitivity (pH 5.5)

97   and prolonged circulation (pH 7.4). The stability studies of these liposomes were

98   performed at a different temperature. Afterward, the NSCLC cells (H-1975 cells) were

99   selected for the assessment of antitumor activity of the optimum liposomes using a

100   colorimetric WST-1 assay and flow cytometry. Moreover, the apoptosis in different cells

101   like H-1975, HCC-827 and H-1650 was established after incubation with liposomes.

102   **2. Materials and methods**

103   2.1. Materials

104   AFT (99.8% purity) was purchased from Green Stone Swiss Co., Limited. 1,2-

105   distearoyl-sn-glycero-3-phosphocholine [18:0] (DSPC), 1,2-dioleoyl-sn-glycero-3-

106   phosphoethanolamine [18:1] (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine [18:1]

107   (DOPC), and 1,2-dioleoy-3-trimethylammonium-propane Chloride salt (DOTAP) were

108   kindly gifted by Avanti Polar Lipid. Cholesteryl hemisuccinate (CHEMS) was purchased

109   from Avanti Polar Lipid. H1975, H-1650, and HCC827 cells were obtained from the

110   American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained

111 at 37°C and 5% CO2 in RPMI 1640 medium (GIBCO®, SIGMA-ALDRICH, Saint Louis,

112 USA) containing 10% fetal bovine serum (FBS) and1% antibiotic/antimycotic which were

113 purchased from (GIBCO®, InvitrogenTM, Carlsbad, USA). All other reagents and

114 chemicals were of analytical grade.

115 **2.2. HPLC assay of AFT**

116 2.2.1. HPLC instrumentation

117 A Water Breeze2TM HPLC system (Waters Corporation, Milford, U.S.A) was used

118 for method development. The HPLC system equipped with an automated sampling system

119 (WatersTM 2695 Plus Autosampler, USA) at 4°C and a photodiode array detector

120 (WatersTM 2998, USA). The HPLC system was examined by "Breeze2 (WaterTM)"

121 software. AFT was analyzed using mobile phase that consisted of A: 0.1% triethanolamine

122 and 1% acetonitrile in HPLC water (pH= 6), and B: acetonitrile and 10% methanol at a

123 flow rate of 1 mL/min. The mobile phase flowed over a reversed-phase C18 column

124 (WaterTM, 3 x 150 mm, 3.5 µm particle size) coupled with a C18 guard cartridge (4×2.0

125 mm) and maintained at 50 °C. The injection volume of each AFT sample was 10 µl and

126 detected by the UV detector at 253 nm. All the operations were carried out at room

127 temperature.

128 2.2.2. HPLC assay

129 A stock solution of AFT was prepared in methanol at a concentration of 1 mg/ml

130 and stored in 4.0 ml amber glass vials at -20°C. Serial dilutions in mobile phase were

131 performed in the range of 0.01 to 25 µg/ml to produce a standard calibration curve and

132 stored at -20°C. A daily standard calibration curve (n=3) ranging from 0.01 to 25 µg/ml

was prepared to determine the unknown AFT concentrations for entrapment efficiency and

drug release.

2.2.3. Method validation

The validation of HPLC method was conducted according to the International

Conference on Harmonization (ICH) guidelines. The following items were considered for

validation: linearity, accuracy, precision, specificity, limit of detection (LOD), limit of

quantification (LOQ) and robustness. Three standard calibration lines were prepared at

different times (3 months) to evaluate the linearity, precision, accuracy, and stability of the

method.

Linearity was assessed by calculating a regression line by plotting the peak area of

AFT vs. the AFT concentration ranging from 0.01 to 25 µg/ml.

The accuracy was determined via the analysis of multiple replicates (n = 6) of AFT

concentration. The accuracy of the method was expressed in term of bias.

The precision of a quantitative method was determined by repeatability as intra-day

precision by an analysis of three replicates of AFT concentrations over the same day. Inter-

day precision was determined by the analysis of three replicates of various AFT

concentrations over three different days. The results were expressed as the relative standard

deviation (RSD%).

Low, medium, and high concentration quality control (QC) samples at

concentrations of (100, 1,000 and 10,000 ng/ml AFT, respectively) were analyzed, on three

distinct occasions within at least 3 months, as before described.

The LOD and LOQ were determined from the calibration curve obtained using six

replicates that were closest to the LOQ. The following equations were used:

$$LOD = 3.3 \ \sigma/S \qquad \qquad \text{Eq. 1}$$

$$LOQ = 10 \ \sigma/S \qquad \qquad \text{Eq. 2}$$

LOD and LOQ were determined based upon the slope (S) of the calibration curve and least standard deviation obtained from the response (σ). It has a low limit of quantitation (5 ng/ml) with satisfactory specificity, no matrix interference was observed. These findings demonstrated that the assay has good selectivity.

2.3. Preparation of liposomes

Different types of AFT-loaded liposomes were fabricated by thin-film hydration method followed by extrusion as described previously [23]. Non-targeting liposomes (NL) were prepared using DSPC, DOPC, and DOPE at molar ratios of 3: 3: 10. Moreover, three parts of the 10 parts of DOPC of the total liposomal contents were replaced by DOTAP or CHEMS to form cationic and pH-sensitive liposomes, respectively. The AFT was added to the lipids at ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1 and 1.5:1 (w:w), respectively. The composition of the liposomes was presented in Table 1. Briefly, AFT and lipids were dissolved in chloroform, in a round bottom flask following by evaporation to obtain dried thin film at 55 °C using a rotary evaporator (Buchi Rotavapor R-200, Switzerland). The resulting lipid film was hydrated with the proper volume of phosphate buffer saline (PBS; pH 7.4) by gently mixing for 30 min at 55 °C to produce multilamellar vesicles (MLVs). The liposomes were subjected to extrusion through polycarbonate membranes with two pore sizes (200 and 100 mm) using a discontinuous extruder (Liposo-Fast™ Avestin Inc., Ottawa, Canada). The liposomes were extruded 5 times through a polycarbonate membrane with a pore size of 200 nm following by 21 times through 100 nm at low pressure (200 psi). The three types of plain liposomes were prepared similarly. The final formulations were stored for overnight at 4°C.

2.4. Physicochemical characterization of liposomes

2.4.1. Particles size distribution and zeta potential

The mean vesicle sizes, size distribution and the zeta potential (ζ) were characterized by dynamic light scattering (DLS) at 25 $^{\circ}$C with a fixed angle of 137° using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The liposomes were appropriately diluted with purified and filtered water (0.2 μm pore size) prior to the measurements. The mean vesicle diameters were the averages of five measurements. All measurements were done in triplicate.

2.4.2. Encapsulation efficiency

Due to the poor solubility of AFT, the free AFT occurred in two forms in the external phase of the liposomal dispersion like free undissolved and free dissolved AFT. The free undissolved AFT was separated from the liposomes using light centrifugation. Meanwhile, the supernatant (encapsulated and free dissolved AFT) were filled into centrifuge tubes and ultra-centrifuged at 40000 rpm at 4 ◦C. The clear supernatant which contained the free dissolved AFT was collected. The total AFT regard to the sum of both encapsulated and free AFT that is existed in the liposomal preparation. The concentration of total AFT was determined after dissolving and disrupting of the liposomal dispersion in methanol and triton x-100 using a vortex mixer, followed by centrifugation for 15 min. The clear supernatant which contained the total AFT was then transferred to a new tube and kept at 4 ◦C until analysis.

The drug encapsulation efficiency (EE%) was calculated using the total drug content of liposomal (AFT$_{total}$) dispersion and un-entrapped drug content of the dispersion (AFT$_{free}$). The EE% of all the formulation was calculated by using the following formula:

204 $$EE\% = \frac{AFT_{total} - AFT_{free}}{AFT_{toal}} \times 100 \qquad \text{Eq. 3}$$

205 2.4.3. Morphology of liposomes

206   The morphology of selected liposomes was visualized by transmission electron

207 microscope (TEM) using a JEM-2100 electron microscope (Jeol, Tokyo, Japan). Briefly, a

208 drop of diluted liposomes was applied to a copper grid and the excess liquid was removed

209 using filter paper. The samples were air-dried for 15 min and observed by TEM.

210 2.4.4. Stability study

211   The physical stability of the selected liposomes was conducted to monitor the

212 physical stability of AFT loaded liposome formulations (Table 2). All liposomal

213 formulations were stored in glass vials at 4±1 °C and 25°C ± 2°C for a period of one month.

214 The stability was evaluated by measuring the average particle size, ζ and PDI during the

215 storage. The AFT content was evaluated by HPLC. The physicochemical stability of the

216 freshly prepared formulation (at day1) was used as the control and the AFT content on day

217 1 was normalized to 100%.

218 2.4.5. *In vitro* drug release

219   *In vitro* release profile of AFT from of NL, CL, and PSL (selected liposomes) were

220 evaluated using the Franz diffusion cell system (FDC-6, LOGAN, Instruments

221 Corporation, USA). The experiments were conducted in 7 ml of PBS buffer (pH 7.4 and

222 5.5) with 0.2% Tween 80 to maintain sink condition. The cellophane dialysis membranes

223 (molecular weight cut off: 12-14 KDa) were soaked before use in distilled water at room

224 temperature for 12 h prior to use to ensure its wetting. An aliquot of 100 μL liposomes was

225 added into donor chambers, ensuring there were no air bubbles under the membrane. The

226 receptor compartment consisted of PBS at pH 7.4 for all liposomes and pH 5.5 for PSL at

227 37 °C, and stirring at 150 rpm. Samples of 500 µL were withdrawn at various time intervals

228 up to 24 h, and replaced immediately with the equal volume of fresh re-heated PBS. The

229 amount of AFT in each sample was analyzed by HPLC. The experiments were performed

230 in triplicate. The data of *in vitro* AFT release were fitted to various kinetic equations,

231 including zero order, first order, Higuchi's model and Korsmeyer Peppas plot and R^2.

232 Then, n values (diffusion exponent) were calculated for each linear curve obtained by the

233 regression analysis of each kinetic equation [24].

2.5. Antitumor activity studies

235 2.5.1. Cell proliferation assay, WST-1

236 The *in vitro* cytotoxicity of AFT compared to different chemotherapeutic agents

237 (carboplatin, gemacitabine and paclitaxel) was determined using WST-1 using NSCLC

238 cells (H-1975 cells). In brief, the cells were seeded into 96-well plates at a density of 10^4

239 cells/well and incubated overnight in culture medium. Afterward, different concentrations

240 of 1, 5, 10, 20, 40 and 80 µM were added to each well and incubated for additional 24 h.

241 At the end of the treatment, a 10 µl of cell proliferation reagent WST-1 kit was added and

242 incubated for 4 h at 37 °C. The intensities of photometric metabolite (formazan) were

243 measured at 450 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-

244 Rad Laboratories, Inc., Hercules, CA, USA). The results are expressed as the IC50, which

245 was obtained graphically using SigmaPlot 10 (SYSTAT Software, Inc., San Diego CA,

246 USA).

247 2.5.2. Annexin-V apoptosis assay

248 Cell death was assessed using the Vybrant® Apoptosis Assay kit and flow

249 cytometry. The H-1975 cells were seeded in six-well plates at 7×10^4 per well and

250 incubated overnight. Then, the cells were treated with pure AFT (control) and AFT loaded

251 PSL at concentrations of 0.5, 1, 3, 5 and 8 µm for 24 h after dilution with culture medium.

252 After treatment, the cells were harvested by trypsinization and centrifuged, and re-

253 suspended in PBS. The cells were stained with Alexa Fluor® 488 Annexin V/propidium

254 iodide (PI) and analyzed using the flow cytometer. The percentage of cell death was

255 determined using FACS-Calibur™ apparatus and CellQuest Pro software (Becton-

256 Dikinson Biosciences, Franklin Lakes. NJ, USA). Moreover, the apoptosis in different cells

257 like H-1975, HCC-827 and H-1650 was tested after application of NL, CL and PSL at 0.25,

258 0.5, 0.75, 1 and 2 µM. Furthermore, the percentage of cell death in the wells containing

259 the free drug and PSL following a 48 and 72h incubation period was subsequently

260 compared with the results of 24 h incubation at 0.25, 0.5, 0.75, 1 and 2 µM.

261 **2.6. Statistical analysis**

262 Quantitative data were expressed as the mean ± SD of at least three replicates. The

263 Student's *t*-test and one-way analysis of variance (ANOVA) using IBMSPSS Statistics 21

264 was used to assess multiple comparisons between different methods and times. The level

265 of confidence was set as 95%.

266 **3. Results**

267 3.1. HPLC assay

268 Three HPLC methods have been described for AFT quantification in dosage forms

269 [25]. This method has been reported by Vejendle *et al*, which demonstrated a lack of

270 sensitivity with LOD (60 ng/ml) and a longer run time (20 min). Therefore, A new sensitive

271 HPLC method for AFT analysis was developed in the current study. This method was used

272   to quantify the AFT concentration for its *in vitro* studies. The method was determined to

273   be specific for AFT in the matrix with no interfering peaks.

274   It was identified in our laboratory that the maximum absorbance for AFT is at 206

275   and 253 nm. While, the HPLC analytical methods reported in the literature detected AFT

276   at 252, 258 or 268 nm. Therefore, the detection wavelength of 253 nm was used for a better

277   sense of AFT in the HPLC in this study. As shown in Fig. 1, the average retention time

278   was 2.4 min, with no interfering peaks in chromatogram A (the blank) and chromatograms

279   B, C (AFT). The obtained results indicated the specificity of the HPLC assay method. It

280   should be mentioned that, during the *in vitro* studies, there was no interfering peaks from

281   the NP ingredients co-eluted with the AFT peak, which further confirmed the specificity

282   of the method.

283   3.2. Method validation

284   A calibration curve of the peak area of AFT vs. the various AFT concentrations, in

285   the range of 0.01 to 25 µg/ml was conducted. The regression equation of the line was

286   obtained ($y = 360232x + 56.95$) resulting in the correlation coefficient (R^2) of 0.9999. The

287   results indicated the quality of the curve (data not shown). Therefore, there was a good

288   linear relationship between the AFT peak area and its tested concentrations.

289   The analytical method was validated in terms of linearity, precision, and accuracy.

290   Linearity was assessed using a calibration curve to investigate the ability of this method to

291   get a proportional response to the different concentrations. Based on the concentrations

292   used ranged from 10 to 250000 ng/m, in triplicate, the linearity was evaluated and a

293   calibration curve was constructed.

294   The LOD was determined to be 10 ng/ml and the LOQ was 5 ng/ml, with the

295   corresponding CV values of 1.8 and 0.93 %, respectively (Table 2).

296   For precision and accuracy of sample analysis, AFT standard solutions of three

297   replicates were prepared in triplicate, and analyzed on the same day (repeatability) or in

298   three different days (intermediate precision). Tables 3 showed that the precision did not

299   exceed the required RSD value with maximum RSD value was < 1.98 %. Analysis of

300   variance of the data indicated no significant difference (p =0.401) in the slopes, intra- and

301   inter-day of the calibration curves. The results confirmed the reproducibility of the assay.

302   The accuracy was more than 99.9 %.

303   The method was found to be robust, since small variations in the method conditions

304   had a negligible effect on the chromatographic behavior of the AFT. The results indicated

305   that changing of the HPLC system or the C_{18} column had no effect on the chromatographic

306   behavior of AFT. Even a small change in the mobile phase composition did not

307   significantly change the peak area of the drug used for this method.

308   3.3. Particle size distribution and zeta potential

309   This study aims to evaluate the incorporation of AFT to CL and PSL compared to

310   NL (control liposomes) for the design of an efficient anticancer delivery system. The

311   obtained liposomes were characterized in terms of the mean particle size, PDI and ζ values

312   using DLS and electrophoretic light scattering (Data not shown). The particle size of the

313   liposomes were ranged from 46 to 57 nm and values of PDI were less than 0.2, which

314   indicate narrower size distribution indicating no aggregation. Regarding ζ, the liposomes

315   presented high values according to the increasing lipid to the drug ratios, until the ratio was

316   1:0.5 (Fig. 2). The AFT-containing liposomes exhibited more positive ζ than liposomes

317 without AFT, which proposes that the addition of DOTAP increased the amounts of AFT

318 in the liposomes. In case of NL, the positive ζ was low and after the incorporation of the

319 drug, ζ increased by approximately two-fold until 1:0.5 of lipid to the drug ratio. CL

320 possessed great values of positive ζ ranging from 38.9 mV for the blank to 48.4 mV for the

321 ratio of 1:0.5. However, PSL exhibited negative values of ζ, due to CHEMS and decreased

322 with the increasing the drug to lipid ratios (Fig. 2).

323 3.4. Encapsulation efficiency

324   The effect of the lipid to drug ratio on the encapsulation of AFT is indicated in Fig.

325 3. As the lipid to AFT ratios increased, the amount of the drug was increased to a certain

326 extent and then decreased. The highest values of the encapsulated AFT were on lipid to

327 drug ratio, 1:0.5, the EE values were 43, 50, and 52 % for NL, PSL, and CL respectively.

328 As expected, the amount of AFT in the liposomes would increase with increasing of drug

329 concentration. After reaching the maximum capacity of AFT, EE values were decreased

330 with more AFT. However, the amount of free drug increased ($P< 0.05$) significantly,

331 thereby notably decreasing the EE%.

332   According to the obtained results, a lipid to drug ratio 1:0.5 in all tested liposomes

333 was selected for further studies due to the high value of EE%. On the contrary, PSL at a

334 lipid to drug ratio of 1:1 showed the lowest EE%.

335 3.5. Morphology of liposomes

336   TEM images of PSL are presented in Fig 4. The images showed that PSL were a

337 spherical shape. In addition, the existence of multilamellar structure was well visualized

338 inside PSL (Fig. 4). It has been reported that the protonated CHEMS showed a lower

339 hydration ability than CHEMS at neutral pH, while the protonated amino group of

340 phosphatidylethanolamine (PE) exhibited greater hydration ability, thus enhancing the

341 immiscibility between PE and CHEMS and causing formation of a heterogeneous system

342 [23].

343 3.6. Stability study

344   The short-term stability of the selected liposomes of lipid:drug ratio of 1:0.5 was

345 investigated for up to 30 days at 4 ± 1 ^{o}C and 25 ± 2 ^{o}C. This ratio was chosen due to the

346 highest EE% of AFT. There was no significant change in the particle size, PDI, ζ and AFT

347 EE% of liposomes during the stability study at 4°C compared to the initial preparation (p

348 > 0.05). However, at 25 ^{o}C, the particle size of the liposomes after storage for 30 days as

349 47.5 ± 2.3 to 75 ± 3.3 nm, 53.8 ± 2.6 to 84 ± 15.9 nm and 55.3 ± 1.2 to 79.5 ± 2.5 for CL, PSL and

350 NL respectively. However, liposomes were still smaller than 100 nm. There was no

351 appreciable change in PDI over 30 days. The ζ at 4°C and 25°C for 30 days exhibited

352 insignificantly different ($p = 0.141$) compared to the initial formulation. The EE% of AFT

353 after storage at 4°C and 25°C for 30 days was slightly decreased but was still higher than

354 90% and 80%, respectively, of the initial formulations (Fig.5).

355 3.7. *In vitro* release study

356   To evaluate the *in vitro* drug release behaviors of AFT from CL and PSL compared

357 to NL, these nanoliposomes were incubated in pH 7.4 PBS solutions at 37 ^{o}C in (Fig. 6).

358 Moreover, to determine the pH sensitivity of PSL, the drug release behavior under pH 5.5

359 was also investigated, and pH 5.5 was selected to mimic the tumor pH (Fig. 6). As weakly

360 acidic environments are presented in the endosomal and lysosomal of tumor cells [26].

361   The AFT release rate was relatively slow in neutral pH 7.4, just reaching 63.6%,

362 28.1% and 35.6 % for CL, NL, and PSL, respectively within 24 h. These data revealed that

363 the liposomes exhibited significantly constant release profiles and AFT was successfully

364 loaded into the liposomes. However, the cumulative release of AFT in PSL at pH 5.5

365 reached to 101 % in 4 h, presenting a burst release phenomenon. The release profiles of

366 AFT from different liposomes apparently biphasic release processes, where rapid release

367 of the surface-adsorbed AFT was observed during the initial phase (first 4 h), followed by

368 a slow release profile for up to 24 h. The AFT release was increased significantly with the

369 pH decreased from 7.4 to 5.5 in case of PSL, and showed reasonably good pH-

370 responsiveness. But, in a physiological environment (pH 7.4), the encapsulated AFT was

371 released at a constant rate. It indicated that the AFT was well protected inside the liposome

372 bilayers at physiological pH. But in acidic condition (pH 5.5) cancer environment, the AFT

373 release was hastened. Therefore, the release of the PSL containing AFT was controlled by

374 the environmental pH. This phenomenon are consistent with the obtained results of

375 doxorubicin release at various pH values [27]. The constant release rate of AFT may

376 conserve a constant contact of the drug to the cancer cell resulted in improved antitumor

377 activity and helpful for drug delivery applications. The PSL system has the greatest

378 potential to improve cancer therapy efficacy. To fit the release kinetics of AFT from

379 liposomes at pH 5.5 and 7.4, different kinetic models viz. Peppas, Higuchi, zero order and

380 first order were exploited to predict the drug release profile. These models depend on the

381 diffusion equations, which based on the composition of liposomes and release conditions.

382 It was reported that the release of the drug from liposomes could be allocated in three

383 different mechanisms: diffusion, erosion and diffusion-erosion [28]. It was found that the

384 results were supported by the Korsmeyer-Peppas model at pH 7.4, which it presented the

385 highest value of R^2. Moreover, the values of n are 0.460, 0.681, 0.431 and 0.599 for CL,

386   NL, PSL and PSL (pH 5.5), respectively, indicated a non-Fickian diffusion kinetics ($0.5 <$

387    $n < 1$) [28]. PSL ($n=0.431$) exposed Fickian diffusion due to slow release at neutral

388   condition [28]. Subsequently, it is concluded that the drug release mechanism was mainly

389   owing to the combination of diffusion and erosion of the liposomes containing AFT (Table

390   4).

391   Moreover, the release profiles of the three AFT-loaded liposomes could be divided

392   into two phases, the first phase from 1 to 4 h and the second phase from 6 and 24 h. The

393   fitting parameters derived from korsmeyer-Peppas were listed in Table 5. n is the release

394   exponent indicating the drug release mechanism and k reflected the rate constant of the

395   release. At pH 7.4, n values of liposomes in the first phase were higher than 0.43, indicating

396   the release behavior of AFT followed a combination of diffusion and erosion control. In

397   the second stage, n values of liposomes were below 0.43, except NL, representing a

398   combination of diffusion and erosion mechanism. The k values for PSL at pH5.5 were

399   much higher than those of pH 7.4, demonstrating the highest release rate in an acidic

400   environment. The difference between k values of the first and second stage were nearly the

401   same, which indicated that the AFT release rate was comparatively slow in a neutral

402   environment.

403

404   3.8. Cell proliferation assay, WST-1

405   In general, it is essential to screen and confirm that the antitumor drugs are potent

406   and efficient for cancer therapy. Therefore, the potency of AFT was evaluated in

407   comparison with selected drugs depending on their activities against lung cancer in an *in*

408   *vitro* cell-based assay. Half-maximal inhibitory concentration (IC_{50}) of these drugs was

409 attained from an experimentally derived dose-response curve. In this study the cytotoxicity

410 was evaluated with a WST-1 assay in H-1975 cells. The cells were incubated with AFT,

411 paclitaxel, carboplatin and gemcitabine for 24 h. As shown in Fig. 7, the results detected

412 that effect of AFT and paclitaxel on H-1975 cells had dose-dependent manner. On the

413 contrary, the data displayed minor cytotoxicity against H-1975 cells, even up to the highest

414 doses of carboplatin and gemcitabine. The IC_{50} values for AFT and paclitaxel were 20 and

415 25 µM, respectively. Particularly, by increasing the concentrations of AFT to 40 µM, H-

416 1975 cells exhibited higher sensitivity than paclitaxel. The cell viability dropped to 2%

417 with 40 µM of AFT and 50% with paclitaxel. Further increasing the concentration of AFT

418 up to 80 µM, the insignificant reduction in the cell viability was observed (Fig. 7). Thus,

419 *in vitro* anticancer activity revealed that AFT had potent cytotoxic (IC50 value; 20 µM) as

420 compared to other drugs.

421 The cell toxicity of the best liposomes PSL, NL, and CL was also measured by a

422 WST-1 assay on H-1975 cells (data not shown). Unfortunately, the reduction of cell

423 viability (H-1975 cells) at any AFT concentrations did not recorded. This behavior

424 confirmed that the WST-1 assay failed to detect any reduction in viable cell numbers. In

425 contrast, the cytotoxicity (dose-dependent) was detected by microscopic examination using

426 1-80 µM of PSL, NL, and CL. Accordingly, the intracellular vacuoles and cell aggregates

427 at concentrations from 1 to 5 µM was appeared. Further increasing the concentrations from

428 10 to 80 µM, indefinite aggregates of damaged and dying cells were perceived. The order

429 of liposomes to kill H-1975 cell was followed as PSL > NL > CL. Therefore, AFT-loaded

430 PSL at concentrations less than 10 µM were performed in the next study.

431 3.9. Annexin-V apoptosis assay

432   Apoptosis-inducing influence of AFT loaded-PSL formulation was evidenced by

433   Annexin V/PI protocol. The extent and the nature of the induced cell death were analyzed

434   by flow cytometry. H-1975 cells were incubated with various concentrations of AFT

435   loaded-PSL (0.5 - 8 µM) for 24 h, which were selected based on WST-1 assay results. The

436   amounts of the early apoptotic and the late apoptotic cells, with necrotic cells were

437   determined after deduction of the proportion of spontaneous apoptosis. The results clearly

438   revealed that the AFT loaded-PSL triggered both apoptosis in H-1975 cells (Fig. 8A).

439   The quantities of apoptotic cells increased from 55 to 58.9 % after exposure to 0.5

440   to 1 µM of AFT loaded PSL. However, increasing the concentration to 8 µM resulted in a

441   reduction of the quantities of apoptotic cells from 30% at 3 µM to 9 % at 8 µM.

442   Furthermore, high cell viability of 87.5 % of free liposomes was observed. Concerning the

443   concentrations of AFT loaded-PSL at 3 to 8 µM, the proportions of necrotic cells increased

444   as the number of apoptotic cells decreased. The proportions of necrotic cells increased from

445   3 to 90%, depending on the concentrations of AFT loaded-PSL at 0.5 and 8 µM (a dose-

446   dependent manner) (Fig. 8B). Consequently, AFT at a concentration of 2.0 µM was

447   selected due to high apoptotic activity for further cytotoxicity studies using different lung

448   cancer cell lines.

449   Moreover, the cell viability of AFT loaded-PSL after H-1975 cells were incubated

450   for 48 and 72 h in addition to 24 h also was investigated (Fig. 9). The significant cytotoxic

451   effect in H-1975 cell at a concentration of 2 µM of AFT loaded-PSL, with the total cell

452   death proportion exceeding 78, 80 and 84 % after 24, 48 and 72 h, respectively. The

453   cytotoxic effect of the AFT-PSL formulation at a concentration of 2 µM was mainly due

454   to induced apoptosis, with slight necrosis. For comparison, the cell viability of free

455 liposomes at 72 h was 90 %. These results revealed that no significant difference in the cell

456 death after 24 and 48 h of exposure with H-1975 cells ($p > 0.05$). Therefore, 24 h of

457 exposure was selected for further study. Free liposomes showed insignificance cytotoxic

458 (apoptosis) after 24 and 48 h of exposure with considerable toxicity (necrosis) after 72 h

459 of exposure.

460   Overall, the obtained results clearly revealed the greater anticancer activity of PSL

461 as shown by a WST-1 assay and apoptosis assay. Therefore, 24 h of exposure was selected

462 for further cytotoxicity study of AFT-loaded PSL, CL and NL by using different lung

463 cancer cell lines. Hence, the anticancer activity of these liposomes was performed using

464 Flow cytometric analysis in the three cell lines as H-1975, H-1650, and HCC-827 (Fig.10

465 A, B & C). This study was conducted to detect the level of apoptosis induced after

466 incubation with various concentrations of 0.25, 0.5, 0.75, 1, and 2 µM of each liposomes

467 for 24 h. While, dimethyl sulfoxide and free liposomes were used as the controls. The

468 incubation of the cells with free liposomes did not induce notable cytotoxicity (data not

469 shown). The viability of H-1975 cells decreased more significantly compared to H-1650

470 and HCC-827 cells ($p < 0.05$). PSL produced highest cytotoxic effect in the different lung

471 cancer cell lines (H-1975 cells and HCC-827) compared to NL and CL, when using

472 concentrations 2 µM AFT (Fig. 10). Overall, the results clearly revealed the superior

473 anticancer activity of PSL as shown by MTT assay and apoptosis assay.

474 **Discussion**

475   It has been reported that the cancer cells exhibited leaky vasculature with gap of

476 ~100 nm, this allows the drug to leak out of the blood vessels and into the cancer cells [29].

477 Additionally, cancer cells have an impaired lymphatic system; therefore, substances such

478   as drugs loaded-liposome can be retained for a relatively longer time [30]. This behavior

479   is commonly referred to EPR effect, which increases the exposure of tumor cells to drug

480   action. The liposomes have been reported as a potential carrier to target cancer cells. The

481   liposomes less 100 nm are able to escape the tumor vasculature and accumulate in the cells

482   by passive targeting [31]. Moreover, the targeted liposomes were designed depending on

483   the type of phospholipids used. In this study, it has been illustrated that modified liposomes

484   as PSL can work as an effective carrier for delivery of AFT *in vitro*. Moreover, this carrier

485   system presented significant antitumor activity. The desired liposomes can obtained by

486   investigating different quantities of the drug to lipid ratios to detect the greatest

487   encapsulated liposomes. Furthermore, the best liposomes in terms of vesicle size, PDI, ζ

488   and EE were selected. The *in vitro* release study and cytotoxicity of the selected liposomes

489   loaded AFT were examined. In addition, AFT is a potent antitumor drug used in clinical

490   oncology against a lung tumor. However, AFT had low specificity, systemic toxicity and

491   indiscriminating of the tumor and healthy tissues [14]. So AFT loaded-lipoosmes were

492   developed for successful cancer therapy that decreases dose-limiting toxicity. In this study,

493   a novel pH-sensitive liposomes-based AFT as targeted delivery (PSL) were used to target

494   tumor cells. It has been observed that PSL has intensely improved the cytotoxicity in

495   comparison to conventional liposomes (NL) or cationic liposomes (CL). Kraft *et al*

496   observed that CL or PSL could accumulate in the lung cells more, compared with NL [32].

497   PSL exposed to the destabilization behavior under acidic condition, which lead to rapid

498   release of its content. It is interesting that the tumor tissues are relatively acidic compared

499   to the normal tissue site [19-21]. The main difference between these two liposomes is the

500   composition of phospholipid used. In general, three lipid components: DSPC, DOPC, and

501 DOPE. DSPC were used. The rationale for the selection of DSPC was its stability against

502 chemical degradation due to saturated lipid, which reduces the drug leakage from

503 liposomes on storage and in vivo transit. To increase the fluidity of liposomal membrane,

504 DOPC was selected due to its high fluidity at room temperature (transition temperature

505 (T_m)= −20°C). While, T_m of DSPC is +55 °C, which remains in the gel phase [33].

506 Moreover, DOPE was combined to provide fusogenic characters to the liposomes, due to

507 the formation of an inverted hexagonal phase upon destabilization of membranes at a

508 mildly acidic pH [34]. Theses lipids possess various chain lengths and degrees of

509 saturation, which can produce fine-tune the membrane dynamics and phase properties [35].

510 The main composition of CL is DOTAP, which is considered as a cationic phospholipid.

511 While PSL composed from pH-sensitive phosphlipid (CHEMS). The film hydration

512 method has been used to actively entrap AFT into liposomes with relatively high

513 efficiencies and small vesicle size (<100 nm) [36]. The PDI values of the obtained

514 liposomes are less than 0.3 indicating narrow size distribution. The higher ζ, of the obtained

515 liposomes provoked the potential stability of a liposome. Patil *et al* investigated the

516 influence of ζ on the cellular uptake of cerium oxide nanoparticles in A549 lung

517 adenocarcinoma [37]. Thus, the charged surface of liposomes has ability to attach the cell

518 membrane. Furthermore, to obtain the liposomes with the highest EE%, the best ratio of

519 drug to phospholipid was selected for further studies. Accordingly, the highest EE% of

520 AFT reached to 43.20%, 50.20%, and 52.01% for NL, PSL, CL, respectively at the 1:0.5

521 ratio of lipid to the drug. Nallamothu *et al* 2006 indicated that the high EE% of

522 combretastatin A4 was obtained by increasing the drug to lipid ratios. By using 1:10 to

523 2:10 of combretastatin A4 to lipid ratios, the amount of the drug increased from 1.05 mg/ml

524 to 1.55 mg/ml, respectively. When the drug to lipid ratios was further increased to 4:10,

525 the EE% of the drug did not increase [38]. Thus, the best liposomes were subjected to

526 stability study, they exhibited better stability at 4°C or at 25°C after storage for 1 month.

527 CL showed the highest stability in term of EE%, particle size and zeta potential. This effect

528 is due to the inclusion of DOTAP in the liposomes, which decreases the rigidity of the

529 liposomes with good loading capacity. *In vitro* drug release data revealed that PSL and NL

530 exposed sustained release profiles due to the presence of DSPC (T_m), which lead to a

531 decrease in leakage of AFT in the circulation or extracellular environment. But in case of

532 CL, AFT exhibited high release rate compared with the other liposomes, at pH 7.4. This is

533 due to the complete protontion of DOTAP at pH 7.4 [39]. By contrast, the fast drug release

534 profile of AFT was found after addition PSL in acidic media, which reached to 100% after

535 4 h. The PSL undergoes destabilization at pH 5.5 and acquire fusogenic properties, thus

536 tended to rupture and quickly release of AFT. The fusogenic performance of PSL is due to

537 the presence of DOPE in the lipid layer, which forms a hexagonal structure instead of a

538 bilayer structure after dispersion in aqueous media. Düzgünes *et al* showed that liposomes

539 composed of CHEMS had high stabilize of EE calcein at pH 7.4 and undergo

540 destabilization and irreversible aggregation under acidic pH [40]. According to the kinetic

541 models, the release of AFT at pH 7.4 displayed release with Korsmeyer-Peppas model.

542 This effect is due to early rapid release followed by slow release of the liposomes [41]. In

543 case of the release pattern of PSL at pH 5.5, AFT release was quite faster with Korsmeyer-

544 Peppas model. This behaviour because AFT exists liposomal membrane, which leaks out

545 at a faster rate in acidic condition [42].

546   Therefore, it was confirmed that the pH-sensitive point in the liposome is close to

547   5 (the tumor microenvironment). PSL might release their content in the acidic environment

548   of the tumor tissues quickly. Therefore, the PSL were fabricated in the current study to

549   achieve a certain active targeting toward the tumor. The TEM of PSL revealed uniform,

550   homogenous and spherical-shaped liposomes with a smooth surface.

551   The potency of free AFT was compared with selected drugs depending on their

552   activity against lung cancer. The cell toxicity was evaluated by WST-1 using H-1975 cells.

553   The WST-1 assay showed that AFT is more effective as a cytotoxic agent compared to

554   other compounds used in lung cancer treatments (H-1975 cells). Furthermore, the anti-

555   proliferative effect of AFT on H-1975 cells was investigated at various concentrations for

556   24 h. The results indicated that the inhibition of cell viability AFT had concentration-

557   dependent manners. Moreover, anticancer activity of the obtained liposomes was also

558   investigated using and H-1975 cells. Unfortunately, WSR-1 assay failed to detect the

559   anticancer activity of the obtained liposomes due level of interference. However, a detailed

560   characterization of this interference was not undertaken here. Therefore, the levels of cell

561   viability between each liposomes were measured by using flow cytometry analysis after

562   Annexin V/PI staining. The results of flow cytometry after the treatment of cells with the

563   AFT loaded-PSL using different concentrations exhibited a comparable level of cell

564   intensity. It was clearly indicated that the uptake of AFT loaded-PSL by H-1975 cells was

565   higher than free AFT. The results revealed a marked decline in cell viability with AFT

566   loaded-PSL up to 60.4% of cell apoptosis at 1 µM after 24 h. The free AFT resulted in

567   apoptosis in 11.88% of the cells after 24 h. The low cytotoxic effect of free AFT could be

568   attributed to the low cellular uptake and poor trans-membrane permeability. Of the three

cancer cell lines tested, H-1975 cells appeared more sensitive to the liposomes. Particularly, the cytotoxicity of PSL is high compared with that of CL and NL. It is possible that PSL released AFT in response to the lowered pH in the endosome, and thus facilitated diffusion of the released AFT from the endosome to the cytosol. It has been suggested that pH-sensitive liposomes are internalized more efficiently than non-pH-sensitive formulations [43]. It is notable that the destabilization of PSL at the endosomal demonstrated that the efficacy of PSL depends on the pH of the tumor tissues [34]. Additionally, the liposomes containing CHEMS release their contents into the cytoplasm from 5 to 15 min upon their incubation with the cells [44]. The destabilization of PSL induced by acidification of the endosomal lumen represents the most important stage in the process of intracellular delivery. Carvalho *et al* developed cisplatin loaded-PSL to treat the SCLC. Compared with free cisplatin, the cytotoxicity of this PSL was significantly enhanced [7]. Kim *et al* developed a PSL with an efficient and targeted delivery system for gemcitabine, and represent a useful, novel treatment approach for tumors that overexpress EGFR [45]. More, the cationic liposomes containing paclitaxel composed of DOTAP were able to significantly decrease tumor perfusion and vascular diameter and the progress of A-Mel-3 melanoma in mouse models [46]. The association of cationic liposomes with surface membrane is due to the presence of anionic glycoproteins, such as sialic acid rich glycoproteins [47]. Furthermore, pH-sensitive liposomes containing cytarabine have been shown greatly antitumor effectiveness in both human HepG2 hepatoma cells and normal human liver L02 cells compared to non-pH-sensitive liposomes [48]. These promising results are required further *in vivo* analysis to understand the biodistribution profile of AFT loaded PSL to achieve new targeted-formulation for the tumor therapy.

4. Conclusion

In this work, a novel AFT-loaded PSL for targeted therapy of lung cancer (NSCLC) were developed. For comparison purpose, AFT-loaded NL, CL and PSL were successfully designed. The obtained liposomes showed small particle less than 100 nm with a low PDI (<0.27) and acceptable zeta potential with a spherical shape. The highest EE% values of the liposomes were achieved according the following order: CL>PSL>NL. The selected liposomes were stable at 4 and 25°C for 1 month. The PSL, CL and NL showed slow release profiles in pH 7.4. However, in acidic pH values, PSL exhibited fast release, which improved its tumor targetability. The selected liposomes revealed efficiency on cancer cells (NSCLC). Moreover, AFT-loaded PSL inhibited the cell growth of lung cancer cells more efficiently than free AFT, CL and NL based on using Annexin V assay. The obtained data indicate that AFT-loaded PSL is a promising a targeted drug delivery for cancer therapy.

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738 **Table 1**

739 Compositions of different types of liposomes.

Phospholipids*	Amount required (µmol/mL)		
	NL	PSL	CL
DSPC	3	3	3
DOPC	10	7	7
DOPE	3	3	3
CHEMS	-	3	-
DOTAP	-	-	3

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741 *NL: Non-targetin liposomes; CL: Cationic liposomes; PSL: pH-sensitive liposomes;
742 DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine[18:0]; DOPC: 1,2-dioleoyl-sn-
743 glycero-3-phosphocholine [18:1]; DOPE:1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
744 [18:1]; CHEMS: Cholesteryl hemisuccinate; DOTAP:1,2-dioleoy-3-trimethylammonium-
745 propane Chloride salt (DOTAP)
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Table 2

Precision of the developed method for analysis of AFT.

Nominal (µg/mL)	Concentrations Mean ± SD	CV%
0.01	0.01 ± 6.09	0.9266
0.05	0.05 ± 6.40	0.8051
0.1	0.1 ± 5.71	1.7143
0.25	0.25 ± 1.76	0.7076
0.5	0.5 ± 5.70	1.1404
1	1 ± 1.77	0.1763
2	2 ± 4.03	2.3016
5	5 ± 5.65	1.0731
10	10 ± 3.76	1.3577
20	20 ± 3.83	0.1992
25	25 ± 2.55	0.1102

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765   SD: standard deviation; CV: Coefficient of variation percetage

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Table 3

Repeatability for different levels of AFT (n = 3).

Concentration (µg/ml)	Mean ± SD[a]	Precision RSD[b] (%)	Accuracy (%)
Inter day			
0.1	99.98 ± 1.98	1.98	99.9
1	1000.72 ± 0.49	0.05	102
10	10000 ± 113.26	1.13	100.1
Intra-day			
0.1	100 ± 1.06	1.07	99.86
1	1000 ± 0.29	0.03	100.5
10	10000 ± 60.08	0.6008	100.2

a   Standard deviation of the mean
b   Relative standard deviation

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Table 1

Release kinetics of AFT release from different liposomes.

pH media	Codes	Zero order	First order	Higuchi (Diffusion)	Korsmeyer Peppas	"n" value
	CL	0.691	0.559	0.871	0.940	0.460
pH 7.4	NL	0.928	0.731	0.988	0.994	0.681
	PSL	0.754	0.634	0.912	0.971	0.431
pH 5.5	PSL	0.647	0.486	0.835	0.889	0.599

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Table 5

Drug release kinetics parameters derived from Korsmeyer Peppas.

pH media	Codes	[a]n_1	[a]k_1	[b]n_2	[b]k_2
	CL	0.675	16.987	0.168	37.619
pH 7.4	NL	0.681	3.606	0.527	5.434
	PSL	0.483	8.809	0.201	16.381
pH5.5	PSL	0.941	20.910	0.181	59.671

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[a]The first stage is 0-4 h. [b]The second stage is 6-24 h.

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827   **Fig. 1**

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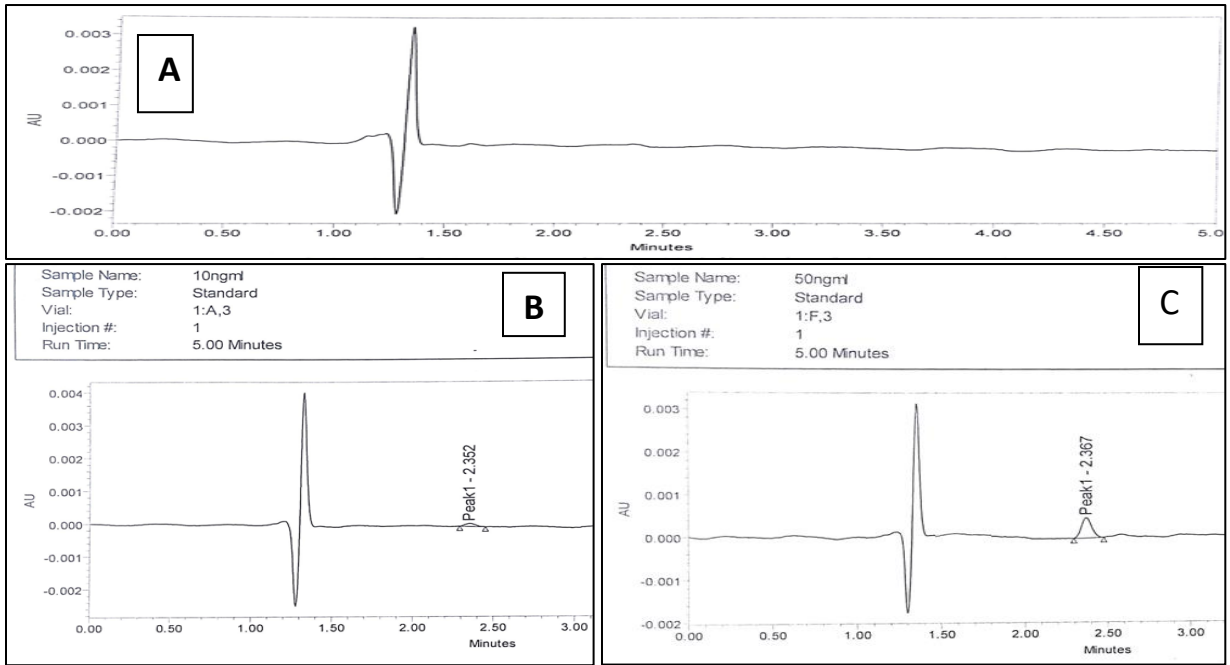
Fig. 1. HPLC chromatograms of the mobile phase (chromatogram A), and HPLC chromatograms of the mobile phase containing (B) 10 ng/ml and (C) 50 ng/ml afatinib.

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839   **Fig. 2**

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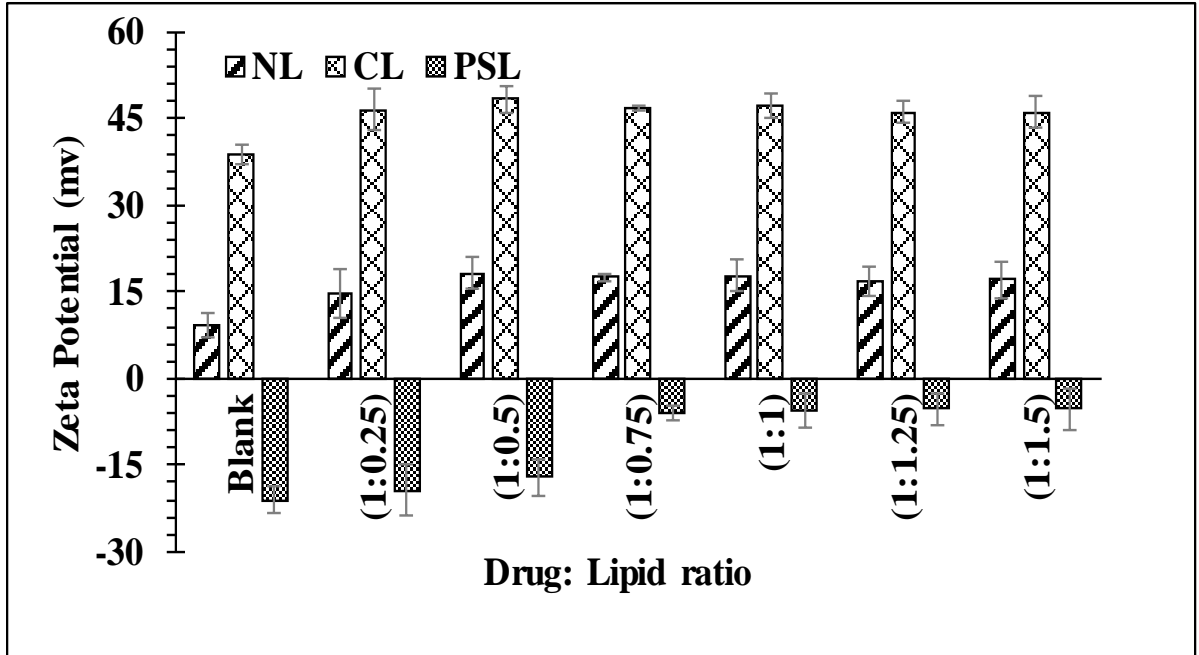
Fig. 2. Zeta potentials of afatinib-loaded liposomal formulations at different lipid to drug ratios.

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852   **Fig. 3**

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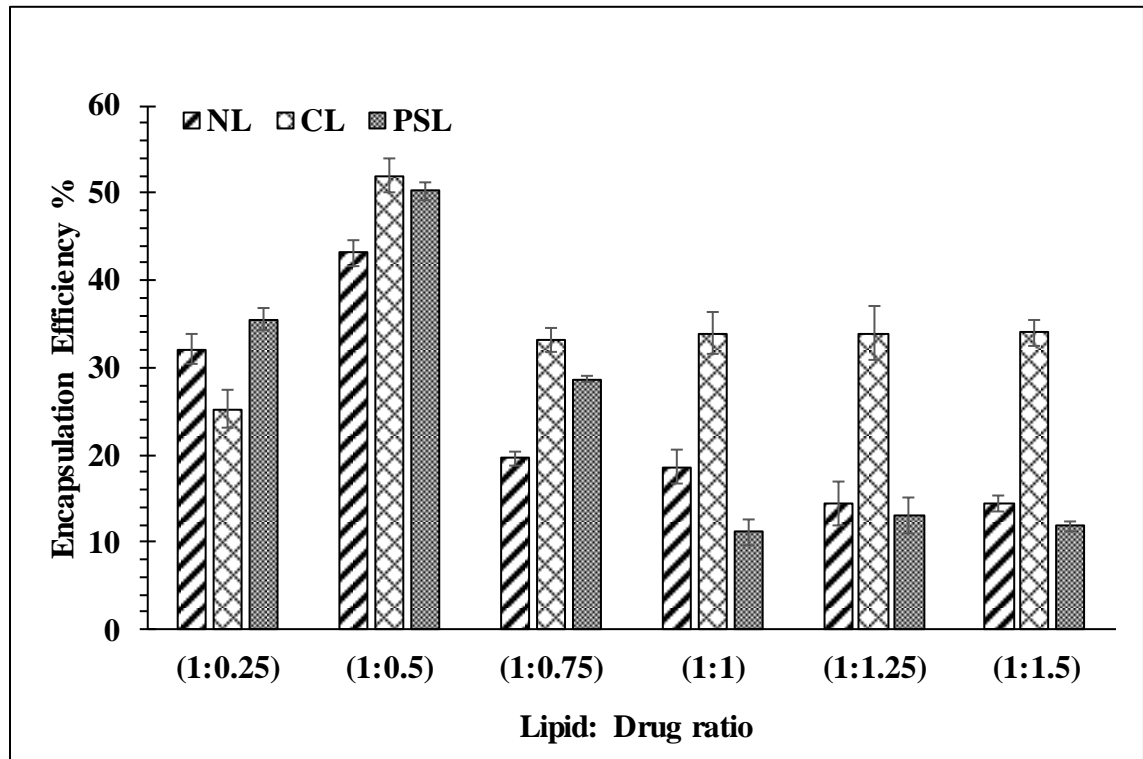
Fig. 3. EE% of afatinib-loaded different liposomal formulations with different lipid to drug ratios.

875   **Fig. 4**

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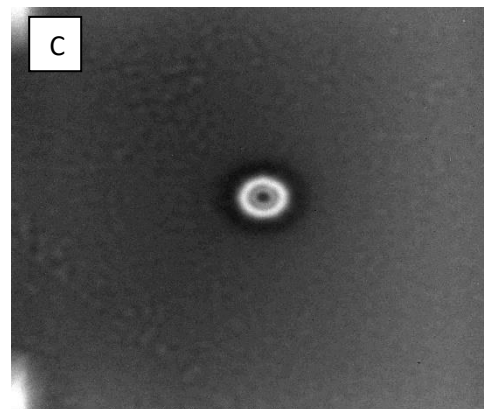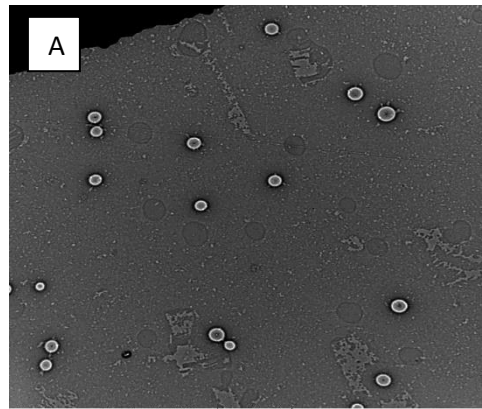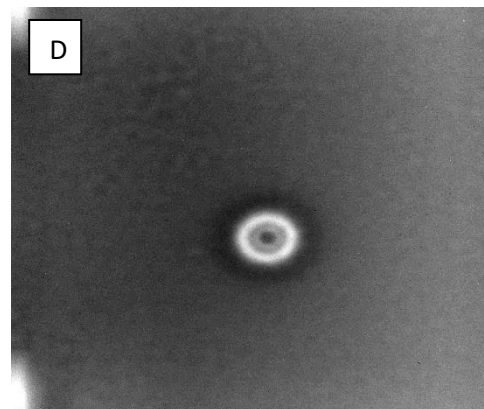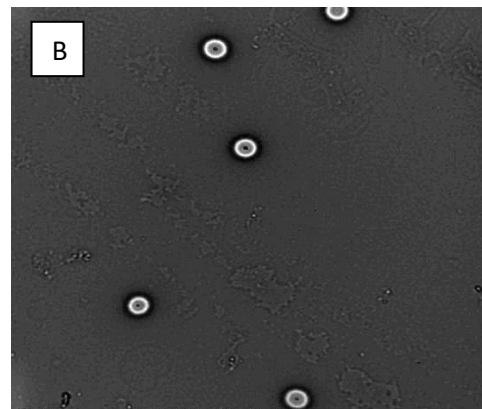
Fig. 4. TEM micrographs of the pH sensitive liposome at a drug to lipid ratio of 1:0.5 (PSL), using varied magnification power.

Fig. 5

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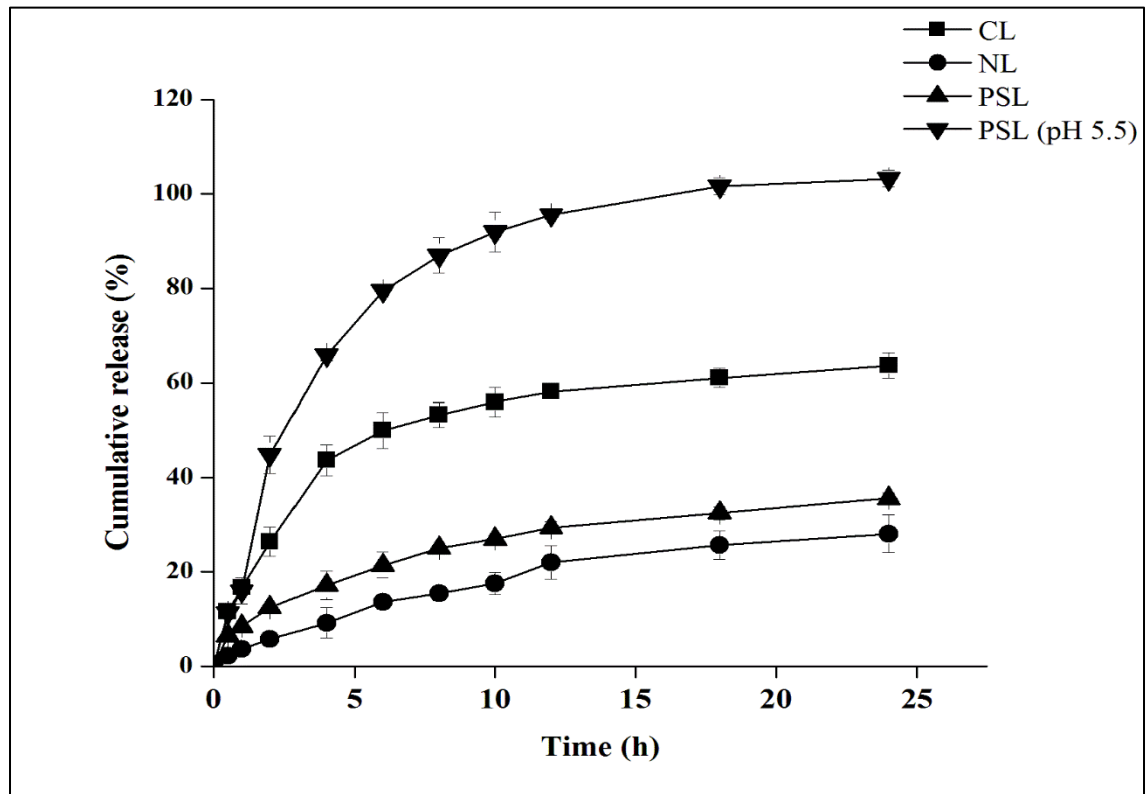
Fig. 5. *In vitro* release profiles of the liposomal formulations loaded with afatinib in phosphate-buffered saline containing 0.2% Tween 80 at pH 7.4 and pH 5.4. Values are presented as the mean ± SD.

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921 **Fig. 6**

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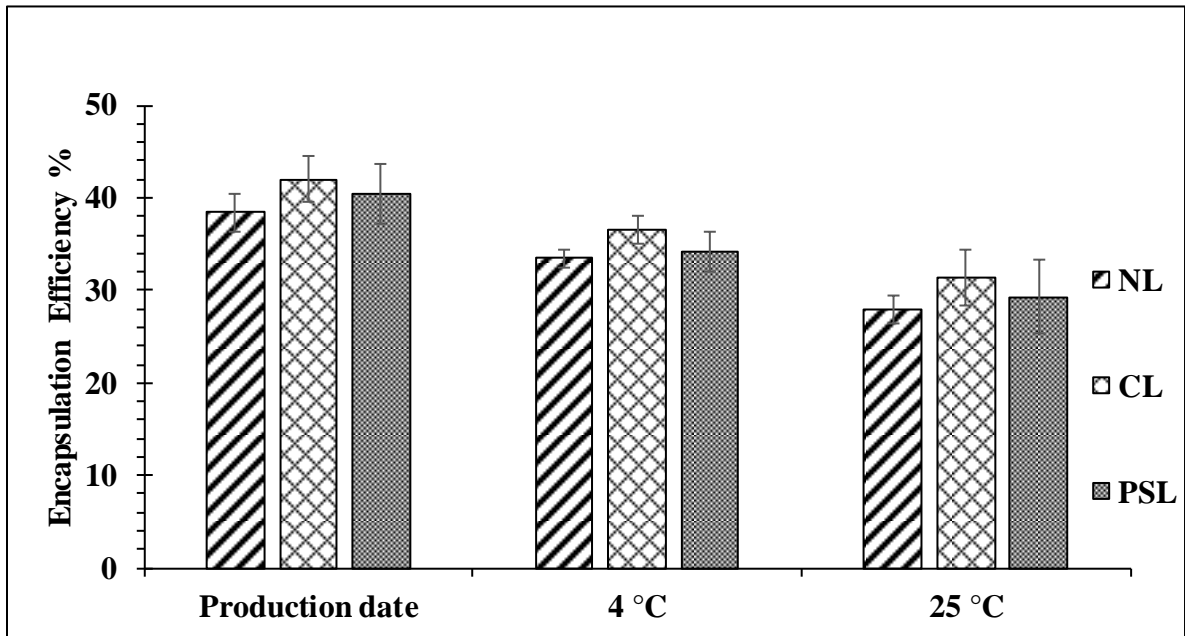
Fig. 6. EE % of afatinib at the ratio 1:0.5, following storage for one month at 4 and 25°C, of NL (nontargeting liposome), PSL (pH sensitive liposome) and CL (cationic liposome), P = 0.141.

Fig. 7

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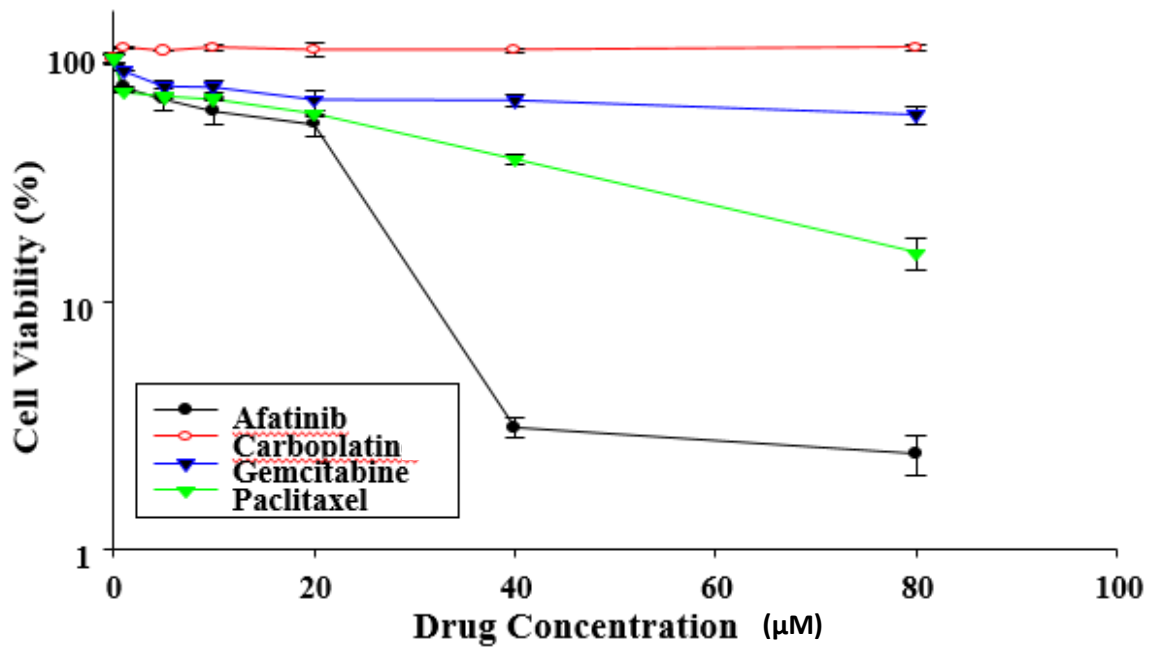
Fig. 7. Cytotoxicity of afatinib, carboplatin, gemcitabine and paclitaxel on H-1975 cells, as
943 determined by a WST-1 assay. Cells were treated with varying concentrations of the drugs for
24 h. Results are from three independent experiments and are expressed as the mean ± SD.
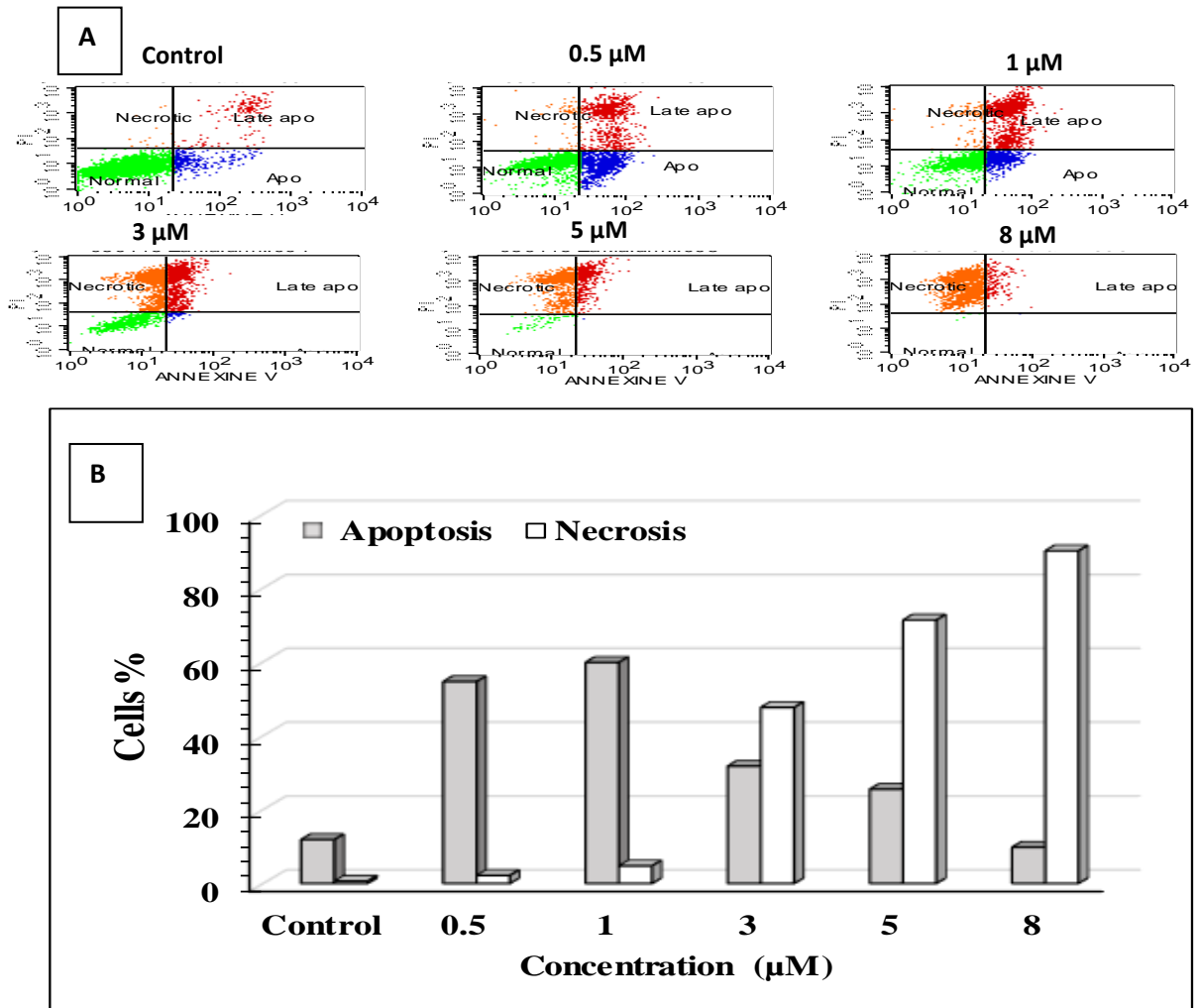
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950   **Fig. 8**

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Fig. 8. H-1975 lung cancer cells were either treated with free liposomes, as controls, or challenged with Afatinib loaded liposomes (PSL2) for 24 hrs, and then the proportion of apoptosis and necrosis was analysed by Annexin V/PI-flowCytometry. four groups of cells, viable cells that excluded both Annexin V and PI (Annexin V/PI), bottom left; early apoptotic cells that were only stained with Annexin V (Annexin V+/PI), bottom right; late apoptotic cells that were stained with both Annexin V and PI (Annexin V+/ P+), top right and necrotic cells that were only stained with PI (Annexin V/PI+), top left. (A) Flow charts. (B) Histogram showing the percentage of induced apoptosis in H-1975cells.

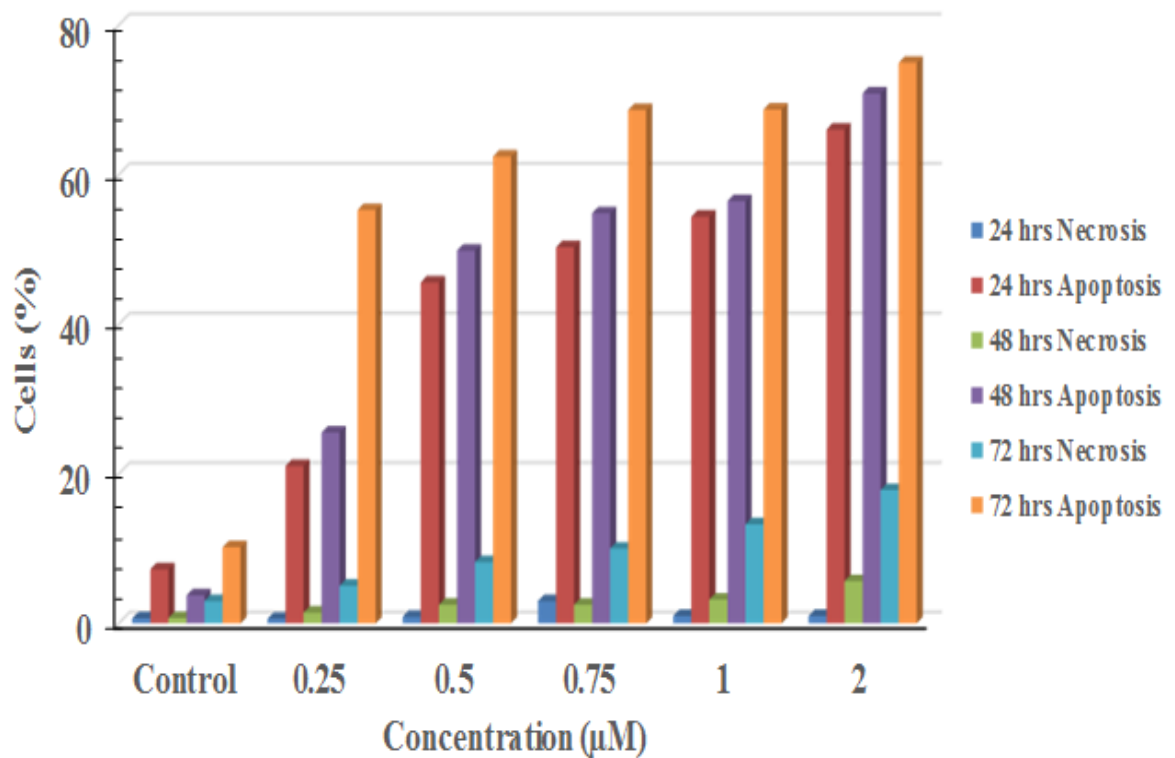956   **Fig. 9**

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Fig. 9. H-1975 cells were challenged with pH-sensitive liposomes (PSL) (0.25-2 μM) for 24, 48 or 72 h, following which apoptosis was analysed with Annexin V/PI-flow cytometry. Each value represents the mean ± SD of three independent experiments performed in triplicate.

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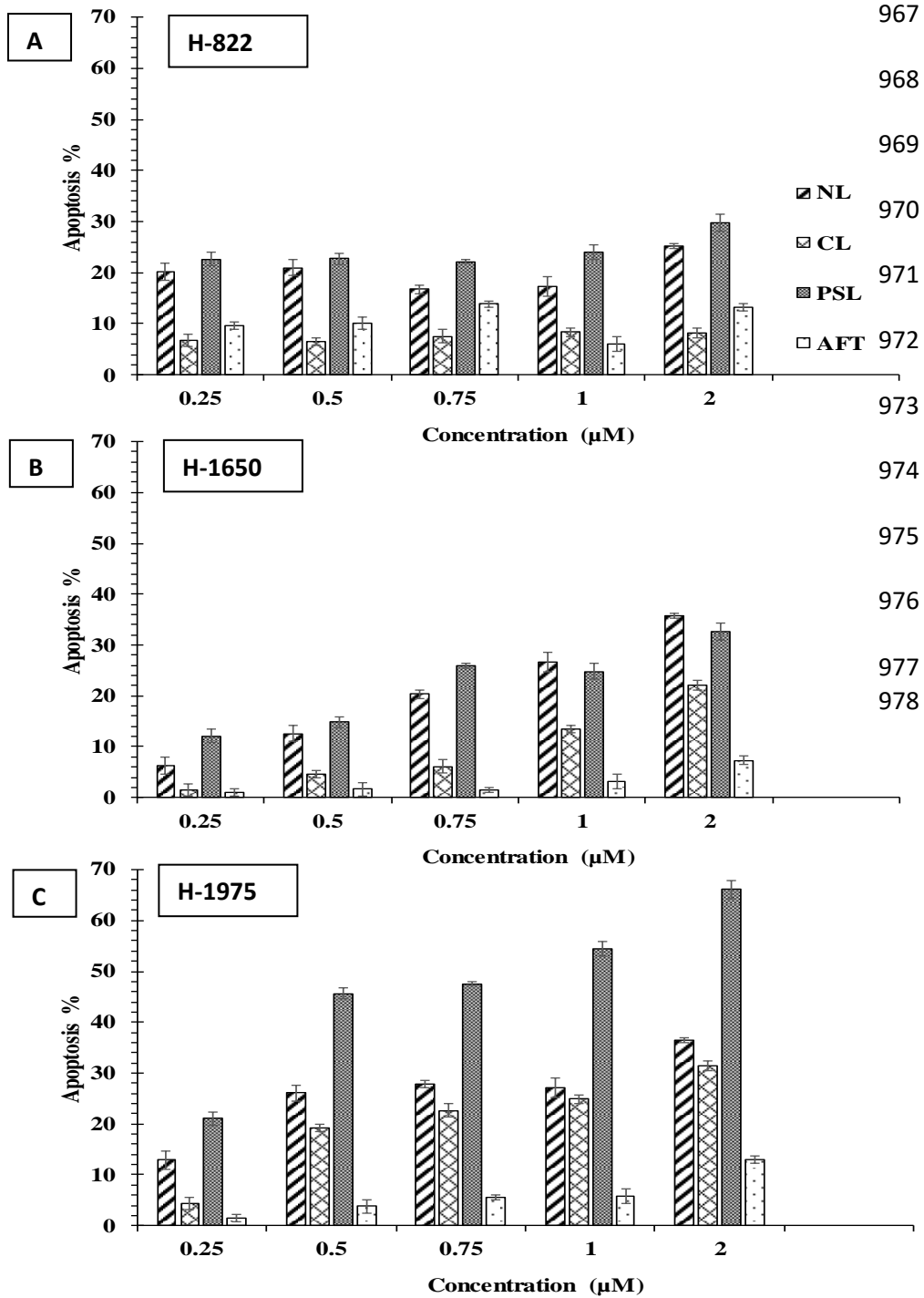
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966   **Fig. 10**

Fig.10. Non-small cell lung cancer cells were either treated with Blank Liposomes, as control, or challenged with AFT and AFT-loaded liposomes (PSL, NL, CL) for 24 h, following which the proportion of apoptotic cells was analysed using Annexin V/PI-flow cytometry. Histogram shows the percentage of induced apoptosis in H-1975 cells. Each point represents the mean ± SD of three independent experiments performed in duplicate.